Archival Report

Biological Psychiatry: GOS

Peripheral MicroRNA Signatures in Adolescent Depression

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ABSTRACT

BACKGROUND: Adolescent depression is linked to enduring maladaptive outcomes, chronic severity of symptoms, and poor treatment response. Identifying epigenetic signatures of adolescent depression is urgently needed to improve early prevention and intervention strategies. MicroRNAs (miRNAs) are epigenetic regulators of adolescent neurodevelopmental processes, but their role as markers and mediators of adolescent depression is unknown.

METHODS: Here, we examined miRNA profiles from dried blood spot samples of male and female adolescents with clinical depression and psychiatrically healthy male and female adolescents (N = 62). We processed and sequenced these samples using a small RNA protocol tailored for miRNA identification.

RESULTS: We identified 9 differentially expressed (DE) miRNAs (adjusted p value $<$ .05), all of which were upregulated in adolescents with depression. At future follow-ups post blood collection, expression of miR-3613-5p, mir-30c-2, and miR-942-5p were positively associated with depression severity but not anxiety, suggesting a stronger link to persistent depression symptoms. Expression of miR-32-5p inversely correlated with hippocampal volume, highlighting a potential neurobiological basis. Common predicted gene targets of the DE miRNAs are involved in neurodevelopment, cognitive processing, and depressive disorders.

CONCLUSIONS: These findings lay the groundwork for identifying adolescent peripheral miRNA markers that reflect neurodevelopmental pathways that shape lifelong psychopathology risk.

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Major depressive disorder (MDD) is a pervasive and debilitating condition, consistently ranked among the leading causes of disability globally. Across the lifespan, the peak period for the onset of depression is adolescence (1,2). Adolescent-onset MDD is associated with significant long-term maladaptive outcomes, including chronic and severe forms of depression, as well as a poorer response to treatment (3). Despite the significant clinical impact of adolescent-onset depression, research into its genetic and molecular underpinnings remains scarce. This research gap is particularly concerning given the distinct differences in the pathophysiology of MDD that originate during adolescence versus adulthood, which are likely due to the neurodevelopmental changes that uniquely influence biopsychosocial systems during younger years (4–7). Subphenotyping MDD by age of onset has shown promise in delineating an age-specific genetic contribution to depression, reducing phenotypic variability (8–13). Defining molecular targets specific to adolescent-onset MDD is critically needed.

Noncoding genomic regions, which comprise most of the genome (14), are emerging as significant biomarkers in psychiatry, particularly microRNAs (miRNAs). miRNAs function as modulators of gene expression posttranscriptionally and have the advantage of being both stable and readily assayable from peripheral biosamples (15). Our studies with rodents have demonstrated that manipulation of a candidate miRNA in the adult brain induces corresponding changes in its expression in peripheral blood (16). Furthermore, circulating levels of this miRNA during adolescence predicted the severity of stress-induced depressive-like behaviors observed later in life (17).

Given that peripheral miRNAs can be informative of their levels in the brain and may index adolescent psychiatric vulnerability, we sought to profile circulating miRNAs in adolescents with and without depression. We used a microsampling technique that preserves dried blood spots (DBSs) for high-throughput miRNA expression analysis. We used a small RNA sequencing (RNA-seq) pipeline that captures unbiased miRNA profiles (18).

METHODS AND MATERIALS

Participants and Study Design

The participants (N = 62) were carefully selected by clinical researchers to closely match 2 cohorts, with the primary difference being that TIGER (Teen Inflammation Glutamate Emotion Research) (19) was a case-control study on adolescent depression, and ELS (Early Life Stress) examined mental health outcomes transdiagnostically following exposure to

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early-life adversity before puberty. The 2 cohorts were drawn from the same geographic regions, were assessed contemporaneously, and were evaluated using the same gold-standard instruments to diagnose clinical conditions, including depression. Therefore, we were able to include both TIGER and ELS participants in our control group (n = 28) and TIGER participants only in the MDD group (n = 34). Both studies were approved by the institutional review boards of their respective institutions (University of California San Francisco and Stanford University). All participants and their parents gave written assent and informed consent, respectively, in accordance with the Declaration of Helsinki, and were financially compensated with gift cards for their participation.

The severity of self-reported symptoms of depression and anxiety was measured using the Reynolds Adolescent Depression Scale, Second Edition (RADS-2) (20) and the Multidimensional Anxiety Scale for Children, Second Edition (MASC-2) (21) at a subsequent time point assessment. Additional sample information can be found in Supplemental Methods.

Sample Processing

Sample collection, RNA extraction, and library preparation were performed using the protocol that we described in detail in (18). Briefly, blood drops were collected from participants' nondominant hand on Whatman filter paper cards (GE Healthcare). The cards were stored at −20 °C and shipped, insulated with dry ice, to Douglas Research Institute, Montreal, where 6-mm diameter punches were taken for subsequent processing. The samples underwent lysis, agitation, and sonication, followed by RNA extraction using the miRNeasy kit (Qiagen) with the small RNA fraction enrichment.

Library Generation. To mitigate the small RNA input that a single punch of DBS poses, small RNA libraries were prepared with a protocol that effectively captures miRNAs and minimizes the selection biases of RNA sequences during ligation with the use of degenerate bases on ends of the Illumina TruSeq small RNA adapters (Galas Lab 4N RNA library prep protocol version 1.0; Pacific Northwest Research Institute).

Sequencing. Libraries were sequenced at the Genome Quebec on NovaSeq6000 S1 (Illumina) (100-bp single-end reads, covering 24 million reads per sample on average).

Bioinformatics Analysis

Sequencing reads were trimmed with cutadapt to remove technical sequences. Following standardization of RNA-seq data analysis, we submitted trimmed reads to the Extracellular RNA Communication Consortium's exceRpt small RNA-seq pipeline (https://github.gersteinlab.org/exceRpt) on an institutional server [Genboree; exceRpt version 4.6.2 (22)], which aligned the reads to the human genome, with a zero-mismatch setting, and quantified miRNAs into counts. miRNAs with 0 reads across all subjects were removed, and the rest were analyzed for differential expression analysis with the DESeq2 package in R (version 1.34.0). miRNAs that were differentially expressed (DE) in the control and MDD groups above the false discovery rate (FDR) threshold ($p_{adjusted} < .05$) are referred to as top DE miRNAs from here on.

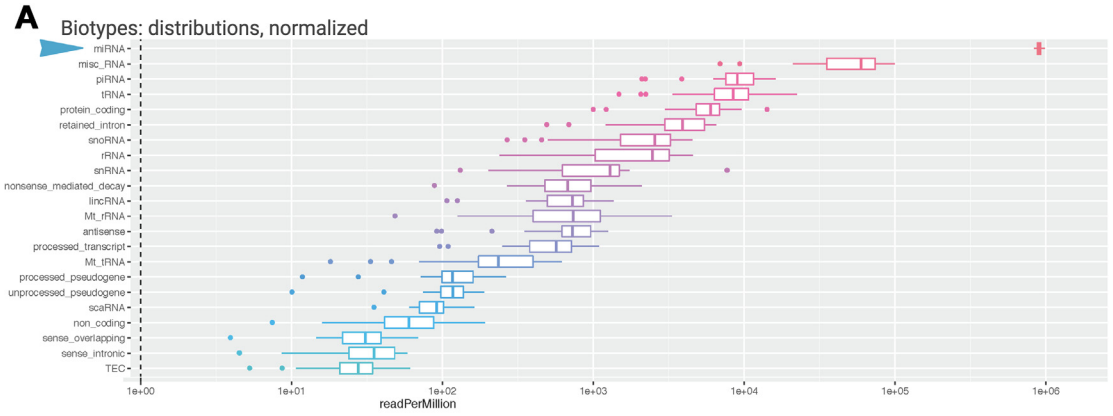
miRNA Pathway Analysis and Tissue Expression Probing

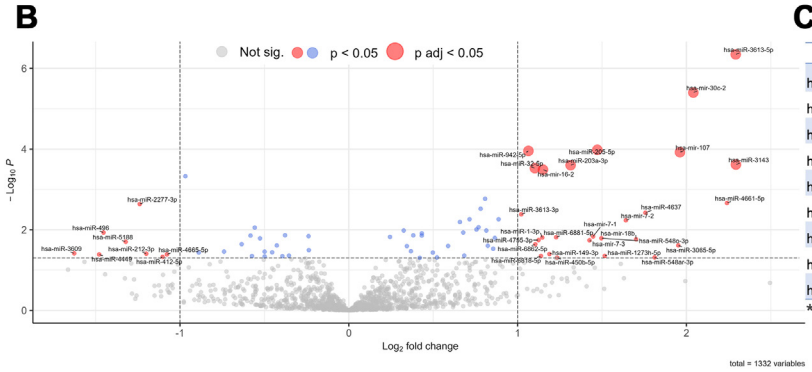
Tissue-based query of top DE miRNAs was conducted using the Tissue Atlas Database (23,24) and the DIANA Tools miRNA Tissue Expression Database (DIANA-miTED) (25) with the following settings: The Cancer Genome Atlas and Sequence Read Archive data collections, $\log_2$ reads per million, all diseases, all health status. Expression values for top DE miRNAs were extracted specifically for regions within the brain regions available in the TissueAtlas database and are depicted in a heatmap. Cell-type enrichment of top DE miRNAs was based on the DIANA miRPath version 4.0 (26) web-based computational tool with the following specifications: TarBase version 8.0, direct targets, Homo sapiens, miRbase-v22.1 annotation, classic analysis with FDR correction, and the Molecular

Table 1. Summary of Cohort Demographic Characteristics

	Control, n = 28	MDD, n = 34	p Value
Age, Years	16.3 ± 1.3	17.0 ± 1.3	.044
Sex			.611
Female	18 (64.3%)	25 (73.5%)	
Male	10 (35.7%)	9 (26.5%)	
Gender			.086
Female	18 (64.3%)	21 (61.8%)	
Male	10 (35.7%)	8 (23.5%)	
Nonbinary	0	5 (14.7%)	
Ethnicity			>.99
Hispanic/Latino	6 (21.4%)	7 (20.6%)	
Non-Hispanic/Latino	22 (78.6%)	27 (79.4%)	
Race			.803
American Indian or Alaska native	2 (7.14%)	1 (2.94%)	
Asian	7 (25%)	7 (20.59%)	
Black or African American	1 (3.57%)	2 (5.88%)	
Multiracial	3 (10.71%)	8 (23.53%)	
Native Hawaiian or other Pacific Islander	0	0	
Other	3 (10.71%)	3 (8.82%)	
White	12 (42.86%)	13 (38.24%)	
Parental Level of Education			.568
High school graduate or equivalent[a]	0	1 (3.0%)	
Some college, no degree	2 (7.1%)	5 (15.1%)	
Associate's degree[b]	3 (10.7%)	2 (6.1%)	
Bachelor's degree[c]	10 (35.7%)	9 (27.3%)	
Master's degree[d]	11 (39.3%)	10 (30.3%)	
Doctoral or professional degree[e]	2 (7.1%)	6 (18.2%)	
Not disclosed	0	1	

Values are presented as mean (SD), n (%), or n.
[a]General Educational Development.
[b]A.A., A.S.
[c]B.A., B.S.
[d]M.A., M.S., M.Ed.
[e]M.D., D.D.S., D.V.M., Ph.D., Ed.D.





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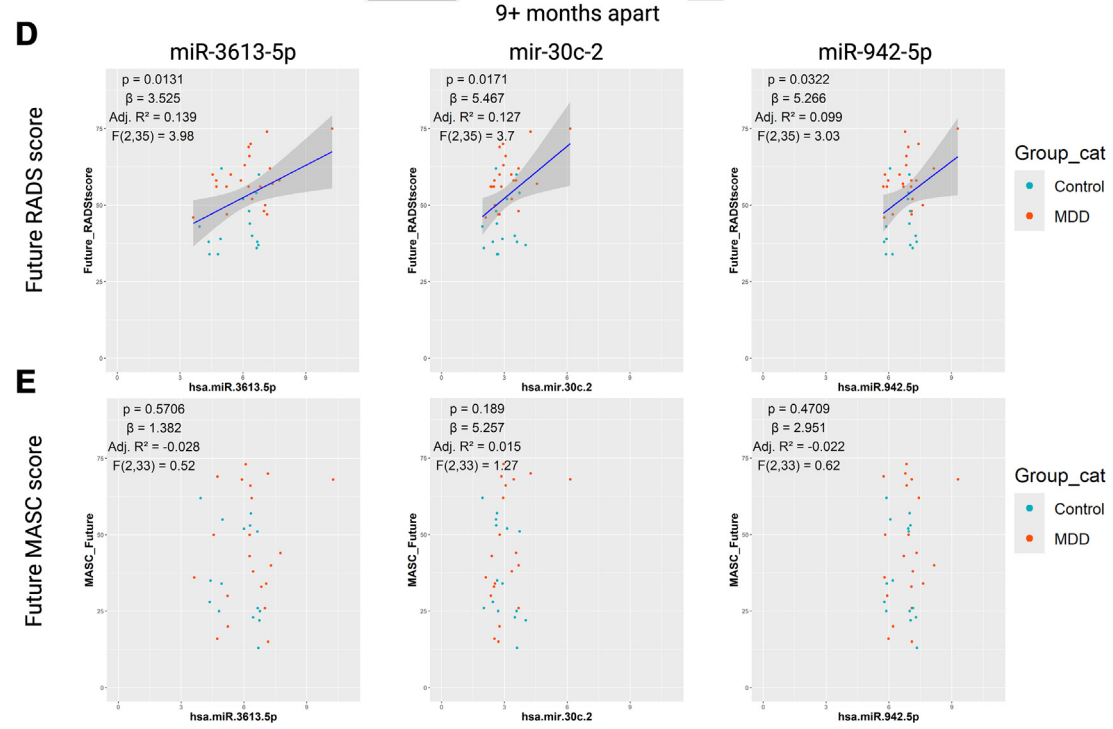
miRNA ID	Log_2 fold change	p adjusted
hsa-miR-3613-5p	2.29	0.0006
hsa-mir-30c-2*	2.04	0.0026
hsa-mir-107*	1.96	0.0317
hsa-miR-205-5p	1.47	0.0317
hsa-miR-942-5p	1.06	0.0317
hsa-miR-203a-3p	1.31	0.0475
hsa-miR-3143	2.29	0.0475
hsa-mir-16-2*	1.15	0.0478
hsa-miR-32-5p	1.10	0.0478

*Precursor



Signatures Database. The DIANA-miTED database for top-miRNAs query was used to assess the expression profile overlap of the top 20 highly expressed miRNAs in the DBS samples with those in plasma, blood (liquid), white blood cell, and serum under healthy status settings.

Logistic Regression on Top DE miRNAs

Logistic regression analysis was conducted to evaluate the diagnostic and classification capabilities of the 9 miRNAs, using categorical diagnosis as the outcome and miRNA expression levels as explanatory variables across all data samples. We compared models selected through both stepwise and backwards selection methods to identify the effective combinations of miRNAs for predictive modeling. For stepwise selection, the Akaike information criterion was used for model comparison, and the final selected model was subsequently used for receiver operating characteristic (ROC) analysis.

Expression of Top DE miRNAs and Future Self-Reported Depression Scores

To assess the potential association between top DE miRNAs and future self-reported depression scores, individual linear regression analyses were conducted using the RADS-2 T scores. We acquired clinical diagnostic data at the follow-up time points and confirmed that diagnosis was stable over time (i.e., no participants with MDD met criteria for full remission, and no control participants met threshold for any Axis I disorder). We also used linear regression analyses to evaluate the associations between top DE miRNAs and MASC-2 scores. Given the variable follow-up period between DBS collection and the subsequent behavioral assessments, we adjusted the analyses for the length of the follow-up interval, subtracting the age at DBS sample collection from the age at follow-up. Cook's distance was used as an estimate to identify potential outliers, with a cutoff value set above 1.

Association Between Top DE miRNAs and Brain Morphology

T1-weighted anatomical magnetic resonance imaging scans were acquired using a spoiled gradient sequence and preprocessed with FreeSurfer 6.0, as described in (27–29). Because the imaging data violated the normality assumption, Spearman correlations were used to perform partial correlations with adjustments for antidepressant medication use (dichotomous factor) and total intracranial volume (ICV) to assess associations between expression levels of top DE miRNA and volumes of brain regions including the amygdala, caudate, hippocampal, putamen, and nucleus accumbens. These results were not corrected for multiple comparison, because these tests were exploratory rather than hypothesis driven. Data were available for 3 ELS and 6 TIGER cohort control participants and 22 participants with MDD from the TIGER cohort.

Gene Target Prediction

Gene target prediction analysis of the top DE miRNAs was done using an integrative database of human miRNA target predictors, the microRNA Data Integration Portal (mirDIP) (version 5.3.0.1; Database version 5.2.3.1), which aggregates target predictors from curated algorithms and validated databases (30–32). We considered genes in the top 1% score class. Genes that predicted targets of ≥2 top DE miRNAs were annotated with gene ontology (GO) analysis using the enrichGO R library and enrichment analysis with the MetaCore database (Clarivate Analytics). We only retained terms that were adjusted for multiple comparisons using FDR correction. Gene localization enrichment was assessed based on the gene2func in the Functional Mapping and Annotation (FUMA) platform.

Statistical Analysis

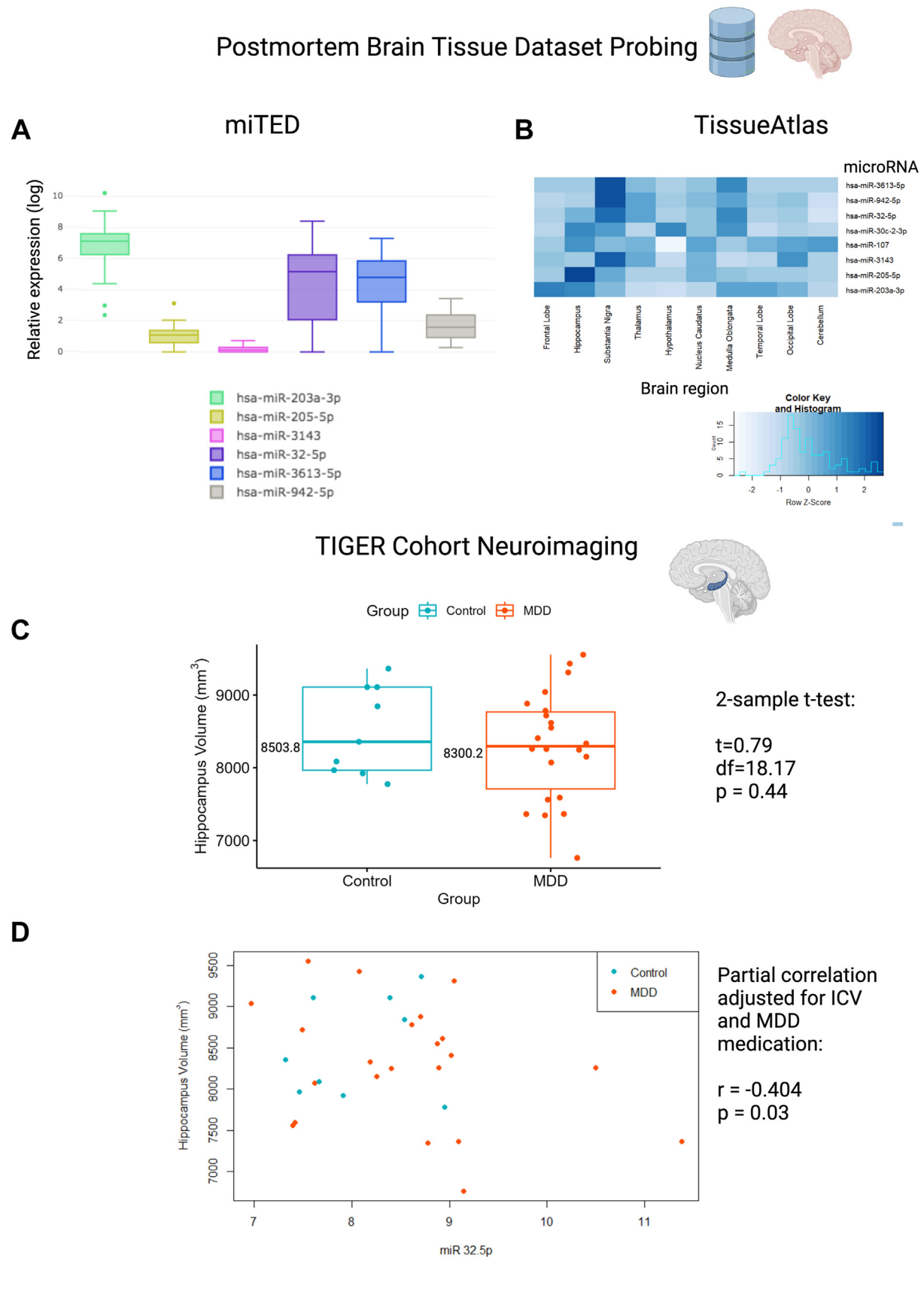
For demographic comparisons between the control and MDD groups, an independent 2-samples *t* test was used for age, Pearson's χ^2 test for sex and gender, and Fisher's exact test for race and parental level of education. Postcounts obtained with DESeq2 were extracted (DESeqDataSet data) and transformed using the regularized logarithm for visualization. Statistical analysis and plots were done using GraphPad Prism version 9.5 and R packages. A significance threshold of .05 was used with 2-tailed tests throughout, with the Benjamini-Hochberg method (FDR) used to correct for multiple comparisons in specified analyses.

RESULTS

Adolescents With Depression Have a Distinct Blood miRNA Profile

A summary of the participant demographic characteristics in healthy control participants (n = 28) and adolescents diagnosed with depression (n = 34) is presented in Table 1. Participants with MDD and healthy control participants did not differ significantly on any variable except for a minor difference in age (p = .04). The age distribution of the 2 groups is depicted in Figure S1A. Participants were predominantly of female sex,

Figure 1. Overview of dried blood spot–derived miRNAs DE between healthy control participants and adolescents diagnosed with MDD. **(A)** The vast majority of mapped reads are dominated by miRNAs. Showing representational image of mapping summary by exceRpt for one-third of the samples combined. **(B)** Volcano plot showing differential expression analysis between control and MDD groups of 1332 miRNAs. In gray are miRNAs that did not pass any significance threshold. In blue are miRNAs that passed the nominal p value but were below an FC of 1. In red are miRNAs that passed the nominal p value and were FC > 1, with top DE miRNAs that passed false discovery rate correction shown with enlarged circles. **(C)** Summary table of the top DE miRNAs by $p_{adjusted} < .05$, with upregulated FC in the MDD group compared with the control group. **(D)** Linear models of miRNAs as predictive values for depression severity, measured with RADS T scores, adjusted for the length of the follow-up interval. **(E)** Linear models of miRNAs as predictive values for anxiety measured with MASC T scores, adjusted for age at follow-up, revealed a lack of prediction. No outliers were detected as per Cook's distance cutoff >1. [Figures were created in BioRender.com under McGill University Department of Psychiatry plan.] DE, differentially expressed; FC, fold change; lincRNA, long intergenic noncoding RNA; MASC, Multidimensional Anxiety Scale for Children; MDD, major depressive disorder; miRNA, microRNA; misc_RNA, miscellaneous RNA; piRNA, PIWI-interacting RNA; RADS, Reynolds Adolescent Depression Scale; rRNA, ribosomal RNA; scaRNA, small Cajal body-specific RNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; TEC, transcription elongation complex; tRNA, transfer RNA.


Postmortem Brain Tissue Dataset Probing
A
miTED
Relative expression (log)
hsa-miR-203a-3p
hsa-miR-205-5p
hsa-miR-3143
hsa-miR-32-5p
hsa-miR-3613-5p
hsa-miR-942-5p
B
TissueAtlas
microRNA
hsa-miR-3613-5p
hsa-miR-942-5p
hsa-miR-32-5p
hsa-miR-30c-2-3p
hsa-miR-107
hsa-miR-3143
hsa-miR-205-5p
hsa-miR-203a-3p
Frontal Lobe
Hippocampus
Substantia Nigra
Thalamus
Hypothalamus
Nucleus Caudatus
Medulla Oblongata
Temporal Lobe
Occipital Lobe
Cerebellum
Brain region
Color Key and Histogram
Count
Row Z-Score
TIGER Cohort Neuroimaging
C
Group
Control
MDD
Hippocampus Volume (mm3)
8503.8
8300.2
Control
MDD
Group
2-sample t-test:
t=0.79
df=18.17
p = 0.44
D
Hippocampus Volume (mm3)
Control
MDD
miR 32.5p
Partial correlation adjusted for ICV and MDD medication:
r = -0.404
p = 0.03

with 5 participants identifying as nonbinary. In Table S1, we present a comparison of demographic characteristics of the 2 cohorts, with statistical tests indicating no significant differences.

DBS samples were divided into 2 library pools and, correspondingly, into 2 sequencing lanes to ensure deep coverage (Supplemental Methods and Figure S1B). The number of mapped reads to the human genome was on average 13 million reads per sample, with ∼79% mapping to miRNA sense and ∼11% mapping to precursor sense sequences. The distribution of mappings by biotype demonstrates distinct miRNA selectivity (Figure 1A). Normalized mappings for each sample, extracted from exceRpt diagnostic plots by 3 batches (due to large data size), are shown in Figure S1C, D. Principal component (PC) analysis of miRNA transcripts by the 2 main variables (PC1 and PC2) did not reveal apparent clustering by age, diagnosis, sex, or sequencing pool batch (Figure S1E). A total of 1332 individual miRNA transcripts with nonzero total read count were processed with DESeq2 for differential analysis between the MDD and control groups, resulting in comprehensive and unbiased miRNA profiling.

Following the differential expression analysis between participants with MDD and control participants, miRNAs were sorted according to their FDR-corrected p values, with the full DESeq2 analysis output provided in Table S2. The volcano plot encompassing all miRNAs ($p < .05$) DE between the MDD and control groups is depicted in Figure 1B. Interestingly, the number of upregulated miRNAs in MDD surpassed (>60% of miRNAs with $p < .05$) those that were downregulated, and the top 9 DE miRNAs ($p_{adjusted} < .05$) were all upregulated in the MDD group. In Figure 1C, the summary table lists the top DE miRNAs, indicating fold change and adjusted p values. Three of the 9 DE miRNAs are precursor sequences, potentially giving rise to mature forms through cleavage at either the 3′ or 5′ end [for process description, see review by (33)]. Details about the 9 DE miRNAs are provided in Table S3. We also tested whether the identified top DE miRNAs were a result of a medication effect and found no expression-level differences between the medicated and unmedicated groups (Table S4).

miRNA Expression During Adolescence Predicts Future Self-Reported Depression Severity

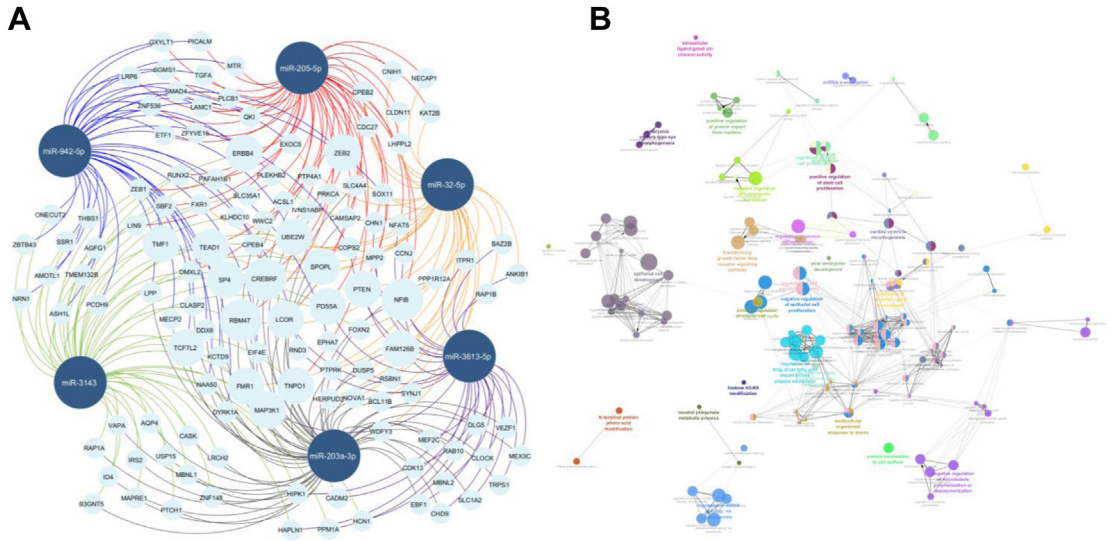
We conducted linear regression analyses to assess the relationships between expression levels of each of the top DE miRNAs and RADS-2 T scores obtained during a follow-up visit after the blood sampling session. Follow-up data were available for most participants (23 MDD and 15 control), with the pandemic impacting the attrition rate. Given the variable follow-up period between blood collection and the subsequent behavioral assessments, we adjusted the analysis for the length of the follow-up interval. The linear model of MDD severity computed as a function of miRNA expression (Figure 1D) revealed statistically significant positive associations for miR-3613-5p ($\beta = 3.525$, adjusted $R^2 = 0.139$, $p = .013$), mir-30c-2 ($\beta = 5.46$, adjusted $R^2 = 0.127$, $p = .017$), and miR-942-5p ($\beta = 5.266$, adjusted $R^2 = 0.099$, $p = .032$). The nonsignificant associations for the other top DE miRNAs are summarized in Figure S2A. None of the points reached a Cook's distance >1. Interestingly, none of the top DE miRNAs were associated with future anxiety scores (quantified by the MASC-2) (Figure 1E and Figure S2B), indicating specificity for depressive symptoms. In Figure S2C, the associations between the depression-related scores and top DE miRNA levels were explored in the control and MDD groups separately.

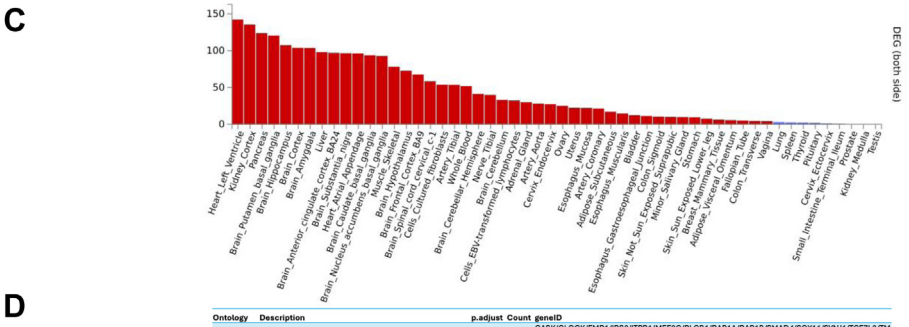
In addition, we assessed the potential of the top DE miRNAs as indicators of diagnostic outcome at the time of sample collection. Therefore, we conducted model selection using stepwise selection and backward elimination methods to ensure that the selection process was reliable. Consistently, these analyses specifically highlighted miRNAs miR-203a-3p and miR-3143 as showing the highest sensitivity and specificity. To evaluate potential predictive utility, we constructed a logistic regression model with categorical diagnosis as the dependent variable and miRNA expression levels as independent variables. The area under the curve of the model for miR-203a-3p and miR-3143 was 0.71, with the 95% CI ranging from 0.58 to 0.84. The specificity and sensitivity of this exploratory model was 0.62 and 0.57, respectively (visualized as ROC in Figure S2D), suggesting the need for caution regarding false positives and false negatives.

Top DE miRNAs Are Expressed in Key Brain Regions Linked to MDD

To examine whether top DE miRNAs are expressed in the brain and whether their relative levels vary according to brain region, we conducted exploratory analysis using the publicly available miRNA datasets, DIANA-miTED (Figure 2A) and TissueAtlas (Figure 2B). The top DE miRNAs with mature sequences are expressed in the human brain; precursor sequences, referenced in Table S3, could not be included in the analysis because they are typically not represented in these datasets. Assessment of relative expression (z score) in the brain showed that miR-203a-3p and miR-3143 had the highest and lowest levels, respectively, suggesting distinct modulatory influences (Figure 2A). A breakdown by brain region showed high expression of miR-3613-5p, miR-942-5p, miR-32-5p, and miR-3143 in the substantia nigra (Figure 2B), an area well known for its enrichment in dopaminergic cells. miR-205-5p showed the highest expression in the hippocampus, while miR-203a-3p had the highest expression in the frontal lobe, with moderate

Figure 2. Top DE miRNAs were expressed in the human brain, and miR-32 was negatively associated with hippocampal volume. **(A)** The miTED revealed human brain expression levels of each of the top DE miRNAs. **(B)** TissueAtlas dataset allowed evaluation of top DE miRNA expression in specific human brain regions. **(C)** Hippocampal volume comparison by group did not show statistical differences. **(D)** Partial correlation of miR-32 by hippocampal volume adjusted by ICV and MDD medication use showed a negative association. [Figures were created in BioRender.com under McGill University Department of Psychiatry plan.] DE, differentially expressed; ICV, intracranial brain volume; MDD, major depressive disorder; miRNA, microRNA; miTED, MicroRNA Tissue Expression Database; TIGER, Teen Inflammation Glutamate Emotion Research.





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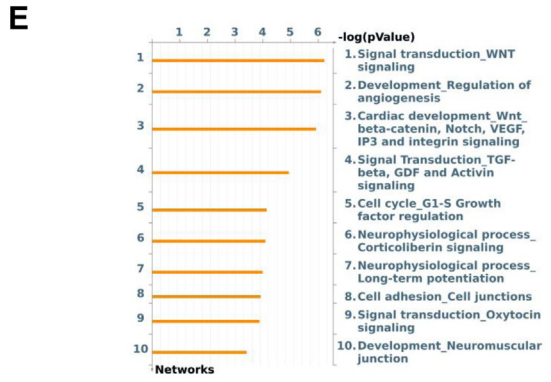
Ontology	Description	p.adjust	Count	geneID
BP	signal release	0.0024	14	CASK/CLOCK/FMR1/IRS2/ITPR1/MEF2C/PLCB1/RAP1A/RAP1B/SMAD4/SOX11/SYNJ1/TCF7L2/TM F1
BP	hippo signaling	0.0047	5	AMOTL1/DLG5/SOX11/TEAD1/WWC2
BP	cellular response to transforming growth factor beta stimulus	0.0047	10	MEF2C/ONECUT2/PPM1A/PTPRK/SMAD4/SOX11/THBS1/USP15/ZEB1/ZEB2
BP	modulation of chemical synaptic transmission	0.0051	13	CASK/EIF4E/EPHA7/FMR1/HCN1/MECP2/MEF2C/MPP2/PAFAH1B1/PLCB1/PTEN/RAP1A/RAP1B
BP	regulation of trans-synaptic signaling	0.0051	13	CASK/EIF4E/EPHA7/FMR1/HCN1/MECP2/MEF2C/MPP2/PAFAH1B1/PLCB1/PTEN/RAP1A/RAP1B
BP	regulation of dendritic spine development	0.0056	5	DLG5/FMR1/MEF2C/PAFAH1B1/PTEN
BP	multicellular organismal response to stress	0.0056	6	EIF4E/HCN1/MECP2/MEF2C/PTEN/THBS1
BP	positive regulation of hormone secretion	0.0063	7	IRS2/ITPR1/PLCB1/SMAD4/SOX11/TCF7L2/TMF1
BP	regulation of neuron differentiation	0.0070	8	BCL11B/DDX6/EIF4E/ID4/MEF2C/SOX11/ZEB1/ZNF536
BP	axon development	0.0098	12	BCL11B/CAMSAP2/CHN1/EPHA7/MTR/NFIB/PAFAH1B1/PICALM/PTCH1/PTEN/RAB10/SMAD4
BP	cell fate commitment	0.0112	9	BCL11B/ERBB4/MEF2C/ONECUT2/PTCH1/RAB10/RUNX2/SMAD4/TCF7L2
BP	vesicle-mediated transport in synapse	0.0112	8	CASK/FMR1/NECAP1/PICALM/PTEN/RAP1A/RAP1B/SYNJ1
BP	regulation of neurotransmitter secretion	0.0154	5	CASK/FMR1/MEF2C/RAP1A/RAP1B
BP	regulation of synaptic vesicle exocytosis	0.0167	4	CASK/FMR1/RAP1A/RAP1B
BP	central nervous system neuron differentiation	0.0355	6	BCL11B/ID4/NFIB/PAFAH1B1/PTCH1/PTEN
BP	forebrain development	0.0375	9	BCL11B/ERBB4/ID4/NFIB/PAFAH1B1/PLCB1/PTEN/SLC1A2/ZEB1
BP	behavioral fear response	0.0447	3	EIF4E/MECP2/MEF2C
BP	ERK1 and ERK2 cascade	0.0451	8	DUSP5/EPHA7/ERBB4/PRKCA/PTEN/RAP1A/RAP1B/SMAD4
BP	behavioral defense response	0.0456	3	EIF4E/MECP2/MEF2C
BP	learning or memory	0.0490	7	MECP2/MEF2C/PAFAH1B1/PICALM/PLCB1/PTEN/SYNJ1
CC	glutamatergic synapse	0.0057	11	DLG5/EIF4E/EPHA7/ERBB4/FXR1/HCN1/PAFAH1B1/PLCB1/RAP1A/RAP1B/SLC1A2
CC	neuron to neuron synapse	0.0075	10	CPEB4/DLG5/EPHA7/ERBB4/FMR1/FXR1/ITPR1/MPP2/PTCH1/PTEN



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Notable diseases	FDR	# of Genes
Psychiatry and Psychology	6.850E-07	75
Mood Disorders	8.553E-07	28
Mental Disorders	9.093E-07	74
Depressive Disorder, Major	2.832E-06	23
Depressive Disorder	3.638E-06	23
Nervous System Diseases	3.864E-06	108
Neurocognitive Disorders	6.683E-06	52
Brain Diseases	8.816E-06	80
Neurodevelopmental Disorders	2.327E-05	34
Neurobehavioral Manifestations	3.476E-05	35
Bipolar and Related Disorders	1.301E-04	17
Child Development Disorders, Pervasive	1.323E-04	12
Cardiomyopathy, Hypertrophic	5.455E-03	6
Cardiovascular Diseases	1.274E-02	52
Substance-Related Disorders	1.399E-02	13

Figure 3. Bioinformatics-based analysis identified highly targeted genes and their association with gene ontologies, pathways, and diseases. **(A)** Illustration of the top DE miRNAs and their shared predicted gene targets (*n* = 127). **(B)** Visualization of the interacting GO terms for the multi-miRNA target genes.

levels also being observed in the hippocampus. Cell-type signature analysis, as per DIANA miRPath, indicated that top DE miRNAs had multiple associations with the midbrain, prefrontal cortex (PFC) neurotypes, and glial cells during early development (Table S5).

Negative Association Between miR-32-5p and Hippocampal Volume

Given the reported volumetric differences in subcortical regions between individuals with depression and healthy control participants (34), we assessed associations between morphological gray matter features and DE miRNA in our study samples. Neuroimaging data were available for 31 participants (control n = 9; no statistical age differences between the groups, p = .07). MDD during adolescence has been linked to alterations in brain morphology, including reduced hippocampal volume (34–36). However, in our sample population, we did not observe differences between the MDD and healthy control groups in the volume of the amygdala, caudate, hippocampus, putamen, or nucleus accumbens (the hippocampus volume comparison is visualized in Figure 2E; Table S6). Then, we examined whether levels of individual top DE miRNAs were correlated with subcortical gray matter volumes (Table S7) and found that higher levels of miR-32-5p were nominally associated with smaller hippocampal volumes (Figure 2D) after adjusting for ICV and medication status at the time of blood collection ($r = -0.404, p = .0299$). This association was not observed for any of the other top DE miRNAs, and there were no significant correlations between top DE miRNAs and amygdala, caudate, putamen, or nucleus accumbens morphometry. Associations between miRNA levels and neuroimaging measures for the control and MDD groups separately are provided in Table S8.

Gene Targets of Top DE miRNAs Are Involved in Neurodevelopmental Processes and Mental Illness

Utilizing mirDIP, a comprehensive tool that amalgamates confidence scores from various prediction algorithms and experimentally validated interactions, we identified 958 gene targets of the top DE miRNAs (Table S9). Common gene targets of at least 2 of the top DE miRNAs (referred to as multi-miRNA target genes) identified 127 genes (Figure 3A). GO terms cluster based on the similarity of the multi-miRNA target genes show large nodes including regulation of neuron differentiation, regulation of cell junction assembly and negative regulation of epithelial cells, regulation of long-chain fatty acid import across the plasma membrane, epithelial cell development, regulation of messenger RNA splicing, and regulation of microtubule polymerization. The localization of multi-miRNA target gene enrichment, as indicated by FUMA database analysis, includes heart, kidney, and pancreas, followed by prominent brain region–specific enrichment, indicating concordance with the brain localization analysis of DE miRNAs described in Figure 2.

GO analysis (as per enrichGO) of multi-miRNA target genes (full analysis output in Table S10) shows associations with several key biological processes (BPs). These BPs include cellular signaling, neurodevelopment and neuronal function, and behavioral and cognitive functions (Figure 3D). Furthermore, cellular component ontology localized multi-miRNA target gene activity to the synaptic region. These results suggest that by regulating these predicted common gene targets, the top DE miRNAs exert control over complex biological systems, ranging from neural development to cellular signaling pathways, potentially influencing diverse aspects of cognitive function across the lifespan.

For a network-centric approach to probe potential roles of the predicted common gene targets, we used the integrated curated knowledge database MetaCore. Top (FDR $<$ .05) network associations from the enrichment analysis consistently encompassed signal transduction, cardiovascular development and signaling, and neurophysiological processes (Figure 3E), consistent with the GO analysis. Importantly, the disease-based analysis identified several psychiatric disorders, most notably depression-related disorders (FDR $<$ .05) (Figure 3F; see Table S11 for all possible disease associations). The top DE expressed miRNAs in adolescent MDD appear to be coordinate biological networks, including those involved in neurodevelopment and mood disorders.

The Expression Pattern of DBS miRNAs Matches Their Distribution Profile in Serum and Plasma

A heatmap depicting the 20 miRNAs with the highest expression levels in the DBS samples, regardless of group, is shown in Figure 4A. To investigate the similarity between the expression of these DBS miRNAs and their levels in whole blood and its fractions (i.e., serum, white blood cells, and plasma), we extracted data available in DIANA-miTED. As shown in Figure 4B, the distribution of the top 20 DBS miRNAs resembles their profile in serum, followed by plasma (see also Figure S3A, B), validating the utility and highlighting the practical advantages of the DBS miRNA sequencing approach.

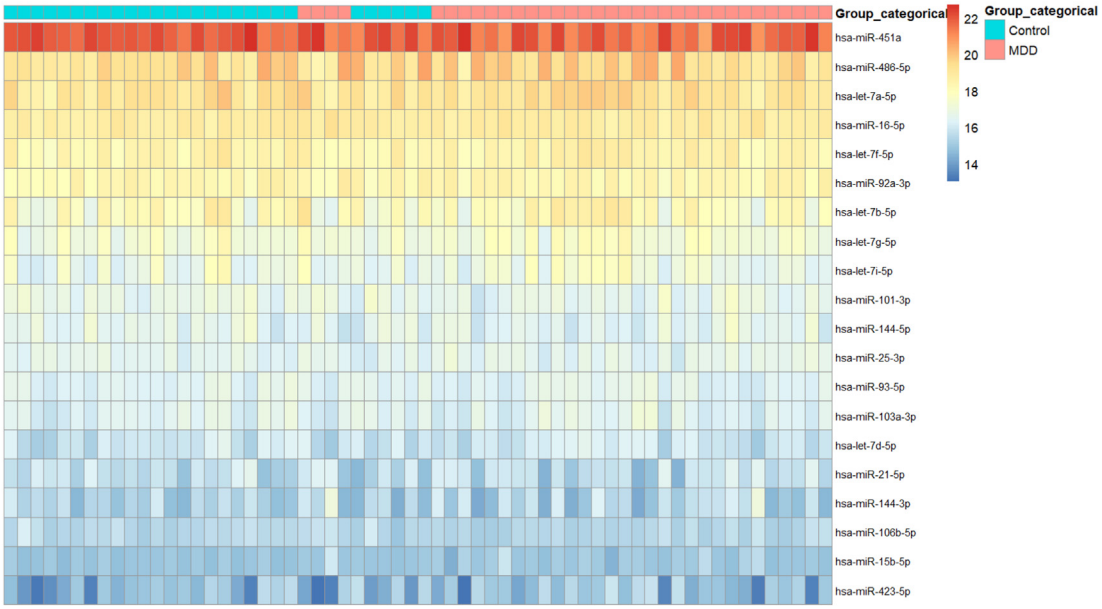
DISCUSSION

In this study, we showed that adolescents diagnosed with MDD have a distinct expression profile of peripheral miRNAs compared with healthy control participants. We found that 9 miRNA transcripts were DE ($p_{adjusted}$ $<$ .05)—miR-3613-5p, mir-30c-2, mir-107, miR-205-5p, miR-942-5p, miR-203a-3p, miR-3143, mir-16-2, and miR-32-5p—all showing upregulated levels in adolescents with depression, suggesting an

(C) Localizing the enrichment of the genes in the body yielded peripheral organ hits as well as some specific brain regions. **(D)** Performing GO analysis for multi-miRNA target genes, we identified many associations, here showing an excerpt of only the notable biological processes (the pathways/processes to which that gene product's activity contributes) and cellular components (where the gene products are active). Notable enriched **(E)** process networks and **(F)** disease associations for multi-miRNA target genes using MetaCore. [Figures were created in BioRender.com under McGill University Department of Psychiatry plan.] BA, Brodmann area; BP, biological process; CC, cellular component; DEG, differentially expressed gene; ERK, extracellular signal-regulated kinase; FDR, false discovery rate; GO, gene ontology; miRNA, microRNA; TGF, transforming growth factor.

A

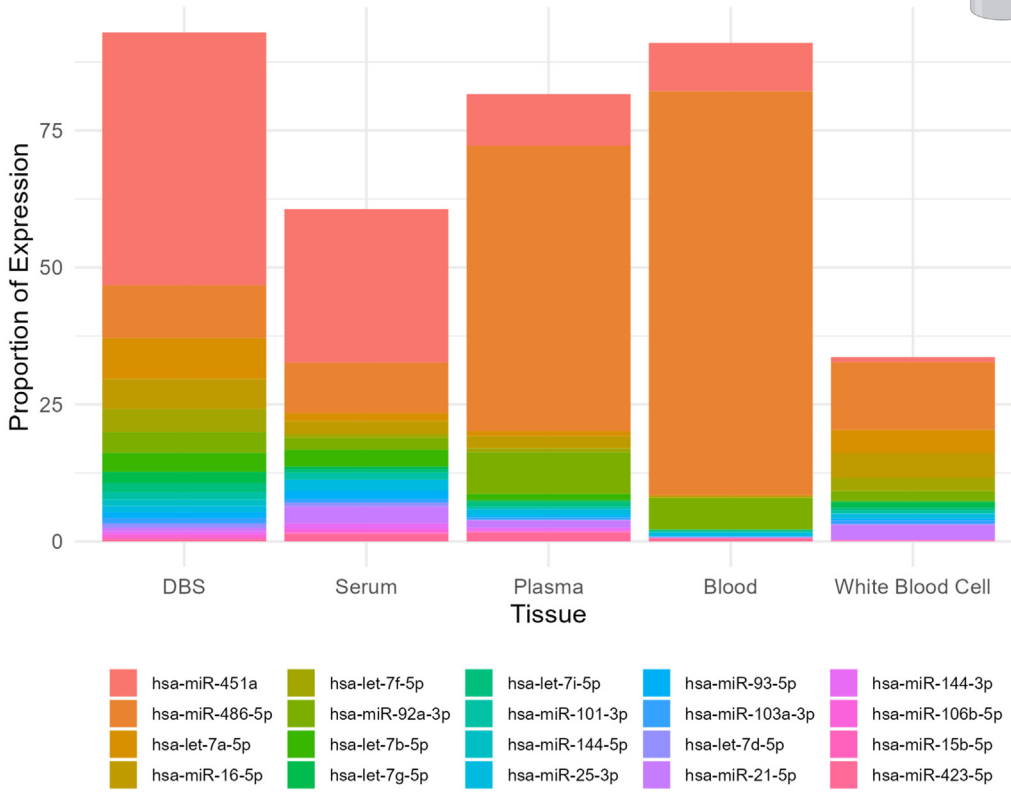
Heatmap of highly expressed miRNAs in TIGER DBS



B

Database probing of highly expressed miRNAs in the periphery

Stacked Column Plot of miRNA Expression Proportions



engagement of active modulatory processes. To learn more about these top DE miRNAs, we used exploratory statistical models and bioinformatics analyses. The identified DE miRNAs showed potential selective clinical prediction value: Levels of miR-3613-5p, mir-30c-2 and miR-942-5p were associated with depressive but not with anxiety symptoms in subsequent follow-ups conducted months later. All DE miRNAs showed expression in brain tissue datasets, particularly within the hippocampus and substantia nigra; we found that the 127 common gene targets of the DE miRNAs are linked to neurodevelopment, cognitive function, and mood disorders. Notably, miR-32-5p expression had a negative and specific association with hippocampal volume, indicating a potential link to neurodevelopment. The definitive impact of the results provided by these explorative assessments requires further validation and replication. Levels of miR-205-5p have previously been found to be increased in exosomes of an adult patient with MDD (37), and expression of mature miR-16, which targets the serotonin transporter gene and is involved in hippocampal neurogenesis, has been shown to be altered in cerebrospinal fluid and serum samples of adult patients with MDD and in response to antidepressant chronic treatment (38). In adolescents with MDD, 2 studies have profiled circulating miRNAs, but from extracellular vesicles and applying less stringent statistical threshold (39,40).

Our initial results suggest that levels of miR-3613-5p, mir-30c-2, and miR-942-5p are associated with the severity of future self-reported depression, but not of anxiety symptoms, suggesting their potential involvement in the pathophysiology of depression rather than general emotional distress. The predicted gene targets of these miRNAs include *EPHA7*, a gene reported to drive neuronal maturation and linked to neurodevelopmental deficits (41–43), and *ERBB4*, a gene that encodes a neuregulin receptor and directly linked with depression pathology in adults (44,45), and also targeted by the DE miRNA, miR-205-5p. Although future studies are needed to validate these miRNA candidates, our data are hypothesis generating and offer a framework for assessing the biomarkers across larger, independent datasets.

The hippocampus has consistently been found to be implicated in MDD (36,46–49). The specific and significant negative association between circulating miR-32-5p and hippocampal volume in our cohort raises the possibility that this miRNA is involved in neurodevelopmental alterations in adolescence. We found BCL11B to be a predicted target of miR-32-5p, which is a critical transcription factor in developmental processes, including long-distance axon guidance and neuron differentiation (50). In rodents, miR-32-5p has been shown to modulate depression-like behaviors via its effects on astrocytes and glutamatergic signaling in the hippocampus and the PFC (51).

Ontologies of the 127 common gene targets of the DE miRNAs spanned different levels of neurodevelopmental processes, including axon development, dendritic spine growth, and forebrain development, and provided support for the idea that peripheral miRNAs in adolescence can serve as both markers and mediators of psychiatric risk. The link between the DE miRNAs and the dysregulation of ongoing maturational processes in the adolescent brain can be investigated further as a polygenic network.

While DBS biosamples have only recently been used for psychiatric research on miRNAs [e.g., (52)], ample literature supports the use of serum or plasma for miRNA profiling. Our comparative analysis of miRNA expression levels in DBSs with existing biofluid datasets suggests similar proportional representation of miRNAs between DBSs and serum, with plasma also showing a notable overlap. DBSs could serve as a reliable alternative to serum and plasma in studying circulating miRNAs in healthy and disease conditions, offering simplified logistical requirements of sample collection and storage. Incorporation of other multi-omics data reliably derived from DBSs (53), including genotyping (54), proteomics (55), and methylome profiling (56), would lead to further improvements in biomarker discovery.

One of the strengths of our study lies in focusing on adolescents diagnosed with depression. The results of the exploratory analysis examining associations between the top DE miRNAs and 1) future behavioral outcomes and 2) neuroimaging measures are limited regarding their interpretation and generalization due to the small sample size. We analyzed the specific roles that these miRNAs may play in biological processes by leveraging the power of advanced bioinformatic tools on big datasets. The investigation of direct mechanistic studies and replication in larger independent cohorts will be performed in future studies. The current study sample consists of participants from educated, mid- to high- socioeconomic status families. Similar to the progress that has been made in large collaborative genome-wide association studies addressing sample size, population heterogeneity, and phenotypic complexity, additional peripheral microRNA studies on adolescents with depression will provide deeper insights into biomarker specificity and effect size.

Conclusions

miRNAs serve dual roles as epigenetic factors that bridge environmental influences and genetic predisposition and as potential messengers from the brain, reflecting regulatory changes in disease states in the peripheral blood. This study stands as one of only a few that have examined the role of miRNAs in adolescent depression, leveraging the advantages of DBS samples for a novel and accessible approach.

Figure 4. Comparison of the most expressed miRNAs in DBSs in comparison to miRNA expression in other peripheral fluids suggests resemblance to the serum profile. **(A)** The heatmap of the top 20 expressed miRNAs in the current DBS samples. **(B)** Based on the additional MicroRNA Tissue Expression Database data, we extracted expression profiles for miRNAs in other whole blood–related samples to compare their similarity with DBS proportions. [Figures were created in BioRender.com under McGill University Department of Psychiatry plan.] DBS, dried blood spot; miRNA, microRNA; TIGER, Teen Inflammation Glutamate Emotion Research.

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GEO accession for sequencing data and miRNA counts will be made accessible.

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