

Research article

SERUM MARKERS OF INFLAMMATION AND ENDOTHELIAL FUNCTION ARE ELEVATED BY HORMONAL CONTRACEPTIVE USE BUT NOT BY EXERCISE-ASSOCIATED MENSTRUAL DISORDERS IN PHYSICALLY ACTIVE YOUNG WOMEN

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ABSTRACT

The purpose of the study was to determine the effects of exercise-associated menstrual disorders and hormonal contraceptives (HC) on systemic inflammatory markers and endothelial function in female athletes. Thirty-nine active women (≥ 5 h of aerobic exercise per wk), aged 18–33 y, participated in this cross-sectional study comparing women with menstrual disorders (MD, $n = 10$; 0–9 cycles \cdot y $^{-1}$), eumenorrheic women (E, $n = 13$; 10–13 cycles \cdot y $^{-1}$), and HC users (HC, $n = 16$; 12 cycles \cdot y $^{-1}$). Fasting serum samples were collected during the early follicular phase (d2-5) for the menstruating women. Tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), C-reactive protein (CRP), soluble vascular adhesion molecule-1 (sVCAM-1), total cholesterol (TC), high- and low density lipoprotein-cholesterol (HDL-C, LDL-C), triglycerides (TG), reproductive hormones, and cortisol were measured in serum. Estradiol, progesterone, and cortisol were not statistically different between MD and E groups; cortisol was significantly greater in the HC versus E group ($p = 0.002$). TC ($p = 0.005$), LDL-C ($p = 0.03$), and CRP ($p = 0.05$) were increased in the HC versus MD and E groups. TNF- α was significantly higher in the HC ($p=0.001$) compared with the E group. There were no significant group differences in the concentrations of sVCAM-1 or IL-6. TNF- α and cortisol were positively correlated ($r=0.31$, $p = 0.058$), as were sVCAM-1 and estradiol ($r = 0.41$, $p = 0.010$). In conclusion, HC use, but not exercise-associated menstrual disorders, is associated with increased TNF- α and LDL-C.

KEY WORDS: Cytokines, soluble vascular adhesion molecule, female reproductive disorders.

INTRODUCTION

Female athletes experience menstrual disorders due to suppression of the hypothalamic-pituitary-ovarian axis by low energy availability, which results from an imbalance between energy intake and expenditure. Hypoestrogenemia causes loss of bone

mass, increasing current bone fragility and risk for future osteoporosis. In addition, risk factors for cardiovascular disease (CVD) are more common in these apparently healthy, premenopausal women than in regularly menstruating athletes (O'Donnell and De Souza, 2004). Amenorrheic athletes have greater total cholesterol (TC), low-density

lipoprotein-cholesterol (LDL-C), and triglycerides (TG) than their regularly menstruating counterparts (Friday et al., 1993). Recently, endothelial dysfunction, manifest as reduced flow-mediated dilation has been identified as another consequence of athletic amenorrhea (Rickenlund et al., 2005a; Zeni Hoch et al., 2003). It is not known if these risk factors that predict CVD in the general adult population, also are predictive in young women with exercise-associated menstrual disorders.

Postmenopausal women and women with anorexia nervosa also exhibit adverse changes in serum lipids and impaired endothelial function, suggesting that low circulating estradiol plays a causal role in these negative events (O'Donnell and De Souza, 2004). Estrogens protect against CVD by: decreasing the concentration of LDL-C in blood; reducing oxidation of LDL and other lipids; and stimulating vasodilation by increasing the production of nitric oxide and prostacyclin, thereby improving blood flow (Taddei et al., 1996) (Kublickiene et al., 2005). Estrogen replacement therapy (ERT) with estradiol or conjugated equine estrogens improves the lipid profile in postmenopausal women. Addition of progestins to the hormonal replacement therapy (HRT) may abrogate the benefits of exogenous estrogens, but the results are equivocal (Dubey et al., 2004; Silvestri et al., 2003).

Monocyte adhesion to vascular endothelial cells is an early critical step in the atherogenic process; attached monocytes differentiate to macrophages, which actively sequester cholesterol, forming fatty streaks in blood vessels. Vascular adhesion molecule-1 (VCAM-1), which is expressed by vascular endothelial and smooth muscle cells, mediates adhesion via binding of leukocytes to integrins (Cybulsky and Gimbrone, 1991), and inflammatory cytokines are known to upregulate VCAM-1 expression (Mukherjee et al., 2003). Estrogen suppresses VCAM-1 in vivo (Nathan et al., 1999) and in vitro (Mukherjee et al., 2003), apparently via an indirect mechanism, as no estrogen response element has been identified in the VCAM-1 promoter. Soluble VCAM-1 (sVCAM-1) arises via proteolytic cleavage from the cell surface; the concentration of sVCAM-1 in serum is proportional to its expression on endothelial cells (Pigott et al., 1992).

Although the deleterious effects of exercise-associated amenorrhea on blood lipids has been appreciated for sometime, impaired endothelial function was only recently described by Zeni-Hoch et al. (2003). Rickenlund et al. (2005b) subsequently investigated the mechanistic links between estrogen, inflammatory cytokines, vascular adhesion

molecules, and endothelial function. In a cross-sectional study and a treatment trial with hormonal contraceptives, impaired flow-mediated dilation was associated with elevated sVCAM-1, but not with TNF- α , CRP or IL-6 in female athletes (Rickenlund et al., 2005a; 2005b). Because the beneficial effect of estrogen on endothelial function is thought to be mediated through cytokine-stimulated expression of adhesion molecules, further investigation of this causative pathway is warranted.

Thus, the purpose of this study was to determine the effects of exercise-associated menstrual disorders and hormonal contraceptive use on TNF- α , CRP, IL-6 and sVCAM-1 and on serum lipids in non-eating disordered female athletes. We hypothesized: 1) menstrual disorders secondary to low energy availability would be associated with increased TC, TG, TNF- α , IL-6, CRP, cortisol, and sVCAM-1; 2) oral contraceptive users would have increased TC, LDL-C, TNF- α , IL-6, CRP, cortisol, and decreased sVCAM-1 compared to regularly menstruating women.

METHODS

Study participants

A total of 39 recreationally-trained (at least 5 hours of aerobic, weight-bearing exercise per week) female athletes, aged 18-33, were recruited from local multi-sport clubs and the University of Missouri in Columbia. Subjects were classified as: having an exercise-associated menstrual disorder (MD, $n = 10$; 0-9 cycles-year⁻¹ during the previous 12 months); eumenorrheic women (E, $n = 13$; 10-13 cycles-year⁻¹); using hormonal contraceptives for at least 12 months (HC, $n = 16$; 12 cycles-year⁻¹) without a history of menstrual disorders. Four of the women in the MD group were amenorrheic (0 cycles-year⁻¹) and six were oligomenorrheic (average 6 cycles-year⁻¹). Nine subjects currently were taking monophasic oral contraceptives (combinations of norgestimate, or levonorgestrel, or norethindrone, or drospirenone and ethinyl estradiol) and 7 subjects currently were taking triphasic oral contraceptives (norgestimate and ethinyl estradiol). Prior to initial screening, all subjects were informed of any risks associated with this study, read a consent form, and gave oral consent as had been approved by the University of Missouri Health Sciences Institutional,

Review Board. Subjects with a current or previous disease, or use of medications, affecting bone including eating disorders, or who were pregnant or lactating, were excluded from the study. Subjects were paid \$25 upon completion of the study. All procedures involving human subjects were in accordance with the ethical standards

Table 1. Characteristics of female athletes with menstrual disorders (MD), regular menstrual cycles (E), or hormonal contraceptive (HC) users. Data are means (\pm SD) or median and range.

	MD ^a (n = 10)	E ^b (n = 13)	HC ^c (n = 16)
Age (yrs)	22.7 (3.2)	21.1 (3.8)	23.2 (3.54)
Menarchal age (yrs)	13.4 (1.5)	13.8 (1.7)	13.0 (1.3)
Weight (kg)	54.2 (6.1) ^b	62.2 (7.8) ^a	61.8 (8.1) ^a
Height (m)	1.66 (.05)	1.68 (.05)	1.67 (.06)
BMI (kg·m ⁻²)	19.6 (1.7) ^b	22.0 (2.2) ^a	22.2 (2.4) ^a
Body fat (%)	18.2 (3.7) ^b	20.4 (5.2) ^{a,c}	23.2 (4.4) ^a
Lean body mass (kg)	41.3 (5.5)	46.4 (5.8)	45.1 (5.8)
Menstrual cycles per year	4 (3) ^b	12 (1) ^a	12 (1) ^a
Missed menstrual cycles (since menarche)	47 (16-57) ^b	4 (0-21) ^a	3.5 (0-36) ^a
Months on hormonal contraceptives	12 (0-84) ^c	0 (0-120) ^c	78 (36-192) ^{a,b}
Physical activity (hrs·wk ⁻¹)	11.2 (3.9)	9.2 (3.0)	11.7 (6.0)
Physical activity energy expenditure (kcal·d ⁻¹)	622 (237)	604 (213)	596 (220)

Superscripts denote significantly ($p < 0.05$) differences between the groups.

of the University of Missouri Institutional Review Board and with the Helsinki Declaration of 1975 as revised in 1983.

Anthropometric data

Subject weight was determined to the nearest 0.05 kg and height was determined to the nearest 0.5 cm, and used to calculate body mass index (BMI; kg·m⁻²). Body composition was determined from three skinfold measurements and percent body fat was calculated by the Jackson-Pollock method (Siri, 1961).

Serum lipids, hormones, and inflammatory markers

Blood (20 ml) was drawn between 0800 and 1000 hours after an overnight fast and prior to exercise from an antecubital vein. Serum was separated by centrifugation at 2000 g for 15 minutes and stored at -80°C. Sample collection occurred during the early follicular phase of the menstrual cycle, defined as days 2-5 of menstruation, for regularly menstruating subjects, i.e., eumenorrheic, and hormonal contraceptive users, and on an arbitrary day for amenorrheic subjects. All of the participants had concentrations of LH and FSH that were less than 15 IU/liter and 11 IU/liter, respectively, consistent with the early follicular phase or anovulatory amenorrhea.

TC, HDL-C and TG were measured using enzymatic methods (Sigma Diagnostics, St. Louis, MO). LDL-C was estimated using the Friedwald equation (Friedwald et al., 1972). TNF- α , IL-6, sVCAM-1, and CRP were measured using ELISA (ALPCO Diagnostics, Windham, NH; intra-assay CVs were 5.5%, 10.2%, 5.7%, 9.1% respectively). LH, FSH, cortisol, progesterone, and parathyroid hormone (PTH) were measured using ELISA (ALPCO Diagnostics, Windham, NH; intra-assay

CVs were 8.6%, 6.6%, 10.4%, 9.1%, 2.7%, respectively). Estradiol, leptin, and prolactin (PRL) were measured using ELISA (Diagnostic Systems Laboratories, Inc, Webster, Texas; intra-assay CVs were 6.5%, 7.9%, 6.8%, respectively).

Questionnaires

All subjects completed a medical, menstrual, and sports history questionnaire. Physical activity was quantified using a 7-day written training log of activity type, duration, and frequency. The Compendium of Physical Activities was used to estimate daily energy expenditure (Ainsworth et al., 2000). Menstrual history was assessed using a written questionnaire that consisted of a timeline on which participants indicated menarche, missed menstrual periods, initiation and cessation of hormonal contraceptive use.

Statistical analyses

CRP, TC, and TG were not normally distributed, so analyses were performed using a logarithmic transformation. One-way ANOVA with the least significant difference technique was used to determine differences among groups. Pearson correlations between inflammatory markers and hormones (cortisol, estradiol, progesterone) were performed. Statistical analyses were conducted using SAS version 8.2 (Cary, 1999) and statistical significance was set at $p < 0.05$.

RESULTS

Participant characteristics

The MD group had significantly lower body weight, BMI, and percent body fat than the E and HC groups (Table 1). The number of missed menstrual cycles since menarche was greater in the MD group than in

Table 2. Hormones, markers of inflammation and endothelial function in serum of female athletes with menstrual disorders (MD), regular menstrual cycles (E), or hormonal contraceptive (HC) users. Data are means (\pm SD) or median and range.

Hormone	MD ^a	E ^b	HC ^c	Normal Range
	(n = 10)	(n = 13)	(n = 16)	
Estradiol (pmol·L ⁻¹)	378 (117)	330 (143)	323 (198)	110–1470
Progesterone (nmol·L ⁻¹)	3.56 (1.23) ^c	3.15 (.62) ^c	2.60 (1.03) ^{a,b}	< 3.2
LH (IU·L ⁻¹)	6.4 (1.5-17.1)	6.8 (4.0-14.1)	2.5 (1.1-17.1)	1.7–15
FSH (IU·L ⁻¹)	4.7 (2.7-6.1)	4.2 (2.7-6.4)	3.5 (1.6-15.0)	1.8–11.2
Leptin (ng·mL ⁻¹)	6.5 (1.7-16.1)	6.8 (2.1-22.8)	12.3 (4.3-27.9)	2.5–9.0
Cortisol (nmol·L ⁻¹)	1137 \pm 448 ^c	1068 \pm 379 ^c	1653 \pm 276 ^{a,b}	170–860
PTH (ng·L ⁻¹)	47 (12)	42 (19)	43 (24)	10–65
Prolactin (pmol·L ⁻¹)	503 (261-1043)	565 (274-1710)	636 (270-1913)	< 4000
TNF- α (pg·mL ⁻¹)	19.1 (17.9-33.2) ^c	12.4 (6.7-33.1) ^c	34.9 (18.6-43.1) ^{a,b}	<2
CRP (mg·L ⁻¹)	.50 (0-5.76) ^c	2.40 (0-13.41) ^c	4.42 (0-13.87) ^{a,b}	<10
IL-6 (pg·mL ⁻¹)	7.1 (2.9-12.4)	9.1 (2.2-36.8)	11.5 (3.5-88.7)	<150
sVCAM-1 (ng·mL ⁻¹)	955 (572-1245)	1072 (753-1726)	970 (790-1194)	400-800

Superscripts denote significantly ($p < 0.05$) differences between the groups.

the E and HC groups, but did not differ between E and HC groups. Finally, the HC group had been using hormonal contraceptives for a significantly greater number of months than the MD and E groups. There was no significant difference between MD and E groups in the number of months of hormonal contraceptive use. No significant differences existed among the menstrual status groups in hours of physical activity participation per week or in energy expended in exercise per day. The HC group had significantly greater concentrations of cortisol and lower concentrations of progesterone in serum than the E and MD groups (Table 2).

We expected that the concentration of estradiol in serum would be low in all groups. The

women with exercise-associated menstrual disorders were presumed to have suppressed ovarian hormone production due to decreased LH secretion. We expected the menstruating women to have low circulating estradiol because we measured reproductive hormones during the early follicular phase of the menstrual cycle when estradiol secretion is lowest. Thus, we did not expect to detect statistically significant differences in estradiol concentrations among groups.

Serum lipids

TC and LDL-C were significantly higher in the HC group compared to the E and MD groups ($p < 0.01$, Table 3). HDL-C and TG did not differ among groups ($p = 0.1$, Table 3). There were no differences in serum lipids between amenorrheic and oligomenorrheic women in the MD group (data not shown).

Inflammatory markers

TNF- α and CRP were significantly elevated in the HC compared to the E group (Table 2, Figure 1). The MD group tended to have elevated TNF- α compared to the E group, but this difference was not statistically significant ($p = 0.062$). sVCAM-1 and IL-6 did not differ among groups ($p > 0.05$). There was a positive correlation between TNF- α and cortisol ($r = 0.31$, $p = 0.058$, $n = 39$; data not shown). This association remained significant when HC users were excluded from the analysis ($r = 0.44$, $p = 0.03$, $n = 23$). sVCAM-1 was positively correlated with estradiol ($r = 0.41$, $p = 0.010$; data not shown), but was not significantly correlated with TNF- α or IL-6. There were no differences in serum markers of inflammation between amenorrheic and oligomenorrheic women in the MD group (data not shown).

Table 3. Cholesterol and triglyceride concentrations in serum of female athletes with menstrual disorders (MD), regular menstrual cycles (E), or hormonal contraceptive (HC) users. Data are means (\pm SD).

Hormone	MD ^a	E ^b	HC ^c	Desirable Range
	(n = 10)	(n = 13)	(n = 16)	
TG (mmol·L ⁻¹)	1.06 (.36)	1.03 (.23)	1.28 (.43)	<1.695
TC (mmol·L ⁻¹)	3.83 (.47) ^c	3.94 (.67) ^c	4.74 (.96) ^{a,b}	<5.18
LDL-C (mmol·L ⁻¹)	2.02 (.72) ^c	2.02 (.60) ^c	2.61 (.76) ^{a,b}	<2.59
HDL-C (mmol·L ⁻¹)	1.33 (.31)	1.45 (.33)	1.53 (.26)	>1.036

Superscripts denote significantly ($p < 0.05$) differences between the groups.

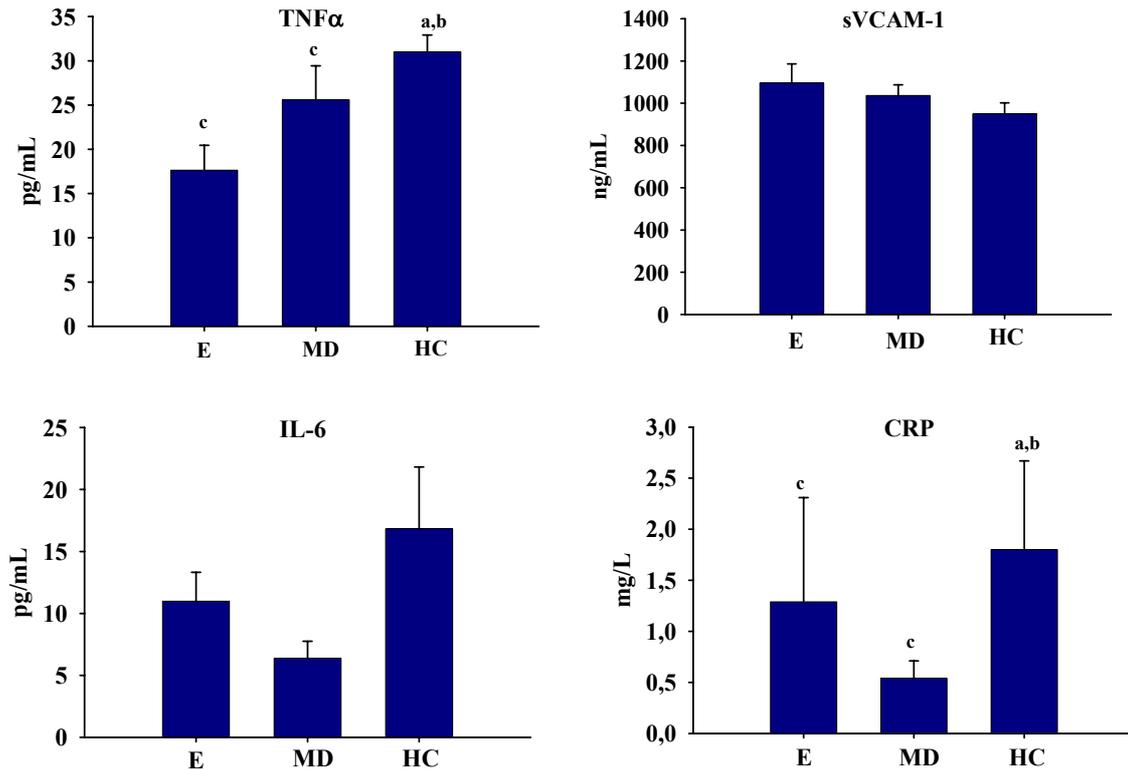


Figure 1. Serum markers of inflammation and endothelial function in female athletes with regular menstrual cycles (E^a), menstrual disorders (MD^b), or hormonal contraceptive users (HC^c). Data are means (\pm SD). Superscripts denote significantly ($p < 0.05$) differences between the groups.

DISCUSSION

Contrary to our hypothesis, female athletes with exercise-associated menstrual disorders did not have significant alterations in serum inflammatory markers or blood lipids. We expected women with menstrual disorders to have significantly greater serum TNF- α , IL-6, TC, LDL-C, and TG than eumenorrheic women. Estrogen suppresses TNF- α and IL-6 gene expression (Salem, 2004) and elevations in TNF- α and IL-6 have been observed in postmenopausal women and in individuals with anorexia nervosa (Gianni et al., 2004; Kahl et al., 2004; O'Donnell and De Souza, 2004). We did not expect to observe group differences in serum estradiol because blood was collected from regularly menstruating subjects during the early follicular phase of the menstrual cycle. Assuming that women with menstrual disorders have lower cumulative estradiol exposure over the course of the menstrual cycle than eumenorrheic women, the lack of group differences in blood lipids was unexpected.

Hormonal contraceptive users had elevated cortisol, CRP, TNF- α , TC, and LDL-C in serum compared to eumenorrheic athletes. Oral estrogens and progestins in hormonal contraceptives previously have been shown to increase TC, LDL-C,

TG (Guazzelli et al., 2005), as well as, TNF- α and CRP in premenopausal women (Rickenlund et al., 2005b). There is evidence that the increase in CRP associated with HC use, results from hepatic metabolism of the steroid hormones rather than induction of an inflammatory response (Silvestri et al., 2003). Transdermal administration of hormonal contraceptives does not result in altered serum lipoproteins or CRP (Dreon et al., 2003).

In the only other published observations of serum and endothelial inflammatory markers in women with exercise-associated menstrual disorders, Rickenlund et al. (2005a) reported that oligomenorrheic and amenorrheic athletes had similar sVCAM-1, TNF- α , IL-6, and CRP compared with eumenorrheic athletes, despite impaired flow-mediated dilation. The results of the present study are consistent with these findings, strengthening the evidence that altered systemic cytokines may not be the link between estrogen inadequacy and impaired endothelial function.

In the current study, sVCAM-1 was not correlated with TNF- α or IL-6, consistent with the findings of Rickenlund et al. (2005a) in female athletes. Souter et al. (2005) reported a significant positive relationship between TNF- α and sVCAM-1 throughout the menstrual cycle in sedentary women.

Although TNF- α and IL-6 induce expression of cellular adhesion molecules, circulating concentrations of these inflammatory markers do not necessarily reflect their bioactivity, which is modulated by soluble receptors, nor are they indicators of localized cytokine autocrine and paracrine activity (Fruhbeck et al., 2001). Exogenous estrogens also cause a divergent response of the cytokines and sVCAM-1 in vivo (Rickenlund et al., 2005b; Souter et al., 2005). Oral contraceptives decreased sVCAM-1, but increased TNF- α and CRP, in amenorrheic and eumenorrheic female athletes (Rickenlund et al., 2005b). In regularly menstruating, sedentary premenopausal women, hormonal contraceptive use decreased sVCAM-1, but had no effect on serum TNF- α (Souter et al., 2005).

The effect of synthetic estrogens (e.g., ethinyl estradiol), commonly found in hormonal contraceptives, on serum sVCAM-1 in vivo is surprising based on regulation of VCAM-1 expression by estrogens in cultured endothelial cells (Mukherjee et al., 2003). In vitro experiments demonstrate that suppression of TNF- α -induced VCAM-1 expression by estrogen is mediated by binding of estrogen receptor β (ER β) but not ER α (Mukherjee et al., 2003). Although 17- β estradiol binds ER α and ER β , ethinyl estradiol only binds ER α and has no effect on VCAM-1 expression in vitro. Our findings are consistent with these in vitro experiments.

The positive correlation between estradiol and sVCAM-1 observed in this study was unexpected and may be an artifact of the study design. Although, hormonal contraceptives suppress sVCAM-1 (Rickenlund et al., 2005b; Souter et al., 2005) in premenopausal women, the amount of this adhesion molecule in circulation does not vary across the menstrual cycle (Souter et al., 2005). Because we measured reproductive hormones during the early follicular phase of the menstrual cycle when estradiol secretion is lowest, we were unable to determine group differences in cumulative estrogen exposure throughout the course of the menstrual cycle. Thus, our ability to examine the relationship between estradiol and sVCAM-1 was impaired. When the number of menstrual cycles per year was used as a proxy variable for cumulative estrogen exposure, the relationship between cycle number and sVCAM-1 was not significant.

A limitation of this study is that the MD groups included women who were amenorrheic and oligomenorrheic. Rickenlund et al. (2005a) reported that flow mediated dilation was more impaired in women with exercise-associated amenorrhea than in

those with oligomenorrhea; however, TNF- α , IL-6 and sVCAM-1 did not differ between groups. Similarly, TNF- α , IL-6, CRP, and sVCAM-1 were not different between amenorrheic and oligomenorrheic subjects in the current study in post hoc analyses.

The relatively small sample size is another limitation of this study, reducing the power to detect group differences. Retrospective power analyses demonstrate that a sample size of 32 subjects is required to detect a significant difference in IL-6 and TNF- α between the eumenorrheic and menstrual disturbance groups at power = 0.80 and $p = 0.05$. However, the required sample sizes to detect group differences in CRP and sVCAM-1 are 137 and 274, respectively, at power = 0.80 and $p = 0.05$. Thus, there may be differences in the inflammatory cytokines associated with menstrual disorders. However, it is unlikely that true differences in CRP and sVCAM-1 exist between the E and MD groups.

CONCLUSION

In conclusion, we did not observe altered serum markers of inflammation and endothelial function in women with exercise-associated menstrual disorders. Regular use of hormonal contraceptives, however, significantly increased total and LDL cholesterol, TNF- α , and CRP in physically active women, as previously reported (Souter et al., 2005). Future studies are needed to determine if the increased proinflammatory cytokines in serum initiate the atherosclerotic process and impair vascular endothelial function. Moreover, the absence of consistent relationships between the inflammatory cytokines, adhesion molecules, and estradiol, indicates that further studies are needed to elucidate the mechanisms by which estrogen regulates endothelial function.

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KEY POINTS

- Serum lipids and markers of inflammation were not altered by exercise-associated oligomenorrhea or amenorrhea.
- Hormonal contraceptive users had elevated total and LDL cholesterol compared with regularly menstruating non-HC users.
- C-reactive protein and tumor necrosis factor- α , but not soluble vascular adhesion molecule-1, were increased in hormonal contraceptive users.
- The long-term effect of these changes on cardiovascular disease is unknown.

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