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The Association of Salivary Estradiol Levels with Anterior Cruciate Ligament Stiffness and Laxity

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BACKGROUND: Females are three to six times more likely to sustain an anterior cruciate ligament (ACL) injury than males. Researchers have previously identified hormonal fluctuations as a possible intrinsic factor related to the higher incidence of ACL injuries among female athletes. Whether hormonal levels, with or without use of oral contraceptives (OC), influence ligamentous mechanical properties resulting in increased ACL injury risk remains unclear. Furthermore, previous studies have mainly focused on anterior tibial translatory (ATT) laxity, with limited research on changes in ATT tissue compliance/stiffness. The purpose of this study is to investigate the influence of estradiol levels, menstrual cycle phases, with and without use of OC on ATT tissue compliance/stiffness and laxity.
METHODS: Fifty-two female participants (age 24-30 years), divided into two groups of 26 each (Group OC using oral contraception, and Group NOC not using oral contraceptive). Data collection was performed on two different dates corresponding to two menstrual cycle phases: follicular or inactive phase (days 1-3) and the ovulation or active pill phase (days 12-14). Estradiol levels via saliva sampling, ATT tissue compliance/stiffness and laxity measures via the GNRB knee arthrometer were collected each testing session. Spearman's rank-order correlation product-moment correlations were run to determine the relationship between follicular and ovulation phase estradiol and GNRB outputs related to translation and compliance.
RESULTS: Estradiol levels showed a significant change between follicular and ovulation phases across both groups combined ($p = 0.01$). There were weak non-significant correlations between measured estradiol levels and GNRB measures of anterior translation and compliance, ($p > 0.05$). No significant relationships were found across subjects whether they were on oral contraceptive or not using hormone regulated birth control.
CONCLUSION: Estradiol level changes in the follicular and ovulation phases and oral contraceptive use did not significantly influence ACL laxity (anterior tibial translation) or compliance/stiffness. Additional studies on hormonal fluctuations as a potential risk factor for higher female ACL injury are indicated.

INTRODUCTION

The integrity of the anterior cruciate ligament (ACL) is crucial to knee stability and function. Injury to the ACL can lead to detrimental functional deficits including loss of playing time amongst athletes, sequalae of osteoarthritis, concomitant injuries such as meniscal damage and need for additional surgical procedures.[1-4] The estimated annual reported ACL injury rate in the United States is 1 in 3500 persons, resulting in approximately 400,000 ACL reconstruction (ACLR) surgeries each year.[5] Females are up to eight times more likely to sustain an ACL injury than males and consequently, much research has focused on identifying risk factors associated with these higher female injury rates.[6–8] A review of the literature suggests there may be several female-biased risk factors associated with ACL injury, including biomechanical structural differences related to kinetic and kinematic pattern alterations, as well as the influence of sex hormones on ACL laxity.[9–15] Understanding the role of each of these female-biased risk factors can ultimately improve strategies to prevent and treat ACL injuries.

Increased ligament laxity is correlated with functional knee instability.[16] Uhorchak et al.

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reported that anterior tibial translatory laxity greater than one standard deviation above the mean was associated with a three-fold increase in the risk of ACL injury in female military cadets.[17] Similarly, Myer et al. reported a four-fold increase in injury rate among female soccer and basketball players for every 1.33 mm increase in anterior tibial translation (ATT) laxity.[18] Fluctuations in female sex hormones, (estrogen, progesterone, and relaxin) have been associated with changes in ligamentous laxity across the menstrual cycle and hypothesized to influence ACL injury via a direct effect on ligamentous properties such as laxity and tissue compliance.[12, 18,19] In a systematic review and meta-analysis by Herzberg, et al., peak estrogen levels during the ovulation phase were associated with a significant increase in ATT laxity compared to the follicular phase when estrogen levels are typically lower.[12] Studies investigating the effects of oral contraceptive (OC) used to stabilize these sex hormone level fluctuations suggest hormonal contraceptive use may serve as a protective measure against ACL injury, with a decrease in ACL laxity and nearly 20% reduction in the incidence of ACL injury in female athletes.[20-22] However, systematic reviews by Hertzberg et al. and Samuelson et al. investigating the effect of the menstrual cycle and contraceptives on ACL injuries and laxity found the potential stabilizing effects associated with OC use vary in study quality and outcomes, creating a challenge to understanding the true impact of OC on ACL laxity, stiffness, and injury incidence.[12,22]

With the invention of newer, more precise robotic knee arthrometers, and more accurate measures of ATT laxity the ability to evaluate the passive compliance of tissues restraining anterior tibial translation states is now feasible.[23,25–27] Bercovy and Weber identified the importance of analyzing ACL stiffness as defined as changes in force divided by changes in displacement rather than laxity measures alone.[16] Bercovy and Weber analyzed ACL tissue compliance (inverse of stiffness) curves in 1500 ACLR patients, reporting a strong correlation between functional knee instability and higher measures of tissue compliance of the ACL graft one year after surgery (p 0.95 value 0.0001).[16] Subsequently, Nouveau et al. analyzed compliance curves and demonstrated the clinical utility of monitoring changes in compliance curves (slope) to optimize rehabilitation programs and patient outcomes.[19] Nouveau et al. tracked ACL laxity and stiffness/compliance in post-op ACLR patients over 2 years and identified thresholds of strong divergent curves/slopes, defined as >2mm displacement differential and >10um/N slope/compliance differential.[19] If these thresholds of divergence were observed during the first 3-6 months of rehabilitation, his team showed it was possible to modify and enhance the ACL tissue compliance by altering the rehab program to decrease excessive stress to the ACL.[19] With the revised rehabilitation approach to decrease excessive load to the ACL graft, the slope of the tissue compliance curve was restored back to mirror the uninvolved knee and was associated with good outcomes (knee stability).[19] In contrast, if the slopes of the compliance curves between the surgical and uninvolved limbs were observed to diverge during the first 3 months post-op, but the rehab program was not modified to decrease stress to the ACL, those patients went on to have poorer outcomes, with knee instability.[16,19] Based on these earlier works of Bercovy and Noveau, there is a need to understand further the influences of menstrual cycle estradiol levels, not only on measures of ATT laxity but to understand the potential influences of estradiol levels on ATT compliance/stiffness.[16] Given the evidence to date, the authors of this study hypothesize that because estradiol levels have been shown to influence measures of tibial laxity, they may also influence robotic arthrometer measurement of anterior tibial compliance.

Therefore, this study aimed to investigate the influence of estradiol levels during menstrual cycle phases, with and without use of OC, on ACL tissue compliance/stiffness and laxity.

METHODS

Study Design

A test-retest cross-sectional study was conducted across adult female participants (18-35 years) using the GNRB® knee arthrometer for measurements of anterior tibial translatory laxity and compliance. GNRB® arthrometer measurements were taken during predicted follicular and ovulation periods of the menstrual cycles of all study participants. Estradiol levels were measured from collected saliva samples at the time of GNRB® measurements.

Participants

After institutional ethics approval, fifty-two participants (18-35 years) were recruited from a local university setting. Participants were recruited via flyer and university email for the study from

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November 2021 through June 2023. Sample size was initially determined with a priori f-test ANOVA using G*Power 3.1.9.4, with an estimated effect size of 0.4, a power of 0.80, and alpha level of 0.05. Minimum sample size and effect size were then confirmed by the works of Smith et al. where calculations were completed using $2x(Za+ Z\beta)^2 x \sigma^2)/d^2$ for a parallel design; a parallel design powered at 0.90 would require 19 participants to detect an absolute change in anterior proximal tibial displacement of 1mm assessed at a test force of 134N.[25]

Participants were provided informed consent and were separated into two groups based on the use of OC birth control. Group NOC (26 participants) included subjects not using OC birth control for a minimum of 6 months prior to participation in the study, and Group OC (26 participants) included subjects currently using oral contraceptives. Exclusion criteria included current knee pain, history of ACL compromise, or irregular menstrual cycle within the past 6 months.

Methodology

Data collection was performed once during the follicular phase of the menstrual cycle (day range 1-3) and once during the ovulation phase (day range 12-14). For both groups, the follicular phase was determined by the onset of menses. For the OC group, the "ovulation phase" was considered to occur between days 12 and 14 of the participant's menstrual cycle. For the NOC group, the ovulation phase was detected using a home ovulation test (Pregmate ® ovulation urine test strips). Once the participant tested positive on the home ovulation test (OC group), or started menses (OC and NOC groups), they contacted the primary investigator via text (used subject ID on text) who then scheduled a data collection visit within 48 hours. The participants were randomized to the order of testing across the two sessions based on their menstrual cycle phase. Participants height and weight were recorded for BMI and group comparison calculations.

Participant Activity Level

The participant's physical activity level was collected through use of the self-report International Physical Activity Questionnaire (IPAQ). The IPAQ is a reliable and commonly used instrument to identify levels of physical activity and exercise prescription for the treatment and prevention of disease.[28] All participants were given a paper copy and instructed on how to complete the IPAQ at their study orientation; the total MET-minutes/week was calculated and used in the data analysis.

Estradiol Level Saliva Testing

Subjects were provided instructions (written and verbal) regarding food and drink diet restrictions on the day of testing based on the Salimetrics Lab testing protocol for best practice sampling (see Table 1 for detailed instructions). After verbal confirmation to adherence of saliva sample instructions, participants provided a passive drool sample of a minimum of 0.5ml of saliva into a sterile test tube collector, which was immediately placed into a freezer at -20 degrees C to preserve the samples for later processing by an offsite certified saliva testing lab, (Salimetrics, LLC Lab, Carlsbad, California). Passive drool saliva sample has been shown to be an accurate and valid assay in measuring estradiol levels.[29,30] Each participant provided a saliva sample for each of the two testing sessions. All samples were assayed twice for estradiol levels, and the average estradiol levels across the two tests was used in the analysis.

Table 1. Saliva sample instructions to participant

1.	Avoid eating foods high in sugar, acidity, or caffeine content immediately before collection
2.	Avoid eating large meal 60 minutes prior to sample collection
3.	Avoid ingesting alcohol, caffeine, nicotine, medications within the prior 12 hours
4.	Avoid brushing teeth within 60 minutes of sample collection
5.	Rinse mouth with water 10 minutes before sample collection

GNRB®

Anterior tibial translatory laxity and compliance/stiffness (slope) measurements were completed using the GNRB® arthrometer. The GNRB® device has previously been shown to be a reliable and valid measure of tibial anterior translation.[25,31,32] Testing was performed by an experienced practitioner and to maximize accuracy and reliability, a standardized GNRB® testing protocol involving precise patient positioning, accurate landmark skin markings, consistent patellar force plate and footplate location values

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was followed; see Figure 1 for participant set up in GNRB® device.[25,31,32]

Based on protocols of prior studies, the GNRB® device was programmed to robotically produce three consecutive anterior tibial translation ramp forces to a maximum of 200N.[32–34] During each testing session, anterior tibial translation values at 134N and 200N and tissue compliance values were recorded. Previous studies investigating changes in ACL laxity include anterior tibial translation testing forces ranging between 89N-250N. This study investigated forces of 134N and 200N to allow comparison of values obtained in previous research using similar forces.[31–33,35] For each testing session (follicular and ovulation) the mean values from the three measurements were used in data analysis.

Statistical Analysis

All data analyses were performed using IBM® SPSS® Statistics (Version 29). Data were screened, and distribution normality was confirmed by calculating skewness, kurtosis, and Shapiro-Wilk tests. For variables that were non-normally distributed, a Wilcoxon signed-rank test or a Mann-Whitney U test was used for within and between group comparisons, respectively. For normally distributed variables, a paired sample or independent t-test was used for within and between group comparisons, respectively. Univariate correlation (Spearman's rank-order) analyses were utilized to determine the levels of association between follicular and ovulation phase estradiol levels and GNRB® outputs related to translation and compliance. Statistical significance was set at $p < 0.05$.

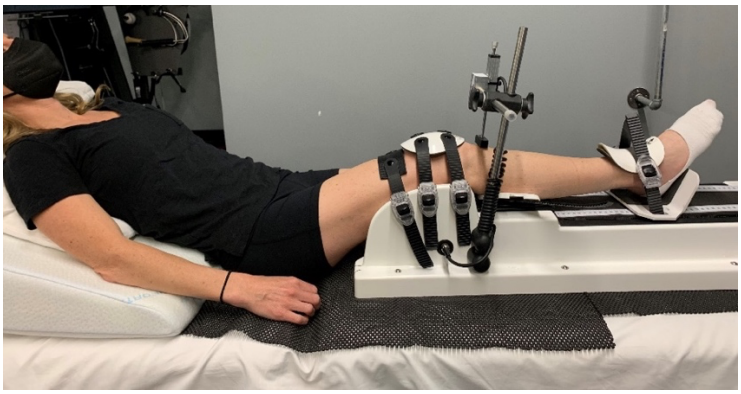

Figure 1. Patient set-up in GNR®B device

RESULTS

Fifty-two participants (age 24 – 30 years; height 57 – 70 inches; weight 91.4 -195.4 pounds) completed this study. No statistical differences were found between groups for activity level IPAQ score of MET-min/wk ($p = 0.992$; mean 3320 ± 2152; range 396-10638), height inches ($p = 0.83$; mean 64.24 ± 2.3 and 64.07± 3.15 for NOC and OC respectively), or BMI ($p = 0.37$; mean 24.4 ± 4.17 and 5.3 ± 3.0 for NOC and OC Groups respectively).

The results of the saliva analysis demonstrated mean estradiol levels were significantly higher during ovulation ($p = 0.01$) across all study participants compared to follicular estradiol levels (Table 2). When analyzing estradiol levels within groups (OC and NOC) both demonstrated increases in estradiol levels in the ovulation phase when compared to the follicular phase. The OC group estradiol levels increased 0.20 pg/ml (Table 3) while the NOC group increased 0.29 pg/ml (Table 4), however, the increases in estradiol levels were not significantly different ($p = 0.22$ and $p = 0.06$ for OC and NOC respectively; Tables 3 and 4). Estradiol levels between OC and NOC groups did not significantly differ during follicular ($p = 0.97$) or ovulation ($p = 0.32$) phases (Table 5).

Mean anterior tibial translatory laxity at 134N and 200 N did not significantly differ between phases or groups (Tables 2-5). Anterior tibial translatory compliance (slope) did not significantly differ between phases or groups (Tables 2-5).

There were weak non-significant correlations between measured salivary estradiol levels and GNRB® measures of anterior tibial translatory laxity at 134N and 200N or with compliance (slope). These non-significant relationships were found across participants whether they were in OC or NOC groups (Table 6).

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Table 2. Combined groups (OC and NOC) descriptives and between phase comparisons

	Mean ± SD	*Range*	*p-value*
Estradiol (pg/mL)			
F	1.61 ± 0.68	0.37-3.83	0.01*
O	1.86 ± 0.82	0.39-4.97	
Laxity 134N			
F	3.48 ± 0.89	1.83-6.37	0.89
O	3.47 ± 0.86	2.00-7.27	
Laxity 200N			
F	5.29 ± 1.07	2.77-8.77	0.73
O	5.26 ± 1.04	3.33-9.53	
Slope (µm/N)			
F	27.32 ± 3.40	21.67-37.73	0.88
O	27.31 ± 3.38	19.77-35.70	

*OC = oral contraceptive; NOC = no oral contraceptive; SD = standard deviation; pg/mL = picograms per milliliter; N= newtons; µm/N = microns per newton; F = follicular phase; O = ovulation phase; * = significance difference between ovulation and follicular phases, significance p< 0.05.*

Table 3. Within group subjects on birth control (OC group) descriptives and between phase comparisons

	Mean ± SD	*Range*	*p-value*
Estradiol (pg/mL)			
F	1.61 ± 0.79	0.37-3.83	0.22
O	1.81 ± 0.96	0.39-4.97	
Laxity 134N			
F	3.36 ± 0.95	1.83-6.37	0.72
O	3.39 ± 1.02	2.00-7.47	
Laxity 200N			
F	5.13 ± 1.17	2.77-8.77	0.74
O	5.16 ± 1.20	3.33-9.53	
Slope (µm/N)			
F	27.00 ± 3.57	21.67-37.73	0.55
O	27.32 ± 3.29	19.77-35.70	

OC = oral contraceptive; SD = standard deviation; pg/mL = picograms per milliliter; N= newtons; µm/N = microns per newton; F = follicular phase; O = ovulation phase

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Table 4. Within group subjects not on birth control (NOC group) descriptives and between phase comparisons

	Mean ± SD	*Range*	*p-value*
Estradiol (pg/mL)			
F	1.62 ± 0.55	0.98 - 3.02	0.06
O	1.91 ± 0.64	0.92 - 3.36	
Laxity 134N			
F	3.62 ± 0.83	2.10 - 5.93	0.76
O	3.56 ± 0.69	2.63 - 5.67	
Laxity 200N			
F	5.49 ± 0.94	3.80 - 8.10	0.64
O	5.36 ± 0.89	4.03 - 7.87	
Slope (µm/N)			
F	27.68 ± 3.37	22.63 -33.83	0.56
O	27.16 ± 3.67	20.97- 34.53	

NOC = no oral contraceptive; SD = standard deviation; pg/mL = picograms per milliliter; N= newtons; µm/N = microns per newton; F = follicular phase; O = ovulation phase

Table 5. Between group comparison (OC vs NOC)

	p-value
Estradiol (pg/mL)	
F	0.97
O	0.32
Laxity 134N	
F	0.15
O	0.08
Laxity 200N	
F	0.17
O	0.14
Slope (µm/N)	
F	0.44
O	0.94

NOC = no oral contraceptive; SD = standard deviation; pg/mL = picograms per milliliter; N= newtons; µm/N = microns per newton; F = follicular phase; O = ovulation phase

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Table 6. Spearman rank-order correlations

	Coefficient	*p-value*
F Laxity 134N vs Estradiol (pg/mL)		
All	0.14	0.33
OC	-0.01	0.97
NOC	0.27	0.18
O Laxity 134N vs Estradiol (pg/mL)		
All	0.07	0.65
OC	-0.04	0.86
NOC	0.12	0.58
F Laxity 200N vs Estradiol (pg/mL)		
All	0.16	0.28
OC	0.05	0.84
NOC	0.26	0.19
O Laxity 200N vs Estradiol (pg/mL)		
All	0.08	0.59
OC	0.09	0.68
NOC	-0.01	0.98
F Slope vs Estradiol (pg/mL)		
All	0.05	0.75
OC	0.03	0.91
NOC	0.02	0.94
O Slope vs Estradiol (pg/mL)		
All	-0.01	0.97
OC	0.15	0.49
NOC	-0.12	0.57

OC = oral contraceptive; NOC = no oral Contraceptive; pg/mL = picograms per milliliter; N= newtons; F = follicular phase; O = ovulation phase.

DISCUSSION

Despite the large number of studies dedicated to understanding ACL injury, it remains unclear as to which mechanical factors, menstrual hormonal influences, types of exercise or training programs directly influence the functional behavior of the ACL in terms of laxity and compliance.[12,18] Contrary to the original hypothesis, this study did not find a significant or meaningful change in ACL stiffness or laxity between the follicular and ovulation phases of the participant's menstrual cycle. While estradiol levels increased during the ovulation phase in both the OC and NOC groups, the lack of significant changes in GNRB® measures suggests the level of estradiol did not influence the tissue laxity or compliance (slope) values as measured.

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Saliva samples are commonly used to assess hormone levels because they are noninvasive, easy to collect, and have been shown to be an accurate source to determine estradiol levels.[29,30] However, hormone levels in saliva are influenced by factors such as momentary emotional states and food intake, and some vary greatly across women's ovulatory cycle.[36] Although attempts were made to standardize and optimize the saliva samples in this study with best practice instructions (Table 1), large standard deviations were observed, which is similar to other studies and likely reflects the inherent large variation in hormone levels among females.[37,38] Normal ranges of estradiol levels in an age-matched female population vary greatly and have been shown to fluctuate up to 17 times throughout the menstrual cycle, challenging the ability to detect consistent hormone levels, and therefore identify and compare significant group estradiol changes.[39] The variation in lab testing procedures also challenges the ability to make comparisons across studies investigating hormone levels. This study found estradiol level fluctuations from 0.37pg/mL – 4.97 pg/mL which is consistent with other studies using the same Salimetrics saliva laboratory, but lower than normal ranges in studies utilizing different laboratory testing facilities; the variety of testing procedures is inconsistent, making it difficult to compare values across studies.[39,40]

The results of this study using the GNRB® instrument align with previous studies which also failed to find a significant correlation between estrogen levels and anterior tibial translatory laxity.[12,15,41] Eiling et al. investigated the effects of menstrual-cycle hormone fluctuations on musculotendinous stiffness (MTS) and knee joint laxity and, like this study, found estrogen fluctuations had no significant effect on anterior knee laxity, however, they did find a significant effect on MTS.[41] It is possible reductions in MTS could result in greater reliance on reflexive response from contractile components of the muscle due to a decreased passive elastic structure, which could also increase electromechanical delay. When high velocity loads are applied to a knee joint, any delay in response within the contractile components of muscles supporting the knee may lead to excessive force transmission through its joint restraining and supporting structures.[41] Furthermore, Behrens et al. found that a delayed reflex response was associated with increased tibial translation, suggesting a sex-specific influence of fatigue on reflex activity and mechanical stability of the knee.[42]

The previous works of Bercovy and Weber and Nouveau et al. suggest a role for monitoring ACL tissue compliance during rehabilitation programs post-ACLR and using thresholds of changes in the compliance to adjust rehab protocols towards optimizing outcomes.[16,19] This study aimed to further investigate ACL stiffness/compliance by investigating the influence of changing estradiol levels on tissue compliance (slope). It is possible this study did not find a significant differences of laxity or compliance (slope) between menstrual phases across the two groups (OC and NOC) due to additional hormonal influences that were not investigated in this study. In addition to estrogen fluctuations, female hormones such as relaxin and progesterone levels have been associated with changes in ligamentous laxity.[15] Although not investigated in this study, the timing of onset of menarche when these hormones are changing drastically in females has been shown to influence ACL laxity.[43] Before puberty, the incidence of female ACL injury is low and equal to males, however, after puberty, the incidence rate in female athletes is estimated to be four and a half times greater than males.[5] It has been suggested that males undergo a "neuromuscular spurt" at puberty that females do not undergo, resulting in female quadriceps and ligament dominance (lower extremity asymmetry) and poor landing mechanics that are both associated risk factors identified with higher ACL injury rates.[9] Neuromuscular control was not specifically investigated in this study but has been postulated to be associated with the hormonal fluctuations that occur during the menstrual cycle.[9] Changes in ligamentous laxity and stiffness influenced by hormonal fluctuations may affect neuromuscular control and account for the variation of research outcomes depending on the combined effect on knee stability on an individual basis. Additional studies looking at the relationship of ACL laxity, stiffness/compliance, and hormone changes throughout the menstrual cycle on neuromuscular control are warranted to better understand this relationship.

Although this study aimed to investigate estradiol influence and the use of oral contraceptives on additional ACL risk factors such as laxity and tissue compliance/stiffness, no significant differences were observed between groups (OC versus NOC) or across menstrual cycle phases (follicular and ovulation). These outcomes

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suggest that additional research is needed to better understand the influence of hormone fluctuations on quantitative measurements of ATT laxity and stiffness, neuromuscular control, and other potential contributors to high female ACL injury rates.

Limitations

The participants were all healthy young (24-30 years) females with similar activity level and no history of knee injury or pain, limiting the ability to generalize findings to other populations. The highest incidence of female ACL injury rate peaks at 16-17 years of age, and this study's age lower limit was 24 years, which does not capture this population.[43] Participants were not screened for systemic laxity, which has been shown to be a possible risk factor for ACL injury, however, post-hoc analysis found no significant difference within or between groups for ATT, lessening the likelihood that systemic laxity, if present, was a factor in the outcomes of this study.[44,45] It is also plausible that differences in participant activity level may influence knee stability measures. In this study, the mean IPAQ exercise scores were similar between groups (p = 0.99), lessening the chance that this affected the outcomes. However, the IPAQ is a self-reported measure that is inherently subjective and, therefore, may not accurately reflect the participant's true activity level. Additional studies with larger, more diverse populations are indicated

Estradiol was the only hormone investigated in this study and the variety of OC was not specified as the participants were grouped only on whether they were using OC. It is possible the oral contraceptives used in the OC group included a combination of estrogen or progesterone, leading to similar levels of estrogen between groups; no significant difference between groups was found on estrogen levels (see Tables 3-6 for specific outcome values). It was initially hypothesized that the changes in estrogen levels between these groups would be significantly different, allowing for a comparison of estrogen levels and the influence of estrogen levels on ACL laxity and compliance (opposite of stiffness) between groups. However, the similar levels of estrogen observed across both OC and NOC groups may have limited the expected outcomes of finding a significant change in ATT displacement and compliance between groups. The addition of testing saliva estradiol levels did help to highlight the OC and NOC estrogen similarities.

CONCLUSION

Estradiol level changes in the follicular and ovulation phases and oral contraceptive use did not significantly influence ACL laxity (anterior tibial translation) or compliance as measured by the GNRB® device. Additional studies on hormonal fluctuations, tissue compliance, and influences on neuromuscular control, kinetics, and kinematics as potential risk factors for higher female ACL injury are needed.

Conflict of Interest Statement

The authors declare the following conflicts of interest: all authors received a grant for study supplies and equipment purchase (knee arthrometer) and use of campus facilities for data collection from University of Saint Augustine for Health Sciences (Internal University Grant).

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