

TRANSLATIONAL PHYSIOLOGY |

Effect of testosterone on the female anterior cruciate ligament

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Submitted 8 December 2004; accepted in final form 15 March 2005

Lovering, Richard M., and William A. Romani. Effect of testosterone on the female anterior cruciate ligament. *Am J Physiol Regul Integr Comp Physiol* 289: R15–R22, 2005. First published March 24, 2005; doi:10.1152/ajpregu.00829.2004.—Injuries to the anterior cruciate ligament (ACL) result in immediate and long-term morbidity and expense. Young women are more likely to sustain ACL injuries than men who participate in similar athletic and military activities. Although significant attention has focused on the role that female sex hormones may play in this disparity, it is still unclear whether the female ACL also responds to androgens. The purpose of this study was to determine whether the female ACL was an androgen-responsive tissue. To identify and localize androgen receptors in the female ACL, we used Western blotting and immunofluorescent labeling, respectively, of ACL tissue harvested during surgery from young women ($n = 3$). We then measured ACL stiffness and assessed total testosterone (T) and free [free androgen index (FAI)] testosterone concentrations, as well as relative estradiol to testosterone ratios (E_2/T and E_2/FAI) at three consecutive menstrual stages ($n = 20$). There were significant rank-order correlations between T ($0.48, P = 0.031$), FAI ($0.44, P = 0.053$), E_2/T ($-0.71, P < 0.001$), E_2/FAI ($-0.63, P = 0.003$), and ACL stiffness near ovulation. With the influences of the other variables controlled, there were significant negative partial rank-order correlations between ACL stiffness and E_2/T ($-0.72, P < 0.001$) and E_2/FAI ($-0.59, P = 0.012$). The partial order residuals for T and FAI were not significant. These findings suggest that the female ACL is an androgen-responsive tissue but that T and FAI are not independent predictors of ACL stiffness near ovulation. Instead, the relationship between T, FAI, and ACL stiffness was likely influenced by another hormone or sex hormone binding globulin.

androgen receptor; gender differences; immunofluorescence; free androgen index

THE ANTERIOR CRUCIATE LIGAMENT (ACL) is the primary restraint of anterior translation of the tibia on the femur (7). Consequently, injuries to the ACL are both debilitating and costly. The annual cost for surgical repair and rehabilitation of ACL injuries has been estimated at over \$850 million (13). These expenses do not include the long-term costs related to any immediate or long-term degeneration of the knee or subsequent development of osteoarthritis (5, 23, 24, 44, 47).

Women are 2 to 10 times more likely to injure the ACL than men who participate in similar military and athletic activities (2, 20). Although gender-specific differences in anatomy, neuromuscular control, and the hormonal milieu have all been suggested as possible causes for the disparity in the ACL injury rate (16, 22), little is known about how sex hormone-mediated

mechanisms influence the physical properties of the ACL or injury risk. Previous in vitro studies have shown estradiol (E_2) to decrease the type I collagen formation that provides a ligament's tensile strength (8, 28, 65, 66). More recent in vivo studies that focused on the relationship among sex hormones, menstrual cycle stage, injury, and the strength of the ACL have been inconclusive (10, 38, 46, 55, 56, 59, 61, 62).

Androgens produced by the female adrenal glands bind directly to receptors on androgen-receptive tissues or serve as substrates for estrogen metabolism (12, 19, 33, 67). Testosterone (T) is the most abundant androgen and has been related to increases in collagen content in prostate, mammary, and capsular tissue (3, 58, 68) and increased knee ligament repair strength (60). Androgen receptors (ARs) have been identified in a variety of female tissues (14, 21, 41, 49), but not yet in the female ACL (21), leaving no definitive evidence that the female ACL is responsive to circulating androgens.

The purpose of this study was to determine whether the female ACL was an androgen-responsive tissue. To do this, we identified ARs in ACLs of young women and conducted an analysis to determine the correlation between T, the free androgen index (FAI), and ACL stiffness at three stages of the menstrual cycle in healthy active women. We hypothesized that higher concentrations of testosterone would be significantly correlated with higher ACL stiffness. Because such a relationship may suggest an antagonistic relationship between testosterone and E_2 , we also examined the relative relationship between the E_2 -to-T ratio and E_2 -to-FAI ratio (E_2/T and E_2/FAI) to determine whether testosterone was an independent predictor of ACL stiffness.

MATERIALS AND METHODS

Androgen Receptor Expression

Western blot analysis. All subjects in this study read and signed a consent form; the consent form and procedures were approved by the university's Institutional Review Board. ACLs were harvested from three female subjects (ages 19, 24, and 32 yr) and one male subject (25 yr old, used as a positive control) at the time of ACL reconstructive surgery. Harvested ligaments were immediately snap-frozen in pentane-cooled liquid nitrogen, cut in half, and stored at -80°C . Western blot analysis was used to determine whether ARs are expressed in ACLs of women.

Samples allocated for Western blotting were homogenized in a solution containing 10 mM sodium phosphate (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2

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mM EDTA, 1 mM *p*-aminoethylbenzenesulfonyl fluoride, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin. Protein concentration was assayed, and 60 μ g of protein homogenate were incubated (5 min, 95°C) with an equal volume of sample buffer (NuPage sample buffer, Invitrogen, Carlsbad, CA) for each sample. Prostate-derived LNCaP cells were used as an additional positive control. LNCaP cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, until 80% confluent. The medium was aspirated, and 1 ml of ice-cold Dulbecco's PBS was added. Cells were scraped into Eppendorf tubes and centrifuged at 3,000 *g* for 5 min, and the supernatant was removed. Lysis buffer (80 μ l) was added to the tubes, and lysates were sonicated and placed on ice for 30 min, after which they were centrifuged at 17,000 *g* for 30 min and supernatant was collected. Samples were subjected to SDS-PAGE (NuPage System, Invitrogen) and transferred to nitrocellulose electrophoretically in blotting buffer at 100 V (constant voltage) for 90 min. The nitrocellulose was stained with Ponceau S (0.1% in 5% acetic acid) for 5 min to verify transfer and to check for equal loading of lanes, blocked for 2 h in 3% milk-0.1% Tween/PBS, 10 mM sodium azide, and then washed and incubated overnight with polyclonal antibodies against AR (N-20, Santa Cruz Biotechnology, Santa Cruz, CA; dilution of 1:200) or normal rabbit IgG serum (negative control). The blots were washed and incubated with donkey anti-rabbit antibodies conjugated to alkaline phosphatase (Jackson Laboratories, Westgrove, PA), and the bands were visualized by chemiluminescence (Tropix, Bedford, MA).

Immunolabeling of sections. For immunofluorescent experiments, frozen tissue was cryosectioned (10 μ m) and collected onto slides. Sections were incubated with 3% BSA/PBS for 1 h, followed by labeling with the primary antibodies for 1 h. Primary antibodies used were a polyclonal (rabbit) antibody against AR (N-20, Santa Cruz Biotechnology; dilution of 1:20) and a monoclonal antibody to type I collagen (Sigma, St Louis, MO). On some tissue sections, normal rabbit IgG serum was substituted for the anti-AR antibodies as a negative control. After the sections were washed, secondary antibodies (Cy5 donkey anti-mouse and fluorescein donkey anti-rabbit; Jackson Immunoresearch Laboratories), mixed with propidium iodide (50 μ g/ml) to label nuclei, were applied for 1 h. Tissue sections were washed and mounted in Vectashield medium (Vector Laboratories, Burlingame, CA). We obtained digital images using a Zeiss 410 confocal laser-scanning microscope and linked software.

Hormone Concentration and ACL Stiffness

Subjects. The 20 subjects used to determine hormone concentrations and ACL stiffness were the same subjects described in a separate project that investigated the influence of four female sex hormones and sex hormone binding globulin (SHBG) on ACL stiffness (46). Before participation in the study, all subjects completed a health history questionnaire, were familiarized with the KT-2000, and were briefly interviewed and examined by a physician to determine whether each met inclusion criteria for the study as reported previously (46).

Experimental procedures. Subjects were randomly designated to begin data collection at the onset of menses, near ovulation, or the luteal phase by drawing numbers. Subjects used the OvuQuick One-Step ovulation predictor (Quidel, San Diego, CA) to identify ovulation according to the manufacturer's instructions. Onset of menses was defined as that point where a subject required feminine protection. Because subjects were likely to be tested with the knee arthrometer and have their blood drawn as many as 24–36 h after the spike in E_2 concentration, the middle stage of the menstrual cycle was called "near ovulation." The "luteal phase" was defined as between days 22 and 24 of the menstrual cycle (46).

Blood and stiffness data were collected as previously reported (46). To test stiffness, the KT-2000 was fastened to the subject's tibia with a plate over the patella to restrict femoral translation. As the examiner applied force through the handle, the tibia was anteriorly translated

relative to the femur. A force-displacement curve based on the force applied through the handle and the anterior translation of the tibia was illustrated by an *x-y* plotter. Three force-displacement curves were generated during each testing session according to the manufacturer's instructions as described previously (46). These data were used to determine the stiffness of the right ACL. Stiffness was defined as the change in force (45 N) between 89 and 134 N divided by the displacement (mm) between 89 and 134 N. We used the stiffness calculation that had the largest displacement of the three curves generated at each menstrual stage in our statistical analysis. The two researchers collecting data with the KT-2000 established their intra-tester reliability above the 0.92 and 0.96 [intraclass correlation coefficient (3,1)] level before data collection was started.

Blood was analyzed via enzyme-linked immunoassay for total testosterone concentration (T; Diagnostic Systems Laboratory, Webster, TX) at each of the three menstrual stages as reported previously (46). Samples were run in duplicate; the minimum detection limits were 0.04 ng/ml, and the intra- and interassay coefficients of variation were 5.3 and 4.8%, respectively. The FAI was calculated to estimate the serum free testosterone in our subjects (36).

Statistical Methods

Because sex hormones seldom act in isolation, we combined the new androgen values into a statistical model that included E_2 , estriol, estrone, progesterone, and SHBG (46) to better represent the normal hormonal milieu and help clarify the interactions between female and male sex hormones. In particular, we were interested in the interaction between relative concentrations of testosterone and E_2 . As a result, we compared E_2/T and E_2/FAI to ACL stiffness. This paper will only report the new androgen and ratio data described above.

ANOVA. All data were transformed to the natural logarithmic scale for the statistical analysis prior to the repeated-measures ANOVA, as was done previously (46). Inter-menstrual stage pair-wise comparisons between T, FAI, E_2/T , and E_2/FAI at the onset of menses, near ovulation, and during the luteal phase were conducted via mixed-effect ANOVA (11) and presented as the ratio of the geometric means. The ratio of geometric mean is often interpreted as the change (fold) in the geometric mean. Under the null hypothesis, we assumed the geometric mean of the distribution was equal at each of the three menstrual stages or equivalently that the ratio of geometric means was equal to one. All of our hypotheses were formulated a priori, and we used a comparison-wise significance level of $P \leq 0.05$ as the criterion for rejecting the null hypothesis.

Spearman correlations. Because of the inherent between-subject variability in hormone concentrations (27), we chose to examine the relationship between T, FAI, E_2/T , E_2/FAI , and ACL stiffness with nonparametric Spearman's rank-order correlation coefficients (r_s) (48) and Spearman's partial rank-order correlation coefficients (r_{sp}) (52) as described previously (46). Because of the small sample size, percentile confidence intervals (CI) for r_s and r_{sp} were estimated by the nonparametric bootstrap resampling method (9), which was based on 1,000 bootstrap random samples from the original sample of data. All of our hypotheses of associations were again formulated a priori, and a univariate significance level of $P \leq 0.05$ was utilized as the criterion for rejecting the null hypothesis of no association. All ANOVA calculations were carried out with SAS version 8.2 (SAS Institute, Cary, NC) with the PROC MIXED procedure, whereas the Spearman correlation analyses were carried out in Splus version 2000 (Insightful, Seattle, WA).

RESULTS

AR Expression

To examine AR protein expression specifically in ACLs of young women, we used Western blots and immunofluorescent labeling of tissue sections (Fig. 1). Western blot analysis of the

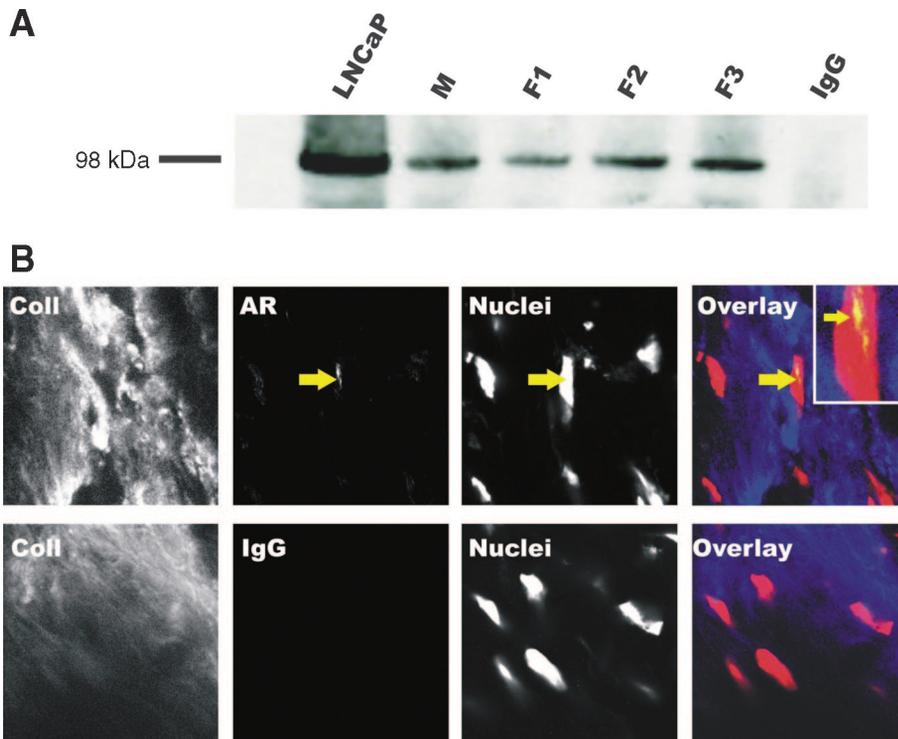


Fig. 1. Localization of the androgen receptor (AR). A: Western blot analysis of AR in human anterior cruciate ligaments (ACLs). Cultured LNCaP cells (prostatic cells that have ARs), as well as male ACL, were used as positive controls. AR was present in both male (M) and female (F1–F3) samples at the predicted weight of 98 kDa (60 μ g of homogenate loaded/lane). The last lane of the nitrocellulose (IgG) was cut off and incubated with normal rabbit IgG serum (negative control) in lieu of the primary antibodies against ARs. B: fluorescent confocal images of tissue sections from a female ACL. Sections were labeled with monoclonal antibodies against collagen (coll) and polyclonal antibodies against ARs or rabbit IgG (negative control), and propidium iodide was used to label nuclei. In the overlay panels, collagen is blue and nuclei are red. Localization of ARs in nuclei appears yellow (*inset*).

AR in male and female ACLs resulted in a band at the predicted molecular mass of 98 kDa (Fig. 1A). Prostate-derived LNCaP cells (see MATERIALS AND METHODS) served as the positive control, and rabbit IgG served as the negative control. Cryosections from ACLs of these same subjects were labeled with antibodies against AR and showed positive nuclear labeling in all subjects (Fig. 1B, yellow in overlay panels and *inset*), which did not occur in negative controls. These data show that ARs are present in the female ACL samples that we evaluated.

Hormone Concentration and ACL Stiffness

The arithmetic means, geometric means, interquartile ranges, and minimum and the maximum values of the distributions of T and FAI at the onset of menses, near ovulation, and during the luteal menstrual phase are presented as distribution summary measures in Table 1. T and FAI values were highest near ovulation (0.76 and 9.23 ng/ml, respectively),

somewhat lower during the luteal phase (0.69 and 5.86 ng/ml), and lowest at the onset of menses (0.59 and 5.92 ng/ml).

There were significant increases in the geometric mean of T, FAI, E_2/T , and E_2/FAI from the onset of menses to near ovulation (change as follows: T = 1.25-fold, $P = 0.001$; FAI = 1.35-fold, $P = 0.002$; $E_2/T = 1.96$ -fold, $P < 0.001$; and $E_2/FAI = 1.81$ -fold, $P = 0.005$) and from the onset of menses to the luteal phase (change as follows: T = 1.17-fold, $P < 0.001$; FAI = 1.14-fold, $P = 0.026$; $E_2/T = 1.67$ -fold, $P = 0.017$; $E_2/FAI = 1.71$ -fold, $P = 0.012$). There was a significant decrease in FAI from near ovulation to the luteal phase (0.86-fold change, $P = 0.004$) but no significant change in the other parameters.

There was a significant relationship in r_s between T and ACL stiffness [$r_s = 0.48$, 95% CI(0.10,0.75), $P = 0.031$] (Fig. 2) and a positive relationship between FAI and ACL stiffness near ovulation [$r_s = 0.44$, 95% CI(-0.05,0.80), $P = 0.053$]. As T and FAI increased, ACL stiffness also increased. Conversely, E_2/T (Fig. 2) and E_2/FAI were negatively correlated with ACL stiffness near ovulation [E_2/T : $r_s = -0.71$, 95% CI(-0.91,-0.33), $P = 0.021$; E_2/FAI : $r_s = -0.63$, 95% CI(-0.89,-0.21), $P = 0.003$]. In these two relationships, subjects with higher concentrations of E_2 relative to T (Fig. 2) or FAI also had lower ACL stiffness. A similar relationship existed between E_2/FAI and ACL stiffness during the luteal phase [E_2/FAI : $r_s = -0.45$, 95% CI(-0.82,0.10), $P = 0.045$], but there was no statistically significant correlation between any of the other hormones or hormone ratios and ACL stiffness at the onset of menses or in the luteal phase of the menstrual cycle. As a follow-up to our initial analyses, there were significant inverse relationships between T and E_2 [$r_s = -0.50$, 95% CI(-0.80,-0.07), $P = 0.025$] and FAI and E_2 [$r_s = -0.44$, 95% CI(-0.87,0.09), $P = 0.050$] and a near signifi-

Table 1. Distribution of testosterone and FAI

	Menstrual Stage		
	Onset of menses <i>n</i> = 20	Near ovulation <i>n</i> = 20	Luteal phase <i>n</i> = 20
<i>Testosterone, ng/ml</i>			
Geometric mean	0.59 \pm 0.04	0.76 \pm 0.07	0.69 \pm 0.04
Interquartile range	0.47, 0.70	0.50, 0.90	0.50, 0.82
Minimum and maximum	0.40, 1.10	0.40, 1.60	0.40, 1.00
<i>FAI, ng/ml</i>			
Geometric mean	5.92 \pm 0.75	9.23 \pm 1.70	7.17 \pm 1.09
Interquartile range	4.23, 7.03	4.14, 11.20	4.01, 7.99
Minimum and maximum	1.74, 14.99	2.33, 25.86	2.26, 18.86

Values are means \pm SE; *n* = no. of subjects. FAI, free androgen index.

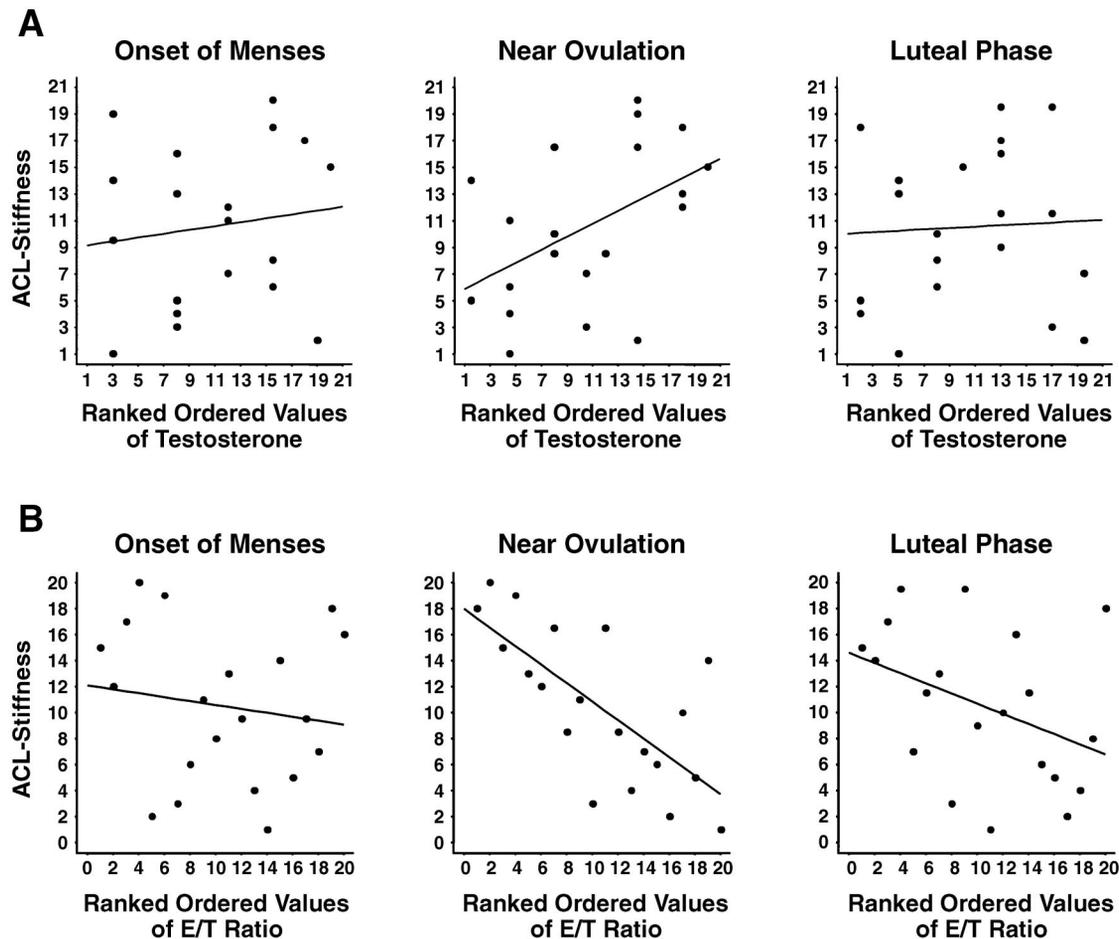


Fig. 2. Scatter plot diagrams showing the linear relationships between Spearman's ranked-order values of testosterone concentration (A; ng/ml) and estradiol-to-testosterone concentration ratio (E/T) and the ranked-order value of ACL stiffness (B; N/mm) at the onset of menses, near ovulation, and at the luteal phase of the menstrual cycle.

cant correlation between T and SHBG [$r_s = -0.44$, 95% CI(-0.79,0.06), $P = 0.051$] near ovulation.

After controlling for the influence of the other hormones and SHBG, we found a significant inverse partial correlation between E_2/T and ACL stiffness near ovulation [$r_{sp} = -0.72$, 95% CI(-0.93,-0.19), $P < 0.001$] (Table 2). Similarly, there was a significant inverse partial correlation between E_2/FAI and ACL stiffness near ovulation [$r_{sp} = -0.59$, 95% CI(-0.88,-0.14), $P = 0.012$] and in the luteal phase [$r_{sp} = -0.50$, 95% CI(-0.84,-0.04), $P = 0.039$]. The partial correlations between T, FAI, and ACL stiffness were no longer

significant near ovulation. Again, there were no significant correlations between these or the other hormones at the onset of menses or in the luteal phase of the cycle. Together, these data indicate that the relationship between T, FAI, and ACL stiffness is not independent but related to the concentrations of other sex hormones or SHBG in our model.

DISCUSSION

The purpose of this study was to determine whether the female ACL was an androgen-responsive tissue. We identified

Table 2. Spearman's partial rank-order correlation coefficients for the assessment of the relationship between the hormone concentrations and ACL stiffness at 3 stages of the menstrual cycle

Parameter	Menstrual Stage		
	Onset of menses	Near ovulation	Luteal phase
Testosterone	0.15 [-0.56,0.70] (0.593)	0.23 [-0.46,0.83] (0.419)	0.02 [-0.65,0.68] (0.952)
Estradiol/testosterone	-0.14 [-0.62,0.32] (0.614)	-0.72 [-0.93,-0.19] (<0.001)	-0.40 [-0.82,0.21] (0.122)
FAI*	0.42 [-0.01,0.83] (0.100)	0.18 [-0.38,0.67] (0.510)	0.32 [-0.30,0.77] (0.224)
Estradiol/FAI†	-0.26 [-0.66,0.14] (0.316)	-0.59 [-0.88,-0.14] (0.012)	-0.50 [-0.84,-0.04] (0.039)

Brackets contain the 95% confidence interval for Spearman's partial rank-order correlation coefficient (r_{sp}); parentheses contain the test probability for the hypothesis that $r_{sp} = 0$. *Statistical model adjusted for estradiol, estriol, estrone, and progesterone. †Statistical model adjusted for estriol, estrone, and progesterone.

the ARs on the ACLs of three young women and found that T and FAI were correlated with ACL stiffness near ovulation and that E_2/T and E_2/FAI were negatively correlated near ovulation. Subjects with higher concentrations of FAI or T near ovulation had higher ACL stiffness. Conversely, subjects with higher E_2/T or E_2/FAI ratios had lower ACL stiffness near ovulation and during the luteal phase. This antagonistic relationship between the androgens and E_2 was consistent with our hypothesis. After controlling for the influence of the other hormones and binding proteins in our model, we found that E_2/T and E_2/FAI maintained a significant negative partial correlation with ACL stiffness near ovulation and the luteal phase; however, the correlations for T and FAI were no longer significant.

The ACL is considered an estrogen- and progesterone-responsive tissue because the receptors for these hormones have been localized on ACL tissue (28, 50) and because in vitro these hormones have influenced proliferation of fibroblasts and synthesis of collagen (28, 65, 66). This hormone-induced reduction of collagen presumably decreases the in vivo ability of ligament tissue to resist tensile loads (45, 55). Because the concentrations of E_2 and progesterone are much higher in women (19), the influence of these hormones on the ACL has been proposed as one explanation for the gender disparity in injury risk (2, 16, 20). Testosterone concentration is normally much higher in men than in women, but its potential role in any gender-specific differences in ACL strength or injury risk is less understood than that of the female sex hormones. Because none of these previous works has successfully localized ARs or the influence of testosterone on the synthesis of type I collagen, it remained unclear whether the female ACL was an androgen-responsive tissue.

The AR is a member of the nuclear receptor super family, and its gene is located on the X chromosome (34). ARs are expressed in many cell types (25, 41, 49), including the male ACL (21) and in some female connective tissues (14). In the present study, we found that ARs were present in the female ACL, suggesting that circulating androgens such as testosterone may play a role in its normal remodeling and tensile strength. This finding is in contrast to an earlier study (21) that localized ARs in the male but not in the female ACL, using immunolabeling of formalin-fixed, paraffin-embedded tissue. However, ACL tissue from only two female subjects under the age of 25 yr was examined in that study, and the authors did not rule out the possibility that their findings were limited by the sensitivity of their techniques. We modified the fluorescent immunolabeling protocol cited above by using a different antibody and unfixed tissue and confirmed our findings with Western blots.

Testosterone potency is commonly influenced by other circulating hormones and its affinity to binding proteins (6, 32, 51). We added new androgen data to our previously reported statistical model (46) because we felt that an analysis that combined T and FAI with the common estrogens, progesterone, and SHBG would provide a more realistic analysis of the in vivo relationship between testosterone and ACL stiffness than an analysis of the androgens in isolation. Spearman's partial rank-order analysis of the expanded hormonal milieu also allowed us to examine whether testosterone concentration might be an independent predictor of ACL stiffness or dependent on other hormones and binding proteins. Adding T and

FAI to Spearman's rank-order analysis did not significantly alter the correlations between the three estrogens, progesterone, SHBG, and ACL stiffness from those previously reported (46).

T concentrations in our subjects were lowest at the onset of menses and were highest near ovulation. This midcycle peak and the overall hormone concentrations were consistent with studies on similar populations (37, 53, 61). Most circulating testosterone is bound to albumin or SHBG, leaving only 1–2% unbound and biologically active (1, 57). To determine whether this active fraction was correlated with ACL stiffness, we calculated the FAI from assay-derived concentrations of SHBG and T to estimate the value for free testosterone ($100 \times T/SHBG$). The FAI has been a valid indicator of changing testosterone concentrations in several female populations (18, 36, 39) and correlated well ($r = 0.93$) with equilibrium dialysis (36), as well as gel filtration and derived methods (18, 39).

We hypothesized that the relationship between the androgen parameters and ACL stiffness would be antagonistic to the negative correlation previously reported for E_2 (46). In fact, T and FAI were significantly correlated with ACL stiffness near ovulation and post hoc analyses indicated that both T [-0.50 , $P = 0.025$] and FAI [-0.44 , $P = 0.050$] were inversely correlated with E_2 at the same menstrual stage. Despite this apparent antagonism, it was not clear whether T and FAI independently influenced ACL stiffness.

To further examine this question, we calculated Spearman's partial correlations for T and FAI and included E_2/T and E_2/FAI in our analysis. If T or FAI were independent predictors of ACL stiffness, we would have expected the partial correlation values for T, FAI, and E_2/T or E_2/FAI to be similar to the significant rank-ordered values for T or FAI alone. Instead, the partial correlations for T and FAI were not significant near ovulation, and the negative correlations for E_2/T and E_2/FAI were similar to the rank-order values for these ratios and similar to those reported previously for E_2 (46). These findings suggest that the relationship between T, FAI, and ACL stiffness was modulated by another variable in our model and that E_2 appeared to be the only independent predictor of ACL stiffness in our subjects.

It is not unusual for testosterone's influence on connective tissues to be directly or indirectly influenced by other hormones or second messengers. Estradiol has been linked to lower testosterone levels (35, 63) and higher concentrations of SHBG (6, 15, 32). Because testosterone has a higher affinity for SHBG than E_2 (6, 30), circulating E_2 may indirectly reduce free testosterone concentrations by modulating an increase in the circulating levels of SHBG (15, 54). In these previous studies, as SHBG concentrations increased, more testosterone was bound to SHBG, leaving less free testosterone available for aromatization to E_2 (26, 33, 40) or to act on target tissues (4, 15).

In our subjects, there was a negative correlation between T and SHBG near ovulation ($r = -0.44$, $P = 0.05$), suggesting that subjects with higher concentrations of SHBG did, in fact, have lower concentrations of T. This relationship is consistent with reports of the antagonistic effect of SHBG levels on testosterone concentration (1, 6, 43) and supports our theory that the relationship between T and ACL stiffness near ovulation was dependent on another variable in our model.

It is still not clear how long it takes ACL tissue to remodel in response to hormonal changes or how long it takes for remodeling to result in measurable changes in ACL stiffness. Previous studies that have examined the relationship between sex hormones and knee laxity or stiffness throughout the menstrual cycle are inconclusive (10, 61). After adding T and FAI to our statistical model, we found that the only significant relationships between rank-ordered androgen concentrations and ACL stiffness were near ovulation. Because there was individual variability in the relationships between the hormone parameters and ACL stiffness in our subjects, it is possible that midcycle variations in sex hormone concentrations between women with normal, consistent menstrual cycles (27) may establish a balance of remodeling or a "baseline" of tensile strength that is unique for each woman. This individualized baseline may be influenced by the ACL's repeated exposure to absolute concentrations, threshold concentrations, or individualized fluctuations of sex hormone concentrations throughout not just one but several menstrual cycles. A limitation of this study is that we only collected data over three consecutive menstrual stages. Thus we do not know whether we would have found any difference in the relationship between androgen concentration and measurements of ACL tensile strength over a longer period of time.

We and other investigators have used the KT-2000 to determine the viscoelastic properties of the intact ACL (10, 46, 61). Contrary to some investigators, our measure of stiffness indexed not only the amount of anterior tibial displacement (laxity) but also the load applied near the end of the force displacement curve (89–134 N). It is at this linear end region of the force-displacement curve where stiffness has been reported to be highest at rates of application similar to those used clinically (17, 29) and more sensitive to measures of small tensile differences than measures of anterior displacement alone (29). A limitation to our characterization of ACL stiffness is that, even though the ACL has been found to provide up to 86% of the passive restraint to anterior tibial translation on the femur, the collateral ligaments, skin, fascia, and muscles may also limit this movement (7). To account for this possibility, we familiarized our subjects with the KT-2000 during a preparticipation training session and oscillated their calf and tibia to minimize apprehension and enhance muscular relaxation during each trial (64). In addition, we calculated stiffness at each menstrual stage from the force-displacement curve with the most tibial translation, as we felt this reflected the trial where any potential muscular restriction of tibial displacement was the smallest.

We have identified the AR on the ACLs of young women and found significant correlations between T, FAI, E_2/T , E_2/FAI , and ACL stiffness near ovulation and, in the case of E_2/FAI , in the luteal phase of the menstrual cycle. It is important to note that the tissue used to identify the AR was from injured ACLs. It is unclear whether AR expression is influenced by the inflammatory responses and remodeling that follow injury.

After adding T and FAI to our statistical model, we found that E_2 remained the only independent predictor of ACL stiffness. Any influence that T had on ACL stiffness appeared to be mediated through SHBG or E_2 . A further limitation to this study is that we did not attempt to quantify changes in AR expression throughout the menstrual cycle or to examine the

relationship between androgens and ACL stiffness with the risks of sustaining an ACL injury or related joint degeneration. We feel that our findings can only be used to further explain the potential influences that circulating levels of sex hormones may have on ACL stiffness and the ability of that ligament to resist the tensile loads that can lead to injury. Moreover, our subjects were young, recreationally active women with a history of regular menstrual cycles. Because age, weight, and activity level have been shown to affect circulating concentrations of sex hormones, our findings may not be similar across other populations of women. Finally, many women who participate in sports that are at high risk for ACL injury may also be using oral contraceptives, which have been shown to influence the concentrations, bioavailability, and metabolism of sex hormones (31, 42, 54). As a result, the findings in our subjects may not be applicable to women who use oral contraception.

In summary, we identified ARs on the ACL of young female subjects. The presence of the AR combined with the correlation between T, FAI, and ACL stiffness strongly suggests that the ACL is an androgen-responsive tissue.

ACKNOWLEDGMENTS

The authors thank Tadas Sean Vasaitis and the laboratories of Dr. Angela Brodie and Dr. Jodi Flaws for assistance with sample specimens; Dr. Leigh Ann Curl, Dr. Jim Heubert, Dr. Nick Kilmer, and Kevin McLaughlin for help with screening subjects and data collection; and James Patrie for contributions to the statistical analysis.

GRANTS

This study was supported in part by a grant from the National Athletic Trainers' Association Research and Educational Foundation (Project 399-D003), the Pfizer Women's Health Research Group (Project 01-023), National Institute of Child Health and Human Development Grant 5 K12 HD-043489, and the Geriatric Research Education and Clinical Center, Veterans Affairs Hospital, Baltimore, Maryland.

REFERENCES

1. **Anderson D.** Sex-hormone-binding globulin. *Clin Endocrinol (Oxf)* 3: 69–96, 1974.
2. **Arendt E, Agel J, and Dick R.** Anterior cruciate ligament injury patterns among collegiate men and women. *J Athl Train* 34: 86–92, 1999.
3. **Asano K, Maruyama S, Usui T, and Fujimoto N.** Regulation of estrogen receptor alpha and beta expression by testosterone in the rat prostate gland. *Endocr J* 50: 281–287, 2003.
4. **Bruch H, Wolf L, Budde R, Romalo G, and Schweikert H.** Androstenedione metabolism in cultured human osteoblast-like cells. *J Clin Endocrinol Metab* 75: 101–105, 1992.
5. **Buckland-Wright J, Lynch J, and Dave B.** Early radiographic features in patients with anterior cruciate ligament rupture. *Ann Rheum Dis* 59: 641–646, 2000.
6. **Burke C and Anderson D.** Sex-hormone-binding globulin is an oestrogen amplifier. *Nature* 240: 38–40, 1972.
7. **Butler D, Noyes F, and Grood E.** Ligamentous restraints to anterior-posterior drawer in the human knee. *J Bone Joint Surg Am* 62-A: 259–270, 1980.
8. **Culav E and Clark C.** Connective tissues: matrix composition and its relevance to physical therapy. *Phys Ther* 79: 308–319, 1999.
9. **Davidson A and Hinkley D.** *Bootstrap Methods and Their Applications*. Cambridge, UK: Cambridge Press, 1997.
10. **Deie M, Sakamaki Y, Sumen Y, Urabe Y, and Ikuta Y.** Anterior knee laxity in young women varies with their menstrual cycle. *Int Orthop* 26: 154–156, 2002.
11. **Diggle P, Liang KY, and Zeger S.** *Analysis of Longitudinal Data*. Oxford, UK: Clarendon, 1994.
12. **Dimitrakakis C, Zhou J, Wang J, Belanger A, LaBrie F, Cheng C, Pow D, and Bondy C.** A physiologic role for testosterone in limiting estrogenic stimulation of the breast. *Menopause* 10: 292–298, 2003.

13. Frank C and Jackson D. The science of reconstruction of the anterior cruciate ligament. *J Bone Joint Surg Am* 79-A: 1556–1576, 1997.
14. Fujimoto J, Nishigaki M, Hori M, Ichigo S, and Morishita S, and Tamaya T. Effects of estradiol and testosterone on the synthesis, expression and degradation of androgen receptor in human uterine endometrial fibroblasts. *J Biomed Sci* 2: 160–165, 1995.
15. Gower B and Nyman L. Associations among oral estrogen use, free testosterone concentration, and lean body mass among postmenopausal women. *J Clin Endocrinol Metab* 85: 4476–4480, 2000.
16. Griffin L, Agel J, Albohm M, Arendt E, Dick R, Garrett W, Garrick J, Hewett T, Huston L, Ireland M, Johnson R, Kibler W, Lephart S, Lewis J, Lindenfeld T, Mandelbaum B, Marchak P, Teitz C, and Wojtyls E. Noncontact anterior cruciate ligament injuries: risk factors and prevention strategies. *J Am Acad Orthop Surg* 8: 141–150, 2000.
17. Gross S, Carcia C, Ganseder B, and Shultz S. Rate of force application during knee arthrometer testing affects stiffness but not displacement measurements. *J Orthop Sports Phys Ther* 34: 132–139, 2004.
18. Guay A, Munarriz R, Jacobson J, Talakoub L, Traish A, Quirk F, Goldstein I, and Spark R. Serum androgen levels in healthy premenopausal women with and without sexual dysfunction. A. Serum androgen levels in women aged 20–49 years with no complaints of sexual dysfunction. *Int J Impot Res* 16: 112–120, 2004.
19. Guyton A and Hall J. Female physiology before pregnancy; and the female hormones. In: *Textbook of Medical Physiology* (10th ed.). Philadelphia, PA: Saunders, 2001, p. 1017–1032.
20. Gwinn D, Wilckens J, McDevitt E, Ross G, and Tzu-Cheg K. The relative incidence of anterior cruciate ligament injury in men and women at the United States Naval Academy. *Am J Sports Med* 28: 98–102, 2000.
21. Hamlet W, Liu S, Panossian V, and Finerman G. Primary immunolocalization of androgen target cells in the human anterior cruciate ligament. *J Orthop Res* 15: 657–663, 1997.
22. Ireland M. Anterior cruciate ligament injury in female athletes: epidemiology. *J Athl Train* 43: 150–154, 1999.
23. Johnson D, Urban W Jr, Caborn D, Vanarthos W, and Carlson CS. Articular cartilage changes seen with magnetic resonance imaging-detected bone bruises associated with acute anterior cruciate ligament rupture. *Am J Sports Med* 26: 409–414, 1998.
24. Kannus P and Jarvinen M. Posttraumatic anterior cruciate ligament insufficiency as a cause of osteoarthritis in a knee joint. *Clin Rheumatol* 8: 251–260, 1989.
25. Kimura N, Mizokami A, Oonuma T, Sasano H, and Nagura H. Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. *J Histochem Cytochem* 41: 671–678, 1993.
26. Labrie F, Luu-The V, Lin S, Labrie C, Simard J, Breton R, and Belanger A. The key role of 17 β -hydroxysteroid dehydrogenases in sex steroid biology. *Steroids* 62: 148–158, 1997.
27. Lenton E, Lawrence G, Coleman R, and Cooke I. Individual variation in gonadotrophin and steroid concentrations and in the lengths of the follicular and luteal phases in women with regular menstrual cycles. *Clin Reprod Fertil* 2: 143–150, 1983.
28. Liu S, Al-Shaikh R, Panossian V, Finerman GA, and Lane J. Estrogen affects the cellular metabolism of the anterior cruciate ligament. *Am J Sports Med* 25: 704–709, 1997.
29. Liu W, Maitland M, and Bell G. A modeling study of partial ACL injury: simulated kt-2000 arthrometer tests. *J Biomech Eng* 124: 294–301, 2002.
30. Longcope C, Femino A, and Johnston J. Androgen and estrogen dynamics in the female baboon (*Papio anubis*). *J Steroid Biochem* 31: 195–200, 1988.
31. Longcope C, Gorbach S, Goldin B, Woods M, Dwyer J, and Warram J. The metabolism of estradiol; oral compared to intravenous administration. *J Steroid Biochem* 23: 1065–1070, 1985.
32. Longcope C, Hui S, and Johnston J, C. Free estradiol, free testosterone, and sex hormone-binding globulin in perimenopausal women. *J Clin Endocrinol Metab* 64: 513–518, 1987.
33. Longcope C, Kato T, and Horton R. Conversion of blood androgens to estrogens in normal adult men and women. *J Clin Invest* 48: 2191–2201, 1969.
34. Lubahn D, Joseph D, Sullivan P, Willard H, French F, and Wilson E. Cloning of human androgen receptor complementary DNA and localization to the x chromosome. *Science* 240: 327–330, 1988.
35. Mall-Haefeli M, Werner-Zodrow I, Huber P, Darragh A, and Lambe R. Effects of various combined oral contraceptives on sex steroids, gonadotropins and SHBG. *Ir Med J* 76: 269–272, 1983.
36. Miller K, Rosner W, Lee H, Hier J, Sesmilo G, Schoenfeld D, Neubauer G, and Klibanski A. Measurement of free testosterone in normal women and women with androgen deficiency: comparison of methods. *J Clin Endocrinol Metab* 89: 525–533, 2004.
37. Mitteilungen K. Total and free testosterone and total and free 17 β -oestradiol in normally menstruating women. *Endokrinologie* 68: 112–114, 1976.
38. Myklebust G, Maehlum S, Holm I, and Bahr R. A prospective cohort study of anterior cruciate ligament injuries in elite Norwegian team handball. *Scand J Med Sci Sports* 8: 149–153, 1998.
39. Nanjee M and Wheeler M. Plasma free testosterone—is an index sufficient? *Ann Clin Biochem* 22: 387–390, 1985.
40. Nimrod A and Ryan K. Aromatization of androgens by human abdominal and breast fat tissue. *J Clin Endocrinol Metab* 40: 367–372, 1975.
41. Pagnotta A, Specchia N, and Greco F. Androgen receptors in du-puytren's contracture. *J Orthop Res* 20: 163–168, 2002.
42. Palatsi R, Hirvensalo E, Liukko P, Malmiharju T, Mattila L, Riihiluoma P, and Ylostalo P. Serum total and unbound testosterone and sex hormone binding globulin (SHBG) in female acne patients treated with two different oral contraceptives. *Acta Derm Venereol* 64: 517–523, 1984.
43. Plymate S, Moore D, Cheng C, Bardin C, Southworth M, and Levin-ski M. Sex hormone-binding globulin changes during the menstrual cycle. *J Clin Endocrinol Metab* 61: 993–996, 1985.
44. Price J, Till S, Bickerstaff D, Bayliss M, and Hollander A. Degradation of cartilage type II collagen precedes the onset of osteoarthritis following anterior cruciate ligament rupture. *Arthritis Rheum* 42: 2390–2398, 1999.
45. Rasanen T and Messner K. Estrogen-dependent tensile properties of the rabbit knee medial collateral ligament. *Scand J Med Sci Sports* 10: 20–27, 2000.
46. Romani WA, Patrie J, Curl L, and Flaws J. The correlations between estradiol, estrone, estriol, progesterone, and sex hormone binding globulin and anterior cruciate ligament stiffness in healthy, active females. *J Womens Health* 12: 240–245, 2003.
47. Roos H, Ornell M, Gardsell P, Lohmander L, and Lindstrand A. Soccer after anterior cruciate ligament injury—an incompatible combination? *Acta Orthop Scand* 66: 107–112, 1995.
48. Rosner B. *Fundamentals of Biostatistics*. Pacific Grove, CA: Duxbury, 2000.
49. Roy A, Tyagi R, Song C, Lavrovsky Y, Ahn S, Oh T, and Chatterjee B. Androgen receptor: structural domains and functional dynamics after ligand-receptor interaction. *Ann NY Acad Sci*: 44–57, 2001.
50. Sciore P, Frank C, and Hart D. Identification of sex hormone receptors in human and rabbit ligaments of the knee by reverse transcription-polymerase chain reaction: evidence that receptors are present in tissue from both male and female subjects. *J Orthop Res* 16: 604–610, 1998.
51. Selby C. Sex hormone binding globulin: origin, function, and clinical significance. *Ann Clin Biochem* 27: 532–541, 1990.
52. Shirahata S. Tests of partial correlation in a linear model. *Biometrika* 64: 162–164, 1977.
53. Sinha-Hikim I, Arver S, Beall G, Shen R, Guerrero M, Sattler F, Shikuma C, Nelson J, Landgren B, Mazer N, and Bhasin S. The use of a sensitive equilibrium dialysis method for the measurement of free testosterone levels in healthy, cycling women and in human immunodeficiency virus-infected women. *J Clin Endocrinol Metab* 83: 1312–1318, 1998.
54. Slater C, Zhang C, Hodis H, Mack W, Boostanfar R, Shoupe D, Paulson R, and Stanczyk F. Comparison of estrogen and androgen levels after oral estrogen replacement therapy. *J Reprod Med* 46: 1052–1056, 2001.
55. Slauterbeck J, Clevenger C, Lundberg W, and Burchfield D. Estrogen level alters the failure load of the rabbit anterior cruciate ligament. *J Orthop Res* 17: 405–408, 1999.
56. Slauterbeck J, Fuzie S, Smith M, Clark R, Xu K, Starch D, and Hardy D. The menstrual cycle, sex hormones and anterior cruciate ligament injury. *J Athl Train* 37: 275–278, 2002.
57. Sodergard R, Backstrom T, Shanbhag V, and Carstensen H. Calculation of free and bound fractions of testosterone and estradiol-17 β to human plasma proteins at body temperature. *J Steroid Biochem* 16: 801–810, 1982.

58. **Srinivasan N, Aruldas M, and Govindarajulu P.** Sex steroid induced changes in collagen of the prostate and seminal vesicle of rats. *J Androl*: 55–58, 1986.
59. **Strickland S, Belknap T, Turner S, Wright T, and Hannafin J.** Lack of hormonal influences on mechanical properties of sheep knee ligaments. *Am J Sports Med* 31: 210–215, 2003.
60. **Tipton C, Tcheng T, and Mergner W.** Ligamentous strength measurements from hypophysectomized rats. *Am J Physiol* 221: 1144–1150, 1971.
61. **Van Lunen B, Roberts J, Branch J, and Dowling E.** Association of menstrual-cycle hormone changes with anterior cruciate ligament laxity measurements. *J Athl Train* 38: 298–303, 2003.
62. **Wojtys E, Huston L, Boynton M, Spindler K, and Lindenfeld T.** The effect of menstrual cycle on anterior cruciate ligament injuries in women as determined by hormonal levels. *Am J Sports Med* 30: 182–188, 2002.
63. **Wreje U, Brynhildsen J, Aberg H, Bystrom B, Hammar M, and von Schoultz B.** Collagen metabolism markers as a reflection of bone and soft tissue turnover during the menstrual cycle and oral contraceptive use. *Contraception* 61: 265–270, 2000.
64. **Wroble R, Van Ginkel L, Grood E, Noyes F, and Shaffer B.** Repeatability of the KT-1000 arthrometer in a normal population. *Am J Sports Med* 18: 396–399, 1990.
65. **Yu W, Liu S, Hatch J, Panossian V, and Finerman G.** Effect of estrogen on cellular metabolism of the human anterior cruciate ligament. *Clin Orthop* 366: 229–238, 1999.
66. **Yu W, Panossian V, Hatch J, Liu S, and Finerman G.** Combined effects of estrogen and progesterone on the anterior cruciate ligament. *Clin Orthop* 383: 268–281, 2001.
67. **Zeleniuch-Jacquotte A, Shore R, Koenig K, Akhmedkhanov A, Afanasyeva Y, Kato I, Kim M, Rinaldi S, Kaaks R, and Toniolo P.** Postmenopausal levels of oestrogen, androgen, and SHBG and breast cancer: long-term results of a prospective study. *Br J Cancer* 90: 153–159, 2004.
68. **Zhou J, Ng S, Adesanya-Famuyi O, Anderson K, and Bondy C.** Testosterone inhibits estrogen-induced mammary epithelial proliferation and suppresses estrogen receptor expression. *FASEB J* 14: 1725–1730, 2000.

