

SERUM CONCENTRATIONS OF PROTEIN
S100B AND THE MENSTRUAL CYCLE

A Thesis
by
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Abstract

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Protein S100B can be found in the serum of blood and is a biomarker for the blood brain barrier. S100B can be found in adipose tissue, skeletal muscle, and brain tissue; therefore, S100B concentrations can increase in the serum with excess adiposity or obesity, exercise, or a traumatic brain injury (TBI). In sport, S100B has been considered a possible indicator of sports related concussion (SRC) independent of exercise. Women tend to have more severe symptoms from SRC than men for a reason that has not been fully explained. The menstrual cycle phase and contraceptive use in women have both shown to have an effect on the severity of symptoms after a TBI. The goal of this study was to determine if protein S100B concentrations were affected by the phases of the menstrual cycle or the use of a contraceptive. A group of men (N=15), women taking a contraceptive (N=15), and women not taking a contraceptive (N=15) were recruited and all participants underwent the same testing protocol. Each participant came to the lab three separate times within 21-30 days depending on cycle length; analysis dates for men was comparable to women. The first visit for each participant was during the week of menses, the next visit was 10-15 days later, and the last visit was 3-7 days after the second. Participants were given a health history form to ensure inclusion criteria. Then they were asked to provide a urine sample to determine the phase of the menstrual cycle based off hormones in the sample. Next participants underwent a body composition analysis to determine body fat percentage using the BodPod. Lastly the

participant endured a blood draw which was processed using a centrifuge to isolate the serum which was then stored at -80 degrees Celcius. After all samples for each participant was collected, an enzyme-linked immunosorbent assay (ELISA) was performed to determine S100B concentrations. A one-way analysis of variance (ANOVA) was used in measuring S100B among the different sample groups, and a repeated measures ANOVA was used to analyze the relationship within groups of S100B concentrations to the menstrual phases.

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Introduction

Serum S100B is a biomarker of blood brain barrier (BBB) disruption that has been highly researched in regard to traumatic brain injury (TBI) and sports-related concussion (SRC). The biomarker has been shown to be of higher concentration systemically after a head injury (Koh & Lee, 2014). Concentrations of S100B can increase simply due to increased adiposity (Pham, 2010) as well as exercise (Hasselblatt et al., 2004; Rogatzki, 2018); however, the incidence of a concussion causes the increase to be of greater magnitude (Kiechle et al., 2014). Therefore, S100B can be considered while diagnosing TBI or SRC (Kiechle et al., 2014).

For reasons unknown, women tend to have worse post-concussive symptoms when compared to men. In a study by Delaney et al. (2002), female soccer players were found to be at greater risk of suffering a concussion than males; the increased risk was attributed to anatomical differences in neck and torso size. Post-concussive symptoms in female athletes have been associated with hormonal fluctuations of the menstrual cycle, suggesting that it should be taken into account while deciding return-to-play (Brown et al., 2015). Even contraceptive use has been examined in regard to its effects in recovery time from SRC symptoms in women. Women who used a form of contraception had less severe post-concussive symptoms than women who were not using a form of contraception (Gallagher et al, 2018). Men do not use contraceptives and therefore their only link to recovery time is the severity of post-concussive symptoms they present. In women the use of contraception can influence the severity of post-concussive symptoms, however contraception use itself does not influence recovery time (Gallagher et al, 2018).

Research needs to be done to determine if the menstrual cycle has any effect on S100B to determine if it plays a role on baseline concentrations. This research would benefit future SRC and TBI studies.

Problem Statement

The purpose of this study is to examine the variation of S100B during different phases of the menstrual cycle and in regards to contraceptive use.

Hypothesis

We hypothesize there will be no change in S100B during the different phases of the menstrual cycle, however we do expect to see a difference in S100B concentrations for women who use hormonal contraceptives.

Summary

One group of men and two different groups of women, all between the ages of 18-30 will be recruited for this study. The two experimental groups will consist of women who may or may not be using a form of contraception that does not alter normal menstruation and have not had a concussion within the past six months. The control group will be men who have not suffered from a concussion within the past six months. A blood sample during the early follicular (menses), late follicular (ovulation), and mid-luteal phases of the menstrual phase will be obtained from the two experimental groups. The control group will be tested three times over a similar 28-day cycle. An enzyme-linked immunosorbent assay (ELISA) will be run on the blood to determine the concentration of S100B at each phase of the menstrual cycle. A comparison of results using a repeated-measures analysis of variance (RMANOVA) within groups will determine if the phases of the menstrual cycle have an effect on the concentration of S100B. A one-way ANOVA will also be used to determine S100B concentration differences among the sample population groups.

Literature Review

Concussion

A concussion is a form of brain injury caused by an external force which effects pathophysiological processes of the brain (McCrorry et al., 2017). When taking into account the number of sports-related TBI that occur each year, ranging from total loss of consciousness to those who seek no medical care, a total of 1.6 to 3.8 million cases have been estimated (Langlois et al., 2006). Considering the numerous symptoms (headache, neurological deficit, amnesia, slowed reaction time, drowsiness, etc.) that can be unique to each individual, diagnosis of severity can be a complicated task (McCrorry et al., 2017). TBI severity can be clinically diagnosed by intracranial computerized tomography (CT), focal neurological signs, post traumatic amnesia, or Glasgow Coma Score (GCS); most tests are for diagnosing more severe TBI even though mild is the most common form in clinical and sport settings (Begaz et al., 2006). In most SRCs, athletic trainers or team physicians are those who diagnose the concussion based on number of present symptoms to decide the severity of the injury to determine further analysis and return to play (Covassin et al., 2009). Specific guidelines are given for reporting a concussion by the National Athletic Training Association (NATA), but there is no standardization in how athletic training students are taught to properly diagnose a concussion. As a result, organizations are expected to keep up to date with new guidelines and teach their students how to diagnose a concussion in whatever manner they please (Covassin et al., 2009).

Athletes can be reluctant to seek medical advice especially when it comes to reporting a concussion. Some athletes are aware of the signs and continue to compete, others may not be aware they have suffered from a concussion and still continue to compete. This has been shown in a study done by Delaney et al. (2002), where out of the 70.4% of football players who acquired at least one concussion within a year, only 23.4% recognized the signs of the concussion. Unfortunately, athletes

do not always realize the repercussions that follow ignorance to report a head injury. Once an individual has suffered from one concussion, they are more susceptible to suffer from another in the incidence of an additional head injury as well as post-concussive symptoms while still trying to heal from the previous injury (Hergenroeder et al., 2008). Athletes are the most at risk for this reason. If an athlete obtained a concussion in a sporting event and continued to play and had any more impacts to the head, they are at greater risk of suffering from a more devastating TBI as well as harsher symptoms.

S100B

S100B is a calcium-binding protein with an intracellular function to stimulate cell proliferation and migration as well as inhibit apoptosis and differentiation (Donato et al., 2009). It also has extracellular tendencies to bind with a receptor for advanced glycation end products (Donato et al., 2009). S100B has a half-life of approximately one hour which has been supported by in previous literature (Jeter et al., 2013; Kawata, Liu et al., 2016; Kawata, Rubin et al., 2017). S100B is also a BBB biomarker that has been well linked with head injuries and TBI. This means if an individual underwent another concussive event post one hour of the original, S100B concentrations would not be affected as the previous increase would have subsequently reached baseline. The biomarker can be found in the glial cells of the central nervous system; however, this is not the only place in the body it is present (Koh et al., 2014).

Pham et al. (2010) compared different types of tissue using different testing methods such as ELISA, Western blot, and mass spectroscopy to support findings that S100B is expressed highest in brain tissue; however, they also found increased concentrations of S100B in skeletal muscle and adipose tissue. S100B could be a good indicator of a concussion as its concentrations do rise following concussion, however exercise alone can cause a rise in concentration as well (Kiechle et al., 2014; Pham et al., 2010; Rogatzki, 2018). In higher intensity mixed with long duration exercises such as a marathon or a long run, S100B concentration appeared to be elevated similar to those who have

experienced a mild TBI even though no head injury had occurred (Hasselblatt et al, 2004; Rogatzki, 2018). Independently of exercise, S100B concentrations still appear to be higher after an SRC; hypothetically stating there could be a threshold to determine if someone has had a concussion whether or not they were participating in a sport (Kiechle et al., 2014).

As supported by previous literature, S100B is widely researched in response to concussion, and its increase when linked with a concussion can also be associated with prognosis and return to play for athletes (Graham et al., 2015; Korfias et al., 2007; Papa et al., 2015). Measuring S100B concentration changes in response to a function of the body, such as the menstrual cycle, is important to determine if the cycle changes the concentration; this knowledge can better benefit future research into the biomarker and provide a stronger reasoning as to why it increases and decreases at different times. Research into the effects of the menstrual cycle on this biomarker has thus far been overlooked, and findings from this research could help shed light into the sex differences that could be present in the concentration of S100B in serum. For reasons that have yet to be thoroughly explained, women have harsher post-concussive symptoms and a longer return-to-play time than men. Previous explanations refer to anatomical differences (Brown et al., 2015), the possibility of contraceptive use (Gallagher et al., 2018), and even the menstrual cycle (Delaney et al., 2002).

The Menstrual Cycle

The menstrual cycle consists of two specific phases; the follicular phase which begins with the onset of menses and ends with the onset of ovulation, and the luteal phase which is approximately 14 days in length and ends upon the first day of menses. Estrogen is secreted throughout the cycle with a rise in the mid-follicular and the mid-luteal phases with a subsequent fall thereafter. The phase of menstruation can be confirmed through biochemical analysis of estrogen and luteinizing hormone (LH) (Reed & Carr, 2000).

When performing a biochemical analysis of hormones, average concentrations at each phase need to be taken into consideration. There appears to be a difference in estrogen secretion between

race according to Marsh et al. (2011) where African American women have higher concentrations of estrogen when compared to Caucasian women. However, both groups still show a rise at similar times during each phase, with African American women synthesizing an overall higher total amount of estrogen. Peak estrogen concentrations appear during the LH pre-ovulation surge at 200 to 500 pg/mL, 20 to 80 pg/mL in the mid-follicular phase, and 60 to 200 pg/mL in the mid-luteal phase in eumenorrheic women (Carmina et al., 2009; Carmina et al., 2019).

There has been evidence to support that the phase of the menstrual cycle a woman is in at the time of a brain injury can have an effect on her recovery and post-concussive symptoms. A study performed on women who had suffered from a TBI showed women who obtained the injury during the luteal phase, experienced harsher post-concussive symptoms as well as lower quality of life compared to those in the follicular phase (Wunderle et al., 2014). In a study by Mihalik et al. (2009), non-concussed women underwent standard concussion tests in different phases of the menstrual cycle to determine if the phase could affect neurocognition, postural stability, or symptom status. This study showed any discrepancies that occurred were independent of menstrual cycle phase (Mihalik et al., 2009).

Contraceptive Use

Hormonal contraceptive use is always a factor for research involving women since it can affect blood proteins, along with behavioral and cognitive traits. Contraceptives have been shown to play a role in SRC recovery in that they can be protective and decrease return to play time in athletes as well as decrease overall symptoms (Gallagher et al., 2018). This is important, as the use of hormonal contraceptives can decrease an athlete's return-to-play and can be used for future research for helping recover from a concussion. It has also been shown that women who are taking contraceptives at the time of injury tend to perform better on post-concussive and return-to-play tests, and return faster than those who are not on any form of contraception (Wunderle et al., 2014).

The effect of hormonal contraceptives on female hormone levels is well understood, but what tends to get overlooked in the process is how each contraceptive can have a different effect on blood proteins. In a double-blinded study, four different contraceptives were given to women and a blood analysis was performed to test their effects on hormones as well as blood proteins. Each contraceptive had a different effect on the tested blood proteins (thyroxine-binding globulin, corticosteroid-binding globulin, and sex hormone-binding globulin), thus indicating contraceptive use alone can alter blood chemistry (Wiegratz et al., 2003). This study only looked at these serum-binding globulins, but the effect of the contraceptives used on proteins like S100B cannot be determined without further research.

Methods

Participants

Subjects were recruited in the local community by flyers, emails, and class announcements. Prior to analysis, all participants were asked to complete a short medical history form and research consent form. The sample included one group of women taking a form of contraception (N=15), a group of women not using a contraceptive (N=15), and one group of men (N=15) with an age ranging from 18-30 years. All women were required to experience a normal menstrual cycle every month and not yet undergone menopause. The study design was a three-group study design, women were divided into contraception and non-contraception users and men were control. An ELISA of S100B was compared during the early and late follicular phases as well as the mid-luteal phase of the menstrual cycle.

Participants were excluded if they were not eumenorrheic, took a form of contraception that prevented menses from occurring, started taking or changed contraceptives within the past year, have been diagnosed with a concussion within the past six months, consumed any medication that alters hormones or the menstrual cycle such as steroids, antidepressants, antipsychotics, and thyroid medication, had a history of fainting while giving blood, had been diagnosed with endometriosis, had

a high percentage of body fat (higher than 40% for women and higher than 30% for men), had been diagnosed with hemophilia or diabetes, weighed less than 110 pounds, or if they were pregnant or had given birth within the last year.

The research was conducted over the course of 12-15 weeks, following a normal menstrual cycle, until all participants had provided all blood and urine samples and anthropometric data required in each menstrual cycle phase. Only participants who completed all mandatory visits were included in the study. The men were tested on a similar day of the month as a woman as if to follow a menstrual cycle. Since men do not experience a menstrual cycle, they began the study at any time of the month and followed the same phase dates as the women. When any man or woman entered the lab, their assigned start date fell on early follicular (day 1-5), their next visit was for late follicular (day 12-14), and last visit for mid luteal (day 17-20). The medications taken by contraceptive participants were a hormonal contraceptive that still allowed for normal menses to occur. Medication name and dosage were provided on the medical background and there were no changes to type during the testing period.

Participants who fit the inclusion criteria were asked each visit to abstain from physical activity 12-hours prior to arriving in the lab and underwent the following procedures: a body composition test was performed using a COSMED BodPod (Life Measurement, Inc; Rome, Italy) to determine differences between men and women. Women underwent ovulation testing to confirm their phase of the menstrual cycle. The participants then underwent a standard venipuncture to a prominent vein in the antecubital space.

Ovulation Testing

Once a woman was recruited and was properly consented, she informed the researcher of the first day of menses so the early follicular stage data could be collected. When the participant finished with menses, she contacted the researcher once more so she could take an ovulation test to pinpoint the exact days of ovulation. After those days were documented the participant came back to the lab

during a given time frame so the late follicular measurement could be obtained. Lastly, the mid-luteal phase was calculated based on the ovulation measurement and the last-first day of menses.

The phases were set apart from another by using an ovulation detection monitor (Clearblue Fertility Monitor, Touch Screen, 1 Count; Bedford, United Kingdom). This monitor used a urine sample to obtain LH and estrogen concentrations during a woman's cycle to track the 36-hour peak ovulation (Fertility Monitor with Touch Screen, 2019). Women came in during the calculated time based off their cycle length to determine when the peak ovulation was in order to give the late follicular measurement. If the calculated time was not the appropriate peak then they came in consistently until they reached peak ovulation.

Body Composition

When participants were enrolled in the study, they underwent a body composition measurement through COSMED BodPod (Life Measurement, Inc; Rome, Italy) to estimate body weight and composition, volume, density, body fat mass, and fat-free mass (Cosmed, 2019). The participants were instructed to wear proper clothing required for the measurement before each visit. Each participant went through standard BodPod protocol with each visit to control for any weight loss or gain that may have occurred during the study or due to the menstrual cycle.

Blood Processing

One serum-separating tube (SST) was filled with four milliliters of venous blood using a standard venipuncture procedure, inverted 8 times to mix the blood with the clotting factor, and then set aside for at least 30 minutes at room temperature, but no longer than one hour for proper coagulation. The blood was then be centrifuged at room temperature for five minutes at 5,600 revolutions per minute to separate the serum. The serum was then removed and stored at -80 degrees Celsius until S100B was analyzed. This procedure was performed three times per participant, one for

each tested phase of the menstrual cycle. Once all samples were taken, S100B was tested against the phases of the menstrual cycle to determine its concentration throughout the cycle.

Medical History

Prior to blood draw, the participant took part in a medical questionnaire. They were asked age, weight, and questions relating to the exclusion criteria to be sure they fit in the study. The rest of the questions were used to determine the use of contraceptive and check for normal menstruation. These questions asked if they experience normal, monthly menstrual cycles for at least three consecutive months, if they use a form of contraception and if so they were required to list the specified type, the length of their average period, etc. All participants were given a code (e.g., MCM01, MCFC02, MCFN03) to impair identifiability and preserve their identity throughout the experiment.

Extended Methods

Recruitment and Scheduling

After a subject was recruited in the Appalachian State and/or Boone community they were informed to either provide an email to be reached or were requested to email the principal investigator. The following email was then sent to potential subjects to better educate them on what would be asked of them before agreeing to consent.

Dear [PARTICIPANT NAME],

Thank you for showing interest in my research, before I confirm schedules with you I wanted to give you a bit more detail as to what would be asked of you and what to expect. Please read the following and if you have any questions about the procedures, please do not hesitate to ask.

First you will come in and we will have you complete a health history and sign an informed consent. Next we will take a urine sample to test for some hormones in your urine. We will collect your height and weight, then take you to a machine called BodPod that will test your body composition. Lastly, we will perform a blood draw of 4 mL (about the size of an index finger) and that will be the end of your visit. You will need to attend 3 visits and we will schedule your next upcoming visit in the lab, but they will all need to be within a 28-day time frame.

When you come to the lab you need to be fasting so no food and only water for 8 hours prior. You cannot have exercised within 12 hours of your visit. You also need to come with either a change of clothes or wearing somewhere under your clothes tight clothing. The body composition test recommends wearing a tight bathing suit, but spandex or tight shorts are fine as well. We will provide a swim cap.

The following questions will be on the health history form when you come into the lab as well, but I need to confirm them before I have you come in for testing. You can simply reply no all or yes to any that apply, if the question does not apply to you then simply answer N/A.

After I get the email confirmation, we can schedule a time for you to come into the lab.

Again, thank you so much for showing interest and if you have any questions about anything please feel free to ask!

Are you taking birth control? If so is it a type that allows you to have a normal period each month? (Ex no IUD)

If you are on birth control have you been on the same kind for at least 6 months?

Have you had a concussion within the past 6 months?

Do you consume any form of steroids, antidepressants, antipsychotics, and/or thyroid medication?

Do you have a history of fainting while giving blood?

The point of this email was to inform potential subjects of what would be asked of them and how to properly prepare before entering the lab. The questions were also inserted in the email to ensure that participants fell within the inclusion criteria of the project and could be scheduled into lab accordingly. For men, a time was scheduled immediately that worked best for them. When women responded to the questions, the following question was asked: “When do you anticipate your next period to start?” Based on the answer to this question, the lab date was scheduled within 5 days of the initial date to start.

Before a time was confirmed that worked with the investigator as well as the participant, a reservation was made on the body composition lab calendar to ensure no other researchers had already reserved the lab during that time frame. Once all schedules matched and an agreed upon time could be scheduled, a confirmation email through google calendar was sent to the subject as well as any lab assistants that may help with data collection. When google calendar confirmation emails were sent, there was an option to agree to the appointment or decline so the investigator could be aware of exactly who would help with each data collection. Lastly, the day before data collection an email was sent to the participant to ensure they prepared to come to lab properly and one last time to confirm the time they needed to be at the lab. This email was simply stating:

Hello [NAME OF PARTICIPANT],

Thank you again for agreeing to be a part of my study. Please be sure to wear a tight bathing suit or tight clothing, do not eat anything for 8 hours before entering the lab, and try to enter the lab ready to use the bathroom. Again, let me know if you have any questions and feel free to text if you need help getting into the building or finding the body composition lab (room 127) [PHONE NUMBER].

This was important as people would forget to fast, forget to bring or wear proper clothing, and most commonly people would urinate prior coming to lab rendering them unable to provide a urine sample. If they were unable to provide a sample when they first entered the lab, it was done at the end of the

blood draw and body composition test. In order to maintain consistency, the urine sample should be collected before the body composition analysis.

Preparation for Participant

On the day a participant was expected in lab, the researcher would get to the body composition lab 1.5 to 2 hours early to properly calibrate equipment and ensure it was working properly. When entering the lab, the BodPod was turned on by flipping the green switch on the white box at the bottom of the cart connected with the BodPod so a light turned on, similar to a surge protector. The BodPod then made a clicking noise to ensure it had been turned on. Next, the computer accompanying the cart was turned on by pressing the power button on the computer box that lays vertically on the second shelf of the cart. A small light came on next to that button and the monitor showed a windows icon to ensure the computer had turned on. Occasionally the computer was forcefully turned off in which case the monitor indicated to start windows normally. When this would happen the instructions on the screen were followed. There were also times that a screen would appear testing the communication between the BodPod and the computer itself, once communication was confirmed “Okay” was clicked.

Once the desktop view was available, the icon with a picture of the egg-shaped BodPod was clicked, the caption was either body composition or BodPod. Once the program was opened, a login screen appeared requiring a username “VBASL” and “Cardiac1” as the password. The username and password were confirmed with the instruction paper under the keyboard of the cart. After logging on, the “QC” on the left side of the screen was located and “Warm-Up” was clicked. A screen appeared asking if a warm-up was to be initiated at a certain time and date; there was also an option “start warm-up now” and the instructions indicated on the screen were followed, it was ensured the volume canister was left sitting on the seat of the BodPod before beginning the warm-up. The volume canister should always be left inside the BodPod for safe keeping, if it was not present there, it was likely located next to the BodPod. Once the warm-up was initiated, the BodPod performed five volume

measurements, this warm-up could take up to one hour. The volumes measured by the BodPod did not always match that of the canister which was the reason for the calibration upon the completion of the warm-up; the importance of the warm-up was to indicate that the standard deviation was low (generally lower than 5, a negative occasionally happened as well and was nothing to worry about) between each measurement. The computer software indicated whether or not the standard deviation was too high and determine whether another warm-up was to be initiated. If the warm-up was considered successful, the calibration process then began. If there is an indication the warm-up was unsuccessful, reach out to the faculty in charge of the body composition lab (Dr. Marco Meucci but subject to change) for further instruction.

In some instances, the warm-up was initiated, and a window appeared indicating the BodPod and computer were not linked or the computer could not find the BodPod. In this case, the BodPod program was closed out on the screen and the program was re-entered. Occasionally that was all that was needed, in other instances the entire mechanism was turned off and back on via the green switch. This was only performed as a last resort to complete the connection. If all programs opened again and the issue was still prevalent, the faculty representative (Dr. Marco Meucci but subject to change) of the body composition lab was alerted to determine if they could assist with the issue. As a last resort, if the faculty representative is not available, then a COSMED representative could be contacted through customer service for further assistance. There should be a booklet on the bottom of the cart with a number for customer service, if it is missing or not able to be found a tollfree number can be called (+1 800-426-3763).

When the warm-up was finished, the calibration process began in the “QC” menu. Each of the following tests were completed: “Analyze Hardware,” “Scale Check,” “Autorun,” and “Volume.” Some tests addressed the calibration had been completed within the past two weeks and was not necessary; however, as good practice, and to ensure each test’s similarity, the full calibration was run for each before every data collection. In each of tests, the screen prompted the exact processes that needed to be done for each procedure; the instructions were followed accordingly – “open BodPod

door,” “close BodPod door,” “place calibrated weights on the scale,” “remove weights from the scale,” “place cylinder inside BodPod,” “remove cylinder from BodPod.” The calibrated weights, gold in color and weighing ten kilograms each, were both placed in the middle of the scale for the calibration. The weights were found sitting either right in front of the BodPod labeled scale or beside the BodPod itself. When doing each of these tasks it was ensured the cylinder was not dropped or harmed as it has been calibrated to the nearest thousandth and any dents could change that number. It was also ensured the BodPod scale was being used and not any others present in the body composition lab. The calibrated weights were stored beside the BodPod, as well as the cylinder inside the BodPod when it was not being used.

Once the BodPod calibration was completed, necessary items from the biochemistry lab for testing were acquired: urine sample cup stored on the shelves at the back of the lab just over the large centrifuge, a urine test stick, and the travel phlebotomy bag. Inside the bag were nitrile gloves, tourniquets, 21-gauge butterfly needles, vacutainer adapters, red-top vacutainers, band-aids, alcohol swabs, gauze pads, and a stress ball. Lastly, an informed consent and a health history form were acquired if it was the subject’s first attendance. The phlebotomy bag and contents were taken to the phlebotomy lab and the rest was taken to the body composition lab where the informed consent took place.

Data Collection with Participant

After consent was signed and the health history form was completed, the Body Composition tab of the BodPod screen was selected and the required information was filled in – Name (subject ID), date of birth, gender, ethnicity (general pop or African American), height (in centimeters to the tenth decimal). The height measurement was taken in the body composition lab using the white stadiometer, and the participant did not have on shoes or socks. The participant was then instructed to provide a urine sample and shown the location of the restrooms. Not a lot of urine was required for analysis; however, if a sample was unable to be produced at the beginning of analysis, the sample was

taken at the end of all measurements. During this time, and after all data was entered, the next screen was prompted and “predicted thoracic measure” was chosen. Another simple calibration was required before starting the measurements. Once the subject re-entered the body composition lab, they were asked to change into their approved clothing, removed all jewelry, and placed the swim cap on their head to cover as much hair as possible. The screen then prompted to have the participant stand on the BodPod scale to measure body mass.

When prompted, the cylinder was removed and the participant was asked to enter the BodPod. The participant sat relaxed with knees pointed forward, feet placed flat on the ground if possible, hands placed on legs and fingers fanned out, sitting straight and looking forward. The procedure was then explained to the participant – “there will be two separate tests, and each will take 20 to 30 seconds long; there will be several ‘washy sounding’ noises during the test, but remain as still as possible and remember to breathe normally.” Between each test there was a prompt to open the door. This was to control for body heat that would get trapped inside the BodPod during each measurement, ensuring the temperature was the same between measurements (Shaw & Kerr, 2018). Occasionally the machine prompted for a third test which was required if the standard deviation between tests was too large. In this case, the participant was not to leave the BodPod until prompted to do so.

After the participant exited the BodPod, the swim cap was removed and they changed back into their clothing. They were then escorted to the phlebotomy lab and instructed to sit in one of the phlebotomy chairs. At this time the standard venipuncture occurred, 4 mL of blood was obtained, and a timer was set for 45 minutes to ensure proper clotting of the blood. The next visit for the participant to enter the lab was scheduled based on the time of their menstrual cycle. If it was their first visit, the next was scheduled within ten to fifteen days to obtain the late follicular phase. If it was their second visit, the next visit was scheduled within four to six days. If the women had knowledge of the phases of their cycle, they were scheduled accordingly. Some women started menstruating earlier than expected; if they had already undergone the first two measures and the last measure was missed, it

was then captured the following month a few days before they menstruated again. This was measured by approximating the length of the previous menstrual cycle they endured to see how long the cycle lasted (time elapsed between the last day of menstruation and the first day of menstruation). By using this information, the best day for measuring the mid luteal phase for the following month was calculated to give at least a three-day buffer before the next estimated menstruation would begin to ensure the measurement was captured.

After the participant exited the lab, the urine was tested to measure ovulation in a measure of either low, high, or peak fertility using a Clearblue fertility monitor. The Clearblue Fertility Monitor was a good measure of LH hormone and estrogen; however, it was made for one individual to use over the course of their own menstrual cycle and has not been shown to be effective in determining the concentration of these hormones in multiple individuals. For this study, a single use ovulation monitor would be more effective to give a more accurate representation of each menstrual cycle phase. The ClearBlue Advanced Ovulation tests would be a better alternative as they provide more individualized and a better indicator of both hormones for each participant.

After the 45-minute timer ended, the blood was centrifuged using a StatSpin Express 3 portable centrifuge (Beckman Coulter, Inc., Brea, CA) for five minutes at 5,600 revolutions per minute (2,685 relative centrifugal force) to separate the serum from the red blood cells. The centrifuge was found in the Biochemistry Laboratory on Dr. Rogatzki's lab bench in a box labeled "Rogatzki." The serum was then pipetted and transferred to a cryotube which was found in the first drawer of Rogatzki's lab bench. A cryobox was obtained from the far-right corner of the lab on the top shelves, many of them have been recycled so labeling them with a sharpie is necessary to not get mistaken with other experiments. The samples were then taken to the freezer closest to the window in the freezer room and in the top left storage box in that freezer; samples were frozen at -80 °C until analysis of all samples could occur. The leftover vacutainer tube and blood inside was then disposed of in biohazardous waste.

Running the ELISA

After 45 participants were recruited and all samples were collected, four ELISA testing kits were ordered allowing each sample to be run in duplicate. For S100B the preferred retailer was millapore sigma (catalog number: EZHS100B-33K); however, due to price, other retailers can be chosen as long as they have good rating from previous literature. All samples were taken from the freezer and stored overnight in the refrigerator to allow them to thaw properly. On the day of analysis, the samples were removed from the refrigerator and incubated at room temperature for at least one hour before running the samples. The storage procedure of the ELISA kits was read and properly followed until date of analysis. The kit also indicated times of incubation in different temperatures before running samples.

In the kits there was a standard, in some cases the standards are prepared, however in other instances a serial dilution was required to create standards using the sample diluent. In the 96 well plate, each sample (including standards) was run in duplicate; meaning one sample took up two wells so each column of sample had another column exactly the same next to it. The sample diluent was not used on the samples, since serum S100B levels are generally within the range of the standard curve. Before the tip was inserted into the sample, the sample was vortexed to allow any proteins that may be at the bottom of the tube to be equally present throughout the serum. To save pipet tips, the wet tip method was used when filling the wells with sample. When this method was performed, some of the sample was drawn into the micropipette then pushed back into the sample container so the tip was considered wet. After being vortexed and wetting the tip, the sample was inserted into both wells. The tip did not touch the bottom of the well as doing so would have scratched the surface and risked detaching the antibody. To ensure the location of the samples for later analysis, when a sample was put into a well the location was noted; an example would be A1 and B1 were NCEFF01. This technique was followed every time a sample was added into a well.

The amount of sample added to the wells, incubation times, washing, creating the buffer, and any other information regarding the testing kit was found in the protocol form of the kit and was

thoroughly reviewed and followed to ensure the integrity of the assay. When the analysis was complete and the stop solution was set, the absorbance of the samples was read by putting the plate in the Eon spectrophotometer (BioTek Instruments, Inc., Winooski, VT USA) located in the sensitive equipment room within the Biochemistry laboratory at the wavelength required by the instructions. Before exiting the program, all information regarding the samples was exported to an external drive on Microsoft Excel. Using Excel, the standard curve, intra-assay coefficient of variation, and inter-assay coefficient of variation were calculated. Intra-assay coefficient of variation should be less than 10% and inter-assay coefficient of variation should be less than 15%. After the concentrations of S100B for each sample were determined they were transferred to SPSS for statistical analysis.

Millipore Sigma ELISA Instructions

The kit in full was stored at 2-8 °C until the date of use, the day before testing the samples were removed from -80 °C and moved to 2-8 °C to allow time to thaw. On day of the assay the standards provided by the kit were reconstituted with 0.5mL of distilled or de-ionized water. After 6 tubes were labeled 1 to 6, 200 µL of assay buffer was added to each. Starting with tube 6, 100 µL of the reconstituted standard was added and mixed well; next 100 µL was taken from tube 6 and added to tube 5 then mixed well, then 100 µL from tube 5 was transferred to tube 4, 100 µL from tube 4 to tube 3, 100 µL from tube 3 to tube 2, and lastly 100 µL from tube 2 to tube 1 and all were mixed well between transfers. Next the lyophilized quality control (QC) vials were both reconstituted with 0.50 mL of distilled or deionized water and mixed until they were fully hydrated.

The HRP wash buffer was created by adding all contents of the concentrated form into 900 mL of deionized water. The 96-well plate was then be washed 3 times with 300 µL of wash buffer with an immediate wash/decant cycle. When the sample was decanted, it was gently tapped on an absorbent towel to remove the fluid from the wells. Next 50 µL of the reconstituted standard, 50 µL from tubes 6 through 1, 50 µL of assay buffer, and 50 µL of QC 1 and 2 was added in duplicate to the

wells on the 96-well plate. Next 50 μL of the samples was added to the wells in duplicate after performing a vortex and wet tip procedure for each sample. Once the plate was full of sample and standards it was covered with a plate sealer and incubated at room temperature (approximately 25 $^{\circ}\text{C}$) for two hours on an orbital plate shaker set to rotate around 500 to 600 rpm or moderate speed.

The plate sealer was then removed and all solutions were decanted, tapping gently on an absorbent towel and removing the samples from the wells. A similar wash cycle as before was performed, 300 μL of wash buffer was added to each well then immediately decanted and this was done 5 times. After a full wash cycle, 100 μL of detection antibody was added to each well, the plate was then recovered and incubated at room temperature for an hour and a half on an orbital shaker set to rotate around 500 to 600 rpm or moderate speed. After incubation, the solution was decanted and the wells were washed again the same as before – 300 μL of wash buffer with an immediate wash/decant cycle for 5 cycles. The last wash cycle was decanted and 100 μL of substrate solution was added to each well, then sealed and set on the shaker plate for 25 minutes. A blue color formed in the standard wells and the intensity of color was proportional to the increase in concentration. These colors were monitored and taken off when the color developed accordingly, they were carefully monitored to ensure they were not incubated for too little or too long of time. The seal was then removed and 100 μL of stop solution was added immediately, the plate was shaken by hand gently to ensure proper mixing. Upon addition of stop solution the wells turned from blue to yellow. The plate was then placed in the spectrophotometer and read at an absorbance of 450 nm and 590 nm within 5 minutes and the absorbance was recorded in units.

All the standards were entered into a sigmoidal 4-parameter logistic regression equation to determine the concentration of all other samples. Both QCs were considered accepted when within the calculated range of 2.7 $\text{pg}\cdot\text{mL}^{-1}$ to 2000 $\text{pg}\cdot\text{mL}^{-1}$. The intra-assay coefficient was calculated to determine the accuracy of the test and remained below 10% using the mean and standard deviation between the duplicate of each sample.

Calculating Results

Serum S100B concentration during the mid-luteal and follicular phases was analyzed with an ELISA kit using a 4-parameter logistic regression equation. Estrogen and LH were also analyzed via the ovulation stick to confirm the menstrual phase of each participant. A RMANOVA was used to analyze the relationship within groups of S100B concentrations to the menstrual phases. A one-way ANOVA was used in measuring S100B among the different sample groups. An RMANOVA was also run for anthropometric measures (height, weight, and body composition) to ensure there was no change within the menstrual cycle groups; there was also a one-way ANOVA performed to confirm no differences of anthropometric measures among the different sample groups. We expected to see no change in S100B concentrations in either the mid-luteal or the follicular phases, however we did expect to see a change in S100B based on contraceptive use. We planned to see a decrease in estrogen in the late-follicular phase and an increase in the early-follicular and mid-luteal phases for confirmation of the menstrual cycle phase.

Reflection

We were unable to finish this study; however, some subjects were recruited, and we were still able to perform some of the tests. Keeping in mind this was a thesis research project, we did not have all the time and money to provide all the resources we would have liked which was the main reason some methods were chosen over others. Measuring hormone concentrations in the blood samples to confirm the phase of the menstrual cycle was our original plan, but the ELISA kits are very expensive, and our funds were limited. This would be a better way to determine the phase of the menstrual cycle, but we used the ovulation test with three values as a substitute. It was still an acceptable estimation and gave a general answer to give us an estimated time frame; however, it is not as accurate at determining menstrual cycle phase as ELISAs would be.

We also used the BodPod to measure body composition. As this is a good and accurate measure compared to other options, the dual-energy X-ray absorptiometry (DEXA) scan gives a precise picture of where the fat is located as well as bone mineral density, along with all the other values that are not analyzed with a BodPod. Since the DEXA provides bone mineral density and a visual of fat distribution, differences among sexes in these categories could better be determined. Matching participants would be another way to improve the methods which could be done by matching age, height, weight, fat distribution, and activity level. The reason we did not use the DEXA scan on participants was due to the radiation expose each participant would have endured over the three visits, but in future studies this could be an alternate route to take from the BodPod.

The menstrual cycle has a constant flow and a constant change in hormones throughout the cycle, and each woman is different on how long the changes can last. Instead of doing only three blood draws over the course of the cycle and attempting to hit the exact 36-hour mark of ovulation, we would prefer to take a blood draw every day from each individual to see how their cycle in whole effects S100B. This would also allow for us to determine if there is a correlation between estrogen/progesterone fluctuations and S100B levels.

One thing that we thought was important to keep in the study was allowing participants to reflect on their lives since the last time we saw them. For example, some participants would explain they had the flu, they may have changed their diet, they changed their exercise plan. These factors were important to note since they could potentially have an effect on S100B.

I think this study was well performed with the few participants that were completely tested through. I feel that the only change I would make to the study would be using another protein or even multiple proteins to test for in serum and see if contraceptive use or the menstrual cycle affects any of them. The point of this research was to see how S100B was affected by something most female athletes endure. If the concentrations of S100B rise or fall during certain points of the menstrual cycle or due to a woman taking a contraceptive, it is a variable that future researchers need in order to diagnose a female with a concussion using a biomarker such as S100B.

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Appendix 1. Summary

- Email participants to determine which are compatible with inclusion criteria
- Schedule an appointment for the participant to come into lab and send a follow up email the day before to remind the participant of how to enter the lab.
- Enter lab at least an hour and a half early to ensure proper warm-up and calibration of BodPod
- Give informed consent and health history form
- Obtain height measurement then give the participant urine cup and escort to restrooms, while they use the restroom run the calibration on the BodPod
- Upon return, allow the participant to change behind the curtain in the body composition lab then take weight using BodPod scale, and once instructed by the computer have the participant enter the BodPod with swim cap, proper clothing, and no jewelry
- Follow instructions and take body composition. When finished print results and label with code for that day so all body composition results can be analyzed later
- After body composition allow participant to change back into the clothes they wore to the lab
- Take participant to phlebotomy lab and perform blood draw using proper equipment and disposal methods. Set a timer for 45 minutes to allow coagulation of blood.
- Schedule the next visit for the participant to come to the lab
- Analyze urine sample with fertility stick
- After timer ends, spin and separate blood then transfer to cryotube to be stored in the freezer at -80 degrees Celsius
- On the day for running ELISAs, be sure to follow instructions from the kit to ensure samples and kit is stored and thawed properly
- Use a wet-tip method and vortex the samples before running a duplicate assay for each sample and be sure to record each sample location

- Be sure to add standards from the kit accordingly and follow all other instructions from the assay kit.
- Enter all samples and locations into excel after analysis to determine concentration of S100B
- Calculate intra-assay and inter-assay coefficients of variation
- Use one-way ANOVA to compare data among groups (contraceptive vs. non-contraceptive vs. men) and a RMANOVA to compare within the phases of the cycle (early follicular, late follicular, and midluteal)

Vita

Jessica Elyn Morgan was born in Summerfield, North Carolina to Eric and Jackie Morgan. She graduated from Northern Guilford High School in Greensboro in June 2014. The following fall, she entered Appalachian State University in Boone to study Exercise Science, and in May 2018 she was awarded the Bachelor of Science degree. In fall of 2018, she accepted a research assistantship in Exercise Science at Appalachian State University and began study toward a Master of Arts degree. The M.A. was awarded in July 2020.

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