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# Evaluation of the relationship of miRNA to head impact exposure and motor control behavior: a preliminary study

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**EVALUATION OF THE RELATIONSHIP OF MIRNA TO HEAD IMPACT  
EXPOSURE AND MOTOR CONTROL BEHAVIOR: A PRELIMINARY STUDY**

by

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B.S., Brandeis University, 2020

Submitted in partial fulfillment of the  
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Master of Science

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**MAX BREITER**

**ABSTRACT**

Brain injuries like concussions have been a prevalent issue in contact sports like football for years. It is well documented that impacts to the head can cause concussions along with clinical manifestation such as impaired balance, memory issues, and headache. The aim of this paper is to explore the impact sub-concussive events have on neuroinflammatory markers as well as motor control. The level of nine miRNA neuroinflammatory markers were measured at preseason and at the end of the season and compared to the number of head acceleration events (HAEs) that took place during practice that year. In addition, four types of virtual reality (VR) testing were conducted in preseason and postseason to determine if there are any motor functions that are impaired by an accumulation of sub-concussive impacts.

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## **LIST OF ABBREVIATIONS**

CBF	Cerebral Blood Flow
HAE	Head Acceleration Event
K	Potassium
PSU	Penn State University
TBI	Traumatic Brain Injury
VR	Virtual Reality

## CHAPTER 1

### SECTION 1: INTRODUCTION

Head Trauma is a complicated process with reported affects ranging across many layers of biological organization (i.e., from behavior to circuit to cell to molecules). How head trauma is framed is also a complex issue with some scientists claiming it can be framed as a cluster of clinical symptoms, like concussion, that can become more severe across various levels of traumatic brain injury (TBI)<sup>1,2</sup>. Other clinicians and scientists point to the incidence of sub-concussive injury, sometimes referred to as head acceleration events (HAE)<sup>3</sup>. HAE's can be thought of as something that gets summed up overtime which can make an individual more susceptible to a concussion inducing injury or become a concussion itself with enough exposure<sup>3,4</sup>. This thesis will integrate one layer of biological organization, the transcriptome and microRNA, with HAE and strong measures of behavior from computational cognitive science. This integration will focus on transcriptome measures of inflammation and determine a casual framework for their elevation in individuals undergoing repetitive HAEs. Specifically, this integration will seek to show that exposure to HAEs across a season of athletic competition can be used with the pre-season miRNA level to predict the post-season miRNA level using moderation analysis. The same will be done with pre- and post-season measures of computational behavior to demonstrate in a mechanistic way that, across time, HAEs alter inflammatory miRNAs and behavior.

One area where head trauma is elevated above the average population is in contact sports such as: football, boxing, soccer, rugby, etc<sup>1</sup>. The American Medical

Society of Sports Medicine estimated that as many as 3.8 million athletes in the United States experience a concussion every year<sup>1</sup>. Of the 3.8 million concussions it is expected that around 50% are undiagnosed, which indicates that about 1.8 million concussions are not being treated each year<sup>1</sup>. Lack of diagnosis can be caused by a variety of issues ranging from no clear clinical manifestations of the injury to individuals not reporting symptoms so they can continue to compete. Brain injuries are a serious issue due to the long-term effects they can have<sup>2</sup>. Traumatic brain injuries have been associated with cognitive impairment including decreased executive functioning, reduced information processing speed, impaired memory, and changes in one's ability to pay attention<sup>2</sup>. Other consequences of head injuries are physical disabilities which can range from deficits in motor and sensory function to immobilization<sup>2</sup>. It is also common for individuals to experience psychiatric disorders such as depression, anxiety, and psychosis<sup>2</sup>. There are a variety of other long-term complications linked to brain issues such as dementia<sup>2</sup>. Multiple head injuries are extremely problematic, if the individual is not given adequate time to heal, because the subsequent injuries can cause the damage from the initial injury to be compounded<sup>3</sup>. Given the potential for long term negative effects from brain injuries, there is a need for better methods of diagnosis. HAEs and virtual reality testing, that monitor changes in motor control, have the potential to improve diagnosis of concussion.

HAEs are important as they represent the average cumulative force an individual experiences during athletic collisions. The measurements are taken using head sensors that determine peak linear, peak transitional, and peak angular accelerations during practices and games<sup>5,6</sup>. This is especially feasible for football players as the sensors can

be placed inside the athlete's helmet in discrete locations. There can be a variety of HAE variables, all of which measure different acceleration events. These HAEs can accommodate variations in G-force athletes experience, take an average of the total HAEs over the course of the season, and measure the total number of HAE in a season<sup>3,4</sup>. One G-force represents the force due to gravity, which is mass multiplied by gravitational acceleration,  $9.8 \text{ m/s}^2$ . This thesis will focus on 25G and 80G forces that collegiate football players are exposed to during practice sessions over the course of the 2015 football season. It is important to note that different positions in American football are more susceptible to impacts. One can separate the positions into speed groups, such as wide-receivers and running backs, and non-speed groups, such as the offensive line, where the speed groups are individuals at greater risk for high magnitude impacts<sup>8</sup>. This thesis does not explore this concept, but it is important to keep in mind when running the tests because non-speed positions, the cohort at less risk, will be grouped with speed positions, the more at-risk cohort. This could result in an under representation of HAEs in the speed positions<sup>8</sup>. Although the sensors are effective at measuring various acceleration events there are some limitations that need to be mentioned. The first being that the sensors were not used over multiple years of athletic competition but rather a short period. A larger sample size is needed to fully understand the sensors' ability to accurately quantify head acceleration events<sup>8</sup>. Another limitation is in the helmet design as there has been evidence that the size and weight of modern helmets might increase the risk of concussion<sup>8</sup>.

To understand how HAEs can cause brain injuries, such as concussion, one must first look at how HAEs might affect the brain and produce their deleterious effects. There are three primary theories surrounding the mechanism for concussion and accumulated HAEs, which are, neurovascular dysregulation, axonal shearing, and neuroinflammation.

Neurovascular dysregulation, also known as neurovascular uncoupling, is the process where the connection between neurons in the brain and their vascular supply is disrupted<sup>9</sup>. A disruption in vascular supply decreases the brain's energy source which affects its ability to function normally<sup>9</sup>. Yamakami and colleagues found that rats exposed to traumatic brain injury (TBI) had significantly reduced regional cerebral blood flow (CBF) in all areas of the brain post injury. Although the hindbrain recovered post injury, CBF to injured and non-injured parts of the forebrain did not. In addition to changes in blood flow, brain injuries affect energy levels by decreasing ATP and increasing lactic acid<sup>10</sup>. Biomechanical injury to the brain disrupts voltage-gated potassium (K) channels which causes a rise in extracellular K levels<sup>10</sup>. Mechanical injury prevents neural glial cells from correcting this issue, and taking up the excess K, which results in excessive depolarization<sup>10</sup>. To restore homeostasis, active transport is utilized which increases glucose usage<sup>10</sup>. Sudden increases in cerebral ATP requirements are usually accommodated by glycolysis because cerebral oxidative phosphorylation is believed to run at a maximum<sup>10</sup>. Excessive glycolysis can result in lactate accumulation which can result in neural uncoupling<sup>10</sup>. Coupled with a decrease in cerebral blood flow this can cause a serious energy crisis<sup>10</sup>. This is considered one of the main methods by which head injuries materialize and affect cognition.

Axonal shearing, also referred to as diffuse axonal injury, is the tearing of the brain's axons which can be caused by the brain moving within the skull<sup>11</sup>. Bazarian and colleagues found axonal swelling, in individuals experiencing TBI, using diffusion tensor imaging<sup>12</sup>. The images were taken within 72 hours of the injury<sup>12</sup>. The significance of this finding is that it shows that head injuries cause axonal swelling which is a preliminary step in axonal injury<sup>12</sup>. Individuals that have diffuse axonal injury will experience a decreased interaction and interconnection between neurons<sup>13</sup>. Decreased neural interconnection has been shown to primarily affect white matter in the frontal lobe, temporal lobe, and brainstem<sup>13</sup>. The frontal lobe is responsible for higher cognitive functioning and voluntary movements while the temporal lobe is associated with processing auditory information, encoding memory, and processing emotion<sup>14</sup>. Damage to the frontal lobe can cause loss of simple movement, difficulty problem solving, and inability to focus on tasks while damage to the temporal lobe causes impaired long-term memory, emotional disturbance, and difficulty learning information<sup>14</sup>. These clinical manifestations overlap with the CDC recommendation for the signs and symptoms of concussion<sup>15</sup>. Injury to the brainstem causes balance problems, nausea, and sleep difficulties which are also signs of concussion<sup>14,15</sup>.

Although these first two theories are significant, the third theory surrounding neuroinflammation, is the one that this thesis will focus on. Neuroinflammation is an inflammatory response that takes place in the spinal cord or brain<sup>16</sup>. The inflammation is mediated by products of central nervous system cells (microglia and astrocytes), immune cells, and endothelial cells<sup>16</sup>. The products of these cells linked to the inflammation are

cytokines, reactive oxygen species, and secondary messengers<sup>16</sup>. The molecules involved in the inflammatory response migrate to the injured site to remove damaged tissue and facilitate healing responses<sup>17</sup>. In chronic stages of inflammation, an anti-inflammatory response can worsen the environment and cause secondary cell death<sup>17</sup>. In this sense, inflammatory markers can be used as a measure for the degree of neuroinflammation in each subject<sup>18,19</sup>. This thesis will focus primarily on microRNA (miRNA), a secondary messenger, which is a known inflammatory marker<sup>20</sup>. MicroRNA is a piece of RNA that can bind to other messenger RNA (mRNA) molecules and causes their destruction, which can prevent protein translation<sup>21</sup>. Papa and colleagues found nine miRNAs to be elevated over the course of a season in a collegiate football team<sup>22</sup>. This is a significant finding as the nine miRNAs have various functions in the inflammatory process. The nine miRNAs are: miRNA-20a, miRNA-505, miRNA-362-3p, miRNA-30d, miRNA-92a, miRNA-486, miRNA-195, miRNA-9-3p, and miRNA-151-5p. Each of these miRNAs have distinct profiles and different responses to trauma.

Li and colleagues found that miRNA-20a plays an important role in the promotion of wound healing by regulating the inflammatory response<sup>23</sup>. Injection of miRNA-20a inhibitors into a wound resulted in increased inflammation and delayed closure of a wound<sup>23</sup>. MiRNA-20a reduces inflammation by decreasing the level of chemokines and cytokines<sup>23</sup>. This occurs when miRNA20-a targets SHCBP1 (a gene that produces SH2 binding proteins for TRKs<sup>24</sup>) and SEMA7A (a membrane anchor involved in cell signaling<sup>25</sup>), resulting in a decrease in TLR3-mediated NF- $\kappa$ B activation, which is a known transcription factor involved in immune and inflammatory responses<sup>26</sup>.

Similarly, miRNA-505 is considered an anti-inflammatory agent through its suppression of the HMGB1/NF- $\kappa$ B pathway<sup>27</sup>. Endometritis is a severe inflammatory issue that results in tissue damage. Liu and colleagues found that mice with endometritis had significant down regulation of miRNA-505<sup>27</sup>. Furthermore, they found that overexpression of miRNA-505 suppressed the production of pro-inflammatory cytokines such as IL-1b, IL-6 and TNF- $\alpha$ <sup>27</sup>. MiRNA-505 was found to target the 3' untranslated region of HMGB1 and inhibit its signaling pathway through negative feedback<sup>27</sup>.

Omidbakhsh and colleagues suggest that miRNA-362-3p plays an important role as a biomarker for inflammatory bowel disease<sup>28</sup>. The study showed that miRNA-362-3p was upregulated in the peripheral blood of patients experiencing inflammation, indicating its involvement in inflammatory pathologies<sup>28</sup>. Additionally, alternative studies have shown miRNA-362-3p acts as a tumor suppressor for various cancers<sup>29</sup>. Inflammation is a component of the carcinoma mechanism which further suggests miRNA-362-3p's involvement<sup>30</sup>.

Similar to the previously discussed miRNA's, miRNA-30d regulates inflammation by keeping proinflammatory cytokine levels low. MiRNA-30d levels were found to negatively correlate with pro-inflammatory cytokines, meaning when miRNA-30d levels are high cytokine levels are low and when miRNA-30d levels are low cytokine levels are high<sup>31</sup>. MiRNA-30d manages cytokine levels by regulating Foxo3a and SOCS3<sup>31</sup>. Foxo3a is a tumor suppressor gene which acts to promote cell death and suppress the cell cycle<sup>32</sup>, while SOCS3 regulates cytokine or hormone signaling levels<sup>33</sup>.

Another anti-inflammatory miRNA is MiRNA-92a. Fu and colleagues found that inhibition of miRNA-92a decreases the repression of proinflammatory cytokines: tumor necrosis factor-alpha, interleukin-1B and IL-6<sup>34</sup>. Inhibition of these inflammatory pathways indicates miRNA-92a plays a role in inflammation suppression.

MiRNA-486-5p has been shown to have elevated levels in individuals experiencing multiple sclerosis (MS)<sup>30</sup>. MS is a disease that impacts the central nervous system, specifically the interactions between the brain and the spinal cord<sup>35</sup>. In MS patients, the immune system attacks the myelin sheath surrounding nerve fibers in the brain and spinal cord. This immune attack causes an inflammatory response which destroys the nerve cells<sup>35</sup>. The severity of destruction and symptoms manifested varies across individuals<sup>35</sup>. Given miRNA-486-5p is known to regulate inflammation<sup>30</sup>, its elevated levels in MS patients suggests it plays a role in the neuroinflammatory process of the disease.

MiRNA-195 is involved in the neuroinflammatory process by inhibiting NF-kb signaling while also decreasing cytokine TNF-alpha expression, which has been known to induce apoptosis in neurons<sup>36</sup>. Down regulation of these known inflammatory molecules indicates miRNA-195 plays a role in neuroinflammatory regulation. Some scientists have suggested miRNA-195-5p can play a role as a potential therapeutic for brain injury<sup>36</sup>.

A study conducted by Sim and colleagues suggested that miRNA-9-3p results in learning and memory deficiencies through hippocampal inhibition<sup>37</sup>. In addition, it was found that miRNA-9-3p is important for hippocampal synaptic plasticity<sup>37</sup>. This suggests

that low levels miRNA-9-3p in collegiate football players could result in decreased memory and neural damage. The neural damage could also cause neuroinflammation.

MiRNA-151-5p is upregulated in the sigmoid colon of individuals suffering from ulcerative colitis as well as the peripheral blood flows of people with Crohn's disease<sup>38</sup>. Both ulcerative colitis and Crohn's disease are inflammatory diseases which suggest miRNA-151-5p acts as a biomarker for inflammation<sup>38</sup>. Ulcerative colitis causes inflammation in the digestive tract which leads to sores known as ulcers<sup>39</sup>. For this reason, it is considered an inflammatory bowel disease for which there is no known cure<sup>40</sup>.

All nine of these miRNAs have been implicated in some measure of inflammation which is why they have been chosen for further examination. It is important to differentiate between two types of neuroinflammation: acute and chronic. Acute neuroinflammation can have restorative benefits for individuals post injury<sup>41</sup>, whereas chronic neuroinflammation can have negative effects such as mitochondrial dysregulation<sup>19,41,42</sup>. When individuals experience mitochondrial dysregulation they have less energy to combat the injury and heal<sup>19</sup>. Papa and colleagues found that football players have elevated levels of these miRNAs preseason indicating they are likely experiencing chronic neuroinflammation<sup>22</sup>. The finding from Papa and colleagues does present a confounding variable, but this would likely cause a type II error and lead to an underrepresentation of the results. Therefore, any significant finding can be assumed to be true. The study below looks at whether changes in preseason and postseason miRNA levels are influenced directly by the number of sub-concussive events, HAE's, the athlete

experiences over the course of a season. This analysis will mechanistically quantify the degree of neuroinflammation that occurs in response to an accumulation of sub-concussive events. Subsequently, the impact of these sub-concussive events on motor function will be examined through VR testing.

Dr. Semyon Slobounov identified VR testing as a means for identifying clinical manifestations of concussion in athletes<sup>43</sup>. VR testing was designed to look at sensory motor reactivity, balance, and spatial navigation<sup>43,44</sup>. These behavioral characteristics were chosen based on the recommendations of Alexander Luria who identified them as features that commonly change when one experiences a head injury<sup>44</sup>. Teel, a colleague of Dr. Slobounov, found that athletes diagnosed with a concussion, in a ten-day window post injury, scored significantly worse on various VR tests<sup>45</sup>. Similarly, Teel and colleagues used VR testing and the framework established by Alexander Luria, to measure spatial navigation, body reaction time, and a combination of all the VR measures<sup>46</sup>. The findings from Teel and colleagues further support the significance of VR testing as a means of concussion diagnosis<sup>45</sup>. All three areas of VR testing were found to be statistically significant. This shows that VR technology can be used to detect residual abnormalities due to neurological alterations that alternative tests, such as neuropsychological tests, cannot find<sup>45,46</sup>. This provides a novel approach to identifying potential brain injuries. In addition, it raises the question as to whether individuals not diagnosed with a concussion, but exposed to multiple high magnitude HAEs, have lingering VR abnormalities after the season. This finding would be significant as it would

suggest concussions and brain injuries exist on a spectrum where sub-concussive impacts, such as HAEs, can have consequences on athletes.

Based on the established role of miRNAs as an indicator of inflammation and the high level of contact in collegiate football, it is reasonable to predict that an increased frequency and magnitude of HAE impacts over the course of the season will result in higher miRNA levels post-season compared to pre-season. All miRNAs are expected to be elevated post-season. In addition, the more sub-concussive HAE's an athlete experiences over the course of a season will cause their post-season VR scores to worsen compared to their pre-season VR scores.

## SECTION TWO: SPECIFIC AIMS

One of the main complications caused by brain injuries is the neuroinflammatory response associated with injury<sup>22</sup>. Chronic neuroinflammation can have detrimental effects which include, fatigue, decreased motor function, and depressed mental speed<sup>19</sup>. Although chronic neuroinflammation can have negative implications, acute inflammation is believed to have some protective benefits<sup>41,42</sup>. It has been shown that neuroinflammatory biomarker levels peak seven days post injury<sup>47</sup>. Studies have also suggested neuroinflammation plays a role in chronic neurodegenerative disease, which can lead to dementia<sup>48</sup>. In contact sports, and daily life, there is a degree of subjectivity when diagnosing brain injuries as not all injuries present clear clinical manifestations<sup>43</sup>. Some scientists have suggested that brain injury lies on a spectrum which varies in severity<sup>1,2</sup>. Based on this, there could be a subset of individuals experiencing neuroinflammation while both the clinician and patient are unaware of the situation. This study aims to investigate whether miRNA levels and exposure to HAEs can provide a potential framework for quantifying the extent of the injury and the degree of neuroinflammation. This study plans to provide a more comprehensive understanding of the following objectives:

1. To determine if any of the following five HAE variables, Average 25G, Average 80G, Total 80G hits, Total 25G hits, Total Sensor Sessions, moderate the relationship between preseason miRNA levels and post-season miRNA levels.
2. To determine if any of the nine miRNA variables: miRNA-20a, miRNA-505, miRNA-362-3p, miRNA-30d, miRNA-92a, miRNA-486, miRNA-195, miRNA-

9-3p, and miRNA-151-5p, are significantly altered over the course of a collegiate football season.

3. To determine whether the relationship between  $\Delta$ miRNA and  $\Delta$ V<sub>R</sub> is significantly moderated by exposure to HAE's.
4. To better understand if there is a threshold at which an accumulation of sub-concussive events affects the transcriptome. In addition, if continued exposure makes one more susceptible to potential damage.

## CHAPTER TWO: METHODS

### *Participants*

A cohort of Penn State University collegiate football players, from the 2015 season, were involved in this study. With IRB approval, these participants submitted various questionnaires while also participating in pre-season and post-season testing. The athletes submitted a standard self-report demographic form which they provided information on their team position, number of years playing football, race, and age. Athletes were grouped into “speed” and “non-speed” positions as a means for minimizing any confounding variables associated with positions. The criteria used for group assignment was based on position and was adapted from Lehman and colleagues<sup>8</sup>. Members of the Penn State football team classified as punters, kickers, and centers/long snappers were not included in the study because the Lehman criteria (2013) does not classify them as speed or non-speed positions<sup>8</sup>. Some individuals were not included in the study because attrition resulted in an inability to collect post-season miRNA levels. This occurred due to athlete injuries or staff shortages at the end of the season. Based on this, 24 of the players from the 2015 season, who underwent data collection, have complete data for inclusion in this study.

In a separate questionnaire, participants were prompted to share the total number of diagnosed concussions they have experienced from high school up until the beginning of the 2015 season. It is important to acknowledge that concussions, particularly at a high-school level, are historically underdiagnosed<sup>49</sup>. To minimize this confounding

variable, and get an accurate measurement on diagnosed concussions, participants were told that the data collected was only for research purposes and would not be shared with the coaching staff or recorded on athlete's medical record. To confirm the results of the data the chief team physician for the Penn State University (PSU) football team performed a chart review. This was done to measure internal validity by comparing responses with the physician's concussion diagnoses while at PSU and records of concussion diagnoses for PSU football players prior to entering PSU. All the procedures and data collection were conducted by the Center for Sports Concussion Research and Services at Penn State University. All studies performed received IRB approval and have subsequently received IRB approval for the same data collection every year since 2015. Dr. Semyon Slobounov, one of the principal investigators at the Center for Sports Concussion Research and Services, granted access for the data to be used in this thesis.

### *Serum*

Two 5mL samples of venous blood was extracted from patients, one during pre-season and the other after the season ended. Samples were allowed to clot at room temperature in a serum separator tube before they were centrifuged. Once the centrifuge was completed the serum was extracted and placed in aliquot tubes with bar codes. The tubes were stored at -70°C and sent to a laboratory for blinded batch analysis<sup>22,50</sup>. From there samples were sent to Metabolon, in Morrisville, NC, USA, for blinded transcriptome analysis.

### *miRNA Quantification*

The two serum samples collected were used to measure RNA levels. A serum isolation kit from Qiagen Inc. was used and protocol instructions were followed. The RNA sample eluted in 20uL DNase and RNAase free water sample where it was stored at -80°C. A droplet digital PCR, from Bio-Rad Inc., was used to measure the levels of nine miRNA: miRNA-20a, miRNA-505, miRNA-362-3p, miRNA-30d, miRNA-92a, miRNA-486, miRNA-195, miRNA-9-3p, and miRNA-151-5p. Prior to the droplet digital PCR usage, the RNA samples were analyzed using a bioanalyzer assay to ensure quality control. Once the RNA quality was ensured, a TaqMan assay from Thermofisher Labs was used and the RNA was reverse transcribed.

### *HAE Monitoring*

HAE measurements were recorded at all contact practice sessions over the course of the season including all preseason sessions. No HAE variables were recorded during games. There was a total of 53 contact practices over the course of the season. The HAE variables were determined using a Boditrak sensor system produced by The Head Health Network. It is important to note that although HAE measurements were not taken during games, studies have found the majority of HAE's take place during contact practices<sup>5,6</sup>. The sensors contained an accelerometer, gyroscope, and thermometer which measured the number of impacts, location of impact, and the helmet fit<sup>51</sup>. The sensors were placed, using a 3M VHB adhesive, on the inner surface of the helmet between the padding and the shell. Two threshold measurements of G-force were used to quantify the force of the

HAEs over the course of the season. The first was 25G, indicating the HAE had a force between  $25G \leq HAE < 80G$ . The second 80G, which was a force of  $80G \leq HAE$ . These forces were determined by the threshold guidelines established by previous brain injury reports<sup>52</sup>. These two force thresholds were used to create five HAE variables: Average 25G, Average 80G, Total 25G hits, Total 80G hits, and Total Sensor Sessions. Average HAE represented the average number of HAEs, at a given threshold, experienced across the total practice sessions for a participant. Total HAE hits represented the cumulative number of HAE for a given threshold an athlete experienced over the course of a season. Total Sensor Sessions indicated the cumulation of all HAEs for both thresholds over the course of the season.

### *Virtual Reality Measurements*

Athletes were required to complete a virtual reality test with a 3D TV system at the start of the season and the end of the season<sup>45,46,53</sup>. The tests focused on three areas of motor control: spatial memory, balance, and sensory-motor reactivity or whole-body reaction time. Luria and colleagues found these three areas to be important when determining balance problems and spatial memory for brain injuries in soldiers<sup>44</sup>. Each test was scored on a scale of 0 to 10, with 0 being the worst score and 10 being the best score.

The spatial memory test put athletes in a virtual corridor where they were shown a randomized pathway with a variety of different turns. The participants were asked to walk down the hallway and then to return. After walking the pathway, the athletes were

requested to recall their trip using a joystick. A score was given to the athletes based on the number of errors they made when recalling their trip and the amount of time it took the participant to complete the trip.

Balance was tested by having athletes complete various trials while holding an adjusted tandem Romberg position. The tandem Romberg position requires participants to place their feet in a heel to toe position where one foot is slightly in front of the other<sup>54</sup>. This is a common position used for determining athlete's ability to balance<sup>54</sup>. The first trial established a baseline balance score for the athlete by having them stand completely still. Subsequently, six additional trials were performed where the virtual room moved in a variety of different directions. An accelerometer was used to determine the degree to which the athletes position changed when exposed to the various situations. The athletes were scored based on their ability to successfully adjust their position to the given environment.

The reaction time test had participants stand with their feet about shoulder width apart and their hands by their side. The participants were told to adjust their body and mimic the changes taking place in the virtual room. The changes could occur in the horizontal and vertical direction. Similar to the balance test, an accelerometer measured the response times to abrupt changes that took place in the VR room.

In addition to the areas outlined by Luria and colleagues, a fourth comprehensive score was determined by combining the scores for spatial memory, balance, and reaction time tests. Similarly, this score had a range from 0 to 10. To ensure the score was easily

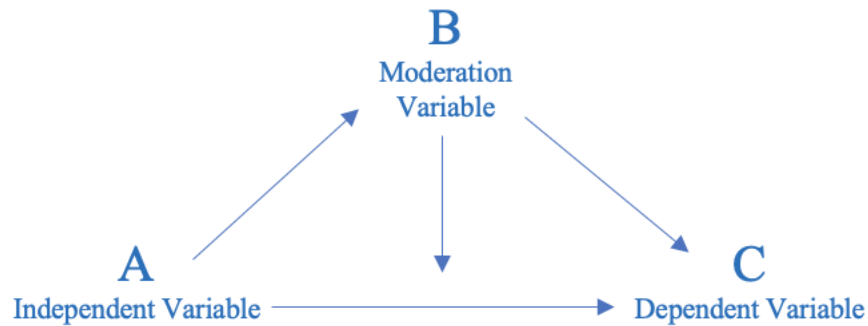
interpretable, it was scaled such that the higher score indicated excellent performance and a low score indicated poor performance<sup>45,46</sup>.

### *Statistical Measurements*

All statistical measurements were done using Stata BE software. For the miRNA comparisons, three regression analyses were done for each set of three variables resulting in ninety-nine regressions. Sets of three were done in an attempt to find significance between a triangulation of variables. Regressions were determined to be significant if  $p < 0.05$ . For the VR comparisons, three regressions were performed for each set of variables, but since there are five VR variables only forty-four regressions were performed.

- 1) Independent Variable → Dependent Variable
- 2) Independent Variable → Moderating Variable
- 3) Moderating Variable → Dependent Variable

Moderation tests were performed to determine if a moderating variable significantly influences the way the independent and dependent variable interact. For a moderation test to be significant both  $pF$  (significance between the DV and IV) and  $pB3$  (significance for the moderating term) need to be  $p < 0.1$ . In addition, cook's outlier test was performed to remove any outliers from both the regression and moderation tests. Figure 1 shows the triangulation model used for the regression tests as well as the framework for the moderation analyses.



**Figure 1: Framework for Moderation Tests and Triangulation of Regressions.**

Variable A is the preseason variable while variable C depicts the post-season change. Variable B is the moderator variable which influences the interaction between variables A and C.

Figure 2 depicts the codes that were plugged into Stata software to run the regression and moderation tests. Code A was used to run a regression test while code B was used to perform a moderation analysis.

- A) `Reg c.DV c.IV## c.Mo`  
`Predict cook, cooks, if e(sample)`  
`Drop if cook>4/_N`  
`Egen z1DV=std(DV)`  
`Egen z1IV=std(IV)`  
`Reg c.z1DV c.z1IV##c.z1Mo.`
- B) `Reg DV IV`  
`Predict cook, cooks, if e(sample)`

```
Drop if coo>4/_N  
Egen z1DV=std(DV)  
Egen z1IV=std(IV)  
Reg z1DV z1IV
```

**Figure 2: Stata Codes for Regression and Moderation Analyses.**

### CHAPTER THREE: RESULTS

A total of 24 players underwent both pre-season and post-season blood tests. Of the 24 athletes, some were missing a few miRNA variables. These individuals were excluded from the miRNA analyses that they did not have data for. This resulted in not all the trials having 24 participants. The majority of the 24 individuals had nine miRNAs measured: miRNA-20a, miRNA-505, miRNA-362-3p, miRNA-30d, miRNA-92a, miRNA-486, miRNA-195, miRNA-9-3p, and miRNA-151-5p. To determine a correlation between pre-season miRNA, post-season miRNA, and HAE exposure, a total of 99 linear regression tests were run. Pre-season miRNA was established as the A variable, HAE was chosen as variable B, and post-season miRNA was the C variable. For a given set of A, B, and C variables three linear regressions were run. The first being, A (pre-season miRNA) to B (HAE). Second, B (HAE) to C (post-season miRNA). Third, A (pre-season miRNA) to C (post-season miRNA). The goal was to find a triangulation between all three regressions for a given set of data. For example, A to B significant, B to C significant, and A to C significant.

**Table 1: Regression Tests for Pre-season and Post-season miRNA Levels.**

Compares pre-season miRNA level to post-season miRNA level, pre-season miRNA to HAE variable, and HAE variable to post-season miRNA level. Regression is significant if  $p < 0.05$ . N represents the number of subjects in each trial. Outliers are the number of participants removed after cook's outlier test performed. The beta coefficient represents the amount of change in the outcome variable for every unit change in the predictor variable.

Trial	IV	DV	N	Outliers Removed	p-value	beta coefficient	95% CI	R2	adjusted r2
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1	miR20a pre	miR20a post	14	4	0.887	0.042	(0.58)- (0.67)	0.0018	-0.0814
2	miR20a pre	Average 25G	16	4	0.573	-0.152454	(-0.72) (0.41)	0.0232	-0.0465
3	Average 25G	miR20a post	17	2	0.677	-0.109158	(-0.66) (0.44)	0.0119	-0.054
4	miR20a pre	Average 80G	17	3	0.007	-0.630611	(-1.06) (-0.203)	0.3977	0.3575
5	Average 80G	miR20a post	17	2	0.081	-0.434837	(-0.93) (0.061)	0.1891	0.135
6	miR20a pre	Total 25G hits	17	3	0.644	-0.121039	(-0.67) (0.43)	0.0147	-0.051
7	Total 25G hits	miR20a post	17	2	0.195	-0.330237	(-0.85) (0.19)	0.1091	0.0497
<b>8</b>	<b>miR20a pre</b>	<b>Total 80G hits</b>	<b>16</b>	<b>4</b>	<b>0.048</b>	<b>-0.50125</b>	<b>(-0.997)</b> <b>(-0.005)</b>	<b>0.2513</b>	<b>0.1978</b>
<b>9</b>	<b>Total 80G hits</b>	<b>miR20a post</b>	<b>16</b>	<b>3</b>	<b>0.002</b>	<b>-0.714239</b>	<b>(-1.12)</b> <b>(-0.313)</b>	<b>0.5101</b>	<b>0.4751</b>
10	miR20a pre	Total sensor sessions	16	4	0.693	-0.107202	(-0.68) (-0.55)	0.0115	-0.0591
11	Total sensor sessions	miR20a post	16	3	0.575	-0.151736	(-0.72) (0.41)	0.023	-0.0468
12	miR505 pre	miR505 post	19	1	0.891	0.0336527	(-0.48) (0.55)	0.0011	-0.0576
13	miR505 pre	Average 25G	19	2	0.639	-0.115119	(-0.62) (0.39)	0.0133	-0.0448
14	Average 25G	miR505 post	16	4	0.061	0.4775851	(-0.026) (0.98)	0.2281	0.173
15	miR505 pre	Average 80G	20	1	0.957	0.0130167	(-0.48) (0.508)	0.0002	-0.0554
16	Average 80G	miR505 post	18	2	0.958	0.0134109	(-0.52) (0.54)	0.0002	-0.0623
17	miR505 pre	Total 25G hits	17	4	0.33	-0.2516	(-0.78) (0.28)	0.0633	0.0009
18	Total 25G hits	miR505 post	19	1	0.478	0.1731	(-0.33) (0.68)	0.03	-0.0271
19	miR505 pre	Total 80G hits	17	4	0.042	-0.497123	(-0.98) (-0.019)	0.2471	0.1969
20	Total 80G hits	miR505 post	16	4	0.507	-0.1788	(-0.74) (0.39)	0.032	-0.0372

21	miR505 pre	Total sensor sessions	19	2	0.057	-0.4437	(-0.902) (0.015)	0.1969	0.1497
22	Total sensor sessions	miR505 post	15	5	0.263	-0.3088	(-0.88) (0.27)	0.0954	0.0258
<b>23</b>	<b>miR362-3P pre</b>	<b>miR362-3P post</b>	<b>17</b>	<b>3</b>	<b>0</b>	<b>0.9048372</b>	<b>(0.671) (1.14)</b>	<b>0.8187</b>	<b>0.8066</b>
24	miR362-3P pre	Average 25G	19	3	0.68	0.10135	(-0.408) (0.61)	0.0103	-0.0479
25	Average 25G	miR362-3P post	16	4	0.366	0.2423	(-0.314) (0.798)	0.0587	-0.0085
26	miR362-3P pre	Average 80G	17	5	0.735	-0.0888	(-0.637) (0.459)	0.0079	-0.0582
27	Average 80G	miR362-3P post	17	3	0.446	-0.1979	(-0.737) (0.342)	0.0392	-0.0249
28	miR362-3P pre	Total 25G hits	18	4	0.684	-0.1031	(-0.630) (0.424)	0.0106	-0.0512
29	Total 25G hits	miR362-3P post	18	2	0.167	-0.3404	(-0.839) (0.158)	0.1159	0.0607
<b>30</b>	<b>miR362-3P pre</b>	<b>Total 80G hits</b>	<b>21</b>	<b>1</b>	<b>0.041</b>	<b>-0.4493</b>	<b>(-0.878) (-0.020)</b>	<b>0.2019</b>	<b>0.1599</b>
<b>31</b>	<b>Total 80G hits</b>	<b>miR362-3P post</b>	<b>16</b>	<b>4</b>	<b>0.032</b>	<b>-0.5359</b>	<b>(-1.019) (-0.052)</b>	<b>0.2872</b>	<b>0.2363</b>
32	miR362-3P pre	Total sensor sessions	19	3	0.92	-0.0246	(-0.536) (0.487)	0.0006	-0.0582
33	Total sensor sessions	miR362-3P post	16	4	0.306	-0.2732	(-0.825) (-0.534)	0.0747	0.0086
34	miR30d pre	miR30d post	15	5	0.066	0.486	(-0.038) (1.009)	0.2363	0.1775
35	miR30d pre	Average 25G	20	2	0.343	0.2236	(-0.259) (0.706)	0.05	-0.0027
36	Average 25G	miR30d post	16	4	0.416	0.2183	(-0.341) (0.778)	0.0477	-0.02
37	miR30d pre	Average 80G	19	3	0.784	0.0675	(-0.443) (0.578)	0.0046	-0.054
38	Average 80G	miR30d post	17	3	0.703	0.0999	(-0.448) (0.647)	0.01	-0.056
39	miR30d pre	Total 25G hits	19	3	0.673	0.1036	(-0.405) (0.613)	0.0108	-0.0474

40	Total 25G hits	miR30d post	17	3	0.751	-0.0831	(-0.632) (0.465)	0.0069	-0.0593
41	miR30d pre	Total 80G hits	18	4	0.087	-0.4145	(-0.897) (0.068)	0.1718	0.1201
42	Total 80G hits	miR30d post	13	7	0.307	-0.3075	(-0.939) (0.324)	0.0946	0.0123
43	miR30d pre	Total sensor sessions	19	3	0.468	-0.1772	(-0.681) (0.326)	0.0314	-0.0256
<b>44</b>	<b>Total sensor sessions</b>	<b>miR30d post</b>	<b>16</b>	<b>4</b>	<b>0.038</b>	<b>-0.5224</b>	<b>(-1.01)</b> <b>(-0.034)</b>	<b>0.273</b>	<b>0.221</b>
45	miR92a pre	miR92a post	16	4	0.46	0.1992	(-0.363) (0.761)	0.0397	-0.0289
46	miR92a pre	Average 25G	18	4	0.203	0.3232	(-0.193) (0.839)	0.0992	0.0429
<b>47</b>	<b>Average 25G</b>	<b>miR92a post</b>	<b>18</b>	<b>2</b>	<b>0.03</b>	<b>0.5125</b>	<b>(0.057)</b> <b>(0.968)</b>	<b>0.2627</b>	<b>0.2166</b>
<b>48</b>	<b>miR92a pre</b>	<b>Average 80G</b>	<b>18</b>	<b>4</b>	<b>0.017</b>	<b>-0.5555</b>	<b>(-0.996)</b> <b>(-0.115)</b>	<b>0.3087</b>	<b>0.2655</b>
49	Average 80G	miR92a post	16	4	0.357	0.2469	(-0.309) (0.802)	0.061	-0.0061
50	miR92a pre	Total 25G hits	19	3	0.968	0.0098	(-0.502) (0.522)	0.0001	-0.0587
51	Total 25G hits	miR92a post	16	4	0.184	0.35	(-0.187) (0.887)	0.1225	0.0598
<b>52</b>	<b>miR92a pre</b>	<b>Total 80G hits</b>	<b>20</b>	<b>2</b>	<b>0.002</b>	<b>-0.644</b>	<b>(-1.023)</b> <b>(-0.265)</b>	<b>0.4147</b>	<b>0.3822</b>
53	Total 80G hits	miR92a post	15	5	0.769	0.0827	(-0.514) (0.68)	0.0068	-0.0695
54	miR92a pre	Total sensor sessions	19	3	0.267	-0.2681	(-0.761) (0.225)	0.0719	0.0173
<b>55</b>	<b>Total sensor sessions</b>	<b>miR92a post</b>	<b>18</b>	<b>2</b>	<b>0.043</b>	<b>-0.48066</b>	<b>(-0.945)</b> <b>(-0.016)</b>	<b>0.231</b>	<b>0.183</b>
56	miR486 pre	miR486 post	18	2	0.319	0.249	(-0.26) (0.76)	0.062	0.0034
57	miR486 pre	Average 25G	19	3	0.743	-0.0807	(-0.591) (0.429)	0.0065	-0.0519
58	Average 25G	miR486 post	16	4	0.186	0.3486	(-0.189) (0.886)	0.1216	0.0588

59	miR486 pre	Average 80G	19	3	0.495	-0.1668	(-0.671) (0.338)	0.0278	-0.0294
60	Average 80G	miR486 post	16	4	0.412	-0.2205	(-0.78) 0.338)	0.0487	-0.0193
61	miR486 pre	Total 25G hits	18	4	0.247	0.2879	(-0.22) (0.795)	0.0829	0.0256
62	Total 25G hits	miR486 post	16	4	0.06	0.4802	(-0.023) (0.983)	0.2307	0.1757
63	miR486 pre	Total 80G hits	19	3	0.85	0.04655	(-0.465) (0.558)	0.0022	-0.0565
64	Total 80G hits	miR486 post	14	6	0.166	0.3916	(-0.187) (0.97)	0.1534	0.0828
<b>65</b>	<b>miR486 pre</b>	<b>Total sensor sessions</b>	<b>19</b>	<b>3</b>	<b>0.037</b>	<b>-0.481</b>	<b>(-0.93) (-0.032)</b>	<b>0.2314</b>	<b>0.1862</b>
66	Total sensor sessions	miR486 post	15	5	0.881	-0.0423	(-0.641) (0.556)	0.0018	-0.075
67	miR195 pre	miR195 post	15	5	0.269	0.3049	(-0.266) (0.876)	0.093	0.0232
68	miR195 pre	Average 25G	19	3	0.873	0.0394	(-0.472) (0.551)	0.0016	-0.0572
69	Average 25G	miR195 post	14	6	0.768	-0.0866	(-0.713) (0.54)	0.0075	-0.0752
70	miR195 pre	Average 80G	18	4	0.074	-0.4308	(-0.909) (0.047)	0.1856	0.1347
71	Average 80G	miR195 post	15	5	0.793	-0.074	(-0.672) (0.524)	0.0055	-0.071
72	miR195 pre	Total 25G hits	20	2	0.634	-0.1134	(-0.605) (0.379)	0.0129	-0.042
73	Total 25G hits	miR195 post	16	4	0.145	-0.381	(-0.911) (0.149)	0.1452	0.0842
<b>74</b>	<b>miR195 pre</b>	<b>Total 80G hits</b>	<b>20</b>	<b>2</b>	<b>0.034</b>	<b>-0.475</b>	<b>(-0.911) (-0.039)</b>	<b>0.2257</b>	<b>0.1827</b>
<b>75</b>	<b>Total 80G hits</b>	<b>miR195 post</b>	<b>14</b>	<b>6</b>	<b>0.029</b>	<b>-0.5809</b>	<b>(-1.09) (-0.069)</b>	<b>0.0338</b>	<b>0.2823</b>
76	miR195 pre	Total sensor sessions	19	3	0.396	-0.2064	(-0.707) (0.294)	0.0426	-0.0137
77	Total sensor sessions	miR195 post	14	6	0.101	-0.4567	(-1.016) (0.103)	0.2086	0.1427

78	miR93p pre	miR93p post	16	4	0.035	0.5299	(0.044) (1.02)	0.2809	0.2295
79	miR93p pre	Average 25G	21	1	0.02	-0.5017	(-0.92) (-0.086)	0.2518	0.2124
80	Average 25G	miR93p post	17	3	0.075	-0.44231	(-0.936) (0.0513)	0.1956	0.142
81	miR93p pre	Average 80G	19	3	0.002	-0.6643	(-1.05) (-0.283)	0.4414	0.4085
82	Average 80G	miR93p post	18	2	0.027	-0.5208	(-0.97) (-0.068)	0.2712	0.2257
83	miR93p pre	Total 25G hits	19	3	0.052	-0.4528	(-0.91) (0.0033)	0.2051	0.1584
84	Total 25G hits	miR93p post	15	5	0.106	-0.4334	(-0.97) (0.106)	0.1879	0.1254
85	miR-9-3p pre	Total 80G hits	18	4	0	-0.7536	(-1.102) (-0.405)	0.5679	0.5409
86	Total 80G hits	miR93p post	16	4	0.017	-0.5856	(-1.05) (-0.121)	0.343	0.2961
87	miR93p pre	Total sensor sessions	21	1	0.39	-0.1978	(-0.669) (0.273)	0.0391	-0.0114
88	Total sensor sessions	miR93p post	17	3	0.07	0.0184	(-0.532) (0.569)	0.0003	-0.0663
89	miR1515p pre	miR1515p post	17	3	0.529	0.1639	(-0.379) (0.707)	0.0269	-0.038
90	miR1515p pre	Average 25G	18	4	0.183	-0.3286	(-0.829) (0.172)	0.108	0.0522
91	Average 25G	miR1515p post	16	4	0.19	0.3451	(-0.193) (0.883)	0.1191	0.0562
92	miR1515p pre	Average 80G	19	3	0.877	-0.038	(-0.549) (0.47)	0.0014	-0.0573
93	Average 80G	miR1515p post	17	3	0.756	0.0813	(-0.467) (0.63)	0.0066	-0.0596
94	miR1515p pre	Total 25G hits	19	3	0.327	-0.2375	(-0.735) (0.26)	0.0564	0.0009
95	Total 25G hits	miR1515p post	16	4	0.402	0.2251	(-0.33) (0.784)	0.0507	-0.0171

96	miR1515p pre	Total 80G hits	20	2	0.131	-0.349	(-0.813) (0.115)	0.1218	0.073
97	Total 80G hits	miR1515p post	15	5	0.308	0.2825	(-0.292) (0.857)	0.0798	0.009
98	miR1515p pre	Total sensor sessions	18	4	0.091	-0.4102	(-0.894) (0.073)	0.1683	0.1164
99	Total sensor sessions	miR1515p post	17	3	0.385	-0.7611	(-0.761) (0.311)	0.0506	-0.0127

Three triangulations were found to have significance, using a  $p < 0.05$ . Other triangulations showed significance for one or two of the regressions, but not all three. All the bolded trials in Table 1 represent a significant regression. The first significant triangulation was (A) pre-season miRNA-362-3p, (B) total 80G hits, and (C) post-season miRNA-362-3p. The second subset was (A) pre-season miRNA-9-3p, (B) Average 80G, and (C) post-season miRNA-9-3p. The third is (A) pre-season miRNA-9-3p, (B) Total 80G hits, and (C) miRNA-9-3p post-season. Excluding the significant regression triangulations, thirteen other regressions had significance.

To further explore the interaction between the three variables a moderation analysis was performed. The independent variable was the pre-season miRNA while the dependent variable was the post-season miRNA level. HAE acted as the moderation variable which represents a variable that might influence the interaction between the IV and DV. If a moderation analysis is found to be significant than the moderating variable can be used with the IV to predict what the DV will be. An alpha value of 0.1 was chosen for the moderation tests, such that  $p$  must be  $p < 0.1$  to indicate significance. For a moderation to be considered significant both  $pF$  and  $pB3$ , the  $p$  value for the interaction

term, need to be below 0.1. If only one p-value is below 0.1, then the moderation is considered a trend effect. A trend effect indicates there is some correlation between the variables, but not enough to be considered significant.

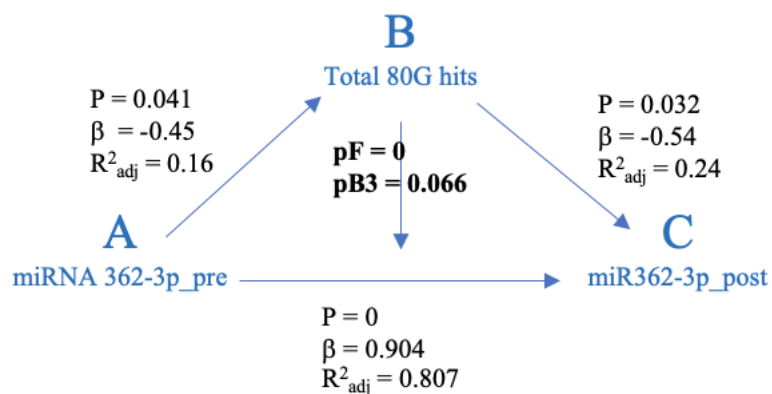
**Table 2: Moderation Tests for Pre-season miRNA, HAE, and Post-season miRNA Levels.** Compares pre-season miRNA to post-season miRNA with HAE acting as a moderating variable. Regression is significant if pF and pB3 < 0.10. N represents the number of subjects in each trial. Outliers are the number of participants removed after cook's outlier test performed. The beta-coefficient represents the amount of change in the outcome variable for every unit change in the predictor variable.

Trial	IV	DV	Mo	pF	pB3	B3 (Coef)	N	Outliers
1	miR3623p_pre	miR3623p_post	Total80Ghits	0	0.066	-0.3571	15	5
2	miR3623p_pre	miR3623p_post	Average25G	0.0035	0.258	0.5754	15	5
3	miR3623p_pre	miR3623p_post	Average80G	0	0.311	-2.652	14	6
4	miR3623p_pre	miR3623p_post	Total25Ghits	0.0006	0.976	-0.0099	14	6
5	miR3623p_pre	miR3623p_post	Total sensor sessions	0	0.852	0.048	16	4
6	miR20a_pre	miR20a_post	Average25G	0.6504	0.493	0.2455	14	4
7	miR20a_pre	miR20a_post	Average80G	0.409	0.667	-1.345	15	3
8	miR20a_pre	miR20a_post	Total25Ghits	0.4195	0.725	0.1299	14	4
9	miR20a_pre	miR20a_post	Total80Ghits	0.0141	0.267	0.2268	13	5
10	miR20a_pre	miR20a_post	Total sensor sessions	0.1407	0.708	0.1616	15	3
11	miR505_pre	miR505_post	Average25G	0.0403	0.984	0.00555	16	4
12	miR505_pre	miR505_post	Average80G	0.944	0.771	0.1342	17	3
13	miR505_pre	miR505_post	Total25Ghits	0.2584	0.336	0.2845	16	4
14	miR505_pre	miR505_post	Total80Ghits	0.9478	0.608	0.07227	14	6

15	miR505_pre	miR505_post	Total sensor sessions	0.262	0.44 2	0.2244	16	4
<b>16</b>	miR30d_pre	miR30d_post	Average25G	0.010 3	0.23 2	-0.5399	15	5
<b>17</b>	miR30d_pre	miR30d_post	Average80G	0.100 1	0.22 5	0.3915	15	5
18	miR30d_pre	miR30d_post	Total25Ghits	0.082 8	0.25 7	-0.6093	14	6
19	miR30d_pre	miR30d_post	Total80Ghits	0.128 5	0.96 5	-0.01602	13	7
<b>20</b>	miR30d_pre	miR30d_post	Total sensor sessions	0.025 2	0.11	0.6806	15	5
21	miR92a_pre	miR92a_post	Average25G	0.151	0.73 6	0.1764	16	4
22	miR92a_pre	miR92a_post	Average80G	0.421 6	0.42 6	0.2964	13	7
23	miR92a_pre	miR92a_post	Total25Ghits	0.282 2	0.38 2	0.3207	15	5
24	miR92a_pre	miR92a_post	Total80Ghits					
25	miR92a_pre	miR92a_post	Total sensor sessions	0.494 2	0.79 4	-0.08999	17	3
<b>26</b>	miR486_pre	miR486_post	Average25G	0.128 8	0.03 6	0.00283 7	17	3
27	miR486_pre	miR486_post	Average80G	0.230 4	0.55 7	-0.1854	18	2
28	miR486_pre	miR486_post	Total25Ghits	0.762 6	0.67 3	0.1538	15	5
29	miR486_pre	miR486_post	Total80Ghits	0.145 1	0.93 6	0.0236	15	5
30	miR486_pre	miR486_post	Total sensor sessions	0.618 5	0.36 7	-0.207	16	4
<b>31</b>	miR195_pre	miR195_post	Average25G	0.032 2	0.91 8	-0.03225	16	4
32	miR195_pre	miR195_post	Average80G	0.830 3	0.55 8	-0.1901	15	5
<b>33</b>	miR195_pre	miR195_post	Total25Ghits	0.011 3	0.53	-0.2072	17	3
<b>34</b>	miR195_pre	miR195_post	Total80Ghits	0.019 2	0.74 9	-0.1091	16	4
<b>35</b>	miR195_pre	miR195_post	Total sensor sessions	0.003 9	0.58 7	-0.2508	15	5
<b>36</b>	miR93p_pre	miR93p_post	Average25G	0.036 6	0.17 1	-0.4198	17	3
<b>37</b>	miR93p_pre	miR93p_post	Average80G	0.056	0.46 9	0.3121	17	3
<b>38</b>	miR93p_pre	miR93p_post	Total25Ghits	0.073 4	0.23 2	-0.5103	16	4
<b>39</b>	miR93p_pre	miR93p_post	Total80Ghits	0.030 2	0.89 3	-0.0631	15	5

40	miR93p_pre	miR93p_post	Total sensor sessions	0.249 6	0.73	0.1214	15	5
41	miR1515p_pre	miR1515p_post	Average25G	0.263 4	0.69 1	-0.1085	18	2
42	miR1515p_pre	miR1515p_post	Average80G	0.280 9	0.17 8	0.4166	15	5
43	miR1515p_pre	miR1515p_post	Total25Ghits	0.469	0.74 9	0.1202	17	3
44	miR1515p_pre	miR1515p_post	Total80Ghits	0.335 7	0.43 7	0.3797	15	5
45	miR1515p_pre	miR1515p_post	Total sensor sessions	0.508 5	0.48 6	0.2353	16	4

Only one moderation test for miRNAs was found to be significant, but there were a variety of trend effects. Trial 1, with (A) pre-season miRNA-362-3p, (B) total 80G hits, (C) post-season miRNA-362-3p had a pF of 0 and a pB3 of 0.066, both of which are below p-value < 0.1 which indicates significance. In addition, all the other miRNA-362-3p moderations produced trend effects as pF < 0.1 for all the trials, but pB3 was not. The pF term was significant for Average 25G, Average 80G, Total 25G hits, and Total Sensor Sessions. In addition, trend effects were found for miRNA-505, miRNA-30d, miRNA-486, miRNA-195, and miRNA-151-5p. MiRNA-30d produced a pF = 0.025 and pB3 = 0.11 indicating that it was close to being considered significant but was still only a trend effect. The beta-coefficient for miR362-3p was -0.357. There was a relatively equal split between positive and negative B3 coefficients for the rest of the trend effects. It is important to note that the B3 coefficient for miRNA-362-3p moderated by Average 80G hits was -2.65.



**Figure 3: Significance of Total 80G Hits on  $\Delta$ MiRNA362-3p Over the Course of the Football Season.**

Shows results from the linear regressions between miRNA 362-3p\_pre and Total 80G hits, Total 80G hits and miR362-3p\_post, and miR362-3p\_pre and miR362-3p\_post. Presents the results from moderation analysis and the degree to which Total 80G hits influences the interaction between miRNA 362-3p\_pre and miR362-3p\_post.

Additional regression and moderation tests were performed using VR scores instead of miRNA levels. With (A) being pre-season VR score, (B) HAE, and (C) post-season VR score. Similar to the previous tests, three regressions were performed for each subset of variables to determine if there was a significant triangulation. A regression with a p-value  $< 0.05$  is considered significant.

**Table 3: Regression Tests for Pre-season and Post-season VR Scores.**

Compares pre-season VR score to post-season VR score, pre-season VR score to HAE variable, and HAE variable to post-season VR score. Regression is significant if  $p < 0.05$ . N represents the number of subjects in each trial. Outliers is the number of participants removed after cook's outlier test performed. The beta-coefficient represents the amount of change in the outcome variable for every unit change in the predictor variable.

<b>Trial</b>	<b>IV</b>	<b>DV</b>	<b>N</b>	<b>Outliers</b>	<b>p-value</b>	<b>beta (Coef)</b>	<b>95% CI</b>	<b>R2</b>	<b>adjusted r2</b>
1	VR Comp_Pre	VR Comp_Post	22	1	0.797	-0.058	(-0.52) (0.41)	0.0034	-0.0465
2	VR Comp_Pre	Average25G	20	3	0.537	-	(-0.64) (0.34)	0.0215	-0.0329
3	Average25G	VR Comp_Post	22	1	0.694	-0.089	(-0.55) (0.38)	0.0079	-0.0417
4	VR Comp_Pre	Average80G	21	2	0.412	-	(-0.66) (0.28)	0.0357	-0.0151
5	Average80G	VR Comp_Post	22	1	0.453	0.1686	(-0.29) (0.63)	0.0285	-0.0201
6	VR Comp_Pre	Total25Ghits	21	2	0.231	0.2731	(-0.19) (0.74)	0.0746	0.0259
7	Total25Ghits	VR Comp_Post	22	1	0.6455	-	(-0.57) (0.36)	0.0108	-0.0387
8	VR Comp_Pre	Total80Ghits	23	0	0.76	-	(-0.52) (0.39)	0.0045	-0.0429
9	Total80Ghits	VR Comp_Post	22	1	0.424	0.1795	(-0.28) (0.64)	0.0323	-0.0161
10	VR Comp_Pre	Total sensor sessions	21	2	0.1658	0.3139	(-0.14) (0.77)	0.0986	0.0511
11	Total sensor sessions	VR Comp_Post	22	1	0.681	-	(-0.56) (0.37)	0.0086	-0.0409
12	VR Spatial_Pre	VR Spatial_Post	21	2	0.187	0.2995	(-0.16) (0.76)	0.0897	0.0418
13	VR Spatial_Pre	Average25G	21	2	0.391	0.1975	(-0.27) (0.67)	0.039	-0.0115
14	Average25G	VR Spatial_Post	22	1	0.144	0.3217	(-0.12) (0.76)	0.1036	0.0587
15	VR Spatial_Pre	Average80G	22	1	0.883	0.0333	(-0.43) (0.49)	0.0011	-0.0488

16	Average80G	VR Spatial_Post	22	1	0.225	0.2698	(-0.18) (0.72)	0.0728	0.0264
17	VR Spatial_Pre	Total25Ghits	20	3	0.8665	0.0402	(-0.455) (0.535)	0.0016	-0.0539
18	Total25Ghits	VR Spatial_Post	23	0	0.37	0.196	(-0.249) (0.641)	0.0384	-0.0074
19	VR Spatial_Pre	Total80Ghits	23	0	0.668	0.0946	(-0.546) (0.357)	0.009	-0.0382
20	Total80Ghits	VR Spatial_Post	22	1	0.174	0.3003	(-0.144) (0.745)	0.0902	0.0448
21	VR Spatial_Pre	Total sensor sessions	21	2	0.782	0.0644	(-0.415) (0.544)	0.0041	-0.0483
22	Total sensor sessions	VR Spatial_Post	23	0	0.316	0.2187	(-0.662) (0.224)	0.0478	0.0025
23	VR Balance_Preview	VR Balance_Post	21	2	0.162	0.3166	(-0.772) (0.139)	0.1003	0.0529
24	VR Balance_Preview	Average25G	21	2	0.968	0.0095	(-0.49) (0.471)	0.0001	-0.0525
25	Average25G	VR Balance_Post	21	2	0.833	0.0489	(-0.529) (0.431)	0.0024	-0.0501
26	VR Balance_Preview	Average80G	21	2	0.976	-0.007	(-0.487) (0.473)	0	-0.0526
27	Average80G	VR Balance_Post	21	2	0.806	-0.057	(-0.536) (0.422)	0.0032	-0.0492
28	VR Balance_Preview	Total25Ghits	20	3	0.611	0.121	(-0.371) (0.613)	0.0146	-0.0401
29	Total25Ghits	VR Balance_Post	20	3	0.417	0.1921	(-0.294) (0.678)	0.0369	-0.0166
30	VR Balance_Preview	Total80Ghits	22	1	0.096	0.364	(-0.0703) (0.798)	0.1325	0.0892
31	Total80Ghits	VR Balance_Post	21	2	0.588	0.1254	(-0.602) (0.351)	0.0157	-0.0361

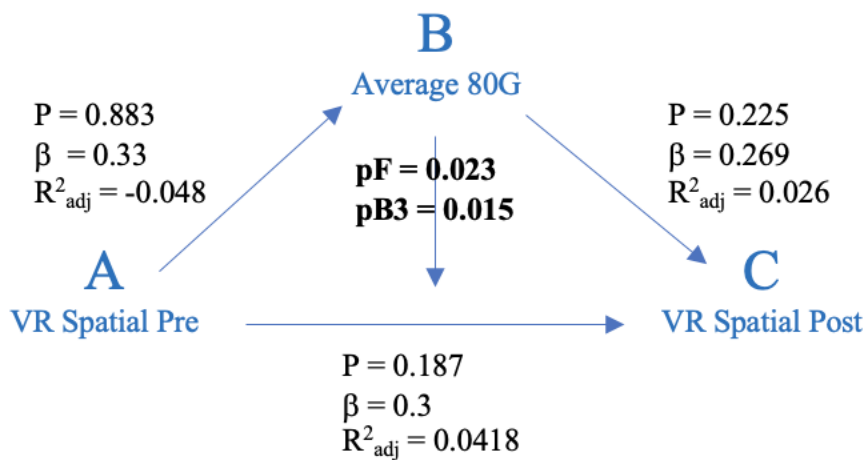
32	VR Balance_Pr e	Total sensor sessions	21	2	0.354	0.2128	(-0.256) (0.682)	0.0453	-0.0049
33	Total sensor sessions	VR Balance_Pos t	19	4	0.248	0.2786	(-0.770) (0.213)	0.0777	0.0234
34	VR Reaction_P re	VR Reaction_Po st	22	1	0.294	0.2344	(-0.219) (0.688)	0.055	0.0077
35	VR Reaction_P re	Average25G	22	1	0.954	0.0129	(-0.479) (0.453)	0.0002	-0.0498
36	Average25 G	VR Reaction_Po st	22	1	0.017	0.5021	(-0.906) (-0.099)	0.2521	0.2147
37	VR Reaction_P re	Average80G	21	2	0.851	0.0438	(-0.523) (0.436)	0.0019	-0.0506
38	Average80 G	VR Reaction_Po st	22	1	0.426	0.1786	(-0.638) (0.280)	0.0319	-0.0165
39	VR Reaction_P re	Total25Ghit s	21	2	0.139	0.3337	(-0.119) (0.786)	0.1114	0.0646
40	Total25Ghi ts	VR Reaction_Po st	22	1	0.07	0.3937	(-0.823) (0.035)	0.1551	0.1128
41	VR Reaction_P re	Total80Ghit s	21	2	0.46	0.1703	(-0.303) (0.644)	0.029	-0.0221
42	Total80Ghi ts	VR Reaction_Po st	22	1	0.253	0.2546	(-0.706) (0.196)	0.0648	0.0181
43	VR Reaction_P re	Total sensor sessions	22	1	0.15	0.317	(-0.125) (0.759)	0.1005	0.0556
44	Total sensor sessions	VR Reaction_Po st	23	0	0.775	0.063	(-0.39) (0.516)	0.004	-0.0435

None of the linear regression triangulations from Table 3, using pre-season VR as variables A and post-season VR as variable C, were found to be significant. Only trial 36, IV, Average 25G and DV, VR score for reaction time was significant. Although there was limited significance for the triangulation of regression tests, a portion of the moderation trials were found to be significant. Table 4 represents the results from the moderation analysis using (A) pre-season VR score, (B) HAE, and (C) post-season VR score.

**Table 4: Moderation Tests for Pre-season VR Score, HAE, and Post-season VR Score.** Compares the pre-season VR score to the post-season VR score with HAE acting as a moderating variable. Regression is significant if  $pF$  and  $pB3 < 0.10$ . N represents the number of subjects in each trial. Outliers are the number of participants removed after cook's outlier test performed. The beta-coefficient represents the amount of change in the outcome variable for every unit change in the predictor variable.

Trial	IV	DV	Mo	pF	pB3	B3 (Coef)	N	Outliers
1	VR Comp_Pre	VR Comp_Post	Average25G	0.8285	0.424	-0.1834	22	1
2	VR Comp_Pre	VR Comp_Post	Average80G	0.9092	0.905	-0.03746	21	2
3	VR Comp_Pre	VR Comp_Post	Total25Ghits	0.9255	0.822	-0.0573	21	2
4	VR Comp_Pre	VR Comp_Post	Total80Ghits	0.9132	0.91	-0.03373	21	2
5	VR Comp_Pre	VR Comp_Post	Total sensor sessions	0.9598	0.928	0.01939	21	2
6	VR Spatial_Pre	VR Spatial_Post	Average25G	0.1549	0.266	-1.385	20	3
7	VR Spatial_Pre	VR Spatial_Post	Average80G	0.0231	0.015	-1.041	20	3
8	VR Spatial_Pre	VR Spatial_Post	Total25Ghits	0.0538	0.045	-2.263	20	3
9	VR Spatial_Pre	VR Spatial_Post	Total80Ghits	0.0176	0.025	-0.4248	20	3

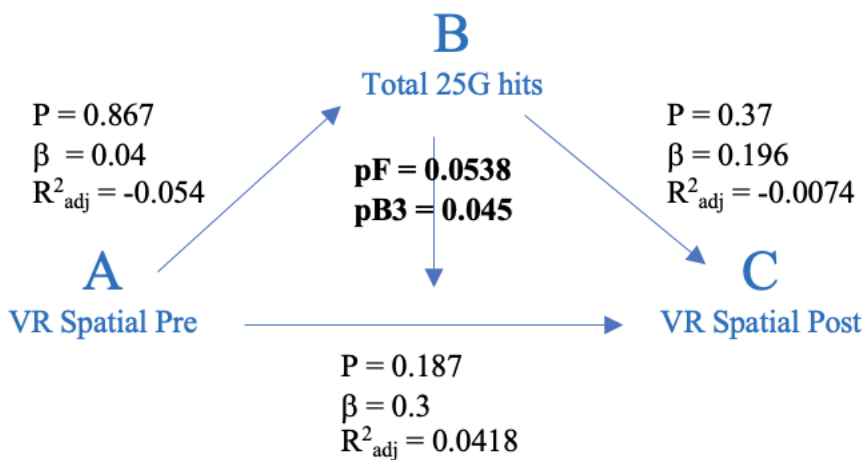
10	VR Spatial_Pre	VR Spatial_Post	Total sensor sessions	0.0308	0.557	-1.03	21	2
11	VR Balance_Pre	VR Balance_Post	Average25G	0.6027	0.892	0.03586	20	3
12	VR Balance_Pre	VR Balance_Post	Average80G	0.6142	0.669	0.1579	20	3
13	VR Balance_Pre	VR Balance_Post	Total25Ghits	0.1798	0.331	-0.2149	21	2
14	VR Balance_Pre	VR Balance_Post	Total80Ghits	0.5491	0.954	0.02571	22	1
15	VR Balance_Pre	VR Balance_Post	Total sensor sessions	0.3514	0.48	0.1866	19	4
16	VR Reaction_Pre	VR Reaction_Post	Average25G	0.0997	0.206	-0.3065	23	0
17	VR Reaction_Pre	VR Reaction_Post	Average80G	0.595	0.303	-1.793	23	0
18	VR Reaction_Pre	VR Reaction_Post	Total25Ghits	0.1064	0.93	0.02625	22	1
19	VR Reaction_Pre	VR Reaction_Post	Total80Ghits	0.2706	0.07	-0.4479	21	2
20	VR Reaction_Pre	VR Reaction_Post	Total sensor sessions	0.7235	0.459	0.2372	23	0



**Figure 4: Significance of Average 80G Hits on  $\Delta$ VR Spatial Navigation Over the Course of the Football Season.**

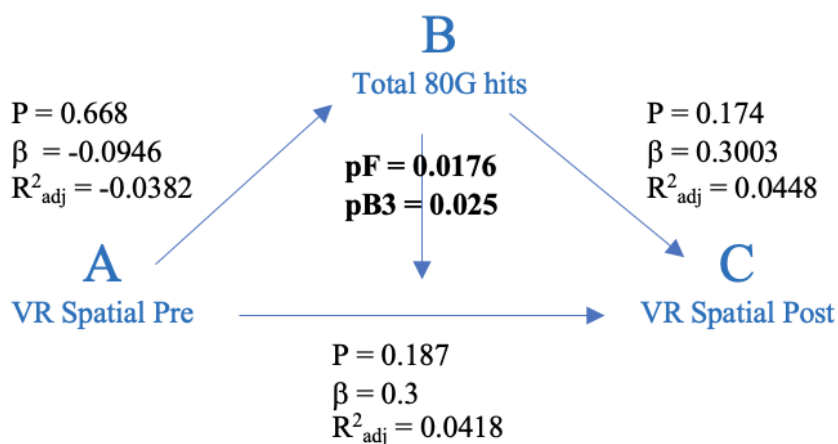
Depicts results from the linear regressions between VR Spatial Pre and Average 80G, Average 80G and VR Spatial Post, and VR Spatial Pre and VR Spatial Post. Presents the

results from moderation analysis and the degree to which Average 80G influences the interaction between VR Spatial Pre and VR Spatial Post.



**Figure 5: Significance of Total 25G hits on  $\Delta$ VR Spatial Navigation Over the Course of the Football Season.**

Shows results from linear regressions between VR Spatial Pre and Total 25G hits, Total 25G hits and VR Spatial Post, and VR Spatial Pre and VR Spatial Post. Presents the results from moderation analysis and the degree to which Total 25G hits influences the interaction between VR Spatial Pre and VR Spatial Post.



**Figure 6: Significance of Total 80G Hits on  $\Delta$ VR Spatial Navigation Over the Course of the Football Season.**

Depicts the findings from the linear regressions between VR Spatial Pre and Total 80G hits, Total 80G hits and VR Spatial Post, and VR Spatial Pre and VR Spatial Post. Displays the results from moderation analysis and the degree to which Total 80G hits influences the interaction between VR Spatial Pre and VR Spatial Post.

VR spatial navigation scores were significantly moderated by Average80G, Total 25G hits, and Total 80G hits,  $p < 0.10$  and  $pB3 < 0.10$ . Three outliers were removed for those tests using cook's outlier analysis. Two interaction effects were found for VR Reaction being moderated by Total 80G hits and Average 25G hits. Interestingly, the  $pF$  for the reaction time moderated by Average 25G hits was significant while the interaction effects moderated by Total 80G hits was not. In contrast, the Reaction Time interaction effects moderated by Total 80G hits had a significant  $pB3$  while the interaction affect for Average 25G hits did not. The  $B3$  coefficient for the three significant spatial navigation tests were negative. The  $B3$  coefficient was also negative for the two VR Reaction trend effects.

## CHAPTER FOUR

### SECTION ONE: DISCUSSION

The main goals from this investigation are to determine if the number and magnitude of HAEs in a season cause significant changes in miRNA levels and VR scores. Of the 99 miRNA regressions, 23 produced significant results, three of which were triangulations between: pre-season miRNA, HAE, and post-season miRNA. There were 45 moderation trials for miRNA of which one was significant. Of the 45 VR regressions, only one was significant. Similarly, 20 moderation trials were performed for: pre-season VR, HAE, and post-season VR. Three moderations looking at VR scores for spatial navigation were significant. The HAEs for these moderations are Average 80G hits, Total 25G hits, and Total 80G hits. The significance of these findings is they suggest that HAEs provide a degree of predictability for certain miRNA levels and one's ability to successfully navigate space.

MiRNA-362-3p is the miRNA that was most affected by the number of HAEs experienced. This RNA's role as a neuroinflammatory marker provides a link between the number of HAEs experienced and the degree of inflammation that individual could be experiencing. The results from the three regression analyses between 1) pre-season miRNA to HAE, 2) HAE to post-season miRNA, and 3) pre-season miRNA to post-season miRNA show that all three variables significantly correlate and interact with one another. In addition, a moderation analyses using Total 80G hits as the moderating variable for pre-season and post-season miRNA was significant. This indicates that the total number of 80G hits an athlete experiences predicts the changes in miRNA-362-3p

levels from pre-season to post-season. In addition, the beta-coefficient comparing pre-season miRNA-362-3p and post-season miRNA-362-3p was 0.905. The positive beta-coefficient indicates that the post-season miRNA levels are consistently higher than the pre-season miRNA levels. Since miRNA-362-3p is a neuroinflammatory marker, this suggests that an accumulation of sub-concussive events can lead to neuroinflammation over the duration of the season. Sustained inflammation can result in long term complications such as dementia and other cognitive disorders<sup>55</sup>. Neuroinflammation has been an indication of concussion in the past so there is the potential that an accumulation of sub-concussive events could potentially result in the same amount of inflammation as a concussion. More research is needed to determine a formal connection between HAEs and concussion, but there is enough evidence to suggest a link might exist.

Another miRNA, miRNA-9-3p, also produced a triangulation of significant regressions. The significant HAEs were average 80G hits and total 80G hits. This shows three important components of the relationships between HAE's and neuroinflammation. First, that HAEs have the potential to be used as a method for determining the presence of neuroinflammation. Second, that elevated levels of miRNA-9-3p, a known inflammatory marker, can be used as a sign of potential inflammation in athletes. Third, that 25G HAE's do not seem to meet the threshold needed to cause long-term neuroinflammation while 80G hits do. Both miRNA-362-3p and miRNA-9-3p only showed significant alterations in miRNA levels when multiple 80G hits were experienced. It is likely that the individuals that experienced 80G hits were also exposed to a significant amount of 25G hits, but it is important to note that a significant alteration

in miRNA levels did not take place when only 25G hits were present. One can hypothesize that this is because one needs to reach a certain threshold of impact for sub-concussive events to cause elevated and prolonged inflammation. If true, this would raise the question as to whether this threshold of impact decreases over time and exposure to injury. If true, this would mean every time one is exposed to a serious brain injury, they need fewer hits at lower magnitudes to experience similar levels of inflammation. This is significant as it would show that one is more susceptible to further cognitive issues after experiencing a certain number of sub-concussive impacts that do not meet the classification as a severe brain injury. Scopaz and colleagues found that someone who has experienced a concussion requires more time to recover and is more likely to experience a concussion in the future, which supports the conjecture that the same could be true for HAEs<sup>56</sup>.

Based on the framework established by Alexander Luria<sup>44</sup>, and previous VR findings from Slobounov and colleagues<sup>43</sup>, one can use VR tests to determine if cognitive injury is impairing motor skills. The VR results for the spatial navigation testing also indicated that exposure to HAEs causes a significant change when comparing pre-season VR scores to post-seasons VR scores. When looking at both average 80G hits and total 80G hits, athlete's spatial navigation scores decreased significantly. In addition, the number of total 25G hits also caused spatial navigation scores to significantly decrease. This indicates that the magnitude and number of HAEs an athlete experiences can affect their ability to recall and navigate space. This link has long since been suggested as Di Virgilio and colleagues found that boxers made more errors in spatial navigation tests,

immediately after a training session, than a control group<sup>57</sup>. Further research is needed to solidify this finding, but the results suggest accumulation of HAEs will decrease one's ability to spatially navigate. In particular, it is important to know more about the time frame in which spatial navigation resolves. The football season is about 3.5 months long and the decrease in VR scores post-season indicates that it will take longer than the duration of the football season for the alterations in spatial navigation to resolve. This is an important concept to investigate more as athletes' spatial navigation abilities should be normal prior to returning to play. If not, athletes will be susceptible to a variety of injuries not all of which are brain related.

Although VR scores for spatial navigation were the only significant findings, there were two trend effects for VR scores examining athletes' reaction time. A trend effect is when one of the p-values is significant, but the other is not. For example, if pF is significant but pB3 is not significant this would be considered a trend effect. A trend effect indicates there is a relationship between variables, but that relationship is not as strong as a significant relationship. Total 80G hits produced a moderating trend effect on pre-season and post-season VR reaction time scores because  $pB3 < 0.1$  but pF was not. This indicates that the total number of 80G hits an athlete experiences has an impact on their reaction time, but not enough to be considered significant. The negative B3 coefficient term indicates that the reaction time post-season is lower than the reaction time pre-season. This supports the claim that a high quantity of 80G impacts affects cognitive functions as their ability to successfully adjust body position in a virtual environment decreases over the course of the season. Similarly, average 25G hits also

produced a trend affect when moderating pre-season and post-season VR reaction scores. The trend effect for the average 25G hits was different from the total 80G hits trend effect because it changes only the pF term and not the pB3 term. This finding is less significant as it shows that pre-season and post-season reaction scores significantly impact each other, but that the average number of 25G impacts does not significantly influence the relationship.

Although these conclusions are supported by the results there are a few procedural areas that could be modified for further investigation. The first change would be the number of athletes included. The average college football team includes roughly 100 players but only starters were included so 24 players participated in data collection<sup>8</sup>. The small sample size leaves the data more susceptible to outliers and gives it reduced power. If the sample size were larger and included multiple football teams, the data would have higher power and be less susceptible to confounding variables. Future studies should look to enroll multiple teams from different programs and group athletes based on height, weight, and position played. Research has shown that different skill positions experience different magnitudes of collisions<sup>8</sup>. In addition, future studies should attempt to keep sensors in athletes' helmets during games and practices. Although it was found that the majority of HAEs occur during practice<sup>5,6</sup>, it is reasonable to assume that there is a decent amount of HAEs that are missed since the sensors are not used in the 12 games during the season. Due to the high level of competition and emotion in games, one might suspect more severe HAEs in games. One approach to achieving this would be to use less invasive sensors.

Another aspect that can be adjusted in future studies is when the post-season VR and miRNA levels are measured. The measurements could be conducted throughout the season with a final measurement taken seven days after the last contact session. The seven-day mark would be an ideal day as neuroinflammation has been shown to peak seven days post contact<sup>47</sup>. The accumulation of sub-concussive head injuries is a potential indicator of neuroinflammation so inflammation levels should be monitored over the course of the season not at the start and end. Having multiple time points would enable one to see the fluctuations in neuroinflammation over the course of the season while also establishing a timeframe for resolution of sub-concussive neuroinflammation. For example, if an athlete has a variety of HAEs in the first two week of the season, and therefore susceptible to more serious injury, but does not undergo miRNA and VR measurements until the end of the season, then the miRNA levels and VR scores might not be an accurate representation of what the results would have been. Lastly, all the analyses that were performed isolated one miRNA and investigated how it was impacted by HAE. One could further explore neuroinflammation in general by testing for an interaction effect between the different miRNAs.

One limitation of the statistical analyses performed is that different significance levels were used for the regression and moderation tests. The moderation significance level was 0.1 while the regression significance was 0.05. The larger significance level for the moderation test can lead to a type I error which is when the null hypothesis is rejected when it is in fact correct.

## SECTION ONE: CONCLUSION

The main takeaway from this study is that miRNA-362-3p and miRNA-9-3p levels are impacted by HAEs more than the other miRNAs. This suggests that their levels, as well as the number of HAEs one experiences, can be used as a measure for the degree of neuroinflammation an athlete is experiencing. Not all sub-concussive events cause clear clinical manifestations but by monitoring HAEs and VR scores medical staff can have a better picture of whether an athlete is experiencing neuroinflammation and the extent to which they are.

An additional confounding variable is that Linda Papa and colleagues found that miRNA, neuroinflammatory biomarkers, have been found to be elevated during pre-season in control subjects prior to any contact<sup>22</sup>. This confounding variable could theoretically be caused by chronic neuroinflammation, but likely cause an underrepresentation of presented results.

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**CURRICULUM VITAE**

