

Salivary Micro RNA as a Biomarker for Concussion Symptoms in Pediatric Patients

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Abstract

This research project employs an untargeted approach using RNA sequencing to identify miRNA in saliva following a traumatic brain injury, specifically a concussion, to better understand how acute brain injuries can be diagnosed and treated. There is currently no biological test in place for diagnosing concussions, which are one of the most frequently observed sport related injuries. This study is invested in determining novel molecular biomarkers in saliva that are associated with concussion so that they can then be correlated with symptoms of concussion. The study predicted that salivary miRNA levels are altered in children with concussion, and there will be a significant difference in levels of miRNA at baseline (pre- injury) and post-injury in athletes. By determining novel biomarkers, noninvasive saliva testing may help to better identify concussions and prevent premature return to play as well as a better understanding of injury mechanisms.

Introduction

According to the CDC, pediatric traumatic brain injury (TBI) is a major cause of death and disability among children that can stunt learning and cause cognitive deficits during those critical developmental years (1). The disorder ranges from mild to severe, and mild traumatic brain injury (mTBI) accounts for over 85% of all TBI cases (2). Concussions are defined as a form of mTBI that embodies any force transmitted to the head, face, or neck that results in the rapid onset of short-lived impairment of neurological function that disrupts cognitive abilities. Concussions can result in the onset of a wide array of either acute or chronic individualized symptoms. Symptoms can range from headache, nausea, vomiting, balance problems, dizziness, fatigue, sleeping difficulties, light and noise sensitivity, emotional imbalance, numbness, mental fog, difficulty concentrating, memory difficulty, or blurred vision. These symptoms are nonspecific and have a weak correlation to the current neurocognitive testing like the Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT) exam.

There is currently no biological test in place to quickly and accurately determine a clinical diagnosis of concussions because the neural mechanisms of mTBI are not well characterized and poorly understood. Previous research has shown that biofluids can be utilized to harness and measure biomarkers released from the brain in response to concussion (3). If diagnostic markers of concussion can be established and correlated to specific symptoms within one of these biofluids, this may aid in the early detection of concussion, preventing increased damage to the brain and improved treatment plans to remediate affected individuals.

Biofluids such as blood, cerebrospinal fluid (CSF), and saliva have been shown to accurately measure biomarkers, like micro RNA (miRNA), released post-concussion (3). Because blood and CSF are invasive and impractical tools of collection for athletes on the field,

saliva has emerged as the prime biomarker for the evaluation of physiological changes in the body after a potential concussion. Previous studies have concluded that saliva accurately reflects the miRNA patterns found in both blood plasma and CSF because saliva can sufficiently detect the sensitive changes of miRNA concentration (4). Additionally, saliva is low-cost, non-invasive, and relatively easy to collect. Biomarkers like miRNA are found within saliva, and they can be measured as indicators of concussion. miRNAs are released from the brain in altered concentrations after TBI because they heavily align with cellular regulatory mechanisms and inflammatory responses of neurodegenerative diseases in the central nervous system (CNS) (5). miRNAs are the ideal biomarker candidates for diagnosing TBI because they are abundant, stable, disease-specific, resistant to enzyme degradation, and they play a key role in regulating molecular pathways throughout the body (6).

The goal of this research is to further examine the miRNA released in saliva and to correlate them with acute symptoms of concussion. This study partners with local high school football teams to collect saliva from players that undergo a concussion. Once the saliva samples are analyzed using RNA sequencing to measure the changes in abundance of each miRNA, they will be correlated to the wide range of concussion symptoms recorded from each patient. The study will incorporate four different timepoints (baseline, time of injury, 1 week, and 4 weeks) to observe and compare how the miRNA concentrations change over time between healthy controls and concussed individuals. Furthermore, the accurate establishment of these biomarkers may lead to the creation of a biological objective tool for assessing and diagnosing mTBIs that can better approximate the recovery time and symptom severity for the affected individuals.

Literature Review

Concussion, a form of mild traumatic brain injury (mTBI), embodies any force transmitted to the head that results in the rapid onset of short-lived impairment of neurological function with an emphasis on the disruption of cognitive abilities over structural damage to the brain (7). Approximately three million concussions occur in the US every year, and 80% are caused by mTBIs (4). A wide range of acute symptoms may evolve after a concussion including neurological, cognitive, somatic, or behavioral symptoms. Due to this high variability, the diagnostic procedure has relied heavily on self-reported symptoms using tests such as ImPACT or SCAT3 to assess the neurocognitive function and symptoms of the affected individuals (8). As a result, researchers have begun exploring the use of biofluids as a potential biological tool to diagnose mTBIs like concussion.

Biofluids such as blood and cerebrospinal fluid (CSF) have been used in previous studies to gather insight on biomarkers released from the brain post-concussion. However, while these collection methods are able to identify changes in the concentrations of biomarkers released from the brain (4,9), they are invasive and are not practical tools to test concussed individuals at the time of injury. A previous study then compared saliva to CSF to determine if saliva could be used as an accurate biofluid, and they found 6 miRNA that had parallel concentration changes between CSF and saliva: miR-182-5p, miR-221-3p, mir-26b-5p, miR-320c, miR-29c-3p, miR-30e-5p (4). The authors argued that saliva accurately reflects the miRNA patterns found in CSF, and they concluded that saliva can be used as a tool to better diagnose mTBI. Furthermore, saliva collection is cost-efficient, non-invasive, easier to collect, and it has a lower risk of pathogenic infection during sampling, which makes it a prime biofluid of study that has been shown to accurately reflect the biomarkers of mTBI found in both blood plasma and CSF (10).

Biofluids contain measurable substances known as biomarkers that can be used as indicators of a mTBI. Examples of biomarkers include metabolites, proteins, and micro RNA (miRNA), the focus of this study. miRNAs are small, noncoding molecules that are involved in post-transcriptional regulation of gene expression and influence protein expression throughout the body (8). Recently, miRNA expression has piqued researchers' interest as a diagnostic tool for central nervous system (CNS) diseases as they are heavily involved in the cellular regulatory mechanisms and inflammatory responses of neurodegenerative diseases like Alzheimer's (5). This finding led researchers to begin evaluating the use of miRNA as a potential biomarker for mTBI. In a 2018 study on salivary miRNA, Hicks et al. argued that miRNAs are the ideal biomarker candidates for diagnosing traumatic brain injury due to the following advantages: abundance, stability, disease-specificity, resistance to enzyme degradation, and importance in regulating molecular pathways throughout the body." (6). An important comparison to note between proteins and miRNA is that due to the larger size and hydrophilicity of proteins, they are unable to cross the blood-brain-barrier while miRNAs can. One study compared and contrasted the sensitivity of miRNA versus blood-based protein biomarkers, and the authors concluded that the miRNAs showed robust concentration changes after a concussion, while potential protein biomarkers did not yield any strong associations to the presence of TBIs, with UCHL1 as a possible exception (11).

While studies have identified miRNA biomarkers that show differences in concentration levels between subjects with concussions and healthy controls, they have not reported the same identification findings due to a low sample number and high variability of the samples. For example, Johnson et al. identified 15 saliva miRNAs associated with prolonged symptoms and neuronal regulation, and after further analysis concluded that 5 of those 15 concentrations

accurately identified patients with post-concussion syndrome (12). The 5 identified saliva miRNA are as follows: miR-320c-1, miR-133a-5p, miR-769-5p, let-7a-3p, and miR-1307-3p. In contrast, Di Pietro and his colleagues detected 5 miRNAs that had a significant upregulation (miR-27b-3p, let-7i-5p, miR-142-3p, miR-107, miR-135b-5p) when correlated with a reduction in the ImPACT test's reaction time (3). The reaction time score is a measure of neurological functioning in the concussed patient, where a decrease in reaction time or average number of correctly chosen matches for a series of modules (X's and O's, symbol match, color match) indicates poor cognitive performance (13).

The first objective of this research uses an untargeted approach to further investigate the miRNAs released from neurons and other CNS cells that are present following a concussion. An untargeted approach analyzes the concentration differences of the identifiable miRNA found in the saliva samples to observe any unexpected changes that occur. This helps clarify what brain injury specific miRNA are present post-concussion. While studies have recognized miRNA as a novel biomarker for concussion, there has been no correlation made to the symptoms that occur post-concussion or to the inability of a concussed individual to resolve their onset of symptoms. The second objective of this study is to correlate either known salivary miRNA biomarkers, or the emergence of novel miRNA biomarkers, to the highly variable and individualized symptoms of concussion with the possibility of discovering a sustained elevation or poor resolution of symptoms. The study utilizes a novel biological tool, RNA sequencing, which has the potential to match identified miRNA to specific symptoms and their severity to better identify the recovery time of concussed patients.

A study by Broshek et al (2015) of the symptoms and psychological factors associated with concussion reviewed the detrimental effects that can result in an athlete post-concussion.

They found that mTBIs can trigger fear and anxiety reactions that prolong the recovery period for the athlete (14). If miRNA biomarkers can be linked to specific symptoms using high resolution technology like RNA sequencing, then clinicians may better understand what patients are predisposed to post-concussion syndrome (PCS). In this novel approach, a library of tens of thousands of miRNAs isolated from a saliva sample from the affected individual is established and sequenced, and then the fragments of RNA are aligned to the genome of a healthy individual (15). This generates a list of the abundance of each miRNA, which can be correlated with the wide range of concussion symptoms. Longitudinal monitoring conducted every few days may also establish whether the biomarker and symptoms disappear at the same rate.

The motivation for the Concussion Biomarker Study is to assemble an accurate tool for diagnosing mTBIs using biomarkers collected from the saliva, and to better approximate the recovery time and severity of symptoms required after a concussion. The relationship between the use of saliva as a sufficiently sensitive biofluid medium and the miRNA that are released as biomarkers post-concussion is unclear, so there is currently no definite identification of these biomarkers that can be traced directly to concussion. This study furthers the explorative field of using saliva as the primary collection method for identifying the miRNA released from concussed athletes and may identify novel biomarkers. This study also lays the groundwork for understanding the biological basis of prolonged concussion symptoms, as well as understanding the diminished neurological function, both of which do not always correlate to current clinical testing like the ImPACT test. Once a spectrum of miRNA molecules is solidified, future studies should attempt to correlate and compare the miRNA to specific symptom types seen in patients with both acute and prolonged symptoms so that treatment plans can be improved and individualized to the patient.

Methodology

Design and Participants

This study uses an untargeted approach to discover what miRNA, and potentially novel miRNA, are released after a concussion to improve the current diagnostic approach. The design for this study takes a longitudinal approach over the course of two football seasons. Data were collected for two separate seasons, 2018 fall and 2019 fall, from local high schools. Data were collected from two separate years to increase the number of recorded concussions and to investigate what miRNA can consistently distinguish between the concussed patients and their controls. The participants were high school male football players, and their demographics and concussion history were accounted for using the ImPACT test. The ImPACT test is a computerized neurocognitive test that provides an analysis of a patient's cognitive ability and symptoms following a concussion. In the first year, there were 8 suspected concussions, but only 3 were confirmed. In the second year, there were 5 confirmed concussions. Verbal consent was obtained from all that participated in the study where they agreed to provide baseline cognitive testing via the ImPACT test and saliva samples for the four timepoints (baseline, time of injury, week 1 follow-up, and week 4 follow-up). The cognitive screening was already put in place as a standard protocol for the team, so the study did not interrupt their pre-season preparations.

Trained sports medicine providers (athletic trainers) monitored the players and were responsible for determining if one sustained a concussion. If an athlete was under question for receiving a concussion, the athletic trainers used the SCAT5 to evaluate their symptoms and whether or not they were able to return to play. If a concussion was suspected, a saliva sample was collected. Athletes were then referred to a physician at a separate facility like Children's

Healthcare of Atlanta (CHOA) to officially diagnose the concussion, and this information was shared and recorded in the data.

ImPACT and BESS Assessments

The ImPACT test gathers data on a patient's Sequencing/Attention, Word Memory, Visual Memory and Reaction Time (13). The Balancing Error Scoring System (BESS) test assesses an individual's postural stability after a concussion. The test uses three different positions: double-leg stance, single-leg stance on nondominant leg, and tandem stance (the toes of the nondominant foot is placed behind the heel of the dominant foot). The balance and postural stability of each stance is tested on both a foam and firm surface with the patient's eyes closed. The patient must remain in the stance for 20 seconds without making errors (opening eyes, falling out of position, etc.). The number of errors is totaled, so lower scores indicate better postural stability (16).

Saliva Collection and Storage

When a concussion occurred, a saliva sample was collected on site from the athlete within 24 hours of the injury using the SalivaBio Passive Drool and the Oragene mRNA kits. The players returned to their school's where the athletic trainers conducted follow-up cognitive testing and saliva sample collection at 1 week post injury and 4 weeks post injury timepoints. The ImPACT data were collected for both years; however, the BESS assessment was only administered to the year 1 athletes due to a change in the protocol. In addition to the concussed players that were removed from play, the substituting players acted as the control samples for this experiment. They were expected to participate in saliva collection for the next 3 timepoints and complete the ImPACT test for the 1- and 4-week timepoints. A patient label was put on all salivary specimens, and they were frozen over the course of the collection phase because saliva

is able to be stored frozen for long periods of time. Once all samples were collected, they were de-identified to protect the participants and transported to the Emory University biorepository where they were stored at -80°C until sample processing.

Sample Processing and Purification

The salivary specimens, which are made up of the Oragene-RNA solution and the participant's saliva, must be processed before total RNA extraction can take place. Once the samples were received in the Emory University biorepository lab, they were thoroughly mixed to ensure maximum RNA recovery and stability. The samples were then incubated in their original vials at 50°C for 1 hour in a water bath or for 2 hours in an air incubator until no longer frozen. Pipettes were used to create 1 x 1 mL aliquots, and they were couriered to Georgia Tech for subsequent purification.

Once the samples arrived at the Georgia Tech lab, they were stored at room temperature for up to 8 weeks or stored frozen at -20°C indefinitely. The following steps describe how the whole RNA was extracted from the samples. A 250-500 μL aliquot was transferred to a 1.5 mL microcentrifuge tube to be incubated in a water bath at 90°C for 15 minutes. After the samples were cooled to room temperature, $1/25^{\text{th}}$ volume of neutralizer solution was added, and the tube was vortexed. The tubes were incubated on ice for 10 minutes to allow impurities and inhibitors to precipitate. The samples were then centrifuged at maximum speed ($> 13,000 \times g$) for 3 minutes. A pipette was used to transfer the clear supernatant to a fresh microcentrifuge tube. Ethanol will precipitate the nucleic acids, so 2 volumes of cold 95% EtOH was added to the fresh tubes and vortexed. The tubes were incubated once more at -20°C for 30 minutes to ensure maximum precipitation of RNA. The samples were centrifuged a second time at maximum speed for 3 minutes. The pellets in the microcentrifuge tube contain the purified nucleic acids, so they

were carefully removed and then dissolved in 350 μ L of buffer RLT found in the RNeasy Micro Kit. The solutions were vortexed until the pellets were completely dissolved. Then, 350 μ L of 70% ethanol was added, and the solutions were vortexed.

The following steps describe the Qiagen RNeasy cleanup procedure. The samples were then transferred into a RNeasy MinElute spin column in a 2 mL collection tube and centrifuged for 15 seconds ($>8,000 \times g$). The flow-through was discarded following each centrifugation for the rest of the protocol. 350 μ L of buffer RW1 was added to the columns and centrifuged again using the same settings listed previously. 10 μ L of DNase I stock solution was added to 70 μ L buffer RDD in a separate tube and mixed gently. The mix was then added to the solutions and incubated at room temperature for 15 minutes. Another 350 μ L of the RW1 buffer was added to the samples and centrifuged. The samples were then transferred to a new 2 mL tube, and 500 μ L of RBE buffer was added and centrifuged. 500 μ L of 80% ethanol was then added to the column and centrifuged for 2 more minutes. The solution was then pipetted into a new 2 mL column and centrifuged for 5 minutes. Finally, the solutions were placed into a fresh 1.5 mL collection tube and 25 μ L of RNase-free water was added directly to the center of the spin column membrane. After the samples were incubated at room temperature for 5 minutes, they were centrifuged one last time at 1 minute to fully extract the RNA. The flow-through was not discarded after this step.

MiRNA Sequencing Library

While saliva samples are noninvasive, low-cost, and relatively easy to collect, they contain high concentrations of bacterial RNA and other forms of human RNA that must be removed. The following protocol was used to extract the miRNA from the saliva samples for future data analysis as well as eliminate the maximum amount of bacterial rRNA. The miRNA

from year 1 and year 2 were prepped using the Illumina TruSeq Small RNA Library Preparation Kits and Qiagen QIAseq miRNA Library Kits, respectively. The Qiagen QIAseq miRNA Library Kit was chosen for year 2 because this kit targets the miRNAs more efficiently than the Illumina kit. Furthermore, it is less labor intensive, more affordable, and there is a decrease in adapter dimer contamination. The Illumina protocol calls for the first-year samples to be run through a gel to read the base pairs (bp) of the RNA. The gel prevents long RNA from being sequenced. The RNA with reads of approximately 25 bp were then cut out.

The Illumina Ribo-Zero Plus rRNA Depletion Kit was added to the year 2 samples to further eliminate the bacterial rRNA in the samples. However, the miRNA from the second-year samples were extracted using the QIAseq® miRNA Library Kit. This protocol was changed to decrease the amount of variance detected in year 1. It used a more targeted approach and allowed for more control over the residual variance. A miRNA database was then used to map the reads. The database identifies the miRNA and indicates what processes they are known to be involved in.

Data Collection:

All of the data were imported into REDCap, an online research forum, where data can be uploaded, stored, and organized. The following forms were created to organize the data. The “Enrollment” form contains the patient ID, whether or not the player was classified as a baseline only, control, or concussed, and all of the dates and times of the follow-up exams. The “RNA Qc” form and “RNA Seq” form display information that quantifies the concentrations of RNA collected from the spit samples of the possibly/confirmed concussed players and their control replacements. The ImPACT form contains the demographic and test information, and shows data collected from the six following categories: verbal memory score, visual memory score, visual

motor score, reaction time, impulse control score, and symptom score. A symptom scale ranging from 0 to 6 was used to determine the symptom score. The “BESS Test” form includes the balance scores for each of the concussed players, but it only was administered for the year 1 participants. Records were created for each player; however, some players required multiple records if they were a confirmed concussion, suspected concussion, or control as one record was created for data collected from each timepoint. There are 71 total records from year 1 and 44 records total from year 2.

Data Analysis:

While data analysis was conducted on the year 1 samples, the year 2 sample data have not yet been collected. However, we plan to perform the same methods of data analysis on the year 2 samples to comprehend what miRNA in the saliva can be linked to concussion.

Principal Component Analysis: These data will be analyzed using a principal component analysis (PCA) to find the axes that show the most variance between the concussed and control subjects. The concentrations of miRNA with the most variation from the control concentrations will have the greatest influence on the principal components. The first principal component (PC1) shows the axis with the greatest amount of variation, and the second principal component (PC2) captures the direction with the second most amount of variation. The PC1 will also be plotted against the individual to depict how the timepoints of both the concussed and control subjects compare to each other over time.

Symptom Analysis: For every concussed subject and their control, their symptoms were categorized in one of the five types: somatic, vestibular-ocular, cognitive, sleep, or emotional. The total number of categories and total symptom score was recorded. These data will be used to analyze how their concussion symptoms resolved across the four timepoints. Because we have

not come to a conclusion on what miRNA constitute as the markers of concussion severity, we cannot yet correlate the symptoms of the concussed subjects to the miRNA. However, once the miRNA that exhibit longitudinal trends following concussion are identified, a regression analysis will be used to correlate the miRNA biomarkers to how the concussion symptoms change over time.

Results

The purpose of this study was to first identify miRNA in saliva that can consistently distinguish between concussed subjects and their controls, which aims to improve the diagnostic process of the affected individuals. Second, the identified miRNA biomarkers are to be compared to the symptoms each subject experienced at the time of impact, 1-week post-concussion, and 4-weeks post-concussion. The year 1 data were successfully analyzed, however the data for year 2 has not yet been completed. The year 1 data collected samples from 3 concussed subjects and their controls. Due to this low number of concussions and a high concentration of bacterial rRNA, the data resulted in a minute amount of sequenced miRNA. The sequenced miRNA from this study was compared to the miRNA cited from prior studies that claimed they were markers of concussion (these miRNA are listed in the literature review), and none of the identified markers were found in this study's samples. The following figures were retrieved from Katie Ferguson, a research assistant in Dr. Gregory Gibson's lab.

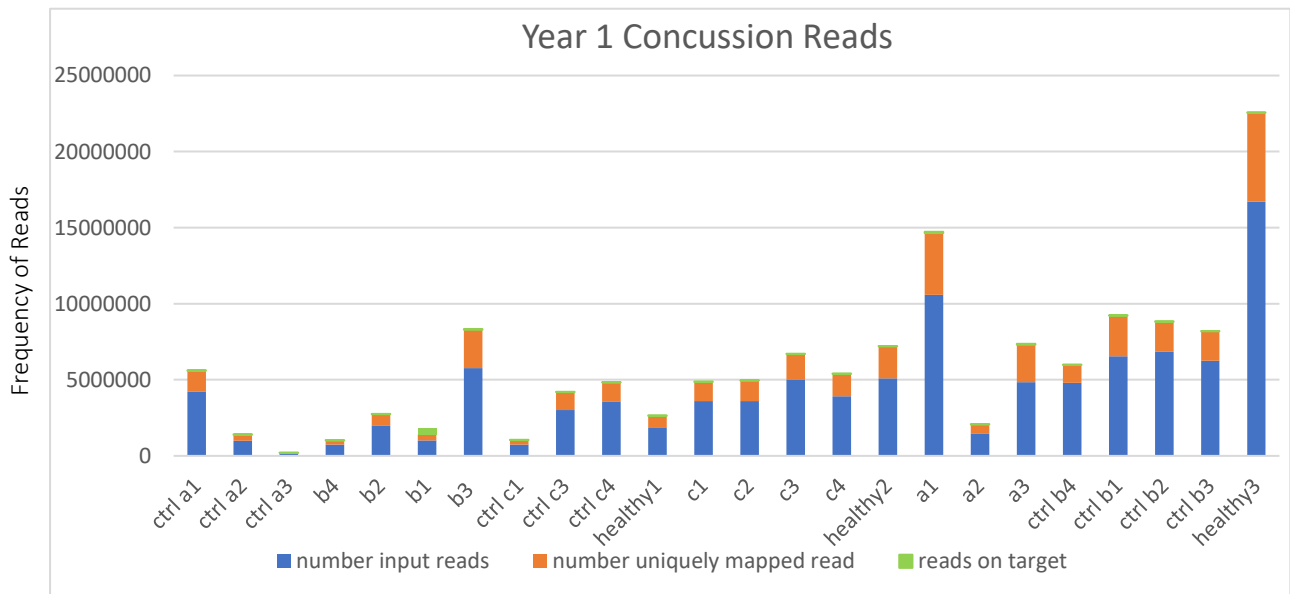


Figure 1. Reads for Year 1 Data. The small RNA extracted from each of the samples were sequenced. The blue bar represents the fragments of reads that were so short they couldn't be

uniquely mapped anywhere. The orange bar represents the small RNA that were successful mapped. The green bar represents the reads that were on target that were identified as miRNA.

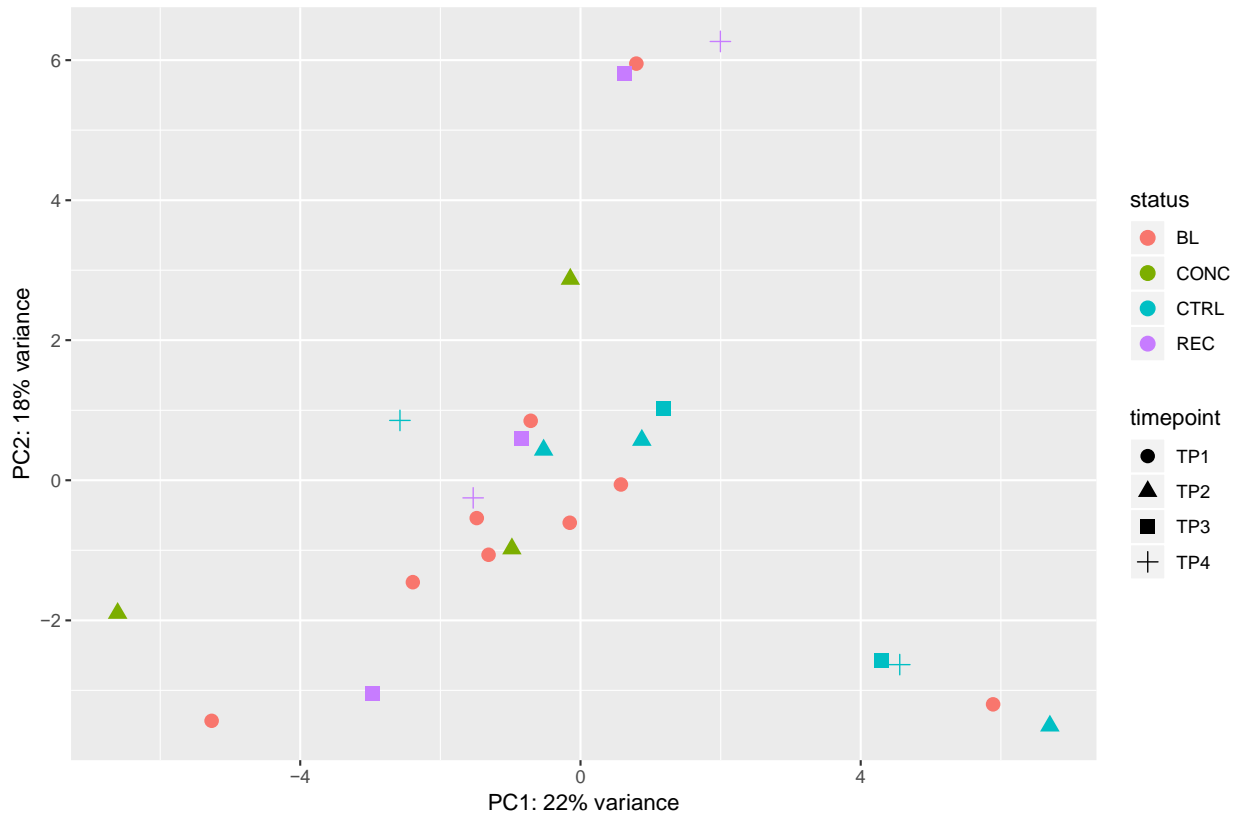


Figure 2. Principal Component Analysis of miRNA in Concussed vs. Control Individuals.

PC1 shows a variance of 22%, while PC2 shows a variance of 18%. Each data point depicts their status (BL = baseline, CONC = concussion, CTRL = control, REC = recovery time) and timepoint (TP1 = Pre-concussion/baseline, TP2 = time of impact, TP3 = 1-week post-concussion, TP4 = 4-weeks post-concussion). No clustering of the points was observed, so no conclusions were made.

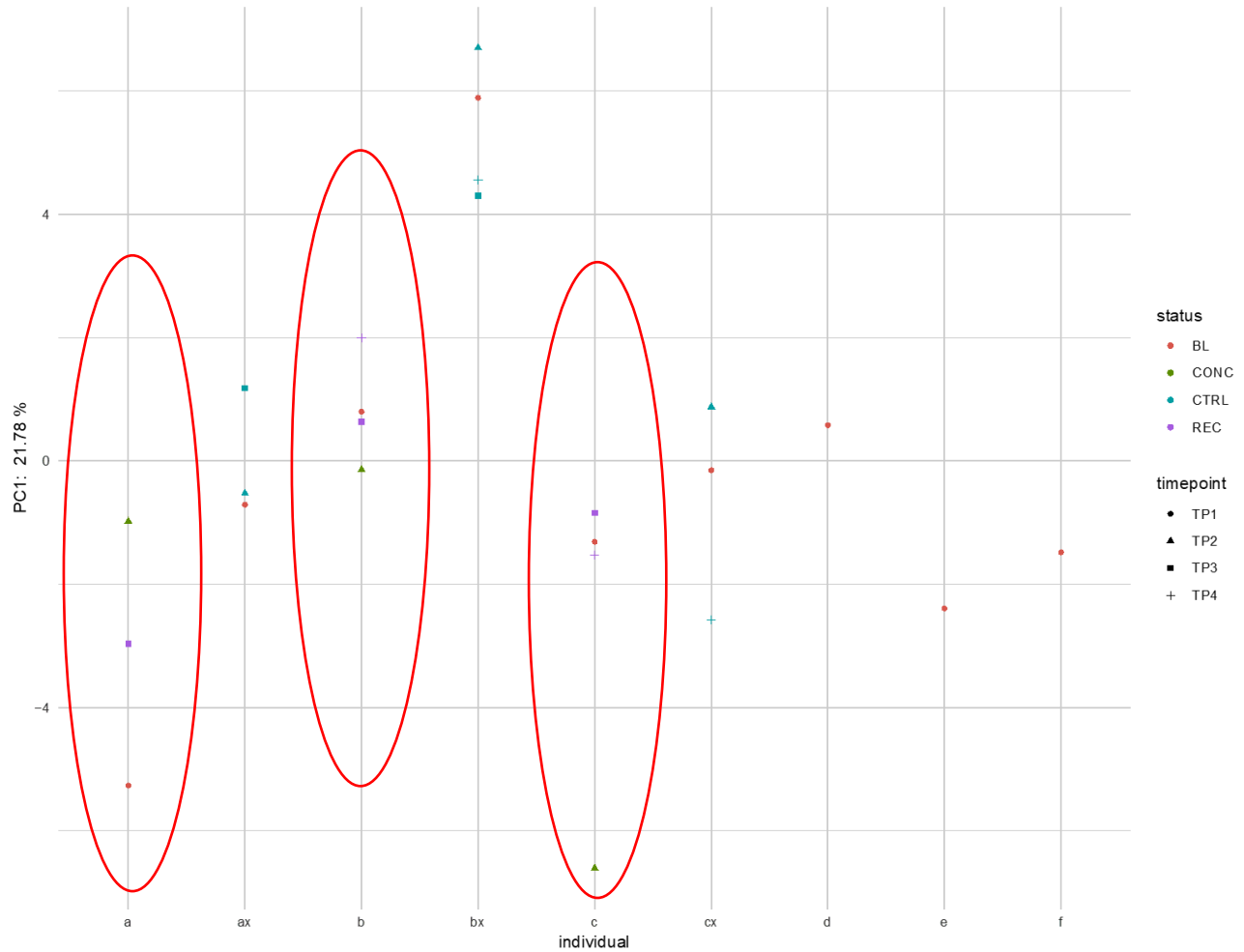


Figure 3. Principal Component Analysis of miRNA Sequenced from Year 1 data. The PC1 that was found to have the greatest variance was 21.78%. This data was inconsistent with the hypothesis because little to no clustering of data points within the same status was seen. The time of impact timepoint (TP2) from the baseline seen in individuals b and c does drop below the baseline; however, the baseline for individual a travels in the opposite direction of b and c, skewing the data. Because of this observation, no trends in the data were confidently seen, so no conclusions can be made at this time.

Discussion

This research utilizes an untargeted approach to identify miRNA that can consistently distinguish between concussed athletes and uninjured controls and then aims to correlate the identified miRNA to the symptoms the patient experiences. The experimental hypothesis states that salivary miRNA levels are altered in children with concussion, and there will be a significant difference in levels of miRNA at baseline (pre- injury) and post-injury in athletes along with observed differences between the concussed and control players. This means that the players with higher symptom scores post-concussion, which suggests a more severe concussion, will display data points with greater distances from their baseline in the PCA plot. The findings from the year 1 show that there were not significant changes in the salivary miRNA of the individuals as seen in Figure 2.

Because data were only collected for 8 concussions, finding trends between the sequenced miRNA and patient type (concussed or control) was challenging. Furthermore, none of the previously identified miRNAs in the literature were found in the year 1 samples. One limitation that occurs when using saliva as the biofluid medium is the large amount of bacterial rRNA found in the saliva samples. As seen in Figure 1, this results in minute concentrations of viable miRNA that were sequenced from the samples and a high level of residual variance. However, this limitation was improved through changes made to the year 2 protocol which used a more targeted approach. The Illumina Ribo-Zero Plus rRNA Depletion Kit was utilized in year 2 to decrease the amount of bacterial rRNA in the samples. By reducing the quantity of bacterial rRNA, the sequencing machine is able to identify more of the reads on target, the miRNA, which would increase the total number of viable miRNA found in the samples.

Figures 2 and 3 used a PCA plot to calculate the variance between each of the samples. Hypothetically speaking, the data in Figure 2 would show the baselines clustered together, TP1 clustered together, and so forth. Due to the low number of concussions and high concentrations of bacterial rRNA, there was high variance between the data points in each status group, meaning there was little change in the miRNA released after the player experienced a concussion. The PC1 and PC2 in Figure 2 were 22% and 18%, respectively. These are relatively low values for the principal components, which means that there are no clear factors separating the miRNA of concussed subjects from their controls. However, the data in Figure 3 shows some promise. The time of impact, TP2, of concussed individuals b and c travels away from the baseline while TP3 and TP4 return closer to the baseline. While this was thought to show a possible trend, the baseline measure for individual a shows a large drop away from the other two baselines, skewing the data.

Once the year 2 data is complete, we hypothesize that there will be an increase in the total of miRNA reads which will in turn improve the number of miRNA that are able to be identified. There were 5 concussions in year 2, so the increase in the number of concussions along with the decrease in bacterial rRNA will elicit higher principal components. If the PC1 and PC2 increase, this will increase the correlation between miRNA associated with concussion. If the miRNAs with altered concentrations post-concussion are found, then they will be compared to the symptom analysis shown in Figure 4 (found in Supplementary Figures). A linear regression will be used to visualize this relationship.

Conclusion/Future Directions

If salivary miRNA levels change in people that undergo an mTBI, then there will be a significant difference in the concentrations of miRNA at baseline (pre- injury) and post-injury between the concussed and control players. However, further investigation is required to pinpoint the spectrum of miRNA that are connected to concussion. Once these miRNA are identified, subjects with higher total symptom scores or a more diverse range of symptoms post-concussion, suggesting a more severe concussion, will display data points with greater distances from their baseline in the PCA plot. The data from year 2 must first be analyzed in order to observe this trend. Another future direction of this study should attempt to correlate and compare the miRNA to specific symptom types seen in patients with both acute and *prolonged* symptoms so that treatment plans can be improved and individualized to the patient. These findings are significant because an establishment of these biomarkers may lead to the creation of a biological objective tool that can better assess and diagnose patients suffering from mTBIs. If the predictability of the recovery time and severity of symptoms is increased, then a better post-concussion management plan can be put in place to help the affected individuals.

Supplementary Figures:

RNA Sample ID	Patient ID	Patient Type	Test Type	Symptom Type	Somatic	Vestibular-ocular	Cognitive	Sleep	Emotional	Total Number	Total Symptom Score
ctrl_a1	mTBI-004	control 033	baseline	fatigue (1), sadness (1), nervousness (1)				1	2	3	3
ctrl_a3	mTBI-004	control 033	post-injury 1	falling asleep trouble (1), sleep less than normal duration (1), light sensitivity (1)	1			2		3	3
b1	mTBI-021	concussion	baseline	headache (5), vomiting (4), sleep less than normal duration (2), light sensitivity (4), sadness (3), memory difficulty (3), visual problem (2)	3	1	1	1	1	7	23
b3	mTBI-021	concussion	post-injury 1	sleep less than normal duration (3), drowsiness (1), light sensitivity (2), mental fog (1)	1		1	2		4	7
b4	mTBI-021	concussion	post-injury 2	headache (1), vomiting (2), fatigue (4), sleep less than normal duration (5)	2			2		4	12
crtl_c1	mTBI-022	control 029	baseline	sleep less than normal duration (2), irritability (2), memory difficulty (3), visual problem (1)		1	1	1	1	4	8
crtl_c3	mTBI-022	control 029	post-injury 1	sleep less than normal duration (3), irritability (2), visual problem (2)		1		1	1	3	7
crtl_c4	mTBI-022	control 029	post-injury 2	sleep less than normal duration (2), sadness (2)				1	1	2	4
c1	mTBI-029	concussion	baseline	balance problem (1), dizziness (1), falling asleep trouble (2), sadness (2), visual problem (3)		3		1	1	5	9
c3	mTBI-029	concussion	post-injury 1	headache (2), sadness (3), slowed down assessment (3)	1		1		1	3	8
c4	mTBI-029	concussion	post-injury 2	none						0	0
a3	mTBI-033	concussion	baseline	balance problem (1), falling asleep trouble (1)	1			1		2	2
a2	mTBI-033	concussion	post-injury 1	none						0	0
crtl_b1	mTBI-042	control 021	baseline	none						0	0
crtl_b3	mTBI-042	control 021	post-injury 1	light sensitivity (1)	1					1	1
crtl_b4	mTBI-042	control 021	post-injury 2	headache (1)	1					1	1
	mTBI-307-P	concussion	baseline	drowsiness (1), light sensitivity (1), sadness (1), visual problems (1)	1	1		1	1	4	4
	mTBI-307-P	concussion	post-injury 1	headache (4), nausea (3), balance problems (1), dizziness (3), fatigue (2), falling asleep trouble (2), sleep less than normal duration (1), drowsiness (4), light sensitivity (6), noise sensitivity (5), irritability (2), sadness (3), nervousness (1), numbness tingling (3), slowed down assessment (4), mental fog (4),	5	3	4	4	3	19	59

				concentration difficulty (4), memory difficulty (3), visual problem (4)							
	mTBI-307-P	concussion	post-injury 2	nervousness (1)					1	1	1
	mTBI-307-P	concussion		none						0	0
	mTBI-308-P	control 307	baseline	drowsiness (1), noise sensitivity (1), slowed down assessment (1), concentration difficulty (1)	1		2	1		4	4
	mTBI-311-P	concussion	baseline	none						0	0
	mTBI-311-P	concussion	post-injury 1	headache (2), dizziness (2), light sensitivity (4), concentration difficulty (1)	2	1	1			4	9
	mTBI-311-P	concussion		none						0	0
	mTBI-311-P	concussion	post-injury 2	none						0	0
	mTBI-314-P	concussion	baseline	sleep less than normal duration (2), drowsiness (1)				2		2	3
	mTBI-314-P	concussion	post-injury 1	headache (1), dizziness (1), fatigue (4), drowsiness (2), light sensitivity (2), noise sensitivity (1), irritability (2), slowed down assessment (3), mental fog (2), concentration difficulty (3), memory difficulty (3)	3	1	4	2	1	11	24
	mTBI-314-P	concussion		fatigue (1), drowsiness (1), slowed down assessment (1), mental fog (1), concentration difficulty (1)			3	2		5	5
	mTBI-314-P	concussion		none						0	0
	mTBI-314-P	concussion	post-injury 2	none						0	0
	mTBI-317-P	concussion	baseline	fatigue (1), sleep less than normal duration (1), nervousness (1)				2	1	3	3
	mTBI-317-P	concussion	post-injury 1	headache (4), nausea (1), balance problem (1), dizziness (3), fatigue (3), falling asleep trouble (3), sleep less than normal duration (4), drowsiness (1), light sensitivity (3), noise sensitivity (1), irritability (1), slowed down assessment (1), mental fog (2), concentration difficulty (6), memory difficulty (1), visual problem (2)	4	3	4	4	1	16	37
	mTBI-317-P	concussion		none						0	0
	mTBI-317-P	concussion	post-injury 2	none						0	0

mTBI-318-P	control 320	baseline	none							0	0
mTBI-320-P	concussion	baseline	none							0	0
mTBI-320-P	concussion	post-injury 1	headache (2), fatigue (2), falling asleep trouble (4), sleep less than normal duration (1), drowsiness (1), light sensitivity (1), noise sensitivity (1), irritability (3), sadness (1), nervousness (1), emotional greater than normal (2), mental fog (2), concentration difficulty (1), memory difficulty (1) visual problem (1)	3	1	3	4	4		15	24
mTBI-320-P	concussion		falling asleep trouble (1), sleep less than normal duration (1)				2			2	2
mTBI-320-P	concussion		none							0	0
mTBI-320-P	concussion	post-injury 2	none							0	0
mTBI-321-P	control 311	baseline	fatigue (1), sleep less than normal duration (1), drowsiness (1)				3			3	3
mTBI-321-P	control 317	baseline	fatigue (1), sleep less than normal duration (1), drowsiness (1)				3			3	3
mTBI-328-P	control 314	baseline	falling asleep trouble (2)				1			1	2

Figure 4. Symptom Analysis of Concussed Patients. The table summarizes the type of symptom and total symptom score that each subject recorded when given the ImPACT test at the baseline timepoint, TP1, or TP2. Once miRNA are identified as biomarkers of concussion, this table will be used to correlate the concentrations of those miRNA found in the subject’s saliva to the severity of their symptoms to investigate if the identified miRNA can be matched to a specific symptom type.

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