A HIGHER PREVALENCE OF IRON DEPLETION IN EUMENORRHEIC EXERCISING WOMEN THAN AMENORRHEIC EXERCISING WOMEN

A Thesis in
Physiology
by
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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 2016
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ABSTRACT

Exercising women are endangered to the health consequences of suboptimal iron status. At the same time, exercising women are also susceptible to declines in menstrual status associated with poor energy status in a condition known as the female athlete triad. Although compromised iron status and declines in menstrual status are common in exercising women, little is known about how these conditions interact in exercising women. The purpose of this thesis was to investigate the effect of menstrual status on iron status in exercising women with amenorrhea (AMEN; n=36) and exercising women with ovulatory, eumenorrheic cycles (OvEU; n=28). As such, we performed a cross-sectional analysis of iron status markers (serum ferritin (Ft), soluble transferrin receptor (sTfR), total body iron (TBI), hemoglobin (Hb), and hematocrit) and the prevalence of iron depletion (Ft <15 μg/L) between AMEN and OvEU aged 18-35 years who performed at least 2 hours per week of exercise. We investigated the relationship of iron status with menstrual bleeding intensity and menstrual bleeding duration in OvEU and amenorrhea duration in AMEN. Exercise parameters and dietary factors were also assessed between groups and as covariates of iron status markers. The main findings were that the prevalence of iron depletion was greater in OvEU at 25% when compared to AMEN at 8%. Despite this finding, no significant differences were observed between AMEN and OvEU for Ft, sTfR, TBI, Hb, and hematocrit. In addition, menstrual status characteristics were not associated with iron status markers or the prevalence of iron depletion. Exercise parameters and dietary factors were neither significantly different between groups nor covariates of iron status markers. Future investigations should aim to increase sample size without compromising the menstrual status classification in this investigation that allowed for a true assessment of the effect of menstrual status on iron status.
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Abbreviations

AGP: alpha-1-acid glycoprotein

AMEN: Exercising women with amenorrhea

ATP: Adenosine Triphosphate

BMD: Bone mineral density

CTx: C-terminal telopeptide

CRP: C-reactive protein

EAMD: Exercise-associated menstrual disturbances

E1G: estrone-1-glucuronide

Ft: Serum ferritin

GH: Growth hormone

GLP-1: glucagon-like peptide 1

HIF-1: Hypoxia-inducible factor-1

IGF-1: Insulin-like growth factor-1

LH: luteinizing hormone

OvEU: Exercising women with ovulatory, eumenorrheic cycles

PdG: pregnanediol glucuronide

PP: pancreatic peptide
P1NP: N-terminal propeptide of type I collagen

NTx: N-terminal telopeptide

sTfR: Soluble transferrin receptor

T3: Triiodothyronine

T4: Thyroxine

TBI: Total body iron

TRH: Thyroid releasing hormone

TSH: Thyroid stimulating hormone

VO_{peak}: peak oxygen consumption
Acknowledgements

Dr. De Souza, thank you for your guidance in this chapter of my life. You have given me more opportunities for personal growth in three years than I could ever imagine. Thanks to you, I can leave Penn State confidently knowing I have the skills needed to succeed in the next chapter in my life.

Dr. Williams, thank you for guidance in this thesis and in research. You’ve taught me by example to question everything and to keep a playful spirit in a field that others often take too serious.

Dr. Murray-Kolb, thank you for your iron expertise and valuable input to my work. I truly valued collaborating with your lab and experiencing the field of nutritional sciences.

Dr. Koehler, thank you for taking me under your wing during your time at Penn State. I owe my current abilities in writing, computer programming, and even shirt ironing to you.

I’d like to thank Jay, Heather, Emily, Clara, Ellen, and especially Sam “the kid” Scott for their continuous support in my work.

I also thank my parents and sisters who have been an endless supply of love and support in my life.

“Keep your nose out the sky, keep your heart to god, and keep your face to the rising sun.”
Chapter 1: Introduction

Iron Status and Menstrual Status in Exercising Women

Exercising women are particularly prone to suboptimal iron status, which can compromise their health and sports performance (Beard, 2000; 2001). Iron status, a representation of whole body iron content, is categorized into four stages of progressively decreasing iron in storage and/or functional compartments: iron sufficiency, iron depletion, iron deficiency without anemia, and iron deficiency with anemia (Chung et al., 2013). Declines in iron status are linked to compromised immune function, decreased metabolic efficiency, and cognitive impairments with more severe health sequelae occurring at more severe stages (Beard, 2000; 2001). In exercising women, iron depletion is linked to decreased endurance performance and suppressed training adaptations (Zhu et al., 1997; Brownlie 2004; DellaValle et al., 2011; Dellavalle & Haas, 2012). With the prevalence of iron depletion ranging from 24 to 47% among exercising women (Rowland, 2012), it is imperative to fully understand the etiology of suboptimal iron status in exercising women to ensure optimal health and performance.

The relatively high prevalence of iron depletion in exercise women can be partly explained by the exercise-associated alterations in iron metabolism. Exercise reduces iron status via increasing iron losses from the body and reducing iron availability within the body (Peeling et al., 2008; Auersperger et al., 2013). Exercise-associated iron losses can occur via increased sweating, exercise induced hemolysis, and gastro-intestinal bleeding (Peeling et al., 2008). Although iron losses through these mechanisms are considered negligible during a single bout of exercise, iron losses of 0.25 mg via hemolysis (Poortmans, 1979), 1.8 mg via sweat (DeRuisseau et al., 2002), and as much as 5 mg via gastro-intestinal bleeding (Nachtigall et al., 1996) during prolonged exercise indicate that exercise-associated iron losses can accumulate through chronic exercise to potentially compromise iron status. Exercise can further
compromise iron status by upregulating hepcidin, a key regulator of iron metabolism that reduces cellular iron availability (Ganz, 2006). These exercise-associated alterations in iron metabolism likely underlie the greater iron deficiency prevalence rates in exercising women of 37% in comparison to the 24% in sedentary women (Fogelholm, 1995). Furthermore, iron deficiency prevalence rates can increase from 7.4% to 18% following only nine weeks of basic combat training (McClung et al., 2009).

In addition to exercise-associated alterations in iron metabolism, iron depletion in exercise women is also driven by menstrual blood loss. Menstrual blood loss account for 14mg of iron loss per menses (Hunt et al., 2009) which underlies the higher prevalence iron deficiency prevalence rates in premenopausal women of 11% in comparison to 1% in men in the general population of the United States (Looker et al., 1997). Further, it is well documented that women with a greater intensity and greater duration of menstrual bleeding are at a greater risk of iron depletion (Harvey et al., 2005; Milman et al. 1998; Heath et al., 2001). Additionally, changes in menstrual function, such as the onset of menses during puberty as well as the cessation of menses during menopause, are associated with concomitant changes in iron status, i.e., a decline in iron status following menarche (Moschonis et al., 2013), and an improvement in iron status following menopause (Kim et al., 2012).

The role of menses in iron status is important for the health of exercising women since up to 56% of exercising women experience exercise-associated menstrual disturbances (EAMD) (Gibbs, 2013). EAMD include both subclinical presentations, luteal phase defects and anovulation, and clinical presentations, oligomenorrhea and amenorrhea (De Souza et al., 1998, 2010). EAMD are associated with decreased bone health, increased incidence of stress fractures (Barrack et al., 2014), infertility (Stafford, 2005), endothelial dysfunction (O'Donnell et al., 2007), and atherogenic blood lipid profiles (Rickenlund et al., 2005). As such, current
treatment strategies for EAMD view the restoration of regular menstrual status as an indicator of good health (Mallinson et al., 2013; Lagowska et al., 2014). However, it is currently unknown if the restoration of menstrual status in women with EAMD can compromise iron status. As such, the purpose of this thesis is to explore the associations of menstrual status and iron status in exercising women.

Aims and Hypotheses

**Aim 1:** To determine if eumenorrheic ovulatory exercising women and amenorrheic exercising women differ with respect to iron status, as indicated by a difference in the prevalence of iron depletion ($Ft < 15\mu g/L$), and in the concentration of iron status markers, to include serum ferritin, serum soluble transferrin receptor, total body iron, hemoglobin and hematocrit.

**Hypothesis 1:** Exercising women with eumenorrheic ovulatory menstrual cycles will exhibit a compromised iron status, as indicated by a higher prevalence of iron depletion, and diminished concentrations of iron status markers, to include a lower serum ferritin, greater soluble transferrin receptor, lower total body iron, lower hemoglobin and lower hematocrit, when compared to exercising women with amenorrhea.

**Aim 2A:** To determine if self-reported menstrual bleeding duration and menstrual bleeding intensity in eumenorrheic ovulatory exercising women are associated with iron status, as indicated by the prevalence of iron depletion, and by the concentrations of markers of iron status.

**Hypothesis 2A:** Women with self-reported menstrual bleeding duration of 5 days or more and self-described menstrual bleeding as “heavy” will exhibit a compromised iron status, as indicated by a higher prevalence of iron depletion, and diminished iron status markers, as indicated by lower serum ferritin, greater soluble transferrin receptor, lower total body iron, lower
hemoglobin, and lower hematocrit concentration, when compared to women with self-reported menstrual bleeding duration of less than 5 days and self-described menstrual bleeding severity described as “light” or “moderate.”

**Aim 2B:** To determine if the duration of amenorrhea in exercising women is associated with compromised iron status, as indicated by the prevalence of iron depletion, and the concentration of markers of iron status.

**Hypothesis 2B:** Women who have been amenorrheic for a longer duration (greater than 6 months) will exhibit a “healthy” iron status, as indicated by a lower prevalence of iron depletion, and higher iron status markers, defined by greater serum ferritin, lower soluble transferrin receptor, greater total body iron, greater hemoglobin, and greater hematocrit concentrations, when compared to women who have been amenorrheic for a shorter duration (less than 6 months).

**Rationale**

The relatively high prevalence rates of both iron depletion and EAMD in exercising women warrants further investigation on the associations between iron status and menstrual status. Knowledge on the association between these conditions would provide critical health information to exercising women of different menstrual status. Exercising women with menses may be at an increased risk of iron depletion and may need to consider supplementation. Exercising women with EAMD may inadvertently increase their risk of iron depletion following restoration of menses. However, it is currently unknown how iron status and menstrual status in exercising women are associated. Thus, the current study aims to evaluate the association between iron status and menstrual status, as indicated by the prevalence of iron depletion (serum ferritin <15μg/L), and by the concentrations of iron status markers: serum ferritin, soluble
serum transferrin receptor, total body iron, hemoglobin, and hematocrit, in eumenorrheic ovulatory exercising women and amenorrheic exercising women.

Several aspects of this thesis will assist in this aim. 1) Each participant’s menstrual status was classified as either ovulatory, eumenorrheic or amenorrheic via analysis of urinary sex hormone metabolites of estrogen and progesterone using methods as described in chapter 3. Previous investigators only classified menstrual status via self-reports. Without hormonal analysis, it is unknown if the menstrual statuses reported were accurate or if subclinical menstrual disturbances may have been present to confound investigation findings. 2) Detailed hormonal classification of menstrual status allows for the comparison of exercising women at opposite ends of the menstrual status spectrum. This comparison should provide the greatest possible difference in the prevalence of iron depletion and iron status markers due to menstrual status based on previous findings in the literature. The presence of menses may compromise iron status as indicated by post-menarcheal adolescents displaying greater iron depletion prevalence rates of 34% in comparison to 16% in premenarcheal adolescents (Moschonis et al., 2013). The absence of menses may improve iron status as indicated by iron depletion prevalence rates in premenopausal women decreasing from 13% to 0% after menopause (Kim et al., 2012). Further, women with amenorrhea display a lower prevalence of iron depletion and improved iron status markers in comparison to women with menses ( Swenne, 2007; Papillard-Marechal et al., 2012). 3) Unlike previous investigations in both general and exercising populations, this thesis excludes users of hormonal contraception, which is known to reduce menstrual blood loss and improve iron status and iron status markers ( Milman et al, 1993; Milman et al., 1998; Heath et al., 2001). 4) This thesis accounts for possible exercise-associated inflammation that may affect iron status (Peeling et al., 2008) by adjusting for falsely elevated ferritin concentrations in individuals with inflammation. 5) Lastly, exercise parameters and dietary iron intake, known to affect iron status biomarkers (Beard, 2000), are included in the
analysis to assess their potential as covariates. Thus, this thesis provides an effective methodology to elucidate associations between iron status and menstrual status in exercising women.

**Expected Findings**

The expected iron depletion (serum ferritin <15 μg/L) (Hallberg, 2001) prevalence rates in our exercising women are based on previous investigations in women with similar menstrual status at the same iron depletion cut-off. Previous investigations observed that exercising women displayed iron depletion prevalence rates of 24% and 28% using the same threshold (Gropper et al., 2006; Mettler et al, 2010). Despite these investigations including users of hormonal contraception, we expect similar iron depletion prevalence rates in our exercising women with ovulatory, eumenorrheic cycles. The iron depletion prevalence rates in our exercising women with amenorrhea are expected to be similar to the 10% observed in women with anorexia and amenorrhea (Swenne, 2007). Since the expected findings are based on study populations slightly different than our exercising women, the iron depletion prevalence rates of exercising women with ovulatory, eumenorrheic cycles and exercising women with amenorrhea will be defined in this study.

The expected difference in iron status markers are based on an investigation similar to this thesis. Wilson et al. (2011) observed that military personnel with amenorrhea displayed a greater serum ferritin concentrations (55.9±2.1 vs. 34.8±2.0 μg/L), similar soluble transferrin receptor concentrations (1.14±1.1 vs. 1.15±0.11 mg/L), and greater hemoglobin concentrations (14.1±0.9 vs. 13.6±1.1 g/dL) in comparison to their counterparts with menses. These findings suggest that amenorrhea has a protective effect on iron status. However, 39% of the menstruating military personnel used hormonal contraception, which are associated with decreased prevalence rates of iron depletion (Larsson et al., 1992; Milman, et al, 1993; Milman
et al., 1998). As such, the findings of Wilson et al. (2011) may have been confounded by hormonal contraception. Regardless, we expect that amenorrhea will have a protective effect on iron status markers.

The expected associations between self-reported menstrual characteristics and iron status in exercising women with ovulatory, eumenorrheic cycles are based on a relatively large investigation in the general population. Milman et al. (1998) observed that the duration and intensity of menses were negatively correlated with Ft ($r=-0.25$, $p=0.001$; $r=-0.27$, $p=0.001$ respectively) in 268 young women. As such, we expect that self-reports of greater duration and intensity to be associated with poorer iron status. The expected associations between the duration of amenorrhea and iron status in exercising women with amenorrhea are based on investigations of amenorrheic women with anorexia nervosa. Amenorrheic women with anorexia nervosa display greater iron stores when compared to healthy controls to suggest that iron stores might accumulate during amenorrhea (Binsbergen et al., 1988, Papillard-Marechal et al., 2012). As such, we expect a greater duration of amenorrhea to be associated with better iron status.
References


Chapter 2: Literature Review

The Unexplored Crossroads of the Female Athlete Triad and Iron Deficiency

A. Introduction

With 24 to 47% of exercising women experiencing iron deficiency non-anemic (Rowland, 2012), exercising women are prone to several health and sports performance detriments. Iron deficiency compromises immune function, decreases metabolic efficiency, and impairs cognitive function (Beard 2000; 2001). Further, iron deficiency non-anemic in exercising individuals is associated with diminished maximum aerobic capacity, decreased metabolic efficiency, and hindered exercise adaptations (DellaValle et al., 2011; Dellavalle et al., 2012; Pasricha et al., 2014). Despite the high prevalence and severe consequences of iron deficiency, little is known about how iron deficiency may interact with another prevalent and severe condition in exercising women: the Female Athlete Triad (Triad). The Triad is a syndrome of three interrelated conditions: poor energy status, compromised reproduction function, and impaired bone health, all of which have profound implications for the health of exercising women (Nazem & Ackerman, 2012; Barrack et al., 2013). The systemic physiological changes associated with iron deficiency and the Triad present ample opportunity for these conditions to interact for adverse health and performance detriments. As such, this review aims to explore how iron deficiency and Triad components can interact.

B. SECTION 1: Iron Deficiency, Energy Status, and the Triad

Chronically poor energy status drives the compromised reproductive function and impaired bone health of the Triad (Nattiv et al., 2007). Energy status can be represented by energy availability, which refers to the energy remaining after exercise energy expenditure is subtracted from energy intake and is calculated as follows: (energy intake – exercise energy
expenditure)/kg lean body mass (Loucks et al., 1998; Loucks & Thuma, 2003). As such, low energy availability can be secondary to inadequate energy intake, with or without disordered eating. Low energy availability triggers energy conservation for vital biological processes by partitioning energy away from energetically intensive and non-vital biological processes, such as reproduction and growth (Wade & Jones, 2004). Low energy availability compromises reproductive function by disrupting the normal function of the hypothalamic-pituitary-ovarian axis (Stafford, 2005). Specifically, low energy availability reduces the pulsatile secretion of hypothalamic gonadotropin releasing hormone from the hypothalamus and decreases the frequency and amplitude of pituitary release of luteinizing hormone. In turn, ovarian sex steroid production and follicular development are suppressed which contributes to poor bone health (Loucks et al., 1998; Williams et al., 2001; Loucks & Thuma, 2003). To further conserve energy, low energy availability also suppresses total triiodothyronine, insulin-like growth factor-1, and leptin serum concentrations (Loucks et al., 1998; Williams et al., 2001; Loucks & Thuma, 2003; Scheid & De Souza, 2010). Further, low energy availability elevates growth hormone, cortisol, and ghrelin serum concentrations to increase the availability of scarce metabolic fuels (Loucks et al., 1998; Williams et al., 2001; Loucks & Thuma, 2003; Scheid & De Souza, 2010). These hormonal aberrations prioritize short-term energy conservation and acquisition while sacrificing long-term physiological processes, most notably reproductive function and bone health. Since poor energy status is fundamental to the Triad, the potential for iron deficiency to reduce energy status should be considered.

B-1. Impaired Thyroid Function

Thyroid metabolism is greatly impaired by iron deficiency (Zimmerman & Köhrle, 2002). Thyroid disorders and iron deficiency are frequent comorbidities with up to 44% of patients with thyroid disorders also presenting with iron deficiency (M’Rabet-Bensalah et al., 2015; Refaat,
Further, individuals with iron deficiency display 40% greater basal TSH concentrations (Gökdeniz et al., 2010; Ipek et al., 2011). Iron deficiency is causative in these associations given that iron deficiency in rodents suppresses the rise in serum thyroid stimulating hormone (TSH) concentrations following thyroid-releasing hormone (TRH) administration and suppresses basal serum TSH, thyroxine (T₄), and triiodothyronine (T₃) concentrations (Beard et al., 1984; Beard et al., 1989). Although it remains unknown how iron deficiency suppresses TSH secretion, it is known that iron deficiency suppresses serum T₄ and T₃ concentrations via two separate mechanisms. First, thyroid peroxidase uses iron as a co-factor in T₄ synthesis and, as a result, the activity of this enzyme is suppressed by 30-50% during iron deficiency anemia (Hess et al., 2002; Zimmermann & Köhrle, 2002). Second, iron deficiency anemia is associated with suppressed hepatic T₄-deiodinase activity such that 40% less T₄ is converted to the more active T₃ (Beard et al., 1989).

Iron deficiency associated impairments in thyroid function may progress the subclinical hypothyroidism present in exercising women with poor energy status, i.e., low energy availability (Berga et al., 1989; Loucks & Callister, 1993, Harber et al., 1998) to clinical hypothyroidism. As such, exercising women may start to suffer from several physical symptoms, such as cold sensitivity, hair loss, coarse skin, and constipation (Brănișteanu et al., 2014; Ghaemi et al., 2015; Keen et al., 2013; Melish, 1990; Neki et al., 2013). In addition, physical performance can suffer because hypothyroidism impairs cardiovascular hemodynamics and increases reliance on glycogen stores during exercise (Bangsbo et al., 2007; Gonçalves et al., 2006; Kahaly et al., 2002; McAllister et al., 1995). Psychological health may also deteriorate as hypothyroidism is linked to impaired cognitive function and increased feelings of anxiety and depression (Ritchie & Yeap, 2015). In sum, iron deficiency may potentiate the hypometabolic state associated with poor energy status by inducing hypothyroidism.
B-2. Reduced Metabolic Fuel Availability

Metabolic fuel availability may be reduced in exercising women via suppressed cortisol synthesis associated with iron deficiency. Cortisol synthesis requires 21-hydroxylase, which uses iron as a cofactor (Danielson, 2002; Ryan & Engel, 1957). As such, iron deficiency in rodents suppresses the rise in cortisol following adrenocorticotropic hormone administration (Saad et al., 1991). Such suppressed cortisol synthesis may reduce the elevated cortisol in exercising women that functions to enhance fuel availability (Dinneen et al., 1993; Djurhuus et al., 2002; Rizza et al., 1982). As a result, the low blood glucose concentrations in women with poor energy status (Berga et al., 1989, Loucks et al., 1989; Loucks et al., 1998) may be driven even lower. These low glucose concentrations may then enhance muscle catabolism due to impaired amino acid metabolism (Battezzati et al., 2000) and impair physical performance (Lambert et al., 2005) in exercising women.

Metabolic fuel availability may be further reduced as iron deficiency may promote growth hormone (GH) dysfunction in exercising women. Iron deficiency suppresses basal serum GH concentrations via suppressing serum ghrelin concentrations, a GH secretagogue. For instance, iron deficiency is associated with 60% lower serum ghrelin concentrations in children with iron deficiency than controls (Isguven et al., 2007) and an increasing severity of iron deficiency is associated with lower basal serum ghrelin concentrations (Akarsu et al., 2013). GH function is further impaired by iron deficiency associated GH resistance. Iron deficiency in calves is associated with a suppressed rise in serum insulin-like growth factor-1 concentrations following GH administration, despite suppressed basal serum GH concentrations (Ceppi & Blum, 1994). Based on these observations, iron deficiency can promote GH dysfunction in exercising women by suppressing basal serum GH concentrations and by promoting GH resistance (Ceppi & Blum, 1994; Gupta & Lee, 2012). Impaired GH function due to iron deficiency may progress the
mild GH resistance observed in exercising women with compromised reproductive function (Waters et al., 2001) to more severe GH resistance observed in adolescent girls with anorexia nervosa (Misra et al., 2003). As a result, iron deficiency associated exacerbations in GH function may reduce free fatty acid availability to further lower serum glucose concentrations and enhance muscle protein degradation (Møller & Jørgensen, 2009) in exercising women.

B-3. Eating Disorders and Disordered Eating

Restrictive eating behaviors, such as eating disorders and disordered eating, are a key determinant of inadequate energy intake in some exercising women. Clinical eating disorders, clinical disordered eating, and subclinical disordered eating prevalence rates among exercising women range from 0% to 49%, 7% to 89%, and 3% to 60%, respectively (Gibbs et al., 2013). Further, clinical eating disorder prevalence rates are higher in exercising women compared to the general public as evidenced by the 14% prevalence rate in elite women athletes in comparison to 3% in sedentary women (Martinsen & Sundgot-Borgen, 2013). These restrictive eating behaviors may underlie the 30% less energy intake relative to body weight in exercising women in comparison to exercising men (Loucks & Nattiv, 2005). The significance of these restrictive eating behaviors in the development of the Triad are further highlighted by disordered eating prevalence rates as high as 50% in exercising women with compromised reproductive function (Gibbs et al., 2013).

Since eating disorders and disordered eating can initiate the development of the Triad, it is important to understand that anxiety can initiate the development of eating disorders and disordered eating (Stice, 2002). Exercising women with eating disorders and disordered eating report greater somatic, trait, and state anxiety than exercising women without eating pathologies (Vardar et al., 2007; Silva, et al., 2011). This association is strengthened by the observation that 65% of women with an eating disorder also present with at least one anxiety disorder.
With reports of anxiety preceding the diagnosis of eating disorders in over 90% of women (Swinbourne et al., 2012), anxiety seems fundamental to the development of restrictive eating behaviors. These observations along with several others (Steere & Cooper, 1993; Evans & Wertheim, 1998; Fiore et al., 2014) demonstrate that anxiety can initiate eating disorders and disordered eating.

By increasing anxiety via neuroendocrine aberrations, iron deficiency may initiate eating disorders and disordered eating in exercising women. The synthesis of the neurotransmitters dopamine, serotonin, and norepinephrine all require iron as a co-factor (Kim & Wessling-Resnick, 2014). As such, iron deficiency anemia in rodents impairs dopamine function by decreasing dopamine receptors which in turn increases brain extracellular dopamine concentrations (Beard et al., 1994; Chen et al., 1995; Kwik-Uribe et al., 2000; Li et al., 2011). Similarly, iron deficiency anemia impairs serotonin and norepinephrine function by decreasing the degradation and transport of serotonin and norepinephrine (Mackler et al., 1978; 1980; Morse et al., 1999; Burhans et al., 2005). The associations of these neuroendocrine factors with anxiety (Durant et al., 2010), eating disorders (Barbarich et al., 2003), and anxiety-related behaviors in rats (Beard et al., 2002; Li et al., 2011) and infants with iron deficiency non-anemic (Deinard et al., 1981; Lozoff et al., 1982) suggest a common etiology among these conditions. Additionally, women with iron deficiency anemia report a reduced perceived quality of life and greater feelings of anxiety and depression than healthy controls (Gulmez et al., 2014; Peuranpää et al., 2014). These markers of well-being improve following treatment of iron deficiency via iron supplementation to further support the notion iron deficiency drives anxiety (Gulmez et al., 2014; Peuranpää et al., 2014). Based on these observations, iron deficiency associated increases in anxiety may play a role in the presence of eating disorders and disordered eating in exercising women.
Inadvertent undereating is another eating behavior that may be responsible for inadequate energy intake (Barrack et al., 2013). Exercise is well-known to suppress compensatory energy intake in response to the energy deficiency incurred by exercise energy expenditure (Blundell & King, 1998). Energy intake is suppressed for up to 48 hours following a single bout of exercise (King et al., 1997; Hagobian et al., 2013; Rocha et al., 2013, 2015) and remains suppressed during periods of habitual exercise of 1 to 3 weeks (Martin et al., 2002; Stubbs et al., 2002; Whybrow et al., 2008; Rosenkilde et al., 2015). More importantly, suppressed compensatory energy intake following exercise decreases the energy intake remaining after subtracting exercise energy expenditure (Schubert et al., 2013; Li et al., 2014); that is, inadvertent undereating reduces energy availability.

Exercise may promote inadvertent undereating by suppressing the hormonal response to an energy deficiency (Stensel, 2010). Energy deficiency via reduced energy intake, i.e. diet, increases serum concentrations of ghrelin, the primary orexigenic hormone (Inui et al., 2004), and decreases serum concentrations of PYY, glucagon-like peptide 1 (GLP-1), and pancreatic peptide (PP), which are hormones associated with satiety (Martins et al., 2008; Austin & Marks, 2009). On the other hand, an energy deficiency achieved via increased exercise energy expenditure suppresses ghrelin and increases PYY, GLP-1, and PP serum concentrations in the hours following exercise (Martins et al., 2007; Deighton et al., 2013; Deighton et al., 2014) and even suppresses serum ghrelin concentrations the day after exercise (King et al., 2015). Exercising women are particularly prone to inadvertent undereating as they display greater changes in ghrelin (Hagobian et al., 2013) and less compensatory energy intake than exercising men following exercise (Alajmi et al., 2015) while consuming foods with low energy density (Reed et al., 2011; Reed et al., 2014).
Inadvertent undereating may become more problematic during iron deficiency due to the reduced appetite associated with iron deficiency. Reduced appetite and iron deficiency are linked such that poor appetite is a clinical symptom of anemia and appetite can be restored in individuals with iron deficiency via iron supplementation (Lawless et al., 1994; Stoltzfus et al., 2004). Reductions in appetite associated with iron deficiency are in part mediated by the suppressed serum ghrelin concentrations associated with iron deficiency anemia previously described (Isguven et al., 2007; Akarsu et al., 2013). Iron deficiency also reduces appetite by increasing serum concentrations of leptin, a hormone associated with satiety (Cowley et al., 2001). For instance, mice with restricted iron intake display 47% greater serum leptin concentrations, despite lower energy intake, than mice with sufficient iron intake (Gao et al., 2015). Moreover, serum ferritin concentrations, an indicator of iron stores, are inversely correlated with serum leptin concentrations independent of body mass index in humans (Gao et al., 2015). By suppressing ghrelin and increasing leptin concentrations, iron deficiency can further promote inadvertent undereating in exercising women.

**B-5. Excessive Exercise Energy Expenditure**

Exercise energy expenditure becomes fundamental to the Triad when exercise energy expenditure is relatively high and not adequately compensated by energy intake (Williams et al., 2001). Excessive exercise energy expenditure, such as the 1222 kcal/day in a group of elite female endurance athletes (Melin et al., 2015) or the nearly 2000 kcal/day in a female triathlete (Vescovi & VanHeest, 2015), increases the difficulty in achieving adequate energy intake. As a result, the excessive exercise energy expenditure associated with endurance sports partly explains the 15.9% prevalence of all three Triad conditions in female endurance athletes (Pollock et al., 2010), which is greater than the 0% to 4.3% in exercising women of other sports (Gibbs et al., 2013). Not only can excessive exercise energy expenditure be secondary to the
demands of sport, but also secondary to body weight and shape concerns. Nearly half of exercising women report body weight and shape concerns as their primary reasons for exercising (Gonçalves & Gomes, 2012). The greater body dissatisfaction experienced by exercising women than exercising men (Tiggemann & Williamson, 2000; Furnham et al., 2002; Strelan et al., 2003) drives some exercising women to use exercise energy expenditure as the primary method to induce an energy deficiency (Hubbard et al., 1998). Further, 45% of women with clinically diagnosed anorexia nervosa and bulimia nervosa use excessive exercise energy expenditure to induce the severe energy deficiency associated with these conditions (Davis & Fox, 1993; Dalle Grave et al., 2008; Mond & Calogero, 2009). In sum, excessive exercise energy expenditure, or any excessive energy expenditure for that matter, can be predominantly responsible for the energy deficiency fundamental to the Triad.

Iron deficiency can worsen energy deficiency by increasing energy expenditure due to poor metabolic efficiency (Haas & Brownlie, 2001). Iron is a vital component of oxygen transport and mitochondrial adenosine triphosphate (ATP) production since the enzymes of the citric acid cycle (Ghosh, 2006) and oxidative phosphorylation (Agarwal, 2007) are comprised of iron-dependent heme groups and iron-sulfur clusters. Accordingly, iron deficiency reduces the content and activity of these enzymes to reduce mitochondrial ATP production efficiency (Blayney et al., 1976; Davies et al., 1982; McKay et al., 1983). Iron deficiency also upregulates iron-independent enzymes of anaerobic ATP production and stimulates mitophagy (Allen et al., 2013). As a result, mitochondria display abnormal metabolism and enzyme composition characterized by an increased reliance on less efficient, anaerobic ATP production (Ohira et al., 1987; Bayeva et al., 2013). Iron deficiency anemia further impairs ATP production by inducing cellular hypoxia by reducing oxygen transport secondary to decreased hemoglobin and myoglobin content (Mackler et al., 1978; Hagler et al., 1981). Hypoxia stabilizes hypoxia inducible factor-1 (Solaini et al., 2010), which stimulates glycolytic and gluconeogenic gene
transcription and inhibits oxidative phosphorylation gene transcription (Hoppeler et al., 2003; Mason et al., 2004; Mason & Johnson, 2007; Kamei et al., 2010). As such, hypoxia suppresses oxidative phosphorylation ATP production while enhancing anaerobic ATP production (Pittman, 2011). In short, iron deficiency reduces metabolic efficiency by shifting the reliance on ATP production from oxidative phosphorylation to anaerobic pathways.

As a result of reduced metabolic efficiency, iron deficiency increases energy expenditure during exercise and rest which can promote energy deficiency in exercising women. During exercise, exercising women with iron deficiency have 2.7% greater oxygen consumptions at a given submaximal workload (Pasricha et al., 2014). These increases in exercise energy expenditure, albeit small, are likely common among exercising women as treatment of the highly prevalent and less severe forms of iron deficiency improves metabolic efficiency (Krayenbuehl et al., 2011; DellaValle & Haas, 2011, 2012). Much more threatening to promote an energy deficiency is the 30% greater resting energy expenditure in individuals with iron deficiency in comparison to healthy controls (Martinez-Torres et al., 1984). Iron deficiency increases energy expenditure at rest such that rodents with iron deficiency must consume 30% more calories to maintain the same weight as healthy controls (Beard, 1987). Further, treating iron deficiency in an exercising woman over 16 weeks decreased resting metabolic rate by 15% (Rosenzweig & Volpe, 2000). In sum, by increasing energy expenditure during exercise and rest, iron deficiency can promote a chronic energy deficiency.

C. SECTION 2: Iron Deficiency, Reproductive Function, and the Triad

Triggered by chronically low energy availability, declines in reproductive function drive the majority of Triad health sequelae (De Souza et al., 2014). Reproductive function can be considered the ability of the hypothalamic-pituitary-ovarian axis to produce estrogen and progesterone profiles conducive to menstrual cyclicity and ovulation (Melmed & Williams, 2011).
As such, reproductive function can be characterized by menstrual status with an optimal menstrual status being the presence of ovulatory along with eumenorrheic cycles, defined as regular menstrual cycles 25-36 days in length (Nattiv et al., 2007). Compromised menstrual status secondary to low energy availability, known as exercise-associated menstrual disturbances (EAMD), is present in up to 56% of exercising women (Gibbs et al., 2013). EAMD include less severe presentations, such as luteal phase defects and anovulation, and more severe presentations, such as oligomenorrhea and amenorrhea (De Souza et al., 1998, 2010). The physiological changes associated with EAMD, most notably estrogen deficiency, have severe consequences such as compromised bone health, increased incidence of stress fractures (Barrack et al., 2014), infertility (Stafford et al., 2005), endothelial dysfunction (O'Donnell et al., 2007), and atherogenic blood lipid profiles (Rickenlund et al., 2005). Given the systemic determinants and effects of reproductive function, there are several mechanisms whereby reproductive function and iron deficiency can interact.

C-1. Fertility Issues

The effects of iron deficiency on reproductive function may be inferred by the effects of iron deficiency on fertility, an indicator of reproductive function (Melmed & Williams, 2011). Besides the well-known associations between iron deficiency and adverse events during pregnancy (Scholl, 2011; Alwan et al., 2015; Shen et al., 2015), iron deficiency is also associated with impaired conception. For instance, rodents with iron deficiency anemia display three fold higher conception failure rates than healthy controls (Li et al., 2014). Similarly, 7 women with iron deficiency and failed conceptions for at least 2 years all conceived within 28 weeks of taking iron supplements (Rushton et al., 1991). Likewise, women that received a nutritional supplement containing iron display greater conception rates than controls (Westphal et al., 2006; Westphal et al., 2004). Further, reduced nonheme iron intake is associated with
greater rates of ovulatory infertility, a condition characterized by frequent anovulatory cycles (Chavarrío et al., 2006). Iron deficiency anemia is also suspected to play a role in the idiopathic infertility associated with celiac disease in women (Sher et al., 1994; Stazi et al., 2000). Since iron deficiency compromises fertility, iron deficiency can likely compromise reproductive function in exercising women.

The high iron requirements of reproductive function underlie the mechanisms that iron deficiency can compromise reproductive function. For instance, transferrin and transferrin receptor synthesis are elevated during follicular development, which indicates high iron demands (Balboni et al., 1987; Aleshire et al., 1989; Briggs et al., 1999). Follicular development iron demands are driven by the rapid cell proliferation and differentiation that occur during follicular development (Robker & Richards, 1998; Le & Richardson, 2002; Ponka, 2004; Quirk et al., 2004; Cuiling et al., 2005). Another process essential for reproductive function with high iron demands is steroidogenesis, which requires the iron-dependent enzymes ferredoxin 1 and 2 (Sheftel et al., 2010). As such, mitochondrial iron restriction suppresses ovarian granulosa cell progesterone synthesis (Palandri et al., 2015). Lastly, endometrial thickness, which is positively associated with fertility (Richter et al., 2007; Habibzadeh et al., 2011), likely requires large amounts of iron for similar reasons as follicular development. In fact, hemoglobin concentrations are positively correlated with endometrial thickness such that women with lower hemoglobin concentrations, but unknown ferritin concentrations, also have thinner endometrial linings (Clancy et al., 2006). All in all, the high iron requirements of reproductive function suggest that iron deficiency compromises reproductive function and thereby can potentiate the progression of EAMD in exercising women.
C-2. Hyperprolactinemia

Another mechanism that iron deficiency compromises reproductive function is by promoting hyperprolactinemia via two mechanisms. First, iron deficiency reduces dopamine inhibition of prolactin (PRL) secretion by impairing brain dopamine metabolism as previously described. Such reduced dopaminergic inhibition of PRL secretion increases serum PRL concentrations and decreases hepatic PRL receptor concentrations in rodents with iron deficiency (Barkey et al., 1985, 1986). Second, iron deficiency associated hypothyroidism stimulates PRL secretion via elevated hypothalamic TRH concentrations. Such TRH stimulation of PRL secretion (Gershengorn et al., 1981) may underlie the 22% hyperprolactinemia prevalence rates in women with hypothyroidism (Bahar et al., 2011) in comparison to 2.4% to 12.1% in age-matched women in the general population (Lee et al., 2012). Released inhibition and increased stimulation of PRL secretion underlie the 50% greater serum PRL concentrations in individuals with iron deficiency in comparison to healthy controls (Calarge & Ziegler, 2013). By increasing serum PRL concentrations, iron deficiency in exercising women may cause hyperprolactinemia, which is well-known to compromise reproductive function (Lechan & Fekete, 2004; Goel et al., 2015).

C-3. Menstrual Blood Loss and Iron Deficiency

Menstrual blood loss is linked to iron deficiency through the iron losses that occur with menstrual blood loss. Menstrual blood loss causes iron losses of 6.4 to 11.2 mg per menses with each 1 mg of iron loss correlating to 6.9 μg/L lower serum ferritin concentrations (Harvey et al., 2005). Self-reported measures of menstrual blood loss are inversely associated with the risk of iron deficiency, such that menstruating women with > 4 days of bleeding have a 65% increased risk of iron deficiency compared to women with shorter bleeding durations (Heath et al., 2001). Furthermore, the onset of menstrual blood loss at menarche doubles the risk of iron
deficiency (Moschonis et al., 2013) and the cessation of menses at menopause reduces iron deficiency prevalence rates from 0% to 13% (Kim et al, 2012). These observations all support the presumption that menstrual blood loss is a key determinant in the 11% iron deficiency non-anemia prevalence rates in women in comparison to only 1% in men (Looker et al., 1997).

Less severe EAMDs, i.e. anovulation and luteal phase defects, are associated with a reduction in menstrual blood loss from 30 to 47 mL in comparison to eumenorrheic cycles (Dasharathy et al., 2012; Schliep et al., 2014). Oligomenorrhea, defined as irregular menstrual cycles between 36 and 90 days (De Souza et al., 1998, 2010), is characterized by infrequent and light menstrual bleeding (Hillard, 2008). Thus, oligomenorrhea is likely associated with a further reduction in menstrual blood loss. Lastly, amenorrhea, defined as an absence of menses for at least 90 days (De Souza et al., 1998, 2010), may be considered a reduction in menstrual blood loss of 46 mL, which is the average menstrual blood loss per menses (Hallberg et al., 1966).

If each 1 mL of blood contains 0.5 mg of iron (Finch et al., 1977), then the reductions in menstrual blood loss associated with EAMD may result in 9 to 23 mg more bioavailable iron. Further, assuming the average dietary absorption rate of 8% in the general population predicted by Collings (2013), these amounts of bioavailable iron would require 113 to 288 mg of iron intake to achieve. In other words, the iron retained via menstrual blood loss reductions secondary to EAMD may represent several days of iron intake at the recommend daily allowance for women of reproductive age (Institute of Medicine, Food and Nutrition Board, 2001).

The menstrual blood loss associated with menstrual status may alter the risk of iron deficiency in exercising women according to their menstrual status. With an absence of menstrual blood loss, amenorrhea appears to be protective against iron deficiency. For instance, deployed female military personnel with amenorrhea display serum ferritin
concentrations of 56 μg/L in comparison to the 35 μg/L in their menstruating counterparts (Wilson et al., 2011). Further, amenorrheic women with eating disorders display a 10% iron deficiency prevalence rate in comparison to 37% in their menstruating counterparts (Swenne et al, 2007). At the other end of the menstrual status spectrum, exercising women with ovulatory, eumenorrheic cycles display a 25% iron deficiency prevalence rate in comparison to only 10% in exercising women with amenorrhea (Petkus et al., unpublished). However, it is currently unknown if the risk of iron deficiency is different across the menstrual status spectrum. Regardless, menstrual status appears to be a primary determinant in the risk of iron deficiency in exercising women.

C-4. Estrogen Regulation of Iron Metabolism

Estrogen (E₂) regulation of iron metabolism may allow reproductive function to alter the risk of iron deficiency. E₂ deficiency, via ovariectomy in rodents, decreases adipose tissue iron regulatory protein-1 and transferrin receptor expression (Mattace Raso et al., 2009) as well as decreases serum iron and hemoglobin concentrations (Haouari et al., 1993, 1994). Moreover, restoring E₂ status promptly reverses these changes (Haouari et al., 1993, 1994; Mattace Raso et al., 2009). Acute rises in serum E₂ concentrations, via subcutaneous E₂ injection, upregulates uterine expression of iron transport genes, such as lactotransferrin, ceruloplasmin, lipocalin 2, and ferroportin, as well as heme biosynthesis genes. Most importantly, E₂ inhibits hepatic expression of the master regulator of iron metabolism: hepcidin (Hou et al., 2012; Ikeda et al., 2012; Yang et al., 2012; Rishi et al., 2015). Hepcidin degrades ferroportin to reduce the export of iron from enterocytes, macrophages, and hepatocytes (Ganz, 2006). As a result, hepcidin reduces dietary iron absorption (Frazer et al., 2002) and iron available for erythropoiesis (Ganz, 2003). Such changes in iron metabolism underlie the association between greater hepcidin concentrations in individuals with iron deficiency who are non-
responsive to iron therapy (Bregman et al., 2013). In general, E₂ deficiency may promote iron deficiency whereas E₂ sufficiency may prevent iron deficiency.

The relationship between E₂ status and the risk of iron deficiency in exercising women is currently not well-understood. E₂ deficiency may decrease iron bioavailability and increase iron metabolism biomarkers associated with iron deficiency based on the above observations. However, it is unknown to what extent the absence of menstrual blood loss during amenorrhea counteracts the effects of E₂ deficiency on iron metabolism. For instance, amenorrheic and eumenorrheic women display similar serum iron and hemoglobin concentrations, despite four-fold greater hepcidin concentrations in amenorrheic women (Papillard-Marechal et al., 2012). This observation suggests that the changes in iron metabolism associated with amenorrhea have a net neutral effect on the risk of iron deficiency. It is also not fully understood how restoring E₂ status may affect the risk of iron deficiency, which is critical knowledge for exercising women with amenorrhea aiming to improve menstrual status. The above observations suggest that increasing serum E₂ concentrations may improve iron bioavailability and decreased iron metabolism biomarkers associated with iron deficiency. Yet, stimulating endogenous E₂ production in women with E₂ deficiency did not alter serum iron or ferritin concentrations despite a three-fold decrease in hepcidin concentrations (Lehtihet et al., 2016). Further, exercising women with amenorrhea and exercising women with eumenorrhea display similar serum ferritin and transferrin receptor concentrations (Petkus et al., unpublished). Regardless, the effects of E₂ on iron metabolism may have implications for exercising women and warrant further investigation.
D. SECTION 3: Iron Deficiency, Bone Health, and the Triad

Driven by poor energy status and compromised reproductive function, impaired bone health can be considered the most deleterious Triad component (Audí et al., 2002; Miller et al., 2006; Nattiv et al., 2007; Misra et al., 2008). Poor energy status (Ihle & Loucks, 2004) and compromised reproductive function (Christo et al., 2008; De Souza et al., 2008; Barrack et al., 2010) in exercising women induces a pro-resorptive bone metabolism characterized by lower serum concentrations of N-terminal propeptide of type I collagen (P1NP), a marker of bone formation, and greater serum concentrations of N-terminal telopeptide (NTx) and C-terminal telopeptide (CTX), markers of bone resorption (Risteli & Risteli, 1993). This pro-resorptive bone metabolism environment drives the 40% prevalence of low bone mineral density (BMD) (Gibbs, et al., 2013) and poorer bone microarchitecture and geometry in exercising women with EAMD (Ackerman et al., 2011, 2012; Duckham et al., 2013). Further, exercising women with EAMD have a three-fold greater risk of stress fractures (Kelsey et al., 2007). Impaired bone health is extremely concerning as osteoporosis prevalence rates of up to 13% in exercising women are observed, in comparison to 2.3% in the general population (Nattiv et al., 2007), and can presumably worsen as exercising women age (Thein-Nissenbaum, 2013).

As an emerging risk factor for osteoporosis (Eastell et al., 1992; Korkmaz et al., 2012; Toxqui & Vaquero, 2015), iron deficiency stands as a serious threat to bone health, especially in combination with other Triad conditions. Although independent of iron deficiency, populations with chronic anemia, such as individuals with hemophilia, sickle cell anemia, and pernicious anemia, display lower BMD, poorer bone microarchitecture, greater fracture risk, and higher osteoporosis prevalence rates than the general population (Goerss et al., 1992; Gallacher et al., 1994; Barnes et al., 2004; Demirbaş et al., 2008;). Iron deficiency plays a role in impairing bone health. For instance, simply inducing iron deficiency with or without anemia via diet in
rodents decreases P1NP, increases NTx, and decreases BMD (Medeiros, et al., 2004; Katsumata et al., 2006; Katsumata et al., 2009; Díaz-Castro et al., 2012). Similar models demonstrate that iron deficiency non-anemia impairs bone strength, bone architecture, and bone geometry (Medeiros et al., 2002; Medeiros et al., 2004). In humans, greater serum transferrin and lower serum ferritin concentrations are correlated with lower P1NP and higher NTx concentrations, respectively (Toxqui et al., 2014). Further, iron supplementation in iron deficient anemic women decreases bone turnover and resorption as indicated by decreased P1NP and NTx concentrations (Wright et al., 2013). These effects of iron deficiency on bone health are secondary to several mechanisms that may interact with several Triad conditions.

D-1. Growth Hormone and Insulin-like Growth Factor-1 Suppression

GH and insulin-like growth factor-1 (IGF-1) are critically important for proper bone growth and development (Kasukawa et al., 2004). IGF-1 stimulates chondrocyte and osteoblast differentiation necessary for bone mineralization (Conover, 2000; Meinel et al., 2003; Lindsey et al., 2015). IGF-1 also enhances renal conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D (Wei et al., 1998), a critical hormone for proper bone metabolism (van Leeuwen et al., 2001). Moreover, IGF-1 enhances type 1 collagen accumulation within bone via mediating parathyroid hormone induced collagen synthesis (Canali et al., 1989) and inhibiting type 1 collagen degradation (McCarthy et al., 1989). Beyond stimulating hepatic and bone IGF-1 secretion (Olney, 2003), GH also has direct anabolic effects on bone. GH directly stimulates osteoblast proliferation (Kassem et al., 1993; Kassem et al., 1994) and the expression of several proteins that promote bone anabolism, i.e. osteoprotegerin, osteocalcin, and alkaline phosphatase (Chenu et al., 1990; Mrak et al., 2007). Further, local GH administration increases collagen synthesis independent of serum IGF-1 concentrations (Vestergaard et al., 2012). All these anabolic effects of IGF-1 and GH underlie the associations of greater fracture rates and
lower BMD with growth hormone deficiency (Rosén et al., 1997; Colao et al., 1999) and IGF-1 deficiency (Garnero et al., 2000; Rucker et al., 2004). As a result, compromised function of GH and IGF-1 should impair bone health.

The effects of GH and IGF-1 are directly and indirectly suppressed by iron deficiency. The previously described compromised GH function associated with iron deficiency suppresses the direct anabolic effects of GH on bone and, more importantly, the indirect effects of GH by suppressing serum IGF-1 concentrations. Iron deficiency is associated with suppressed serum IGF-1 concentrations as evident by a three-fold higher prevalence of iron deficiency anemia associated with the lowest quintile of serum IGF-1 concentrations in comparison to the highest quintile (Choi & Kim, 2004). Further, children with iron deficiency anemia display 40% lower serum IGF-1 concentrations than healthy controls (Isguven et al., 2007). Iron deficiency also undermines the GH and IGF-1 mediated accumulation of type I collagen crucial for bone growth and development (Viguet-Carrin et al., 2006). The primary component of type 1 collagen, 4-hydroxyproline (Tredget et al., 1990), is synthesized by the iron-dependent enzyme prolyl 4-hydroxylase (Gorres & Raines, 2010). The activity of this enzyme is inhibited by cellular iron restriction (Wang et al., 2002) which likely drives the lower collagen formation in individuals with iron deficiency anemia in comparison to healthy controls (Wright et al., 2013). Such compromised GH and IGF-1 function secondary to iron deficiency are concerning since the suppressed IGF-1 in rodents with iron deficiency non-anemia was associated with a 17% lower BMD in only 4 weeks (Katsumata et al., 2009).

Iron deficiency may exacerbate the already compromised GH and IGF-1 function associated with the Triad. Similar to iron deficiency associated IGF-1 suppression, chronically suppressed IGF-1 in exercising women is partly mediated by GH resistance (Waters et al., 2001). However, GH resistance in this scenario is secondary to chronic energy deficiency.
(Soyka et al., 2002). As such, iron deficiency may provide an additional pathway to promote GH resistance in exercising women to suppress the effects of GH on bone and IGF-1 secretion. Further reductions in IGF-1 may prove disastrous given the already suppressed IGF-1 in some exercising women such as the 40% lower serum IGF-1 concentrations in exercising women with amenorrhea than healthy controls (Christo et al., 2008). The consequences of such low serum IGF-1 concentrations in exercising women with EAMD are severely suppressed bone formation and lower BMD (Zanker & Swaine, 1998; Christo et al., 2008; Russell et al., 2009). In sum, iron deficiency may suppress serum IGF-1 concentrations further in some exercising women for even worse both health.

D-2. Hypoxia

Hypoxia associated with iron deficiency anemia promotes several physiological processes deleterious to bone health. Acute hypoxia directly inhibits osteoblast proliferation and stimulates osteoclast proliferation (Warren et al., 2001; Utting et al., 2006). Prolonged hypoxia induces cellular acidosis in bone (Arnett, 2010), which subsequently decreases BMD in rodents (Assapun et al., 2009; Gasser et al., 2014). Acidosis promotes bone resorption at the level of osteoclasts and osteoblasts. Osteoclast differentiation, activity, and survival are enhanced during acidosis (Nordström et al., 1997; Pereverzev et al., 2008; Ahn et al., 2012; Li et al., 2013). Acidosis also stimulates osteoblast expression of receptor activator of nuclear factor κ ligand to further enhance osteoclast activity (Okito et al, 2015). Stabilization of hypoxia-inducible factor-1 (HIF-1) during hypoxia directly stimulates osteoclast bone resorption and inhibits osteoblast proliferation (Knowles et al., 2010). HIF-1 further impairs bone health by upregulating erythropoietin gene transcription (Weidemann & Johnson, 2008). Erythropoietin impairs bone as evident by erythropoietin administration increasing osteoclast cell count by
almost 90%, which is associated with a 70% reduction in bone formation and a 60% reduction in trabecular bone mass (Hiram-Bab et al., 2015).

In addition to these effects, hypoxia can be especially deleterious to bone health in exercising women given the relationship between HIF-1 and E₂ deficiency. Since E₂ is one of the most important hormones involved in optimal bone health, E₂ deficiency, in part, associated with EAMD, drive the low BMD observed in these women (Meczekalski et al., 2010). For instance, exercising women with an E₂ and energy deficiency display 30% greater serum CTx concentrations, 40% lower serum P1NP concentrations, and 6% lower lumbar spine BMD than exercising women with an E₂ and energy sufficiency (De Souza et al., 2008). Iron deficiency may exacerbate this condition as the effects of E₂ deficiency are mediated via HIF-1 (Miyauchi et al., 2013; Miyamoto, 2015). Specifically, E₂ deficiency decreases inhibition on HIF-1 expression (Miyauchi et al., 2013; Miyamoto, 2015). Thus, the presence of both an iron and an E₂ deficiency in exercising women may allow even greater HIF-1 expression for further impairments in bone health.

D-3. Hypothyroidism and Bone Health

Another consequence of the hypothyroidism associated with iron deficiency is impaired bone health. Thyroid hormones play critical roles in bone health and development such as stimulating osteoblast activity and increasing the expression of several growth factors (Harvey et al., 2002). Accordingly, hypothyroidism in rodents impairs bone geometry, chondrocyte differentiation, and bone extracellular matrix formation (Bassett & Williams, 2003). Hypothyroidism also lowers BMD by 37% and reduces bone loading capacity by 50% (Conti et al., 2009). Humans with hypothyroidism display more than twice the fracture risk as those with euthyroid function (Vestergaard & Mosekilde, 2002).
Additional certain aspects of the relationship between hypothyroidism and bone health may be critical to exercising women. Poorer indices of bone microarchitecture are associated with the elevated serum TSH concentrations in postmenopausal women with hypothyroidism (Nagata et al., 2007) whereas there is no association between bone health and thyroid hormones in premenopausal women with hypothyroidism (Tuchendler & Bolanowski, 2013). These observations suggest that \( E_2 \) deficiency potentiates the effects of hypothyroidism on bone health. As such, iron deficiency induced hypothyroidism in combination with the \( E_2 \) deficiency in exercising women with EAMD may severely impair bone health. Another aspect of interest is that sudden reductions in thyroid function, such as when thyroid medication is withdrawn, dramatically increases bone turnover and resorption (Toivonen et al., 1998; Gao et al., 2010). Thus, the reductions in energy availability that occur during the competitive season (Reed et al., 2013) may cause sudden, albeit less extreme, reductions in thyroid function accompanied by acute increases in bone resorption in exercising women.

**E. Conclusion and Future Directions**

This review of the literature offers numerous potential mechanisms for iron deficiency and each Triad component to interact. Energy status can be affected by iron deficiency in several ways. Iron deficiency may potentiate the hypometabolic state associated with poor energy status, promote inadequate energy intake, and promote energy deficiency. Reproductive function may be compromised by iron deficiency, and vice versa. Iron deficiency may accelerate, or even initiate, declines in reproductive function and reproductive function may promote or prevent iron deficiency. Lastly, bone health may be further impaired by iron deficiency. The already compromised GH, IGF-1, and thyroid functions associated with the Triad may be further suppressed by iron deficiency and compounded by iron deficiency associated hypoxia.
These potential interactions between iron deficiency and the Triad generate several directions for future investigations. In regards to energy status, the extent that iron deficiency increases resting metabolic rate and, thus, promotes energy deficiency, warrants further investigation. Further, it is important to elucidate how iron deficiency may contribute to inadvertent undereating in exercising women. In regards to reproductive function, it should be determined if ovarian sex steroid production is suppressed during iron deficiency. Future investigations should also aim to determine how the restoration of menstrual status may affect the risk of iron deficiency. In regards to bone health, it should be determined if iron deficiency and E2 deficiency can synergistically impair bone health. In conclusion, we hope to have provided a unique perspective of the Triad through the lenses of iron metabolism and hope that future investigations will explore the potential mechanisms discussed.
References


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Chapter 3: Methods

This cross-sectional study uses baseline data from “REFUEL,” a randomized controlled trial that assessed the effects of a nutritional intervention of increased caloric intake on menstrual status and bone health among exercising women with EAMD. “REFUEL” was conducted at two sites, the University of Toronto and The Pennsylvania State University over 9 years, and was approved by the Institutional Review Boards at both institutions. Prior to entering the study, all participants underwent a screening period which was followed by a baseline phase that lasted the duration of one menstrual cycle or, if amenorrheic, 28 days. During this baseline phase, each participant self-collected daily urine samples, provided weekly serum samples, completed menstrual and health questionnaires, kept dietary logs that included supplement use, received calcium and vitamin D supplementation, and performed an exercise test to assess aerobic fitness. Upon completion of baseline, participants then entered the intervention phase. The additional procedures of the intervention phase are outside the context of this thesis. But, it should be noted that the blood draw and dietary logs from the first week of the intervention were used in the present analysis.

Screening Procedures

Participants were recruited on a rolling basis and initially screened via phone and online surveys. Initial inclusion criteria for study eligibility were as follows: 1) age 18-35 years, 2) body mass index 16-25 kg/m², 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 hrs/wk purposeful exercise, and 9) no history of a clinical diagnosis of polycystic ovarian syndrome.
The initial criteria for inclusion for exercising women with amenorrhea was a self-reported absence of menses within the past 3 months or less than 6 menses in the past 12 months (De Souza et al., 2004). The initial inclusion criteria for women with ovulatory, eumenorrheic menstrual cycles were based on the self-reported occurrence of ≥ 9 menses in the past 12 months and a menstrual cycle length between 25 and 36 days (De Souza et al., 2004). These criteria were later confirmed by analyzing daily urinary hormone metabolites of estrogen and progesterone as described below.

After providing informed consent, height and weight were measured and medical, exercise, and menstrual histories were assessed. Screening fasting venous blood samples were collected between 0700 and 1000 h. Whole blood was analyzed for hemoglobin concentrations via HemoCue Hb 201 (HemoCue, Inc. Lake Forest, CA). Whole blood was analyzed for hematocrit by drawing blood into a capillary tube, centrifuging at 15000g for 8 minutes, and then measuring (Oxford Labware CritoCaps Micro-hematocrit). Anemic participants (hemoglobin<12 g/dL and/or hematocrit <35% ; n=5) were excluded from the present analysis because their anemia was not accompanied by iron depletion, which indicates that their anemia may be caused by other factors (WHO, 2001). Screening blood samples were also sent to Quest diagnostics for additional analysis. Participants with abnormal serum concentrations of follicle stimulating hormone, luteinizing hormone, prolactin, thyroid stimulating hormone, thyroxin, total testosterone or free testosterone were excluded to rule out causes of menstrual disturbances unrelated to exercise (De Souza et al., 2014). A physical examination was conducted by a Clinical Research Center clinician and women with high androgen phenotypes (clinical symptoms and free androgen index) were excluded (n=6) (The Practice Committee of the American Society for Reproductive Medicine, 2008). Several participants (n=6) had a history of using iron supplements, but were not taking them at the time of the study and were included in the present analysis. All participants were supplemented with calcium (1000 mg/day; Caltrate
Baseline Urinary Hormone Analysis and Menstrual Classification

During the baseline period of the study, which lasted for the duration of one menstrual cycle for participants with menses or 28 days for participants with amenorrhea, participants collected daily urine samples so that menstrual function could be confirmed with urinary profiles of estrone-1-glucuronide (E1G), pregnanediol glucuronide (PdG), and luteinizing hormone (LH) using methods described below.

Data Collection

Urinary Measurement of E1G and PdG

The validity of the urinary technique as representative of the 24-h pattern of E1G and PdG excretion has been reported by other investigators (Beitins et al., 1991; Munro et al., 1991) and the secretion of these metabolites in the urine parallels serum concentrations of the parent hormones. Microtiter plate competitive enzyme immunoassays were used to measure the urinary metabolites E1G and PdG, respectively, using the techniques of Munro et al. (1991). The E1G (R522-2) and PdG (R13904) assays utilized a polyclonal capture antibody supplied by Coralie Munro at the University of California (Davis, CA, USA). The competitors for these assays are E1G or PdG conjugated to horseradish peroxidase (E1G-HRP and PdG-HRP, respectively). An end-point substrate color reaction is developed with azino-bis-ethylbenzthiazoline sulfonic acid and peroxidase. E1G and PdG standards (Sigma) were used for the standard curve, and high and low internal controls from in-house samples were used. The inter-assay coefficients of variation for high and low internal controls for the E1G assay were 14.7% and 13.1%, respectively. The inter-assay coefficients of variation for high and low
internal controls for the PdG assay were 15.6% and 12.9%, respectively. The PdG intra and inter-assay variability was determined in-house as 15.6% and 12.9%, respectively. The sensitivity of the E1G and PdG assays were 7.8 ng/ml and 0.15 mg/ml, respectively. Values below the sensitivity were reported at the minimum detection limits. All urine samples were corrected for specific gravity (NSG Precision Cells, Inc. Farmingdale, NY, USA) to account for hydration status. Specific gravity has been reported to perform as well as creatinine correction for adjusting urinary hormone concentrations (Miller et al., 2004). Specific gravity measurements were taken with a hand-held urine specific gravity refractometer (Atago Uricon-PN; NSA Precision Cells, Inc., Framingham, NY). Urinary LH was determined by double antibody radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA, USA). The sensitivity of the LH assay is 0.6 mIU/L. The intra-assay and inter-assay coefficients of variation were 1.6% and 7.1%, respectively.

Menstrual Status Classification

Classification as OvEU required the following characteristics from menstrual cycle monitoring and daily urinary hormone analysis: 1) a self-reported presence of ≥ 9 menses in the past 12 months, 2) a menstrual cycle length between 26 and 35 days, 3) an E1G peak concentration above 35 ng/ml, 4) a peak PdG concentration above 5 μg/mL during the luteal phase, and 5) a LH surge concentration above 25 mIU/ml to confirm ovulation (De Souza et al., 1998, 2010). Classification as AMEN required the following characteristics: 1) absence of menses and menstrual bleeding 2) a negative pregnancy test, and 3) E1G and PdG profiles with suppressed concentrations that lack cyclicity (De Souza et al., 1998, 2010).
Menstrual Bleeding Characteristics

The following self-reported menstrual data was included from screening questionnaires: number of periods in the last 12 months, duration of menstrual bleeding, menstrual bleeding intensity, rated as either “light,” “moderate,” or “heavy” (OvEU only), and duration of amenorrhea prior to the intervention (AMEN only).

Exercise Testing and Logs

Exercise parameters were assessed to control for their potential confounding effects on iron status. Peak oxygen consumption (VO_{2peak}), an indicator of aerobic fitness, was measured during a progressive treadmill test modified from Åstrand and Saltin (1961) to volitional exhaustion during baseline on an arbitrary day using breath by breath open-circuit spirometry. The VO_{2peak} test is initiated by the volunteer selecting a comfortable running speed at 0.0% grade. The grade of the treadmill was increased 2.0% after every 2 min for the first 8 min of the test, after which the grade then was increased 1.0% for each subsequent minute. VO_2 and VCO_2 were measured using a Sensor Medics Vmax metabolic cart (Yorba Linda, CA). We considered VO_{2peak} to be achieved if three of the following four criteria were obtained: (1) attainment of age predicted maximal heart rate (208-(0.7\times age)); (2) respiratory exchange ratio ≥ 1.1; (3) plateau in oxygen consumption despite an increase in exercise workload; (4) attainment of a rating of perceived exercise score ≥ 18 (Saltin et al., 1967). Participants kept logs of their purposeful exercise training for seven-days throughout baseline. The durations of exercise were then summed for each week and then averaged to produce the average weekly exercise duration of each participant.
Dietary Information

Participants completed 3-day diet logs on 2 weekdays and 1 weekend day during the third week of baseline and the first week of the intervention for a total of 6 days of dietary data. Participants were provided with detailed instructions on how to record types and quantities of foods eaten. Diet analysis of the 3-day food logs were performed using Nutrition Data System for Research (NDS-R 2008, Minneapolis, MN) to provide estimates of 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. Dietary factors were assessed to control for their potential confounding effects on iron status.

Serum Sampling and Assay Analysis

Fasting venous blood samples were collected between 0700 and 1000 h in the third week of the baseline period and one week after completion of baseline. Participants were asked to abstain from exercise for 24 hours prior to blood sampling to control for possible effects of exercise on iron status markers (Schumacher et al., 2002). Samples were allowed to clot for 30 min and then centrifuged at 4°C for 15 min at 1600g. Serum aliquots were stored at -80°C until analysis. Prior to performing assays, each participant’s serum from the third week of baseline and one week after the completion of baseline were pooled to minimize possible variations in serum ferritin (Ft) and soluble transferrin receptor (sTfR) concentrations across the menstrual cycle (Kim, 1993).

Ft and sTfR concentrations were assessed separately 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%, and sensitivities of 0.59 μg/L and 0.07 mg/L respectively. Inflammation status was assessed via serum alpha-1-acid glycoprotein (AGP) concentrations.
(radial immunodiffusion, Kent Laboratories, Bellingham, WA) and serum C-reactive protein (CRP) concentrations (qualitative measurement with threshold of 10 mg/ml, IMMUNEX CRP, Alere, Waltham, MA). Ft concentrations were adjusted according to inflammation status as described by Thurnham et al. (2010). Based on these classifications, individuals were categorized into one of four inflammation statuses: reference (normal CRP and AGP), incubation (elevated CRP only), early convalescence (elevated CRP and AGP), and late convalescence (elevated AGP only). With no individuals in the early convalescence group phase, adjustment factors derived by Thurnham et al. (2010) of 0.77 and 0.75 were applied to the log Ft of individuals within the early convalescence and late convalescence, respectively. Values were then reverse log transformed and used to compare group differences in Ft. Due to observations that inflammation may also falsely elevate sTfR (Grant et al., 2012; Kasvosve et al., 2006), we assessed for differences in sTfR concentrations based on inflammatory status. As we observed no mean differences between groups, no correction factors were applied. Total body iron was estimated with the inflammation adjusted Ft using Cook’s equation (2003):

\[
\text{Total body iron (mg/kg)} = \frac{-\log(s\text{TfR}/Ft) - 2.8229}{0.1207}.
\]

**Data analysis**

Statistical analysis was performed using R version 3.1.0 (The R Foundation for Statistical Computing, 2014). Geometric means and geometric standard deviations were calculated for skewed biomarkers, Ft and sTfR (Wilson et al. 2011, Worwood, 2011), for a measure of central tendency that is less influenced by extreme outliers. Arithmetic means and standard deviations were calculated for all other values. Variables were assessed for normality with the Shapiro-Wilk test. Differences between group means were assessed with independent T-tests for normally distributed variables or with Wilcoxon rank-sum tests for non-normally distributed variables. The difference in the prevalence of iron depletion (Ft<15 μg/L; Hallberg,
2001) between groups was assessed with Fisher’s exact test. Correlations between iron status indices (Ft, sTfR, total bod iron, hemoglobin, and hematocrit) and menstrual characteristics (bleeding duration and bleeding intensity in OvEU only and duration of amenorrhea in AMEN only) were assessed with Spearman’s rank-order correlation coefficients. Generalized linear models were used to determine if exercise parameters, dietary factors, and differences between groups, such as age, were significant predictors of individual iron status biomarkers. P-values less than 0.05 were considered statistically significant. Alpha levels were adjusted to account for multiple comparisons with Bonferroni’s correction for correlations.

**Power analysis**

Sample size calculations for group differences in ferritin and soluble transferrin were performed using data from previous investigations on physically active women with power set to 0.80 that could detect differences at α=0.05. Specifically, data was used from an investigation that compared iron status between menstruating and amenorrheic female military personnel (Wilson et al., 2011) and an investigation that compared iron status between physically active and sedentary women (Woolf et al., 2009). The sample size required to detect a group difference in serum ferritin concentrations based on a group difference of 21μg/L and a standard deviation of 28μg/L was determined to be 29 women in each group. The sample size required to detect a group difference in soluble transferrin receptor concentrations based on a group difference of 1.5mg/L and a standard deviation of 2.1mg/L was determined to be 31 women in each group. The sample size required to detect a group difference in hemoglobin concentrations based on a group difference of 0.5g/dL and a standard deviation of 1.0g/dL was determined to be 64 women in each group.

The sample size calculation for a difference in the prevalence of iron depletion was performed using data from a previous investigation that assessed the prevalence of iron
depletion in menstruating and amenorrheic women with anorexia (Swenne, 2007). The sample size required to detect a similar difference in the prevalence of iron depletion with power set to 0.80 that could detect differences at $\alpha=0.05$ was determined to be 36 women in each group.

The sample size calculations for the correlation between menstrual bleeding duration and intensity depletion were performed with power set to 0.80 that could detect differences at $\alpha=0.05$ based on a previous investigation that the correlations between menstrual characteristics and iron status in the general population (Milman et al, 1998). The sample size required to detect a similar correlation between menstrual bleeding duration and serum ferritin concentrations was determined to be 123 women, based on the correlation coefficient between menstrual bleeding duration and serum ferritin ($r=-0.25; p<0.001; n=174$). The sample size required to detect a similar correlation between menstrual bleeding intensity and serum ferritin concentrations was determined to be 105 women, based on the correlation coefficient between menstrual bleeding intensity and serum ferritin ($r=-0.27; p<0.001; n=174$).
References


Chapter 4: Manuscript

ABSTRACT:

Objectives: To investigate the effect of menstrual status on iron status in exercising women with amenorrhea and exercising women with ovulatory, eumenorrheic cycles.

Design: Cross-sectional analysis of iron status markers, exercise parameters, and dietary factors.

Methods: Women aged 18-35 years with at least 2 hours 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=36). Women with ovulatory eumenorrheic cycles (OvEU) were defined by the presence of ovulatory cycles of 26-35 days in length for the past 6 months (n=28). Group differences in serum ferritin (Ft), soluble transferrin receptor (sTfR), total body iron (TBI), hemoglobin (Hb), hematocrit, and the prevalence of iron depletion (Ft <15 μg/L), exercise parameters, and dietary factors were assessed.

Results: The prevalence of iron depletion was greater in OvEU when compared to AMEN (25% vs 8%, p=0.03). No significant differences were observed between AMEN and OvEU in Ft (33.9±1.95 vs 31.5±2.41 μg/L; p=0.24), sTfR (5.4±1.3 vs. 5.7±1.4 mg/L; p=0.51), TBI (5.1±2.5 vs. 4.4±3.9 mg/kg; p=0.22), Hb (13.3±0.7 vs. 13.4±0.6 g/dL; p=0.60), hematocrit (39.2±0.5% vs. 40.0±4.0%; p=0.91), exercise parameters, or dietary factors.

Conclusion: Our primary finding was that 7/28 (25%) of women with ovulatory, eumenorrheic cycles were iron depleted compared to 3/36 (8%) of amenorrheic women. Yet, there were no significant difference in iron status markers in the two groups which was likely attributable to an insufficient sample size to detect differences in iron status markers.
Introduction

Exercising women are at risk for suboptimal iron status, and therefore are more likely to experience compromised immune function, decreased metabolic efficiency, and cognitive impairment (Beard, 2001). Iron status, a representation of whole body iron content, is categorized into four stages of decreasing iron in storage and functional compartments: iron sufficiency, iron depletion, iron deficiency without anemia, and iron deficiency with anemia (Gibson et al., 2005). Iron status can be assessed using markers of iron metabolism, such as serum ferritin (Ft), which relates to iron in storage compartments, and serum soluble transferrin receptor (sTfR), which correlates with intracellular iron demand (Skikne et al., 1990; WHO, 2007). As such, reductions in Ft concentrations, increases in sTfR concentrations, or a combination of both indicate diminishing iron status (Skikne et al., 1990; WHO, 2007).

The iron status of exercising women are susceptible to the well documented associations between exercise and changes in iron status markers indicative of compromised iron status (Rowland et al., 1987; Rowland et al., 1989; Fogelholm, 1995; Beard et al., 2000; Dubnov et al., 2004; Sinclair et al., 2005; Fallon, 2008; Mettler et al., 2010; Woolf et al., 2009;). For example, in physically active women, as little as 10 days of exercise training was associated with a 33% reduction in Ft and a 8% increase in sTfR (Auersperger et al., 2013). In a separate study, Ft decreased 25% and sTfR increased 30% after women soldiers completed nine weeks of basic combat training (McClung et al., 2009). Exercise can compromise iron status by increasing iron losses from the body due to several factors including sweating, exercise-induced hemolysis in high-impact exercise modes, and gastro-intestinal bleeding (Peeling et al., 2008). Exercise can further compromise iron status by decreasing cellular iron availability via upregulating hepcidin (Peeling et al., 2009), leading to iron sequestration in macrophages and hepatocytes (Ganz et al., 2006). These detrimental effects of exercise on iron status may
accumulate over time, as demonstrated by a 60% greater risk of iron depletion in chronically exercising women when compared to their sedentary counterparts (Fogelholm, 1995).

Menstrual bleeding may further compromise iron status in exercising women. On average, women lose about 14 mg of iron per menses such that monthly iron losses are 41% greater in women than in men (Hunt et al., 2009). Menstrual iron losses are considered the primary cause for the higher prevalence of iron deficiency in premenopausal women compared to age-matched men (Looker et al., 1997). Moreover, self-reports of greater menstrual bleeding, such as that reported in one third of exercising women (Bruinvelts et al., 2016), are correlated with a higher prevalence of iron depletion (Milman et al., 1998; Heath et al., 2001; Harvey et al., 2005). Changes in menstrual status, such as the onset of menses during puberty as well as the cessation of menses during menopause, are associated with concomitant changes in iron status: a decline in iron status following menarche (Moschonis et al., 2013) and an improvement in iron status following menopause (Kim et al., 2012).

Understanding the association between menstrual status and iron status in exercising women would provide critical health information for this population, particularly given that as many as 56% of exercising women have been reported to suffer from exercise-associated menstrual disturbances (EAMD) (Gibbs et al., 2013). EAMD are one component of the Female Athlete Triad, a medical condition often observed in exercising women driven by chronic energy deficiency (De Souza et al., 2014). EAMD may range from subclinical presentations, such as luteal phase defects and anovulation, to clinical presentations, such as oligomenorrhea and amenorrhea (De Souza et al., 1998, 2010). EAMD are associated with decreased bone health, increased risk of stress fractures (Barrack et al., 2014), infertility (Stafford et al., 2005), endothelial dysfunction (O’Donnell et al., 2007), and atherogenic blood lipid profiles (Rickenlund et al., 2005). Current treatment strategies for EAMD view the restoration of regular menstrual
status as an indicator of good health (Mallinson et al., 2013; Lagowska et al., 2014). However, it is currently unknown if the resumption of menses in these women with EAMD increases iron losses sufficiently to further compromise iron status in this high risk population. Further, it remains to be determined if the association between menses and iron status observed in the general population (Milman et al., 1998; Heath et al., 2001; Harvey et al., 2005) persists in the background of exercise-associated iron losses due to limited comparisons of iron status among exercising women of different and well-defined menstrual status, such as ovulatory, eumenorrhea vs. amenorrhea.

As such, the purpose of this investigation was to assess the association between menstrual status and iron status by comparing exercising women at opposite ends of the menstrual status spectrum. Iron status was compared between exercising women with ovulatory eumenorrheic menstrual cycles (OvEU) and exercising women with amenorrhea (AMEN). In contrast to previous studies addressing this question, we confirmed menstrual status using urinary reproductive hormone analysis, and excluded women using hormonal contraceptives. We excluded users of hormonal contraception, which are known to alter iron status (Milman et al., 1998; Heath et al., 2001; Casabellata et al., 2007). We hypothesized that OvEU would exhibit a higher prevalence of iron depletion (Ft < 15 μg/L) as well as poorer iron status, as indicated by lower Ft, higher sTfR, lower hemoglobin concentrations, and lower TBI and hematocrit values when compared to AMEN.

An exploratory aim of this investigation was to assess the associations between menstrual bleeding characteristics and iron status. In OvEU, we hypothesized that a menstrual bleeding duration of 5 days or more and menstrual bleeding described as “heavy” would exhibit a more compromised iron status and diminished iron status markers when compared to women with self-reported menstrual bleeding duration of less than 5 days and menstrual bleeding
severity described as “light” or “moderate.” In AMEN, we hypothesized that women with amenorrhea for a shorter duration (less than 6 months) would exhibit a more compromised iron status when compared to women with amenorrhea for a longer duration (at least 6 months).

Methods

This cross-sectional study uses baseline data from “REFUEL,” a randomized controlled trial that assessed the effects of a nutritional intervention of increased caloric intake on menstrual function and bone health among exercising women with EAMD. “REFUEL” was conducted at two sites, the University of Toronto and The Pennsylvania State University over 9 years, and was approved by the Institutional Review Boards at both institutions. Prior to entering the study, all participants underwent a screening period which was followed by a baseline phase that lasted the duration of one menstrual cycle or, if amenorrheic, 28 days. During this baseline phase, each participant self-collected daily urine samples, provided weekly blood samples, completed menstrual and health questionnaires, kept dietary logs, received calcium and vitamin D supplementation, and performed an exercise test to assess aerobic fitness. Upon completion of baseline, participants then entered the intervention phase. The additional procedures of the intervention phase are outside the context of this thesis. But, it should be noted that the blood draw and dietary logs from the first week of the intervention were used in the present analysis.

Screening Procedures

Participants were recruited on a rolling basis and initially screened via phone and online surveys. Initial inclusion criteria for study eligibility were as follows: 1) age 18-35 years, 2) body mass index 16-25 kg/m², 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 hrs/wk purposeful exercise, and 9) no history of a clinical diagnosis of polycystic ovarian syndrome.

The initial criteria for inclusion for exercising women with amenorrhea was a self-reported absence of menses within the past 3 months or less than 6 menses in the past 12 months (De Souza et al., 2004). The initial inclusion criteria for women with ovulatory, eumenorrheic menstrual cycles were based on the self-reported occurrence of ≥ 9 menses in the past 12 months and a menstrual cycle length between 25 and 36 days (De Souza et al., 2004). These criteria were later confirmed by analyzing daily urinary hormone metabolites of estrogen and progesterone as described below.

After providing informed consent, height and weight were measured and medical, exercise, and menstrual histories were assessed. Anemic participants (hemoglobin<12g/dL and/or hematocrit < 35%; n=5) were excluded from the present analysis because their anemia was not accompanied by iron depletion, which indicates that their anemia may be caused by other factors (WHO, 2001). Participants with abnormal serum concentrations of follicle stimulating hormone, luteinizing hormone, prolactin, thyroid stimulating hormone, thyroxin, total testosterone or free testosterone were excluded to rule out causes of menstrual disturbances unrelated to exercise (De Souza et al., 2014). A physical examination was conducted by a Clinical Research Center clinician and women with high androgen phenotypes (clinical symptoms and free androgen index) were excluded (n=6) (The Practice Committee of the American Society for Reproductive Medicine, 2008). Several participants (n=6) had a history of using iron supplements, but were not taking them at the time of the study and were included in the present analysis. All participants were supplemented with calcium (1000 mg/day; Caltrate 600 + D, Wyeth Consumer Health Care Products) and vitamin D3 (400 IU; Wyeth Consumer Health Care Products) during the study.
Baseline Urinary Hormone Analysis and Menstrual Classification

During the baseline period of the study, which lasted for the duration of one menstrual cycle for participants with menses or 28 days for participants with amenorrhea, participants collected daily urine samples so that menstrual function could be confirmed with urinary profiles of estrone-1-glucuronide (E1G), pregnanediol glucuronide (PdG), and luteinizing hormone (LH) using methods previously described (De Souza et al., 2010).

Menstrual Status Classification

Classification as OvEU required the following characteristics from menstrual cycle monitoring and daily urinary hormone analysis: 1) a self-reported presence of ≥ 9 menses in the past 12 months, 2) a menstrual cycle length between 26 and 35 days, 3) an E1G peak concentration above 35 ng/ml, 4) a peak PdG concentration above 5 μg/mL during the luteal phase, and 5) a LH surge concentration above 25 mIU/ml to confirm ovulation (De Souza et al., 2010; De Souza et al., 1998). Classification as AMEN required the following characteristics: 1) absence of menses and menstrual bleeding 2) a negative pregnancy test, and 3) suppressed E1G and PdG profiles (De Souza et al., 2010; De Souza et al., 1998).

Menstrual Bleeding Characteristics

The following self-reported menstrual data was included from screening questionnaires: number of periods in the last 12 months, duration of menstrual bleeding, menstrual bleeding intensity, rated as either “light,” “moderate,” or “heavy” (OvEU only), and duration of amenorrhea prior to the intervention (AMEN only).
Exercise Testing and Logs

Peak oxygen consumption (VO\textsubscript{2peak}), an indicator of aerobic fitness, was measured during a graded exercise test to volitional fatigue with an on-line SensorMedics Vmax metabolic cart (Yorba Linda, CA) using methods previously published (Scheid et al., 2009). Participants kept logs of their purposeful exercise training for each week of baseline. The exercise duration from all four weeks of baseline were averaged to produce the average weekly exercise duration of each participant.

Dietary Information

Participants completed 3-day diet logs on 2 weekdays and 1 weekend day during the third week of baseline and the first week of the intervention for a total of 6 days of dietary data. Participants were provided with detailed instructions on how to record types and quantities of foods eaten. Diet analysis of the 3-day food logs were performed using Nutrition Data System for Research (NDS-R 2008, Minneapolis, MN) to provide estimates of 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. Dietary factors were assessed to control for their potential confounding effects on iron status.

Serum Sampling and Assay Analysis

Fasting venous blood samples were collected between 0700 and 1000 h in the third week of the baseline period and one week after completion of baseline. Participants were asked to abstain from exercise for 24 hours prior to blood sampling to control for possible effects of exercise on iron status markers (Schumacher et al., 2002). Samples were allowed to clot for 30 min and then centrifuged at 4°C for 15 min at 1600g. Serum aliquots were stored at -80°C until analysis. Prior to performing assays, each participant’s serum from the third week of baseline
and one week after the completion of baseline were pooled to minimize possible variations in Ft and sTfR across the menstrual cycle (Kim, 1993).

Ft and sTfR concentrations were assessed separately 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%, and sensitivities of 0.59 μg/L and 0.07 mg/L respectively. Inflammation status was assessed via serum alpha-1-acid glycoprotein (AGP) concentrations (radial immunodiffusion, Kent Laboratories, Bellingham, WA) and serum C-reactive protein (CRP) concentrations (qualitative measurement with threshold of 10 mg/ml, IMMUNEX CRP, Alere, Waltham, MA). Ft concentrations were adjusted according to inflammation status as described by Thurnham et al. (2010). Based on these classifications, individuals were categorized into one of four inflammation statuses: reference (normal CRP and AGP), incubation (elevated CRP only), early convalescence (elevated CRP and AGP), and late convalescence (elevated AGP only). With no individuals in the early convalescence group phase, adjustment factors derived by Thurnham et al. (2010) of 0.77 and 0.75 were applied to the log Ft of individuals within the early convalescence and late convalescence, respectively. Values were then reverse log transformed and used to compare group differences in Ft. Due to observations that inflammation may also falsely elevate sTfR (Grant et al., 2012; Kasvosve et al., 2006), we assessed for differences in sTfR concentrations based on inflammatory status. As we observed no mean differences between groups, no correction factors were applied. TBI was estimated with the inflammation adjusted Ft using Cook’s equation (2003).

Data Analysis

Statistical analysis was performed using R version 3.1.0 (The R Foundation for Statistical Computing, 2014). Geometric means and geometric standard deviations were calculated for skewed biomarkers, Ft and sTfR (Wilson et al. 2011, Worwood, 2011), for a
measure of central tendency that is less influenced by extreme outliers. Arithmetic means and standard deviations were calculated for all other values. Variables were assessed for normality with the Shapiro-Wilk test. Differences between group means were assessed with independent T-tests for normally distributed variables or with Wilcoxon rank-sum tests for non-normally distributed variables. The difference in the prevalence of iron depletion (Ft<15 μg/L; Hallberg, 2001) between groups was assessed with Fisher’s exact test. Correlations between iron status markers (Ft, sTfR, TBI, hemoglobin, and hematocrit) and menstrual characteristics (bleeding duration and bleeding intensity in OvEU only and duration of amenorrhea in AMEN only) were assessed with Spearman’s rank-order correlation coefficients. Generalized linear models were used to determine if exercise parameters and dietary factors were significant predictors of individual iron status biomarkers. P-values less than 0.05 were considered statistically significant. Alpha levels were adjusted to account for multiple comparisons with Bonferroni’s correction for correlations.

Results

Participant Anthropometrics

Complete data were available for 36 AMEN and 28 OvEU individuals. Body mass index, VO2peak, and exercise duration were not significantly different between AMEN and OvEU, but AMEN were significantly younger than OvEU (p<0.05; Table 1). Energy and iron intake were not significantly different between AMEN and OvEU. Heme iron, non-heme iron, servings of meat, fish, or poultry, cups of tea and coffee, carbohydrate, protein, and fat intakes were also not significantly different between AMEN and OvEU. The proportion of women with dietary iron intakes above the RDA of 18 mg/day (Institute of Medicine, Food and Nutrition Board, 2001) was not significantly different between AMEN vs. OvEU (39% vs. 60%, X^2=2.42, p=0.12). Neither exercise parameters nor dietary factors were significant predictors of any iron status
biomarker (p>0.05). Since age was significantly different between groups, age was assessed as a covariate and found to not be a significant predictor of any iron status biomarker (p>0.05).

Comparison of Iron Status Biomarkers between AMEN and OvEU

The prevalence of iron depletion was greater in OvEU compared to AMEN (25% vs 8%, p=0.03; Fig. 1) at a Ft threshold of 15μg/L. When 6 participants who reported taking iron supplements were excluded, this group difference lost significance (p=0.10). There were no significant differences between AMEN and OvEU with regard to Ft (33.9±1.95 vs 31.1±2.41 μg/L; p=0.24), sTfR (5.4±1.3 vs. 5.7±1.4 mg/L; p=0.51), TBI (5.1±2.5 vs. 4.4±3.9 mg/kg; p=0.22; Fig. 2), hemoglobin concentrations (13.3±0.7 vs. 13.4±0.6 g/dL; p=0.60), and hematocrit (39.2±0.5% vs.40.0±4.0%; p=0.91).
Table 1. Anthropometric, dietary, and iron status characteristics of exercising women with amenorrhea (AMEN) and exercising women with eumenorrheic, ovulatory cycles (OvEU)

<table>
<thead>
<tr>
<th></th>
<th>AMEN (n=36)</th>
<th>OvEU (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anthropometric</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age; years</td>
<td>21.4±3.2</td>
<td>24.1±4.8*</td>
</tr>
<tr>
<td>Body-mass index; kg/m²</td>
<td>20.5±1.9</td>
<td>21.2±2.5</td>
</tr>
<tr>
<td>Average duration of amenorrhea; days</td>
<td>320±281</td>
<td>NA</td>
</tr>
<tr>
<td>Menses in past 12 months</td>
<td>NA</td>
<td>12±1</td>
</tr>
<tr>
<td>VO₂peak; mL/kg/min</td>
<td>49.6±8.4</td>
<td>48.0±8.7</td>
</tr>
<tr>
<td>Average exercise duration; min/week</td>
<td>332±236</td>
<td>271±193</td>
</tr>
<tr>
<td><strong>Dietary</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy intake; kcal/d</td>
<td>2005±1010</td>
<td>1973±467</td>
</tr>
<tr>
<td>Dietary iron intake; mg/d</td>
<td>19.6±10</td>
<td>19.0±8.5</td>
</tr>
<tr>
<td>Heme iron; mg/d</td>
<td>2.2±3.1</td>
<td>1.9±3.6</td>
</tr>
<tr>
<td>Non-heme iron; mg/d</td>
<td>15±7.2</td>
<td>15.6±8.5</td>
</tr>
<tr>
<td>Meat, fish, or poultry; g/d</td>
<td>138±186</td>
<td>161±112</td>
</tr>
<tr>
<td>Tea and coffee; cups/d</td>
<td>2.2±2.7</td>
<td>1.5±2.1</td>
</tr>
<tr>
<td>Vitamin C; mg/d</td>
<td>248±202</td>
<td>179±169</td>
</tr>
<tr>
<td>Calcium; mg/d</td>
<td>1815±957</td>
<td>1626±1053</td>
</tr>
<tr>
<td>Carbohydrate intake; g/d</td>
<td>286±150</td>
<td>277±83</td>
</tr>
<tr>
<td>Protein intake; g/d</td>
<td>85±43</td>
<td>78±20</td>
</tr>
<tr>
<td>Fat intake; g/d</td>
<td>63±37</td>
<td>64±15</td>
</tr>
<tr>
<td><strong>Iron Status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron-Depleted</td>
<td>3(8%)</td>
<td>7(25%)*</td>
</tr>
<tr>
<td>Iron-Sufficient</td>
<td>33(92%)</td>
<td>21(75%)*</td>
</tr>
<tr>
<td>Ferritin; μg/L</td>
<td>33.9±2.0</td>
<td>31.5±2.4</td>
</tr>
<tr>
<td>Soluble transferrin receptor; mg/L</td>
<td>5.4±1.3</td>
<td>5.7±1.4</td>
</tr>
<tr>
<td>Total body iron†; mg/kg</td>
<td>5.1±2.5</td>
<td>4.4±3.9</td>
</tr>
<tr>
<td>Hemoglobin; g/dL</td>
<td>13.3±0.7</td>
<td>13.4±0.6</td>
</tr>
<tr>
<td>Hematocrit; %</td>
<td>39.2±0.5</td>
<td>40.0±4.0</td>
</tr>
<tr>
<td>Alpha-1-acid glycoprotein; g/L</td>
<td>0.42±0.3</td>
<td>0.39±0.2</td>
</tr>
<tr>
<td>Elevated C-reactive protein††</td>
<td>2(5.5%)</td>
<td>1(3.5%)</td>
</tr>
</tbody>
</table>

*Significantly different from AMEN (p<0.05)
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
† Total body iron was calculated as -[log(soluble transferrin receptor/ferritin)-2.8299]0.1207
†† C-reactive protein was determined qualitatively at a threshold of 10 mg/ml
Figure 1. Prevalence of iron depletion (Ft < 15μg/L) in exercising women with amenorrhea (AMEN; n=36) and exercising women with eumenorrheic, ovulatory cycles (OvEU; n=28).

Iron-Depleted: Ft < 15 μg/L; Iron-Sufficient: Ft ≥ 15 μg/L
Figure 2. Comparison of serum ferritin, soluble transferrin receptor, and total body iron in exercising women with amenorrhea (AMEN; n=36) and exercising women with eumenorrheic, ovulatory cycles (OvEU; n=28).

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.

Correlation of Iron Status Biomarkers with Menstrual Characteristics

Menstrual bleeding intensity, menstrual bleeding duration, and amenorrhea duration were not significantly correlated with any iron status biomarkers (Table 2).
Table 2. Correlations of iron status biomarkers with menstrual bleeding duration and menstrual bleeding intensity in exercising women with eumenorrheic, ovulatory cycles and correlations of iron status markers with duration of amenorrhea in exercising women with amenorrhea.

<table>
<thead>
<tr>
<th>Iron Status Biomarkers</th>
<th>Menstrual Bleeding Duration</th>
<th>Menstrual Bleeding Intensity</th>
<th>Amenorrhea Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin; μg/L</td>
<td>-0.30</td>
<td>-0.35</td>
<td>0.05</td>
</tr>
<tr>
<td>Transferrin Receptor; mg/L</td>
<td>-0.14</td>
<td>0.40</td>
<td>0.27</td>
</tr>
<tr>
<td>Total Body Iron (mg/kg)</td>
<td>-0.25</td>
<td>-0.36</td>
<td>-0.04</td>
</tr>
<tr>
<td>Hemoglobin; g/dL</td>
<td>0.39</td>
<td>0.39</td>
<td>0.09</td>
</tr>
<tr>
<td>Hematocrit; %</td>
<td>0.13</td>
<td>0.19</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Menstrual bleeding and amenorrhea duration are in days. Menstrual bleeding intensity was self-reported as “light,” “moderate,” or “heavy.” Correlations are displayed as Spearman’s correlation coefficients (rho).

Two OvEU did not report menstrual bleeding characteristics. The number of OvEU that reported “light,” “moderate,” and “heavy” bleeding menstrual bleeding intensity were 4, 9, and 13, respectively. Thirteen OvEU reported 5 or more days of menstrual bleeding, 7 OvEU reported 4 days of menstrual bleeding, 4 OvEU reported 3 days of menstrual bleeding, and 1 OvEU reported 1 and 2 days of menstrual bleeding, each. The average frequency of menses in the past 12 months was 12±1 menses with 50% reporting a menstrual bleeding duration of at least 5 days or describing their menstrual bleeding intensity as “heavy.” 13 (46%) self-reported menstrual bleeding duration of 5 days or more combined with menstrual bleeding described as “heavy” and 15 (54%) self-reported menstrual bleeding duration of less than 5 days combined with menstrual bleeding severity described as “light” or “moderate.” The prevalence of iron depletion was not significantly different between these groups (31% vs 20%, p=0.41; Table 3).
There were no significant differences in iron status markers between these groups (data not shown).

Table 3. Prevalence of iron depletion in exercising women with eumenorrheic, ovulatory cycles by menstrual bleeding grouping

<table>
<thead>
<tr>
<th></th>
<th>Low (n=15)</th>
<th>High (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron Depleted</td>
<td>3(20%)</td>
<td>4(31%)</td>
</tr>
<tr>
<td>Iron Sufficient</td>
<td>12(80%)</td>
<td>9(69%)</td>
</tr>
</tbody>
</table>

Iron Depleted: Ft < 15 μg/L; Iron Sufficient: Ft ≥ 15 μg/L
Low: menstrual bleeding intensity of “moderate” or “light” and a duration less than 5 days; High: menstrual bleeding intensity of “heavy” and a duration of 5 days or more

In AMEN, the average duration of amenorrhea was 320±281 days and the duration of amenorrhea was not significantly correlated with any iron status biomarkers (Table 4). In AMEN, 27 (75%) were amenorrheic for at least 6 months and 9 (25%) were amenorrheic for less than 6 months. The prevalence of iron depletion was not significantly different in AMEN who were amenorrheic for a longer duration than AMEN who were amenorrheic for a shorter duration (11% vs 0%, p=0.41; Table 4). There were no significant differences in iron status markers between these groups (data not shown).

Table 4. Prevalence of iron depletion in exercising women with amenorrhea by duration of amenorrhea grouping

<table>
<thead>
<tr>
<th></th>
<th>Short-term (n=9)</th>
<th>Long-term (n=27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron Depleted</td>
<td>0(0%)</td>
<td>3(11%)</td>
</tr>
<tr>
<td>Iron Sufficient</td>
<td>9(100%)</td>
<td>24(89%)</td>
</tr>
</tbody>
</table>

Iron Depleted: Ft < 15 μg/L; Iron Sufficient: Ft ≥ 15 μg/L
Short-term: amenorrhea duration < 6 months;
Long-term: amenorrhea duration ≥ 6 months
Discussion

Main Findings

Our study was the first to compare iron status in exercising women at opposite ends of a menstrual status spectrum, i.e. ovulatory, eumenorrheic vs, amenorrhea, that was characterized by daily measures of urinary ovarian steroids using previously established methods. Our primary finding was that 3/36 (8%) of amenorrheic women were iron depleted compared to 7/28 (25%) of women with ovulatory eumenorrheic cycles at a cut-off of 15μg/L for Ft. Even so, Ft, sTfR, TBI, Hb, and hematocrit values were not significantly different between groups. Furthermore, we found that the iron depletion prevalence and levels of iron status markers were not significantly associated with the intensity and duration of menses in exercising women with ovulatory, eumenorrheic cycles and with the duration of amenorrhea in exercising women with amenorrhea.

Difference in the Prevalence of Iron Depletion

The 25% prevalence of iron depletion in our exercising women with ovulatory eumenorrheic cycles women was similar to the 24% prevalence rate with the same Ft cut-off observed in the exercising women by Grooper et al. (2006). Interestingly, the 8% prevalence of iron depletion in our exercising women with amenorrhea was in the same magnitude as the prevalence of iron depletion in exercising men, which ranges from 0-17% (Rowland, 2012). Moreover, Swenne et al. (2007) observed a 37% prevalence rate in menstruating women, comparable to the 25% in OvEU, and a 10% prevalence rate in amenorrheic women, just as the 10% in AMEN. These observations suggest that amenorrhea in exercising women is associated with a decreased prevalence of iron depletion.
Similar Iron Status Markers According to Menstrual Status

Despite a higher prevalence of iron depletion, exercising women with ovulatory eumenorrheic cycles and exercising women with amenorrhea did not differ in any iron status markers. This finding is atypical as other investigations that observed a difference in iron depletion prevalence rates between exercising women and sedentary women and amenorrheic women and menstruating women also observed a difference in iron status markers. For instance, iron depletion prevalence rates, as determined by TfR index, of 50% in active women in comparison to 17% in sedentary women were substantiated by active women displaying significantly lower Ft and greater sTfR concentrations than sedentary women (Woolf et al. 2009). Furthermore, the 27% higher iron depletion prevalence rates in menstruating women than amenorrheic women observed by Swenne et al. (2007) was substantiated by a 77% lower Ft concentration in menstruating women. Our findings were in contrast to a similar investigation by Wilson et al. (2011) that observed that female military personnel with amenorrhea had a significantly greater Ft and Hb than their menstruating counterparts.

Several factors may explain our discrepant findings. The above investigations, and others not described in detail (Rowland et al., 1987; Rowland et al., 1989; Fogelholm, 1995; Beard et al., 2000; Dubnov et al., 2004; Fallon, 2008; Mettler et al., 2010; Kim et al., 2012; Moschonis et al., 2013), did not all consider inflammation, diet, exercise, and menstrual function as this investigation has. Inflammation, exercise, and diet have been known to affect iron status (Beard, 2000; McClung et al., 2009; Koehler et al., 2012; Auersperger et al., 2013) and should be accounted for accordingly. These investigations did also not confirm menstrual status by hormonal evaluation nor exclude users of hormonal contraception. Not considering such factors may have confounded their results given that menstrual status is associated with iron status and hormonal contraception can decrease menstrual blood loss (Casbellata et al., 2007). In fact,
41% of the menstruating women took hormonal contraception in the study by Wilson et al (2011), which may have profoundly confounded their results. Lastly, the greater Ft concentration and lower prevalence of iron depletion observed by Swenne et al. (2007) in the amenorrheic women may be attributed to these women’s diagnosis of anorexia nervosa, a condition associated with dramatically elevated Ft concentrations secondary to elevated hepcidin concentrations when compared to healthy controls (Papillard-Marechal et al., 2012).

Iron Status and Menstrual Bleeding Characteristics

We found that menstrual bleeding intensity and duration were not associated with iron status. This finding was demonstrated by the similar prevalence of iron depletion and similar levels of iron status markers in exercising women reporting more intense and longer menstrual bleeding vs. those reporting less intense and shorter menstrual bleeding. These findings were contrary to a previous investigation in the general population (Milman et al., 1998). However, it should be note that the correlations coefficients of Ft with menstrual bleeding duration and intensity in the present study were similar to that observed by Milman et al. (1998). Further, these correlations trended towards significance (p<0.10) prior to Bonferroni adjustment. As such, it appears that our sample size was insufficient to detect significant associations of menstrual bleeding intensity and duration with iron status.

Similarly, the prevalence of iron depletion and iron status markers did not vary by amenorrhea chronicity. The purpose of this exploratory aim was to determine if iron stores would accumulate over time due to a chronic absence of menses. Our results suggested that this is not the case but, rather, that iron regulatory mechanisms may act to prevent an accumulation of iron stores in amenorrheic women.
Limitations and Strengths

The quantity of phytate, a key inhibitor of iron absorption, consumed was not available and intake was not recorded on a meal-by-meal basis. Thus, iron bioavailability, the proportion of dietary iron absorbed (Hurrell & Egli, 2010), could not be assessed retrospectively. This limitation could be concerning as amenorrheic women with anorexia nervosa have been shown to consume significantly more phytate than healthy controls (Misra et al., 2006). Additionally, the heterogeneity in exercise modality in our exercising women may potentially confound our findings as exercise-associated iron losses depend on the intensity, duration, and mode of exercise (Peeling et al., 2008). However, previous studies have found no difference in iron status among individuals participating in different sports (Gropper et al., 2006; Ostojic & Ahmetovic, 2008). Lastly, the sample size may have been insufficient to detect subtle differences in iron status biomarkers between our groups of varying menstrual status and correlations between iron status biomarkers and menstrual bleeding characteristics. Yet, power analysis revealed that our study was adequately powered to detect group differences in the same magnitude as those previously reported for Ft (Wilson et al. 2011) and sTfR (Woolf et al. 2009).

Our findings are strengthened by several aspects of the study design. The use of well-established methods for classification of menstrual status (De Souza, 2004) allowed for the comparison of exercising women at opposite ends of the menstrual status spectrum. Since menstruation is negatively associated with iron status (Kim et al., 2012; Moschonis et al., 2013), a comparison of exercising women with ovulatory eumenorrheic menstrual cycles and exercising women with amenorrhea should have provided the greatest possible difference in iron status due to menstrual status. Additionally, several confounders of iron status were accounted for by the design of this investigation. Exercise and dietary characteristics between
groups were found to have no significant effect on iron status and similar between groups. Excluding users of hormonal contraception allowed for a true investigation of the effect of menstrual status on iron status. Lastly, Ft was adjusted based on inflammation status to reduce any confounding effects of exercise-associated inflammation on iron status (Peeling et al., 2008; Thurnham et al., 2010).

Conclusion

The primary finding that exercising women with ovulatory, eumenorrheic menstrual cycles have a higher prevalence of iron depletion than exercising women with amenorrhea supports the notion that menses is a primary determinant of the relatively high prevalence of iron depletion in exercising women. Although we found no difference in iron status markers by menstrual status or menstrual characteristics, these findings may be the result of our small sample size. Nevertheless, exercising women with ovulatory, eumenorrheic cycles should be informed of their increased risk of iron depletion.
References


in two exercising women with amenorrhea of varying duration. *Journal of the International Society of Sports Nutrition, 10*, 34.


Chapter 5: Conclusion

Despite high prevalence rates of compromised menstrual status and suboptimal iron status in exercising women, it is unknown how menstrual status affects iron status in exercising women. As such, the primary aim of this thesis was to determine the effect of menstrual status on iron status in exercising women by assessing and comparing the iron status of exercising women with ovulatory, eumenorrheic cycles and exercising women with amenorrhea. This thesis also aimed to determine if menstrual status characteristics were associated with iron status in exercising women with ovulatory, eumenorrheic cycles. An exploratory aim of this thesis was to determine if the duration of amenorrhea was associated with iron status in exercising women with amenorrhea. More specifically, this thesis tested the following hypotheses:

1) Exercising women with ovulatory, eumenorrheic cycles will display a more compromised iron status, as indicated by a higher prevalence of iron depletion (Ft <15 μg/L) and poorer iron status markers, i.e. lower serum ferritin concentrations, greater soluble transferrin receptor concentrations, lower total body iron values, lower hemoglobin concentrations and lower hematocrit, when compared to exercising women with amenorrhea.

2) Exercising women with ovulatory, eumenorrheic cycles with self-reported menstrual bleeding duration of 5 days or more and self-described menstrual bleeding as “heavy” will exhibit a more compromised iron status when compared to exercising women with ovulatory, eumenorrheic cycles with self-reported menstrual bleeding duration of less than 5 days and self-described menstrual bleeding severity described as "light" or “moderate.”
3) Exercising women with amenorrhea who have been amenorrheic for less than 6 months will display a more compromised iron status when compared to exercising women with amenorrhea who have been amenorrheic for at least 6 months.

Findings

The main findings of the study, which are discussed in greater depth in chapter 4 in the discussion section, are briefly summarized here:

1) Exercising women with ovulatory, eumenorrheic cycles displayed a higher prevalence of iron depletion than exercising women with amenorrhea, yet exercising women with ovulatory, eumenorrheic cycles and exercising women with amenorrhea had similar iron status markers.

2) Exercising women with ovulatory, eumenorrheic cycles with self-reported menstrual bleeding duration of 5 days or more and self-described menstrual bleeding as “heavy” did not display a significantly different iron status than exercising women with ovulatory, eumenorrheic cycles with self-reported menstrual bleeding duration of less than 5 days and self-described menstrual bleeding severity described as “light” or “moderate.”

3) Exercising women with amenorrhea who have been amenorrheic for less than 6 months did not display a significantly different iron status than exercising women with amenorrhea who have been amenorrheic for at least 6 months.

Limitations

The quantity of phytate, a key inhibitor of iron absorption, consumed was not available and intake was not recorded on a meal-by-meal basis. Thus, iron bioavailability, the proportion of dietary iron absorbed (Hurrell & Egli, 2010), could not be assessed retrospectively. This limitation could be concerning as amenorrheic women with anorexia nervosa have been shown
to consume significantly more phytate than healthy controls (Misra et al., 2006). Additionally, the heterogeneity in exercise modality in our exercising women may potentially confound our findings as exercise-associated iron losses depend on the intensity, duration, and mode of exercise (Peeling et al., 2008). However, previous studies have found no difference in iron status among individuals participating in different sports (Gropper et al., 2006; Ostojic & Ahmetovic, 2008). Lastly, the sample size may have been insufficient to detect subtle differences in iron status biomarkers and correlations between iron status biomarkers and menstrual bleeding characteristics. Yet, power analysis revealed that our study was adequately powered to detect group differences in the same magnitude as those previously reported for Ft (Wilson et al. 2011) and sTfR (Woolf et al. 2009).

Implications and Future Directions

Our main findings have implications for iron intake guidelines and future investigations. Exercising women with ovulatory, eumenorrheic cycles should be informed of their higher risk of iron depletion and may need to consider iron supplementation under physician guidance. Further, since amenorrhea appears to be protective against iron depletion, exercising women who are aiming to restore menstrual status should be aware of the higher risk of iron depletion associated with menses. However, exactly how the restoration of menstrual status affects iron status is unknown. As such, future investigations should determine how restoration of menstrual status affects iron status.

Concluding Remarks

The field of women’s health and exercise research has provided valuable information that has assisted exercising women in achieving peak performance without compromising health. In particular, the field has made great strides in understanding the etiology of several
deleterious conditions so that have been critical in the prevention and treatment of these conditions. This thesis on menstrual status and iron status provides a valuable addition in understanding how to maintain and achieve optimal health for exercising women.
References


