Estrogen and Cortisol Fluctuations Throughout the Menstrual Cycle in Athletes in Association with ACL Injuries

By
Emily Dean Herrold
April 1, 2024



Allegheny College
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By
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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

_____
Pledge

Approval by the Senior Thesis Committee:

_____
Dr. Becky Dawson

_____
Dr. Mahita Kadmiel

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ABSTRACT

Athletes who menstruate are two to eight times more likely to experience an ACL injury as a result of biomechanical, anatomical, and neuromuscular differences between the sexes. Hormonal fluctuations throughout the menstrual cycle also have a prominent role in this uptick of injuries, specifically those of the sex hormone estrogen. Estrogen receptors have previously been found in ACL tissue and are associated with ACL injuries; however, estrogen's relationship and effect on other hormones, like the stress hormone cortisol, is not well understood. A self-report survey was distributed to collegiate student-athletes across NCAA divisions to gather more information on menstruating athletes' ACL injuries and menstrual cycle history. Additionally, a sputum study was conducted at Allegheny College, a Division III liberal arts college, amongst student-athletes to observe the hormonal fluctuations of estradiol and cortisol. Statistical analyses of the survey determined that there was a significant association between the day of the menstrual cycle and when menstruating athletes experienced their ACL injury, and that birth control has protective effects against ACL injuries. The sputum study analyses found that menstruating athletes on birth control may experience a blunted cortisol response. While this study could not determine the relationship between estradiol and cortisol due to multiple limitations, its results indicate that hormonal fluctuations throughout the menstrual cycle in association with ACL injuries have further points of study that need to be investigated and lay the ground for future research.

DEI STATEMENT

An important note about this topic is that most scientific papers written about this subject use gendered terms like “women " and “girls” when referring to people who menstruate. It’s crucial to recognize that people who menstruate may not identify as women or have female-typical anatomy. Gender and sex do not exist within binaries. Gender is based on social norms that we perform repeatedly, leaving room for it to change over the course of a lifetime (Lindqvist et al., 2021). Sex is generally defined by the chromosomes you have, either XY or XX, but other factors involved in sex determination, like hormone production, hormone responsiveness, external genitalia, internal reproductive organs, and gonads (testes or ovaries), are not always consistent between the chromosome pairings (King, 2022). It is important for everyone, but especially those within the scientific community, to recognize this and use inclusive language whenever possible in their research, as language is a powerful tool. In this paper, I will strive to use terms like “people who menstruate” and “female-typical reproductive systems/bodies” instead of the terms “women" and “female” that are most often used to refer to gender and the reproductive system associated with the XX chromosomal arrangement. For the scope of my study specifically, when I am referring to people who have a uterus and menstruate (female-typical reproductive systems), I will say “people who menstruate.” When referring to those who don’t have a uterus and do not menstruate (male-typical reproductive systems), I will say “people who do not menstruate.”

INTRODUCTION

The anterior cruciate ligament (ACL) (Figure 1) is located in the knee joint and contributes to knee stability during jumping, running, and pivoting or cutting movements (Nessler et al., 2017). Injuries to the ACL are associated with both short- and long-term financial burdens including the costs of surgery and rehabilitation. ACL injuries also result in other long-term consequences like an increased retear rate and the injury's impact on an individual's mental health. Given these impacts in addition to the fact that ACL injuries can be career-ending, it is especially concerning that ACL injuries are becoming more common in athletes (Herzberg et al., 2017).

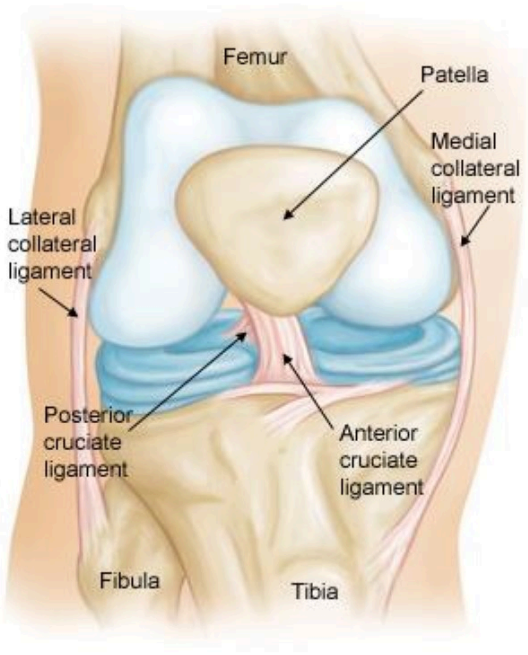

Figure 1. A diagram of the normal human knee anatomy that includes the major bones, tendons, ligaments, and cartilage (Mulcahey, 2022).

Athletes who menstruate have an increased risk of ACL injury that is two to eight times greater than that of athletes who do not menstruate (Gould et al., 2016). This increased risk is a

result of the hormonal fluctuations of estrogen and progesterone, the main hormones involved in the menstrual cycle (Herzberg et al., 2017). Numerous studies looking at multiple variables such as cycle phase, contraceptives, level of physical activity, and knee laxity have been conducted to determine the correlation between hormonal fluctuations and ACL injuries (Gould et al., 2016; Herzberg et al., 2017; Stijak et al., 2014); however, little research has been done to determine if stress and the primary stress hormone, cortisol, affects these hormonal fluctuations and ACL injuries, specifically in student-athletes. Cortisol is a steroid hormone that affects nearly every organ system, including the reproductive system (Thau et al., 2022). The menstrual cycle has been determined to influence the hypothalamic-pituitary-adrenal axis (HPA axis), which is responsible for cortisol secretion. Understanding how this relationship affects athletes who menstruate and their increased risk for ACL injuries is important for the future of these athletes in sports.

The Menstrual Cycle

The events that occur within the menstrual cycle to prepare female-typical reproductive systems for reproduction are regulated by hormones associated with the hypothalamic-pituitary-ovarian (HPO) axis. These hormones fluctuate within a 28-day cycle on average, although cycle lengths vary between individuals and can be between 23 and 38 days (Reilly, 2000). Within the HPO axis, the gonadotropin-releasing hormone (GnRH), which is released from the hypothalamus, stimulates the production of two gonadotropic hormones: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Fox and Rompolski, 2022). GnRH controls the secretion of LH and FSH, which promote the structural and functional changes of the ovaries through the cycle, via feedback from the main steroid hormones of the

ovaries, estrogen and progesterone (Fox and Rompolski, 2022).

Estrogen, within both male and female-typical reproductive systems, is important for sexual development and cell proliferation (Otağ et al., 2016). The main form of estrogen within the body is estradiol, which is important in the development of female-typical secondary sex characteristics during puberty. Estrogen also has systemic effects on multiple systems of the body such as the musculoskeletal system, central nervous system, cardiovascular system, and immune system (Otağ et al., 2016; Fox and Rompolski, 2022). Progesterone is another important hormone that helps regulate the menstrual cycle and is also involved in the development of female-typical secondary sex characteristics and structures during embryogenesis (Rodney & Sataloff, 2016; Fox and Rompolski, 2022). Lastly, testosterone also plays an important role in the menstrual cycle, as it is the precursor in the synthesis of estradiol (Davis & Wahlin-Jacobsen, 2015). Testosterone also increases muscle mass and bone tissue (Stijak et al., 2014).

These hormones are essential for the menstrual cycle to occur and fluctuate throughout it. The menstrual cycle has more simplistic models that are split into two phases and highly detailed models that are split into seven phases; however, research conducted on athletes is most often split into a three-phase model: the follicular phase, ovulation, and the luteal phase (Bruinvels et al., 2022; Fox and Rompolski, 2022). The early follicular phase starts with a period of menstruation, where the lining of the uterus, the endometrium, is shed, and normally lasts four to five days (Reilly, 2000; Fox and Rompolski, 2022). FSH stimulates the growth of follicles throughout the follicular phase, which in total lasts for about the first 14 days of the cycle, and these follicles stimulate the secretion of estrogen. Estrogen levels peak right before ovulation occurs around day 12 and an LH surge that follows triggers ovulation (Figure 2). The cycle now has entered the luteal phase, which is characterized by an increase in progesterone (Reilly, 2000).

If the egg released is not fertilized, estrogen and progesterone levels fall in the late luteal phase and the withdrawal of these hormones prepares for and begins the next cycle (Reilly, 2000; Fox and Rompolski, 2022).

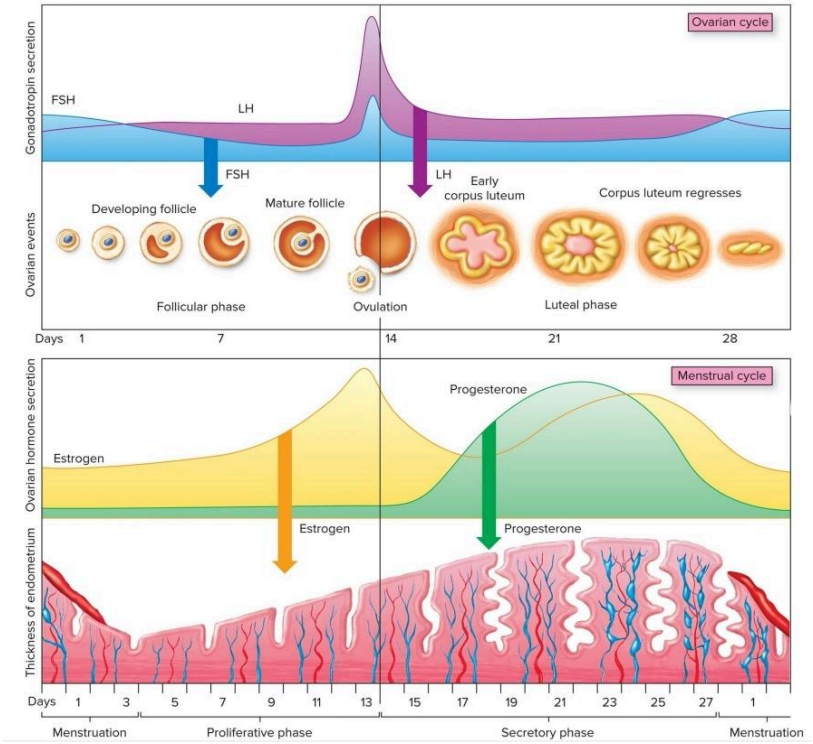

Figure 2. This image is representative of the different phases and hormonal fluctuations throughout the menstrual cycle (Fox and Rompolski, 2022). The top panel represents FSH (blue) and LH (purple) levels throughout the follicular and luteal phases. The bottom panel represents estrogen (yellow) and progesterone (green) levels, as well as endometrium thickness, throughout a menstrual cycle.

As previously mentioned, the hormones involved in the menstrual cycle also impact other major systems in the body, one of them being the central nervous system. GnRH is regulated by higher brain centers, like the hypothalamus. It is well known that stress hormones can impact the menstrual cycle and emotions (Fox and Rompolski, 2022). Cortisol is one of the stress hormones that is regulated by the hypothalamus.

Cortisol

Cortisol is a glucocorticoid that helps regulate metabolism, immune function, the inflammatory response, and the stress response (Fox and Rompolski, 2022; Thau et al., 2022). In normal individuals, cortisol levels remain low or undetectable at night and start building to reach a peak in the early morning between 7-10 AM (Figure 3). The hypothalamus produces corticotropin-releasing hormone (CRH) that stimulates the production of adrenocorticotropic (ACTH) hormone from the anterior pituitary gland. ACTH triggers the production of cortisol from the adrenal cortex (Smith & Vale, 2006). There is evidence from human and animal studies that there is a sex difference between HPA response patterns (Kirschbaum et al., 1999). It is suggested that the female adrenal cortex is more sensitive as males secrete more ACTH than females with similar cortisol levels (Kirschbaum et al., 1999).

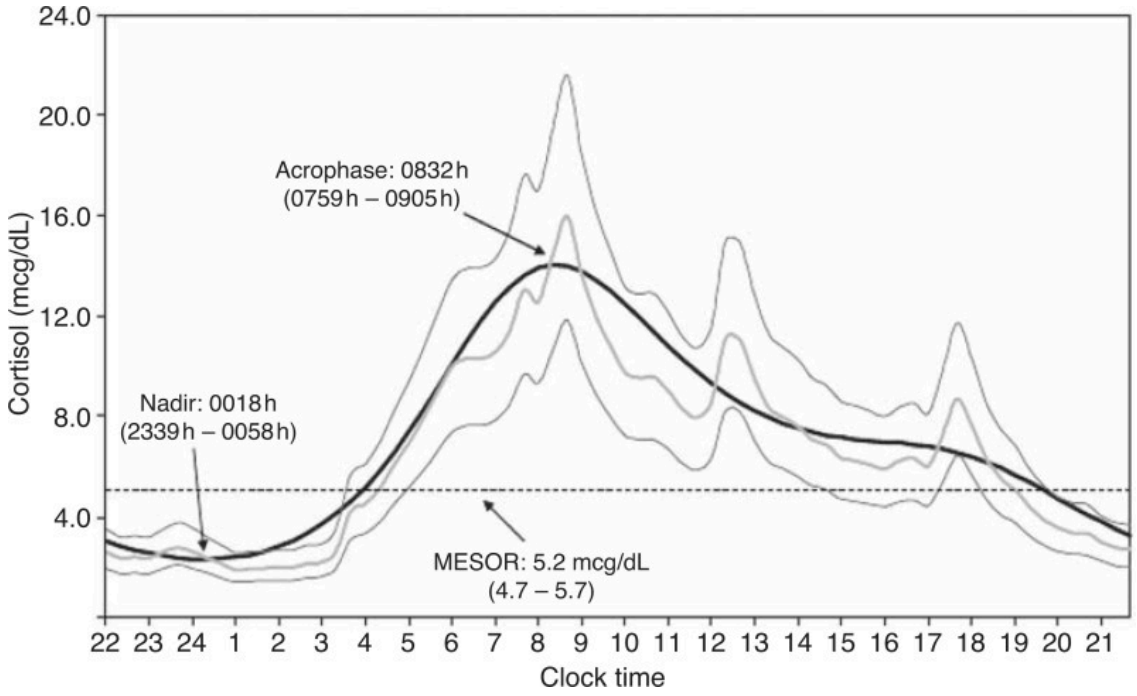

Figure 3. Circadian rhythm of cortisol (Chan & Debono, 2010). Cortisol levels fluctuate throughout a 24-hr day with acrophase being the peak of cortisol levels, mesor being the adjusted mean, and nadir being the lowest point of cortisol levels.

The relationship between cortisol production and the menstrual cycle has not been widely studied and needs to be better understood; however, cortisol has been documented to be affected by the menstrual cycle. Kirschbaum and colleagues (1999) conducted a psychosocial stress test called the Trier Social Stress Test (TSST) on four groups: a non-menstruating group with male-typical reproductive organs, menstruators in the follicular and luteal phases, and menstruators using oral contraceptives. The authors discovered that there are lower free cortisol levels during the follicular phase. Additionally, they noted that cortisol secretion levels are similar between the sexes and that there is a clear difference in available free cortisol between sexes and different menstrual cycle phases (Kirschbaum et al., 1999). The authors suggested that the difference in cortisol levels may be explained by estradiol. Another study that investigated the vulnerability of people who menstruate to psychosocial stress by exposing them to the Montreal Imaging Stress Test (MIST) confirmed these results (Albert et al., 2015). A third study that investigated how cortisol affects memory and might be influenced by sex hormones during the menstrual cycle further confirmed that there were lower cortisol levels when estrogen levels were high in the follicular phase (Andreano et al., 2008). All three studies call for additional research that explores this relationship and how it affects an individual's health outcomes.

Hormonal Fluctuations in Correlation with ACL Injuries

It is well established that athletes who menstruate are at a substantially higher risk for ACL injury than those who do not as a result of hormonal fluctuations that occur throughout the menstrual cycle, in addition to biomechanical, anatomic, and neuromuscular differences between the sexes (Gould et al., 2016). Both estrogen and progesterone receptors have been found within ACL tissues and cells (Herzberg et al., 2017; Gould et al., 2016; Faryniarz et al., 2006).

Increased estrogen levels have been associated with a decrease in fibroblasts, which are responsible for collagen and procollagen production, the latter of which makes up 85% of the collagen within the ACL (Slauterbeck et al., 2002; Perini et al., 2022; Mancino et al., 2024). The effects of testosterone on ACL tissues and injuries are less researched; however, a study that used western blotting on ACL tissue harvested during surgeries to identify and locate androgen receptors found that testosterone is not an independent predictor of ACL stiffness close to ovulation after they measured ACL stiffness and testosterone concentrations (Lovering & Romani, 2005). A separate study investigated the concentrations of estradiol, testosterone, and progesterone in people who menstruate with and without ACL tears via saliva specimens that were collected within 72 hours after an ACL tear and determined that subjects with ACL tears had significantly lower concentrations of all three hormones in the immediate aftermath of their injury (Stijak et al., 2014). Overall, the fluctuations of estradiol and progesterone throughout the menstrual cycle seem to have more of an impact on the probability of an ACL injury than testosterone.

Furthermore, the menstrual cycle phase and specific cycle days have been of interest to researchers trying to determine when athletes are most at risk for ACL injuries. It was discovered that there is a significant correlation between days 1 and 2 of the menstrual cycle and ACL injuries (Slauterbeck et al., 2002). Out of the 37 athletes who participated in this study, 27 tore their ACLs during the follicular phase of their cycle (Slauterbeck et al., 2002). Further research on the hormonal fluctuations that occur during the menstrual cycle and their impacts on ACL injuries is needed to provide better prevention and treatment plans for athletes who menstruate.

Low-quality data and research have made it difficult to determine what phase of the menstrual cycle is associated with an increased risk of ACL injury. A meta-analysis that

reviewed twenty-one studies, totaling 68,758 participants, called for this increase of better quality research in this area, as the current research available is of low quality. Some of the reviewed studies did not include all possible participants who met the criteria of the study and others did not adjust for criteria that may have affected their results (e.g. prior ACL injury) (Herzberg et al., 2017). Herzberg et al. reviewed the menstrual cycle, contraceptives, and ACL injuries and indicated that the lowest risk for ACL injuries occurs during the luteal phase. Additionally, the researchers were able to conclude from their review that oral contraceptives (OCs) decrease the risk of ACL injuries by nearly 20% (2017). This conclusion is supported by a more recent study done on mice, where mice treated with 1 of 5 clinically relevant OCs had a significant increase in ACL strength relative to control groups (Konopka et al., 2020). The authors suggested that OCs with higher progestin-to-estrogen ratios may be more protective against and reduce ACL injury.

Currently, there is limited research that investigates the relationship between hormonal fluctuations during the menstrual cycle and cortisol in athletes. A study that monitored salivary cortisol concentration in female collegiate soccer players during games and practices found that cortisol levels increased by 250% for starters and 140% for non-starters (Haneishi et al., 2007). Cortisol deficiencies have been cited in veterinary medicine to elevate estrogen levels and dysregulate the production of B and T cells, therefore negatively impacting immune function (Plechner, 2004). Cases of elevated estrogen contributing to adrenal dysfunction occur in spayed females or neutered males 90% of the time, so normal hormonal fluctuations cannot be credited for these deficiencies. The author of this study questions the implications his research has on human health, asking if estrogen is involved in similar diseases and conditions he investigates in his paper. As previously discussed, low levels of estrogen, progesterone, and testosterone in the

early follicular phase are correlated with an increase in ACL injuries (Stijak et al., 2014; Slauterbeck et al., 2002). With ACL injury rates increasing steadily over the past 20 years and an annual incidence rate of 200,000 injuries, the costly financial burden of surgery and rehabilitation, and the return to sports rate being less than 50%, it has become increasingly important to further our understanding of what puts athletes who menstruate at an increased risk for ACL injuries (Herzberg et al., 2017). I hypothesize that there is an indirect relationship between cortisol levels and estrogen levels that fluctuate throughout the menstrual cycle which increases the risk of ACL injury. Studying the relationship between hormonal fluctuations and cortisol levels throughout the menstrual cycle is important in order to increase the knowledge of why people who menstruate are more likely to experience ACL injuries, and how we can prevent and treat these injuries.

METHODS

Self-Report Survey

A self-report survey was produced and approved by the Internal Review Board (IRB) at Allegheny College. It was distributed via emails to athletic directors within the PAC and NCAC conferences. To increase the number of participants and to obtain a larger data sample on this topic -- emails and advertisements were also sent to friends, teammates, and other individuals who could participate in the study. Some survey questions were adapted from Jessie Theissen's comp (2017) and the survey included questions about age, age of first period, type of birth control taken (if any), prior ACL injuries, and if they were menstruating when they experienced an ACL injury. Individuals were able to report more than one injury, as the survey captured the number of participants and the total number of injuries sustained by those participants.

Sputum Study

This study determined the levels of estradiol and cortisol in athletes who have uteruses and menstruate and athletes who don't have uteruses and don't menstruate. The IRB at Allegheny College reviewed and approved this study, ensuring the safety of the participants. Participants in this study were between the ages of 18 and 22. Each participant signed a consent form at the first sample collection in order to voluntarily participate. Additionally, at the first sample collection, participants were also asked multiple verification questions to link their self-report survey responses to their samples. It was also ensured that none of the participants were actively receiving treatment in the form of cortisol shots which might have affected the results of this study.

Five menstruating athletes and five non-menstruating athletes participated in the study. Samples were collected in Dr. Humphreys's level two biosafety lab. For six participants (three menstruating and three non-menstruating), three saliva samples of about 1 mL each were collected from each participant throughout their menstrual cycle. From the remaining four participants (two menstruating and two non-menstruating), only two samples of about 1 mL each were collected, on days one and seven. Samples from athletes who don't menstruate were collected on the same days and within the same time frame as a sample from an athlete who does menstruate.

The first spit sample was collected on day one of menstruation. The second sample was collected one week after the first sample was collected (day seven) and the third sample was collected a week after the second one was obtained (~day fourteen). The samples were collected in sterile 15 mL screw-cap conical tubes and split evenly between two 1 mL microcentrifuge

tubes to make the assays of each hormone easier. The tubes were labeled with the participant's assigned letter and the date the sample was collected. The date and time for each sample collection from each participant were also documented on a Google spreadsheet. Each sample was stored in a -20°C freezer in Dr. Humphreys's lab within 30 minutes of collection.

Salimetrics Enzyme-linked Immunosorbent Assays (ELISAs)

The saliva collected from each participant was used to complete estradiol and cortisol ELISAs in order to visualize the concentrations of each hormone throughout different phases of the menstrual cycle. Per the manufacturer's directions, participants were instructed to avoid eating a major meal 60 minutes before the time of collection and to rinse their mouths with water 10 minutes before the sample was collected. Additionally, spit samples were not collected within 12 hours of any consumption of alcohol. The samples were placed in the freezer within 30 minutes of collection in order to preserve the specimen. On the day of the assay, the saliva samples were thawed completely, vortexed, and centrifuged for approximately 15 minutes at 1500 x g.

Enzyme-linked immunosorbent assays measure the absorbance of a substance by using precoated plates with antibodies bound to the bottom of each well to detect target antigens (Boguszewska et al., 2019). Once a sample has been added to a well, the target protein (cortisol or estradiol in this experiment) will bind to the antibody (Figure 4). An enzyme-labeled antibody and a substrate are added to the well in order to cause an enzyme reaction, usually a colored one, that will make the concentration levels of each hormone visible (Boguszewska et al., 2019). A color change indicates that the test is negative because the enzyme-conjugated antibody bound to the antigen and not the antibodies of the saliva while the absence of color indicates a positive

test, as the antigens bound to the saliva's antibodies (Alhajj et al., 2023). The ELISA kits that were used are the 17β-estradiol (Item No. 1-3702) and cortisol (Item No. 1-3002) salivary ELISA kits from Salimetrics.

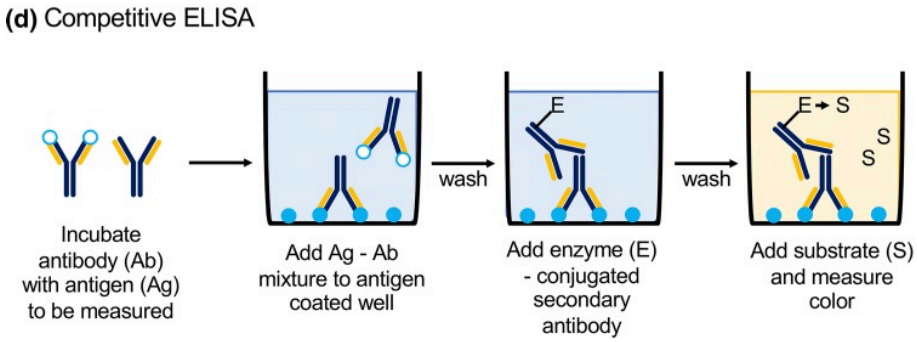

Figure 4. This figure is adapted from (Boguszewska et al., 2019) and shows a basic schematic representation of a competitive ELISA.

The estradiol ELISA was the first assay run. The plate layout was made prior to beginning the assay with manufacturer recommendations in mind (Figure 5). All reagents were allowed to come to room temperature for at least 1.5 hours and mixed before use. The Microtitre Plate was kept in the foil pouch to prevent humidity from affecting the coated walls while being brought to room temperature for use. First, 1L of 1X wash buffer was prepared by diluting the Wash Buffer Concentrate 10-fold with room-temperature deionized water. Serial dilutions of the estradiol standard were prepared as seen in Figure 6.

	1	2	3	4	5	6	7	8	9	10	11	12
A	32 Std	32 Std	Ctrl-H	Ctrl-H	C2	C2	G2	G2	J2	J2		
B	16 Std	16 Std	Ctrl-L	Ctrl-L	E1	E1	G3	G3	K1	K1		
C	8 Std	8 Std	A1	A1	E2	E2	H1	H1	K2	K2		
D	4 Std	4 Std	A2	A2	E3	E3	H2	H2	K3	K3		
E	2 Std	2 Std	A3	A3	F1	F1	I1	I1				
F	1 Std	1 Std	B1	B1	F2	F2	I2	I2				
G	Zero	Zero	B2	B2	F3	F3	I3	I3				
H	NSB	NSB	C1	C1	G1	G1	J1	J1				

Figure 5. 96-well plate layout for the estradiol ELISA adapted from the Salimetric suggested layout. NSB stands for non-specific binding well. These may serve as blanks. Each participant was assigned a letter and the number correlates with the sample collection number. Unused wells are represented by blank, white cells.

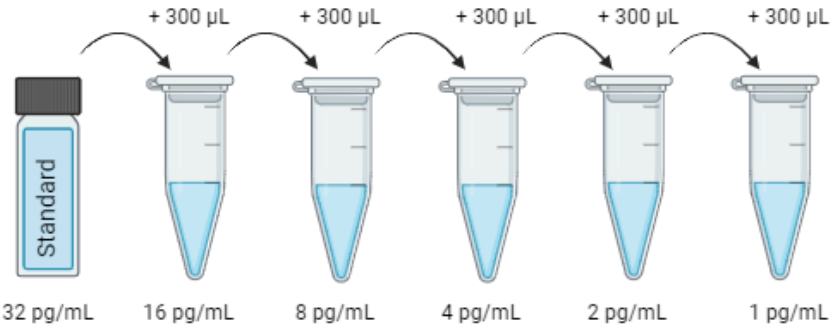

Figure 6. Serial dilution of HS Estradiol Standard. Created with BioRender.com.

Once the reagents and serial dilutions were prepped, 12 mL of the HS Estradiol Assay Diluent was pipetted into a disposable tube for a later step. Then, 100 µL of standards, controls, and saliva samples were pipetted into the appropriate wells. All standard, controls, and saliva

samples were assayed in duplicate. Then, 100 µL of the assay diluent was pipetted into wells G1 and G2 to serve as the zero or blank, and 100 µL of the assay diluent was pipetted into each NSB well. Next, the enzyme conjugate was diluted 1:800 by adding 15 µL of the conjugate to the disposable tube of assay diluent that was previously prepared. The diluted conjugate solution was immediately mixed and 100 µL of the solution was added into each well using a multichannel pipette. An adhesive covering that was provided was placed over the plate. The manufacturer's directions recommended that the plate should be mixed on a plate rotator for 5 minutes at 500 rpm; however, the plate was mixed on a Lab-Line Plate Rotator (model number 1314) for 5 minutes at 220 rpm, as this was the only plate rotator available for this experiment. The plate was then incubated at room temperature for two hours.

After the incubation period, the plate was washed four times with 1X wash buffer by pipetting 300µL of the wash buffer into each well and discarding the liquid into a biohazard container. The plate was blotted with paper towels between each wash before turning upright. After the last wash, 200 µL of a TMB Substrate Solution was pipetted into each well using a multichannel pipette. The plate was placed on a plate rotator for 5 minutes at 220 rpm and then placed in a lab drawer to be incubated in the dark at room temperature for 25 minutes. After the second incubation period, 50 µL of the Stop Solution was added to each well using a multichannel pipette. The plate was mixed on a plate rotator for 3 minutes at 220 rpm until the wells turned from a green color to yellow. Before placing the plate in the plate reader, the bottom of the plate was wiped with a water-moistened cloth and then wiped dry. The plate was read at 450 nm within 10 minutes of adding the Stop Solution for the best results.

The cortisol ELISA was the second assay run. The plate layout was made before the assay began with manufacturer recommendations in mind (Figure 7). All reagents were allowed

to come to room temperature for at least 1.5 hours and mixed before use. The Microtitre Plate was kept in the foil pouch to prevent humidity from affecting the coated walls while also being brought to room temperature for use. First, a 1X wash buffer was prepared by diluting the Wash Buffer Concentrate 10-fold with room-temperature deionized water. Serial dilutions of the HS Cortisol Standard were provided by the manufacturer.

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.000 Std	3.000 Std	Ctrl-H	Ctrl-H	C2	C2	G2	G2	J2	J2		
B	1.000 Std	1.000 Std	Ctrl-L	Ctrl-L	E1	E1	G3	G3	K1	K1		
C	0.333 Std	0.333 Std	A1	A1	E2	E2	H1	H1	K2	K2		
D	0.111 Std	0.111 Std	A2	A2	E3	E3	H2	H2	K3	K3		
E	0.037 Std	0.037 Std	A3	A3	F1	F1	I1	I1				
F	0.012 Std	0.012 Std	B1	B1	F2	F2	I2	I2				
G	Zero	Zero	B2	B2	F3	F3	I3	I3				
H	NSB	NSB	C1	C1	G1	G1	J1	J1				

Figure 7. 96-well plate layout for the cortisol ELISA adapted from the Salimetric suggested layout. NSB stands for non-specific binding well. These may serve as blanks. Each participant was assigned a letter and the number correlates with the sample collection number.

Once the reagents and serial dilutions were prepped, 24 mL of the HS Cortisol Assay Diluent was pipetted into a disposable tube for a later step. Then, 25 µL of standards, controls, and saliva samples were pipetted into the appropriate wells. All standard, controls, and saliva samples were assayed in duplicate. Then, 25 µL of the assay diluent was pipetted into wells G1

and G2 to serve as the zero or blank, and 25 µL of the assay diluent was pipetted into each NSB well. Next, the enzyme conjugate was diluted 1:1600 by adding 15 µL of the conjugate to the disposable tube of the previously prepared assay diluent. The diluted conjugate solution was immediately mixed and 200 µL of the solution was added into each well using a multichannel pipette. An adhesive covering that was provided was placed over the plate. The plate was mixed on a plate rotator for 5 minutes at 220 rpm and incubated at room temperature for two hours.

After the incubation period, the plate was washed four times with the 1X wash buffer by pipetting 300µL of the wash buffer into each well and discarding the liquid into a biohazard container. The plate was blotted with paper towels between each wash before turning upright. After the last wash, 200 µL of a TMB Substrate Solution was pipetted into each well using a multichannel pipette. The plate was placed on a plate rotator for 5 minutes at 220 rpm and then placed in a lab drawer to be incubated in the dark at room temperature for 25 minutes. After the second incubation period, 50 µL of the Stop Solution was added to each well using a multichannel pipette. The plate was mixed on a plate rotator for 3 minutes at 220 rpm until the wells turned from a green color to yellow. Before placing the plate in the plate reader, the bottom of the plate was wiped with a water-moistened cloth and then wiped dry. The plate was read at 450 nm within 10 minutes of adding the Stop Solution for the best results.

Statistical Analysis

The absorbance of the spit samples was measured by a plate reader. Two standard curve graphs were made in Google Sheets that are representative of the standards of estradiol and cortisol against their absorbances. The concentrations of each hormone standard are on the x-axis and the mean absorbance is on the y-axis. The standard curve generated an equation. The

absorbance of each sample was entered into the equation and I solved for the concentration of the hormone in each sample. The statistical significance of the concentrations of each hormone was calculated. The broader self-report survey that was used to collect data on menstruating athletes who had torn their ACLs was analyzed and the statistical significance of this was calculated with a chi-square test in a free statistical analysis software, SAS Online for Academics. A chi-square is used to determine if categorical variables, like if an athlete was menstruating during the time of their ACL tear or what day of their cycle the injury occurred on, are statistically significant in correlation to the population group. Statistical significance for this study was set at $p < 0.05$.

RESULTS

Utilization of the Self-Report Survey

Of the original 56 responses to the self-report survey distributed to student-athletes across multiple divisions, only 30 responses were utilized; however, 38 responses were included in the chi-square analysis, as some participants in this sample were double counted if they reported experiencing multiple ACL tears in their response. A total of 10 participants completed the survey in order to participate in the sputum study. None of these participants had experienced an ACL injury at the time of completing the survey or first sample collection. The remaining 16 were excluded from the survey analysis because they did not fit the study criteria (e.g. the participant did not report an ACL injury, reported age did not fit within the age range of the typical collegiate student-athlete, or did not menstruate).

Self-Report Survey

Characteristics of the survey participants are displayed in Table 1. Only those menstruating athletes who had reported an ACL injury were included in this analysis. The mean age of all analyzed participants was 20.3 ± 1.26 years. Of the 38 reported ACL injuries, 14 of these injuries occurred while the participant was menstruating while the other 23 injuries did not occur during menstruation. Whether or not participants had a regular cycle was not deemed significant to whether they were menstruating at the time of their injury ($p = 0.1733$). The day of the menstrual cycle participants were on at the time of injury was not reported in every response; however, significance was still found in association with whether or not participants were menstruating at the time of injury and cycle day ($p = 0.0346$). A significant relationship between taking birth control and whether or not participants were menstruating at the time of injury ($p = 0.0098$) was also found from this analysis; however, after reviewing each response, it was determined that there was differential misclassification bias among the group that responded that they were on birth control. For participants who reported the year that they experienced their ACL injury and how long they had been on birth control, a few of them had not been on birth control at the time of their injury. The type of birth control participants were taking was insignificant ($p = 0.3262$).

Variable	Sample	P-Value*
Age (in years)		
Mean	20.3	
Standard Deviation	1.26	
Sex		
Female	38	
Male	0	
Gender		
Woman	38	
Man	0	
Menstruating at the time of injury		
Yes	14	
No	23	
Regular Cycle		
Yes	27	0.1733
No	10	
Day of Cycle		
Day 1	3	**0.0346**
Day 2	1	
Day 3	7	
Day 5	1	
Day 10	3	
Day 16	1	
Birth Control (BC)		
Yes	19	**0.0098**
No	19	
Type of Birth Control		
The Pill	13	0.3262
IUD (Hormonal)	3	
IUD (Non-hormonal)	3	
Shot	2	
Arm Implant	0	
Vaginal Ring	0	
The Patch	0	

Table 1. Descriptive data for the self-report survey. The sample included only menstruating athletes who had torn their ACLs. All other individuals were excluded from this specific analysis. Indicated p-values were generated by a chi-square test.

Salimetrics Enzyme-linked Immunosorbent Assays (ELISAs)

All participants completed the self-report survey to determine the characteristics of the sample. Table 2 summarizes this data. All participants were between the ages of 18 and 22. The mean age of the menstruating participants was 20.2 ± 1.30 years and the mean age of the

non-menstruating participants was 20.8 ± 0.84 years. There were a total of 5 participants in each group. In the menstruating group, only two participants were taking birth control during the time of sample collection and both were taking a pill form of birth control.

Variable	Menstruating	Non-Menstruating
Age (in years)		
Mean	20.2	20.8
Standard Deviation	1.30	0.84
Sex		
Female	5	0
Male	0	5
Gender		
Woman	5	0
Man	0	5
Birth Control (BC)		
Yes	2	N/A
No	3	N/A
Type of Birth Control		
The Pill	2	N/A

Table 2. Descriptive data for the sputum study.

Visualizations of both the estradiol and cortisol ELISA are seen in Figures 8 and 9. These show the concentration of hormones in each sample via a color reaction. A lighter color indicates a higher concentration of the hormone, while a darker color indicates a lower concentration of the hormone.

	1	2	3	4	5	6	7	8	9	10	11	12
A	32 Std	32 Std	Ctrl-H	Ctrl-H	Y	Y	N	N	N	N		
B	16 Std	16 Std	Ctrl-L	Ctrl-L	Y	Y	N	N	N	N		
C	8 Std	8 Std	N	N	Y	Y	N	N	N	N		
D	4 Std	4 Std	N	N	Y	Y	N	N	N	N		
E	2 Std	2 Std	N	N	N	N	N	N				
F	1 Std	1 Std	N	N	N	N	N	N				
G	Zero	Zero	N	N	N	N	N	N				
H	NSB	NSB	Y	Y	N	N	N	N				

Figure 8. Visualization of the concentration of estradiol in each sample. "Y" indicates that the participant did take birth control while "N" indicates they did not. Unused wells are represented by blank, white cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.000 Std	3.000 Std	Ctrl-H	Ctrl-H	AM	AM	AM	AM	AM	AM		
B	1.000 Std	1.000 Std	Ctrl-L	Ctrl-L	AM	AM	AM	AM	AM	AM		
C	0.333 Std	0.333 Std	PM	PM	AM	AM	PM	PM	AM	AM		
D	0.111 Std	0.111 Std	AM	AM	AM	AM	AM	AM	AM	AM		
E	0.037 Std	0.037 Std	AM	AM	PM	PM	PM	PM				
F	0.012 Std	0.012 Std	AM	AM	AM	AM	AM	AM				
G	Zero	Zero	AM	AM	AM	AM	AM	AM				
H	NSB	NSB	AM	AM	PM	PM	AM	AM				

Figure 9. Visualization of the concentration of cortisol in each sample. "AM" indicates that the sample was collected between 8:00-11:45 AM while "PM" indicates that the sample was collected between 2:00-5:00 PM. For exact collection times, see Appendix Table 1. Unused wells are represented by blank, white cells.

Standard curves for both estradiol and cortisol were produced (Figures 10A and 11A). Samples were collected on days 1, 7, and 14 of a menstrual cycle to determine if there was any difference in estradiol as well as if estradiol and cortisol fluctuations had a relationship.

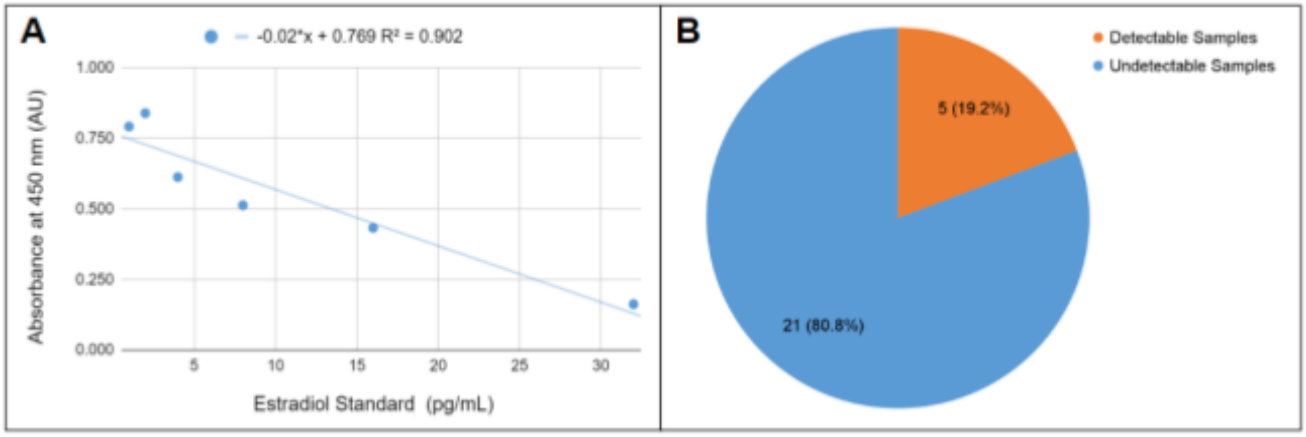

Figure 10. (A) Standard curve for completed estradiol ELISA. **(B)** Amount of samples with detectable and undetectable concentrations of estradiol. The orange pie slice represents the samples with detectable concentrations of estradiol and the blue pie slice represents the samples with undetectable concentrations of estradiol.

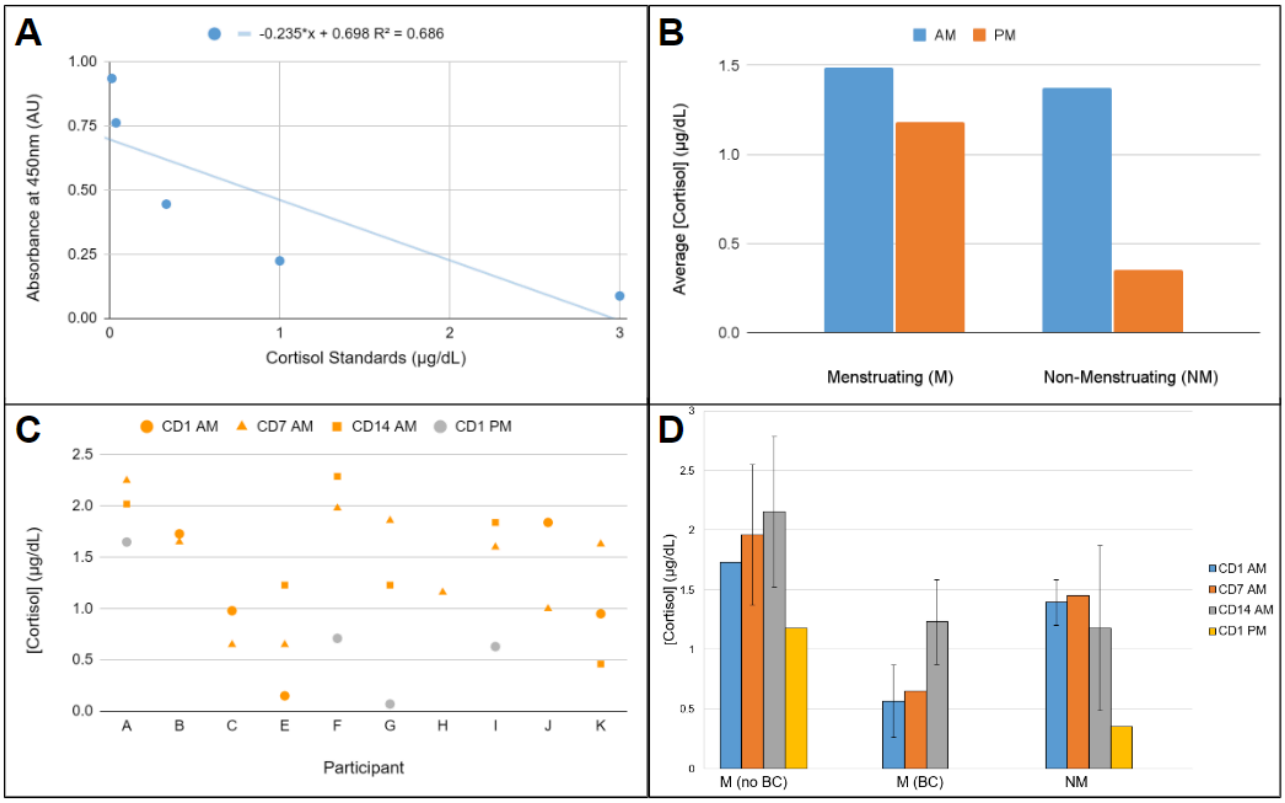

Figure 11. (A) Standard curve for completed cortisol ELISA. **(B)** Averages of all AM and PM sample concentrations for both the menstruating and non-menstruating groups. The average AM concentration is represented by the blue bar and the average PM concentration is represented by the orange bar. **(C)** The concentration of cortisol by participant's cycle day (CD1, CD7, or CD14) is represented by different shapes (CD1 is a circle, CD7 is a triangle, and CD14 is a square), and time of collection (AM or PM) is represented by different colors (AM in orange and PM in gray). **(D)** The average concentration of cortisol for each group (menstruating not on birth control, menstruating on birth control, and non-menstruating) by cycle day and collection time group. Error bars represent standard deviations for the concentration levels for each group. CD1 (AM) is represented by the blue bar, CD7 (AM) is represented by the orange bar, CD14 (AM) is represented by the gray bar, and CD1 (PM) is represented by the yellow bar.

The estradiol ELISA results of this study can not be reported because the concentrations of estradiol in 21 out of 26 samples (80.8%) were too low to be detectable (Figure 10B). The concentration of cortisol in each sample is shown in Figure 11C. Data points are shown by the participant and the cycle day and time the sample was collected. Overall, the concentrations of cortisol in each sample followed typical trends of daily cortisol fluctuations - higher concentration levels in the morning and lower levels in the afternoon. There are no large differences in cortisol levels between different cycle days. Figure 11B represents the average AM and PM concentrations of cortisol in all samples by group (menstruating and non-menstruating). These levels are relatively consistent, with the menstruating group having slightly elevated levels of cortisol in comparison to the non-menstruating participants. Figure 11D shows the average concentration of cortisol by cycle day and collection time between all groups (menstruating not on birth control, menstruating on birth control, and non-menstruating). This analysis shows that the menstruating group not on birth control has higher cortisol levels than the non-menstruating group but also that the menstruating group on birth control has lower levels than both groups.

DISCUSSION

This investigation utilized a self-report survey and sputum study to evaluate the role of hormonal fluctuations in ACL injuries. Previous research has shown that athletes who menstruate are two to eight times more likely to experience an ACL injury as a result of these hormone fluctuations, in addition to biomechanics, neuromuscular, and anatomical differences between the sexes (Herzberg et al., 2017; Gould et a.. 2016). This study firstly aimed to collect background information from athletes who menstruate and have experienced an ACL injury, and

secondly explore the hormonal fluctuations of estradiol and cortisol throughout one menstrual cycle in student-athletes via hormone levels in spit.

(1) The day of the menstrual cycle, birth control, and ACL injuries.

The results of the self-report study revealed that the type of birth control participants took was not found to be associated with whether or not participants were menstruating when their ACL injury occurred, nor was having a regular cycle. It also confirmed that both the day of the menstrual cycle ($p = 0.0346$) and birth control ($p = 0.0098$) are significant factors for ACL injuries in athletes who menstruate. These findings suggest that there are certain days when athletes who menstruate are more at risk for an ACL injury. This increased risk is likely caused by estrogen fluctuations, specifically, due to the presence of estrogen receptors in ACL tissue. Typically, estrogen improves muscle mass and strength in musculoskeletal tissues, but in tendons and ligaments, like the ACL, estrogen decreases stiffness and therefore the strength of these tissues (Chidi-Ogbolu & Baar, 2019). This directly impacts injury and performance rates in athletes and supports the hypothesis that estrogen fluctuations increase the likelihood of an ACL injury. Additionally, these results suggest that birth control helps act as a protective measure against ACL injuries in this population. This is most likely because contraceptives modulate the normal hormonal fluctuations that those who menstruate experience in a normal cycle.

Previous literature aligns with the findings of this study that indicate that the menstrual cycle day and birth control impact the likelihood of ACL injuries in menstruating athletes. Several studies have reported that ACL injuries are associated with hormonal fluctuations (Herzberg et al., 2017; Stijak et al., 2014; Mancino et al., 2024; Gould et al., 2016). The most prominent idea behind this hypothesis is that ACL tissues have estrogen receptors and when there are elevated estrogen levels in the ovulatory and luteal phases, there is a decrease in

fibroblast production within ACL tissue (Gould et al., 2016). Fibroblasts are important for the collagen production of ACL tissue so if their production is decreased as a result of high estrogen levels, athletes may be more vulnerable to injury. A study by Slauterbeck and colleagues (2002) reported that 27 out of 37 menstruating athletes had torn their ACL during the follicular phase of their menstrual cycle, a phase where estrogen levels are low and follow the periods of elevated estrogen levels. From these studies, it can be concluded that it is the rapid change of estrogen levels that contributes to menstruating athletes' increased likelihood of ACL injury.

Given that most of the estradiol levels in the samples of this study were undetectable and cannot be reported, this study is not generalizable to what previous studies have found on hormonal fluctuations of estrogen during a menstrual cycle. Although specific days during a menstrual cycle where athletes are more at risk cannot be determined from this analysis, 11 of 16 participants who reported the day of their cycle indicated that their ACL tear occurred within the first 3 days of their cycle (Table 1). Further research on when ACL injuries occur during menstrual cycles is needed to provide athletes and trainers with beneficial health information.

Literature on contraceptives also validates the results of this study. A meta-analysis found that oral contraceptives may provide up to a 20% reduction in the risk of ACL injury (Herzberg et al., 2017). Another study that specifically investigated ACL tears in the 15-19-year-old age group via data provided by a medical research database called PearlDiver and performed a chi-square test that compared the number of menstruating athletes on and off oral contraceptives who experienced ACL tears (DeFroda et al., 2019). This study indicated that there was a 63% reduction in the rate of tears among this population as a result of oral contraceptives.

Overall, the self-report survey distributed and analyzed in this study further contextualized and validated the findings of previous studies; however, more research needs to

be done on what specific days of the menstrual cycle athletes who menstruate are more at risk for an ACL injury and if there are specific types of birth control that are more protective than others.

(2) Cortisol fluctuations follow the reported circadian rhythm

The results from the cortisol ELISA assay revealed differences between morning and afternoon levels of salivary cortisol. In all three figures used to compare morning and afternoon times (Figures 11B-D), morning levels were shown to be consistently higher than the afternoon levels. This is consistent with previous studies that have investigated the normal cortisol circadian rhythm (Chan & Debono, 2010; Pearlmutter et al., 2020; Mohd Azmi et al., 2021). The salivary cortisol levels found in this study are within the ranges considered normal for both morning and afternoon salivary cortisol (Pearlmutter et al., 2020).

Another finding from this study is that there appears to be a sex difference between the menstruating and non-menstruating groups. Figure 11B compares the average cortisol levels in the morning and afternoon for each group. The menstruating group has higher cortisol levels in comparison to the non-menstruating group. There is some evidence that biological sex influences cortisol responses and levels; however, most studies report that males have an elevated response of ACTH and cortisol after the TSST, a type of stress test (Kirschbaum et al., 1999; Liu et al., 2017; Zimmer et al., 2003). The results of this study directly contradict what is normally expected for sex differences in cortisol, but it is important to note that the participants in this study did not undergo a stress test to trigger a cortisol response. Further research is needed to understand the stress responses in athletes on and off the field and to determine if there is a sex difference in cortisol response outside of experiencing a stress-inducing event.

(3) The relationship between estradiol and cortisol fluctuations undefined.

The ELISA assays of estradiol and cortisol were inconclusive regarding whether any association between the two hormones exists. The main reason for this is because the estradiol results could not be reported, due to undetectable levels producing negative concentrations in post-analysis, and therefore cannot be generalized. Undetectable levels of estradiol levels in most of the samples collected can be explained by a variety of reasons, the two biggest being that hormonal fluctuations are variable from cycle to cycle and the assay kit's binding affinity to different types of estrogen. Two participants were taking an oral contraceptive at the time of the study. The type of estrogen used in these pills was ethynylestradiol which has a low cross-reactivity of 0.189% and therefore a low binding affinity (Salivary 17β-Estradiol Enzyme Immunoassay Kit, n.d). Additionally, with artificial hormones, there will be competition between them and the naturally occurring hormone, which also might cause lower or undetectable levels in samples.

Although the cortisol assay produced results that were expected based on previous literature, this study is unable to propose a relationship between these two hormones and their fluctuation patterns. Prior studies in humans and animals have indicated that estradiol and cortisol share an indirect relationship, meaning that when one hormone was elevated, the other was low (Plechner, 2004; Andreano et al., 2008). Additional research, with a larger sample size, that investigates these two hormones and their relationship throughout multiple menstrual cycles is needed for a better understanding of how they affect ACL injuries in athletes.

(4) Cortisol levels could be influenced by birth control.

The menstruating group on birth control had lower average levels of cortisol, relative to both the menstruating group not on birth control and the non-menstruating group (Figure 11D).

These results corroborate previous findings on how birth control affects cortisol levels. Cortisol responses have been shown to be blunted in menstruators who are on hormonal birth control in comparison to menstruators who are not on hormonal birth control. A study demonstrated that menstruators on hormonal birth control have a blunted cortisol response relative to menstruators not on hormonal birth control, by observing glucocorticoid (cortisol) responses during a cold pressor stress test (Nielsen et al., 2013). Other studies confirmed this finding and even indicated that menstruators on hormonal contraceptives had low cortisol levels that were comparable to menstruators not on contraceptives in the follicular phase of their menstrual cycle (Gervasio et al., 2022; Kirschbaum et al., 1999). This response in cortisol was significantly blunted in comparison to non-menstruators and menstruators in the luteal phase of the menstrual cycle (Kirschbaum et al., 1999).

While the results from my study are exciting and align with previous literature, it is important to note that only two participants were taking hormonal birth control in this experiment. A small sample size is not conducive of significant data nor can it fully validate previous literature. Cortisol levels can also be influenced by a variety of factors like food intake, metabolism, the immune system, physical activity, and sleep (Azmi et al., 2021). Future research should strive to have a larger sample size of both menstruators on birth control and not on birth control in order to see if these results can be replicated and therefore validate previous literature.

(5) Strengths and Limitations

There are several strengths to this study, the first being that there is little research done on collegiate student-athletes, especially those playing at the Division III level, as most research done on athletes is conducted on professional athletes. The self-report survey reached athletes across multiple NCAA divisions and the sputum study sampled Division III student-athletes at a

small liberal arts college. Specifically for the sputum study, Division III student-athletes are an underserved and understudied population that should be included and studied in injury prevention research. Additionally, another strength of this study was that it highlighted DEI issues within the scientific community surrounding inclusive language that have not been previously addressed. Sex does not equal gender and neither exists within a binary. It is my hope that by recognizing this and using more inclusive language in this analysis, future research can use the language used in this study or adapt it to better represent everyone in the collegiate student-athlete population. All in all, this study included a self-report survey, sputum study, statistical analysis of data, and laboratory work (ELISAs) with biohazardous materials to observe hormonal fluctuations throughout a menstrual cycle.

This analysis also has multiple limitations that should be noted. First, the small sample sizes of both the self-report survey and the sputum study made it difficult to collect enough data to make conclusions about the larger population of NCAA student-athletes. During the analysis of the self-report survey, it was revealed in the analysis of the results that several questions needed a follow-up response. For example, participants were not asked to specify the date of their injury nor were they asked if they were on birth control at the time they experienced their ACL injury. Additionally, several participants reported multiple ACL tears in one survey response. These responses were double-counted but not all of them had the desired level of specificity for this study. For example, some participants provided dates and what day of their menstrual cycle they were on for each tear, while others only stated how many times they tore each ACL with no further details.

For the sputum study, time constraints and sample collection scheduling challenges resulted in multiple samples being collected on days other than days 1, 7, and 14, as described in

the methods portion of this paper. These samples were still categorized as day 1, 7, or 14 in the results portion of this paper for simplicity and data analysis purposes. Time constraints included working with a student-athlete schedule (practices, classes, traveling for games, work, etc.) and Allegheny College's spring break.

Sample collection began roughly four weeks before the week-long spring break. The sputum study participants were unable to be recruited until the IRB application was approved in early/mid-January and samples could not be collected until participants in the menstruating group began their regular cycle. Four participants (two menstruating and two non-menstruating) only contributed two samples each as a result of the spring break beginning before the fourteenth day of the menstrual cycle. Additionally, one of the participants in the non-menstruating group experienced an ACL tear between the collection of samples one and two. This participant was still included in the analysis of the study because they were in the non-menstruating group and therefore their estradiol levels were not fluctuating based on a menstrual cycle; however, this should be kept in mind when considering the validity of this participant's results. See Appendix Table 1 for more information on the sputum study and its participants.

A small sample size was especially impactful on the results and analysis of the sputum study, as significant data cannot be collected, determined, or validate previous literature. The ideal collection time for cortisol is in the morning between 7-10 AM when cortisol levels are the highest (Chan & Debono 2010). Only five samples were collected in the afternoon as a result of scheduling conflicts, but this could cause the averages in Figure 10 to not be representative of the sample and therefore the population.

Another limitation of this study is the lack of access to lab equipment. The manufacturer of the ELISA kit used (Salimetrics) recommended a plate rotator that had the capacity to mix a

plate at 500 rpm. In the Biology department at Allegheny College, the plate rotators available for this project only could mix a plate at 220 rpm. It is unclear how much the mixing of assay reagents and samples was affected by this, but it is something to take into consideration when reviewing these results.

(6) Future directions

For future works, the ideal study design would involve a larger sample size for both the self-report and sputum study. As mentioned above, adjustments should be made to questions on the self-report survey to gain more specific information and clarity on ACL injuries. Participants should be asked to record a response for each ACL injury they had and specify all details for each injury (e.g. date, if they were menstruating, cycle day, if they were on birth control at the time of injury, type of birth control, etc.).

Future sputum studies should aim to collect samples over multiple menstrual cycles to gain a better understanding of hormonal fluctuations, as hormone variability is high, and observing more than one allows researchers to account for this. This would be a much bigger study than it is possible to do at a liberal arts college, but this idea should be taken into account for larger-scale research endeavors.

Furthermore, future research could investigate which specific days athletes are more at risk for ACL injury by taking daily samples or exploring which types of birth control work the best as injury prevention. This could be done by choosing to investigate 3-5 of the most commonly used types of birth control. Exploring specific types of birth control by having a control group of menstruating athletes not on birth control and an experimental group of menstruating athletes on birth control could also help determine if cortisol levels are influenced by estradiol levels. Additionally, samples could be collected from participants in both the

morning and afternoon of chosen collection days to get a more well-rounded understanding of how cortisol fluctuates based on birth control.

Generally speaking, more research on collegiate student-athletes, specifically within the division III athlete population, and the hormonal fluctuations they experience throughout the menstrual cycle in association with ACL injuries is needed to better prevent and treat injuries.

(7) Conclusion

It is clear that hormonal fluctuations throughout a menstrual cycle increase the likelihood of ACL injuries in menstruating athletes. The self-report analysis determined that there are certain days and phases of the menstrual cycle where menstruating athletes are more at risk and that birth control has protective effects against ACL injuries. The sputum study found that cortisol levels are impacted by birth control in menstruating athletes. Further research is needed to determine if estradiol and cortisol share a relationship and what impact this relationship might have on the increased incidence rate of ACL tears in menstruating athletes. This research needs to be conducted on collegiate student-athletes, specifically on Division III athletes as they are underrepresented in research on this topic. This study can be used as a guide for future research and sets the stage for future analysis. Injury prevention amongst collegiate student-athletes is a prominent issue that requires more research for the benefit of all athletes.

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APPENDICES

Appendix Table 1: Sputum study sample collection information

Participant	Birth Control (BC)	Sample 1 Date	Sample 1 Time	Sample 2 Date	Sample 2 Time	Sample 3 Date	Sample 3 Time
A	No	**2/13/2024**	**4:27:00 PM**	2/19/2024	8:43:00 AM	2/26/2024	8:48:00 AM
B	No	2/22/2024	9:14:00 AM	2/29/2024	8:57:00 AM	X	X
C	Yes - Pill	2/21/2024	11:25:00 AM	2/28/2024	10:29:00 AM	X	X
E	Yes - Pill	2/17/2024	11:25:00 AM	2/24/2024	10:33:00 AM	**2/29/2024**	**11:03:00 AM**
F	No	2/20/2024	3:54:00 PM	2/27/2024	10:54:00 AM	**3/4/2024**	**8:17:00 AM**
G	N/A	2/13/2024	4:48:00 PM	2/19/2024	8:51:00 AM	2/26/2024	8:54:00 AM
H	N/A	2/22/2024	2:55:00 PM	2/29/2024	9:03:00 AM	X	X
I	N/A	2/20/2024	4:01:00 PM	2/27/2024	10:46:00 AM	3/1/2024	11:03:00 AM
J	N/A	2/21/2024	11:37:00 AM	2/28/2024	10:10:00 AM	X	X
K	N/A	2/17/2024	11:42:00 AM	2/23/2024	7:33:00 AM	2/29/2024	10:53:00 AM

*** Bolded sample collection dates and times indicate that the sample was not collected following the day 1, 7, and 14 schedule due to time restraints and schedule conflicts that come with working with student-athletes. Sample A1 was collected on day 2 of the participant's menstrual cycle. Sample E3 was collected on day 12 of the participant's menstrual cycle. Sample F3 was collected on day 13 of the participant's menstrual cycle. Additionally, the underlined text in participant K's row indicates that he experienced an ACL tear in between sample collections 1 and 2.

Appendix 2: Consent Form

By signing this consent, you are volunteering to take part in a research study. The purpose of this experiment is to identify hormonal fluctuations of estrogen and cortisol that occur in athletes who menstruate and athletes who don't, and which phases of the menstrual cycle they occur in. The purpose is to also determine whether or not these fluctuations increase the likelihood of ACL injury and the impact of hormonal contraceptives on hormonal fluctuations. It is important to understand the role of estrogen and cortisol in athletes who menstruate in order to predict and prevent possible effects that estrogen might have on the rest of the body. We ask that you sign below where you are comfortable and after you are sure you understand the scope, procedures, and purposes of this project. Specifically, we want you to understand the following:

- This study will determine estrogen and cortisol levels in athletes who menstruate and athletes who do not and investigate estrogen and cortisol fluctuations in the menstrual cycle. You will be one of approximately 20 participants in this study.
- If you feel uncomfortable in any way during the interview session, you have the right to decline to answer any question or to end the interview.
- There will be no more risk other than what is normally experienced in everyday life.
- Your participation involves the collection of saliva into small test tubes. You will spit into a tube three times throughout your menstrual cycle (days 1, 7, and 14, roughly). Lastly, you will complete a survey about your injury and menstrual cycle history. The study will last about a month (the length of a menstrual cycle).
- The research team will not identify you by name in any reports using information obtained from the survey or saliva collection, and your confidentiality as a participant in this study will remain secure. For the purposes of this research study, your comments will not be anonymous. Every effort will be made by the researcher to preserve your confidentiality.
- You are entitled to a copy of the results once this study is completed. You can request a summary via email or view the full project on D-Space.
- This research study has been reviewed and approved by the Institutional Review Board (IRB) at Allegheny College (if you want more information, please contact Becky Dawson at 814-332-2196)

I have read and understand the explanation provided to me. I have had all my questions answered to my satisfaction, and I voluntarily agree to participate in this study. Additionally, I affirm that I am at least 18 years of age.

Printed Name of Participant: _____
Signature of Participant: _____
Date: _____

Appendix 3: Self-Report Survey Questionnaire

Self-Report Survey

Many risk factors for injuries in athletes are unknown or not well understood. One of the risk factors for knee injury in athletes who menstruate are hormonal fluctuations throughout the menstrual cycle. The goal of this survey is to collect data on knee injuries in menstruating and non-menstruating athletes and use it to better understand the risk factors associated with hormonal fluctuations.

By completing this survey, I recognize that I am at least 18-years-old.

This survey has been approved by the Allegheny College IRB.

What is your age?
Short answer text

What is the sex you were assigned at birth?
- ☐ Female
- ☐ Male
- ☐ Intersex

How would you describe your gender identity?
Short answer text

What college sport (NCAA or NAIA) do you play? Please specify men's or women's.
Short answer text

Injury Background
Please answer the following questions to the best of your ability.

Have you ever experienced an ACL injury?
- ☐ Yes
- ☐ No

Please describe your injury.
Short answer text

If applicable, were you on your period when you experienced your injury?
- ☐ Yes
- ☐ No

What day of your menstrual cycle did your injury occur on? (With Day #1 being the first day of menstruation.)

Short answer text

Menstrual History

If you do not have a uterus and do not menstruate, please feel free to disregard this section.

Age of first menstrual period?

Short answer text

Date of last period

Month, day, year

Do you have a regular cycle? A regular cycle can be defined as a period occurring for 2-7 days every 21-35 days.

- ☐ Yes
- ☐ No

Do you currently take oral contraceptives or any form of birth control?

- ☐ Yes
- ☐ No

Please specify the type of birth control

- ☐ IUD (Non/hormonal)
- ☐ IUD (Hormonal)
- ☐ Arm Implant
- ☐ Shot
- ☐ Vaginal Ring
- ☐ The Patch
- ☐ The Pill
- ☐ Other…

What is the brand of birth control you are on?

Short answer text

How long have you been on birth control?

Short answer text

Continued from ACL Injury Survey

Thank you for completing this survey! If you are submitting BOTH of the following forms, copy and paste the links into a separate browser.

If you are interested in being entered into a raffle to win a $25 Amazon gift card, click the link below! Three winners will be randomly selected and contacted when the survey closes (early April). Fill out this form if you are interested: *Form link*

If you are an Allegheny College student-athlete and are interested in participating in an on-campus spit study, please fill out this form: *Form link*

This work is mine unless otherwise cited: Emily Herrold

Allegheny College, 2024