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Association of Salivary MicroRNA Changes With Prolonged Concussion Symptoms

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IMPORTANCE Approximately one-third of children who experience a concussion develop prolonged concussion symptoms. To our knowledge, there are currently no objective or easily administered tests for predicting prolonged concussion symptoms. Several studies have identified alterations in epigenetic molecules known as microRNAs (miRNAs) following traumatic brain injury. No studies have examined whether miRNA expression can detect prolonged concussion symptoms.

OBJECTIVE To evaluate the efficacy of salivary miRNAs for identifying children with concussion who are at risk for prolonged symptoms.

DESIGN, SETTING, AND PARTICIPANTS This prospective cohort study at the Penn State Medical Center observed 52 patients aged 7 to 21 years presenting for evaluation of concussion within 14 days of initial head injury, with follow-up at 4 and 8 weeks.

EXPOSURES All patients had a clinical diagnosis of concussion.

MAIN OUTCOMES AND MEASURES Salivary miRNA expression was measured at the time of initial clinical presentation in all patients. Patients with a Sport Concussion Assessment Tool (SCAT3) symptom score of 5 or greater on self-report or parent report 4 weeks after injury were designated as having prolonged symptoms.

RESULTS Of the 52 included participants, 22 (42%) were female, and the mean (SD) age was 14 (3) years. Participants were split into the prolonged symptom group (n = 30) and acute symptom group (n = 22). Concentrations of 15 salivary miRNAs spatially differentiated prolonged and acute symptom groups on partial least squares discriminant analysis and demonstrated functional relationships with neuronal regulatory pathways. Levels of 5 miRNAs (miR-320c-1, miR-133a-5p, miR-769-5p, let-7a-3p, and miR-1307-3p) accurately identified patients with prolonged symptoms on logistic regression (area under the curve, 0.856; 95% CI, 0.822-0.890). This accuracy exceeded accuracy of symptom burden on child (area under the curve, 0.649; 95% CI, 0.388-0.887) or parent (area under the curve, 0.562; 95% CI, 0.219-0.734) SCAT3 score. Levels of 3 miRNAs were associated with specific symptoms 4 weeks after injury; miR-320c-1 was associated with memory difficulty (*R*, 0.55; false detection rate, 0.02), miR-629 was associated with headaches (*R*, 0.47; false detection rate, 0.04), and let-7b-5p was associated with fatigue (*R*, 0.45; false detection rate, 0.04).

CONCLUSIONS AND RELEVANCE Salivary miRNA levels may identify the duration and character of concussion symptoms. This could reduce parental anxiety and improve care by providing a tool for concussion management. Further validation of this approach is needed.

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Editorial

Journal Club Slides and Supplemental content

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Nearly two-thirds of concussions take place in children and adolescents,[1] and more than 80% of concussions result from mild traumatic brain injuries (mTBIs).[2,3] For most children, concussion symptoms resolve within 2 weeks, but one-third may experience prolonged concussion symptoms (PCS).[4-10] To our knowledge, there are currently no objective tools that identify children at risk for PCS.[11,12] The absence of accurate, objective measures delays specialist referral and prevents individualized treatment plans.[13]

Studies have identified associations between PCS and some factors, such as sex, age, headache, and hospital admission.[4,11,14] Clinical risk scores using these features demonstrate modest ability to determine PCS risk.[15] However, the feasibility of administering multiple age-specific questionnaires within the time constraints of a clinical encounter poses challenges.[12] An alternative approach uses biomarkers.

MicroRNAs (miRNAs) are small, noncoding molecules that influence protein translation throughout the body.[15] They are transported through the extracellular space protected in exosomes and microvesicles, which allows them to be easily measured in biofluids, including serum, cerebrospinal fluid, and saliva.[16,17] Because of their abundance, stability in fluctuating pH levels, resistance to enzymatic degradation, and essential role in transcriptional regulation, miRNAs make ideal biomarkers.[18]

To our knowledge, 7 previous studies have examined miRNA expression in individuals with TBIs.[16,19-24] These investigations have demonstrated that concentrations of peripheral miRNAs are altered in adults with TBI[18] and vary with severity of head injury.[16,21] Changes in miRNA expression occur within the first 24 hours after injury[20] and reflect cerebrospinal fluid alterations in severe TBI.[16,24] Alterations in the circulating miRNA profile may also reflect specific TBI symptoms, such as amnesia.[23]

Although many studies have identified miRNA targets that are dysregulated in adult TBI, to our knowledge, none have examined their usefulness in identifying PCS or have focused solely on children. We recently investigated the biomarker potential of salivary miRNAs in 60 children with mTBI and identified 6 miRNAs dysregulated in the cerebrospinal fluid after severe TBI and the saliva after mTBI.[24] Here, we assess the clinical accuracy of salivary miRNA levels to detect children with PCS relative to symptom burden on the Sport Concussion Assessment Tool (SCAT3). This study tests the hypothesis that salivary miRNA levels are altered in children with PCS and have prognostic value that exceeds subjective survey tools.

Key Points

Question Can salivary microRNA levels be used to identify prolonged concussion symptoms in children?

Findings In this prospective cohort study of 52 children with mild traumatic brain injury, concentrations of 5 salivary microRNAs identified prolonged concussion symptoms with 85% accuracy and outperformed standard survey measures of symptom burden.

Meaning Salivary microRNA levels may represent an accurate, objective, and easily collected measure of prolonged concussion symptom risk.

Methods

Ethics Statement

This study was approved by the Penn State Hershey Medical Center Institutional Review Board. Written informed consent was obtained for all participants.

Study Population

The study included participants aged 7 to 21 years with a clinical diagnosis of mTBI. These ages were selected to represent typical concussion patients at outpatient pediatric clinics and to capture a period of critical neuroanatomical and psychosocial development that persists into early adulthood. The mTBI group was initially composed of 61 participants presenting to the Penn State Hershey Medical Center for evaluation of mTBI within 14 days of injury. This 14-day cutoff was chosen based on previous research indicating that symptoms and biomarkers return to baseline within 2 weeks of concussion.[25] Patients with a Glasgow Coma Scale score of 12 or less at injury, skull fracture, or intracranial bleeding were excluded from the study. Additional exclusion criteria included periodontal disease, respiratory infection, focal neurologic deficits, and history of migraine.

Data Collection

Medical and demographic characteristics of participants were recorded, including age, weight, height, sex, race/ethnicity, allergies, psychiatric history, sensorineural deficits, medication history, and dental fillings. Concussion history was also recorded, including time since injury, mechanism of injury, immediate symptoms (eg, amnesia, loss of consciousness, emesis, seizures, fractures, or weakness), time of nonsteroidal anti-inflammatory drug or acetaminophen use, and previous concussion history. The symptom evaluation portion of the SCAT3 was administered to children and parents at the time of enrollment.[26] This measure was repeated via telephone 4 weeks after injury. Thirty participants with a SCAT3 score of 5 or greater on child report and/or parent report at 4 weeks were classified as having PCS. When possible, the presence of PCS at a follow-up clinical visit was confirmed through medical record review. Participants with SCAT3 scores less than 5 four weeks after injury or with absence of symptoms at follow-up clinical encounter were classified as having acute concussion symptoms (ACS). Participants with PCS were contacted again 8 weeks after injury for a third SCAT3 evaluation. Six participants who failed to complete a follow-up SCAT3 at 4 weeks and lacked a follow-up clinical visit were excluded from the study.

RNA Collection, Processing, and Quantification

Nonfasting saliva was collected from each participant at enrollment following orally rinsing with tap water. Participants expectorated into Oragene-RNA RE-100 Expression Analysis Self-Collection Kit (DNA Genotek). RNA was extracted with Plasma/Serum Circulating and Exosomal RNA Purification Kits

(Norgen Biotek), as previously reported.[24] RNA yield and quality were assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies). Sequencing of salivary RNA occurred at the Penn State Genomics Core Facility using a NEXTflex Small RNA Sequencing Kit version 3 (Bioo Scientific), a HiSeq 2500 Instrument (Illumina), and a targeted depth of 3 million reads. Reads were aligned to the hg38 build of the human genome using Partek Flow (Partek) and the SHRiMP2 aligner. Total miRNA counts within each sample were quantified with miRBase microRNA version 21. Three saliva samples with less than 2.5×10^4 total miRNA counts were excluded from the final analysis, resulting in 52 participants with mTBI. Individual miRNAs with raw read counts greater than 10 in at least 22 of 52 samples (42%) were evaluated for differential expression. This criterion was based on the ratio of participants with ACS vs PCS and the possibility that an miRNA might be present in only 1 group. Raw read counts were quantile-normalized, mean-centered, and divided by the standard deviation of each variable. The data set for this study will be made available in the NCBI GenBank.

Statistical Analysis

Salivary miRNAs with differential expression between PCS and ACS groups were identified with a Mann-Whitney test and false detection rate (FDR) correction. A 2-dimensional partial least squares discriminant analysis (PLSDA) was used to investigate the prognostic potential of salivary miRNA profiles for PCS. The variable importance in projection, a weighted sum of squares of PLSDA variables accounting for dimensional variance, was determined for each miRNA in Metaboanalyst software.[27] The 15 miRNAs with the largest variable importance in projection scores were reported. A multivariate logistic regression analysis was used to evaluate the PCS classification accuracy of those 15 miRNAs. Concentrations of miRNAs were used in the regression as ratios, providing a second level of control for variation in total miRNA across samples. Accuracy was determined by measuring area under the curve (AUC) on a receiver operating characteristics plot and validated with 10-fold Monte Carlo cross-validation. Area under the curve for the top performing miRNA group was compared against the AUC for 3 clinical measures: (1) total symptom score on the child-response portion of the SCAT3; (2) total symptom score on the parent-response portion of the SCAT3; and (3) modified PCS risk score using sex, age, previous concussion history, headache, fatigue, processing difficulty, and migraine history, as previously described by Zemek et al.[28] This last tool was limited in part by an absence of a balance error score and noise sensitivity report.

Associations between the 15 salivary miRNAs (measured at time of injury) and PCS characteristics (measured 4 weeks after injury) were evaluated with Pearson correlations. Pearson correlations were also used to examine associations between salivary miRNAs and medical/demographic variables. A 2-tailed *t* test compared medical and demographic variables across PCS and ACS groups, and significance was set at $P < .05$. The top 15 miRNAs were inspected for functional relevance to brain injury and repair using DIANA mirPath version 3 (http://snf-515788.vm.okeanos.grnet.gr/). Human-specific, high-confidence gene targets for each miRNA were identified with DIANA's microT-CDS algorithm (score ≥ 0.90).[29] Gene ontology and KEGG pathway categories overrepresented by the miRNA gene targets (FDR < 0.05; Fisher exact test) were reported.

Results

Participants

Fifty-two participants (mean [SD] age, 14 [3] years; 22 [42%] female) were included in the analysis. There were no differences between participants in the ACS group (n = 22) and PCS group (n = 30) in demographic, medical, or concussion characteristics (**Table 1**). Forty-eight participants (92%) were white, and 13 (25%) had used a nonsteroidal anti-inflammatory drug within 6 hours of saliva collection. Eight participants (15%) were taking a stimulant or selective serotonin reuptake inhibitor at the time of enrollment. Most participants were enrolled within 1 week of their concussion, and the most common mechanisms of injury were sport participation (42%) or motor vehicle collision (15%). Nearly half had experienced a previous concussion (24 participants [46%]). The most commonly reported symptoms at the time of injury were amnesia (25 participants [48%]) and loss of consciousness (14 participants [27%]).

Symptom Reporting

Symptom burden was measured with the SCAT3 within 2 weeks of injury and again 4 weeks after injury (**Table 2**). At the initial assessment, children who went on to develop PCS reported a higher symptom severity score but no difference in the total number of symptoms. Parents of children who developed PCS reported no initial difference in their child's symptom severity or total symptom number. Five of the 20 queried symptoms were more severe in participants with PCS on initial child survey. On the initial parental survey, 2 of 20 symptoms were more severe in the PCS group.

Four weeks after injury, the PCS group had a mean severity score of 18, endorsing 11 of 20 concussive symptoms. "I get tired a lot" and "I get tired easily" were the most commonly reported PCS symptoms. Fifteen participants (29%) continued to have concussive symptoms 8 weeks after injury. The most commonly reported symptom at that time was "I have problems remembering what people tell me" (48 participants [92%]).

MiRNA Expression

Among the 52 saliva samples analyzed, the mean read count was 2.1×10^5 reads per sample, and 437 miRNAs were detected in at least 22 of 30 samples. Among these 437 miRNAs, 14 demonstrated nominal differences in participants with PCS (eTable 1 in the Supplement). None survived FDR correction. Of these 14 miRNAs, 3 were downregulated in participants with ACS, and 11 were upregulated. A PLSDA using miRNA expression levels for all 437 miRNAs achieved partial separation of ACS and PCS groups while accounting for 21.5% of the variance in the data set (**Figure 1**A). The 15 miRNAs most critical for ACS/PCS separation were identified by variable importance in projection score (Figure 1B). Two of these miRNAs (miR-30e and miR-320c) have previously been identified as sig-

Table 1. Participant Characteristics

Clinical Characteristic	No. (%) Total (n = 52)	ACS (n = 22)	PCS (n = 30)	*P* Value[a]
Demographic				
Female	22 (42)	7 (32)	15 (50)	.19
Age, mean (SD), y	14 (3)	14 (3)	14 (3)	.48
White	48 (92)	20 (91)	28 (93)	.76
Height, percentile (SD)	61 (28)	55 (26)	65 (29)	.24
Weight, percentile (SD)	68 (28)	67 (26)	69 (29)	.82
Medical characteristics				
NSAID use	13 (25)	3 (14)	10 (33)	.09
Acetaminophen use	6 (12)	2 (9)	4 (13)	.64
Ondansetron use	0	0	0	>.99
Stimulant or SSRI use	8 (15)	4 (18)	4 (13)	.64
Concussion characteristics				
Days since injury, mean (SD)	6.8 (3.8)	6.4 (3.7)	7.1 (3.9)	.52
Sport participation	22 (42)	11 (50)	11 (37)	.35
MVC	8 (15)	3 (14)	5 (18)	.77
LOC	14 (27)	8 (36)	5 (18)	.21
Amnesia	25 (48)	9 (41)	16 (53)	.39
Bony injury	5 (10)	1 (5)	4 (13)	.26
Emesis	12 (23)	6 (27)	6 (20)	.56
Previous concussion	24 (46)	12 (55)	12 (40)	.31
No. of previous concussions, mean (SD)	1.5 (2.0)	1.4 (1.0)	1.6 (2.5)	.78

Abbreviations: ACS, acute concussion symptoms; LOC, loss of consciousness; MVC, motor vehicle collision; NSAID, nonsteroidal anti-inflammatory drug; PCS, prolonged concussion symptoms; SSRI, selective serotonin reuptake inhibitor.

[a] *P* values based on 2-tailed *t* test.

nificantly changed in the saliva and cerebrospinal fluid following pediatric TBI. Five of 15 miRNAs have been identified in prior TBI investigations.[24,30-32]

MiRNA Function

The top 15 miRNAs on PLSDA were interrogated for functional targets. They targeted 2429 genes (micro-c-tds > 0.90) implicated in 62 gene ontology and 22 KEGG pathways (eTable 2 in the Supplement). Among the targeted pathways were a number of signaling cascades related to synaptic development, neuronal migration, and repair (eTable 3 in the Supplement). Targeted gene ontology pathways included neurotrophin TRK signaling (34 genes), axon guidance (61 genes), and nervous system development (56 genes). KEGG pathways of interest included glioma (14 genes), Forkhead box O signaling (29 genes), and Wnt signaling (22 genes). Hierarchical clustering of the 15 miRNAs demonstrated 3 distinct miRNA clusters based on target function: miR-629-3p/miR-133a-5p; let-7a-5p/let-7b-5p; and miR-320c/miR-200b-3p (eFigure 1 in the Supplement).

Symptom and miRNA Correlations

Associations between concentrations of the 15 salivary miRNAs (at the time of injury), symptom characteristics (4 weeks after injury), and medical/demographic characteristics were evaluated. Nominal correlations ($P < .05$) were identified between 12 miRNA symptom pairs (**Figure 2**). Three of these correlations survived multiple testing corrections; miR-320c-1 was positively associated with "I have problems remembering what people tell me" (*R*, 0.55; FDR, 0.02), miR-629 was positively associated with "I have headaches" (*R*, 0.47; FDR, 0.04), and let-7b-5p was positively associated with "I get tired a lot" (*R*, 0.45; FDR, 0.04). There were also associations between individual miRNAs. Let-7b and let-7a (which showed hierarchical clustering based on target gene function) demonstrated a direct association in expression among participants with PCS. There were no associations between salivary miRNA concentrations and participant age, sex, race/ethnicity, medication use, days since injury, or sports participation (eFigure 2 in the Supplement). A history of previous concussion was nominally associated with salivary levels of miR-320c (*R*, 0.27; $P = .048$; FDR, 0.42) and participant age (*r*, 0.35; $P = .01$; FDR, 0.13).

Prognostic Potential

A multivariate logistic regression analysis was used to evaluate PCS classification accuracy of the 15 miRNAs. A model using 5 miRNAs (miR-320c-1, miR-133a-5p, miR-769-5p, let-7a-3p, and miR-1307-3p) demonstrated the highest classification accuracy (AUC, 0.856; 95% CI, 0.822-0.890) with a sensitivity of 80% and a specificity of 75% for PCS status (**Figure 3**A). To prevent overmodeling the data, 2 validation techniques were tested. A 10-fold cross-validation technique demonstrated an AUC of 0.812 (95% CI, 0.691-0.893). In addition, the first 20% of samples in each group were held out, producing an initial AUC of 0.792 (95% CI, 0.673-0.958) with an AUC of 0.933 (95% CI, 0.787-0.960) in the holdout set (Figure 3B and C). Logistic regression models using the child SCAT3 severity score or the parent SCAT3 severity score demonstrated AUCs of 0.649 (95% CI, 0.388-0.887) and 0.562 (95% CI, 0.219-0.734), respectively (Figure 3D and E). A modified version of the PCS predictive tool[15] was calculated for each participant, with 7 of 9 available risk factors. This risk score demonstrated an AUC of 0.625 (95% CI, 0.093-0.848) for PCS status (Figure 3F), performing similar to the original study.

Discussion

This investigation identified 15 salivary miRNAs associated with PCS and functionally associated with neuronal regulation. Five of these miRNAs accurately identified PCS status, and 3 were associated with specific concussion symptoms.

Concentrations of 5 miRNAs determined PCS status in 42 of 50 participants, an accuracy of more than 85%. The misclassified participants had nominally higher rates of sports participation and had saliva collected slightly sooner following injury. However, neither of these factors displayed statistical significance. Further studies will need to evaluate the influence of exercise on circulating miRNA levels and investigate longitudinal expression of salivary miRNA following mTBI. Of note, there were no differences in age, sex, race/ethnicity, nonsteroidal anti-inflammatory drug use, or

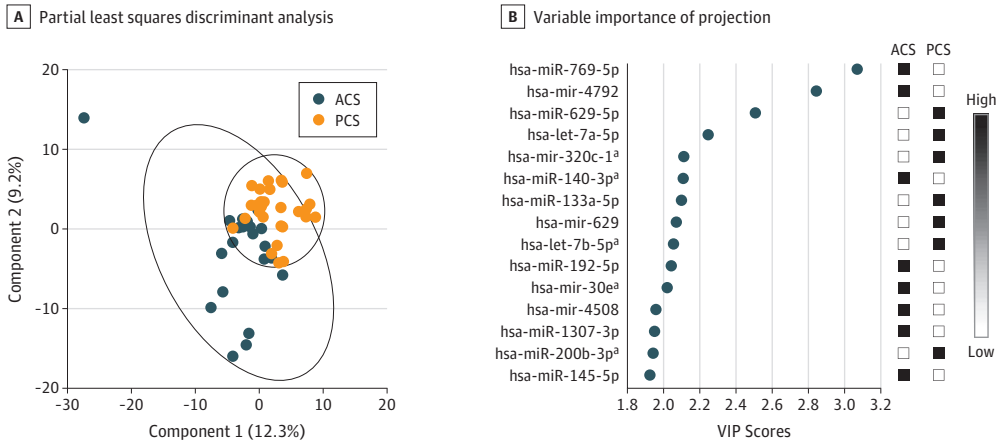
Table 2. Concussion Symptom Characteristics[a]

Concussion Symptom Characteristic	Mean Total Population	Mean ACS	Mean PCS	*P* Value
At enrollment (0-14 d after injury)				
Child symptom severity score	23	19	26	.04
Child total symptoms reported	12	11	13	.11
"I have a hard time concentrating" response	1.6	1.2	1.9	.03
"I have problems remembering what people tell me" response	1.3	0.9	1.6	.03
"I daydream too much" response	1.2	0.8	1.4	.047
"I have headaches" response	2.2	1.7	2.5	.005
"I get tired a lot" response	1.7	1.1	2.1	.001
Parental symptom severity score	22	20	23	.30
Parent total symptoms reported	12	11	13	.22
"The child has difficulty concentrating" response	1.5	1.1	1.8	.02
"The child feels dizzy" response	1.3	1.0	1.6	.045
4-wk Follow-up (28-34 d after injury)				
Child symptom severity score	11	0.8	18	<.001
Child total symptoms reported	6.9	0.8	11	<.001
"I get tired a lot" response (% positive)	0.9	0	1.6 (90)	<.001
"I get tired easily" response (% positive)	1.0	0.2 (18)	1.6 (85)	<.001
Parental symptom severity score	8.8	0.5	13	.005
Parent total symptoms reported	4.6	0.3	7.1	<.001
8-wk Follow-up (56-62 d after injury)				
Child symptom severity score	NA	NA	11	NA
Child total symptoms reported	NA	NA	10	NA
Parental symptom severity score	NA	NA	16	NA
"I have problems remembering what people tell me" response (% positive)	NA	NA	1.3 (92)	NA
Parent total symptoms reported	NA	NA	8.4	NA

Abbreviations: ACS, acute concussion symptoms; NA, not applicable; PCS, prolonged concussion symptoms.

[a] Average symptom scores on the symptom scale of the child Sports Concussion Assessment Tool are shown. Parent and child reports of symptoms were collected at enrollment (0 to 14 days after injury), 4 weeks after injury, and 8 weeks after injury (PCS group only). At each assessment, 20 concussive symptoms were rated on a Likert scale (0 = never, 1 = rarely, 2 = sometimes, and 3 = often experiencing symptoms) by both children and parents, yielding a maximum possible severity score of 80 and a maximum total of 20 symptoms reported. Of the 20 symptoms assessed at each encounter, only those with significant differences between ACS and PCS groups (at enrollment) or those most commonly reported (at 4 and 8 weeks) are shown.

Figure 1. Discriminant Analysis of Prolonged Concussion Symptom (PCS) and Acute Concussion Symptom (ACS) Groups and 15 MicroRNAs (miRNAs) With Highest Variable Importance

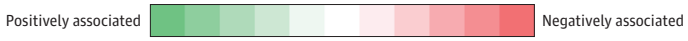


A, Partial least squares discriminant analysis of total miRNA expression in participants with ACS and PCS. The 2-dimensional partial least squares discriminant analysis accounts for 21.5% of the total variance in the miRNA data. The small and large ellipses indicate 95% CIs for the dispersion of participants with PCS and ACS, respectively. B, Variable importance of projection score identifying the 15 miRNAs most important for separating participants with ACS from those with PCS. Seven miRNAs were found to be increased in the PCS group (black boxes), and 8 miRNAs were found to be decreased in the PCS group (white boxes).

[a] Identified in previous studies of traumatic brain injury.

Figure 2. Pearson Correlation Analysis Between Salivary MicroRNA Concentrations and Concussive Symptoms

	miR-769-5p	mir-4792	miR-629-5p	let-7a-5p	mir-320c-1	miR-140-3p	miR-133a-5p	let-7b-5p	miR-192-5p	mir-30e	mir-4508	miR-1307-3p	miR-200b-3p	miR-145-5p	mir-629	PCS Risk Score	Parent SCAT3 Score	Child SCAT3 Score	Attention at w 4	Memory at w 4	Confusion at w 4	Learning at w 4	Headache at w 4	Dizziness at w 4	Fatigue at w 4	Nausea at w 4
miR-769-5p	1.00	-0.07	-0.13	**-0.41**	-0.35	**0.49**	-0.01	**-0.37**	0.28	0.49	-0.27	0.04	**-0.47**	**0.71**	-0.17	-0.16	0.07	-0.13	-0.29	-0.30	-0.09	-0.01	-0.30	-0.27	**-0.39**	-0.09
mir-4792	-0.07	1.00	**-0.41**	-0.22	-0.12	**0.43**	0.17	-0.28	0.09	0.10	**0.46**	0.34	-0.11	0.06	-0.23	-0.08	-0.28	-0.20	-0.17	-0.30	-0.28	-0.23	-0.20	-0.15	-0.25	-0.35
miR-629-5p	-0.13	**-0.41**	1.00	**0.46**	0.09	-0.32	0.01	**0.50**	-0.09	**-0.38**	-0.04	-0.13	0.27	-0.15	**0.91**	0.07	0.20	0.32	0.29	0.21	-0.02	0.18	**0.42**	-0.01	**0.38**	0.28
let-7a-5p	**-0.41**	-0.22	0.46	1.00	0.27	**-0.48**	-0.18	0.85	-0.15	**-0.63**	0.23	0.02	**0.54**	-0.30	**0.52**	-0.02	0.04	0.33	0.24	**0.40**	-0.05	-0.05	0.32	0.16	0.33	-0.10
mir-320c-1	-0.35	-0.12	0.09	0.27	1.00	-0.21	-0.13	0.01	-0.06	-0.24	0.01	-0.17	0.02	-0.16	0.09	-0.30	-0.07	-0.11	0.35	**0.55**	0.08	-0.09	0.03	-0.01	0.32	-0.09
miR-140-3p	**0.49**	**0.43**	-0.32	**-0.48**	-0.21	1.00	-0.04	-0.35	0.02	**0.60**	-0.04	-0.01	**-0.64**	**0.62**	-0.19	0.03	-0.03	-0.22	-0.29	-0.35	-0.18	-0.15	-0.29	-0.20	-0.33	-0.07
miR-133a-5p	-0.01	0.17	0.01	-0.18	-0.13	-0.04	1.00	-0.11	-0.12	0.17	-0.16	0.12	-0.07	0.07	-0.05	0.23	-0.02	0.14	0.10	0.08	0.14	-0.06	0.08	0.01	0.14	0.06
let-7b-5p	**-0.37**	-0.28	**0.50**	**0.85**	0.01	-0.35	-0.11	1.00	-0.25	**-0.55**	0.20	-0.03	**0.51**	-0.34	**0.54**	0.10	-0.03	0.35	0.18	0.30	0.09	0.07	0.27	0.23	**0.45**	0.06
miR-192-5p	0.28	0.09	-0.09	-0.15	-0.06	0.02	-0.12	-0.25	1.00	0.17	0.17	-0.02	-0.27	0.13	-0.15	0.08	0.24	0.24	-0.13	-0.13	-0.09	-0.07	-0.13	-0.16	-0.34	-0.02
mir-30e	**0.49**	0.10	**-0.38**	**-0.63**	-0.24	**0.60**	0.17	**-0.55**	0.17	1.00	**-0.39**	-0.09	**-0.65**	0.48	-0.37	0.03	0.22	-0.03	-0.25	-0.34	-0.06	-0.03	-0.31	-0.21	**-0.37**	0.01
mir-4508	-0.27	**0.46**	-0.04	0.23	0.01	-0.04	-0.16	0.20	0.17	-0.39	1.00	0.63	0.18	-0.18	0.09	0.07	-0.08	0.15	-0.22	-0.24	-0.27	-0.12	-0.15	0.00	-0.21	-0.25
miR-1307-3p	0.04	0.34	-0.13	0.02	-0.17	-0.01	0.12	-0.03	-0.02	-0.09	**0.63**	1.00	0.07	0.16	-0.09	-0.03	-0.28	0.01	-0.29	-0.33	-0.18	-0.06	-0.20	0.07	**-0.37**	-0.16
miR-200b-3p	**-0.47**	-0.11	0.27	**0.54**	0.02	**-0.64**	-0.07	**0.51**	-0.27	**-0.65**	0.18	0.07	1.00	**-0.52**	0.35	-0.18	-0.02	0.05	0.24	0.24	0.15	-0.09	**0.39**	0.30	**0.39**	-0.01
miR-145-5p	0.71	0.06	-0.15	-0.30	-0.16	**0.62**	0.07	-0.34	0.13	**0.48**	-0.18	0.16	**-0.52**	1.00	-0.18	0.01	0.02	-0.09	-0.26	-0.23	-0.08	-0.22	-0.23	-0.21	-0.32	0.03
mir-629	-0.17	-0.23	**0.91**	**0.52**	0.09	-0.19	-0.05	**0.54**	-0.15	**-0.37**	0.09	-0.09	0.35	-0.18	1.00	0.08	0.19	0.32	0.35	0.27	0.01	008	**0.47**	0.14	**0.39**	0.18
PCS Risk Score	-0.16	-0.08	0.07	-0.02	-0.30	0.03	0.23	0.10	0.08	0.03	0.07	-0.03	-0.18	0.01	0.08	1.00	**0.38**	**0.53**	0.24	0.21	0.21	0.29	0.20	0.30	0.15	0.21
Parent SCAT3 Score	0.07	-0.28	0.20	0.04	-0.07	-0.03	-0.02	0.03	0.24	0.22	-0.08	-0.28	-0.02	0.02	0.19	**0.38**	1.00	**0.69**	0.05	0.00	-0.25	-0.01	0.11	-0.08	-0.08	-0.05
Child SCAT3 Score	-0.13	-0.20	0.32	0.33	-0.11	-0.22	0.14	0.35	0.24	-0.03	0.15	0.01	0.05	-0.09	0.32	**0.53**	**0.69**	1.00	0.12	0.15	-0.08	0.16	0.13	0.13	0.13	0.01
Attention at w 4	-0.29	-0.17	0.29	0.24	0.35	-0.29	0.10	0.18	-0.13	-0.25	-0.22	-0.29	0.24	-0.26	0.35	0.24	0.05	0.12	1.00	**0.83**	**0.63**	**0.45**	**0.69**	**0.65**	**0.80**	0.07
Memory at w 4	-0.30	-0.30	0.21	**0.40**	**0.55**	-0.35	0.08	0.30	-0.13	-0.34	-0.24	-0.33	0.24	-0.23	0.27	0.21	0.00	0.15	**0.83**	1.00	**0.64**	0.27	**0.41**	**0.54**	**0.73**	0.10
Confusion at w 4	-0.09	-0.28	-0.02	-0.05	0.08	-0.18	0.14	0.09	-0.09	-0.06	-0.27	-0.18	0.15	-0.08	0.01	0.21	-0.25	-0.08	**0.63**	**0.64**	1.00	**0.42**	**0.44**	**0.70**	**0.69**	**0.54**
Learning at w 4	-0.01	-0.23	0.18	-0.05	-0.09	-0.15	-0.06	0.07	-0.07	-0.03	-0.12	-0.06	-0.09	-0.22	0.08	0.29	-0.01	0.16	**0.45**	0.27	**0.42**	1.00	0.27	**0.39**	**0.43**	0.21
Headache at w 4	-0.30	-0.20	**0.42**	0.32	0.03	-0.29	0.08	0.27	-0.13	-0.31	-0.15	-0.20	**0.39**	-0.23	**0.47**	0.20	0.11	0.13	**0.69**	**0.41**	**0.44**	0.27	1.00	**0.49**	**0.60**	**0.38**
Dizziness at w 4	-0.27	-0.15	-0.01	0.16	-0.01	-0.20	0.01	0.23	-0.16	-0.21	0.00	0.07	0.30	-0.21	0.14	0.30	-0.08	0.13	**0.65**	**0.54**	**0.70**	**0.39**	**0.49**	1.00	**0.56**	0.23
Fatigue at w 4	**-0.39**	-0.25	**0.38**	0.33	0.32	-0.33	0.14	**0.45**	-0.34	**-0.37**	-0.21	**-0.37**	**0.39**	-0.32	**0.39**	0.15	-0.08	0.13	**0.80**	**0.73**	**0.69**	**0.43**	**0.60**	0.56	1.00	0.30
Nausea at w 4	-0.09	-0.35	-0.28	-0.10	-0.09	-0.07	0.06	0.06	-0.02	0.01	-0.25	-0.16	-0.01	0.03	0.18	0.21	-0.05	0.01	0.07	0.10	**0.54**	0.21	**0.38**	0.23	0.30	1.00



Pearson correlation with *R* values depicting associations between salivary concentrations of the 15 microRNAs of interest (at initial assessment) and concussive symptom severity (at 4 weeks after injury) measured via Sport Concussion Assessment Tool (SCAT3) symptom report. Bolded values indicate nominal significance ($P < .05$), and highlighted boxes indicate significance ($P < .05$) following multiple testing corrections. Color-scale values indicate Pearson correlation between 2 features, where red indicates an inverse association and green indicates a direct association. PCS indicates prolonged concussion symptom.

rates of previous concussion between misclassified and correctly classified participants.

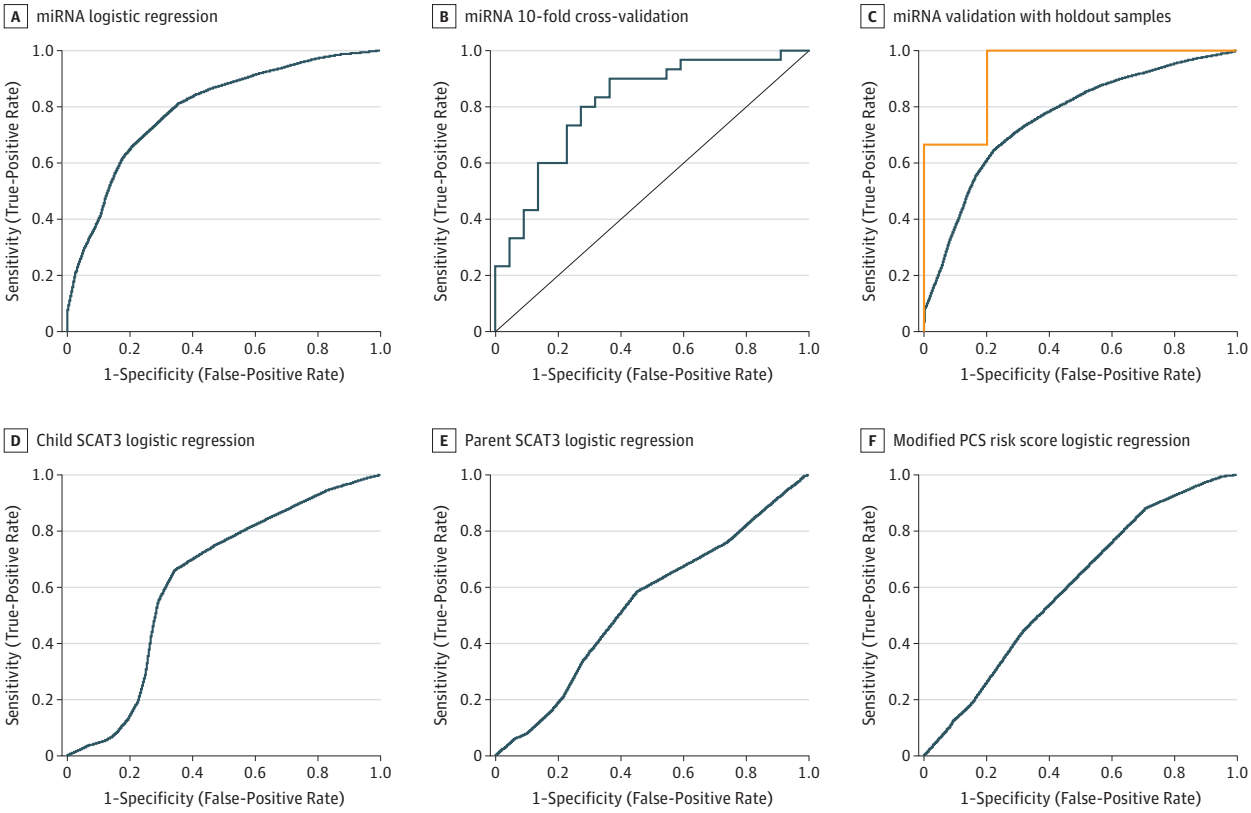
Clinical Implications

Currently, to our knowledge, no objective, easily administered tool exists for identifying PCS.[33] Application of clinical factors in PCS prediction models has limited efficacy.[4,11] In this cohort of 52 children, the classification accuracy of salivary miRNA exceeds that of symptom burden and the modified PCS risk score.

The miRNAs associated with PCS have potential utility as a toolset for facilitating concussion management. This tool could ease parental anxiety about expected symptom duration.[34] An objective PCS tool could also inform clinical recommendations about return-to-play and school-based accommodations. For example, we have previously reported that miR-320c levels are reduced in both saliva and cerebrospinal fluid of children with TBI (relative to healthy controls).[24] Here, we show that reductions in miR-320c (which targets genes involved in synaptic plasticity) are pronounced in children with ACS, suggesting this change may serve a neuroprotective role. Indeed, miR-320c levels in participants with PCS are directly associated with difficulties in attention 1 month after injury. This group might benefit from cognitive rest, supplemental test time, or school-based tutoring.

Pediatricians typically avoid initiating medications in mTBI, given that most concussion symptoms self-resolve over time.[35,36] Therapies such as trazodone or amitriptyline have demonstrated mixed efficacy in pediatric concussion and are generally reserved for refractory symptoms.[36] Biomarkers that identify PCS risk could expedite referral to concussion specialists and individualized therapies. For example, steroids,

Figure 3. Multivariate Logistic Regression Analyses for Salivary MicroRNAs (miRNAs) and Subjective Symptom Reports



The 5 miRNAs of interest accurately identified prolonged concussion symptoms in a multivariate regression analysis. A, A receiver operator characteristic curve using salivary concentrations of 5 miRNAs (miR-320c-1, miR-133a-5p, miR-769-5p, let-7a-3p, and miR-1307-3p) demonstrated an area under the curve (AUC) of 0.856 (95% CI, 0.822-0.890) for prolonged concussion symptom (PCS) status. B, 10-Fold cross-validation of this tool demonstrated an AUC of 0.812 (95% CI, 0.691-0.893) for identifying PCS status. C, 10-Fold cross-validation of this tool holding out 20% of participants with acute and prolonged concussion symptoms at random demonstrated an AUC of 0.792 (95% CI, 0.673-0.958) in the cross-validation set and an AUC of 0.933 (95% CI, 0.787-0.960) in the holdout set. D, Logistic regression model using the total child Sport Concussion Assessment Tool (SCAT3) severity score demonstrated an AUC of 0.649 (95% CI, 0.388-0.887) for determining PCS status. E, Logistic regression model using total parent SCAT3 severity score demonstrated an AUC of 0.562 (95% CI, 0.219-0.734) for identifying PCS status. F, Modified clinical risk score including sex, age, previous concussion history, headache, fatigue, processing difficulty, and migraine history demonstrated an AUC of 0.625 (95% CI, 0.093-0.848) for determining PCS status.

amitriptyline, and triptans have been investigated in concussion management.[36] Targeting these therapies toward patients with alterations in miR-629c (associated with headache severity 1 month after injury) could help determine their efficacy and provide clinical benefit.

Physiologic Implications

Of the 15 miRNAs associated with PCS, 9 target neurotrophin TRK signaling. This pathway includes 119 genes, and 34 (29%) are targeted by the 9 miRNAs. Understanding the role these genes play in brain repair could help explain how miRNA levels influence PCS risk. For example, the sortilin-1 gene (*SORT1*) controls neuronal function and viability by regulating protein transport critical for signal transduction. *SORT1* mutations lead to dysregulated neurotransmission.[37] SORT1 is targeted by miR-30e, which is downregulated in children with PCS. Thus, PCS-induced changes in miR-30e may lead to increased SORT1 and promote neuronal apoptosis. Another neurotrophin TRK member, calcium/calmodulin-dependent protein kinase type IV (CAMK4), is also a target of miR-30e. CAMK4 is associated with memory, and its disruption impairs long-term memory through deficits in consolidation and retention.[38]

Limitations

The major limitation of the current study is its reliance on a derivation cohort to assess classification accuracy. Validation of these miRNAs in an independent, larger cohort will be necessary to discern predictive power of the proposed toolset. To our knowledge, the current investigation constitutes the largest study of miRNA in pediatric concussion and the only study of miRNA expression in PCS. The sample size for this investigation was informed by a priori power analysis providing greater than 80% power to detect changes in 50 miRNAs. The tight age range used in this investigation (mean [SD] age, 14 [3] years) also helps mitigate age-induced differences in adult miRNA studies. None of the 5 miRNAs in our classification model were correlated with age or change over time in the developing brain.[39]

This study uses a validated tool to measure subjective concussion symptoms but does not provide functional measurements (eg, balance or processing speed). Cognition was assessed in 16 participants using the standardized assessment of concussion on the child SCAT3 and showed no difference between the PCS and ACS groups on orientation, immediate memory, or concentration (eTable 4 in the Supplement). Concussion is a clinical diagnosis relying on subjective reports. Functional assessments are not essential to establish the diagnosis. However, future studies should assess miRNA signatures alongside functional measures and neuroimaging.

We acknowledge that SCAT3 is most accurate acutely (ie, administered 1 to 3 days after injury) and is best applied in the context of a baseline assessment. Participants in this study were not evaluated at baseline (average evaluation, 6 days after injury). However, the portion of the SCAT3 used in this study is a valid assessment tool in PCS, and symptom burden has been associated with poor outcomes in concussion.[40]

Prolonged concussion symptoms are generally present in 15% to 30% of patients with concussion. However this data set was collected to overrepresent PCS and allow for balanced PCS/ACS comparison. Thus, the participants in this study may not accurately reflect a typical cross-section of pediatric concussion.

Conclusions

Salivary miRNA measurement may provide an accurate, noninvasive technique for identifying children with PCS. Such information could reduce parental anxiety and improve care for patients by providing a simple tool for concussion management.

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Study concept and design: Johnson, Loeffert, Stokes, Hicks.
Acquisition, analysis, or interpretation of data: Johnson, Olympia, Bramley, Hicks.
Drafting of the manuscript: Johnson, Stokes, Hicks.
Critical revision of the manuscript for important intellectual content: Johnson, Loeffert, Olympia, Bramley, Hicks.
Statistical analysis: Hicks.
Obtained funding: Hicks.
Administrative, technical, or material support: Stokes, Hicks.
Study supervision: Johnson, Bramley, Hicks.

Conflict of Interest Disclosures: Dr Hicks is a coinventor of preliminary patents for microRNA biomarkers in disorders of the central nervous system that is assigned to the State University of New York Upstate Medical University and Penn State Research Foundations and licensed to Quadrant Biosciences. Dr Hicks is also a consultant for Quadrant Biosciences and a shareholder in the company. These financial interests are currently managed by the Penn State College of Medicine. No other disclosures were reported.

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