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Salivary MicroRNA as a Concussion Biomarker and the Implications for Athletic Trainers' Practices

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SALIVARY MICRORNA AS A CONCUSSION BIOMARKER AND THE IMPLICATIONS FOR ATHLETIC TRAINERS' PRACTICES

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ABSTRACT

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Many concussions are sustained globally each year; however, research suggests that a large number may go undiagnosed due to a reliance on subjective information from patients and limitations of current objective measures. Recently, efforts have been focused on identifying clinical biomarkers of concussions, including salivary microRNA (miRNA), to improve healthcare professionals' concussion management practices. Even if salivary miRNA were shown to be a valid and reliable measure for managing concussions, healthcare professionals, such as athletic trainers (ATs), must be familiar with the tool and have positive attitudes toward the implementation into clinical practice. Therefore, the purpose of this dissertation was to better understand salivary miRNA as a concussion biomarker through three studies.

In our first study, we performed a scoping review to identify specific salivary miRNA that have demonstrate potential in acting as a diagnostic or prognostic indicator of concussions. Overall, we found forty-nine salivary miRNA across nine studies throughout the literature. Of those forty-nine, thirty-four show potential for being a concussion diagnostic marker and twenty-one correlated to multiple concussions or symptom trends. Of those identified, five were identified in multiple studies.

As previously stated, even if identified as a valid and reliable tool for concussion management, healthcare professionals such as ATs should demonstrate familiarity with these tools and show positive attitudes toward the implementation into clinical practice. Therefore, the

second study of this dissertation aimed to evaluate ATs familiarity with biomarkers of concussion and their attitudes toward the future implementation into clinical practice. Overall, the results of this study indicated that ATs self-reported a lack of familiarity with current literature surrounding the use of biomarkers for concussion management but a large majority self-reported positive attitudes towards the future implementation of such a tool into clinical practice.

Lastly, to address a limitation for the application of salivary miRNA as a concussion biomarker, we aimed to evaluate the effects of one NCAA Division I football season. In this study, we found that although simple inferential statistics revealed no significant differences between pre- and post-season expression of salivary miRNA, further analysis through intraclass correlation coefficient statistics revealed a lack of reliability in each of the six target salivary miRNA. Although these target miRNA did not demonstrate reliability from pre- to post-season, sub-analyses revealed a ratio of two miRNA that showed high sensitivity when identifying concussed individuals. Overall, research should continue to evaluate the potential for salivary miRNA to act as a concussion biomarker by evaluating other target miRNA.

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CHAPTER 1

INTRODUCTION

1.1 Background

Annually, more than 1.6 million concussions are sustained in the United States, but research has demonstrated that this number may underrepresent the true occurrence of this injury due to the underreporting of symptoms by patients.[1,2] Despite the short-term effects of concussions being well established, the long-term effects of concussions may be unclear. However, recent research has suggested that concussions may lead to long-term effects such as declines in cognitive health as indicated by decreased memory, impaired psychomotor function, and may even lead to other conditions such as Alzheimer's disease or chronic traumatic encephalopathy (CTE).[3–6]

With the typical lack of structural damage to the brain following a concussion, current standard neuroimaging techniques cannot detect abnormalities with this injury.[7] So in response, it is suggested that health care providers use multiple tools to evaluate an individual's history, including symptoms, neurological function, motor control, neurocognitive function, and physical function.[7–9] Evaluations should occur during pre-season for athletes to obtain baseline measurements, and whenever a concussion is suspected. For the general population, however, pre-season evaluations are not possible, making reliance on patient symptom reporting even more crucial.

Clinical tests will typically include assessments of memory, balance, and sensation.[7–9] However, recent research has shown that current tools may not be very reliable and in fact, evaluations truly rely heavily on a patient's self-reported symptoms.[10–13] The current lack of objective measures for concussion management, and reliance on patient reporting of symptoms

for diagnosis, makes it essential that research is aimed at identifying an objective tool, such as a clinical biomarker, to improve patient outcomes. One possible clinical biomarker that has been identified in the literature is salivary microRNA (miRNA). If proven to be a valid and reliable tool for diagnosing and managing concussions, salivary miRNA may in turn improve patient outcomes following a concussion by providing clinicians a more objective measure of injury status.

One of the first health care professionals to perform concussion assessments and implement return to activity protocols are certified athletic trainers (ATs).[14] ATs can be employed within various settings, and ultimately many of the decisions surrounding the diagnosis, management, and return to activity rely on the knowledge and familiarity of current best practices by ATs.[14–16] If an AT lacks knowledge or familiarity with best practices, this may result in the misdiagnosis or delayed diagnosis of a concussion, ultimately leading to a patient's prolonged recovery or further complications.[14]

1.2 Statement of the Problem

Concussions are a highly prevalent injury that not only results in short term effects such as decreased reaction time, memory disfunction, and increased risk of further musculoskeletal injury, but may also lead to further complications later in life such as Alzheimer's disease and CTE.[3–6,17–20] Despite the known and possible effects of these injuries, procedures used by health care providers, such as ATs, are highly subjective due to the reliance of patient input, particularly patient self-reported symptoms. Of the tools used by health care providers that provide objective measures, many have been shown to lack reliability and validity for the use of diagnosis or management of concussions. A topic of interest in the current literature is the identification of a clinical biomarker of concussions which would provide an objective measure for health care

providers to determine a patient's concussion status. However, if health care providers, particularly ATs, lack knowledge of or familiarity with these clinical biomarkers, or are unwilling to implement these tools into their practice, discovery of such a tool would not be useful. One possible clinical biomarker target is salivary miRNA but there are still limitations surrounding the applicability of this tool for concussion practices. One of the biggest limitations is the effects of time and impacts sustained during a contact sport on expressions of miRNA.

Due to the relatively novel approach of salivary miRNA acting as a concussion biomarker, the literature surrounding this topic lacks synthesis. Although showing some overlap in methods and results, there has been only one research article which aimed to synthesize the research surrounding salivary miRNA and concussions.[21] The authors of this publication review the findings from 14 studies which examined the relationship of miRNA and traumatic brain injury. However, of the 14 studies, 12 studied miRNA from peripheral blood, and only three examined miRNA from saliva. Since publication, the number of publications which further evaluate the relationship of salivary miRNA and concussions has more than tripled.[21]

While ATs familiarity with and attitudes toward topics have been an interest within the literature over the years, to my knowledge, there have been no studies which aimed to evaluate ATs familiarity with and attitudes towards concussion biomarkers. It is crucial to evaluate this topic due to the likelihood of ATs implementing these tools into their current concussion practices and the frequency at which ATs diagnose and treat concussions.

As previously mentioned, one of the largest limitations surrounding the applicability of salivary miRNA as a concussion biomarker is the effects of time and impacts sustained during a contact sport on expressions of miRNA. To our knowledge, there has been only one study that has analyzed these effects on salivary miRNA expressions, however, the activities in which

participants were participating in included basketball, lacrosse, soccer, hockey, or mixed martial arts training.[22] No studies have analyzed the effects of one football season on salivary miRNA expressions. Additionally, the athletic seasons varied from 30 days to 115 days, as compared to a traditional intercollegiate football season which can last over 120 days from start to finish. Lastly, throughout those seasons, the authors note that none of the participants were diagnosed with a concussion.[22] The proposed study aims to follow a large cohort of Division I American football players for a full competitive season. This will likely result in several concussion diagnoses, ultimately yielding valuable information regarding the effects of concussions on miRNA.

1.3 Purpose of the Dissertation

There were multiple purposes to this dissertation surrounding the use of salivary miRNA as a clinical biomarker of concussions. The first purpose was to review the current literature surrounding salivary miRNA and the relationship to concussions. The second purpose was to explore ATs knowledge of and familiarity with concussion biomarkers. The third purpose was to evaluate the effects of one NCAA Division I football season on previously identified salivary miRNA that have shown potential as a concussion biomarker.

1.4 Specific Aims and Hypotheses

Aim 1: Identify salivary miRNA within the research literature that have been shown to be sensitive to concussion status

Aim 2.1: Identify current tools used by athletic trainers for the diagnosis and management of concussions

Aim 2.2: Assess certified athletic trainers' familiarity with potential concussion biomarkers

Aim 2.3: Examine certified athletic trainers' attitudes towards the future use potential concussion biomarkers

Aim 3.1: Determine the effects of one NCAA Division I football season on previously identified salivary miRNA as biomarkers for concussion diagnosis

Hypothesis 3.1: Previously identified salivary miRNA which have shown promise as a diagnostic concussion biomarker will not significantly change over the course of one season in individuals not diagnosed with a concussion

Aim 3.2: Investigate the effects of one NCAA Division I football season on salivary miRNA which have been identified as potential biomarkers for concussion management

Hypothesis 3.2: Salivary miRNA which have been previously identified as potential concussion biomarkers associated with concussion management will show no significant change over the course of one season in individuals not diagnosed with a concussion

Aim 3.3: Validate previously identified salivary miRNA as concussion biomarkers for diagnosis

Hypothesis 3.3: If a patient is clinically diagnosed with a concussion, expressions of salivary miRNA which have shown promise as a concussion diagnostic tool will be significantly altered

Aim 3.4: Compare post-concussion return to participation performance on the Sway tool with salivary miRNA expressions

Hypothesis 3.4: Salivary miRNA collected once a participant is cleared for full participation via the Sway tool, which demonstrated a significant change as compared to baseline measures due to a concussion diagnosis, will demonstrate no significant difference compared to baseline measures

1.5 Operational Definitions

1. Clinical biomarker: A objective measure which can be used to identify the presence or severity of a condition, injury, or disease state.[23]

2. Cognitive health: An individual's ability to learn, recall, and think clearly.

3. Concussion: Term to describe the sequalae of symptoms associated with a mild traumatic brain injury (mTBI). This injury often negatively affects an individual's psychological, physical, and cognitive function.[21,24]

4. Mild traumatic brain injury (mTBI): Injury to the brain as the result of a traumatic event which causes an acceleration or deceleration of the brain. Injuries may occur as a result to a direct or indirect blow to an individuals' head.[23]

5. Motor control: The development of movement through deliberate initiation, organization, direction, and control.

6. Neurocognitive function: Skills associated with an individuals' ability to think and reason which are related to specific areas or neural pathways of the brain.

7. Neurological function: The ability of an individual's neurological system, including the central and peripheral nervous systems, to perform.

5. Physical function: The ability to perform simple and more complex activities associated with daily living.

9. Psychomotor function: The combination of attention, processing of problem-solving skills, and specific motor function.

10. Salivary microRNA: Abundant non-coding fragments of RNA, which consist of approximately 22 nucleotides. MicroRNA (miRNA) affect protein expression through

post-transcriptional processes. One biofluid in which these RNAs can be measured is human saliva.

1.6 Assumptions

The primary assumptions of this dissertation are as follows:

Chapter 3:

1. Information in selected published articles was accurate and contained no errors
2. Data, including salivary miRNA, timing of collection, and participant demographics was accurate and contained no errors

Chapter 4:

1. Participants who completed the survey provided honest and accurate answers

Chapter 5:

1. Participants reported symptoms immediately following a trauma potentially resulting in a concussion
2. Participants' medical history was accurate and free from error
3. Healthy matched controls were free from concussion at time of data collection as a match-control for an individual who sustained a concussion

1.7 Delimitations

Chapter 3:

1. Only studies using salivary miRNA were included, narrowing the methods of miRNA measurement

Chapter 4:

1. Participants were currently practicing certified athletic trainers

2. Recruitment was limited to members of the National Athletic Trainers' Association (NATA) and individuals who were involved in social media

Chapter 5:

1. Participants were college-aged athletes

2. Participants included only football athletes

3. Only the Sway concussion tool was used as a current concussion tool for comparison to salivary miRNA

1.8 Limitations

Chapter 3:

1. Our review is limited to available peer-reviewed literature

2. Procedures for sample processing are not well reported in some literature

Chapter 4:

1. Randomization of survey questions did not occur

Chapter 5:

1. Only one method of salivary miRNA sample processing was used

2. Participants completed Sway analysis on their own time

3. Collection of saliva samples occurred within 72 hours

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Epidemiology

2.11 Mild Traumatic Brain Injury Epidemiology

Each year, approximately 69 million individuals worldwide will suffer from a traumatic brain injury (TBI), which can be expressed as a global incidence rate of approximately 939 cases per 100,000 people.[25] Of these TBIs, mild traumatic brain injuries (mTBI) affects approximately 55.9 million individuals each year globally, or 740 cases per 100,000 people.[25] In the United States alone, estimates suggest that approximately anywhere from 1.6-3.2 million mTBI are sustained annually.[26–28] A contributing factor for this wide range estimate can be attributed to many mTBI going undiagnosed due to patients underreporting symptoms.[1,2] However, TBIs, including mTBI, place a significant burden on the health care system.[27] In 2010, the United States Centers for Disease Control and Prevention (CDC) reported 275,000 hospitalizations and approximately 1.4 million emergency room visits due to TBI.[28] These numbers represent a continually growing number of emergency room visits and hospitalizations, which increased 14.4% and 19.5%, respectively, from 2002 to 2006.[28]

2.12 Epidemiology in Sport

More than 200 million individuals partake in organized sports or other physical activities annually in the United States.[29] Approximately 38 million of these individuals are children or adolescents, while the other approximately 170 million are adults.[29] These individuals who partake in contact sports such as boxing, mixed martial arts, ice hockey, soccer, and particularly American football are at an increased risk for suffering from mTBI.[30]

According to the National Electronic Injury Surveillance System (NEISS), which is comprised of a sample of approximately 100 hospitals from across the United States, participation in individual sports from 1997 to 2019 resulted in an estimated 753,295 sport-related concussions which resulted in a visit to the emergency department (ED).[31] During that time, cycling was responsible for the most ED visits related to concussions, representing 38.8% (292,111) of cases from an individual sport.[31] Other individual sports which resulted in concussions were horseback riding (11.3%; 85,285), skateboard/scooter riding (10.3%; 77,671), snowboarding (8.9%; 67,179), snow skiing (8.0%; 60,262), wrestling (5.4%; 40,979), exercise without equipment (3.0%; 22,848), martial arts (1.7%; 12,549), boxing (1.5%; 11,313), in-line skating (1.5%; 11,252), exercise equipment (1.4%; 10,761), gymnastics (1.4%; 10,356), dancing (1.2%; 9,139), and swimming (1.1%; 8,824).[31]

Participation in team sports resulted in approximately 1,139,529 concussions which resulted in visits to the ED from 1997 to 2019.[31] As suspected, football was the team sport which was responsible for the most concussion related ED visits, representing approximately 40.3% (458,613) of all cases.[31] Other team sports which contributed to concussions that resulted in ED visits included basketball (18.5%; 211,139), soccer (16.2%; 184,385), baseball/softball (12.9%; 146,405), hockey (3.7%; 42,261), cheerleading (2.7%; 30,968), volleyball (2.2%; 25,556), lacrosse (1.8%; 20,962), and rugby (1.6%; 18,351).[31] Most shockingly, hockey and rugby did not contribute some of the highest amounts of ED visits from concussions, despite the contact nature of both sports. Additionally, Reid et al. reported that the patients who presented to the ED with concussion-like complaints were significantly younger than those who reported as a result of participation in individual sports ($p<0.001$).[31] The mean age of the individuals who reported

from team sports was 16.1 years old and the mean age of individuals who reported from individual sports was 22.8 years old.[31]

2.13 Age and Sex Considerations

A total of 136,592 patients reported to emergency departments from 1997 to 2019 according to the NEISS. This number represents a national estimate of approximately 4,471,431 patients reporting to the emergency department for a concussion.[31] The NEISS also reports that the estimated incidence of concussions reported to emergency departments experienced a 3-fold increases from 1997 to 2019.[31] Interestingly, this increase was most prominent in individuals aged 5-24 years old, however, this increase was seen through all age groups.[31]

Interestingly, data from many studies support the claim that this age range experiences a significant amount of concussions in sports.[32] However, the exact age ranges from each study can vary. For example, some studies found that the age range with the highest rate of concussions in sports comes from the 10-14 year old age range while others have found that the 12-14 year old range, 14-19 year old range, or 15-24 year old range experience the highest rate of concussions in sports.[32–36]

Reports on the differences in concussion rates between sexes vary throughout the literature. However, in a study of 193,757 high school student-athletes, it was found that the incidence rate of sport related concussions for male athletes was 1.9 per 100 player-seasons (95% CI, 1.8-2.0) and for female athletes was 1.5 per 100 player-seasons (95% CI, 1.4-1.6).[37] Overall, however, a clinical incidence rate of 1.7 per 100 player seasons (95% CI, 1.6-1.8) was found for all sports.[37] Additionally, it was found that when comparing males and females in sex-comparable sports, females were at a 1.9 (95% CI, 1.8-2.2) times greater risk of sustaining a

sports-related concussion. In the general population, however, it is suggested that males sustain concussions at about double the rate as females.[38]

2.2 Pathophysiology of Concussions

Despite the increase in research surrounding concussions, much is still misunderstood about the condition. One of the most recent commonly accepted definitions of a concussion comes from the 5th International Conference on Concussion in Sport:

> "*Sport related concussion (SRC) is a traumatic brain injury induced by biomechanical forces. Several common features that may be utilized in clinically defining the nature of a concussive head injury include:*
>
> - *SRC may be caused either by a direct blow to the head, face, neck or elsewhere on the body with an impulsive force transmitted to the head.*
> - *SRC typically results in the rapid onset of short-lived impairment of neurological function that resolves spontaneously. However, in some cases, signs and symptoms evolve over a number of minutes to hours.*
> - *SRC may result in neuropathological changes, but the acute clinical signs and symptoms largely reflect a functional disturbance rather than a structural injury and, as such, no abnormality is seen on standard structural neuroimaging studies.*
> - *SRC results in a range of clinical signs and symptoms that may or may not involve loss of consciousness. Resolution of the clinical and cognitive features typically follows a sequential course. However, in some cases symptoms may be prolonged.*
>
> *The clinical signs and symptoms cannot be explained by drug, alcohol, or medication use, other injuries (such as cervical injuries, peripheral vestibular*

dysfunction, etc) or other comorbidities (et, psychological factors or coexisting medical conditions).”[7]

As previously stated, concussions are not well understood, and this includes the pathophysiological effects of trauma causing a concussion.[39–41] What is known is the neurometabolic cascade which occurs after a head trauma. After initial trauma, cellular membranes are disrupted which results in neuronal depolarization from intracellular potassium moving across voltage-gated channels.[41] In response, the sodium-potassium pump attempts to restore the potential of the neuronal membrane by working excessively.[41] This, in turn, requires an increased amount of adenosine trisphosphate (ATP) which requires an increase in metabolism of glucose, often referred to as “hypermetabolism”.[41] This increase in metabolism occurs in an area where there is already decreased cerebral blood flow, and ultimately the supply and demand of glucose for the “hypermetabolism” results in cellular energy crisis. It is suggested that this cellular energy crisis may be a leading cause of a condition known as “second impact syndrome” where a second impact prior to resolution of a concussion results in further complications and lasting effects.[41]

After the initial spike in use of glucose, metabolism in a brain which has been concussed decreases.[41] In addition to this, calcium will continue to increase, and results in the impairment of oxidative metabolism of mitochondria, and ultimately increases the energy crisis within the brain.[41] This unrestrained accumulation of calcium may ultimately lead to cellular death.[41] Furthermore, lactic acid has been shown to generate within the brain, intracellular magnesium decreases, free radicals are produced, neurotransmission is altered, and other responses to inflammation are increased.[41]

2.3 Current Practices of Athletic Trainers

According to current position and consensus statements, healthcare providers with appropriate training in concussion recognition, diagnosis, and treatment should be providing care for individuals with these injuries.[7,9] Among those healthcare providers are certified athletic trainers.[42] In addition to outlining the individuals who should be providing care for concussions, the same position statements and consensus statements say that the recognition, diagnosis, and management of concussions should include a multiple tool approach.[7,9,42] This section will discuss current practices and common tools used by athletic trainers for concussion recognition, diagnosis, and management.

A recent study by Lempke et al. (2019) looked to assess the techniques used to assess and manage concussions by ATs.[16] The researchers were able to contact 8,777 ATs from the National Athletic Trainers' Association and had a response rate of 15% (n=1307). Of the ATs who responded, the median number of concussions assessed each year was 12 (range = 0-218), and a total of 52.7% reported using a multifaceted concussion assessment plan.[16] Additionally, the ATs reported that a concussion assessment tool, followed by a concussion symptom assessment was the most common method for assessing concussion status. In relation to participation practices, 91% of ATs reported the use of published guidelines pertaining to RTP as the most common method, followed by a clinical examination (88.2%).[16]

As mentioned, it is suggested that concussion assessments should use a multimodal approach.[7,9,42] Common domains that will be assessed include an evaluation of symptoms, balance, and neurocognitive performance.[16] When questioned about evaluation techniques of these domains, 81.8% (n=709) of ATs reported that the most common tool used for assessing symptoms was the symptom checklist from the Sport Concussion Assessment Tool (SCAT).[16]

When assessing balance, 78.7% (n=671) of ATs reported that the most common tool used was the Balance Error Scoring System.[16] A total of 83.5% (n=501/600) reported the use of the Immediate Post-Concussion Assessment and Cognitive Test (ImPACT; ImPACT Applications, Inc, Pittsburgh, PA) as the most commonly used tool for neurocognitive performance, and 57.3% (n=476/830) reported the Sport Concussion Assessment Tool – 5th edition (SCAT5) as the most commonly used standardized assessment tool.[16]

The SCAT5 has been recommended for use by the 2017 Concussion in Sport Group (CISG) as a result of the 5th international conference on concussion.[7] This tool can be used as a sideline evaluation tool for cognitive function and is a neuropsychological test battery. An individual's memory and attention function along with orientation and self-reported symptoms can all be assessed by a health care provider when administering this test.[7,43] However, research has demonstrated that this test may not be a reliable tool, particularly when it comes to patient self-reported symptoms.[44] Despite this, it is still a highly regarded tool when used as part of a multimodal approach to concussion management.[7,44] Additionally, a Child SCAT5 is widely available to use for the evaluation of children aged 5 to 12.[45]

The ImPACT tool is a computerized neurocognitive test that can be used for baseline examinations, post-injury examinations, and examinations to determine return to activity.[46] This test is comprised of questions regarding patient demographics, symptoms, and tests for neurocognitive function.[46] The modules within this tool evaluate a patient's verbal recognition memory, visual processing speed, visual working memory, attentional processes, numerical sequencing ability, learning, and reaction time.[46] Although this computerized neurocognitive test is the most widely used in the athletics setting, results from the research regarding reliability and validity are conflicting. Few studies have found the ImPACT tool to be a reliable test[47,48], while a

more recent study has provided evidence conflicting those findings.[49] Regarding validity of the ImPACT tool, a study performed by Alsalaheen et al. (2016) found that validation studies for this tool are inconclusive and may not provide significant data to support the widespread use of this tool.[50]

Relative to ATs making return to participation decisions, the two most used guidelines were from the 2014 NATA position statement on sport concussion[42] and the consensus statement from International Conference on Concussion and Sport[7] which was published in 2017.[16] Comparatively, both sets of guidelines recommend a graduated return to participation protocol and suggest that each step of the return to progression protocol increases in intensity.[7,42] If a patient experiences concussion related symptoms during a stage, they are to immediately stop activity for the day and will challenge the protocol once symptom free again.[7,42] Interestingly, 61.3% (n=579/944) of respondents selected the use of the NATA position statement on sport concussion[42], while the consensus statement from the International Conference on Concussion and Sport[7] was selected by 36.9% (n=348/944) of respondents.[16]

2.4 Biomarkers of Concussions

Methods for detecting biomarkers may include the use of imaging or laboratory analysis.[23] Imaging techniques may include computed tomography (CT), magnetic resonance imaging (MRI), diffusion tensor imaging (DTI), functional MRI (fMRI), magnetic resonance spectroscopy (MRS), positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic source imaging (MSI), and magnetoencephalography (MEG).[23,51,52] Biological indicators which may be detected through laboratory analysis include lipids, proteins, peptides, metabolites, ribonucleic acid (RNA), or protein autoantibodies which may be released injured or diseased tissue.[21,23]

2.41 Non-Biologic Measures

Despite the use of imaging techniques for the diagnosis of many injuries and illnesses, abnormalities associated with concussions are often not found on traditional imaging techniques, such as CT or MRI, due to the typical lack of structural damage from the injury.[7,23,51,52] These tools, however, may be used in cases where further injury, such as intracranial hemorrhage are suspected.[23,51,52] Due to the cost of testing, radiation exposure concerns associated with CT, and using resources appropriately, the use of CT or MRI are typically not warranted unless the patient demonstrates a decreased neurological status, persistent focal deficit, or symptom severity is increasing. Additionally, with the lack of accessibility to a scanner by many, and testing duration, these tools are often not employed.[23]

An additional imaging technique which may be used in the evaluation of concussions is DTI. This technique is an MRI-based technique that measures water molecule diffusion and can generate structural images of white matter tracts in the brain.[23,51–53] Additionally, DTI has the capability of detecting axonal injury.[23,52] Overall, DTI may have the ability to measure specific brain region abnormalities and predict deficits associated with concussions.[23] Although promising in the identification of axonal injury, even in the chronic stages of concussion, it is worthy to note that most studies analyzing DTI and concussions have been performed on concussed patients who have Glasgow Coma Scale (GCS) scores between 13-15, which is typical of patients whose extent of impaired consciousness is minor. Lastly, it is worthy to note that not all persons who have sustained a concussion have any abnormalities structurally.[23]

fMRI is a noninvasive technique which does not require exposure to radiation.[51,52] One of the most widely researched neuroimaging techniques in regards to concussion diagnosis and management, this technique has been used to measure functional activation patterns in patients at

various stages of concussions. To accomplish this, an individual will perform various tasks while undergoing an MRI, to identify the specific areas of their brain that are active when performing the various tasks. This tool has demonstrated the ability to be useful for determining when a patient has returned to baseline brain functioning, particularly with patients suffering from post-concussion symptoms.[52] According to a review by McCrea, et al., however, the results from fMRI studies are variable. Lastly, when compared to a traditional MRI technique, fMRI procedures require additional equipment, further analysis of the data, increased time requirements, and increased expenses, leaving this technique unlikely to become a common approach to concussion diagnosis anytime soon.

According to a review by Hunter, et al., MRS may provide a sensitive assessment of neurometabolite changes when traditional imaging techniques show no visible injury. Additionally, this tool has provided information regarding the prognosis of pediatric traumatic brain injury.[51] The challenges with the application of this tool lies in the analysis of MRS. Analysis requires complicated programs with individuals who are experts in employing these programs, which is not available at many institutions, ultimately making this technique inaccessible to many healthcare providers who may want to use it.[51] Additionally, this tool may not be readily available at many institutions for the use on patients with suspected concussions.[51]

Electroencephalography (EEG) is a clinical technique used to measure brain electrical activity.[51] In fact, EEG was the first tool, when used to analyze brain activity following a TBI, to detect abnormal brain activity.[54,55] A recent study demonstrated that when compared to traditional cognitive assessment tools, EEG analysis identified significant differences between individuals who had suffered from one or more previous concussions and individuals with no history of concussions.[56] Interestingly, the traditional cognitive assessment tools were not able to

identify any significant differences between the two groups.[56] Although promising as a potential tool to assist in the diagnosis and management of concussions, there is still research needed.[57,58] According to Nuwer et al., the relationship between clinical symptoms and EEG findings in later stages of head injury is questionable.[57] Additionally, although potentially identifying changes in brain activity initially following a brain injury, these changes may return to normal within hours, making rapid evaluation vital.[57] When compared to other neuroimaging techniques such as CT or MRI, EEG analysis is a much more affordable procedure, however, as mentioned previously, there is still much research needed to evaluate the likelihood of this tool which can aid health care clinicians in the diagnosis and management of concussions.[59]

PET and SPECT imaging are two additional methods which involve nuclear medicine to capture imaging of the brain. Although no studies have utilized these tools for the investigation of sport related concussions, there have been studies which have used these tools for the investigation of non-sports related concussions.[51,52] Both PET and SPECT imaging can be used to measure brain activation patterns, which may be useful in the diagnosis and management of sport concussions, however, there are limitations with the use of these tools. The biggest limitations associated with the use of these tools are the exposure to radiation by the patient, expense of imaging, and the amount of time it takes to perform and analyze images from these tools.[52] Abnormal brain activities can also be detected through the use of MEG and MSI.[51,52] The limitations associated with the use of these tools include access to gradiometers or magnetometers associated with these tools, along with the lack of studies using these tools for the diagnosis or management of sport related concussions.[51,52]

2.42 Biologic Indicators

Biological indicators such as microRNA (miRNA), S100 calcium binding protein B (s100B), neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP), ubiquitin carboxy-terminal hydrolase L1 (UCHL1), and myelin basic protein (MBP) may be quantified in cerebrospinal fluid (CSF), serum, plasma, saliva, and urine.[21,23] Of the above biological indicators, s100B is the most studied and can be quantified in CSF and serum. In serum, this biomarker has been shown to be increased immediately after injury and eventually decreases in patients with concussions.[23] The largest concern surrounding the use of this biomarker is the protein's inability to cross an individual's intact blood-brain barrier (BBB).[23] This raises the question as to whether the increases shown in patients who have suffered a concussion actually correlate to the amount of brain damage suffered from the injury.

NSE can be quantified in serum within 6 hours after injury but has a half-life of only about 24 hours. Although demonstrating high sensitivity to mortality, it has not demonstrated the same with neuropsychological outcomes. Jeter, et al. states that this tool should not be used in isolation as an indicator of brain trauma, as it has also been shown to be a marker for other various conditions such as stroke, bladder tumors, lung cancer, and neuroblastomas.[23]

A protein that has demonstrated the ability to distinguish patients who have suffered a concussion from uninjured patients in GFAP. This protein is released after a concussion, is brain-specific, and has been shown to be a predictor of reduced arterial pressure, elevated intracranial pressure, and poor GCS scores.[21,23] Although this protein can be quantified in serum, and has shown utility in concussion diagnosis, there is a lack of evidence related to the application for injury monitoring or return-to-activity considerations. A similar biomarker, UCHL1, has also demonstrated the ability to distinguish patients who have suffered brain injury. UCHL1 has been

quantified in CSF and serum, and has shown a significant correlation with the permeability of one's BBB.[23]

If demyelination is suspected due to a neurological condition, a MBP test can be used to assess the patient. Interestingly, MBP may cause the BBB to become permeable, allowing for the entry of this protein into circulation. This capability of MBP may indicate the ability of this tool as an indicator of concussion, however, no studies have measured this protein in individuals who have sustained a concussion.[23]

MiRNA can be quantified in CSF, serum, plasma, saliva, and other body fluids.[21,23] These ribonucleic acids are found in humans, animals, and even plants.[60,61] MiRNA have been shown to activate gene expression, including neuronal gene expression, are crucial for natural development, cell proliferation, and muscle differentiation.[60,61] Naturally, with these roles, any alterations in the expression of miRNA, sequence of miRNA, or target sites for miRNAs could indicate genetic disorders or diseases, most notably conditions such as cancer.[60,61] Recent research has demonstrated that these biomarkers may be an ideal indicator of brain injury such as concussions, and may provide other information about the injured brain which can assist in the tracking of brain recovery.[21,24,62] Recent large studies by Hicks, et al. and Di Pietro, et al. have identified specific miRNA targets that can potentially assist in diagnosing concussions, relationships with the number of previous concussions sustained, and relationships with the amount of head impacts an individual sustains over the course of a practice.[24,62,63]

Of the biomarkers which have been described above, salivary miRNA is arguably the most favorable candidate for a concussion biomarker. In addition to the evidence provided above in relation to the tool's utility, there are a few characteristics of salivary miRNA which add to the potential of the tool's utility. Unlike circulating proteins, miRNA are resistant to degradation, are

abundant, remain stable despite pH levels which may fluctuate, and do not require a disruption of an individual's BBB.[21,64] Additionally, it is highly likely that within the oropharynx, there will be rapid expression by the cranial nerves (V,VII, IX, X, XII). Lastly, with the appropriate collection devices, which are non-invasive unlike the methods to quantify blood-based proteins, miRNA are stable at room-temperature.[21]

CHAPTER III

PROJECT I: SALIVARY MICRORNA AS A PROSPECTIVE TOOL FOR CONCUSSION DIAGNOSIS AND MANAGEMENT: A SCOPING REVIEW

3.1 Introduction

Estimates indicate that over 2 million concussions occur annually in the United States alone, and over 42 million occur worldwide.[30,65] Despite so many occurrences, it is hypothesized that this number underrepresents the actual number of occurrences due to underreporting of patients to healthcare professionals.[2,3] With concussion diagnosis and management relying heavily on subjective input, specifically self-reported symptoms and other assessment tools which lack exclusive effectiveness, patients must report their symptoms accurately.[2,66,67] Signs and symptoms of concussions may vary between individuals. However, the most common symptoms associated with concussions include dizziness, headache, loss of consciousness, and amnesia.[67]

Concussions result from forces caused by a direct or indirect blow to an individual's head, ultimately resulting in a rapid acceleration or deceleration of the individual's brain within the skull.[7,8,40,67] Despite concussions occurring from a mechanism which can be seen across many activities such as motor vehicle accidents, falls, assaults, etc. much of the research surrounding concussions involves mechanisms seen in sporting events. This may be attributed to concussions being such a common sport injury.[9,68] These injuries can affect individuals of all ages ranging from youth to elderly. [69–74] Due to the typical lack of macroscopic structural damage sustained by the brain, objective tools such as magnetic resonance imaging (MRI) or computed tomography (CT) scans cannot identify concussions.[66,67,75] Instead, the use of these tools can rule out a more

significant injury such as a severe traumatic brain injury like subdural hematomas or epidural hematomas.[66,67,75]

The most recent international consensus statement on concussion in sport and the National Athletic Trainers' Association position statement on managing sport concussions suggest using multiple assessment tools to diagnose and manage concussions.[7,8] Some of the most common tools athletic trainers use to manage concussions include the Sport Concussion Assessment Tool 5th edition (SCAT5), Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT) tool, and the King-Devick test.[16,46] Although widely used, none of these tools have shown exclusive effectiveness and must be used in conjunction with other instruments.[66,67] Despite the ImPACT tool yielding promising sensitivity and specificity when used to assess high school and collegiate athletes,[76,77] the tool has also demonstrated poor reliability and validity, especially with test-retest protocols.[10,11,13,49,50,78] With the limitations of current assessment tools used to identify concussions, there is a need to identify novel objective tools.

Recently, research has focused on identifying biomarkers as an objective tool for diagnosing and managing concussions; such biomarkers include neuronal imaging, proteins, and metabolites.[21] With the discovery of chronic traumatic encephalopathy (CTE) and the condition's potential link to concussions, significant research over the past decade has focused on proteins such as Tau proteins, glial fibrillary acidic protein (GFAP), and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) as potential biomarkers of concussions.[6,21] Although showing potential, proteins are subject to degradation, making the identification of their presence often complex and highly time-sensitive. Additionally, they may require the disruption of an individual's blood-brain barrier (BBB) to present in biofluids, making collection difficult, ultimately hindering their applicability.[21,60,75,79–82] More recently, the use of microRNAs (miRNA) as an objective tool for

diagnosing and managing concussions has also shown promise due to the ability to resist degradation, abundance, and stability despite fluctuating pH levels, and the role they play in the regulation of transcription. Furthermore, miRNAs play a significant role in neuronal injury and repair.[21]

Mature miRNAs are small (19-28 nucleotides), non-coding, endogenous molecules that regulate the synthesis of protein by targeting messenger RNA (mRNA) post-transcriptionally.[21,80,81,83] Initial transcription occurs in the nucleus and forms primary microRNAs (pri-miRNA) greater than 1000 base pairs in length and shaped in a hairpin structure. While still in the nucleus, pri-miRNAs are shortened into precursor microRNAs (pre-miRNA), which are approximately 60-100 nucleotides in length, by a type of ribonuclease (RNase) called Drosha. Pre-miRNAs move into the cytoplasm where another enzyme, Dicer, removes the loop structure and forms miRNA:miRNA duplexes. One-half of the miRNA:miRNA duplex will become a mature miRNA, whereas the other half will be subject to degradation. Mature miRNAs will then be bound within a microRNA-induced silencing complex (miRISC) and target mRNA to inhibit protein synthesis. The targeted mRNA will then be subject to degradation.[21,60]

It has been shown that saliva, serum, plasma, urine, and cerebrospinal fluid (CSF) are biofluids where miRNA can be quantified.[21] Of the more than 2000 miRNAs identified in humans, approximately 70% are estimated to be expressed in the CNS.[21,84] Of those 2000 identified, 291 miRNAs show a promising role associated with concussion diagnosis and management. However, only 17 miRNAs have been shown to be altered consistently across multiple studies, using three biofluids (blood, CSF, saliva).[21,82,85] Unlike blood-based miRNAs that must cross the BBB, salivary miRNAs show potential in the acute diagnosis of concussions

due to the likelihood of the rapid expression of miRNA by the cranial nerves (V, VII, IX, X, XII) in the oropharynx. If proven correct, this would allow for clinicians to perform rapid, non-invasive sideline assessments. Additionally, salivary miRNAs can remain stable at room temperature when proper RNA collection devices are used.[21]

The availability of an objective method of concussion assessment that clinicians can use in a variety of non-clinic settings would drastically improve the diagnosis and management of concussions, especially in athletic and tactical settings. Therefore, the purpose of this scoping review is to thoroughly evaluate the existing literature on salivary miRNAs use during concussion evaluation, diagnosis, and management. Furthermore, we chose to deem this a scoping review rather than a systematic review due the aim of salivary miRNA and the potential use for concussion practices being a broad topic.

3.2 Methods

3.21 Search sources and eligibility criteria

We completed a scoping review using PubMed, CINAHL, and SPORTDiscus to identify the use of salivary miRNAs as objective tools for concussion diagnosis and management. The search terms included "concussion", "traumatic brain injury", "TBI", "mTBI", "microRNA", and "miRNA". Only studies using human subjects and those published in English were included in this review. Original research articles were deemed appropriate if they included an analysis of the utility of salivary miRNAs as a tool for concussion diagnosis or management. Articles were accepted if the researchers examined miRNA expression levels from other biofluids only if compared to salivary miRNAs.

3.22 Study selection

This review was focused on saliva as a biofluid as compared to other biofluids due to its applicability to clinical practice. The use of other biofluids, such as blood or CSF, is much more invasive and requires further training when compared to saliva collection, making these biofluids more difficult to implement in certain practice settings. After removing duplicates from the list of articles, each article's reference list was hand searched for other relevant articles.

3.23 Quality appraisal

The Strengthening the reporting of Observational Studies in Epidemiology (STROBE) statement[86] was used to appraise the quality of all articles in this review. Results of each articles appraisal can be found in Table 1. The search and appraisal of all articles was deemed complete on September 7, 2021.

3.24 Data extraction

Data extracted from each article included the number of subjects enrolled in each phase of the study, the time of saliva sample collection during each phase of the study, and the salivary miRNA identified as potential concussion biomarkers from the results of each study.

3.25 Data Availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

3.3 Results

The search parameters described above resulted in 293 total articles, from which only nine were deemed appropriate for this review. All articles included in this review were published between 2018 and 2021. During the initial screening phase, fourteen articles were removed due to duplication (*n*=279). Upon further investigation bsy reading titles and abstracts, 269 additional

articles were eliminated for the following reasons: only analysis of miRNA found in blood (*n*=166); only analysis of miRNA found in CSF (*n*=102); non-human subjects (*n*=10). See Figure 1 for a description of screening methods as presented in a PRISMA-style diagram.[87]

Critical appraisal was performed by two readers using the STROBE statement for cohort studies.[86] After full article reading, one article was removed due to a lack of reported results (*n*=9). Reviewers met with a third-party arbitrator to discuss the scoring of each article. In scenarios where we disagreed on scores, a discussion occurred, and a consensus was determined. Upon reading nine articles, specific miRNAs relating to the diagnosis or management of concussions were identified. The results of each article can be found in Table 1 and will be further described later in this review.

3.31 Salivary miRNA as potential diagnostic tools

A total of thirty-four salivary miRNAs were identified across six studies as showing potential for being an objective concussion diagnostic biomarker.[22,63,75,80,81,83] A study performed in 2018 by Di Pietro et al. resulted in the following five miRNAs being significantly upregulated, as indicated by greater concentrations being present, in six concussed patients when compared to six non-concussed matched controls: miR-27b-3p, let-7i-5p, miR-142-3p, miR-107, miR-135b-5p.[75] In that same year, the following six salivary miRNAs were identified as showing parallel changes in saliva and CSF in a study of pediatric traumatic brain injury patients (control (*n*=18), mTBI (*n*=60)): miR-182-5p, miR-221-3p, miR-26b-5p, miR-320c, miR-29c-3p, miR-30e-5p.[80] Hicks et al. later identified the following four miRNA which accurately distinguished concussed individuals from non-concussed individuals: miR-34a-5p, miR-192-5p, miR-27a-5p, miR-4510.[81] Additionally, LaRocca et al., identified that the expression levels of the following twelve salivary miRNAs were significantly altered from pre- to post-fight in adult martial artists (*n*=50):

let-7b-3p, miR-2682-5p, miR-3118, miR-3170, miR-3919, miR-433-3p, miR-4632-3p, miR-4660, miR-4760-5p, miR-601, miR-608, miR-6870-3p.[83] Interestingly, the authors of this study did not state whether any adult MMA fighters were clinically diagnosed with a concussion.[83]

A recent study by Di Pietro et al. looked to identify salivary miRNA, which accurately distinguished concussed from non-concussed elite professional rugby players. From this study of over 1,000 participants, the researchers identified the following miRNA: let-7f-5p, let-7a-5p, miR-143-3p, miR-103a-3p, miR-34b-3p.[63] This panel of miRNA was able to differentiate between concussed and non-concussed individuals with a 0.91 area under the curve (AUC) immediately following a competition and 0.94 AUC 36-48 hours following competition.

To refine the list of miRNAs that have been discovered as being associated with concussion diagnosis, Hicks et al. analyzed 455 saliva samples from 314 individuals across ten different sites.[22] Using these samples, the researchers performed within-subjects testing to eliminate miRNA that significantly changed over the course of a single exercise session or from participation in a season of contact sports. This study revealed that a ratio between two salivary miRNA (miR-27a-5p/miR-30a-3p) was able to distinguish between concussed and non-concussed participants with high accuracy (AUC = 0.81). This ratio yielded a sensitivity of 82.4% and a specificity of 73.3%.[22]

3.32 Salivary miRNA as potential management tools

Johnson et al., identified the following salivary miRNA in patients who suffered from prolonged concussion symptoms (*n*=52, mean age=14): miR-320c-1, miR-133a-5p, miR-769-5p, let-7a-3p, miR-1307-3p.[84] The researchers collected initial saliva samples within fourteen days of the injury and performed follow-up measurements four weeks post-injury.[84] Interestingly, the salivary miRNAs mentioned previously which were identified as a potential diagnostic tool by

Hicks et al.,[80] also showed longitudinal trends that may infer the utility of those salivary miRNAs as a prognostic indicator. If shown to be a reliable indicator of concussion prognosis, this could mitigate an early return to activity and potentially decrease the likelihood of further complications associated with a brain that has not fully recovered from a concussion.

More recently, an additional study by Hicks et al. revealed three miRNA which differed between individuals with a history of concussions as compared to individuals with no history of concussions: miR-28-3p, miR-339-3p, miR-361-5p.[62] The results from this study indicate that there are significant physiological changes within an individual as a result of multiple concussions. Lastly, a recent study by Fedorchak et al. identified seven salivary miRNA that, when combined with nine other non-coding RNAs (ncRNAs), balance, and cognition measures in an algorithm, was able to accurately predict the presence of persistent post-concussion symptoms (PPCS) in patients who had suffered a concussion with an AUC of 0.86.[88] When samples were obtained within 14 days of the initial injury, the ncRNAs themselves, without the inclusion of balance and cognition measures, demonstrated utility for prognosis of PPCS.[88] The researchers compared the performance of the algorithm against solely performance of balance and cognitive tests, as well as a validated clinical prediction rule for concussions.[89]

3.33 Overlapping miRNA

As previously mentioned, over 2000 miRNA have been identified in humans, all having various functions throughout the body. Due to the novelty of this topic, research included in this review analyzed a large amount of those miRNA as potential targets in hopes to narrow the target miRNAs and identify a smaller subset which may act as a concussion biomarker. Throughout the nine studies presented in this paper, there were five families of miRNA which demonstrated a relationship to concussion diagnosis and/or management across multiple studies.

The miR-27, miR-30, and miR-34 families have demonstrated the ability to act as a diagnostic biomarker for concussions across multiple studies.[22,63,75,80,81] Additionally, the miR-320 family has shown potential as acting as a prognostic indicator of concussions across two studies presented in this paper.[80,84] Interestingly, the let-7 family has demonstrated the ability to act as a potential tool to diagnose and manage concussions across four different studies.[63,75,83,84] With the demonstrated utility for both diagnosis and management of concussions, the researchers suggest that the let-7 family should be a target miRNA in future studies analyzing the relationship with concussions.

3.4 Discussion

This scoping review found that forty-nine salivary miRNAs have been identified as potential biomarkers of concussions which can be used for diagnosis and management (Table 2). With further research, these miRNAs could provide more objective measures that clinicians along with other healthcare clinicians could use to improve patient outcomes after sustaining a concussion. The saliva collection method is non-invasive and requires minimal training, allowing for many healthcare providers to use this potential tool in practice.[21] Additionally, miRNAs are rapidly expressed through the cranial nerves and remain stable at room temperature, making these molecules a promising concussion diagnostic and management tool.[21]

Concussions result in long-term effects on individuals, including impairments on cognitive and motor function. There is also potential that repeated concussions may lead to more severe conditions such as CTE.[20,85,90] Despite the efforts of many to improve the current knowledge of concussions, improve athlete safety, and improve concussion management tools, concussions are becoming a significant health issue globally.[7]

Current measures used by clinicians for diagnosing and managing concussions rely heavily on subjective patient input and lack reliable, objective measures.[2,66,67] Research on these current measures has shown that individuals may demonstrate suboptimal performances during baseline assessments.[10] In turn, these baseline assessments could cause some concussions to go undiagnosed when compared to post-injury assessments that demonstrate truly suboptimal performances. With the articles presented in this scoping review demonstrating the ability of salivary miRNA as a biologic marker to distinguish between concussed and non-concussed individuals, this may potentially limit the number of concussions that may go undiagnosed.[22,63,75,80,81]

This scoping review identified forty-nine salivary miRNA across nine studies which may prove to be helpful concussion diagnostic and/or management biomarkers with continued research. Of the forty-nine different salivary miRNA, thirty-four correlated to the diagnosis of concussions,[22,63,75,80,81,83] while twenty-one correlated to symptom trends or multiple concussions.[62,80,84,88] Interestingly, six of the twenty-one which correlated to symptom trends also accurately distinguished concussed subjects when compared to non-concussed subjects: miR-182-5p, miR-221-3p, miR-26b-5p, miR-320c, miR-29c-3p, miR-30e-5p.[80] Despite the overlap of miRNA being identified, if salivary miRNA are identified as a valid and reliable tool for concussion diagnosis, it would still require clinicians to perform multiple swabs to track a patient's injury progression. Even though the specific miRNA associated with concussion diagnosis may not be present in a follow up test, this may not truly indicate resolution of injury, as demonstrated by the prolonged expressions of salivary miRNA associated with concussion symptom resolution.

Previous research has shown individuals may demonstrate a learning curve with current assessments.[91] However, with the ability of salivary miRNA to identify long-term changes in previously concussed individuals, this may improve patient outcomes by clinicians a biologic marker that may assist in the prognosis of concussions.[62,84,88] This characteristic of salivary miRNA may better predict recovery time and return to activity timelines.

3.41 Limitations

Despite a large amount of research efforts focused on concussions, much of the literature focuses on sport-related concussions, specifically research surrounding salivary miRNA. With the novelty of salivary miRNAs as an objective measure for concussion diagnosis and management, there are still a limited number of studies published. There are quite a few limitations of the studies published that need addressing before this tool can be universally accepted. Most of the articles described in this review lacked information on the effects associated with orthopedic injuries at the time of sample saliva collection and the use of oral equipment such as mouthguards commonly used in athletics. Additionally, the sample sizes used in these studies are smaller, making it important for future studies to recruit larger samples when analyzing the utility of salivary miRNAs as a potential concussion biomarker. Furthermore, although collection of salivary miRNA samples is non-invasive, current procedures for processing samples can be time consuming and potentially expensive. Lastly, despite some saliva sample collection kits having the capability to stabilize samples and store them for extended periods of time, if not analyzed in a reasonable amount of time, samples must be frozen in order to ensure miRNA stability. With future efforts focused on research and development, processes may be developed which may decrease the time of sample processing or increase collection kits' abilities to stabilize samples for extended periods of time.

3.5 Conclusions

Despite the limitations previously mentioned, salivary miRNAs may act as a concussion biomarker. Before these can be used to assist clinicians in diagnosing or managing concussions, the limitations must be addressed. Importantly, research should be expanded to include concussions resulted from other mechanisms of injury other than sport such as, falls, motor vehicle accidents, assaults, etc. If identified as a reliable tool, this objective measure could add to many clinicians' toolboxes improve the accuracy of diagnosis and subsequent management of concussion injuries, ultimately improving patient outcomes after concussions.

Many healthcare clinicians diagnose and manage concussions throughout the world. Current research has identified the limitations of using many current measures used to diagnose and manage concussions.[2,10,67,91] However, with the ease of saliva collection and the ability to accurately diagnose concussions and predict recovery times, salivary miRNA could become another tool added to current practices of clinicians. In general, the addition of this biomarker could aid in improving concussion diagnostic and management practices, limit the number of individuals with concussions who go undiagnosed, and ensure that individuals do not return to activity before full recovery from a concussion.

	STROBE raw score (/22)	STROBE percent score	Number of subjects	Time of sample collection	Salivary miRNA identified
Di Pietro et al. 2018[75]	21	95	20 (only 12 analyzed for salivary miRNA, all 20 for proteins) (half control)	48-72 hours from concussion	miR-27b-3p, let-7i-5p, miR-142-3p, miR-107, miR-135b-5p (5)
Di Pietro et al. 2021[63]	20	91	1,028 (included were 102 uninjured players, 66 players with musculoskeletal injury)	Pre-season, in-game, post-game, and 36-48 hours post-game	let-7f-5p, let-7a-5p, miR-143-3p, miR-103a-3p, miR-34b-3p (5)
Fedorchak et al. 2021[88]	17	77	505 samples from 112 individuals	PRE $\leq$ 14 days post-injury; POST $\geq$ 21 days post-injury	miR-486-5p, miR-1246, miR-92b-3p, miR-203a-5p, miR-148a-5p, miR-100-5p, miR-148-3p (7)
Hicks et al. 2018[80]	21	95	89 (CSF = 8/3; Saliva = 60/18)	<24 hours after injury	miR-182-5p, miR-221-3p, miR-26b-5p, miR-320c, miR-29c-3p, miR-30e-5p (6)
Hicks et al. 2020[81]	20	91	538 (251 with concussion)	$\leq$3, 4-7, 8-14, 15-30, 31-60 days post-concussion	miR-34a-5p, miR-192-5p, miR-27a-5p, miR-4510 (4)
Hicks et al. 2020[62]	19	86	310 (230 control, 56 single concussion, 24 recurrent concussion)	No information regarding time after concussion	miR-28-3p, miR-339-3p, miR-361-5p (3)
Hicks et al. 2021[22]	21	95	455 samples from 314 individuals	15-60 min before exercise and within 20 min post-exercise; before contact sports season then within 30-115 days following; within 24 hr of concussion	miR-27a, miR-30a (2)
Johnson et al. 2018[84]	20	91	61 initially recruited, 52 included in the analysis	Within 14 days of initial injury; 4-8 week follow-up	miR-320c-1, miR-133a-5p, miR-769-5p, let-7a-3p, miR-1307-3p (5)
LaRocca et al. 2019[83]	19	86	50 (218 samples – 87 saliva, 131 serum)	PRE (1 week or 1 hour); POST (15-30 min, 2-3 days, 1 week, 3+ weeks)	let-7b-3p, miR-2682-5p, miR-3118, miR-3170, miR-3919, miR-433-3p, miR-4632-3p, miR-4660, miR-4760-5p, miR-601, miR-608, miR-6870-3p (12)

Table 1. Results of reviewed articles, including identified salivary miRNAs as diagnostic or management tools for concussions.

Saliva miRNA	*Diagnosis*	*Management*
miR-27a[22]	X	
miR-30a[22]	X	
miR-486-5p[88]		X
miR-1246[88]		X
miR-92b-3p[88]		X
miR-203a-5p[88]		X
miR-148a-5p[88]		X
miR-100-5p[88]		X
miR-148-3p[88]		X
let-7f-5p[63]	X	
let-7a-5p[63]	X	
miR-143-3p[63]	X	
miR-103a-3p[63]	X	
miR-34b-3p[63]	X	
miR-34a-5p[81]	X	
miR-192-5p[81]	X	
miR-27a-5p[81]	X	
miR-4510[81]	X	
miR-28-3p[62]		X
miR-339-3p[62]		X
miR-361-5p[62]		X
let-7b-3p[83]	X	
miR-2682-5p[83]	X	
miR-3118[83]	X	
miR-3170[83]	X	
miR-3919[83]	X	
miR-433-3p[83]	X	
miR-4632-3p[83]	X	
miR-4660[83]	X	
miR-4760-5p[83]	X	
miR-601[83]	X	
miR-608[83]	X	
miR-6870-3p[83]	X	
miR-27b-3p[75]	X	
let-7i-5p[75]	X	
miR-142-3p[75]	X	
miR-107[75]	X	
miR-135b-5p[75]	X	
miR-320c-1[84]		X
miR-133a-5p[84]		X
miR-769-5p[84]		X
let-7a-3p[84]		X
miR-1307-3p[84]		X
miR-182-5p[80]	X	X
miR-221-3p[80]	X	X
miR-26b-5p[80]	X	X
miR-320c[80]	X	X
miR-29c-3p[80]	X	X
miR-30e-5p[80]	X	X

Table 2. Salivary miRNA which have been identified as potential biomarkers for concussion diagnosis and/or management.

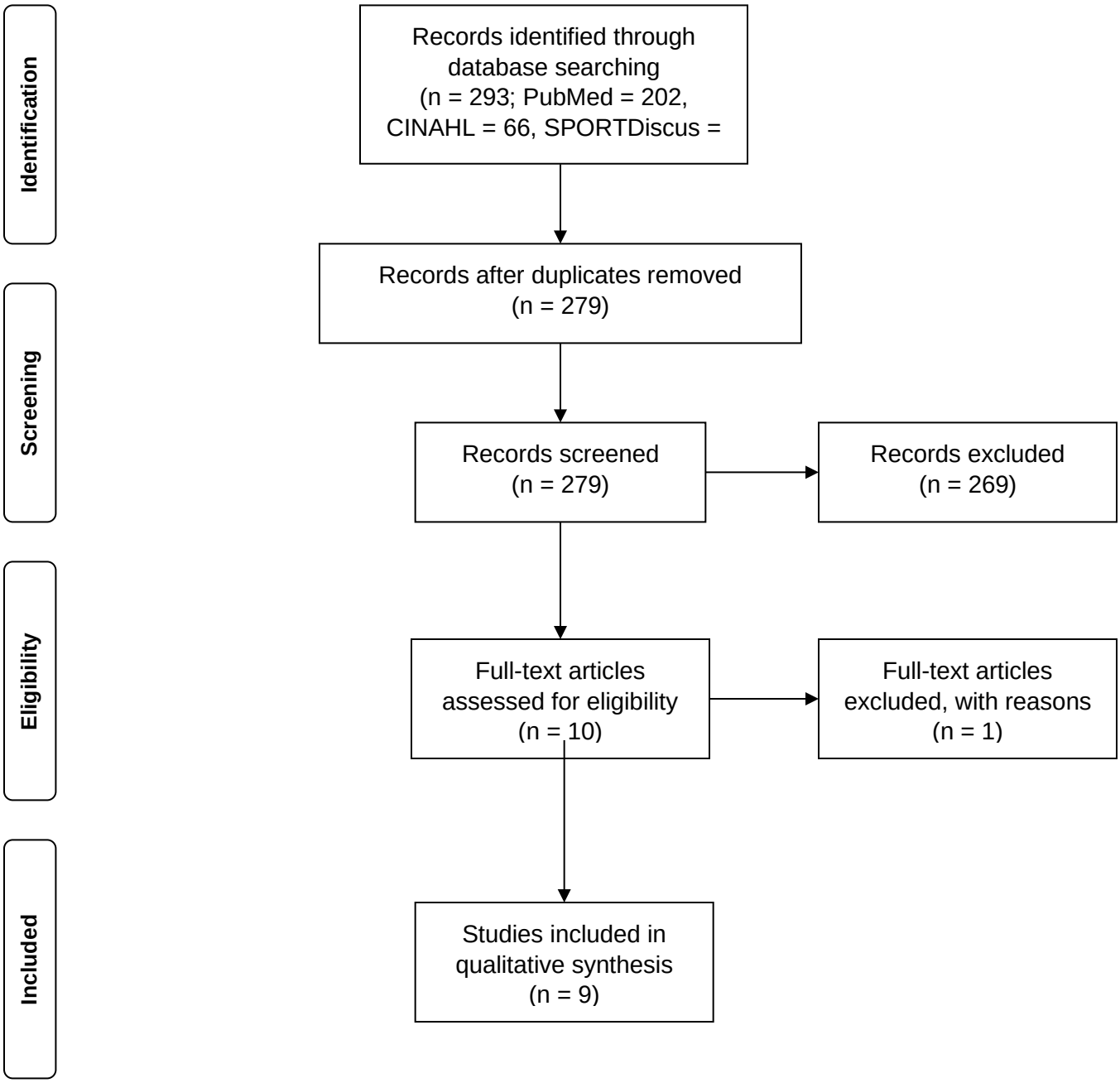

Figure 1. PRISMA style flow diagram of included and excluded research articles.[87]

CHAPTER IV

PROJECT II: ATHLETIC TRAINERS' FAMILIARITY WITH AND ATTITUDES TOWARDS THE FUTURE IMPLEMENTATION OF POTENTIAL CONCUSSION BIOMARKERS

4.1 Introduction

Athletic trainers (ATs) are highly qualified and skilled health care professionals who receive thorough training in the recognition, evaluation, and management of concussions.[42] It is suggested that ATs should be present at all organized sport events throughout all levels of competition, so, many times concussion assessments and return to activity protocols for patients are often first performed by ATs.[14,42] In collaboration with a physician, the diagnosis, management, and return to activity following a concussion rely on ATs being knowledgeable, comfortable, and familiar with current best practices.[14–16] A lack of knowledge, comfort, or familiarity with these best practices may result in the misdiagnosis, delayed diagnosis, or mismanagement of a concussion, ultimately leading to a patient's prolonged recovery.[14] It is imperative that ATs are knowledgeable with the current best practices for the prevention, evaluation, diagnosis, and management of concussions.

Current concussion diagnosis and management strategies rely heavily on patients self-reporting symptoms and research has demonstrated that athletes in particular may underreport symptoms.[1] Ultimately, this may hinder a clinician's ability to effectively manage a concussion, contributing to potential long term effects such as mental or behavioral health problems, cognitive impairment, and potentially long-term conditions such as chronic traumatic encephalopathy (CTE) or Alzheimer's disease.[1,5] Additionally, immediate effects following concussion, such as balance and other motor control disturbances, and increased risk for further

injury may be exacerbated if not recognized and treated appropriately.[19,42,92,93] With the reliance on subjective measures for concussion diagnosis and management, recent research has been focused on the identification of reliable and valid objective measures of concussions, including concussion biomarkers.[23] However, even if identified and found to be reliable and valid, the implementation into clinical practices would be easier and potentially quicker if healthcare providers, including ATs, were knowledgeable about them and demonstrated positive attitudes toward their implementation.

A biomarker is defined as a "distinctive biological or biologically derived indicator (such as a metabolite) of a process, event, or condition (such as aging, disease, or oil formation)."[94] Methods for detecting biomarkers may include the use of imaging or laboratory analysis.[23] Imaging techniques may include computed tomography (CT), magnetic resonance imaging (MRI), diffusion tensor imaging (DTI), functional MRI (fMRI), magnetic resonance spectroscopy (MRS), positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic source imaging (MSI), and magnetoencephalography (MEG).[23,51,52] Biological indicators which can be detected through laboratory analysis include lipids, proteins, peptides, metabolites, ribonucleic acid (RNA), or protein autoantibodies which may be released as a result of injured or diseased tissue.[21,23] These biological indicators can be measured in bodily fluids, or biofluids, such as cerebrospinal fluid, plasma, whole blood, serum, urine, and/or saliva.[21,23,95]

With the increased emphasis of research aimed at discovering a reliable and valid concussion biomarker, the implementation of such a tool would require all healthcare professionals, in particular ATs, to be knowledgeable with biomarkers, how they are assessed, and be willing to implement the measure into practice. Therefore, the purpose of our study was

to assess ATs' familiarity with potential concussion biomarkers and evaluate ATs' attitudes toward the future implementation of a biomarker assessment into their concussion practices.

4.2 Methods

We used a cross-sectional, online survey design to achieve our research aim. The Old Dominion University College of Health Sciences Human Subjects Review Committee determined this study to be exempt research, and all participants were required to provide electronic consent prior to beginning the survey.

4.21 Instrumentation

We performed a literature review to identify previous surveys that aimed to evaluate participants' familiarity with and attitudes towards concussion biomarkers. Due to the lack of preexisting instruments that achieved this aim, we developed a new instrument. The survey consisted of 3 sections, (1) demographics (2) current concussion practices, and (3) biomarker familiarity and attitudes toward future implementation. Demographic questions included current and previous job settings, years of experience, highest degree earned, and NATA district (regional geographic location). Question formatting varied to achieve the aims of the study and answer research questions, and included Multiple Choice, Select All That Apply and Likert scale responses. The survey was created and distributed using Qualtrics (Qualtrics LLC, Provo, UT).

4.22 Survey Validation

Questions were created and refined by the research team, then reviewed for content validity by three content experts. The experts consisted of two athletic trainers with previous clinical experience, one having expertise on the epidemiology of sport-related concussion, the other with expertise in clinical measures of sport concussion, and the third being a pediatrician with expertise in biomarkers of concussion. All three are leading researchers in the field of

concussion and mild traumatic brain injury. Each expert was contacted via e-mail to participate in the validation process.

Content validity of the survey was assessed by using an item-level content validity index (I-CVI) tool.[96,97] The requested validation process included reviewing the survey, completing a validity rubric which graded the importance and clarity of each question, and to rate its relevance toward the aim of the study. Experts rated each survey item on a 4-point scale (1 being not relevant/clear and 4 being very relevant/clear) and were asked to provide comments to improve the clarity or relevance of each item that was rated below a 4.[96,97] Once all validity rubrics were returned, all suggested edits and revisions were applied to the questions and survey, which included the elimination of open-ended questions that were determined to not contribute to the aim of the study. Additionally, further choice options for multiple choice and select all that apply questions were included. The final I-CVI score, once all revisions were made, was 0.90, signifying consistent agreement across all reviews for the remaining items.[96,97]

4.23 Current Concussion Practices

Two questions aimed to summarize participants' current concussion practices. Participants were first asked to identify all measures used to determine a concussion diagnosis in their current practice (select all that apply). Next, participants were asked to indicate their level of comfort in diagnosing and managing concussions as well as their level of comfort in returning a patient to activity following a concussion in their current clinical practice (7-point Likert scale: 1. Extremely comfortable; 2. Moderately comfortable; 3. Slightly comfortable; 4. Neither comfortable nor uncomfortable; 5. Slightly uncomfortable; 6. Moderately uncomfortable; 7. Extremely uncomfortable).

4.24 Biomarker Familiarity and Attitudes

Participants' familiarity with biomarkers and their attitudes towards including biomarker assessments in their clinical practice were assessed via nine questions. Participants were first asked to identify biofluids and biomarkers that they were currently familiar with (select all that apply). Following each of these questions were follow-up questions in which the participant was asked to identify the mechanism by which they learned about those biofluids and biomarkers (select all that apply). Next, participants were asked to self-rate their knowledge of the current literature surrounding the use of biomarkers for concussion diagnosis and management via 5-point Likert scale (1. Extremely knowledgeable; 2. Very knowledgeable; 3. Moderately knowledgeable; 4. Slightly knowledgeable; 5. Not knowledgeable at all). Finally, participants were asked to rate their level of agreement with statements about their attitudes toward the future implementation of concussion biomarkers into clinical practice via a 5-point Likert scale (1. Strongly agree; 2. Somewhat agree; 3. Neither agree nor disagree; 4. Somewhat disagree; 5. Strongly disagree).

4.25 Procedures

The survey was distributed to 1000 clinically practicing ATs via the National Athletic Trainers' Association (NATA) Research Survey Service and was also posted on social media outlets. Participants recruited via social media were asked to verify that they had not received the survey from the NATA prior to accessing the survey. Participation recruitment for this investigation occurred during spring 2021.

Participants were given 8 weeks to complete the survey (February 2021 to April 2021), and follow-up e-mails from the NATA distribution were sent biweekly to encourage participation of those who had not completed the survey. Inclusion criteria required participants

to be current, clinically practicing, certified athletic trainers and in good standing with the Board of Certification. Exclusion criteria included individuals who were not current, clinically practicing certified athletic trainers, retired certified athletic trainers, or non-credentialed athletic training students.

4.26 Data Analysis

Responses were exported to IBM SPSS Statistics software (version 27.0.0; IBM Corporation, Armonk, NY) for statistical analyses. Descriptive statistics were used to characterize the data.

4.3 Results

A total of 238 participants submitted our survey, however, not every participant elected to answer every question. Details regarding participant demographic data can be found in Table 1. Partial responses were included in the data analysis, and where applicable, specific participant numbers will be identified throughout the remainder of this section. Participants included ATs from all 11 NATA districts with a majority coming from district 3 (*n*=56; 23.8%). The districts with the least number of respondents were district 7 (*n*=9; 3.8%), district 8 (*n*=9; 3.8%), and district 10 (*n*=9; 3.8%). Respondents reported a mean of 11.16 $\pm$ 10.30 years of experience practicing as a certified athletic trainer (*n*=222) with the maximum years of experience being 45 years (*n*=1). Regarding route of athletic training credentialling, 61.8% (*n*=147) of participants graduated from a CAATE-accredited undergraduate athletic training program (post 2003), while only 10.9% (*n*=26) of participants graduated from a NATA approved curriculum route (prior to 2003).

4.31 Current Practices

The most selected tool by ATs for concussion diagnosis or management was the Sport Concussion Assessment Tool – 5th edition (SCAT5) (*n*=191; 81.28%). The second most selected tool was the Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT) tool (*n*=158; 67.23%). The tool that was selected the least was the EyeBOX® Assessment tool (*n*=1; 0.43%). Additionally, 73.19% (*n*=172) of participants reported using a multi-tool approach for concussion diagnosis and/or management. Of the respondents who reported their current tools for concussion practices, 36.57% (*n*=93) identified two tools, 22.75% (*n*=58) identified three tools, 7.45% (*n*=19) identified four tools, and 0.78% (*n*=2) identified five tools. Other concussion assessment tools were reported by participants including the Sway Balance (Sway Medical, Tulsa, Oklahoma) assessment tool (*n*=13; 5.53%) and the Concussion Vital Signs® (CNS Vital Signs, LLC, Morrisville, NC) instrument (*n*=4; 1.70%). Table 2 presents the instruments used by athletic trainers to diagnose and/or manage concussions.

In reference to level of comfort with diagnosing and managing a concussion, participants reported mean scores of 1.67±.74 (*n*=234) and 1.67±.71 (*n*=233), respectively. This score indicates an extreme or moderate level of comfort in diagnosing and managing concussions. Related to the return of a patient to activity following a concussion, ATs reported a mean score of 1.60±.695 (*n*=232), indicating that ATs are even more comfortable with returning a patient to activity following a concussion as compared to diagnosing or managing a concussion.

4.32 Familiarity with Potential Biomarkers and Biofluids

The most common response selected when asked about familiarity of potential biomarkers was "*I am not familiar with any substances"* (*n*=96; 44.86%). The potential biomarker that ATs were most familiar with was protein (*n*=80; 37.38%), and the least familiar

potential biomarker was DNA (*n*=7; 3.27%). All participants were also questioned on potential biofluids associated with concussions. Most participants selected blood (*n*=110; 50.93%) as a potential biofluid associated with concussions and the least selected biofluid was urine (*n*=7; 3.24%). Summary of responses regarding potential biomarkers and biofluids can be found in Figure 1 and Figure 2.

4.33 Knowledge of biomarker literature

Participants were questioned on their knowledge of the literature surrounding concussion biomarkers. Of the 215 participants who answered the question regarding knowledge of current literature surrounding biomarkers and their use for concussion diagnosis, 55.35% (*n*=119) self-rated as having *"No knowledge at all"*, while only 0.47% (*n*=1) self-rated as being *"Extremely knowledgeable"*. Similarly, of the 216 participants who answered the question regarding their knowledge of current literature surrounding biomarkers and their use for concussion management, 62.04% (*n*=134) self-rated as having *"No knowledge at all"*, and only 0.93% (*n*=2) self-rated as being *"Extremely knowledgeable"*. Figure 3 and Figure 4 detail all responses for both questions regarding participant knowledge of concussion biomarker current literature.

4.34 Attitudes towards concussion biomarkers

The mean score reported for ATs attitudes toward the identification of a biomarker adding to the ability to diagnose a concussion was 2.30±.87 (*n*=217) and the ability to manage a concussion was 2.33±.86 (*n*=217). These scores indicate that ATs somewhat agree that the identification of a biomarker would improve their ability to diagnose and manage concussions. Additionally, when questioned about the likelihood of implementing such a tool into clinical practice if widely available, the mean score reported was 1.95±.867 (*n*=217) indicating a strong likelihood for implementation.

4.4 Discussion

4.41 Current practices

Our study found that 73% of respondents identified more than one tool used for their concussion practices. We can postulate that these respondents are currently using multiple tools to diagnose and manage concussions in their clinical practices. These findings concur with the findings of Lempke et al in which 52.7% (527/1000) of ATs used a 3-domain concussion assessment battery, and 86.4% (864/1000) of ATs used a 2-domain concussion assessment battery in their current clinical practices.[16] From this study, we can postulate that an overwhelming majority of ATs are practicing in accordance with current practice recommendations relative to concussion evaluation and diagnosis by using multiple tools, indicating multiple domains are being assessed.[9,42] If a valid and reliable biomarker were discovered and properly vetted, this tool should be included in all healthcare professionals' concussion practices. These tools would provide healthcare professionals with an additional measure for diagnosing concussions and tracking the progress of a patients' return to activity.

Unlike previous studies, this study aimed to identify specific tools used by athletic trainers for the diagnosis and management of concussions. The two most commonly used tools by ATs were the SCAT5 and ImPACT. The use of the SCAT5 coincides with the recommendations from the *Consensus Statement on Concussion in Sport – the 5th International Conference on Concussion in Sport Held in Berlin, October 2016* (2017).[7] Although many ATs reported using a multi-tool approach for concussion diagnosis and management, there were over ten instruments which were identified as being used in current practices. This speaks to the fact that there is not a single gold standard tool readily available, such as a biologic marker of concussions, specific to the diagnosis and management of concussions.

This finding points to potential difficulties for all healthcare professionals in reference to standardizing techniques used to diagnose and manage concussions. If many different tools are being used in concussion practices, it may be difficult for healthcare providers to potentially transfer care of a concussed patient to each party. Additionally, when considering the perceptions of concussed patients and their families, if varying approaches are being used by multiple clinicians, the stakeholders may lose trust in the providers. Practices may improve if a select number of tools are identified and suggested for use in future consensus or position statements from multiple healthcare organizations, such as the NATA.

4.42 Knowledge of literature and familiarity with biofluids and biomarkers

Many targets for concussion biomarkers are in the early stages of research, which is why it is not surprising that many ATs feel as though they have "*No knowledge at all*" of literature surrounding the use of biomarkers for concussion diagnosis or management. However, it was an interesting finding that ATs reported protein as the most familiar biomarker. This can likely be attributed to the protein S100B, as it is the most extensively studied biomarker of traumatic brain injuries.[23] In addition to the large number of research publications, we believe that this familiarity can also be attributed to the attention given by the media to the use of S100B as a concussion biomarker.[95,98,99] Rather than relying on various media outlets, healthcare providers should receive their information about this topic through peer reviewed content. However, due to the intangible nature of concussion biomarkers, it is likely that clinicians may feel the research is unapproachable and therefore seek out information from sources which are written in laymen's terms. To combat this, efforts should be made to better market and direct research regarding this topic toward clinicians.

If a biomarker is identified and readily available for use in clinical practice, many steps will need to occur prior to the implementation into ATs or other healthcare workers clinical practices. As with other new treatments or diagnostic instruments, limitations for the application must first be addressed, validity and reliability must be established, and stakeholders must become aware of the instrument. Currently, research on concussion biomarkers is aimed at addressing limitations and establishing validity and reliability. With the findings of this study indicating that ATs are not familiar with the literature on concussion biomarkers, we would suggest that, once the limitations are addressed and validity and reliability established, an effort should be made to make ATs aware of the biomarker(s) through targeted continuing education efforts. Such efforts may include the distribution of infographics and structured seminars surrounding the topic which can be distributed by professional membership organizations, like the NATA, to their members.

4.43 Attitudes towards concussion biomarkers

The results of this study indicate that ATs demonstrate favorable attitudes towards the prospect of adding a measurable biomarker to their current concussion practices. The overwhelming majority of AT respondents (75.5%, n=162) indicated that they would welcome the opportunity to add such a measure to their practices, demonstrating that the outlook on the acceptance of such a tool is positive. This fact bodes well for the inclusion of such a tool into many clinical practices as ATs manage a large amount of these injuries daily in their clinical practices.

As part of the NATA education competencies, formal instruction on evidence-based practices should be given to students. Additionally, the intent of continuing education requirements are to ensure that ATs are abreast on current and future concepts related to

evidence-based practices.[100] If found to be a valid and reliable measure of concussions, a clinical biomarker could ultimately improve evidence-based practices surrounding concussion diagnosis and management. According to Bridges et al[101], it is crucial that stakeholders' attitudes are considered when implementing evidence-based practices. Much like McCarty et al[102] suggested with the implementation of evidence-based practice into AT education, when considering the positive attitudes of ATs demonstrated towards the implementation of a concussion biomarker into clinical practice, efforts can be made toward improving ATs' knowledge of such a biomarker by providing appropriate resources and tools, as well as addressing any barriers or limitations that may prevent the use of these tools.

4.44 Limitations and future research

There are inherent limitations related to data collection with our study. ATs were asked to self-report familiarity and knowledge, and we did not implement any mechanism to attempt to triangulate or confirm their self-perceptions. Additionally, we relied on ATs fully comprehending each question. To combat this, contact information was provided to clarify questions; however, the researchers received no inquiries to clarify questions. Lastly, due to the aim of this study inquiring about attitudes towards the future use of a biomarker, there is the likelihood of acquiescence bias.

Overall, research should continue to investigate potential biomarkers of concussions, with the hopes of addressing the current limitations and barriers to their current use in practices. Targeted continuing education efforts are necessary for ATs in order to improve familiarity with biomarkers and biofluids that are likely to be used in future clinical practices, if found to be valid and reliable. In addition, once found to be reliable, valid, and all limitations addressed, efforts should be made to improve ATs familiarity and knowledge of a biomarker by membership

organizations like the NATA. This, in turn, may increase ATs use of evidence-based practices surrounding concussion management and diagnosis, ultimately improving the outcomes following a concussion.

4.5 Conclusions

Many ATs within the NATA reported practices that align with current consensus and position guidelines[7,42] for the management of concussions. ATs identified numerous potential biomarkers and biofluids of concussion that they were familiar despite many ATs self-reporting having *no knowledge at all* of current literature surrounding potential concussion biomarkers and biofluids. An overwhelming majority of AT respondents reported that they would respond favorably to implementing these tools in their clinical practices if one became readily available.

Despite the favorable response of ATs being willing to implement such a tool into practice, there may be additional training that could accompany the use of these tools. Such training could include proper techniques for blood draws, oropharyngeal or nasopharyngeal swabbing, as well as proper administration of neural imaging techniques. The Commission on Accreditation of Athletic Training Education has already implemented some of these possible techniques into standards required of education programs, however, ATs who have not recently graduated from these accredited programs may not possess those skills. Thus, it is imperative that those ATs partake in continuing education that contain training for those various skills.

	Mean ± St. Dev.	
Years of Experience *(n=221)*	11.21 + 10.28 yrs.	
	Frequency	**Percent**
Route of Credentialing *(n=238)*		
Internship route (prior to 2003)	34	14.3
NATA approved curriculum route (prior to 2003)	26	10.9
CAATE-accredited undergraduate athletic training program (post 2003)	147	61.8
CAATE-accredited graduate athletic training program (post 2003)	31	13
Highest Level of Degree Attained *(n=238)*		
Bachelor's Degree (e.g. BS, BA)	63	26.5
Master's Degree (e.g. MS, MSAT)	156	65.5
Clinical Doctorate (e.g. DAT, DPT, DHSc)	7	2.9
Academic Doctorate (e.g. PhD, EdD, ScD)	11	4.6
Professional Degree (e.g. MD, DO)	1	0.4
NATA District where currently practicing *(n=235)*		
1 (Connecticut, Maine, Massachusetts, New Hampshire, Rhode Island, Vermont)	11	4.7
2 (Delaware, New Jersey, New York, Pennsylvania)	32	13.6
3 (District of Columbia, Maryland, North Carolina, South Carolina, Virginia, West Virginia)	56	23.8
4 (Indiana, Michigan, Ohio)	32	13.6
5 (Iowa, Kansas, Missouri, Nebraska, North Dakota, Oklahoma, South Dakota)	16	6.8
6 (Arkansas, Texas)	6	2.6
7 (Arizona, Colorado, New Mexico, Utah, Wyoming)	9	3.8
8 (California, Guam, American Samoa, Hawaii, Nevada)	9	3.8
9 (Alabama, Florida, Georgia, Puerto Rico, Virgin Islands, Kentucky, Louisiana, Mississippi, Tennessee)	45	19.1
10 (Alaska, Idaho, Montana, Oregon, Washington)	9	3.8
11 (Illinois, Minnesota, Wisconsin)	10	4.3

Table 3. Project II participant demographics.

Tools	Frequency (n=235)	Percent
ImPACT	158	67.2
SCAT5	191	81.3
King-Devick	16	6.8
VOMS	88	37.4
ACE	7	3.0
EyeBOX	1	0.4
Other	34	14.5
No tools used	14	6.0

Table 4. Tools used in clinical practice by athletic trainers for concussion diagnosis and management.

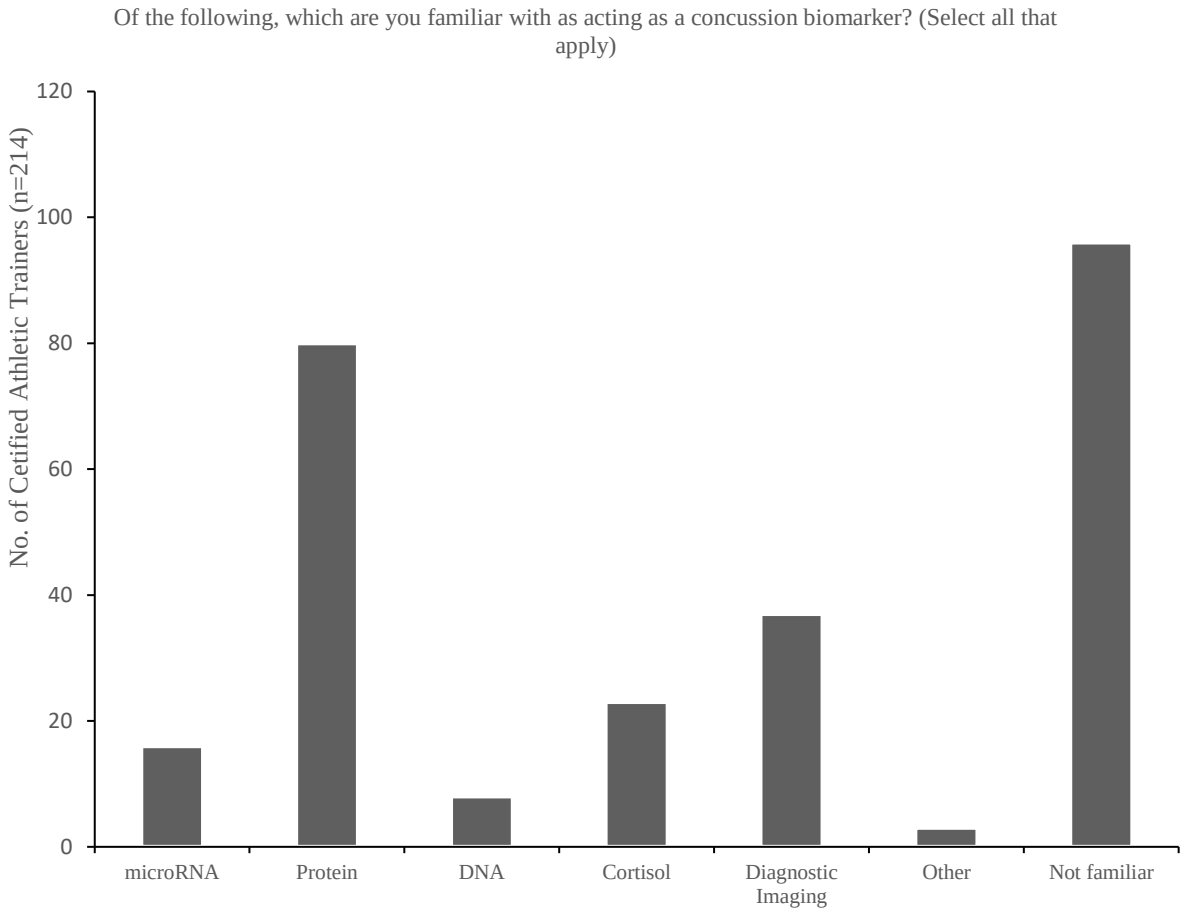

Figure 2. Familiarity of potential concussion biomarkers by athletic trainers.

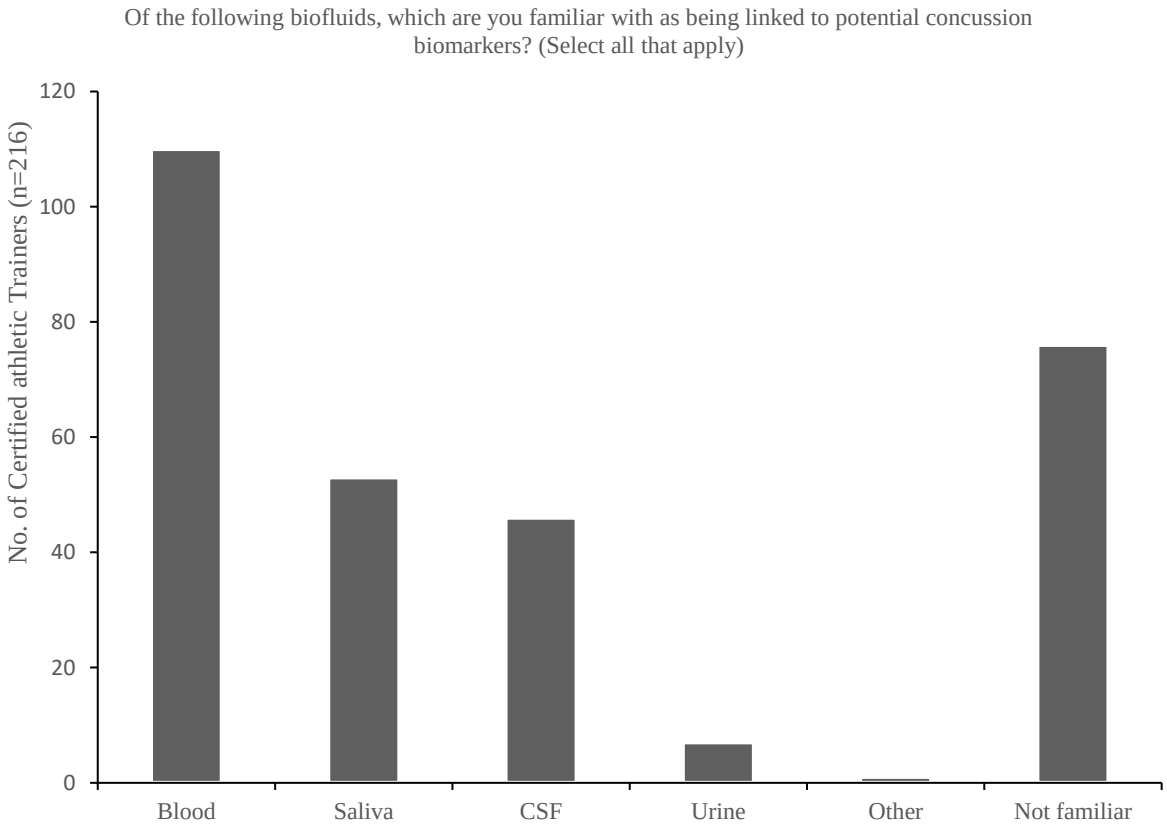

Figure 3. Familiarity of potential concussion biofluids by athletic trainers.

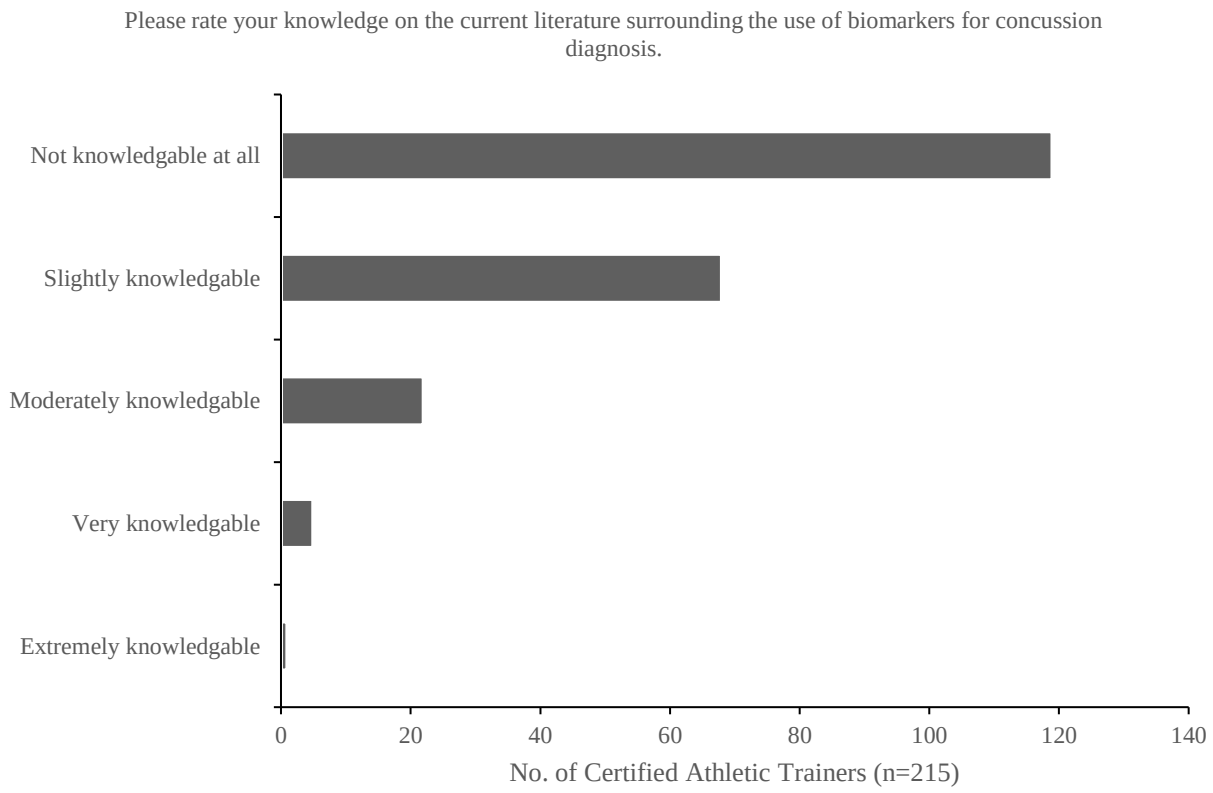

Figure 4. Athletic trainers' self-reported knowledge on current literature surrounding use of biomarkers for concussion diagnosis.

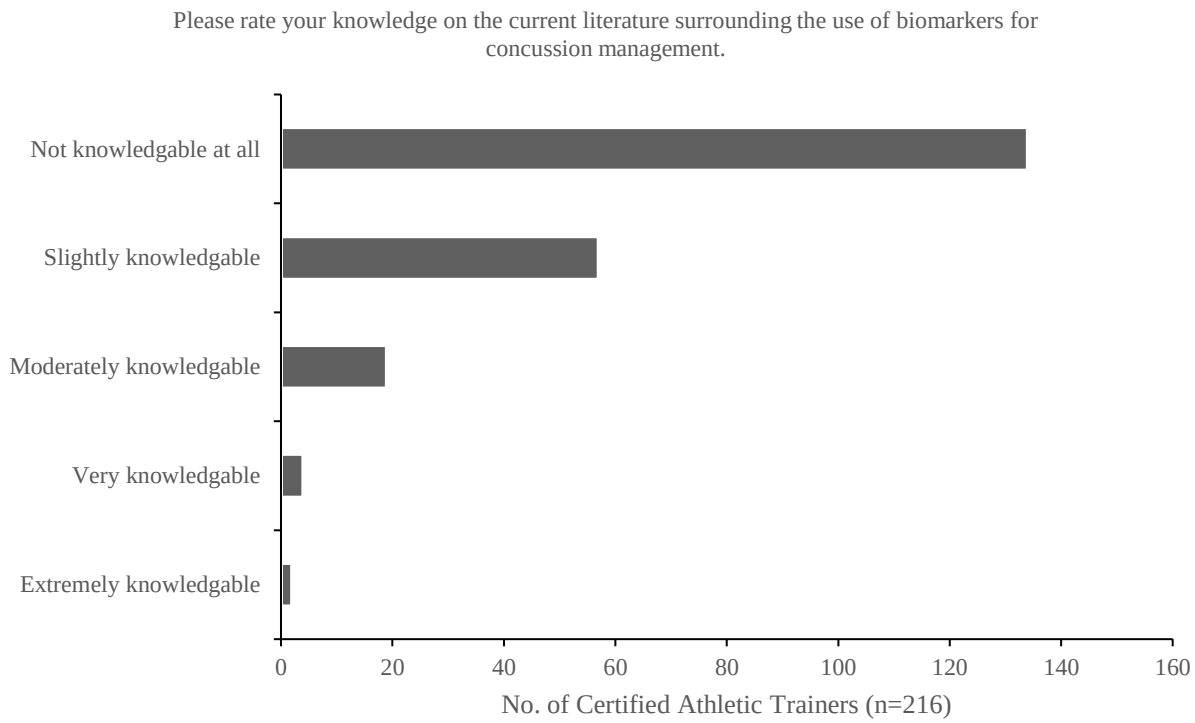

Figure 5. Athletic trainers' self-reported knowledge on current literature surrounding use of biomarkers for concussion management.

CHAPTER V

PROJECT III: THE EFFECTS OF ONE NCAA DIVISION I FOOTBALL SEASON ON SALIVARY MICRORNA

5.1 Introduction

Estimates indicate more than 2 million concussions are sustained in the United States annually.[30,65] However, research has demonstrated that this number may not accurately depict the true occurrence of this injury due to patients not self-reporting symptoms associated with concussions and a reliance on objective measures which lack reliability and validity, such as the Immediate Post-Concussion Assessment and Cognitive Test (ImPACT) and Standardized Assessment of Concussion (SAC).[1,2,10–13,44] Due to the limitations of current practices, the diagnosis and management of concussions may provide difficulties to healthcare clinicians. With the difficulties of concussion diagnosis and management, and the potential long-term effects of concussions such as chronic traumatic encephalopathy (CTE) or early onset Alzheimer's disease, it is crucial that valid and reliable objective measures of concussions are identified.[3–6] If discovered, concussion diagnosis and management protocols will be improved, limiting missed diagnoses by improving clinicians' abilities to diagnose and manage, and ultimately limiting the potential for long-term effects following a concussion. A group of markers which have preliminarily been identified in the research over the past few years is salivary microRNA (miRNA). Despite the increases in research focusing on this potential tool, there are still limitations surrounding their use as a tool for concussion diagnosis and management.

MiRNA are non-coding, small molecules that regulate protein synthesis and can be quantified in urine, serum, plasma, cerebrospinal fluid, and saliva.[21,24,83,103] Research evaluating these molecules' abilities to act as a biomarker of concussion has increased due to the potential

of the molecules being rapidly released into the oropharynx by cranial nerves (V, VII, IX, X, XII).[21] Additionally, collection of these molecules can be non-invasive, particularly when collecting saliva. Healthcare professionals may also use collection kits that allow miRNAs to remain stable at room temperature, making sideline collections possible.[21]

Previous research has identified over 2000 miRNA that can be quantified in humans, with estimates indicating approximately 70% being expressed in the central nervous system.[21,84] Throughout the current concussion literature, approximately 290 miRNAs have been identified as being a marker of concussions, with overlapping changes being shown in only seventeen miRNA, across multiple biofluids (cerebrospinal fluid, saliva, blood). However, forty-nine miRNAs collected through saliva have been identified as showing potential as a concussion biomarker.[22,24,62,63,75,83,84,88,103] Of those forty-nine identified, twenty-one have been linked to concussion management, and thirty-four have been linked with concussion diagnosis.[22,24,62,63,75,83,84,88,103] For the purposes of this study, a subset of previously identified target miRNA were selected to evaluate their utility as biomarkers of concussion.

Despite favorable evidence throughout the literature of salivary miRNA as a concussion biomarker, there are still several limitations which need to be addressed. Notably, the stability of miRNA measures over time. If miRNA is to be used to identify concussions in sport, it is necessary to assess it over the course of one impact sport season. This would aim to observe how miRNA respond to repeated impacts sustained over the season regardless of concussion occurrence. If one contact sport season does not significantly alter miRNA expressions, this would further ensure that changes seen in expressions at time of concussion are due to the injury rather than natural fluctuations from one contact sport season. Given the high incidence of

concussions in collegiate football, the purpose of this study was to examine the effects of one NCAA Division I football season on salivary miRNA.

5.2 Methods

Ethical approval for this study was provided by the University's Institutional Review Board (IRB 1563355). Written, informed consent was obtained for all participants.

5.21 Design and Participants

This prospective, case study included a convenience sample of 50 individuals who were active participants on a NCAA Division I Football team and were medically cleared for intercollegiate athletic competition by the institution's sports medicine staff members. We also included a case-control component of the study for participants who were diagnosed with a concussion during the season. Participants in this study were enrolled from July 2021 through December 2021. All participants were case control matched based on height, weight, sport position, musculoskeletal injury status/history, and concussion history. Exclusion criteria included being currently concussed at time of enrollment and any significant neurological disorders (e.g., seizure disorder, Guillain-Barre syndrome, stroke).

5.22 Demographics and Medical History

Participants self-reported age, sex, playing position, and medical history, which consisted of seizure disorder (e.g., epilepsy), Guillain-Barre syndrome, and stroke diagnoses; concussion history, including number of diagnosed, number of undiagnosed, most recent concussion, and time to full recovery following most recent concussion; musculoskeletal injury history, including whether the injury restricted the participant from participation, and if so, how long.

5.23 Saliva Sample Collection

All saliva samples were collected using ORAcollect-RNA ORE-100 swab kits (DNA Genotek, Ottawa, Ontario, Canada) by the principal investigator to ensure consistency with collection procedures. Per manufacturer's guidelines, collection involved swabbing between the participant's cheek and gums on the lower jaw, ten times on each side, avoiding the teeth as best as possible and once finished, the swab is then placed into a storage tube and shaken vigorously fifteen times. Sample collections occurred between 7AM and 7PM, and all participants performed an oral rinse 15 minutes prior to collection. Samples were then stored at -80°C until sample preparation.

5.24 Fatigue Assessment Scale (FAS)

To address the potential confounding variable of fatigue associated with ongoing physical activity, we chose a previously validated survey. The Fatigue Assessment Scale[104–106] (FAS) was used to assess individuals' change in fatigue over the course of one NCAA Division I football season. Additionally, previous research has demonstrated that expressions of salivary miRNA demonstrate changes associated with diurnal oscillations.[107] The FAS questionnaire asks individuals to grade ten statements which pertain to fatigue on a 5-point Likert type scale (never - always). Individuals who are "never fatigued" on any of the ten statements receive a score of 10, while individuals who are "always fatigued" receive a score of 50. Participants completed this survey during pre-season and at the conclusion of the season.

5.25 Timing of data collection

One week prior to the first structured pre-season practice of the 2021 football season, all participants reported to the athletic training facility and completed a demographics questionnaire,

medical history questionnaire, and the FAS. Following completion, baseline saliva samples were collected.

Throughout the course of the season, if a participant was suspected of sustaining a concussion, the sports medicine staff would determine concussion status using the Sway (Sway Medical, Tulsa, Oklahoma) assessment tool. If a participant was diagnosed with a concussion, a saliva sample was then collected within 27 hours of clinical diagnosis. Additionally, a healthy matched control was then identified from the participants based on playing position, height, and weight. Saliva samples were then collected for the healthy matched control. Saliva samples were also collected within 72 hours of full return to activity for the concussed individual and healthy control. Return to play decisions were determined using the Sway assessment tool as well by the sports medicine staff. At the conclusion of the athletic season (within 72 hours of the conclusion of the last competition), participants reported back to the athletic training clinic to provide post-season measures of saliva, medical history questionnaire, and the FAS.

5.26 Saliva Sample Processing

RNA was isolated from each saliva sample per manufacturer instructions using the miRNeasy Kit (Qiagen, Inc., Germantown, MD, USA). The quality of RNA was evaluated using a Thermo Scientific Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and a Thermo Fisher Qubit 4 Fluorometer (Thermo Fisher Scientific, Wilmington, DE, USA). MiRNA was extracted using the MagMAX mirVana Total RNA Isolation kit (Thermo Fisher Scientific, Wilmington, DE, USA) and a KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, Wilmington, DE, USA). Following manufacturer's protocols, all complementary DNA (cDNA) templates were created using a TaqMan® Advanced miRNA cDNA synthesis kit (Thermo Fisher Scientific, Wilmington, DE, USA). The following

TaqMan advanced miRNA assays (Thermo Fisher Scientific, Wilmington, DE, USA) were used: miR-29c-3p, miR-26b-3p, miR-192-5p, miR-27a-5p, miR-30e-5p, miR-7-1-3p, miR-361-5p (endogenous miRNA control). Quantitative real time polymerase chain reaction experiments were conducted using a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Conditions for PCR consisted of: 95.0°C for 20 seconds, then forty cycles of 95.0°C for 1 second and 60°C for 20 seconds.

5.27 Statistical Analysis

Expression fold changes were calculated for saliva samples using the $2^{-\Delta\Delta CT}$ method with miR-361-5p as the reference gene.[108] Three PCR tests were run on each target miRNA from each sample, then a mean cycle threshold (CT) was calculated. Next, the ΔCT (change in CT) was calculated by subtracting the mean CT of each target gene from the mean CT of the reference gene. For all samples other than baseline samples, a ΔΔCT was calculated by subtracting the ΔCT of subsequent samples from the baseline ΔCT of each target miRNA. Lastly, the expression fold change was calculated using $2^{-\Delta\Delta CT}$.[109]

Paired t-tests were used to compare individuals' pre- and post-season performance on the FAS. Intraclass correlation coefficients (ICC) were calculated to determine individual consistency between pre-and post-season saliva samples. Estimates of ICC and their 95% confident intervals were calculated using IBM SPSS Statistics (IBM Corporation, version 28.0, Armonk, NY, USA) based on an absolute-agreement, 2-way mixed-effects model. Consistent with the literature, non-parametric tests (Wilcoxon signed-rank test) were used to compare individuals' ΔCT values from pre-season collection to post-season collection of salivary miRNA, pre-season collection to concussion diagnosis collection, and concussion diagnosis collection to return to play (RTP) collection. Additionally, a Mann-Whitney *U* test was employed to compare

the miRNA expression fold changes ($2^{-\Delta\Delta CT}$) in two independent groups (concussed vs. healthy control) at concussion diagnosis collection, RTP collection, and post-season collection.

GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, CA, USA) was used to calculate expression fold changes, Metaboanalyst v4.0 online biomarker toolkit software[110] was used to examine the accuracy of target miRNAs using area under the receiver operating characteristic (ROC) curve (AUC) analysis, and all other statistical analyses were carried out using IBM SPSS Statistics. The ROC curve analysis aimed to assess each individual miRNA and ratios between miRNA markers' ability to distinguish between concussed and healthy patients.[24] For the purpose of this study, the following parameters were considered for effect size: small (d = .2 or r = .1), medium (d = .5 or r = .3), or large (d = .8 or r = .5).[111]

5.3 Results

5.31 Participant Characteristics

Descriptive statistics can be found in Table 1. Participants were college-aged males (n=50), aged 18-24 years (21 $\pm$ 1.6 years). All individuals denied having a history of significant neurological disorders (seizures, Guillain-Barre syndrome, stroke). Thirty-four percent (17/50; 34%) participants had suffered a previous concussion at time of pre-season data collection. One participant was not able to partake in post-season data collection due to a season ending injury and subsequent temporary withdrawal from the institution.

5.32 Fatigue Assessment Scale (FAS)

There were no statistically significant differences found between self-reported fatigue using the FAS during pre-season (M = 18.94, SD = 4.15) and post-season (M = 18.67, SD = 4.09), $t(48) = .409$, $p = .684$. The effect size was less than small, $d = .06$.

5.33 Effects of One NCAA Division I Football Season

A poor degree of reliability was found between pre- and post-season measurements of salivary miRNA for all target miRNA. However, miR-29c-3p and miR-27a-5p demonstrated a moderate degree of reliability. The average measure ICC for miR-29c-3p was .52 with a 95% confidence interval from .16 to .73 ($F(49, 49) = 2.09$, $p < .01$); miR-26b-3p was .21 with a 95% confidence interval from -.42 to .55 ($F(49, 49) = 1.25$, $p = .22$); miR-192-5p was .19 with a 95% confidence interval from -.43 to .54 ($F(49, 49) = 1.24$, $p = .23$); miR-27a-5p was .65 with a 95% confidence interval from .39 to .80 ($F(49, 49) = 2.89$, $p < .01$); miR-29c-3p was .52 with a 95% confidence interval from .16 to .73 ($F(49, 49) = 2.09$, $p < .01$); miR-30c-5p was -.04 with a 95% confidence interval from -.78 to .40 ($F(49, 49) = .96$, $p = .55$); miR-7-1-3p was -.16 with a 95% confidence interval from -.94 to .34 ($F(49, 49) = .88$, $p = .68$). Any ICC values less than 0.5 were deemed poor reliability while values between 0.5 and 0.75 were deemed moderate reliability, values between 0.75 and 0.9 were deemed good reliability, and values greater than 0.9 were deemed excellent reliability.[112]

Pre-season and post-season salivary miRNA expression levels were compared for all participants ($n = 50$) to determine the effects of one NCAA Division I football season. No significant differences were found between pre-season (*Mdn = -3.58*) and post-season (*Mdn = -4.19*) levels of miR-29c-3p, $z = -.95$, $p = .34$, $r = -.13$; pre-season (*Mdn = -.08*) and post-season (*Mdn = 1.79*) levels of miR-26b-3p, $z = -.74$, $p = .46$, $r = -.10$; pre-season (*Mdn = -2.50*) and post-season (*Mdn = -.96*) levels of miR-192-5p, $z = -1.82$, $p = .07$, $r = -.26$; pre-season (*Mdn = 2.06*) and post-season (*Mdn = 2.57*) levels of miR-27a-5p, $z = -1.09$, $p = .28$, $r = -.15$; pre-season (*Mdn = -.54*) and post-season (*Mdn = -.95*) levels of miR-30c-5p, $z = -1.04$, $p = .30$, $r = -.15$;

pre-season (*Mdn = 1.26*) and post-season (*Mdn = 1.36*) levels of miR-7-1-3p, z = -1.02, p =.31, r = -.14. The effect size was small for all miRNA targets. Figure 1.

5.34 Concussion Diagnosis

Salivary miRNA expressions collected within 72 hours of a participant being diagnosed with a concussion (n = 6) were compared to baseline expressions using a Wilcoxon Sign Rank test. No statistically significant differences were found between pre-season (*Mdn* = -3.58) and time of concussion diagnosis (*Mdn* = -4.02) expression levels of miR-29c-3p, z = -.94 p = .35, r = -.38.; pre-season (*Mdn* = -.08) and time of concussion diagnosis (*Mdn* = 2.14) expression levels of miR-26b-3p, z = -1.57 p = .11, r = -.64; pre-season (*Mdn* = -2.50) and time of concussion diagnosis (*Mdn* = -.85) expression levels of miR-192-5p, z = -.74 p = .46, r = -.30; pre-season (*Mdn* = 2.06) and time of concussion diagnosis (*Mdn* = 2.57) expression levels of miR-27a-5p, z = -1.15 p = .25, r = -.47; pre-season (*Mdn* = -.54) and time of concussion diagnosis (*Mdn* = -.90) expression levels of miR-30c-5p, z = -.31 p = .75, r = -.13; pre-season (*Mdn* = 1.26) and time of concussion diagnosis (*Mdn* = 1.36) expression levels of miR-7-1-3p, z = -.31 p = .75, r = -.13. The following miRNA targets demonstrated a small effect size: miR-30c-5p and miR-7-1-3p. A medium effect size was demonstrated by miR-192-5p, miR-29c-3p and miR-27a-5p. Lastly, miR-26b-3p demonstrated a large effect size.

When a ROC analysis was employed, it was found that a ratio of miR-192-5p and miR-7-1-3p achieved the highest accuracy for differentiating between concussion status (AUC=.97) followed by a ratio between miR-26b-3p and miR-7-1-3p (AUC=.92), and a ratio between miR-27a-5p and miR-7-1-3p (AUC=.86). However, when compared between concussed and healthy individuals, a ROC analysis identified that the ratio of miR-26b-3p and miR-7-1-3p performed

less accurately (AUC=.64). Additionally, the accuracy of the ratio between miR-27a-3p and miR-7-1-3p also decreased (AUC=.53). Figure 2.

5.35 Return to Play

Using a Wilcoxon Sign Rank test (n=6) revealed no statistically significant differences between salivary miRNA expressions collected within 72 hours of concussion diagnosis (*Mdn.* = -4.02) and 72 hours of return to play (*Mdn.* = -4.48) for miR-29c-3p, z = -1.78, p = .08, r = .62; salivary miRNA expressions collected within 72 hours of concussion diagnosis (*Mdn.* = 2.15) and 72 hours of return to play (*Mdn.* = 1.37) for miR-26b-3p, z = -.52, p = .60, r = .28; salivary miRNA expressions collected within 72 hours of concussion diagnosis (*Mdn.* = -.85) and 72 hours of return to play (*Mdn.* = -1.61) for miR-192-5p, z = -.31, p = .75, r = .37; salivary miRNA expressions collected within 72 hours of concussion diagnosis (*Mdn.* = 2.57) and 72 hours of return to play (*Mdn.* = 1.87) for miR-27a-5p, z = -1.15, p = .25, r = .44; salivary miRNA expressions collected within 72 hours of concussion diagnosis (*Mdn.* = -.90) and 72 hours of return to play (*Mdn.* = -.95) for miR-30c-5p, z = -.16, p = .92, r = .01; salivary miRNA expressions collected within 72 hours of concussion diagnosis (*Mdn.* = 1.36) and 72 hours of return to play (*Mdn.* = 1.45) for miR-7-1-3p, z = -.31, p = .75, r = .11. A small effect size was demonstrated by miR-26b-3p, miR-30c-5p, and miR-7-1-3p; a moderate effect size was demonstrated by miR-192-5p and miR-27a-5p; a large effect size was demonstrated by miR-29c-3p.

A ROC analysis identified that the ratio of miR-192-5p and miR-7-1-3p achieved the highest accuracy for differentiating between samples collected at time of concussed and RTP (AUC=.75) for concussed participants only. This was followed by miR-29c-3p (AUC=.72) and miR-27a-5p (AUC=.69). However, when performing a ROC analysis comparing the ratios for

concussed and healthy individuals, the ratio of miR-192-5p and miR-7-1-3p performed less accurately (AUC=.50). Additionally, the accuracy of miR-29c-3p (AUC=.61) also decreased. Interestingly, the accuracy of miR-27a-5p (AUC=.78) increased when comparing concussed to healthy individuals. Trajectory plots (pre-season – concussion – RTP – post-season) which show individuals' salivary miRNA expressions throughout the course of one can be found in Figures 3-8.

5.36 Comparison of Expression Fold Change

No statistically significant differences were found when comparing $2^{-\Delta\Delta CT}$ values from pre-season (*Mdn.*=1.29) to post-season (*Mdn.*=1.31) salivary expressions between concussed individuals and their healthy matched controls for miR-29c-3p, $U = 14.00$, $z = -.18$, $p = .86$, $r = -.64$; pre-season (*Mdn.*=.38) to post-season (*Mdn.*=1.16) salivary expressions between concussed individuals and their healthy matched controls for miR-26b-3p, $U = 6.00$, $z = -1.64$, $p = .10$, $r = .51$; pre-season (*Mdn.*=.47) to post-season (*Mdn.*=.93) salivary expressions between concussed individuals and their healthy matched controls for miR-192-5p, $U = 9.00$, $z = -1.10$, $p = .27$, $r = .47$; pre-season (*Mdn.*=1.30) to post-season (*Mdn.*=.72) salivary expressions between concussed individuals and their healthy matched controls for miR-27a-5p, $U = 11.00$, $z = -.73$, $p = .47$, $r = .88$; pre-season (*Mdn.*=.87) to post-season (*Mdn.*=.97) salivary expressions between concussed individuals and their healthy matched controls for miR-30c-5p, $U = 14.00$, $z = -.18$, $p = .86$, $r = .94$; pre-season (*Mdn.*=.75) to post-season (*Mdn.*=1.26) salivary expressions between concussed individuals and their healthy matched controls for miR-7-1-3p, $U = 8.00$, $z = -1.28$, $p = .20$, $r = -.48$. The effect size was large for all miRNA targets except miR-192-5p and miR-7-1-3p which demonstrated medium effect sizes. Demographics of concussed and healthy controls can be found in Table 2.

When comparing expression fold changes between concussed patients and their healthy matched controls at the time of concussion diagnosis, no statistically significant differences were seen; pre-season (*Mdn.*=1.29) and within 72 hours of concussion diagnosis (*Mdn.*=1.05) expressions for miR-29c-3p, $U = 15.00$, $z = -.44$, $p = .63$, $r = -.35$; pre-season (*Mdn.*=.41) and within 72 hours of concussion diagnosis (*Mdn.*=1.35) expressions for miR-26b-3p, $U = 9.00$, $z = -1.44$, $p = .20$, $r = .23$; pre-season (*Mdn.*=.83) and within 72 hours of concussion diagnosis (*Mdn.*=1.07) expressions for miR-192-5p, $U = 12.00$, $z = -.96$, $p = .34$, $r = -.21$; pre-season (*Mdn.*=.67) and within 72 hours of concussion diagnosis (*Mdn.*=1.47) expressions for miR-27a-5p, $U = 13.00$, $z = -.80$, $p = .42$, $r = -.25$; pre-season (*Mdn.*=1.87) and within 72 hours of concussion diagnosis (*Mdn.*=.70) expressions for miR-30c-5p, $U = 11.00$, $z = -1.12$, $p = .26$, $r = -.53$; pre-season (*Mdn.*=1.12) and within 72 hours of concussion diagnosis (*Mdn.*=.99) expressions for miR-7-1-3p, $U = 16.00$, $z = -.32$, $p = .75$, $r = -.70$. The effect size was small for miR-26b-3p, miR-192-5p, miR-27a-5p; moderate for miR-29c-3p; large for miR-30c-3p, miR-7-1-3p.

5.4 Discussion

Concussion management techniques rely heavily on subjective input from patients and objective tools which lack reliability.[10–13] This reliance may lead to the underreporting of concussions, potential mismanagement, and ultimately potential long-term effects associated with improper management of these injuries.[1–5] To improve management techniques, research has aimed to identify valid, reliable, objective measures called biomarkers. Of the many potential biomarkers, salivary miRNA has demonstrated potential for the ability to distinguish between concussed and non-concussed states, and the correlations with typical concussion symptom resolution.[22,24,62,63,75,83,84,88,103] However, limitations still prevent the implementation of such a

tool into clinical practices. One such limitation is the effects of one contact sport season on target miRNA's expressions. Addressing this limitation will help clinicians' abilities to interpret changes in salivary miRNA expressions following a concussion as compared to natural variations in expressions due to time. Therefore, we aimed to address the effects of one season of NCAA Division I football on salivary miRNAs which have been identified as potential biomarkers of concussions. Additionally, we aimed to validate previously identified salivary miRNA as concussion diagnosis and management biomarkers when comparing to time of diagnosis and return to play. Time of diagnosis was determined by a clinical decision which used the Sway concussion tool and return to play was determined in the same manner.

5.41 Effects of One NCAA Division I Football Season

The target salivary miRNAs chosen for this study did not demonstrate significant changes between pre-season (n=50) and post-season (n=49) measures after one season of contact sport. However, ICC values indicated that low to moderate reliability was found between pre- and post-season ΔCT values for all target miRNA. This finding shows that individuals' measures of salivary miRNA demonstrate variation in ΔCT values from pre- to post-season. The findings from this study indicate that salivary miRNA may indeed demonstrate natural variations over the course of one season, possibly due to head impacts sustained throughout the course of one season. With this finding, we cannot conclude that alterations in miRNA expressions demonstrated at the time of concussion may not truly be a result of the injury itself and may in fact be due to other factors. Despite previous research[22,81,103] identifying these miRNA as potential biomarkers of concussion due to their relationship between concussion diagnosis and/or management, the presented findings do not support the use of the subset of miRNA analyzed in

this study as a biomarker of concussion for current clinical practices. However, research should continue to aim at validating these markers as concussion diagnostic or prognostic measures.

5.42 Concussion Diagnosis

Clinical diagnosis of concussion was determined by the comparison of pre-season performance of the Sway concussion tool to performance at time of injury. This procedure is consistent with recommendations from multiple position statements surrounding concussion care.[7–9] Similarly, saliva samples were collected at baseline (prior to first contact practice of season) and within 72 hours of injury (diagnosed concussion). Although there were no significant differences found between pre-season samples and those collected at time of concussion for individual salivary miRNA targets, when combined, the ratio of miR-192-5p and miR-7-1-3p was able to accurately identify 97% of the samples collected at time of concussion as compared to those collected at pre-season for just concussed patients (n=6). However, when the samples of healthy matched controls (n=6) were included, the accuracy of the ratio to distinguish between samples collected at pre-season and time of concussion decreased drastically. In turn, this indicates that the ratio is highly sensitive, but lacks specificity. When considering this for clinical application, this indicates that more concussions could be identified, and less would go undiagnosed, ultimately increasing the chances of better outcomes following a concussion. However, due to the lack of specificity, individuals who are not actually concussed may be deemed concussed by this ratio. In turn, this may cause issues for clinicians when attempting to determine concussion status. Individuals may unnecessarily be held from competition or activity despite not having sustained an injury that would preclude them from participating in activities.

5.43 Return to Play

Recommendations suggest return to play decisions are made based on a patient's performance on various batteries (balance, cognitive performance, etc.) as compared to baseline measures. For participants enrolled in this study, full return to play was decided when performance on the Sway concussion tool was within normal ranges of their baseline performance on the same tool. However, comparisons of samples were made between saliva samples collected within 72 hours of concussion diagnosis and 72 hours of full return to play. Although there were no significant differences found between samples collected at the time of concussion and the time of return to play for individual miRNA targets, once again, the ratio of miR-192-5p and miR-7-1-3p was able to accurately identify 75% of the samples collected at time of concussion as compared to those collected at return to play for concussed patients (n=6) only. However, the accuracy of that ratio also decreased to 50% when ratios from healthy matched controls were also placed into the analysis. With this finding, we do not believe that the clinical utility of the miR-192-5p and miR-7-1-3p ratio would be clinically appropriate for determining return to play status. Although demonstrating high sensitivity, indicating the ratio's ability to identify many individuals with concussions, the lack of specificity may result in individuals who have been deemed concussed being held from participation longer than necessary. Individuals return-to-activity timelines may be prolonged due to the ratio's inability to accurately separate out healthy individuals from concussed individuals.

5.44 Limitations and Future Research

This study was not conducted without several limitations. First, this study involved participation from individuals from one team across one season from one institution, ultimately limiting the sample size to only male participants from an intercollegiate football team.

Additionally, we were only able to account for six concussions over the course of one season, potentially limiting our ability to fully examine the effects of concussion on the previously identified salivary miRNA. Our analysis on those effects involved only six concussive injuries, however, we were able to include the same amount of healthy matched controls. Additionally, we were unable to account for the number of head impacts each participant experienced throughout the season, so the effects of sub-concussive impacts sustained was not able to be examined.

Future research should aim to enroll larger cohorts to further validate the use of salivary miRNA as a concussion biomarker. Additionally, the use of head accelerometers or video recordings can be incorporated to estimate head impacts sustained by participants. This may give us further insight as to how these salivary miRNAs are affected by sub-concussive traumas. Lastly, future research should aim to enroll larger populations of subjects as well as address other limitations surrounding the use of salivary miRNA as a concussion biomarker to enhance the evidence for their clinical utility in concussion diagnosis and management. These studies should aim to recruit participants of varying ages, female, and from other activities.

5.5 Conclusions

This study aimed to address one large limitation surrounding the clinical utility of salivary miRNA as a concussion biomarker by examining the effects of one contact sport season. We established that there were no significant differences noted between pre-season and post-season expressions. However, ICC values indicated poor to moderate reliability between pre- and post-season measures of target miRNA. This finding indicates that individuals' salivary miRNA may exhibit natural variations over the course of one NCAA Division I season. The reason for these variations may be due to natural variations from time or be due to impacts sustained over

the course of the season. Additionally, although further analysis did not find any significant differences between saliva samples collected at pre-season and samples collected within 72 hours of concussion diagnosis, a ratio of two miRNAs (miR-192-5p and miR-7-1-3p) displayed very high sensitivity toward concussion status.

	Pre-Season	Post-Season
Demographics		
Age (year)	21 ± 1.64	
Height (cm)	187.45 ± 6.99	
Weight (kg)	103.14 + 19.83	
Medical History		
Seizure Disorder	0	0
Guillain-Barre Syndrome	0	0
Stroke	0	0
Head Injury Details		
No. Participants with Previous Concussions	17	20
Mean Previous Concussions Dx	1.47	1.40
Mean Previous Concussions Un-Dx	0.19	0.00
Fatigue Assessment Scale		
Composite Score	18.96	18.67

Table 5. Project III participant demographics.

	Concussed	Healthy Controls
Demographics		
Age (year)	21 ± 1.41	21 ± 2.09
Height (cm)	190.50 ± 7.70	188.38 ± 7.25
Weight (kg)	105.21 ± 9.54	98.18 ± 9.07
Medical History		
Seizure Disorder	0	0
Guillain-Barre Syndrome	0	0
Stroke	0	0
Head Injury Details		
No. Participants with Previous Concussions	2	3
Mean Previous Concussions Dx	1.50	0.67
Mean Previous Concussions Un-Dx	0	0
Fatigue Assessment Scale		
Composite Score	21.00	19.50

Table 6. Demographics of concussed individuals and healthy matched controls at pre-season.

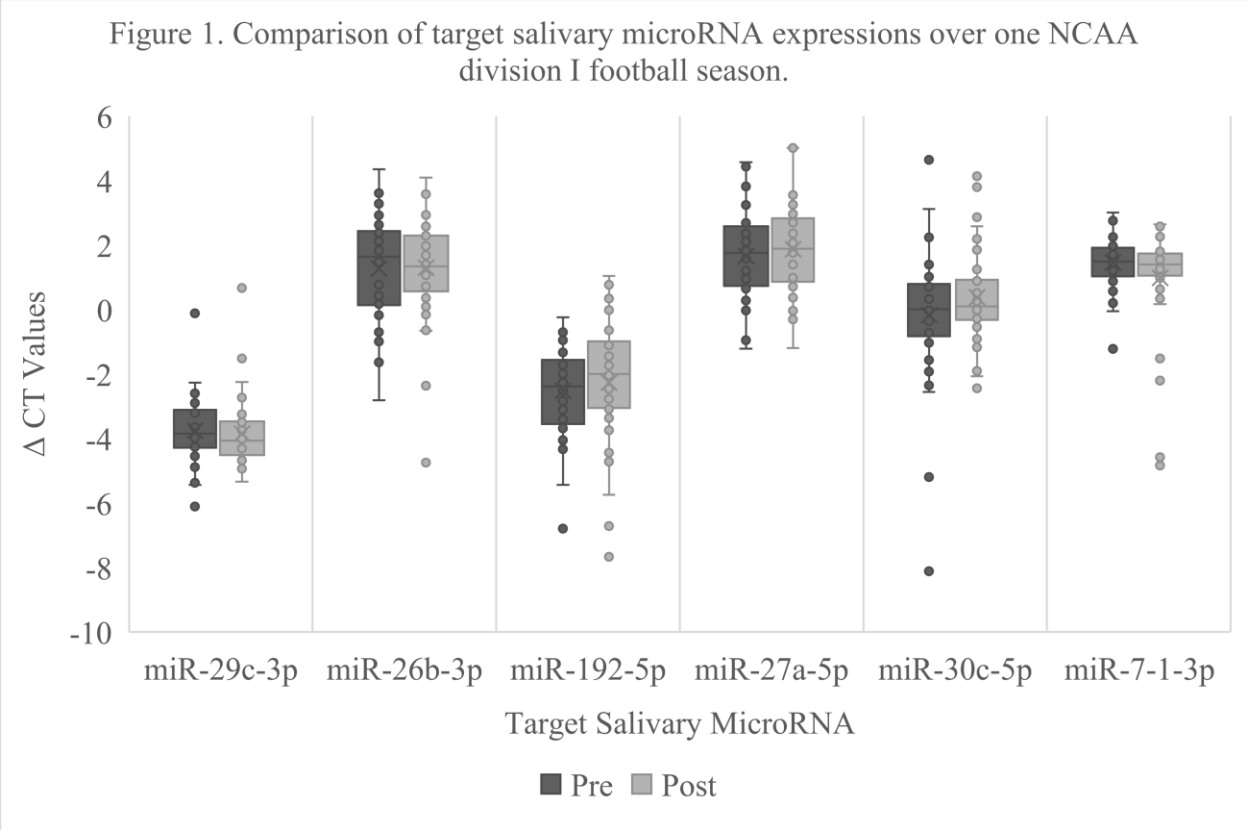

Figure 6. Comparison of target salivary miRNA expressions over one NCAA Division I football season.

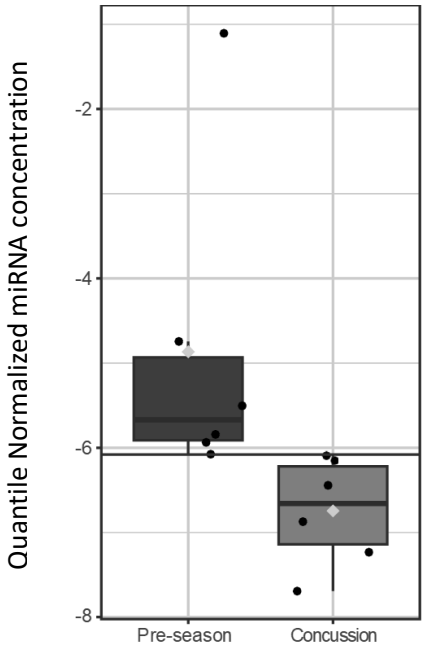

Figure 7. Box-plot representation of quantile normalized concentrations of ratio between miR-192-5p and miR-7-1-3p comparing samples collected during pre-season and samples collected within 72 hours of concussion diagnosis.

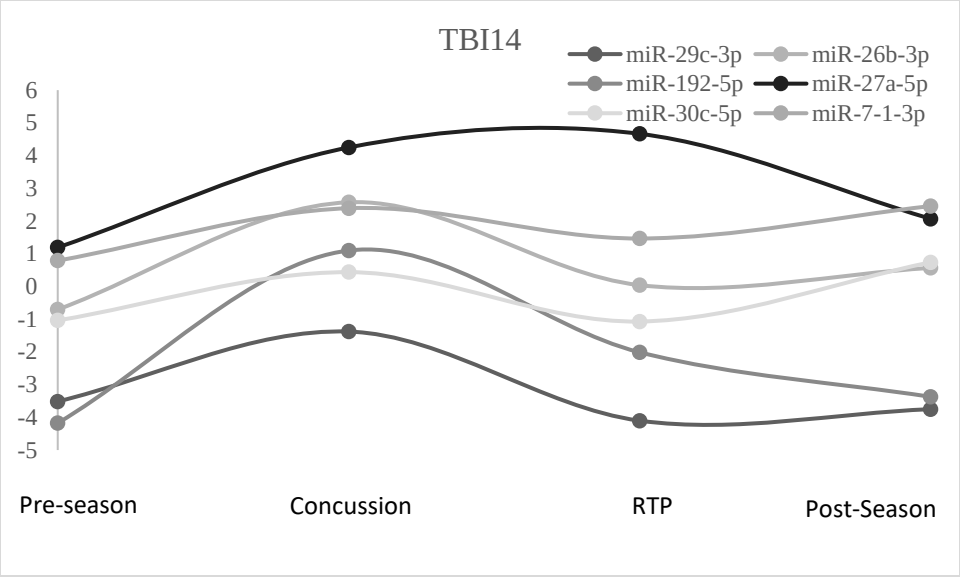

Figure 8. Trajectory plots of target miRNA for participant TBI14, who suffered a concussion while enrolled in this study.

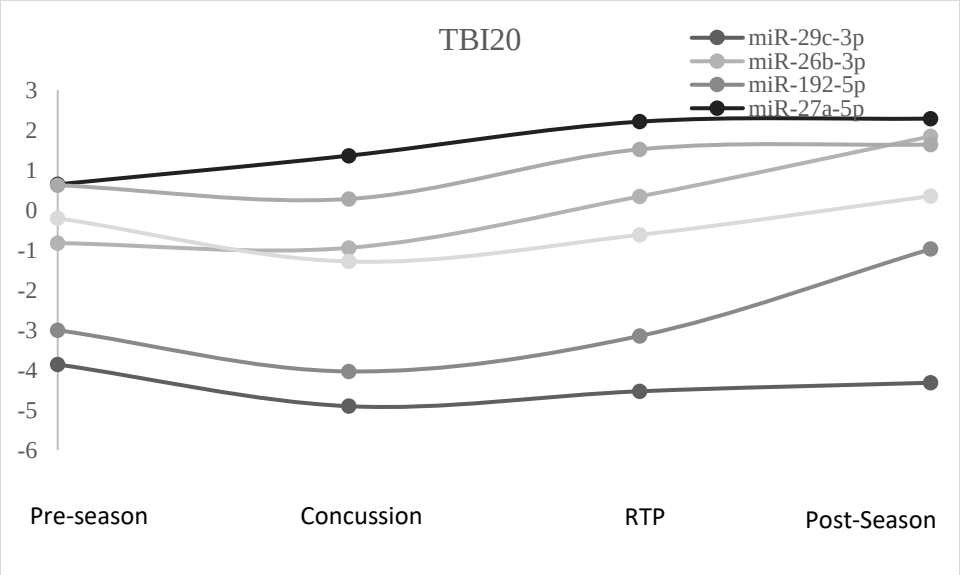

Figure 9. Trajectory plots of target miRNA for participant TBI20, who suffered a concussion while enrolled in this study.

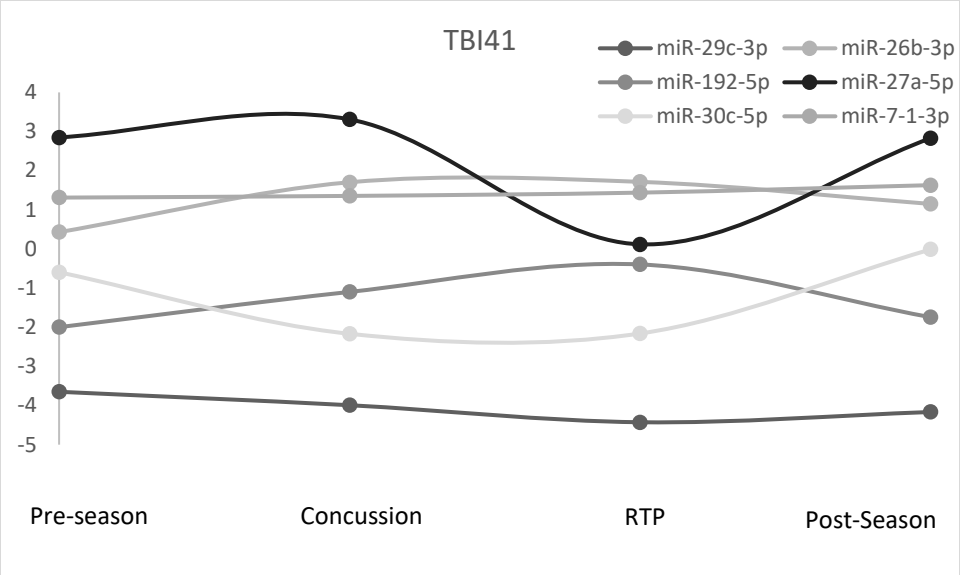

Figure 10. Trajectory plots of target miRNA for participant TBI41, who suffered a concussion while enrolled in this study.

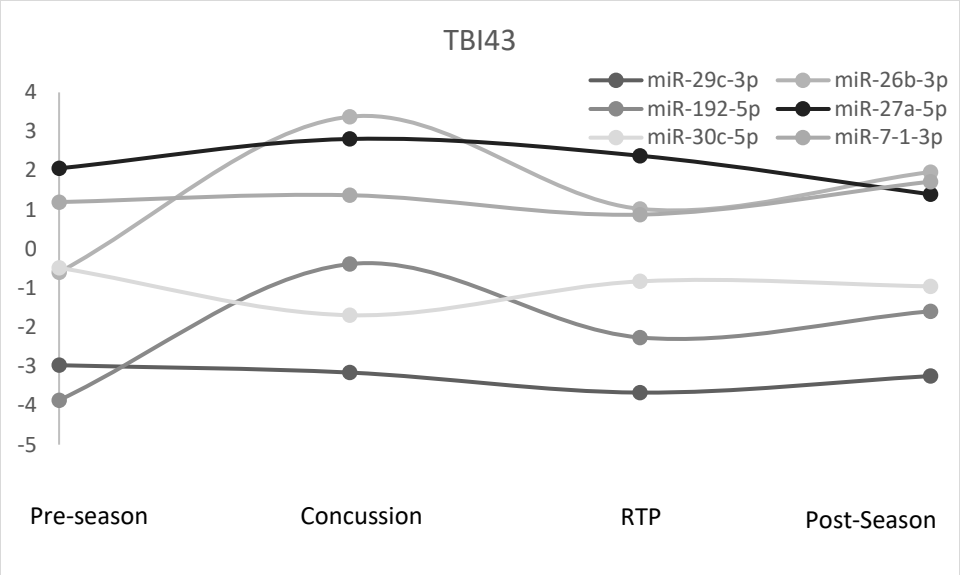

Figure 11. Trajectory plots of target miRNA for participant TBI43, who suffered a concussion while enrolled in this study.

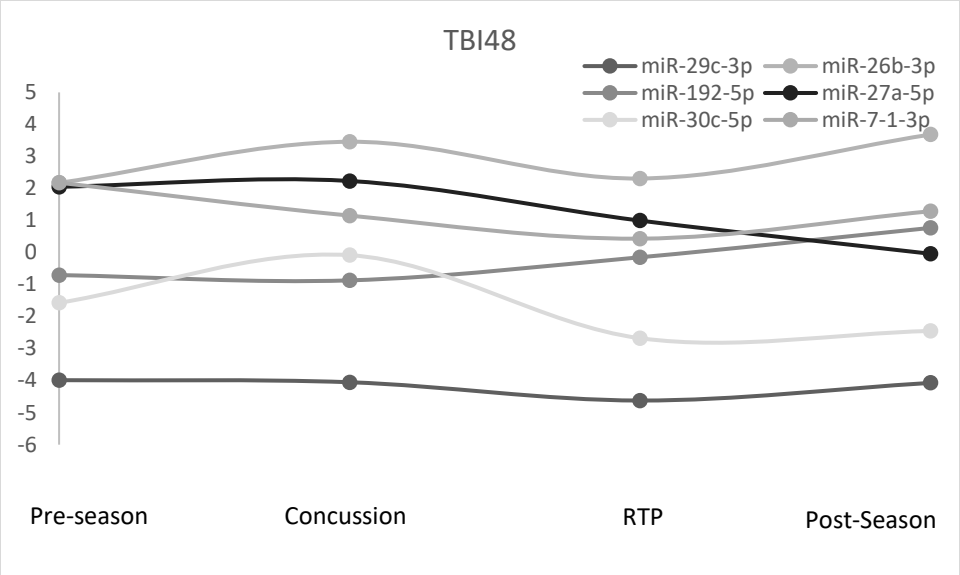

Figure 12. Trajectory plots of target miRNA for participant TBI48, who suffered a concussion while enrolled in this study.

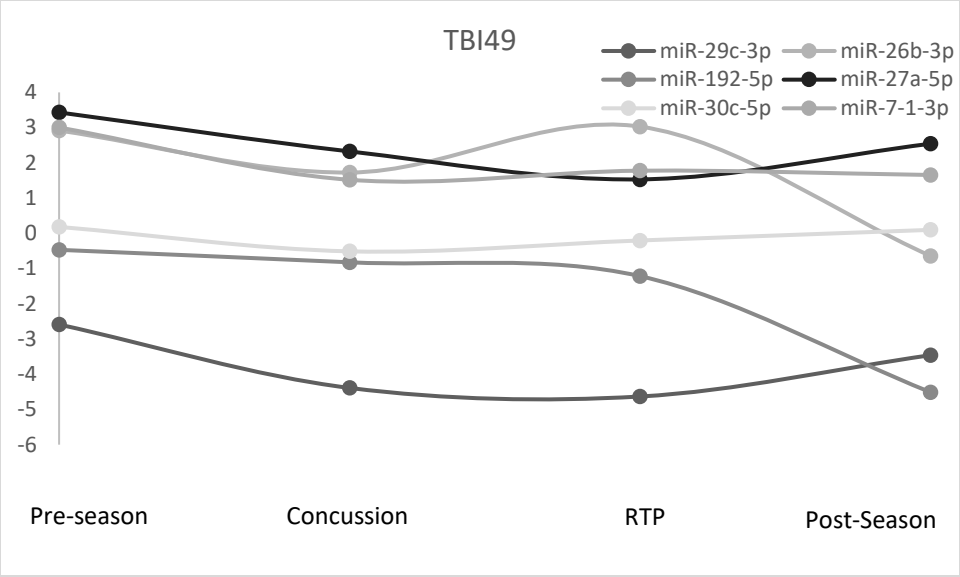

Figure 13. Trajectory plots of target miRNA for participant TBI49, who suffered a concussion while enrolled in this study.

CHAPTER VI

CONCLUSIONS

This dissertation's overall purpose was to examine salivary miRNA's potential to act as a clinical biomarker of concussions. To achieve this overarching purpose, we conducted three studies; the first study examined the current literature regarding salivary miRNA as a potential concussion biomarker, the second study explored ATs familiarity with and attitudes toward potential concussion biomarkers, and the third study examined the effects of one NCAA Division I football season on salivary miRNA which have been identified as a concussion biomarker. Overall, the first study identified forty-nine salivary miRNAs throughout the literature which may potentially act as a biomarker of concussion. Of the forty-nine, thirty-four demonstrated significant changes with the diagnosis of concussion, and twenty-one correlated with symptom trends following a concussion or multiple concussions. Additionally, six of the forty-nine accurately distinguished between concussed and non-concussed individuals.

With the novelty of salivary miRNA as a concussion biomarker, it is essential to note that many of the 2,000 different miRNAs are evaluated for their potential as a biomarker in current research. Despite this, it is very promising that only forty-nine have demonstrated potential as a concussion biomarker. Additionally, five families of miRNA overlapped across multiple studies, making them prime targets for future studies. Salivary miRNAs are an ideal candidate for a biomarker of concussion due to the ease and lack of invasiveness of collection, and the potential for immediate release into the oropharynx following trauma.[21] Future research should focus on further narrowing the list of potential miRNA targets by addressing current limitations. The impact of musculoskeletal injury, diverse populations, collection time, and various activities are not well understood and should be addressed in future research.

Regardless of the abilities of salivary miRNA to act as a concussion biomarker, it is still important to also consider the healthcare professionals who could potentially implement this tool into their clinical practices. Even if this tool were shown to be a valid and reliable measure of concussion injuries and clinicians were not aware of the tool, had negative attitudes toward the implementation, or lacked the skills to implement the tool, it would hinder the use for clinical practice. The findings from the second study of this dissertation found that a majority of responding ATs were not familiar with many potential biomarkers or the knowledge surrounding concussion biomarkers. However, most respondents agreed that identifying a valid, reliable biomarker of concussion would aid their ability to diagnose and manage concussions. Additionally, an overwhelming majority indicated they would implement such a tool into their clinical practice if it were available. These findings could potentially be correlated to the difficulties that concussions bring most healthcare professionals today due to the lack of valid and reliable objective measures for concussion management.

The results from the third study indicated that a subset of previously identified potential salivary miRNA concussion biomarkers did not differ significantly over the course of one season of contact sport. However, further analysis through intraclass correlation coefficient statistics revealed that the expressions from the subset of salivary miRNA were not reliable across one football season. Additional findings revealed a lack of significant differences between individual miRNA expressions from samples collected during pre-season and samples collected at the time of injury. However, when creating a ratio between two target miRNAs (miR-192-5p and miR-7-1-3p), we could distinguish between samples collected during pre-season and samples collected at the time of injury with 97% accuracy. Additionally, when samples collected from healthy matched controls were included in the comparison for the ratio of the two target miRNAs, the

ratio's accuracy decreased to 64%. This indicates that the ratio has a high sensitivity but lacks specificity. With the capability to accurately diagnose concussions, fewer concussion diagnoses could go potentially undiagnosed, ultimately improving the outcomes following an injury. However, due to the lack of specificity, many individuals who are not concussed may be identified as concussed or despite being completely recovered from the injury, the ratio may indicate they are not recovered, ultimately withholding them from activity longer than necessary.

Overall, biomarkers of concussion are a novel yet important topic that needs to be explored further through continued research. Concussions often provide difficulties for healthcare professionals and can potentially lead to long-term effects, which makes it important to identify a valid and reliable objective measure of concussions. The results from this dissertation indicate that ATs are open to implementing such a tool and even believe that it will improve their ability to manage concussions, ultimately improving outcomes post-injury. Additionally, since ATs are not the only healthcare providers who diagnose and manage concussions, it is important to consider other healthcare providers' knowledge of, familiarity with, and attitudes toward potential concussion biomarkers. We aimed to address one limitation surrounding the use of salivary miRNA as a concussion biomarker by establishing the effects of one NCAA Division I football season on a subset of previously identified miRNA. Although the findings indicated that this specific subset of salivary miRNA were not reliable over one season, a ratio of two salivary miRNA demonstrated high sensitivity to a concussion diagnosis. Overall, we believe research should continue to address limitations surrounding salivary miRNA as a concussion biomarker for other subsets that have been identified throughout the literature.

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PUBLICATIONS AND PRESENTATIONS

Refereed Manuscripts

1. **Campbell TR**, Reilly N, Zamponi M, Leathers D, Mollica P, Cavallario JM, Martinez JC (2023). Salivary microRNA as a prospective tool for concussion diagnosis and management: A scoping review. *Accepted.* Brain Inj.
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2. **Campbell TR**, Boyd JD, Davis BJ, Martinez JC, Cavallario JM. "Athletic trainers' perceptions of concussion biomarkers" Mid-Atlantic Athletic Trainers' Association Annual Symposium. May, 2022.