

## Changes in serum collagen markers, IGF-I, and Knee joint laxity across the menstrual cycle

By: [Sandra J. Shultz](#), [Laurie Wideman](#), Melissa M. Montgomery, Kathleen N. Beasley and Bradley C. Nindl

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### **Abstract:**

Variations in serum markers of collagen production (CICP) and degradation (ICTP), insulin-like growth factor I (IGF-I) and anterior knee laxity (AKL) were measured in 20 women [10 with spontaneous cycles (eumenorrheic), 10 using oral contraceptives] over 5 consecutive days at menses (M1–M5, 1st pill week), the initial estrogen rise near ovulation (O1–O5, 2nd pill week), the initial progesterone rise of the early luteal phase (EL1–EL5, 3rd pill week) and post-progesterone peak of the late luteal phase (LL1–LL5, 4th pill week). ICTP was higher in oral contraceptive women ( $5.3 \pm 1.7$  vs.  $3.7 \pm 1.3$   $\mu\text{g/L}$ ;  $p = 0.030$ ), primarily during days near ovulation and the early luteal phase when concentrations decreased in eumenorrheic women ( $p = 0.04$ ). IGF-I concentrations increased during menses then decreased and remained lower during the early and late luteal phase in oral contraceptive women, resulting in lower concentrations compared to eumenorrheic women at EL2 and LL1 ( $p = 0.03$ ). CICP decreased in early and late luteal days ( $p < 0.01$ ), and there was a trend toward lower concentrations in eumenorrheic versus oral contraceptive women ( $85.7 \pm 35.7$  ng/ml vs.  $123.2 \pm 49.8$  ng/ml;  $p = 0.07$ ). Lower CICP and greater IGF-I concentrations predicted greater AKL across the 20 cycle days in both groups ( $R^2 = 0.310$  and  $0.400$ ). Sex hormone concentration changes across the menstrual cycle are of sufficient magnitude to influence collagen metabolism, and may indirectly influence knee structure and function.

**Keywords:** collagen markers | growth factors | menstrual cycle | sex hormones | knee laxity | ACL

### **Article:**

Cyclic increases in anterior knee laxity (AKL) observed across the menstrual cycle<sup>1-5</sup> are of sufficient magnitude to alter knee joint biomechanics.<sup>37</sup> While cyclic changes in sex hormone concentrations appear to partially mediate these effects<sup>5,6</sup> the underlying mechanisms remain unclear. This information is important as ACL injury risk may vary disproportionately across the menstrual cycle,<sup>16</sup> with a higher than expected proportion of ACL injuries observed in the follicular phase around the time of menses<sup>7,8</sup> and ovulation<sup>9</sup> (time points in the cycle where sex hormones are rapidly decreasing and increasing, respectively) compared to the luteal phase.

AKL represents the combined resistance of the ligament, muscle, and capsule to a displacing load. While the ACL provides approximately 85% of the static restraint to anterior tibial translation,<sup>10</sup> it is difficult to elucidate whether cyclic increases in AKL are specifically due to changes in the local metabolism and structure of the ligament, or due to other local factors. With that in mind, sex hormone receptors have been identified on the human ACL, and the effect of sex hormones (primarily estrogen) on collagen metabolism has been examined in both animal<sup>11,12</sup> and human<sup>13,14</sup> ACLs, yielding conflicting results. In studies of human ACL tissue in cell culture,<sup>13,14</sup> progressive decreases in fibroblast proliferation and type 1 procollagen synthesis were observed as estradiol concentrations progressively increased. This decrease in collagen synthesis occurred regardless of progesterone concentration; however, the decrease was somewhat attenuated at higher progesterone concentrations.<sup>14</sup> Further, these hormone effects were most pronounced immediately after hormone exposure (days 1 and 3), and attenuated with sustained exposure (7 days).<sup>14</sup> While this led to the suggestion that acute fluctuations in hormone concentrations across a woman's menstrual cycle (specifically higher estradiol levels) have the potential to inhibit collagen synthesis and reduce ligament strength,<sup>14</sup> they are largely limited to cell culture models that include both physiologic and supraphysiologic hormone concentrations. Research examining associations between sex hormones, collagen metabolism and ligament structure/behavior in physically active women who experience normal physiological fluctuations in sex hormone concentrations across the menstrual cycle are largely lacking.<sup>15</sup>

Also unknown is whether oral contraceptives, commonly used by physically active women, minimize or exacerbate these potential associations. Depending on the type of oral contraceptive administered, the levels of exogenous estradiol and progesterone can be 3–5 times and 1–2 times higher, respectively, than normal endogenous levels.<sup>16</sup> Women on oral contraceptives have been shown to have lower concentrations of serum and local markers of collagen production and degradation, and smaller collagen fibril diameter in tendons, together suggesting an inferior collagen structure.<sup>17-19</sup> However, comparisons are limited to a single time point (mid luteal phase), which may not reflect concentrations at other times in the cycle.<sup>20</sup> Comparisons are also limited to women using specific monophasic brands, and the influence of oral contraceptives may depend on the type administered (monophasic or triphasic), the concentration of ethinyl estradiol delivered, as well as the type, potency and androgenicity of the progestin compound delivered, which ultimately determines its ability to counteract the estrogenic effects.<sup>16</sup> These differences among oral contraceptives may in part explain why studies comparing AKL in eumenorrheic women (normal menstruating women who are not using oral contraceptives) versus women using oral contraceptives are inconclusive.<sup>21,22</sup> These later comparisons were also based on a single measurement of AKL that was collected at a random time of the cycle (i.e., the hormone milieu was not controlled for).

To begin to address these unknowns, our primary purpose was to initially determine whether normal physiological variations in hormone concentrations across the menstrual cycle are of sufficient magnitude to stimulate changes in collagen metabolism as measured by serum collagen markers and mediators. Specifically, we compared eumenorrheic women to women using oral contraceptives on daily changes in their insulin-like growth factor I (IGF-I; a mediator of collagen production<sup>23</sup>), C-terminal propeptide of collagen type-I (CICP; a direct marker of collagen production), and carboxyterminal telopeptide of type I collagen (ICTP; a direct marker of collagen degradation) concentrations over the course of one menstrual cycle. A secondary purpose was to examine whether cyclic changes in serum CICP, ICTP, and IGF-I were associated with cyclic changes in AKL within each group. While acknowledging that (1) the underlying physiological mechanisms for these associations are likely quite complex and involve more than type I collagen, and (2) that serum concentrations reflect changes in type I collagen from a variety of tissues (e.g., bone, ligament, etc.) and may or may not adequately represent the local environment of the ligament,<sup>17, 18</sup> we felt this was a reasonable first step before moving on to more invasive sampling of collagen markers within the knee joint given the daily sampling required to achieve our primary purpose. Based on findings of prior research,<sup>14, 17-19</sup> we hypothesized that concentrations of CICP, ICTP, and IGF-I would generally be lower in both groups during days of the cycle when estradiol levels were elevated, and that greater cyclic variations in CICP, ICTP, IGF-I, and AKL would be observed in eumenorrheic women versus those using oral contraceptives during and immediately following the peri-ovulatory days of the cycle (when estradiol is rising unopposed in eumenorrheic women). We further expected that individuals who experienced greater decreases in concentrations of CICP, ICTP, and IGF-I (suggesting reduced collagen synthesis and inferior collagen structure<sup>14, 18, 19</sup>) would experience greater increases in AKL.

## **METHODS**

Blood samples and AKL data were accessed from ten healthy, eumenorrheic women ( $23.5 \pm 3.9$  years;  $63.0 \pm 9.7$  kg;  $163.0 \pm 5.6$  cm) measured daily across one complete menstrual cycle in a previous study.<sup>5, 6, 24</sup> All had normal spontaneous menstrual cycles lasting 26–32 days that varied no more than  $\pm 1$  day between months, and did not use oral contraceptives. They were compared to 10 women using oral contraceptives for at least 3 months ( $20.7 \pm 2.2$  years;  $67.3 \pm 8.5$  kg;  $164.6 \pm 5.4$  cm) and underwent daily measurements identical to eumenorrheic women (see Table 1 for details of oral contraceptives used). All subjects had healthy knees, were recreationally active 2½–10 h/week for the past 3 months, were non-smokers, had a body mass index ( $\text{BMI} = \text{weight}/\text{height}^2$ )  $< 30$ , and were otherwise free of known medical conditions or connective tissue disorders. All subjects signed an informed consent approved by the University's institutional review board.

**Table 1.** Description of Oral Contraceptives Used by Oral Contraceptive Women

N	Brand	Type	Ethinyl Estradiol Dose (mg)	Progesterin/Dosage (mg)	Potency <sup>a</sup>	Androgenicity <sup>a</sup>	
3	Ortho Tri-Cyclen	Tri	0.035/0.035/0.035	Norgestimate	0.180/0.215/0.250	0.33/0.33/0.33	0.48/0.48/0.48
2	Nortrel	Tri	0.035/0.035/0.035	Norethidrone	0.500/0.750/1.000	0.50/0.75/1.00	0.50/0.75/1.00
1	Trivora	Tri	0.030/0.040/0.040	Levonorgestrel	0.050/0.075/0.125	0.27/0.40/0.66	0.42/0.62/1.04
1	Ortho Tri-Cyclen Lo	Tri	0.025/0.025/0.025	Norgestimate	0.180/0.215/0.250	0.33/0.33/0.33	0.48/0.48/0.48
1	Mercilon	Mono	0.02	Desogestrel	0.150	0.80	1.25
1	Femiane	Mono	0.02	Gestodene	0.075	0.68	0.26
1	Aviane	Mono	0.02	Levonorgestrel	0.100	0.53	0.83

<sup>a</sup>From Ref. 16

Blood samples (10 cc) were collected from the antecubital vein between 7:00 a.m. and 9:00 a.m. each morning to assay estradiol (DSL-4400, Diagnostic Systems Laboratories, Webster TX), progesterone (Coat-A-count, Diagnostic Products Corporation, Los Angeles, CA), testosterone (Coat-A-count; Diagnostic Products Corporation), IGF-I (IDS, Fountain Hills, AZ), CIGP (Quidel, San Diego, CA), and ICTP (IDS). Assay sensitivity (unit value), and mean intra- and inter-assay percent coefficient of variations (%), respectively were 1.5 pg/ml, 3.9–14.1% and 2.8–16.3% for estradiol; 0.1 ng/ml, 3.4–10.0% and 3.8–12.0% for progesterone; 10 ng/dl, 4.5–11.3% and 5.2–13.8% for testosterone; 1.9 ng/ml, 3.0% and 7% for IGF-I; 0.2 ng/ml, 6.5% and 6.1% for CIGP; and 0.3 µg/L, 9.8% and 7.6% for ICTP.

AKL was measured with the KT-2000 Knee Arthrometer (MedMetric Corp, San Diego, CA) as the anterior displacement of the tibia relative to the femur (mm) under an applied anterior directed load of 133 N. Three measures were recorded and averaged for each day. One tester completed all measures for a single subject. Because of the time lapse between studies, different testers measured AKL for eumenorrhic (T1) and oral contraceptive (T2) cohorts. Both were trained in an identical fashion, and established strong intra-tester reliability [T1 ICC<sub>2,3</sub>(SEM) = 0.97 (0.38 mm); T2 ICC<sub>2,3</sub> (SEM) = 0.96 (0.30 mm)] as well as strong inter-tester reliability with others trained in our laboratory [T1 ICC<sub>2,3</sub> (SEM) = 0.92 (0.50); T2 ICC<sub>2,3</sub> (SEM) = 0.96 (0.5 mm)].<sup>4, 5</sup>

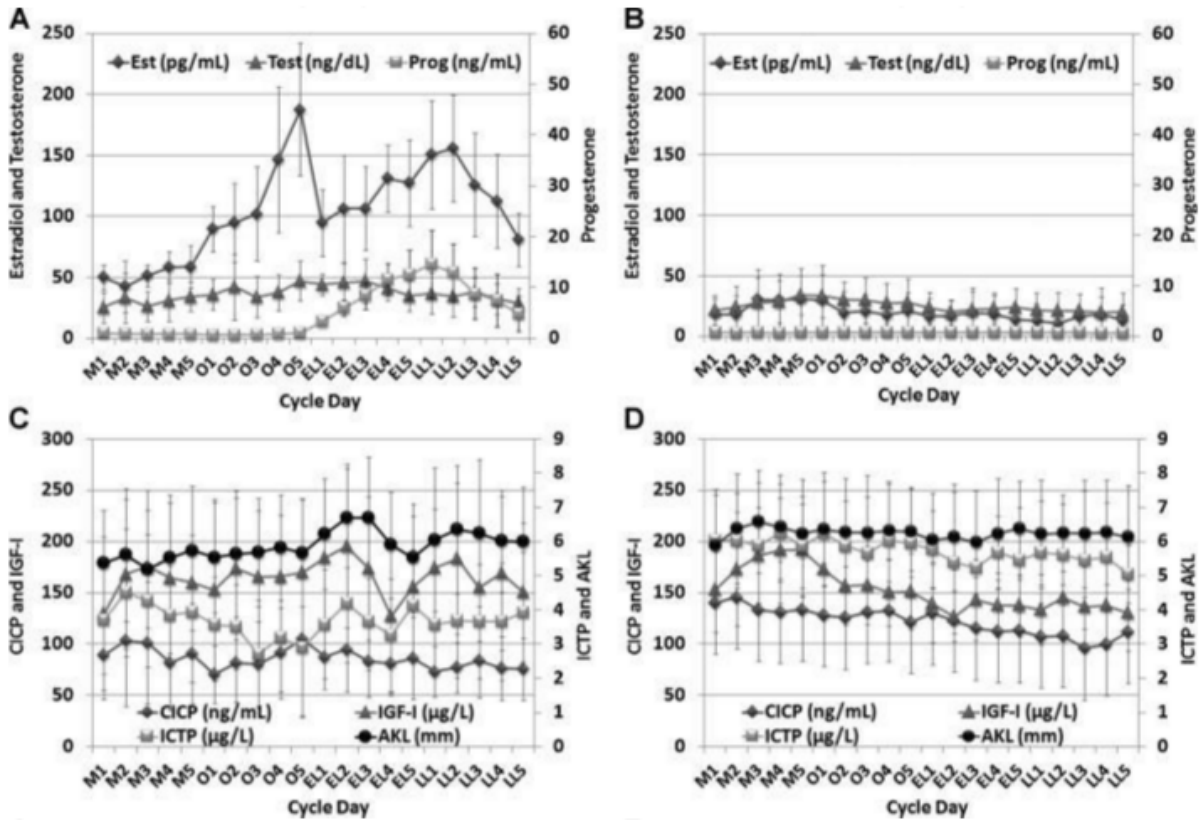
To test the first hypothesis, daily CIGP, ICTP, IGF-I, and AKL values were first aligned for eumenorrhic women based on their sex hormone concentrations for the first 5 days of menses (M1–M5) based on self report; the first 5 days of the initial estradiol rise near ovulation (O1–O5) based on a 2 SD increase above mean menses concentrations; the first 5 days of the early luteal phase (EL1–EL5) based on the initial rise in progesterone (>2 ng/ml); and the first 5 days of the late luteal phase (LL1–LL5) starting with the day of peak progesterone. Because women vary from one another in the timing and phasing of their hormone concentration changes<sup>5</sup>, aligning data in this manner ensured that the variables of interest were compared across women on days representative of a similar hormone milieu. For women using oral contraceptives, the CIGP, ICTP, IGF-I, and AKL values obtained on the first 5 days of each pill week were used to represent their M1–M5, O1–O5, EL1–EL5, and LL1–LL5 test measures. The two groups were then compared across these 20 cycle days on their CIGP, ICTP, IGF-I, and AKL values using a multivariate 2 (group) by 20 (cycle days) repeated measures ANOVA to control for type I error. Post hoc comparisons consisted of simple main effects testing.

To test the second hypothesis, separate multiple, stepwise removal linear regression analyses determined the extent to which the daily change in the group mean values for CICIP, ICTP, and IGF-I predicted daily change in AKL. CICIP, ICTP, and IGF-I were first entered simultaneously, then variables were removed in a stepwise fashion (tolerance = 0.20) if they did not contribute significantly to the model.<sup>25</sup> Assuming that the patterns of change in variables would be different for eumenorrhic women versus those using oral contraceptives, separate analyses were run within each group. Alpha level was set at  $p < 0.05$ .

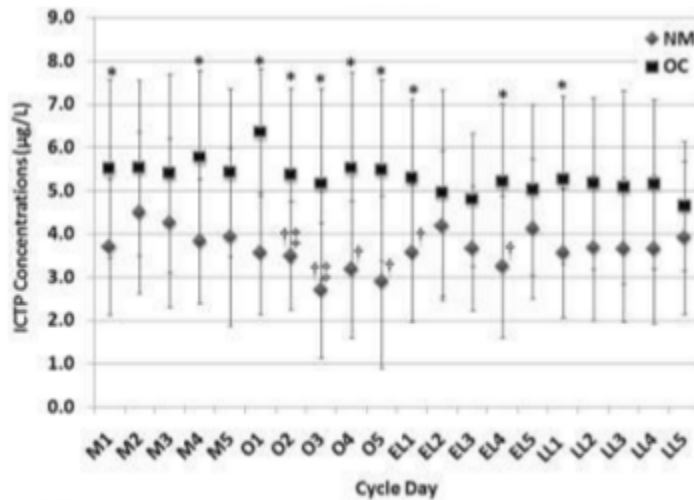
## RESULTS

Mean endogenous hormones and collagen marker/mediator concentrations and anterior knee laxity are graphically displayed in Figure 1, stratified by group [note: current assay techniques do not allow for quantification of exogenous (synthetic) hormone concentrations delivered to oral contraceptive women, which are also biologically active]. The multivariate results were significant for cycle day and group by cycle day effects ( $p < 0.001$ ). Follow-up univariate results revealed significant differences between eumenorrhic and oral contraceptive women in their daily changes in ICTP, IGF-I, and AKL (all  $p < 0.044$ ), but not in their CICIP ( $p = 0.266$ ) (Figures 2–5). ICTP concentrations were stable in oral contraceptive women across cycle days ( $p = 0.174$ ), but decreased in eumenorrhic women during days O2–EL1 and EL4 ( $p = 0.022$ ). While ICTP concentrations were generally higher in oral contraceptive women ( $5.3 \pm 1.7 \mu\text{g/L}$  vs.  $3.7 \pm 1.3 \mu\text{g/L}$ ;  $p = 0.030$ ), this difference was most pronounced during days of O and EL ( $p < 0.05$ ). IGF-I concentrations remained stable across days for eumenorrhic women ( $p = 0.558$ ), but decreased in oral contraceptive women during days of EL and LL ( $p < 0.001$ ; Figure 3). This resulted in oral contraceptive users having lower IGF-I values than eumenorrhic women at EL2 and LL1 ( $p < 0.05$ ). CICIP concentrations generally decreased in days of EL and LL compared to days of M and O ( $p < 0.001$ ; Figure 4). While this change was similar across days for both groups, eumenorrhic women tended to have lower overall CICIP concentrations than oral contraceptive women ( $85.7 \pm 35.7 \text{ ng/ml}$  vs.  $123.2 \pm 49.8 \text{ ng/ml}$ ;  $p = 0.07$ ; Cohen's  $d = 1.1$ ). Lastly, AKL was stable across days in oral contraceptive women ( $p = 0.429$ ), but increased in eumenorrhic women during the first 3 days of EL, and days 2, 4, and 5 of LL ( $p = 0.029$ ).

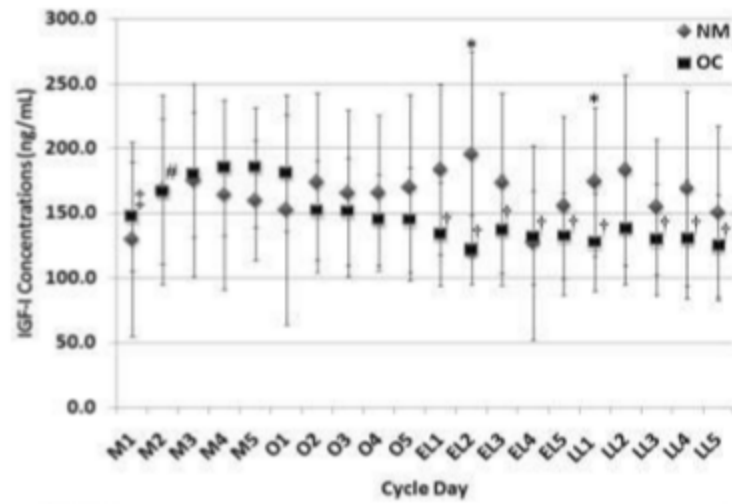
When comparing patterns of variability between cycle days for CICIP, ICTP, and IGF-I with AKL, bivariate correlations are listed in Table 2. For both groups, decreases in CICIP concentrations and increases in IGF-I concentrations predicted increases in AKL across the 20 days of the menstrual cycle, explaining 31% ( $p = 0.043$ ) and 40% ( $p = 0.014$ ) of the variance in eumenorrhic and oral contraceptive women, respectively (Figure 6). The final regression models were  $\text{AKL} = 4.95 - 0.014 (\text{CICIP}) + 0.013 (\text{IGF-I})$  for eumenorrhic women and  $\text{AKL} = 5.9 - 0.005 (\text{CICIP}) + 0.006 (\text{IGF-I})$  for oral contraceptive women. Change in ICTP concentrations was not a significant predictor in either model (part correlation 0.025 and 0.184, respectively, for eumenorrhic and oral contraceptive women;  $p > 0.465$ ), and was therefore excluded from the final model.



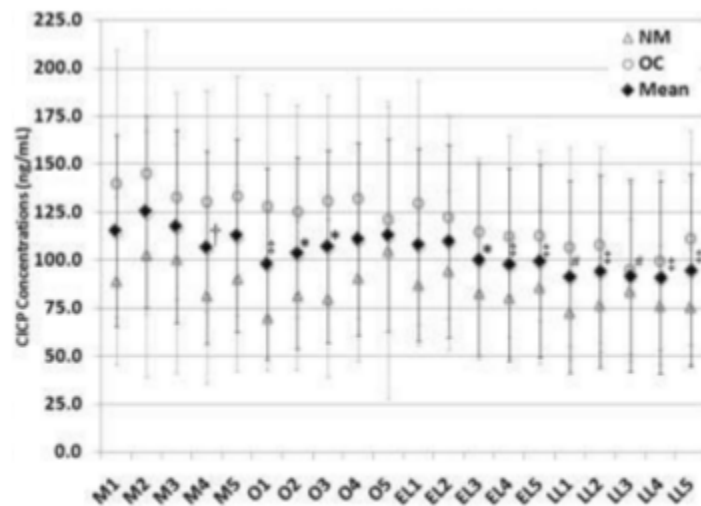
**Figure 1.** Mean endogenous sex hormone concentrations for (a) eumenorrheic (NM) and (b) oral contraceptive (OC) women and mean collagen markers and mediators and anterior knee laxity for (c) eumenorrheic (NM) and (d) oral contraceptive (OC) women during the 5 days for each of the four defined phases (M1–M5, O1–O5, EL1–EL5, LL1–LL5).



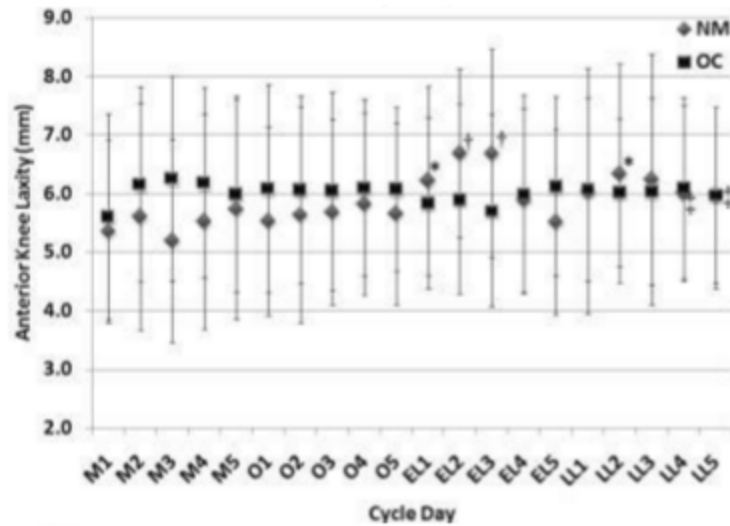
**Figure 2.** Comparisons of ICTP concentrations by group and day. Symbols indicate \*eumenorrheic (NM) < oral contraceptive (OC) women, †value < 1 or more days of M, ‡value < EL2 and EL5.



**Figure 3.** Comparisons of IGF-I concentrations by group and day. Symbols indicate \*eumenorrheic (NM) < oral contraceptive (OC) women, †value < M1–O5, ‡value < M1–M5, and #value < M4, M5.



**Figure 4.** CICP concentrations by day (cycle day main effect). Symbols indicate \*value < M2, †value < M2 and M3, ‡value < M1, M3, and M5, and #value < M1–M5. Data stratified for oral contraceptive (OC) and eumenorrheic (NM) women are also provided for descriptive purposes.

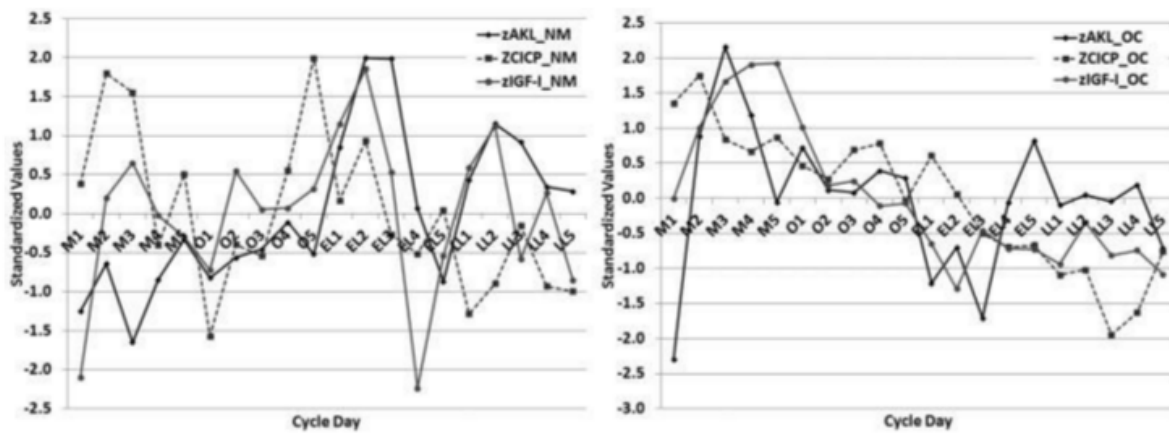


**Figure 5.** Comparisons of AKL by group and day. Symbols indicate \*value > M1, M3, O1, and EL5, †value > M1–O5, and ‡value > M3 for eumenorrheic (NM) women.

**Table 2.** Bivariate Correlations Comparing the Patterns of Variability between CICP, ICTP, CICP:ICTP and IGF-I with AKL within Eumenorrheic (*Upper Grid*) and Oral Contraceptive (*Lower Grid*) Women

	AKL	CICP	ICTP	IGF-I
AKL	1.000	-0.226	-0.017	<b>0.452</b>
CICP	0.087	1.000	0.246	0.203
ICTP	0.389	<b>0.648</b>	1.000	0.162
IGF-I	<b>0.527</b>	<b>0.655</b>	<b>0.713</b>	1.000

Bolded values  $p < 0.05$ .



**Figure 6.** Patterns of change in CICP, IGF-I, and AKL across the menstrual cycle in (a) eumenorrheic (NM;  $R^2 = 0.309$ ) and (b) oral contraceptive women (OC;  $R^2 = 0.395$ ).



## DISCUSSION

Eumenorrheic versus oral contraceptive women differed substantially in their daily changes in AKL, serum IGF-I (a mediator of collagen production) and ICTP (marker of type I collagen degradation) concentrations across the menstrual cycle. Despite these group differences, daily changes in serum C1CP and IGF-I were strong predictors of daily changes in AKL for both eumenorrheic and oral contraceptive women.

Our hypotheses that C1CP, ICTP, and IGF-I concentrations would be lower in both groups during days when estradiol levels were elevated, and that greater cyclic variations in C1CP, ICTP, IGF-I, and AKL would be observed in eumenorrheic women during and immediately following the peri-ovulatory days of the cycle (when estradiol is rising unopposed) were only partially supported. Specifically, C1CP and ICTP concentrations tended to be higher during the days of menses in both groups (Figure 1c and d), with eumenorrheic women decreasing both serum C1CP and ICTP concentrations (but not IGF-I values) during the peri-ovulatory and luteal days compared to menses, while oral contraceptive women decreased both C1CP and IGF-I concentrations (but not ICTP) later in the cycle. However, consistent with our hypothesis, we did observe more acute variability across time in C1CP, ICTP, IGF-I, and AKL in eumenorrheic women (Figure 1c vs. Figure 1d), particularly around the peri-ovulatory days, although this did not reach a level of significance for IGF-I.

The findings in eumenorrheic women are consistent with a prior investigation of serum carboxy-terminal propeptide of type I procollagen (C1CP) and ICTP concentration changes when sampled on multiple days of the cycle.<sup>20</sup> We further observed that decreases in ICTP were sustained for a longer period of time (into days of the early luteal phase) and to a somewhat greater magnitude than C1CP (40% vs. 32% reduction, respectively). As can be seen in Figure 1c, these decreases in C1CP (O1–O3, then again on EL3–LL5) and ICTP (O2–EL1, and again on EL4) occurred just prior to days of the cycle when AKL increased (EL1–EL3, and again on LL2, LL4, and LL5). This is further reinforced by our secondary findings of lower C1CP levels (but not ICTP levels) predicting greater AKL increases. As greater ligament laxity and reduced stiffness have been associated with decreases in ultimate tensile forces,<sup>26, 27</sup> these findings would appear consistent with the suggestion posed by Yu et al.<sup>14</sup> that higher estradiol levels have the potential to inhibit collagen synthesis and ligament strength. However, the current study only measured knee laxity (where other capsular tissues may also contribute to the resistance to joint motion), and ligament properties were not directly measured.

IGF-I did not change appreciably across the menstrual cycle in eumenorrheic women, although their values tended to vary more than oral contraceptive women (Figure 3). This was contrary to our hypothesis and may in part be due to the interplay between sex steroids and IGF-I which is poorly understood and further complicated by the normal feedback regulation observed in the GH–IGF-I axis. Research has shown that estradiol, while increasing GH release, inhibits the hepatic action of GH<sup>28</sup> and that elevated estradiol reduces IGF-I responsiveness, such that only modest elevations in IGF-I occur from midcycle onward despite increased endogenous estrogen levels,<sup>29</sup> exactly as observed in our eumenorrheic women. In addition, although both testosterone and estradiol have been shown to increase GH release, only testosterone consistently elevates

IGF-I.<sup>28</sup> In the current study, increases in testosterone and IGF-I followed a similar pattern in eumenorrheic women.

While oral contraceptive women experienced a similar pattern of decrease in CIGP across the 20 days, they had no change in ICTP or AKL, but experienced progressive consistent depressions in testosterone and IGF-I across the latter half of the cycle. The use of exogenous estradiol in a variety of protocols has resulted in significant reductions in IGF-I across the latter part of the cycle.<sup>17–19, 29</sup> The progressive decreases and subsequent recovery of CIGP concentrations at the onset of menses in women using oral contraceptives is consistent with our hypotheses, and is also consistent with the suppression of collagen synthesis in response to the supraphysiological dosing of estradiol delivered in the 2nd–4th pill weeks as suggested by the findings of Yu et al.<sup>14</sup> However, contrary to our hypotheses (and our findings in eumenorrheic women), ICTP levels remained unchanged while IGF-I levels increased. While the maintenance of relatively higher ICTP concentrations in oral contraceptive women is contrary to previous reports in young women,<sup>18, 19</sup> the relatively higher ratio of ICTP to CIGP concentrations in oral contraceptive women as compared to eumenorrheic women may explain the greater suppression in IGF-I in oral contraceptive women.<sup>30</sup>

IGF-I was a strong positive predictor of cyclic AKL changes (and as can be seen from Figure 1c, track closely together). IGF-I is known to play an important role in the regulation of collagen protein synthesis.<sup>31</sup> Specifically, IGF-I and IGF binding proteins (IGFBPs) have been shown to regulate collagen synthesis and degradation in vitro and IGF-I administration in adults has been shown to increase collagen synthesis and increase type I collagen mRNA in cultured cells.<sup>32</sup> IGF-I is also considered essential for wound healing as decreased IGF-I concentrations profoundly inhibited tissue repair.<sup>33</sup> Additionally, IGF-I increased both PINP and ICTP concentrations and an inverse correlation was observed between the ICTP:PINP ratio and IGF-I.<sup>30</sup> Together, these findings suggest that while IGF-I favors collagen synthesis/deposition—or at the very least, protects against excessive breakdown of collagen—it clearly plays a role in collagen turnover, altering both synthesis and degradation. When considering this literature, the positive associations we observed between IGF-I and AKL may represent a compensatory increase in IGF-I following suppression of both ICTP and CIGP in order to stimulate collagen synthesis and prevent further tissue breakdown, or it may reflect the fact that increased IGF-I results in greater turnover of collagen. Further, it is well accepted that type I collagen turnover originates from several tissues, with ligament being just one, relatively small, contributor. Thus, it is possible that the association we are observing simply represents a data “artifact” due to IGF-I being a major controller for collagen turnover in general (bone, as well as ligament), and that both IGF-I and AKL are not directly related, but rather are responding to a similar, unidentified underlying process. Further work is needed to understand the complex role of IGF-I (i.e., local vs. systemic) in collagen homeostasis and ligament integrity local to the knee joint.

Comparisons of our findings in oral contraceptive women to prior research is difficult given the different types of oral contraceptives the women under study were using (e.g., monophasic vs. triphasic; the varying potency and androgenicity of progestogen compounds in different preparations; and the varying dosages of estradiol),<sup>16</sup> the limited number of time points compared, and difficulty in quantifying serum concentrations of the exogenous hormones delivered. For example, Hansen et al.<sup>18</sup> compared days 18–21 in oral contraceptive women to

early follicular days in eumenorrheic women. They reported lower concentrations of a urine marker of type I collagen synthesis and similar concentrations of a urine marker of type I collagen degradation in oral contraceptive versus eumenorrheic women. As noted in Figures 4 and 5, the lower type I collagen synthesis reported in oral contraceptive women may have simply resulted from the different test days examined, as CICIP values were substantially lower at the end of the cycle compared to menses in both groups. In other work, a clear decrease in markers of collagen synthesis and degradation were observed in women on days 21–24 of their cycle from before to after 3 months of oral contraceptive administration.<sup>19</sup> In this study, all women used the same monophasic oral contraceptive (desogestrel 150 mg and ethinyloestradiol 30 mg), where the progestogen has relatively high potency and androgenicity that can produce more potent masculine effects and counteract estrogenic effects to a larger extent.<sup>16, 34</sup>

In the current study, women used a variety of oral contraceptives that included progestogens with lower potency and androgenicity. To examine whether these different progestogens may in part explain the higher overall serum collagen marker concentrations we observed in oral contraceptive versus eumenorrheic women, we ran secondary bivariate correlations between the mean potency and androgenicity of the oral contraceptive used by each woman and her mean CICIP, ICTP, and IGF-I concentrations. While there was little to no relationship between the potency of the progestogen and these serum concentrations ( $r$  range =  $-0.092$  to  $-0.247$ ,  $p > 0.492$ ), progestogens with a greater androgenicity were associated with lower mean ICTP ( $-0.751$ ,  $p = 0.012$ ) and IGF-I ( $-0.605$ ,  $p = 0.064$ ) concentrations, but not CICIP concentrations ( $r = 0.183$ ,  $p = 0.462$ ). Hence, the magnitude of suppression in collagen production and degradation mediated by oral contraceptives may depend on the progestogen compound used, and should be appropriately accounted for in future research.

Despite these differences, the suppressed IGF-I concentrations we observed in our oral contraceptive women have been associated with lower collagen synthesis and a smaller collagen fibril diameter in tendons in other oral contraceptive women, which is thought to be suggestive of a more inferior collagen structure.<sup>17, 18</sup> Thus, it is somewhat surprising that we did not observe cyclic changes in AKL in our oral contraceptive women, despite the fact that CICIP concentrations decreased in both groups, and lower CICIP concentrations and higher IGF-I concentrations were strongly associated with AKL within both groups. This would seem to suggest that mechanisms involving the inter-play between serum CICIP and IGF-I concentrations may be important. This is based on observations that increases in AKL were primarily observed in eumenorrheic women following the peri-ovulatory days of the cycle when CICIP (as well as ICTP) concentrations were suppressed, which were followed by an observable (but statistically insignificant) increase in both AKL and IGF-I (Figure 1c). Further, the suppression in CICIP and ICTP were of greater magnitude and variability than what was observed in oral contraceptive women (see Figure 6). Conversely, both CICIP and IGF-I concentrations gradually decreased in oral contraceptive women, with no corresponding change in AKL. These differential findings were initiated in the peri-ovulatory days when only eumenorrheic women experienced unopposed increases in estradiol, while oral contraceptive women were exposed to elevated levels of both estradiol and progesterone that remained relatively consistent across the pill days as compared to menses. Thus, the inhibitory effect of estradiol on collagen synthesis (and other important mechanistic processes not studied) may have been attenuated in oral contraceptive women given their concurrent rises in progesterone and their relative consistency in

concentration levels across multiple days.<sup>14</sup> However, because the pharmacokinetics and biologic effects of the various oral contraceptive preparations are not well known,<sup>16</sup> and our assay techniques did not allow us to account for both endogenous and exogenous hormone concentrations, these interpretations remain largely theoretical. Further research should confirm the direct and indirect mechanisms that may underlie these associations, as well as the extent to which the type of exogenous progestin delivered in the oral contraceptive influences the attenuation of estrogenic effects<sup>16</sup> on collagen metabolism.

In summary, although this study represents a fairly rudimentary first step in attempting to understand the mechanisms by which hormone concentration changes lead to changes in knee laxity, our findings tend to support (more than refute) the theories emanating from *in vitro* models that increasing estradiol concentrations have the potential to influence collagen synthesis and ligament integrity in a negative manner. However, much remains unknown about these underlying mechanistic processes, which limits clear interpretation of these findings. First, the associations observed in this study are limited to serum (systemic) biomarkers, which may or may not represent the local environment of the ligament, as changes in type I collagen from other tissues (e.g., bone) largely contribute to circulating serum concentrations.<sup>17, 18</sup> This study is also limited to the assessment of biomarkers for type I synthesis and degradation, and biomarkers for other collagen types relevant to ligament structure and function (e.g., type III, V, XII) should also be examined in future studies. The temporal sequencing of changes in sex hormones, changes in serum collagen markers, and changes in knee laxity also cannot be adequately determined from the current study (this would likely require continuous collection over three consecutive cycles), which limits the interpretation of our findings, particularly for the regression analyses. It is appreciated that complex intermediate processes may drive these associations, and that there could reasonably be time lags between respective changes in sex hormones, collagen metabolism and soft tissue behavior. While a 3- to 5-day time lag was observed between sex hormone changes and knee laxity changes in prior work,<sup>5</sup> this was quite variable among women and was limited to time lags within a single cycle. Further, we were unable to locate literature that has examined the temporal changes between serum sex hormone concentration changes and serum collagen marker changes, *in vivo*. Because of these limitations, it is difficult to fully rectify the sequencing of these events to the times in the cycle when ACL injury risk appears to be elevated.<sup>16</sup> Despite these limitations and the many unknowns that remain, our initial findings suggest that further research examining these mechanisms is warranted, as cyclic increases in knee laxity (and decreases in stiffness) across the menstrual cycle may be characteristic of a weaker ligament<sup>26, 27, 35</sup> and lead to higher risk knee joint biomechanics,<sup>36</sup> which together may explain the disproportional rates of ACL injury across the menstrual cycle.<sup>16</sup>

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