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# Iron status at opposite ends of the menstrual function spectrum

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ABSTRACT

*Objectives:* Although exercising women are at high risk of poor iron status, it is unknown how non-pathological, physiological menstrual function affects iron status. As such, this study investigates the association between menstrual function and iron status in exercising women with amenorrhea and exercising women with ovulatory, eumenorrheic menstrual cycles.

*Design:* Cross-sectional analysis of iron depletion prevalence, iron status indices, exercise parameters, and diet composition.

*Methods:* Women aged 18–35 years performing at least 2 h per week of aerobic exercise were recruited. Women with amenorrhea (AMEN) were defined by the absence of menses for at least 90 days or less than 6 menses in the past 12 months (n = 82). Women with ovulatory, eumenorrheic menstrual cycles (OvEU) were defined by the presence of ovulatory cycles of 26-35 days in length for the past 6 months (n = 109). Group differences in serum ferritin (Ft), soluble transferrin receptor (sTfR), total body iron (TBI), hemoglobin (Hb), hematocrit (Hct), iron depletion prevalence (Ft < 15 μg/L), peak oxygen consumption ($VO_{2peak}$), exercise minutes per week, and diet logs were assessed.

*Results:* The prevalence of iron depletion was greater in OvEU when compared to AMEN (26% vs. 15%, p = 0.04). No significant differences were observed between AMEN and OvEU in Ft (30.2 ± 2.2 vs. 24.9 ± 2.6 μg/L; p = 0.62), sTfR (5.2 ± 1.4 vs. 4.9 ± 1.5 mg/L; p = 0.95), TBI (5.3 ± 2.7 vs. 4.8 ± 3.7 mg/kg; p = 0.42), Hb (13.2 ± 0.4 vs. 13.4 ± 0.6 g/dL; p = 0.80), Hct (39.5 ± 0.8% vs. 39.8 ± 4.1%; p = 0.93), or exercise parameters. AMEN consumed more vitamin C than OvEU (269 ± 180 vs. 129 ± 141 mg/day, p < 0.001), but all other dietary factors were similar between AMEN and OvEU.

*Conclusion:* Exercising women with ovulatory, eumenorrheic cycles are at a greater risk of iron depletion than exercising, amenorrheic women. Thus, menstrual function must be considered when screening for poor iron status in exercising women.

## 1. Introduction

Exercising women are at high risk of iron deficiency (ID) which can compromise immune function, impair cognitive function, and reduce athletic performance [1,2]. Further, ID may exacerbate menstrual dysfunction, low energy availability, and low bone mineral density associated with the Female Athlete Triad [3,4]. ID is characterized by stages of increasing severity. The first stage is iron depletion, which is characterized by depletion of bone marrow iron stores, minimal changes in red blood cell indices, and a reduction of serum ferritin (Ft) below a given threshold. The second stage is ID without anemia, which is characterized by depletion of total body iron that results in diminished red blood cell indices. The final stage is ID with anemia, which is characterized by the depletion of total body iron stores that significantly reduces red blood cell indices below established values associated with clinical sequelae [5]. Iron depletion is typically

*Abbreviations:* AMEN, exercising women with amenorrhea; OvEU, exercising women with ovulatory, eumenorrheic cycles; Ft, ferritin; sTfR, soluble transferrin receptor; TBI, total body iron; Hb, hemoglobin; Hct, hematocrit; ID, iron deficiency; EAMD, exercise-associated menstrual disturbances; AWS, The Active Women's Study; TEF, Thermic Effect of Food Study; E1G, estrone-1-glucuronide; PdG, pregnanediol glucuronide; LH, luteinizing hormone; $VO_{2peak}$, peak oxygen consumption; RDA, recommended daily allowance

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asymptomatic with significant clinical symptoms and sequelae occuring as ID progresses [6].

One determinant of iron status in exercising women is exercise-associated iron loss which underlies the 60% greater risk of iron depletion (Ft < 20 μg/L) in exercising women compared to sedentary women [7]. Exercise elevates iron loss above basal levels via increased iron loss in sweat, intravascular hemolysis, and gastro-intestinal bleeding [8]. Exercise can further compromise iron status by upregulating hepcidin via exercise-induced inflammation [9], which greatly reduces dietary iron absorption and availability for erythropoiesis [10]. These mechanisms likely underlie the report that ten days of exercise training in physically active women was associated with a 33% reduction in Ft, a marker of iron storage content [11], and an 8% increase in soluble transferrin receptor (sTfR), a marker of intracellular iron demand [12]. Likewise, nine weeks of basic combat training in female soldiers was associated with a 25% reduction in Ft and a 30% increase in sTfR [13].

Another determinant of iron status in exercising women is menstrual blood loss. The average woman loses 14 mg of iron per menses [14]. These iron losses likely underlie the correlation between a greater duration and intensity of menstrual bleeding with a higher prevalence of iron depletion [15–17]. To better understand the effects of menstrual blood loss on iron status, it may be useful to consider two naturally occurring physiological models: the onset of menses at menarche and the cessation of menses at menopause. Menarche is associated with a near double increased prevalence of iron depletion [18] and menopause is associated a decrease in iron depletion prevalence from 13% to 0% [19]. These observations suggest that menstrual iron losses are a primary determinant of the 10–20% greater prevalence of ID in exercising women compared to exercising men [20]. Lastly, menstrual blood loss likely impacts iron status in menstruating, exercising women, as one third of exercising women experience heavy menstrual bleeding [21].

Despite the association with menstrual bleeding, iron status is rarely considered within the context of menstrual function. This research gap is noteworthy since as many as 56% of exercising women experience menstrual dysfunction, referred to as exercised-associated menstrual disturbances (EAMD) [22]. EAMD range from subclinical presentations, such as luteal phase defects and anovulation, to clinical presentations, such as oligomenorrhea and amenorrhea [23,24]. More severe EAMD are associated with decreased bone health, increased risk of stress fractures [25], endothelial dysfunction [26], and atherogenic blood lipid profiles [27]. As such, menstrual function restoration is prioritized in EAMD treatment [28,29]. Yet, it is unknown how iron status is affected by EAMD. Such knowledge may have implications for EAMD treatment strategies, such as supplementation or dietary recommendations.

As such, this investigation aimed to determine the association between menstrual function and iron status by comparing exercising women at opposite ends of the menstrual function spectrum. That is, iron status was compared between exercising women with ovulatory, eumenorrheic menstrual cycles (OvEU) and exercising women with complete loss of menstruation, i.e. amenorrhea (AMEN). We hypothesized that OvEU would exhibit a higher prevalence of iron depletion (Ft < 15 μg/L) and poorer iron status indices in terms of higher sTfR and lower Ft, total body iron (TBI; a calculated index of body iron stores), hemoglobin (Hb), and hematocrit (Hct), compared to AMEN women. As a secondary analysis, we explored associations between menstrual bleeding characteristics and iron biomarker concentrations. We hypothesized that iron status would decrease with increasing bleeding intensity and duration.

## 2. Methods

This cross-sectional study used data from three studies: REFUEL (n = 157), The Active Women's Study (AWS; n = 19), and the Thermic Effect of Food Study (TEF; n = 15). REFUEL was a longitudinal study investigating the effects of increased caloric intake on menstrual function and bone health among exercising women with EAMD. AWS was a cross-sectional study investigating associations between menstrual function and factors such as bone density and metabolic hormones in exercising and sedentary women. TEF was a cross-sectional study investigating postprandial thermogenesis and gut peptide secretion in exercising women according to menstrual function. REFUEL and AWS were conducted at the University of Toronto and The Pennsylvania State University. TEF was conducted at The Pennsylvania State University. All participants provided written informed consent before study procedures were conducted. All studies were approved by the Institutional Review Boards at their respective institutions.

### 2.1. Screening

Participants were recruited on a rolling basis and initially screened via phone, in-person, or online surveys. Initial inclusion criteria for REFUEL and TEF eligibility were as follows: 1) age 18–35 years, 2) body mass index 16–25 kg/m$^2$, 3) weight stable ( ± 2 kg) for the past 6 months, 4) no history of any serious medical conditions, 5) no current clinical diagnosis of an eating or psychiatric disorder, 6) non-smoking, 7) no medication use that would alter metabolic, bone or reproductive hormone concentrations, 8) ≥ 2 h/wk purposeful exercise, and 9) no history of a clinical diagnosis of polycystic ovarian syndrome. AWS inclusion criteria included the above except for the following differences: 1) body mass index of 16–30 kg/m$^2$, 2) no weight stability requirement, and 3) no exercise requirement.

After obtaining informed consent, height and weight were measured and medical, exercise, and menstrual histories were collected. Participants in the REFUEL and TEF provided a fasted venous blood sample for a screening panel. For the current analyses, REFUEL and TEF participants with abnormal follicle stimulating hormone, luteinizing hormone, prolactin, thyroid stimulating hormone, thyroxin, total testosterone or free testosterone serum concentrations were excluded to rule out non-exercise related causes of menstrual disturbances [30]. Women with high androgen phenotypes (clinical symptoms and free androgen index) were excluded (n = 6) [31]. Participants in AWS were not screened for these factors. For REFUEL and TEF participants, a physical examination was conducted by a Clinical Research Center clinician at the University of Toronto or The Pennsylvania State University. No participants were taking iron supplements at the time of the study.

Participants were assigned to groups based on their respective study design and retrospectively assigned as either AMEN or OvEU for the present study. Participants eligible to be assigned into these groups met specific criteria during the screening procedures. Inclusion as AMEN required a self-reported absence of menses within the past 3 months or less than 6 menses in the past 12 months [32]. Inclusion as OvEU required a self-report of ≥ 9 menses in the past 12 months and a menstrual cycle length between 25 and 36 days [32].

### 2.2. Post-screening

In each study, screening was followed by a baseline phase lasting one menstrual cycle or, if amenorrheic, 28 days. During baseline, each participant self-collected daily first-morning void urine samples, provided a blood sample, completed menstrual and health questionnaires, recorded diet logs, and performed an exercise test. For REFUEL participants, baseline was followed by a nutritional intervention phase, but only data from the first week of the intervention were used in the present study to avoid potential confounding effects of improved nutrition on iron status.

### 2.3. Menstrual function classification and characteristics

Baseline daily urine samples were analyzed for estrone-1-glucuronide (E1G), pregnanediol glucuronide (PdG), and luteinizing hormone

(LH) using methods previously described [24]. Retrospective assignment to OvEU required the following: 1) an E1G peak concentration above 35 ng/ml, 2) a peak PdG concentration above 5 μg/mL during the luteal phase, and 3) an LH surge concentration above 25 mIU/ml to confirm ovulation [23,24]. Retrospective assignment to AMEN required the following: 1) absence of menses and menstrual bleeding, 2) a negative pregnancy test, and 3) suppressed E1G and PdG profiles [23,24]. Self-reports of number of menses in the last 12 months, duration of menstrual bleeding, and menstrual bleeding intensity were collected in OvEU. Self-reported duration of amenorrhea prior to study participation was collected in AMEN.

### 2.4. Exercise and diet

Peak oxygen consumption ($VO_{2peak}$), an indicator of aerobic fitness, was measured during a graded exercise test to volitional fatigue with a SensorMedics Vmax metabolic cart (Yorba Linda, CA) using methods previously published [33]. Participants recorded their purposeful exercise for at least one consecutive 7-day period. Baseline exercise logs were averaged to determine each participant's average weekly exercise duration.

All participants completed 3-day diet logs on 2 weekdays and 1 weekend day during baseline; REFUEL participants completed another diet log during the first week of the intervention. Participants were provided with detailed instructions on how to record types and quantities of foods eaten. Diet analysis of the 3-day diet logs were performed using Nutrition Data System for Research (NDS-R 2008, Minneapolis, MN) or Nutritionist Pro (Axxya Systems, Woodinville, WA) to obtain energy, protein, carbohydrate, fat, iron, heme-iron, nonheme-iron, calcium, and vitamin C intakes. The presented values are the average of the two 3-day diet logs for REFUEL participants and a single 3-day diet log for AWS and TEF participants.

### 2.5. Serum sampling and analysis

Fasting venous blood samples were collected between 0700 and 1000 h during the early follicular period for all menstruating participants and on an arbitrary day for all AMEN and one week after baseline in REFUEL participants. Participants were asked to abstain from exercise for 24 h prior to blood sampling. Samples clotted for 30 min prior to centrifugation (1600 *g*) at 4 °C for 15 min. Serum aliquots were stored at −80 °C until analysis. Each REFUEL participant's samples were pooled to minimize possible variations in Ft and sTfR across the menstrual cycle [34].

Ft and sTfR concentrations were assessed via enzyme immunoassay (Ramco, Houston, TX) with intra-assay coefficient of variance of 6.0% and 4.2% and inter-assay variability of 9.8% and 14.3%, respectively. Inflammation status was determined via serum alpha-1-acid glycoprotein (radial immunodiffusion, Kent Laboratories, Bellingham, WA) and qualitative serum C-reactive protein assays with a threshold of 10 mg/mL (IMMUNEX CRP, Alere, Waltham, MA). Ft concentrations were corrected as described elsewhere [35]. A correction factor of 0.76 was applied to Ft concentrations in the early convalescence group and group sizes were insufficient in the incubation and late convalescence groups to calculate a correction factor. A similar procedure was performed for sTfR [36] and no correction factors were required. TBI was calculated with the inflammation-adjusted Ft as described elsewhere [37]. Anemic participants (Hb < 12 g/dL and/or Hct < 35%) that were iron replete (Ft ≥ 15 μg/L) were excluded from the present analysis (n = 7) due to the possible presence of pre-existing and unknown medical conditions [5].

### 2.6. Data analysis

Statistical analyses were performed using R version 3.1.0 (The R Foundation for Statistical Computing, 2014). Geometric means and geometric standard deviations were calculated for Ft and sTfR [38,39] to provide a more robust measure of central tendency in these skewed biomarkers and to allow for direct comparison to a similar investigation [39]. Arithmetic means and standard deviations were calculated for all other values. Variable normality was assessed via Shapiro-Wilk tests. Differences between group means were assessed via independent T-tests for normally distributed variables or via Wilcoxon rank-sum tests for non-normally distributed variables. Difference in iron depletion prevalence (Ft < 15 μg/L) [40] between groups was assessed via a one-tailed $X^2$ test. Difference in proportion of individuals that met the recommended dietary allowance (RDA) of iron for women ages 19–50 (18 mg/day) [41] was assessed via a two-tailed $X^2$ test. Correlations between iron status indices and menstrual characteristics were assessed with Spearman's rank-order correlation coefficients. Regression coefficients for exercise parameters and dietary factors as part of generalized linear models for individual iron status indices were determined to assess for possible confounding variables. Alpha levels were set at 0.05 and adjusted with Bonferroni's correction for correlations.

A sensitivity power calculation for the minimal detectable effects for group mean differences of Ft and sTfR was performed using data from an investigation of iron status in exercising women without regard for menstrual function [42]. With a sample size of N = 191, alpha = 0.05 and power = 0.80, the minimal detectable differences between OvEU and AMEN for Ft and sTfR were 1.36 μg/L and 0.07 mg/L, respectively. These differences are below what would be considered clinically meaningful and smaller than previously reported differences in exercising women with and without menstrual function [39].

## 3. Results

### 3.1. Anthropometrics, exercise, and diet

Complete data were available for 82 AMEN and 109 OvEU. Age, body mass index, $VO_{2peak}$, and exercise duration were similar between AMEN and OvEU (Table 1). $VO_{2peak}$ for both groups was approximately equal to the 80th percentile of women aged 18–25 [43]. AMEN consumed more vitamin C than OvEU (269 ± 180 vs. 129 ± 141 mg/day, $p < 0.001$), but all other dietary factors were similar between AMEN and OvEU. Exercise parameters and dietary factors were not significant confounders of any iron status index in AMEN, OvEU, or both groups combined. A greater proportion of OvEU (61%) than AMEN (42%; $X^2 = 6.83$, $p < 0.01$) met the RDA for dietary iron.

### 3.2. Iron depletion and iron status indices

The prevalence of iron depletion was greater in OvEU compared to AMEN (26% vs. 15%, $X^2 = 3.45$, $p = 0.04$; Fig. 1). Ft (30.2 ± 2.2 vs. 24.9 ± 2.6 μg/L), sTfR (5.2 ± 1.4 vs. 4.9 ± 1.5 mg/L), TBI (5.3 ± 2.7 vs. 4.8 ± 3.7 mg/kg; Fig. 2), Hb concentrations (13.2 ± 0.4 vs. 13.4 ± 0.6 g/dL), and Hct (39.5 ± 0.8% vs. 39.8 ± 4.1%) were not significantly different between OvEU and AMEN.

### 3.3. Menstrual function characteristics and iron status indices

The average frequency of menses in the past 12 months was 12 ± 1 menses. Four OvEU did not report menstrual bleeding characteristics. Twenty, 33, and 52 women in OvEU reported "light," "moderate," and "heavy" menstrual bleeding intensity, respectively. Fifty-one, 27, 22, 3, and 2 women in OvEU reported 5 or more, 4, 3, 2, or 1 day(s) of menstrual bleeding, respectively. The "high" menstrual bleeding group (5 or more days of menstrual bleeding and "heavy" menstrual bleeding) contained 45/109 (43%) of the women in OvEU. The "low" menstrual bleeding group (less than 5 days of menstrual bleeding and "light" or "moderate" menstrual bleeding) contained 60/109 (57%) of the women in OvEU. There was not a significant difference in iron depletion

**Table 1**
Anthropometric, dietary, and iron status characteristics of exercising women with amenorrhea (AMEN) and exercising women with eumenorrheic, ovulatory cycles (OvEU).

| | AMEN (n = 82) | OvEU (n = 109) |
|---|---|---|
| *Anthropometric* | | |
| Age; years | 22.4 ± 2.2 | 23.1 ± 2.7 |
| Body-mass index; kg/m$^2$ | 20.1 ± 1.1 | 21.5 ± 2.1 |
| Average duration of amenorrhea; days | 305 ± 124 | NA |
| Menses in past 12 months | NA | 12 ± 1 |
| $VO_{2peak}$; mL/kg/min | 47.6 ± 6.4 | 46.0 ± 5.7 |
| Average exercise duration; min/week | 340 ± 136 | 271 ± 153 |
| *Dietary* | | |
| Energy intake; kcal/d | 1877 ± 805 | 1999 ± 317 |
| Dietary iron intake; mg/d | 18.6 ± 8 | 19.5 ± 7.2 |
| Iron RDA met | 66 (61%) | 34 (42%)** |
| Heme iron; mg/d | 2.0 ± 2.1 | 1.8 ± 2.8 |
| Non-heme iron; mg/d | 14.0 ± 3.2 | 14.6 ± 7.8 |
| Meat, fish, or poultry; g/d | 148 ± 146 | 158 ± 102 |
| Tea and coffee; cups/d | 2.8 ± 2.1 | 1.8 ± 2.5 |
| Vitamin C; mg/d | 269 ± 180 | 129 ± 141*** |
| Calcium; mg/d | 1785 ± 817 | 1826 ± 1011 |
| Carbohydrate intake; g/d | 246 ± 112 | 287 ± 93 |
| Protein intake; g/d | 87 ± 37 | 85 ± 18 |
| Fat intake; g/d | 61 ± 29 | 68 ± 19 |
| *Iron Status* | | |
| Iron-Depleted | 12(15%) | 28(26%)* |
| Iron-Sufficient | 70(85%) | 81(74%)* |
| Ferritin; μg/L | 30.2 ± 2.2 | 24.9 ± 2.6 |
| Soluble transferrin receptor; mg/L | 5.2 ± 1.4 | 4.9 ± 1.5 |
| Total body iron[a]; mg/kg | 5.3 ± 2.7 | 4.8 ± 3.7 |
| Hemoglobin; g/dL | 13.2 ± 0.4 | 13.4 ± 0.6 |
| Hematocrit; % | 39.5 ± 0.8 | 39.8 ± 4.1 |
| Alpha-1-acid glycoprotein; g/L | 0.38 ± 0.2 | 0.39 ± 0.2 |
| Elevated alpha-1-acid glycoprotein[b] | 3 (3.7%) | 0 (0%) |
| Elevated C-reactive protein[c] | 2(2.4%) | 1(3.5%) |

Ferritin and soluble transferrin receptor are geometric mean ± geometric SD; all other values are arithmetic mean ± SD.
Iron-Depleted: Ft < 15 μg/L; Iron Sufficient: Ft ≥ 15 μg/L.
* Significantly different from AMEN ($p < 0.05$; one-tailed $X^2$ test).
** Significantly different from AMEN ($p < 0.01$; two-tailed $X^2$ test).
*** Significantly different from AMEN ($p < 0.001$; two-tailed T-test).
[a] Total body iron was calculated as −[log(soluble transferrin receptor/ferritin) − 2.8299]/0.1207.
[b] Elevated alpha-1-acid glycoprotein was > 1 g/L.
[c] C-reactive protein was determined qualitatively at a threshold of 10 mg/ml.

prevalence (25% vs. 27%, p = 0.85) or iron status indices between "high" and "low" menstrual bleeding groups (data not shown). Menstrual bleeding intensity and menstrual bleeding duration were not significantly correlated with any iron status indices (data not shown).

In AMEN, the average duration of amenorrhea prior to baseline was 305 ± 124 days and the duration of amenorrhea was not significantly correlated with any iron status indices. In AMEN, 60/82 (73%) were amenorrheic for at least 6 months and 22/82 (27%) were amenorrheic for less than 6 months. Between these groups, there was not a significant difference in iron depletion prevalence (15% vs. 14%, p = 0.88) or iron status indices (data not shown).

## 4. Discussion

The present study is the first to compare iron status in exercising women with rigorously defined menstrual function and exclusion of individuals with non-physiological menstrual function (e.g. use of hormonal contraception). This analysis revealed that 28/109 (26%) of ovulatory, eumenorrheic exercising women were iron depleted compared to 12/82 (15%) of amenorrheic exercising women at a Ft threshold of 15 μg/L. Yet, iron status indices (Ft, sTfR, TBI, Hb, and Hct) were not significantly different between groups. This study also

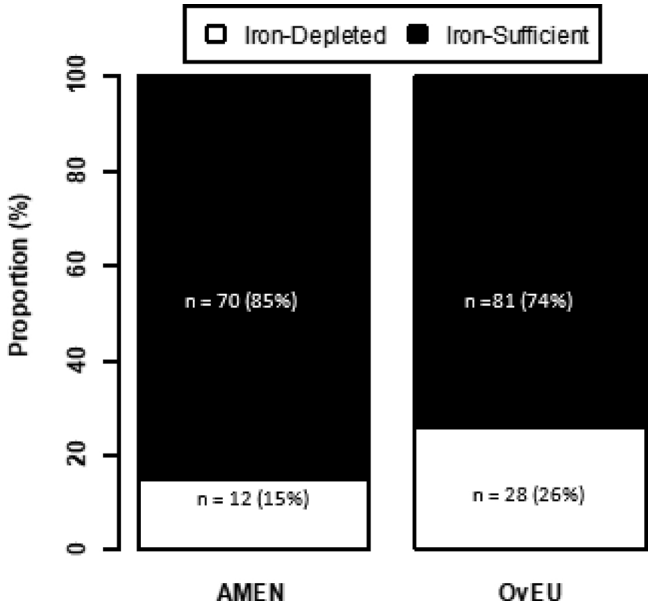


**Fig. 1.** Prevalence of iron depletion (Ft < 15 μg/L) in exercising women with amenorrhea (AMEN; n = 82) and exercising women with eumenorrheic, ovulatory cycles (OvEU; n = 109). Iron-Depleted: Ft < 15 μg/L; Iron-Sufficient: Ft ≥ 15 μg/L.

revealed that menstrual bleeding characteristics, duration of amenorrhea, exercise parameters, and dietary factors were not associated with differences in iron depletion prevalence or iron status indices.

Previous investigations support our findings of iron depletion prevalence in exercising women. Another investigation in exercising women using the same iron depletion threshold reported a similar prevalence of 24% [44]. As might be expected, the exercising women with amenorrhea in the present study displayed a similar iron depletion prevalence as exercising men [20]. An investigation of iron status in menstruating women and women with amenorrhea by Swenne revealed similar prevalence between groups as observed in the present study [45]. Thus, it seems that menses is associated with a greater risk of iron depletion and/or amenorrhea reduces the risk of iron depletion.

Despite a higher prevalence of iron depletion, iron status indices did not differ between the OvEU and AMEN groups. Previous investigations that observed a difference in iron depletion prevalence between groups also observed differences in iron status indices. For instance, the sTfR index-determined iron depletion prevalence of 50% in active women was accompanied by significantly lower Ft and higher sTfR when compared to sedentary women with an iron depletion prevalence of 17% [42]. Likewise, the findings by Swenne were substantiated by a 77% lower Ft concentration in menstruating women when compared to their amenorrheic counterparts [45]. Furthermore, the findings of the present study that iron status indices were not different between our groups contradict the findings by Wilson et al. that female military personnel with amenorrhea had a significantly greater Ft and Hb than their menstruating counterparts [39].

Several factors suggest that this secondary finding is novel rather than erroneous. The aforementioned investigations, as well as others not described in detail [46–50], did not control and assess for diet, exercise or inflammatory status. Further, these investigations did not confirm menstrual function via hormonal evaluation nor exclude users of hormonal contraception. Not controlling for these factors that affect iron status [51–53] may limit or confound the conclusions of prior investigations. For example, the Ft and Hb of the menstruating women studied by Wilson [39] may have been artificially elevated, as 41% of these women were taking hormonal contraception. Likewise, the amenorrheic women studied by Swenne [45] also had a diagnosis of anorexia nervosa, which is a condition associated with dramatically

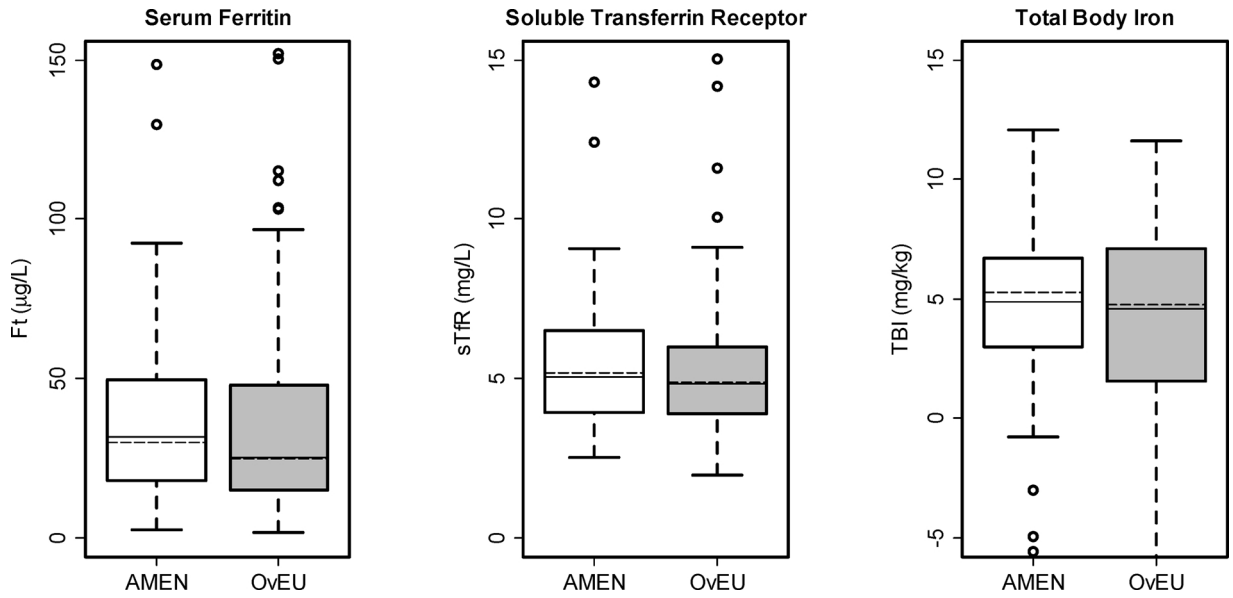


**Fig. 2.** Comparison of serum ferritin, soluble transferrin receptor, and total body iron in exercising women with amenorrhea (AMEN; n = 82) and exercising women with eumenorrheic, ovulatory cycles (OvEU; n = 109) Solid lines represent the median. Horizontal dashed lines represent the geometric mean for serum ferritin and soluble transferrin receptor; and the arithmetic mean for total body iron.

elevated Ft concentrations secondary to elevated hepcidin concentrations [54]. The methodological shortcomings of prior investigations may have led to inaccurate conclusions on the true relationship between physiological menstruation and iron status.

Given the robust study design, the secondary findings of the present study may indicate that menses plays a lesser role in iron stores in exercising women than previously thought. The average 14 mg of iron loss per menses accounts for only 0.4% of total body iron stores assuming an average body iron store of 3500 mg in women [55]. The only available evidence for the direct effect of menses on iron loss are large epidemiological studies, which have found that iron status indices change only slightly [34] or not at all [56–58] across the menstrual cycle. Furthermore, two smaller studies demonstrated menstruation to be associated with better iron status as indicated by greater Hb concentrations. Clancy et al. reported that higher Hb concentrations were associated with a thicker endometrium lining, even though greater endometrial thickness is correlated with greater menstrual blood loss [59,60]. Miller reported that women who resumed menstruation postpartum had a higher Hb concentration than those who had not yet resumed menstruation [61]. Other research has indicated that non-pathological menstrual blood loss, i.e. less than 80 mL, is not associated with an increased risk of iron depletion [62–64]. Thus, it appears that the direct effect of menstrual blood loss on iron status may be less understood than is currently presumed [65].

### 4.1. Iron status and menstrual bleeding characteristics

We demonstrated that menstrual bleeding intensity and duration were not associated with iron status. There was a similar prevalence of iron depletion and similar levels of iron status indices in exercising women reporting more intense and longer menstrual bleeding versus those reporting less intense and shorter menstrual bleeding. Additionally, menstrual bleeding intensity and duration were not correlated with any iron status indices. These findings were contrary to a previous investigation in the general population [17] that found menstrual bleeding and intensity were inversely associated with Ft. Given that the present investigation is adequately powered, it is possible that the relationship of self-reported menstrual bleeding intensity and duration with iron status in exercising women may be different in the exercising population.

Similarly, the prevalence of iron depletion and iron status indices did not vary by the duration of amenorrhea. This finding suggests that iron stores in our pre-menopausal, amenorrheic women do not accumulate over time due to a chronic absence of menses as is the case with post-menopausal women. It is possible that similar physiological mechanisms that prevent iron overload in men also prevent the accumulation of iron stores in exercising, amenorrheic women [2].

### 4.2. Limitations

We were unable to determine consumption of phytate, an inhibitor of iron absorption, and dietary intake was not recorded on a meal-by-meal basis. Thus, iron bioavailability, the proportion of dietary iron absorbed [66], could not be calculated. This limitation is noteworthy, as it has been reported that women with amenorrhea secondary to anorexia nervosa consume more phytate than healthy controls [67]. Iron bioavailability calculations would also determine if the greater dietary vitamin C in AMEN women increased iron bioavailability. Additionally, the CRP assessment was qualitative with a threshold of 10 mg/mL and thus unable to detect smaller differences in inflammation status. Greater sensitivity in CRP measurement may have provided insight regarding the relationship between inflammation associated with exercise and menstruation and iron status [68].

### 4.3. Strengths

The use of well-established methods for classification of menstrual status [32] allowed for the comparison of exercising women at opposite ends of the menstrual function spectrum. Since menstruation has been reported to be associated with poorer iron status, a comparison of exercising women with ovulatory, eumenorrheic menstrual cycles and exercising women with amenorrhea allowed for the greatest possible difference in iron status secondary to menstrual function to be assessed. The physiologic validity of this comparison was further strengthened by excluding users of hormonal contraception. Further, exercise duration and dietary factors between groups were assessed and demonstrated neither a significant effect on iron status nor differences between groups. Additionally, Ft was adjusted based on inflammation status to reduce any confounding effects of exercise-associated inflammation on iron status. Lastly, the statistical analysis was sufficiently powered to

observe small differences in iron status indices.

### 4.4. Future directions and conclusion

Overall, the findings in the present study demonstrate the effect of physiologic menstrual function on iron status which substantiates previous literature on menstrual blood loss and iron status and provide new insight on iron metabolism in women. The primary finding was that iron depletion was higher in exercising women with ovulatory, eumenorrheic cycles compared to exercising women with amenorrhea. Exercising women with ovulatory, eumenorrheic cycles should be made aware of their increased risk of ID, though the risk should not supercede the importance of regular menstruation.

Other physiologic variables associated with menstrual function in exercising women likely play underappreciated roles in iron metabolism and should be investigated. Low energy availability, defined as energy intake minus exercise energy expenditure [69], triggers biological conservation of energy, such as reproductive suppression resulting in EAMD [32]. Low energy availability may also result in iron conservation, given that obesity (a state of high energy availability) affects iron metabolism [70,71]. Lastly, it should be determined if progesterone and estradiol exposure are associated with iron status indices, given that these hormones regulate hepcidin [72,73].

In conclusion, the present study demonstrated that exercising women with ovulatory, eumenorrheic menstrual cycles have a greater risk of iron depletion when compared to amenorrheic exercising women. The present study was the first to confirm that physiological menstrual function is linked to poorer iron status in an exercising population. As a result, the development of iron depletion and subsequent progression to ID may be avoided in a population already at risk of other significant health sequelae.

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## Declarations of interest

None.

## References

[1] J. Beard, B. Tobin, Iron status and exercise, Am. J. Clin. Nutr. 72 (2) (2000) 594s–597s.
[2] J.L. Beard, Iron biology in immune function, muscle metabolism and neuronal functioning, J. Nutr. 131 (2) (2001) 568S–580S.
[3] T.G. Nazem, K.E. Ackerman, The female athlete triad, Sports Health 4 (4) (2012) 302–311.
[4] D.L. Petkus, L.E. Murray-Kolb, M.J. De Souza, The unexplored crossroads of the female athlete triad and Iron deficiency: a narrative review, Sport. Med. (2017) 1–17.
[5] WHO, Iron Deficiency Anaemia: Assessment, Prevention and Control: a Guide for Programme Managers, [cited 2017 August 25]; Available from: (2001) http://www.who.int/nutrition/publications/micronutrients/anaemia_iron_deficiency/WHO_NHD_01.3/en/.
[6] F. Bermejo, S. García-López, A guide to diagnosis of iron deficiency and iron deficiency anemia in digestive diseases, World J. Gastroenterol. WJG 15 (37) (2009) 4638–4643.
[7] M. Fogelholm, Indicators of vitamin and mineral status in athletes' blood: a review, Int. J. Sport Nutr. 5 (4) (1995) 267–284.
[8] P. Peeling, et al., Athletic induced iron deficiency: new insights into the role of inflammation, cytokines and hormones, Eur. J. Appl. Physiol. 103 (4) (2008) 381.
[9] P. Peeling, et al., Effects of exercise on hepcidin response and iron metabolism during recovery, Int. J. Sport Nutr. Exerc. Metab. 19 (6) (2009) 583–597.
[10] T. Ganz, Hepcidin and its role in regulating systemic iron metabolism, ASH Educ. Prog. Book 2006 (1) (2006) 29–35.
[11] I. Cavill, Iron status as measured by serum ferritin: the marker and its limitations, Am. J. Kidney Dis. 34 (4 Suppl 2) (1999) S12–7.
[12] A. Akesson, et al., Serum transferrin receptor: a specific marker of iron deficiency in pregnancy, Am. J. Clin. Nutr. 68 (6) (1998) 1241–1246.
[13] J.P. McClung, et al., Longitudinal decrements in iron status during military training in female soldiers, Br. J. Nutr. 102 (4) (2009) 605–609.
[14] J.R. Hunt, C.A. Zito, L.K. Johnson, Body iron excretion by healthy men and women, Am. J. Clin. Nutr. 89 (6) (2009) 1792–1798.
[15] L.J. Harvey, et al., Impact of menstrual blood loss and diet on iron deficiency among women in the UK, Br. J. Nutr. 94 (4) (2005) 557–564.
[16] A.-L.M. Heath, et al., The role of blood loss and diet in the aetiology of mild iron deficiency in premenopausal adult New Zealand women, Public Health Nutr. 4 (2) (2001) 197–206.
[17] N. Milman, J. Clausen, K.-E. Byg, Iron status in 268 Danish women aged 18–30 years: influence of menstruation, contraceptive method, and iron supplementation, Ann. Hematol. 77 (1) (1998) 13–19.
[18] G. Moschonis, et al., Association of Iron depletion with menstruation and dietary intake indices in pubertal girls: the healthy growth study, Biomed Res. Int. 2013 (2013) 423263.
[19] C. Kim, et al., Changes in iron measures over menopause and associations with insulin resistance, J. Womens Health 21 (8) (2012) 872–877.
[20] T. Rowland, Iron deficiency in athletes, Am. J. Lifestyle Med. 6 (4) (2012) 319–327.
[21] G. Bruinvels, et al., The prevalence and impact of heavy menstrual bleeding (menorrhagia) in elite and non-elite athletes, PLoS One 11 (2) (2016) e0149881.
[22] J.C. Gibbs, N.I. Williams, M.J. De Souza, Prevalence of individual and combined components of the female athlete triad, Med. Sci. Sports Exerc. 45 (5) (2013) 985–996.
[23] M.J. De Souza, et al., High frequency of luteal phase deficiency and anovulation in recreational women runners: blunted elevation in follicle-stimulating hormone observed during luteal-follicular transition, J. Clin. Endocrinol. Metab. 83 (12) (1998) 4220–4232.
[24] M.J. De Souza, et al., High prevalence of subtle and severe menstrual disturbances in exercising women: confirmation using daily hormone measures, Hum. Reprod. 25 (2) (2010) 491–503.
[25] M.T. Barrack, et al., Higher incidence of bone stress injuries with increasing female athlete triad–related risk factors: a prospective multisite study of exercising girls and women, Am. J. Sports Med. 42 (4) (2014) 949–958.
[26] E. O'Donnell, et al., Long-term estrogen deficiency lowers regional blood flow, resting systolic blood pressure, and heart rate in exercising premenopausal women, Am. J. Physiol.-Endocrinol.Metab. 292 (5) (2007) E1401–E1409.
[27] A. Rickenlund, et al., Amenorrhea in female athletes is associated with endothelial dysfunction and unfavorable lipid profile, J. Clin. Endocrinol. Metab. 90 (3) (2005) 1354–1359.
[28] K. Łagowska, K. Kapczuk, J. Jeszka, Nine–month nutritional intervention improves restoration of menses in young female athletes and ballet dancers, J. Int. Soc. Sports Nutr. 11 (1) (2014) 52.
[29] R.J. Mallinson, et al., A case report of recovery of menstrual function following a nutritional intervention in two exercising women with amenorrhea of varying duration, J. Int. Soc. Sports Nutr. 10 (1) (2013) 34.
[30] M.J. De Souza, et al., Female Athlete Triad Coalition Consensus Statement on treatment and return to play of the female athlete triad: 1st International Conference held in San Francisco, California, may 2012 and 2nd International Conference held in Indianapolis, Indiana, May 2013, Br. J. Sports Med. 48 (4) (2014) 289–289.
[31] Medicine, T.P.C.o.t.A.S.f.R., Current evaluation of amenorrhea, Fertil. Steril. 82 (2004) 33–39.
[32] M.J. De Souza, N.I. Williams, Physiological aspects and clinical sequelae of energy deficiency and hypoestrogenism in exercising women, Hum. Reprod. Update 10 (5) (2004) 433–448.
[33] J.L. Scheid, et al., Elevated PYY is associated with energy deficiency and indices of subclinical disordered eating in exercising women with hypothalamic amenorrhea, Appetite 52 (1) (2009) 184–192.
[34] I. Kim, E.A. Yetley, M.S. Calvo, Variations in iron-status measures during the menstrual cycle, Am. J. Clin. Nutr. 58 (5) (1993) 705–709.
[35] D.I. Thurnham, et al., Adjusting plasma ferritin concentrations to remove the effects of subclinical inflammation in the assessment of iron deficiency: a meta-analysis, Am. J. Clin. Nutr. 92 (3) (2010) 546–555.
[36] I. Kasvosve, et al., Association of serum transferrin receptor concentration with markers of inflammation in Zimbabwean children, Clin. Chim. Acta 371 (1) (2006) 130–136.
[37] J.D. Cook, C.H. Flowers, B.S. Skikne, The quantitative assessment of body iron, Blood 101 (9) (2003) 3359–3363.
[38] M. Worwood, *Indicators of the Iron Status of Populations: Ferritin.* WHO, CDC. Assessing the Iron Status of Populations, 2nd ed., World Health Organization, Geneva, 2007, pp. 35–74.
[39] C. Wilson, et al., Iron status of military personnel deployed to Afghanistan, Mil. Med. 176 (12) (2011) 1421–1425.
[40] L. Hallberg, Perspectives on nutritional iron deficiency, Annu. Rev. Nutr. 21 (1) (2001) 1–21.
[41] ODS. Iron: Dietary Supplement Fact Sheet. 2016 [cited 2018 January 17]; Available from: https://ods.od.nih.gov/factsheets/Iron-HealthProfessional/#h2.
[42] K. Woolf, et al., Iron status in highly active and sedentary young women, Int. J. Sport Nutr. Exerc. Metab. 19 (5) (2009) 519–535.
[43] Medicine, A.C.o.S., ACSM's Guidelines for Exercise Testing and Prescription, Lippincott Williams & Wilkins, Baltimore, MD, 2014.
[44] S.S. Gropper, et al., Iron status of female collegiate athletes involved in different sports, Biol. Trace Elem. Res. 109 (1) (2006) 1–13.
[45] I. Swenne, Haematological changes and iron status in teenage girls with eating disorders and weight loss—the importance of menstrual status, Acta Pædiatrica 96 (4) (2007) 530–533.

[46] T.W. Rowland, J.F. Kelleher, *Iron deficiency in athletes. Insights from high school swimmers*, Am. J. Dis. Child. 143 (2) (1989) 197–200.
[47] T.W. Rowland, S.A. Black, J.F. Kelleher, Iron deficiency in adolescent endurance athletes, J. Adolesc. Health Care 8 (4) (1987) 322–326.
[48] G. Dubnov, N.W. Constantini, Prevalence of iron depletion and anemia in top-level basketball players, Int. J. Sport Nutr. Exerc. Metab. 14 (1) (2004) 30–37.
[49] K.E. Fallon, Screening for haematological and iron-related abnormalities in elite athletes—analysis of 576 cases, J. Sci. Med. Sport 11 (3) (2008) 329–336.
[50] S. Mettler, M. Zimmermann, Iron excess in recreational marathon runners, Eur. J. Clin. Nutr. 64 (5) (2010).
[51] K. Koehler, et al., Iron status in elite young athletes: gender-dependent influences of diet and exercise, Eur. J. Appl. Physiol. 112 (2) (2012) 513–523.
[52] G. Casabellata, et al., Evaluation of iron deficiency in young women in relation to oral contraceptive use, Contraception 76 (3) (2007) 200–207.
[53] I. Auersperger, et al., Exercise-induced changes in iron status and hepcidin response in female runners, PLoS One 8 (3) (2013) e58090.
[54] S. Papillard-Marechal, et al., Iron metabolism in patients with anorexia nervosa: elevated serum hepcidin concentrations in the absence of inflammation, Am. J. Clin. Nutr. 95 (3) (2012) 548–554.
[55] S.S. Gropper, Advanced Nutrition and Human Metabolism, (2012), pp. 481–498.
[56] J. Puolakka, Serum ferritin in the evaluation of iron status in young healthy women, Acta Obstet. Gynecol. Scand. Suppl. 95 (1980) 35–41.
[57] O.D. Vellar, *Changes in hemoglobin concentration and hematocrit during the menstrual cycle. I. A cross-sectional study*, Acta Obstet. Gynecol. Scand. 53 (3) (1974) 243–246.
[58] A. Belza, et al., Day-to-day variation in iron-status measures in young iron-deplete women, Br. J. Nutr. 94 (4) (2005) 551–556.
[59] K.B. Clancy, Reproductive ecology and the endometrium: physiology, variation, and new directions, Am. J. Phys. Anthropol. 140 (Suppl 49) (2009) 137–154.
[60] K.B. Clancy, I. Nenko, G. Jasienska, Menstruation does not cause anemia: endometrial thickness correlates positively with erythrocyte count and hemoglobin concentration in premenopausal women, Am. J. Hum. Biol. 18 (5) (2006) 710–713.
[61] E.M. Miller, Maternal hemoglobin depletion in a settled northern Kenyan pastoral population, Am. J. Hum. Biol. 22 (6) (2010) 768–774.
[62] R.L. Cheong, M.D. Kuizon, R.T. Tajaon, Menstrual blood loss and iron nutrition in Filipino women, Southeast Asian J. Trop. Med. Public Health 22 (4) (1991) 595–604.
[63] A.T. Andrade, et al., Menstrual blood loss and body iron stores in Brazilian women, Contraception 43 (3) (1991) 241–249.
[64] J. Gao, et al., Menstrual blood loss and hematologic indices in healthy Chinese women, J. Reprod. Med. 32 (11) (1987) 822–826.
[65] E.M. Miller, The reproductive ecology of iron in women, Am. J. Phys. Anthropol. 159 (Suppl 61) (2016) S172–95.
[66] R. Hurrell, I. Egli, Iron bioavailability and dietary reference values, Am. J. Clin. Nutr. 91 (5) (2010) 1461S–1467S.
[67] M. Misra, et al., Nutrient intake in community-dwelling adolescent girls with anorexia nervosa and in healthy adolescents, Am. J. Clin. Nutr. 84 (4) (2006) 698–706.
[68] K.B.H. Clancy, A.R. Baerwald, R.A. Pierson, Systemic inflammation is associated with ovarian follicular dynamics during the human menstrual cycle, PLoS One 8 (5) (2013) e64807.
[69] A.B. Loucks, Energy Balance and Energy Availability, in The Encyclopaedia of Sports Medicine, John Wiley & Sons Ltd., 2013, pp. 72–87.
[70] C. Becker, et al., Iron metabolism in obesity: how interaction between homoeostatic mechanisms can interfere with their original purpose. Part I: underlying homoeostatic mechanisms of energy storage and iron metabolisms and their interaction, J. Trace Elem. Med. Biol. 30 (2015) 195–201.
[71] C. Becker, et al., Iron metabolism in obesity: how interaction between homoeostatic mechanisms can interfere with their original purpose. Part II: epidemiological and historic aspects of the iron/obesity interaction, J. Trace Elem. Med. Biol. 30 (2015) 202–206.
[72] Y. Hou, et al., Estrogen regulates iron homeostasis through governing hepatic hepcidin expression via an estrogen response element, Gene 511 (2) (2012) 398–403.
[73] X. Li, et al., Progesterone receptor membrane component-1 regulates hepcidin biosynthesis, J. Clin. Invest. 126 (1) (2016) 389–401.