BIOCHEMICAL MARKERS OF IRON STATUS IN RECREATIONAL FEMALE RUNNERS

THESIS

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Abstract

**Purpose:** To describe markers of iron status in recreational female runners and the relationship of iron intake and iron status in this population.

**Methods:** Of the 121 consented participants, 93 met the inclusion criteria and were included in the final analyses. After refraining from running for 24 hours and fasting for 8 hours, venous blood was drawn for markers of iron status (hemoglobin (Hgb), hematocrit (Hct), ferritin (Ferr)). Ferritin and soluble transferrin receptor (sTfR) concentrations were measured using the ELISA method (ALPCO Diagnostics). Dietary data were collected and analyzed (ESHA Research). Statistical analysis was conducted with SPSS version 20.

**Results:** The mean values for markers of iron status ± standard deviation are as follows: Hgb 13.02 ± 1.00 g/dL, Hct 38.21 ± 2.84 %, Ferr 17.22 ± 6.15 ng/mL, sTfR 4.29 ± 2.15 mg/mL, and soluble transferrin receptor/log (Ferr) ratio (TfR ratio) 3.68 ± 2.03. Twelve runners had clinically low Hgb (<12 g/dL). Ferritin was below the literature defined (<20ng/mL) ‘normal-low’ in 63 runners while values outside the normal range for sTfR were found in 30 runners. There was a significant inverse relationship between ferritin and sTfR (p <0.001) with a Pearson correlation coefficient of -0.482. Additionally, there was a significant inverse relationship between ferritin and TfR ratio (p< 0.001). When using 3 literature based clinical ferritin categories, the associated sTfR were found to be significantly different (p<0.001) in two of the three ferritin groups (Ferr <12) and (Ferr >20). Iron intake did not correlate with ferritin or TfR ratio and iron intake did not improve the correlation between ferritin and sTfR or ferritin and TfR ratio; however, the correlation between ferritin and sTfR did improve when vitamin C was added to the
model. Inadequate dietary iron intake correlated with iron status markers ferritin and sTfR indicating it is influential in iron status.

**Conclusions:** Although only 12 of 93 recreational female runners had low Hgb, the majority (n=63) had significantly low iron stores as noted by sTfR and ferritin. This suggests improved early prediction of iron status. Use of sTfR and TfR ratio highlight the significant negative relationships to the clinical categories of iron deficiency (without anemia) when assessed with ferritin. We conclude that the additional iron status markers, ferritin, sTfR, and TfR ratio demonstrated more precision in identifying low iron stores and set the stage for additional work to evaluate the impact of iron supplementation on the iron status of recreational female runners and their performance.
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Publications


Fields of Study

Major Field: Health and Rehabilitation Sciences
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<td>Anemia of chronic disease</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>Fe/d</td>
<td>Iron per day</td>
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<tr>
<td>Fe</td>
<td>Iron</td>
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<td>ID</td>
<td>Iron deficiency</td>
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<td>IDA</td>
<td>Iron deficiency anemia</td>
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<td>IRB</td>
<td>Institutional Review Board</td>
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<td>mg</td>
<td>Milligrams</td>
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<tr>
<td>mg/d</td>
<td>Milligrams per day</td>
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<tr>
<td>ml</td>
<td>Milliliters</td>
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<tr>
<td>RBC</td>
<td>Red blood cells</td>
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<td>RDA</td>
<td>Recommended dietary allowances</td>
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<td>sTfR</td>
<td>Soluble transferrin receptor</td>
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<tr>
<td>TRR</td>
<td>Transferrin receptor ratio</td>
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<tr>
<td>TS</td>
<td>Transferrin saturation</td>
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<td>TIBC</td>
<td>Total Iron Binding capacity</td>
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Glossary of Terms

Anemia
A condition marked by a deficiency of red blood cells or of hemoglobin in the blood, resulting in weariness.

Anemia of Chronic Disease (ACD)
A form of anemia seen in chronic illness, e.g. from chronic infection, chronic immune activation, or malignancy. Also referred to as anemia of inflammatory response.

C-Reactive Protein (CRP)
A globulin that, in the presence of calcium ions, precipitates the C substance of pneumococcal cells. CRP is an abnormal protein detectable in the blood only during the active phase of certain acute illnesses and inflammatory processes.

Cortisol
Steroid hormone produced by the adrenal gland and is released in response to ACTH, which is released due to tumors, illness, physical and emotional stress.

Erythropoietin
Hormone secreted by the kidneys that increases the rate of production of red blood cells in response to falling levels of oxygen in the blood.

Ferritin (Ferr)
The cellular storage protein for iron, found in the liver, spleen, intestine and bone marrow. Is also an acute phase protein and, hence, serves as a clinical marker of body iron content after significant inflammation has been excluded.

Hemochromatosis
also called iron overload, is a disorder that results in too much iron being absorbed from the gastrointestinal tract. Patients with hemochromatosis have hyperferritinemia.

Hepcidin
hormone that acts as the main negative regulator of iron metabolism by inducing the internalization and degradation of the iron transporter ferroportin, thereby reducing intestinal absorption of iron and inhibiting the release of iron from macrophages.

Hyperferritinemia
Indicates high serum ferritin levels found in genetic and acquired conditions that may or may not be associated with iron overload. Patients with hyperferritinemia do not necessarily have hemochromatosis.
Iron (Fe)
Trace element essential for human life, participates in many reactions because of its ability to cycle from ferrous to ferric forms. Free iron is highly toxic because of the potential for generating free radicals by Fenton reaction.

Iron Deficiency Anemia (IDA)
A form of anemia due to lack of iron in the diet or to extreme iron loss from bleeding.

Reticulocyte
An immature red blood cell.

Transferrin
A glycoprotein mainly produced in the liver that serves as iron transporter in circulation, may bind one or two ferric ions.

Soluble Transferrin receptor (sTfR)
Binds diferric transferrin in a complex that enters the cell by endocytosis. The soluble form is released into the circulation when the receptor is not bound to diferric transferrin, and serves as a marker of the rate of erythropoiesis.

Total Iron Binding Capacity (TIBC)
Medical laboratory test which measures the blood’s capacity to bind iron with transferrin. High TIBC is associated with iron deficiency, low is associated with excess iron.

Transferrin
A protein in the blood that binds and transports iron.

Transferrin receptor index (TRI)
See transferrin receptor ratio

Transferrin receptor ratio (TfR ratio)
Also referred to as the transferrin receptor index, calculated by the soluble transferrin receptor concentration divided by the log (ferritin).

Transferrin saturation
Ratio of plasma iron to transferrin (usually between 20% and 50%), increases as a function of iron burden.
Chapter One:
Introduction

BACKGROUND AND SIGNIFICANCE

Running for physical fitness and enjoyment, also known as recreational running, is a growing activity for men and women. There are over 12 million recreational runners in the United States who run at least 100 days out of every year (Running USA 2007). Recreational runners average 10-12 hours of training per week and often participate in a long run every other week (Deitrick R, 1991; Mechelen W, 1992).

Athletes focus on many areas to improve their sports performance such as: training, nutrition, diet, metabolism, and bone health. Research in these areas has led to evidence-based recommendations for elite sports performance. Runners often attempt to enhance their performance by utilizing an optimal training plan, maintaining personal peak performance, following nutritional guidelines and taking preventative health measures.

While running offers extensive benefits, there can be harm if one fuels or trains improperly. Female runners specifically, may experience unique challenges with respect to poor performance, repetitive stress, and acute injuries impacted by the anatomic, biomechanical, hormonal, nutritional, and functional factors. Research regarding
recreational female athletes, remains limited (Ireland, 2004). Most often, extrapolation is made from studies of collegiate athletes, Norwegian elite athletes and high intensity high school athletes (DiSantolo 2008; Sundgot-Borgen J, 1998; Rowland T, 2012). While the study of elite athletes can shed light on the health of female runners, recreational running potentially across the lifespan may have long-term health impacts yet to be studied in women (Prather, 2005).

It has been reported in several studies that female athletes who are involved in heavy training may be more prone to specific nutrient deficiencies (Clement 1984; Gabel 2006). Due to the female physiology, women are most at risk for iron deficiency; this was recently found as high as 46% in active female adolescents (Clement 1984; Constantini et al 2000). Iron deficiency anemia is the most prevalent micronutrient deficiency in the world (Beard and Stoltzfus, 2001). The commonly used definition for anemia is low hemoglobin, which comes from a decrease in number and size of red blood cells and is defined clinically by a hemoglobin level below 12 g/dL. There are two main forms of anemia – iron deficiency anemia (IDA) and anemia of chronic disease (ACD). ACD occurs when there is a suppression of erythropoiesis by mediators of a chronic inflammatory response. IDA is anemia secondary to a lack of adequate iron in red blood cells (Cook and Skikne 1989; Wians et al. 2010). The study of IDA in athletes is common and the focus of this study. Anemia inversely affects oxygen and carbon dioxide exchange between blood and tissues which impairs endurance athletic performance by decreasing the oxygen delivery and uptake by muscles. The reversal of IDA usually requires at least 3 to 6 months of iron supplementation, therefore early
detection for athletes is critical (Iron Disorders Institute, 2004). Understanding the incidence and predictability of iron deficiency in athletes is imperative to optimizing performance. Athletes who train at 65-75% of their VO\(_2\) max are primarily utilizing oxidative metabolism in which iron plays a critical role in producing ATP for useful energy. Celsing and colleagues (1988) showed that the activity of iron-dependent enzymes and cytochromes needed for oxidative metabolism are decreased in athletes with iron deficiency and thereby lead to impaired endurance performance. These enzymes are iron dependent, therefore the lower the available iron in the body the more the rate of oxidative metabolism decreases and the more it negatively impacts performance (Beard 2001).

The prevalence of iron deficiency anemia is estimated at 3-5% of premenopausal women in the United States and iron deficiency without anemia is 12-16% of premenopausal women (Eichner, 2010). Because regular exercise can result in a greater depletion of iron stores, the prevalence of iron deficiency is reported to be 25%-35% of female athletes competing in a variety of sports (Malczewska et al 2001). There is little to no research available on the prevalence of iron deficiency without anemia in a population of recreational female runners. It is known that iron deficiency affects more women than any other condition and puts them at higher risk for multiple health consequences (Khalafallah 2012).

While adequate iron can be defined as a normal hemoglobin concentration, optimal iron nutrition should be regarded as sufficient body iron to avoid any limitation in tissue iron supply. Low tissue iron supply before hemoglobin falls below normal is
termed iron deficient erythropoiesis or iron deficiency without anemia and when hemoglobin concentration declines to below normal the term is iron deficient anemia. A variety of lab measures have been used to identify this milder form of iron deficiency including transferrin saturation, mean corpuscular volume and total iron binding capacity. Another biomarker of iron depletion often used is serum ferritin. Ferritin is an iron transport protein whose blood level reflects the body's iron stores, and has been reported consistently lower than normal in athletes even when hemoglobin concentrations are normal (Sinclair and Hinton 2005). The existence of low iron stores, (ferritin < 20 µg/L) can cause immediate and long term effects (Constantini 2004; Rodenberg and Gustafson 2007). Specifically, low iron increases muscle fatigability, decreases endurance performance and energy efficiency (Brutsaert et al 2003). While iron deficiency is the most common cause of anemia, many of the symptoms of low iron can and do exist in women without anemia.

In the sports health care environment, it is generally agreed that low serum ferritin levels are frequently found in athletes, yet there is no agreement as to the significance of these findings (Eichner 1992 and Sherman 1990), i.e., whether it represents true iron deficiency or a physiological response to training. Being an acute phase protein, ferritin increases with various conditions. Inflammation may mask the actual iron deficiency when iron deficiency exists without anemia. There is controversy surrounding the clinical definition of significant iron deficiency in athletes. Rowland (2012) defines iron deficiency as the level of iron deficit that will negatively impact exercise performance prior to the more severe state which is the onset of iron deficient anemia (Rowland 2012).
Specifically, there is a debate on the ferritin cutoff for iron deficiency in athletes and there are conflicting results about the effect on performance of iron deficiency without anemia (Eichner, 2000; Hinton et al., 2000; Brownlie et al., 2004; Rodenberg and Gustafson, 2007.) There have been a few iron studies in elite athletes but none in the recreational female runner.

Serum ferritin, which is stored in the liver, is recognized as the most commonly used index for the body’s iron storage and reference value for iron status; this marker is elevated following endurance exercise. It has been demonstrated that physical activity is accompanied by inflammation-like reactions in the joints and muscles, which may induce a rise of ferritin in plasma persisting for a few days following strenuous exercise. Therefore false elevations may be observed in athletic populations. With this false elevation, iron deficiency may go unrecognized. Additionally, serum ferritin is a reflection of hepatic iron stores rather than functional iron present in myoglobin, iron-dependent enzymes, and other iron-dependent proteins such as the cytochromes. A more direct indicator of functional iron would enhance the assessment of iron status.

Recent work with iron deficiency provides evidence that the soluble transferrin receptor (sTfR) may be a more sensitive marker for evaluating iron status because it is not an acute phase protein like ferritin; hence, its concentration is not affected by infections and inflammatory states. STfR has low-biological variability and remains quite stable after exercise or a brief training period (Malczewska et al 2000). The appropriate clinical strategies for screening athletes for iron deficiency remain undetermined, but the sTfR and the ratio between sTfR and log ferritin may prove to be a
supplemental method and the optimum identifier of low iron status in recreational athletes (Sinclair and Hinton, 2005; Mettler and Zimmermann, 2010).

According to most relevant studies, the concentration of the sTfR in serum appears to be valuable in detecting iron deficiency (Suominen et al 1998; Koulaoudis et al, 2009). The sTfR is a transmembrane protein present in all cell types (Cook et al, 1993). Recent studies indicate that the soluble transferrin receptor is the preferred measurement because enhanced synthesis of the transferrin receptor represents the initial cellular response to a declining iron supply (Skikne et al, 1990). The transferrin receptor ratio defined as the ratio of serum transferrin receptor to log serum ferritin has been shown in recent relevant studies to be useful in identifying functional iron depletion in athletes whose serum ferritin concentrations are marginal but do not fall below the threshold for iron depletion (Malczewska et al 2000; Peterson et al, 2006). This ratio would also detect false ferritin elevation from exercise because it incorporates the relationship to the sTfR levels.

Diet is another critical aspect to optimal health and performance. Only a few studies have been conducted with recreational female runners and none have evaluated iron status related to diet in this population. A more comprehensive evaluation of iron status and dietary intake from a population of recreational female runners is needed to understand and explain the prevalence of iron depletion and other iron status abnormalities. Additionally, this dietary iron intake data could also help to identify potential nutrition related risk factors for iron depletion in these recreational runners.
Research Questions and Hypotheses

The objectives of this study are to describe and evaluate the serum iron levels in recreational female runners using multiple measures including ferritin, hemoglobin, hematocrit soluble transferrin receptor levels and the transferrin receptor ratio. Our research questions are:

1. Does the ratio of soluble transferrin receptor to log ferritin correlate with the ferritin values? Does the correlation with ferritin improve when controlled for cortisol?

2. Using literature defined ferritin values clinically categorized into normal-low, low and deficient reference ranges, does the soluble transferrin receptor (continuous or categorical) correlate with the same clinical categories?

3. Does the average daily intake of iron (in mg) correlate with ferritin or soluble transferrin ratio? Is this correlation influenced when modeled with dietary vitamin C as an influence on iron absorption?

We hypothesized that the soluble transferrin receptor log ferritin ratio would match with the clinical categories of iron deficiency without anemia as well as correlate with dietary iron, thus enhance our ability to more precisely identify those with low iron status in this population.
Chapter Two

LITERATURE REVIEW

The literature reviewed for this thesis included the following general subcategories related to iron status and performance in recreational female runners: Iron function, metabolism, status and biochemical markers, iron intake and food, sports nutrition topics relative to exercise and gender, iron status in female athletes, other factors in female athletic performance involving iron, soluble transferrin receptor, and iron deficiency classifications for this study.

Currently, the available research concerning iron status in female athletes is focused on elite and competitive athletes more than on recreational runners. There are studies in the literature on recreational females ranging a variety of topics. DeSouza et al evaluated the luteinizing hormone pulsatility and luteal phase iron deficiency found in recreationally active women (DeSouza et al, 1998. DeSouza et al 2003). Schmid et al (2012) limited their work to an evaluation of anthropometrics and training schedule influence on marathon time performance. To our knowledge there is currently no published research that has effectively measured the prevalence of iron deficiency or the
method by which to best evaluate iron levels in either gender of adult recreational runners.

IRON: FUNCTION, METABOLISM AND STATUS MARKERS

Iron is a trace mineral and an essential component of red blood cells (RBC), working specifically within the RBC’s hemoglobin and the muscle cell’s myoglobin to assist in their function as oxygen-carrying proteins. In order to transport oxygen to the exercising muscle, an adequate quantity of circulating hemoglobin and iron are critical. Hemoglobin production is the primary physiological need for iron in the body. The synthesis of hemoglobin takes place in the bone marrow during the late stages of RBC production. During hemoglobin synthesis, iron travels to the bone marrow via the protein carrier, transferrin, and is released into the RBC to form heme. Heme consists of a ferrous (Fe²⁺) iron complex within the precursor protein called protoporphyrin IX (Baynes and Stipanuk 2000). Porphyrin is a macromolecule that binds divalent and trivalent metals like iron to form complexes. Hemoglobin carries about 98.5% of the total oxygen found in the blood and is responsible for the RBCs’ red color. When iron is bound to hemoglobin, the protein’s oxygen carrying capacity is increased by 65 times making it evident that iron is essential for maximal oxygen transport.

Once at the muscle, oxygen is used to generate ATP through the oxidative degradation of substrate in the Tricarboxylic Acid (Krebs) cycle. Ideally for exercising athletes, this substrate is primarily carbohydrates and fats. Oxidative metabolism contributes to sustained exercise such as distance running and cycling. Because iron is an essential component of both hemoglobin and the cytochromes of the electron transport
chain, without it cells rely more heavily on anaerobic metabolism and have a more limited ability to make enough ATP to sustain activity. Even with a normal level of hemoglobin the body may be completely depleted of iron stores. Research shows that low hemoglobin is also expected to impair endurance exercise and physical performance. A previous research study of distance runners found that even small reductions in hemoglobin can negatively affect exercise capacity (Rowland, 2012). They demonstrated a 20% decrease of treadmill endurance time in athletes with hemoglobins of 11.0 to 11.9 g/dL compared to athletes with hemoglobins greater than 13.0 g/dL (Rowland, 2012).

Hemoglobin is not the only molecule that uses iron, iron is used to make enzymes needed for energy metabolism, growth, and cell and nerve protection. It is a component of many heme enzymes including cytochromes, catalase, and peroxidase and a component of non-heme enzymes such as NADH, succinic dehydrogenase, and xanthine oxidase. Though iron is well known for the role in hemoglobin and myoglobin, the role in these oxidative enzymes and systems may be more important to performance than widely realized.

To emphasize the roles of iron, the total iron content of the human body is between 3 and 5 grams (Wick et al 2003). Of this total iron pool, it is present as active iron in the following percents: hemoglobin, 67%; myoglobin, 3.5%; enzymes and proteins, 0.2%; as the transport iron, transferrin, 0.08% and some is deposited in tissues as, hemosiderin-ferritin, 27% (Wick et al. 2003) We cannot limit the role of iron to hemoglobin and myoglobin in athletic performance.
Iron absorption is regulated by transport through the small intestine, which depends on the rate of erythropoiesis, liver-derived iron regulatory protein, hepcidin, and the reduction in body iron stores (Baynes and Stipanuk, 2000). Erythropoiesis takes place in the bone marrow. The ferrous iron absorbed from the diet and transported by transferrin, as well as iron recycled by reticuloendothelial macrophages, is taken up by the bone marrow erythroblasts. Transferrin distributes the iron throughout the body to meet various metabolic needs, including storage in the form of ferritin. Serum iron and transferrin vary considerably on a day to day basis and do not reflect the body’s total iron stores (Cooper and Zlotkin, 1996). These indicators can be influenced by multiple factors including food intake, gender, and acute and chronic diseases (Miller, 2013).

Overall iron status is usually described with an assessment of iron stores, often hemoglobin and hematocrit then ferritin and transferrin saturation are evaluated and lastly, MCV which is not used nearly as often as the previous. Victor Herbert (Herbert et al, 1997) first depicted the stages of declining iron status through a 3-stage model which progressed from negative iron balance (iron depletion) through iron deficient erythropoiesis to iron deficiency anemia (figure 1).
Along the course of this progression, a variety of associated iron-containing blood proteins and markers can be traced including ferritin, transferrin (saturation %), RBC protoporphyrin, total iron binding capacity (TIBC), serum iron (SI), marrow sideroblasts, mean cell volume (MCV), and mean cell hemoglobin (MCH). While Herbert’s work served to inform the clinical path of progression from iron depletion to anemia, the exactness of each step is vague due to overlapping values for the above parameters. The number of abnormalities increases as the progression of iron depletion moves closer to
iron deficiency anemia. Most often clinically, hematocrit and hemoglobin are used to accurately verify anemia, but the stages prior to anemia are not well classified.

This leads to controversy over when to begin treatment and is especially challenging in female athlete populations. Recent work suggests that additional markers such as ferritin, soluble transferrin receptor, and ratio of sTfR to log ferritin (i.e. the transferrin receptor ratio), could provide more specific details to the overall assessment and treatment options for iron status. The role of these markers in assessment is further examined.

Ferritin

Ferritin is the storage biomarker protein for iron which is accessed for making new RBCs and hemoglobin. Ferritin is primarily stored in the liver (about 60%) and in muscle tissue and cells of the reticuloendothelial system (about 40%). Serum ferritin is viewed as an indirect marker of “stored iron”. Ferritin is in a constant state of flux between degradation and resynthesis to provide a continuous reservoir or intracellular iron pool. A value of 1 ng/mL of serum ferritin corresponds to about 5-9 mgs of stored iron (Valberg, 1980). The average stored iron in males is around 1000 mgs, whereas females store between 100 and 400 mgs (Gledhill et al 1999). Initially, during periods of low iron intake, less iron is stored and with very little being incorporated into RBCs and hemoglobin there is no influence on hemoglobin concentration until iron deficiency anemia is reached. This innate conservation masks a worsening state of iron depletion because the lack of iron in these proteins can go undetected for months until enough RBCs with low iron are in large enough numbers to be detected. With prolonged low
iron consumption, total RBCs (hematocrit) and hemoglobin concentration decline. Once in full iron deficiency anemia, the size (microcytic) and color (hypochromic) of the RBC are classically impacted. If a healthcare professional evaluates an individual’s iron status by only looking at RBC concentration, more specifically hemoglobin and hematocrit, early signs of iron depletion can go undetected. Due to the metabolic demands of long distance runners and other endurance athletes, storage iron can be used up quite rapidly. By monitoring the status of this storage iron, detection of iron depletion can occur earlier and prevention of iron deficiency can be initiated. This is the basis for using the storage protein ferritin as the preferred marker for total body iron status (Beard and Tobin, 2000).

Unfortunately, ferritin cannot be treated as the gold standard for measuring iron status for several reasons. Ferritin is an acute phase protein and varies in certain conditions when there is not a change in iron stores; such as infection, inflammation, disorders of the liver and malignancies all can cause increases in ferritin and mask potential iron depletion. Exercise is known to impact iron metabolism to a great extent because exercise-induced inflammation is similar to inflammation seen in chronic disease. Inflammation that occurs with exercise may cause changes in iron status like increased ferritin, decreased serum iron, TIBC, transferrin, and transferrin saturation (Fallon et al. 2001).

Running is proposed to have some unique influences on iron status related to red blood cell turnover. During the process of running, foot strike hemolysis occurs and increases iron loss through the bursting of red blood cells under the heel as the runner lands with each step (Telford et al 2003). RBC survival rate with the continuous foot
pounding declines with the stress of exercise and specifically, the impact of running. Weight et al re-infused autologous $^{51}$Cr-labeled RBCs and found that RBCs survived for an average of 74 days in runners compared with 114 days in sedentary controls, indicating that running reduces RBC life span. This is consistent with a higher rate of RBC destruction. In a similarly designed trial, Telford et al, 2003 suggested this declining life span of runners' RBC might increase the susceptibility of anemia for all athletes involved in running sports. Heel strike hemolysis is one theory of anemia specific to sports.

Physical activity has been shown to initiate inflammation-like reactions by triggering acute phase proteins to respond. The physiologic response in the body includes ferritin levels rising and possibly staying at persistently high levels for days (Weight et al, 1991). Using ferritin levels in routine athlete assessments of iron status may be misleading and provide a false impression of actual iron status. While low ferritin indicates a body is exhausted of its iron reserves, normal or high levels of ferritin do not necessarily correlate with adequate iron stores. In a study of training female collegiate swimmers, researchers found no difference in ferritin levels between the collegiate female athletes and sedentary subjects, $20 \pm 9$ ng/mL and $17 \pm 11$ ng/mL, respectively (Braun 2000). It is difficult to justify if sport-related training inflammation could have artificially elevated the ferritin levels of the swimmer group.

Inflammatory Markers

Strenuous exercise has been shown to induce several aspects associated with the acute phase response and is frequently used as a model to investigate acute phase
response by looking at acute phase proteins including ferritin, creatine phosphokinase, haptoglobin, and CRP. (Fallon, 2001; Pederson 2000). It is typically thought that the alterations in the acute phase reactants are brought on by muscle damage resulting from strenuous exercise. Consequently exercise that produces a greater amount of muscle damage would be expected to produce a greater inflammatory response. Controversy still exists as to whether exercise at different intensities truly exemplifies the acute phase response and how factors such as duration and type of exercise mediate the response (Fallon 2001). Because the acute phase response has been shown to exist in exercise it is important to understand and evaluate markers of inflammation.

C Reactive Protein

In many of these studies of the acute phase response in athletes, an acute phase protein and most frequently used marker evaluated is C-reactive protein, CRP. CRP responds to various threats to normal homeostasis within the body and may become elevated from infection, malignancies, illnesses, forms of stress and inflammatory states. Research has shown the elevation in CRP that takes place in athletes and that the CRP will remain high for a few days after training. Serum ferritin levels may be elevated with CRP and inflammation as both are acute phase proteins and will show short term responses in similar manner.

Cortisol

Cortisol is a hormone produced by the adrenal gland and similar to acute phase proteins, cortisol is released in response to various kinds of stress. One of the main jobs
of cortisol is to increase the glucose concentration in the blood to make more energy readily available to the muscles. Oxidative stress and inflammation caused by high volume and high intensity exercise result in elevated levels of cortisol in athletes. In a study by Taylor et al (1987) they measured acute phase protein responses in triathletes and found that cortisol significantly increased after the race and there was also a 40% rise in the plasma ferritin. This study provides evidence that exercise induced inflammation, evidenced by an elevated cortisol, can cause a temporary rise in ferritin and indicate a false measurement of iron stores. It is therefore important to measure a marker of inflammation in athletes when measuring ferritin levels to confirm the accuracy of the ferritin reading.

Utilization of a marker of inflammation can help better predict the activity of acute phase response proteins. If an acute phase protein is elevated it can be expected that ferritin, also being an acute phase protein would be exhibiting an acute phase response, also be elevated and not exhibit true iron storage status. With this understanding the measurement of ferritin will be more accurate. Another non-sport limitation for using ferritin as a biomarker of iron status is that a wide ‘normal range’ of ferritin, 20 – 150 ng/mL has been cited in the literature and may not be sensitive to iron status across the spectrum ranging from iron depletion to iron deficiency anemia. A recent randomized controlled trial by Vaucher et al (2012) evaluated the relationships between ferritin levels and fatigue in a population of women. Almost 200 women between the ages of 18 and 53, suffering from fatigue were included in the study. Participants were classified as non-anemic with hemoglobin levels ≥ 12.0 g/dL and these
women had ferritin levels lower than 50 ng/ml. The study also evaluated the effects of iron supplementation. Women were treated with ferrous sulfate (most common iron prescribed by doctors) or placebo for 12 weeks (Vaucher et al 2012). In the women taking the iron supplement, fatigue scores were significantly reduced by half at the end of 12 weeks. Physiologically, the group receiving iron supplementation showed an increase in hemoglobin, a significant increase in ferritin (mean initial level of 22.5 ng/mL with average increase of 11.4 ng/mL) and also a significant decrease in soluble transferrin receptor levels (mean initial level 3.44 ng/mL to an average decrease of 0.54 ng/mL). This study suggests that in testing iron status in women, just being in the ‘normal range’ for ferritin is not enough to completely verify iron status. These authors suggested that an additional marker of iron status would be beneficial to more accurately evaluate all women in this age range. In another study, Berra et al, (2004) found a similar conclusion suggesting that for premenopausal women to feel properly energized, ferritin levels should be above 50 ng/mL. Research has not provided clear clinical guidelines on the ferritin levels to define thresholds for the level of supplementation.

Soluble Transferrin Receptor

Plasma iron transport is carried out by transferrin, which delivers iron into cells through its interaction with a specific membrane bound receptor, the transferrin receptor. The transferrin receptor is a homodimeric protein that mediates iron uptake into cells via endocytosis. It is present on all cell types, including erythrocyte precursors and skeletal muscle. A soluble form of the transferrin receptor (sTfR) has been identified in human
serum. Soluble transferrin receptor is a truncated monomer of tissue receptor, lacking its first 100 amino acids, which circulates in the form of a complex of transferrin and its receptor (Beguin, Y, 2003). As the transferrin receptor-transferrin-iron complex reaches the cell membrane it is internalized via an endocytic vesicle and then iron is released from the transporter. The transporter then gets recycled back to the cell surface. During the recycle process as fraction of the transferrin receptor proteins are shed into the blood and appear as soluble transferrin receptors which can then be measured (Gupta S et al 2009). The surface density of this receptor is regulated by the iron stores of the cell and the intracellular iron turnover.

The transferrin receptor is a direct indicator of cellular iron status and starts to increase when iron stores are depleted or iron turnover is stimulated. As the need for iron increases, the number of transferrin receptors also increase. Traditionally, numerous clinical indicators such as hemoglobin, mean red blood cell volume (MCV), total serum iron, total iron binding capacity serum ferritin, and free erythrocyte protoporphyrin, have been used to evaluate iron status. However, physical activity may confound the assessment of some of these parameters, thus female runners may have different criteria for evaluation of an accurate iron status marker. MCV may confirm a microcytic, hypochromic state with serum ferritin often low, but unfortunately these tests are a lot less reliable with athletes due to changes in plasma volume (Koulaouzidis et al 2009). The metabolism of the receptor is closely related to transferrin, and a general thought of experts is that soluble transferrin receptor (sTfR) elimination from circulation is closely related to transferrin degradation. When contrasted with ferritin levels, sTfR levels are
not affected by inflammatory reactions, chronic diseases, or in response to exercise (Malczewska et al. 2000, Schumacher et al. 2002), and can be used for diagnosing iron deficiency in all populations including those which would stimulate a positive acute phase protein (Beguin et al, 1998).

As a result, the soluble transferrin receptor has been introduced as a sensitive, new diagnostic tool for differentiating between iron deficiency anemia (IDA) and anemia of chronic disease (ACD) (Baillie et al 2003). When iron deficiency exists, the soluble transferrin receptor concentration in serum rises even before the hemoglobin concentration is significantly depressed as a mechanism for capturing more of the desired iron. The sTfR concentration can describe the functional iron status, while ferritin reflects the iron storage status (Berlin, Meyer. 2011). Extensive research has shown the value of utilizing the soluble transferrin receptor as a tool for interpreting and diagnosing iron deficiency status (Ahluwalia, 1998; Beguin, 2003; Cook, 1999).

Figure 2 sTfR Levels in Groups of Patients with Abnormal Iron Status

Figure includes non anemic iron deficiency (ID), iron deficiency anemia (IDA) and idiopathic hemochromatosis (IH). The gray zone represents the reference interval in normal subjects. Mean ±1 standard deviation (Baillie et al 2003).
Since sTfR is not an acute phase protein, it does not fluctuate as a result of complex pathologies and is a more stable quality indicator for interpreting iron status (Figure 2). The changes in sTfR and the variables of iron status can be mainly attributed to exercise induced changes in plasma volume. Taking these limitations into account, sTfR has been recommended as a more reliable marker of iron deficiency in athletes (Schumacher et al. 2002).

Both serum ferritin and the sTfR undergo a sequence of changes as the body iron stores decrease from normal iron levels to the most extreme decreased iron state of iron deficiency anemia. In a study by Suominen et al (1998) they demonstrated these changes in a population of healthy, non-anemic men and women (Figure 3).
Figure 3 Phases of Advancing Iron Deficiency

Criteria defining stages of iron deficiency appear in boxes. Prevalence of each stage from healthy, non-anemic men and women population are indicated. Suominen et al. 1998.

It is clear the sTfR levels are influenced by iron status due to the fact that they are considerably elevated in iron deficiency anemia but remain normal in the anemia of inflammation. The latter represents a body’s response mechanism and not a change in iron status. Ferritin levels on the other hand, are elevated in both situations. For this reason, sTfR levels may be of considerable help in the differential diagnosis of true iron deficient anemia versus inflammation from another cause.

There are multiple studies in the literature supporting the usefulness of the sTfR as a marker of iron status. Skikne et al (1990) published a study with group of normal volunteers who underwent graded phlebotomy. In this study, ferritin decreased
progressively while sTfR did not change much during the phase of storage iron depletion. However, sTfR increased significantly when marrow functional iron deficiency and anemia developed (figure 4). This study confirms that iron status may be more comprehensively assessed by using 1) serum ferritin as a measure of iron stores and storage iron depletion, 2) sTfR as a measure of functional tissue iron deficiency and iron deficient erythropoiesis, and 3) hemoglobin as a measure of advanced iron deficiency.

![Figure 4 Changes in Body Iron During Repeated Phlebotomy](image)

Functional tissue iron deficiency, resulting in elevated sTfR is a situation defined by tissue iron deficiency despite adequate iron stores. Because of the reciprocal relationship between sTfR and ferritin measurements, the ratio of sTfR and ferritin describes a perfect log-linear relationship to body iron over a wide range of normal and
depleted iron stores. This same relationship can be described by the transferrin receptor ratio. This ratio increases the sensitivity of sTfR in detecting latent iron deficiency. In a study of iron status in cross-country skiers, researchers found that in both male and female subjects the increase in sTfR was accompanied by very low ferritin levels and, in consequence, the values of the transferrin receptor ratio were elevated (Malczewska-Lenczowska et al. 2010). This evidence further supports our testing of these various status markers in a population of recreational female runners and supports the necessity of taking into account at least two indices of iron status, not only ferritin.

Hepcidin Influence on Iron Absorption

Hepcidin is a liver-derived iron regulatory protein that plays a crucial role in iron metabolism and absorption. It is the key regulator of iron homeostasis. It acts by regulating the export of intestinally absorbed iron. This mechanism can be explained by the binding of hepcidin to the iron channel ferroportin which is located on the luminal surface of the gut as well as at macrophages. After it binds, the entire complex is internalized and degraded by lysosomes (figure 5). The degradation of this iron channel ferroportin inhibits the exit of iron from the duodenal enterocytes and into circulation (Piperno et al 2011). Hepcidin’s release from the liver is regulated by different stimuli including iron, anemia and erythropoietic need. Anemia could mediate hepcidin suppression through multiple mechanisms including increased iron demand which occurs during exercise. When iron is in demand, hepcidin is down regulated thereby elevating iron outflow into circulation. It is known that hepcidin is released from the liver in the acute-phase response that follows either hard training or inflammation. By regulating the
number of iron exporters, hepcidin is involved in the control of iron uptake and release from enterocytes or macrophages. It is conceivable that processes that affect these regulatory mechanisms can result in defective iron mobilization without deficiency in the iron stores (Kemna et al 2008). During inflammation, hepcidin is up-regulated as a defense system. Elevated hepcidin levels, through the down regulation of ferroportin, cause iron to be isolated in enterocytes and macrophages, which inhibits iron release into the plasma. This results in reduced plasma iron levels, reduced erythropoiesis, and consequently anemia. Researchers have sought to determine the properties of hepcidin as a diagnostic test for iron deficiency due to its significant role in regulating iron metabolism.

Figure 5 Hepcidin Iron Regulation

Hepcidin binds to ferroportin and induces its internalization and degradation. By this mechanism, the interaction of hepcidin with ferroportin regulates the flow of iron into plasma, and thereby regulates the distribution of iron in the body (Nemeth et al. Science 2004)
Roeker et al conducted a study with female marathon runners and found that urine hepcidin levels rose for a day or so in 60% of the females after the marathon (Roecker L et al. 2005). Another group evaluated the effects of long-term endurance exercise on hepcidin concentrations and iron status in moderately trained female long distance runners. These investigators found that hemoglobin decreased throughout the eight weeks of training even with a recovery week taking place mid study whereas hepcidin decreased with the first three weeks of training, increased with the recovery week and then decreased after week eight. The main finding of the study is that serum hepcidin is affected after 8 weeks of endurance running in women (Auersperger et al. 2012).

In some long distance female runners, chronic increase in hepcidin could contribute to the development of iron deficiency through the inhibitory effects of hepcidin on intestinal absorption and on the release of iron from macrophages that recycle old red blood cells. There is still much to learn about the role of hepcidin and its impact in recreational female runners.

Transferrin and Total Iron Binding Capacity

With hemoglobin, ferritin, soluble transferrin receptor and hepcidin there are additional proteins evaluated in iron studies. Transferrin, a liver-derived protein, transports iron through the body. Transferrin in the blood is typically around one third saturated with iron and thus low transferrin saturation is used as an indicator of iron deficiency. Transferrin has been shown to decrease with various types of exercise: Fallon et al (2008) studied ultra marathoners and observed a significant decline in transferrin saturation, 22% to 8% by day five. These changes in transferrin saturation were
accompanied by decreases in serum iron (17.3 µmol/L to 10.9 µmol/L). Total iron binding capacity (TIBC) is measured to determine the amount of iron in the blood by assessing the ability of the protein transferrin to transport iron to the blood. Transferrin is typically around one third saturated with iron, and thus low transferrin saturation is used as an indicator of iron deficiency. Birth control pills and fluorides may raise TIBC and chloramphenicol may lower TIBC. Along with serum ferritin, transferrin saturation continually decreases when the requirements for iron increases in individuals.

IRON INTAKE AND FOOD INFLUENCES

The average western diet supplies about 6 mg of iron per 1,000 kcal. If one considers that the recommended daily allowance (RDA) is 18 mg per day for menstruating women, women who consume less than 2,000 kcal are susceptible to insufficient iron intake. Athletes who are involved in sports such as gymnastics, ballet, or long-distance running, in which low body weight is an asset, run the highest risk of inadequate iron intake. Vegetarian athletes are at further risk, due to the poor bioavailability of iron in vegetable foods.

Iron absorption is affected by the lining of the intestine, the amount of iron in the ingested food, and the source of the food (Monsen 1988.) The source of the food is key because that affects how much iron is biologically available for absorption. Heme iron, mainly found in animal products and derived from hemoglobin is the most efficiently absorbed type of iron ranging from 15%-35%. The other type of iron found in plant foods and grains is non-heme iron and is only absorbed at a rate of 2% to 20%.
Only about 15% of the iron consumed in the diet can be absorbed. The average American diet for the woman should contain 9.45 mg of iron for every 1000 kcal if 2000 kcal/day are consumed (Hallberg 1991). This total intake may be exceeding what the average female recreational runner is consuming. Manore et al (Manore 1989) showed that an average of 6 mg/1000 kcals was ingested by female long distance runners, who also tend toward vegetarian eating habits due to the persistent quest for carbohydrate in the diet. The absorbable iron intake of female runners has been reported to be around 1.0 mg/day and in some instances may be even lower (Haymes et al 1989). If this represents female recreational athletes as well, it would be difficult for them to consume enough iron to satisfy the RDA.

Anschuetz et al evaluated the hematological status of middle distance collegiate runners in relation to meal composition demonstrate the impact of the iron source. This study revealed that measures of iron intake alone do not provide an accurate picture of overall iron status and that there is an independent effect of endurance exercise training on an individual’s iron status. There were significant correlations noted between absorbable dietary iron and serum iron levels in the body of female endurance runners. Authors suggested that meal composition may influence the amount of iron available for absorption and for maintaining iron status over time (Anschuetz and Rodgers. 2010.)

Ascorbic acid

The bioavailability of the dietary iron can be enhanced by consumption of ascorbic acid also known as Vitamin C. Vitamin C is thought to enhance iron bioavailability by reducing insoluble ferric iron into ferrous form, which leads to
increased absorption. In previous studies, radioactive iron administered with vitamin C in a single meal showed enhanced iron absorption as compared to the long term supplementation of vitamin C. One such study by Cook et al (2001) showed a 100% increase in iron absorption when vitamin C intake was increased from 50 to 250 mg with no other changes in the diet so they concluded a positive correlation between log of iron absorption and log of vitamin C consumption (Cook and Reddy 2001).

The bioavailability of non-heme iron can also be enhanced by addition of meat to the meal. This was tested in a Latin-American basal meal, to which 75 g of ground beef meat was added, nearly tripling non-heme absorption from 0.17mg to 0.45 mg (Hallberg and Rossander 1984). Although the factors underlying this enhancing effect have not been identified yet, some studies have concluded that isolated protein extracts from muscle proteins present in chicken and beef enhance non-heme iron absorption by 100% and 180%, respectively (Hurrell and others 2006).

Reduced dietary iron consumption and increased iron requirements have been identified as the underlying cause of the prevalence of iron depletion in athletes. Iron levels may be diminished as a result of inadequate intake, inefficient absorption, increased iron loss or the increase in iron demands of the body (additional sweat loses, gastro-intestinal bleeding, foot strike RBC hemolysis, and exercise induced inflammation) not being completely met (Peeling et al. 2008.) According to Benardot (2011) athletes lose approximately .2 mg iron per liter of sweat and may lose up to 2 liters of sweat per hour depending on the intensity of the activity and the individual. This translates to 1.0-1.5 mg of iron during a three hour training run on a mild summer day.
Foot-strike hemolysis can contribute to an additional 1.0 mg loss of iron and gastrointestinal bleeding that has been shown to exist in endurance runners can result in another 0.5 mg of iron lost. All of these factors add up to a significant amount of iron making it clear that female athletes especially, are at high risk for iron depletion.

The most obvious preventative measure for iron depletion would be optimal iron intake in the diet and then next step would be supplementation. There is little understanding of the relationships between dietary iron intake, voluntary supplementation and performance in high-performance female athletes. There is even less known about the female recreational athlete (Klimis-Zacas, D and Wolinsky, I. 2004). Additional research is necessary in this population in order to provide evidence based analyses and recommendations.

EXERCISE AND NUTRITION INFLUENCE IN FEMALE ATHLETES

Women work in many ways to stay healthy and get in shape, one popular method is through running. Pate et al (Pate, 1990) observed the female recreational runner population and defined them as those who ran regularly but were not competitive. For the purpose of this study ‘recreational runner’ is defined by someone who has been running at least 15 miles per week for the past 6 weeks. There are over 12 million recreational runners in the United States alone that run at least 100 days out of every year (Running USA 2007). Of these recreational runners, 48% are women (Running USA 2007). Some of the major medical concerns for female runners include injury to hips,
shins and feet while running where it has been found that they are four times more likely
to develop stress fractures than recreational male runners (Rauh et al 2000, Zifchock et al
2006). However, even with the prevalence of female runners and the higher injury rates
amongst them, few studies look solely at evaluating the health status of this large
population of female runners (Christina et al 2001, Gerlach et al 2005, and Zifchock et al
2006).

An essential component to having optimal health status is proper fueling. All
women require fuel to support their body’s needs and the needs are even greater for
athletes due to physical activity. According to the American College of Sports Medicine
(ACSM) as well as Rodriguez et al (2009), not only does athletic performance benefit
from high quality nutrition, but general physical activity and recovery are optimized as
well. Burke and colleagues (2006) evaluated a group of female athletes and showed that
decreasing energy intake causes the body to use fat and lean muscle tissue for its primary
fuel, decreasing overall lean mass, which ultimately will result in decreased strength,
muscle endurance, immunity and musculoskeletal dysfunction.

Macronutrients are the primary fuel for performance. Upon assuming minimal
caloric intake at 2,000 calories/day, the Academy of Nutrition and Dietetics as well as the
ACSM suggest that 40-65% of total calories should come from complex carbohydrate
sources in order to maintain maximum glycogen stores as well as protect immune and
antioxidant systems (Rodriguez et al. 2009). It has been found that glycogen depletion is
the main inhibitor of exercise performance because it ultimately results in muscle fatigue
(Hawley and Burke, 2010).
When considering physiological and performance effects of diet, adequate dietary fat intake for female athletes is also important to achieve. The Food and Nutrition Board of the Institutes of Medicine acknowledged that active individuals have unique nutritional needs and their recommendation for fat is that 25-30% of one’s daily caloric intake should be from fat with limitations on saturated and trans fats (Manore, 2005). Fat is a primary energy source during rest and exercise and also provides the body with essential fatty acids which are crucial for metabolic processes and function (Manore, 2005).

Dietary protein contributes to the synthesis of muscle tissue while minimizing the loss of amino acids during oxidation and is also important for cell-make up (Gaine et al 2007). Although all females metabolize protein at different rates in comparison to one another, it is recommended to consume at least 1.2- 1.7 g/kg per day (Phillips, Moore, & Tang, 2007). In order to repair, maintain and synthesize skeletal muscle tissue, one must be consuming adequate amounts of protein (Tipton & Witard, 2007).

An energy balance is when one equally matches the energy that is consumed with the body’s energy expenditure. In order to achieve supreme performance that energy balance is necessary and if the balance is not there then the athlete’s performance will suffer.

Micronutrients are also an important consideration to the female athlete’s diet. Micronutrients are involved with energy production, maintaining bone health and a component of muscle synthesis during recovery. Female athletes experience a higher incidence of iron deficiency than their male counterparts. This is not only due to higher iron losses in females because of menses, but also due to a lower and possibly inadequate
intake of iron. An average 28-day menstrual cycle increases iron loss from a typical 1 mg of iron per day a range of 1.6mg – 2.5 mg/day (Killip et al 2007). This increase in iron loss is more than double the daily iron loss of male athletes creating a slow drain for the iron stores of the female. In total, an average 132 lb woman might lose an extra 10 mg of iron per menstruation cycle and that loss is more than 42 mg per cycle depending on how heavily she menstruates (Wintrobe 1999.) Accompanied with this higher iron loss in premenopausal women, dietary iron intake is frequently less than the RDA of 18 mg. This is likely related to a caloric intake less than the 2,000 calories that are necessary to supply this quantity of iron. Furthermore, females may eat less meat thus their diet tends to have a high proportion of non-heme iron foods, resulting in lower iron absorption. On top of all of those contributing factors to decreased iron status there is the loss of iron in sweat. It was estimated by Waller et al (1996) that 5.7% of daily absorbed iron, or 1.2 mg/dL, is lost by exercising females during the first hour of exercise, and that this could contribute to depletion of iron stores (Waller 1996). Therefore, it is not surprising that, when these factors add up, iron deficiency is more common in female athletes than trained males.

IRON STATUS IN FEMALE ATHLETES

Athletes make adaptations to running consistently. The athlete’s body requires an increase in blood volume through an increase in number of red blood cells (which contain hemoglobin), stored fuel in muscles and oxygen reserve capabilities in muscles through myoglobin. This ability to carry and use of oxygen for muscular work and endurance is
measured through a test procedure called maximal oxygen consumption, also known as the VO$_2$ max. It has been reported that iron deficiency greatly decreases VO$_2$ max due to a decrease of hemoglobin and O$_2$ carrying capacity (Sinclair and Hinton 2005). There is a general consensus that depleted iron stores without clinical symptoms occur more frequently in athletes than in the general population (Rodenberg and Gustafson 2007). Recent reviews have concluded that athletes with suboptimal iron status may experience reduced exercise capacity and impaired sports performance (Rodenberg and Gustafson 2007). Prevalence of iron depletion as high as 58% have been reported for at-risk groups including the female population (Petersen et al. 2006). In larger cross-sectional studies, the pooled mean prevalence was 37% in athletes and 23% in sedentary controls (Fogelholm 1995). This data has been developed from many female athlete populations but the prevalence and understanding of iron status in the recreational female runner population has yet to be reported.

The development of iron deficiency indicates a negative iron balance. This may be due to either an imbalance between iron requirements and iron intake, an increased loss of iron, decrease GI iron absorption, increased menstrual loss or to a combination of these factors. Aerobic exercise stimulates an increase in both the mass and enzyme content of muscle; consequently, it increases the iron requirements of athletes. As a result, athletes may have lower hemoglobin levels when compared to general population norms. Aerobic exercise expands the baseline plasma volume thereby reducing the concentration of red blood cells and causing an illusion of low hemoglobin. Because low hemoglobin levels have been one of the defining indicators of anemia it becomes difficult
to get a clear understanding of iron concentration based on hemoglobin. Exercise causes changes in plasma volume by 10–20% expansion thereby diluting both hemoglobin and ferritin and causing ‘sports anemia’. This is a transient effect that typically dilutes ferritin and hemoglobin by 15% (Haymes 1998). It is an inaccurate measurement for anemia because the total volume of red blood cells in this population is in fact normal, not low. A 15% dilution in ferritin also results in ferritin levels being observed lower than the amount of iron stores that actually exist. The existence of sports anemia has been found as a normal adaptive response to endurance exercise in ultra-endurance exercise and elite marathoners but hasn’t been fully studied in recreational athletes (Fink et al 2006).

In general iron status and the effects of iron from recreational running have not been thoroughly studied, specifically the female recreational runners (Beard and Tobin 2000). One study in premenopausal women performing recreational aerobic training evaluated the prevalence of iron deficiency and found 11% with iron deficiency anemia, 30% iron deficient without anemia and a total ID was 41% in this population (Sinclair and Hinton 2005). In comparison, a US general population study found 11% iron deficiency in women of 20–49 years old (Looker et al 1997). With respect to iron depletion, several recent studies have shown that even small changes in the available body iron can have a positive effect on exercise performance, even though the mechanisms are not fully understood (Hinton and Sinclair 2007; Brutsaert et al. 2003; Friedmann et al. 2001; Hinton et al. 2000). Researchers have conducted studies looking into iron therapy and its ability to enhance aerobic training and improve energetic
efficiency. With iron therapy studies what results is the iron-treated group ends up with a slightly higher hemoglobin level than the placebo group so “non anemia” is no longer being studied rather it is more of a “relative anemia”. There is no specific cutoff for defining anemia in physically active individuals – a female athlete with a normal hemoglobin level of 14 g/dL may feel a lower stamina at a hemoglobin of 13 g/dL and characteristically be anemic. (Hinton PS 2007). In a major study (Suominen et al 1998), healthy non anemic adults were supplemented with oral iron for 3 months to evaluate its effect on various parameters of iron status. Iron supplementation reduced sTfR levels and increased ferritin levels in a significant number of women. Changes in sTfR occurred mainly in subjects with elevated values before supplementation.

ERYTHROPOIESIS

Erythropoietin is a hormone secreted by the kidneys to increase the rate of production of red blood cells. It is triggered by a decrease in oxygen delivery to the tissues which could happen for a number of reasons including chronic kidney disease, excessive bleeding and prolonged vigorous exercise. The body uses 80% of the body iron for erythropoiesis, and almost the same proportion of soluble transferrin receptor is found in erythroid cells. Reticulocytes entering the bloodstream carry a high surface concentration of the receptor; as the cells mature the receptors are shed into circulation (Koulaouzidis et al 2009). It was documented very early that an intensification of erythropoiesis and change iron status had the biggest influence on soluble transferrin
receptor levels (Huebers et al. 1990). As erythropoietic activity diminishes there is depletion in bone marrow iron and then the sTfR level increases in attempt to bring more iron into the bone marrow. Thus, numerous studies have been and are still being carried out on the usefulness of sTfR in the diagnostics of iron deficiency (Beguin, 2003; Mast, 2001).

The erythroblasts are the main source of serum sTfR and mature erythroblasts, erythrocytes, require iron to perform their role as oxygen carriers. Serum sTfR levels average 5.0 +/- 1.0 ng/mL in normal subjects but the various commercial assays give incongruent values and there has yet to be any clinical standards established with this marker alone (Beguin, 2003; Suominen 1997). The most important determinant of sTfR levels appears to be erythropoietic activity in the bone marrow which can cause variations resulting in levels ranging from 0.5 ng/mL when erythropoiesis is totally absent to 100 ng/mL in severely anemic thalassemia patients. Soluble transferrin receptors levels are decreased in situations characterized by diminished erythropoietic activity such as chronic renal failure, and are increased when erythropoiesis is stimulated by hemolysis or intense exercise (figure 6).
Measurements of sTfR are very helpful to investigate the pathophysiology of anemia, erythropoietin levels increase exponentially in proportion to the degree of anemia and erythropoiesis increases in proportion to erythropoietin stimulation therefore sTfR also increases exponentially in response to the severity of the anemia. The sTfR levels also help in quantitatively evaluating the rate of erythropoiesis, the adequacy of bone marrow proliferative for any degree of anemia. In particular, the early responses of sTfRs are helpful in allowing to predict changes in the body’s iron stores when changes in hemoglobin are not yet apparent.
An advantage of using the sTfR:log(Ferr) ratio is that, unlike ferritin alone, it is sensitive to a wider range of body iron status, as it includes a measure of iron deficient erythropoiesis. In iron-depleted athletes without anemia, only those with elevated sTfR values showed impaired performance (Hinton et al., 2000; Brownlie et al., 2004). Moreover, sTfR may decrease in athletes who have an accompanying improvement in body iron, even though ferritin decreases (Petersen et al., 2006). In a study by Mettler et al, 7% of the women had ferritin <15 ng/mL, but had normal sTfR. This suggests these athletes with low iron stores were maintaining normal rates of erythropoiesis. In contrast, two female subjects had ferritin between 15 and 40 ng/mL, but had elevated sTfR suggesting impaired erythropoiesis, a condition that has been associated with impaired athletic performance (Hinton et al., 2000; Brownlie et al., 2002, 2004). Thus, the sTfR:log(Ferr) ratio may be preferable to ferritin alone to assess iron status and the need for supplementation in athletes (Petersen et al., 2006).

In iron deficiency anemia, the numbers of sTfR increase significantly. Values of sTfR are elevated due to the up regulation of synthesis of transferrin receptors on the erythrocytes so the cells can compete for iron more efficiently. While the serum concentration of sTfR is an indicator of the iron supply available for erythropoiesis; sTfR reflects erythropoiesis and inversely correlates with the amount of iron available for erythropoiesis (Clark 2008). The literature has shown that sTfR is not influenced by chronic or acute inflammation; therefore it could be a more reliable measure and clinically beneficial (Gibson 2005).
Classification of Iron Deficiency

Development of iron deficiency arises when the intake of dietary iron is not enough to replace the amount that the body requires and the amount that it loses through sweating, menstrual cycle, foot-strike hemolysis and urine. Iron deficiency is classified in three stages consisting of storage iron depletion, iron deficiency erythropoiesis and iron deficient anemia (Rodenberg and Gustafson 2007; Herbert 1997). When iron stores are depleted in the bone marrow, liver, and spleen it is the stage of pre latent iron deficiency. While there is still normal iron-dependent protein production and normal hemoglobin concentrations this stage may only be determined by a serum ferritin level below “normal range” without any other conditions or inflammation. Marginal or latent iron deficiency is also characterized by low ferritin indicating depleted iron stores but there is also reduced iron-dependent protein production, impairment of transport iron and then still normal hemoglobin concentrations. Iron deficiency anemia, the most severe stage of deficiency is characterized by depleted iron stores, reduced hemoglobin concentrations, and reduced iron-dependent oxidative enzyme concentrations (Table 1).

In a review by Rodenberg and Gustafson (2007) they explained that currently there is no consensus as to specific values being established to define “low ferritin”. They also concluded that there is no standardized level of ferritin concentration at which iron supplementation is recommended.

In addition to using ferritin concentrations to classify stages of iron deficiency the soluble transferrin receptor is being looked at as an additional reference marker. With it being relatively new to the study of iron status in athletes there also has yet to be any
standards set. Koulaouzidis et al (2009) conducted a systematic review on the use of sTfR as a marker for the evaluation of iron stores and concluded that it has high sensitivity and improves the clinical diagnosis of iron deficiency anemia. Gimferrer et al (1997) conducted a study with a diverse population of patients exhibiting the different stages of iron deficiency and evaluated sTfR levels. They found that sTfR levels continuously increased with the accompanying progressive iron deficiency and reported that the sTfR level could be of diagnostic value in patients in the intermediate stages of iron deficiency especially in female populations.
LOW IRON STAGES WITH CLASSIFICATIONS*

<table>
<thead>
<tr>
<th>Iron depletion without anemia: pre latent iron deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low serum ferritin levels AND Normal total iron-binding capacity</td>
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<tr>
<td>Normal percent transferrin saturation</td>
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<td>Normal serum iron concentration</td>
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<td>Normal Hemoglobin</td>
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<table>
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<th>Iron Depletion: latent iron deficiency</th>
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<tbody>
<tr>
<td>Low serum ferritin levels AND Increased iron-binding capacity</td>
</tr>
<tr>
<td>Decreased percent transferrin saturation</td>
</tr>
<tr>
<td>Normal Hemoglobin</td>
</tr>
<tr>
<td>OR</td>
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<tr>
<td>Decreased percent transferrin saturation</td>
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<td>Normal Hemoglobin</td>
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<table>
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<td>Low serum ferritin levels AND Increased total iron-binding capacity</td>
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<td>Decreased percent transferrin saturation</td>
</tr>
<tr>
<td>Decreased serum iron concentration</td>
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<tr>
<td>Low Hemoglobin</td>
</tr>
</tbody>
</table>

Table 1. Low Iron Stages with Classifications.


For the purpose of statistical evaluation of this research we staged iron status based on common values from the literature. The subject was categorized as iron deficient with anemia if hemoglobin values were less than 12 g/dL, iron deficient without anemia if the runner presented a hemoglobin greater than 12 g/dL along with a serum ferritin $\geq$12 ng/mL and ferritin $\leq$20 ng/mL and iron depletion as a runner with a hemoglobin greater than 12 g/dL and a serum ferritin $>20$ ng/mL and ferritin $\leq$30 ng/mL. The runner was considered in good iron status if the hemoglobin was greater than 12 g/dL and a serum ferritin $>30$ ng/mL (Fallon 2008). The transferrin receptor–ferritin
ratio (soluble transferrin receptor: log serum ferritin \(\geq 3.6\)) was used as an alternative
determination of iron deficiency without anemia (Suominen P et al. 1998).

Summary

The prevalence of iron deficiency is found to be the highest among the women of
child bearing age at 17.8% (WHO 2002). In this age group, maintaining normal levels of
iron is critical for the physiological functions of body. At the same time, there is
evidence that physical activity, specifically running can adversely affect iron deficiency
in this age of women adults. Spodaryk et al (2004) showed that endurance running is
tougher on iron status than almost any other sport so it is especially important to
understand iron status in runners. Results can potentially vary depending on the study
population, race, dietary habits, physical activity and other lifestyle factors. Therefore we
proposed to study a population of female recreational runners and evaluate the potential
benefits of using additional biochemical markers to better assess this population’s overall
iron status.

We hypothesize that the soluble transferrin receptor and ferritin ratio will provide
a more accurate assessment of iron status in recreational female runners. Additionally,
we hypothesize that the evaluation of dietary iron intake will correlate with the iron status
and further enhance our ability to more precisely identify those with low iron levels.
MATERIALS AND METHODS

The parent study was approved by the Institutional Review Board of The Ohio State University (2009H0177). The athletes in this study were initially 125 female recreational runners recruited and assessed in the Labs in Life at COSI. Participants were recruited from December 2009 thru November 2010 and were 18 years of age or older. Recruitment took place through various venues; IRB-approved fliers posted in running stores and approved oral scripts presented at local running events. The aim of the larger parent study was to analyze a large cohort in order to investigate the components of the female athlete triad. Additional inclusion criteria for participants were that they were running at least 15 miles per week (33 kilometers) for at least the past 6 weeks which we classified as a recreational runner. This was established by using a prescreening form completed by participants during the consent process (Appendix D). Participants were screened in person or via an online pre-participation questionnaire and the exclusion criteria included any runner who was pregnant, had known thyroid or adrenal abnormalities, or any known metabolic disorder affecting bone metabolism, or those with regular corticosteroid usage.
Pre Screening

Participants completed a series of questionnaires (eating disorder examination, beck depression inventory, multidimensional body self relations questions, menstrual status, calcium intake, tendency to diet scale and quick calcium screen) which were used as a SelectSurvey tool. They were provided instructions for and completed a three day dietary record that included all food and beverage intake for two week days and one weekend day. Specificity for portion size and accuracy and completeness of the diet records were facilitated by interview at the laboratory visit. Diet records were analyzed using a computer program (ESHA Food Processor professional grade software) that provided data for total energy, carbohydrate, fat, protein, dietary fiber, calcium and total iron. Average daily iron bioavailability was evaluated based on the specific intake of each subject from that 3-day record. This was done by going through the individual food records and reviewing the consumption of animal products, the amounts and the intake of the non heme iron food sources which are the plant products and grains. Food records were submitted to researchers by the participant prior to the blood collection.

Once the participant was on site for evaluation, they were first tested for sufficient hydration status. This was measured through the collection of a urine sample and the use of a refractometer which measures osmometry. To avoid undue radiation from the iDXA to an unborn fetus, pregnancy tests were also used at this time to confirm using urine that the sample was negative for Human chorionic gonadotropin, hCG. Anthropometric assessments including body mass (kg) and height (cm) were obtained on a standard physician scale with height rod (Healthometer). Body composition and bone density
were obtained using dual energy X-ray absorptiometry (iDXA GE Lunar). Quality assurance tests on the DXA machine were performed each morning. Venous blood samples were drawn from each participant (antecubital venipuncture, three evacuated tubes) after an 8 hour fast and between the hours of 6:00-9:00 a.m. to document the following biochemical markers: hematocrit/hemoglobin, cortisol, thyroid stimulating hormone, thyroid hormone, prealbumin, vitamin D, and parathyroid hormone. Complete data was collected from a total of 116 runners in the parent study. This evaluation of iron status only included premenopausal women (n=101) where the blood sample could be tested for ferritin and sTfR (n=93).

Enzyme-linked immunosorbent assay method, ELISA

Serum samples were recovered through centrifugation, stored at -80° C, and later assayed for: serum ferritin and soluble transferrin receptor. Serum ferritin concentration was measured using a solid-phase enzyme-linked immunosorbent assay method (ELISA) (ALPCO Diagnostics, Salem, New Hampshire). Soluble transferrin receptor was assayed with an immunoassay (Ramco Laboratories). Serum control samples (Lyphochek, Bio-Rad) were used along with the kit set of standards for the calibration curves. The purified antigen for each assay was carefully standardized to reduce variability between tests. Fresh standard material was thawed at the beginning of each experiment to be used with the particular kit from which they came from. The ELISA system utilizes one rabbit anti-ferritin antibody for the solid phase (micro titer wells) immobilization and a mouse monoclonal anti-ferritin antibody in the antibody-enzyme conjugate system. The 96 well
plate is coated with the solid phase antibody and to each well, 100µL of each participants’ serum was added. A typical example for the arrangement of the samples on the plate is shown in figure 7. The samples react simultaneously with the antibodies, resulting in the ferritin molecules being sandwiched between the solid phase and the enzyme-linked antibodies. After 45 minutes of incubation, the wells were washed to remove unbound-labeled antibodies. A solution of tetramethylbenzidine was added to the wells and with 20 minutes of incubation, a blue color develops. The color development is stopped with the addition of a stop solution and the color changes to yellow. The color intensity is measured spectrophotometrically at 450 nm. The concentration of ferritin is directly proportional to the color intensity of the samples. All blood assays were performed in duplicate, the results being expressed as means.

The soluble transferrin receptor assay was also standardized. To minimize errors, all samples from the same subject were assayed on the same day.

<table>
<thead>
<tr>
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<th>1</th>
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<td>18</td>
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</tr>
<tr>
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<td>3</td>
<td>7</td>
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<tr>
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<td>C3</td>
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<tr>
<td>H</td>
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<td>22</td>
<td>26</td>
<td>30</td>
<td>34</td>
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</table>

Figure 7 Plate Set-Up for ELISA Experiments
Additional Iron Biochemical Markers

Hemoglobin and hematocrit were analyzed using (The Ohio State University Medical Center).

Statistical analyses

The collected data was analyzed in order to answer and discuss the proposed research questions. The analyses were conducted using SPSS version 20.0 (IBM© Chicago, IL, 2012). Descriptive statistics were utilized to determine the characteristics of the runners including age, height, weight, years running and miles run per week. Descriptive methods were also used to describe the runners’ dietary intake through carbohydrates, protein, fat, iron and vitamin C. Energy availability is defined as the amount of dietary energy remaining after exercise training to support all other physiological processes. The threshold necessary to maintain daily physiological functions is 30 kcal·kg⁻¹·FFM·d⁻¹ (Loucks and Thuma 2003). Analysis of variance (ANOVA) was used to evaluate the agreement between iron status classification as judged by the 3 clinical ferritin groupings compared to groups determined by soluble transferrin receptor. A one-way ANOVA was used to test for significant relationships with the clinical ferritin level categories as the variable of interest analyzed with the soluble transferrin receptor and the soluble transferrin receptor/log ferritin ratio as the dependent variables. Linear and logarithmic correlation analyses between ferritin and the soluble transferrin receptor were carried out by Pearson’s correlation tests to determine the strength and significance of their relationship. The level of statistical significance
was set at $\alpha = 0.05$ ($P<0.05$ is significant). A linear regression model was also made to evaluate the relationship between daily intake of iron and ferritin levels.

Linear regression analysis was used to test whether iron intake modified the effects of iron status as measured by ferritin. The general linear model was used to test for differences in iron status indicators (Hemoglobin, serum ferritin, serum transferrin receptor, soluble transferrin receptor:log ferritin ratio) among subjects with normal iron status, iron deficiency without anemia (using serum ferritin), and iron deficiency with anemia, controlling for cortisol. Statistical significance was indicated at $P$ value $<0.05$. Results - are presented as means and standard deviations (s.d.). Data were tested for normality of distribution. Previous literatures have found the distributions of ferritin and sTfR concentrations were not normal and therefore the values were subjected to logarithmic transformation before data processing (Punnonen et al 1997). Thus, the soluble transferrin receptor ratio was calculated according to the following formulas: soluble transferrin receptor concentration divided by ferritin concentration logarithm and multiplied by 100 (Punnonen K et al. 1997). Statistical analyses were performed on natural-log-transformed data.
Chapter Four

RESULTS

One hundred twenty five women consented to participate in this study which was approved by the Institutional Review Board of The Ohio State University. Of these participants, 93 were included in the final iron analyses. Participants were excluded due to post menstrual status, missing laboratory, or missing dietary data.

The demographic information for the 93 participants included in the analysis is in Table 2. The women runners were, on average, 64.5 ± 2.6 inches tall and weighed 130.6 ± 19.0 pounds. They ranged in age from 18.4 to 51.5 years (mean age = 34.1 ± 8.4 years) and reported running 23.4 ± 10.3 miles per week for an average of 10.3 ± 8.1 years.
BASIC CHARACTERISTICS (MEAN ± SD AND RANGES) OF RECREATIONAL FEMALE RUNNERS

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Runners (Range); N=93</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34.1 ± 8.4 (18.4-51.5)</td>
</tr>
<tr>
<td>Height (in)</td>
<td>64.5 ± 2.6 (57.5-71.3)</td>
</tr>
<tr>
<td>Weight (lb)</td>
<td>130.6 ± 19.0 (89.2-203.6)</td>
</tr>
<tr>
<td>Running Experience (years)</td>
<td>10.3 ± 8.1 (1-31)</td>
</tr>
<tr>
<td>Running Volume (miles/wk)</td>
<td>23.4 ± 10.3 (11-70)</td>
</tr>
</tbody>
</table>

Table 2. Basic Characteristics of Recreational Female Runners for Iron Analysis.

The clinical data are listed in Table 3 and include mean and standard deviation for all the iron related indices. The values of the hematological indices, hemoglobin and hematocrit were within normal limits with the exception of 12 runners in whom the hemoglobin level was found to be below the clinical level of 12 g/dL. Levels of ferritin below the normal low limit of <20 ng/mL (in reference to Suominen et al) were found in 63 recreational runners while values outside the normal ranges (as defined by the manufacturer kit) for the soluble transferrin receptor were found in a total of 30 runners (68% compared to 32%). Consequently, 39 recreational runners had elevated transferrin receptor ratio values using a defined value from previous literature and this was 42% of the total studied.
HEMATOLOGICAL AND IRON METABOLISM INDICES (MEAN ± SD AND RANGES) IN RECREATIONAL FEMALE RUNNERS

<table>
<thead>
<tr>
<th>Variable</th>
<th>Average± SD N=93 (range)</th>
<th>Clinical Normal</th>
<th>Runners outside of norm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hemoglobin (g/dL)</strong></td>
<td>13.02 ± 1.00 (10.1 – 16.0)</td>
<td>12-16*#</td>
<td>N = 12</td>
</tr>
<tr>
<td><strong>Hematocrit (%)</strong></td>
<td>38.21 ± 2.84 (29.7 - 47.2)</td>
<td>35-47#</td>
<td>N = 11</td>
</tr>
<tr>
<td><strong>Ferritin (ng/mL)</strong></td>
<td>17.22 ± 6.15 (8.91 - 47.49)</td>
<td>20-140@^</td>
<td>N = 63</td>
</tr>
<tr>
<td><strong>Soluble transferrin receptor (ng/mL)</strong></td>
<td>4.29 ± 2.15 (1.04 – 11.16)</td>
<td>2.9-8.3@</td>
<td>N = 24/ N = 6</td>
</tr>
<tr>
<td><strong>Soluble transferrin receptor/ log ferritin (TfR ratio)</strong></td>
<td>3.68 ± 2.03 (0.68 – 10.73)</td>
<td>&lt;3.6^</td>
<td>N = 39</td>
</tr>
<tr>
<td><strong>Cortisol (mcg/dL)</strong></td>
<td>18.20 ± 5.95 (6.8 – 35.4)</td>
<td>5-25*</td>
<td>N = 13</td>
</tr>
</tbody>
</table>

Table 3. Hematological and Iron Metabolism Indices in Recreational Female Runners.

**LEGEND**

# - normal levels as given by Mayo Medical clinical guidelines
@ - normal ranges as given by kit manufacturers ALPCO
$ - lower normal limit after Malczewska et al. (2002)
^ - Suominen et al. (1997)
* - Pagana et al. (2010)

Additionally, Table 3 lists the number of women runners with values outside the normal ranges for these clinical parameters. It is important to note that the clinical normal ranges for ferritin have been determined from a population of healthy, non-anemic adult women. The clinical values for the soluble transferrin receptor come solely from the manufacturer kit and there has yet to be detailed literature on the normal ranges for a female athlete population and, more specifically, a population of female recreational runners. This is also true for the normal value given for the transferrin receptor ratio, this
was determined from a general population of healthy non-anemic women and there is no additional, more specific marker established for recreational female runners.

A correlation model was created with the soluble transferrin receptor as the independent variable and ferritin as the dependent variable. A graph depicting these two variables with a linear line of fit is shown in figure 8. The correlation was strongly significant between the two variables, p <0.001 and the relationship was found to be inversely moderate with a Pearson correlation coefficient (r) of -0.482 (table 4). We also evaluated the correlation of ferritin as the dependent variable and the soluble transferrin ratio as the independent variable which we also found to be significant, p< 0.001 with a Pearson correlation coefficient r = -0.579 (table 4).

A linear regression model with ferritin and soluble transferrin receptor was found to have an R squared value of .232 and adjusted R square value of .224. When cortisol was added into the regression model the adjusted R square value decreased to .220 which demonstrates that controlling for cortisol does not contribute materially to the correlation relationship between ferritin and the soluble transferrin receptor.
Figure 8. Correlation Between Ferritin and Soluble Transferrin Receptor Concentration in Recreational Female Runners.
Table 4. Correlation Summary of Ferritin with sTfR and TfR ratio in Recreational Female Runners.

R value indicates the Pearson correlation coefficient. P value indicates significance. R square value and adjusted R square value indicate coefficient of determination with sTfR and transferrin ratio.

Descriptive statistics were compared within the clinical ferritin categories to understand if the independent variables of the soluble transferrin receptor and the transferrin ratio showed a definable range that also separated them into significantly different categories (table 5). A graph depicting the clinical ferritin categories and mean values of the sTfR is shown in figure 9.

The clinical ferritin categories are divided out in the ‘ferr012’ column of the Table 5 where Ferr <12=0; 12≤ Ferr ≤20=1; Ferr >20=2. The one way ANOVA indicates there is a significant difference found in the values of sTfR when compared between groups (p<0.001). In the post hoc analysis using Tukey HSD we found that the significant difference lies in the lowest ferritin group (ferr <12) and the highest ferritin group (ferr >20) at the 0.05 level (groups labeled in figure 9). The post hoc analysis revealed that the middle ferritin group (12≤ ferr ≤20) does not reach statistical significance for the sTfR levels between groups with p = 0.167. The relationship of sTfR reflected the ferritin value categories as expected.
<table>
<thead>
<tr>
<th></th>
<th>N</th>
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<td>12 ≤ Ferr ≤ 20</td>
<td>46</td>
<td>4.6</td>
<td>1.9</td>
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<tr>
<td>Ferr &gt;20</td>
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<td>13.0</td>
<td>1.0</td>
<td>10.1</td>
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</tbody>
</table>

Table 5. Clinical Low Iron Categories of Ferritin with Independent Variables in Recreational Female Runners.

Means and standard deviations of each independent variable are shown: sTfR, HGB and TfR ratio (sTf:log(Ferr)) found within each category.
Figure 9. Soluble Transferrin Receptor Values for Clinical Categories of Ferritin Status in Recreational Female Runners.

Iron deficient without anemia, moderate iron deficiency and iron depletion without anemia (labeled as groups ferr < 12; 12 ≤ ferr ≤ 20; ferr > 20) with the y axis labeled as mean soluble transferrin receptor levels within each iron status classification group.

* = significantly different from Ferr >20 group.

From table 5 a graph was also created showing mean hemoglobin levels in each clinical ferritin category (figure 10). The one way ANOVA indicates there is not a significant difference in levels of Hgb when compared between groups (p=.091). In the post hoc analysis using Tukey HSD we found that the lowest ferritin group (ferr <12) and

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the highest ferritin group (ferr >20) had the strongest difference with p = .080. The relationship of hemoglobin reflected the ferritin value categories as expected showing that hemoglobin does not vary significantly between groups.

![Bar chart showing hemoglobin levels for different ferritin categories](chart.png)

**Clinical Ferritin categories**

<table>
<thead>
<tr>
<th>Ferritin Category</th>
<th>Mean HGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferr &lt;12</td>
<td>12.6</td>
</tr>
<tr>
<td>12 ≤ Ferr ≤ 20</td>
<td>13.0</td>
</tr>
<tr>
<td>Ferr &gt;20</td>
<td>13.3</td>
</tr>
</tbody>
</table>

**Error Bars:** +/- 2 SE

Figure 10. Hemoglobin Levels for Clinical Categories of Ferritin Status in Recreational Female Runners.

Iron deficient without anemia, moderate iron deficiency and iron depletion without anemia (labeled as groups ferr < 12; 12≤ ferr ≤ 20; ferr > 20) with the y axis labeled as average hemoglobin levels within each iron status classification group.
Dietary data collected and analyzed is presented in Table 6 as the combined means and standard deviations. On average, energy availability amongst the runners was 34.0 ± 14.1 kcal*kg\(^{-1}\) FFM*d\(^{-1}\), with an average daily carbohydrate intake of 260.0 ± 69.6 grams. The runners consumed a daily average of 80.3 ± 26.1 grams of protein, 64.8 ± 20.7 grams of fat and 15.9 ± 6.4 mg consumed of iron from diet alone and total iron intake which includes vitamin supplements 24.3 ± 14.6 mg. Average vitamin C intake including vitamin supplements was 208.0 ± 351.5 mg/day.

### Table 6. Dietary Intake of Recreational Female Runners.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Mean ± SD (Range)</th>
<th>RDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kcal/day)</td>
<td>1941.7 ± 451.1 (894.4 – 2917.4)</td>
<td></td>
</tr>
<tr>
<td>Energy Availability (kcal<em>kg(^{-1}) FFM</em>d(^{-1}))</td>
<td>34.0 ± 14.1 (-10.3 – 65.4)</td>
<td>&gt;30(^@)</td>
</tr>
<tr>
<td>Carbohydrates (g/day)</td>
<td>260.0 ± 69.6 (118.0 – 426.2)</td>
<td>Na</td>
</tr>
<tr>
<td>Protein (g/day)</td>
<td>80.3 ± 26.1 (17.1 - 196.7)</td>
<td>Na</td>
</tr>
<tr>
<td>Fat (g/day)</td>
<td>64.8 ± 20.7 (13.1 - 112.5)</td>
<td>Na</td>
</tr>
<tr>
<td>Iron intake (mg/day) Food + supplements</td>
<td>24.3 ± 14.6 (4.7 – 74.3)</td>
<td>&gt;18(^S)</td>
</tr>
<tr>
<td>Iron intake (mg/day) Food only</td>
<td>15.9 ± 6.4 (3.6 – 32.5)</td>
<td>&gt; 18(^S)</td>
</tr>
<tr>
<td>Vitamin C (mg/day)</td>
<td>208.0 ± 351.5 (18.4 – 3225.1)</td>
<td>&gt;75(^#)</td>
</tr>
</tbody>
</table>

Average over three days, includes mean and standard deviation from 93 recreational female runners. Clinical values listed are for non anemic, healthy adult women.

Legend
\(^@\) - from Loucks A. B. 2003
\(^S\) - from Office of Dietary Supplements. Iron.
\(^#\) - from Office of Dietary Supplements. Vitamin C.
A correlation model was created with average daily iron intake and ferritin levels. The correlation was found to be not significant between the two variables, \( p = 0.115 \) and a Pearson correlation coefficient of 0.165 (table 7). We also created a correlation model with transferrin receptor ratio and average daily iron intake which we found to be significant, \( p = 0.013 \) where \( p < 0.05 \) is significant and the Pearson correlation coefficient of -0.257 (table 7).

<table>
<thead>
<tr>
<th>Iron Intake</th>
<th>R</th>
<th>P</th>
<th>R Square</th>
<th>Adj R Square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin</td>
<td>.165</td>
<td>.115</td>
<td>.027</td>
<td>.016</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>.044</td>
<td></td>
<td></td>
<td>.023</td>
</tr>
<tr>
<td>sTfR/logFerr</td>
<td>-0.257</td>
<td>0.013</td>
<td>.066</td>
<td>.056</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>.122</td>
<td></td>
<td></td>
<td>.103</td>
</tr>
</tbody>
</table>

Table 7. Correlation Summary of Iron Intake with Ferritin and TfR Ratio in Recreational Female Runners.

R value indicates Pearson correlation coefficient. P value indicates significance of relationship. R square value and adjusted R square value indicate coefficient of determination with ferritin and transferrin ratio.

A linear regression model with ferritin and iron intake was found to have an adjusted R square value of .016, \( p = .115 \). When vitamin C was added into the regression model the adjusted R square value increased to .023 which may indicate that vitamin C enhanced the correlation-relationship between ferritin and iron intake. When the same model relationship is looked at between the TfR ratio and iron intake the R square value is .056 and when Vitamin C is added to the regression model the R square value goes up.
to .103. While these values do not show a strong linear relationship, the increase in the R square by adding Vitamin C to the model with iron intake and both ferritin and the TfR ratio shows that vitamin C contributes positively to the relationship.

DISCUSSION

For the hundreds of thousands of recreational female runners, inadequate iron status could compromise their magnitude of training and performance. Low iron limits the quantity and quality of workload capacity. In any group of training endurance athletes, 1 out of every 3 or 4 females can be expected to meet the criteria for iron deficiency without anemia (Fallon 2008). Our study specifically evaluated the iron status of this exercising population of 93 women runners by using serum markers including ferritin, hemoglobin, hematocrit, soluble transferrin receptor and the transferrin receptor ratio.

The measurement of hemoglobin has always been the test to reference as the indicator of iron deficiency anemia. While it clearly shows how much iron is being incorporated into hemoglobin there is a lot more iron in that body that doesn’t get accounted for including iron in the muscles and iron dependent enzymes. Famous Olympian and sports medicine expert Doug Clement conducted an epidemiological study of female endurance runners and identified 82% of the female athletes as iron deficient (Clement and Sawchuck 1984.) Not one of these runners, however, had a hemoglobin level less than 12 g/dL. This study is one of many that made it evident that other markers are necessary to fully assess iron status. Other groups have evaluated anemia and iron
status in elite runners but ours is the first study evaluating this array of iron status markers in recreational female runners. Our data indicate that by utilizing these additional status markers, we can make a distinction between iron deficiency with anemia, severe iron deficiency without anemia and mild iron deficiency without anemia. Biochemical criteria have been used by others to classify individuals as having iron deficiency with anemia, iron deficiency without anemia and iron depletion (Suominen et al 1998). By utilizing additional markers to assess iron status, the athlete, coach, and the associated healthcare team may be better equipped for addressing iron status on a continuing basis and for averting long term negative consequences of low iron stores and/or anemia.

We found that there was a statistically significant negative relationship between the ratio of soluble transferrin receptor to log ferritin and ferritin. When we incorporated cortisol into the correlation model as a potential marker for stress and inflammation, we found that controlling for cortisol did not improve the correlation. The negative relationship would be expected because the soluble transferrin receptor is inversely related to iron status.

Previous studies have demonstrated the activity of the sTfR in response to decreasing iron status. The Colgan Institute (2010) previously reported on this physiological phenomena, explaining that the lower the iron supply in the muscles, the higher the number of transferrin receptors expressed, and the higher their number in serum. This also agrees with the findings of Malczewska et al. (2000) and Schumacher et al. (2002) who both conducted studies demonstrating that the sTfR levels increase when
iron stores diminish and how, unlike ferritin, sTfR is not an acute phase protein, therefore it remains stable after acute exercise and serves as a valuable marker in the diagnosis of iron deficiency. Iron deficiency causes over expression of transferrin receptor and therefore an increase in sTfR levels. According to Mayo Medical Laboratory guidelines, while ferritin measurement is the accepted method for assessment of iron deficiency, ferritin is an acute-phase reactant and elevates in response to processes that do not correlate with iron status, including inflammation and malignancy. Soluble transferrin receptor is not an acute-phase reactant and the interpretation of iron status using sTfR measurement is not affected by these confounding pathologies.

Establishing reliable reference values for ferritin and sTfR to study subclinical stages of low iron has been a challenge (Schumacher et al. 2000; Rodenberg and Gustafson 2007.) Others report these challenges are due to the strict concepts of predictable reference values and to reference limits that have been designed to distinguish between non-anemic patients and patients with iron deficiency anemia and have also been generalized to healthy adults, both male and female. One advantage offered by these assessments of serum ferritin in combination with the sTfR is that this study demonstrates the prevalence of low iron in a population that, until now, had not been evaluated. Serum ferritin reflects the storage iron and sTfR reflects the functional iron which is why the combined ratio provides significant information on the entire range of the body’s iron stores in this population. It was expected that we would find a significant correlation between ferritin and the transferrin ratio because the ratio includes ferritin.
Previous authors have evaluated the relationship between ferritin and the soluble transferrin receptor. Ferritin levels of 20 ng/mL or less have been shown to be diagnostic of iron deficiency (Bakers 2000) but even at higher ferritin levels, ~40 ng/mL, erythropoiesis may be affected which is detected by the soluble transferrin receptor (Miller 2013). Cable et al (2011) explained how iron deficiency causes an increased release of soluble transferrin from erythroblasts. Therefore, ratios of soluble transferrin receptor and ferritin are used to detect iron-deficient erythropoiesis (Cable et al. 2011). When significant inflammation is present, serum ferritin levels may not reflect accurate iron stores. This population of runners did not show this extremely high level of ferritin but it has been found in other populations making it important to assess multiple biochemical markers.

Wians et al (2001) showed that the measurement of serum soluble TfR levels is effective for evaluating the severity of iron deficiency in combination with ferritin because the expression of TfR by cells of the reticuloendothelial system is directly proportional to cellular iron demand, and the serum level of soluble TfR is directly proportional to the total amount of cellular TfRs and the ferritin exhibits the iron that is in storage so in total these two markers can provide a complete understanding of iron status.

Mean concentration of ferritin levels were rather low (17.2 ng/mL). The low average was due to all the women in the study had relatively low ferritin, all subjects fell below 50 ng/mL. Previous studies have reported low ferritin levels in female endurance athletes (marathoners, cyclists and swimmers) and elite runners and now these results indicate that while this population has a less intense training regime, they still exhibits
similar low ferritin results (Mettler and Zimmermann 2010; Fallon 2008; Zotter 2004; Beard 2000). Beard and Tobin (2000) found serum ferritin concentrations in female athletes to be < 12 ng/mL in 35%, < 25 ng/mL in 82%, and < 30 ng/mL in 60%, as compared with their sedentary counterparts from the nonathletic female population. From this study they concluded that an athlete is considered iron deficient with serum ferritin levels less than 10-12 ng/mL but this is a collection of data from all different types of female athletes. Our results are very comparable, with the breakdown of percentages in recreational female runners to be < 12 ng/mL in 18.3%, between 12 and 20 ng/mL in 67.7%, and > 20 ng/mL in 31.2%.

As others have done (Suominen, Sinclair and Hinton, Rodenberg), we evaluated the usefulness of the transferrin receptor ratio for matching and for differentiating between the clinical categories of ferritin values (normal, low, and deficiency). Our results indicate that when ferritin was split into clinical categories and the transferrin receptor ratio was compared there was a significant distinction between two of the iron status categories. Each of the categories was based upon ferritin levels as described in the iron status table (table 1). Using the soluble transferrin receptor:log ferritin ratio as the criterion for evaluating stages of iron status subclinical to iron deficiency anemia, resulted in a greater proportion of recreational runners being classified as iron deficient without anemia. The results also showed that the transferrin receptor ratio was significantly difference between the two extreme stages of iron deficiency (ferritin >20 ng/mL and ferritin <12 ng/mL). Individuals classified within each group had significant differences in both the soluble transferrin receptor values between groups and the soluble
transferrin ratio between groups \( (p<0.01) \). Alternatively, there was not a significant
difference in the transferrin receptor ratio for the two lower classification stages
(ferritin\(<12\) ng/mL and \(12<\) ferr \(<20\) ng/mL). The combination of higher sTfR levels
with lower ferritin was exhibited in each of the clinical groups which confirm the
presence of latent iron deficiency in these subjects (Malczewska et al. 2001; Nikolaidis et
al 2003). There were some cases of subjects with elevated sTfR as well as high ferritin
levels (i.e. ferritin 24.1 ng/mL and sTfR 8.8 ng/mL) in which the use of the transferrin
receptor ratio was needed to determine specific iron status of this subject. As concluded
in previous literature findings, non-anemic stages of iron depletion are most readily
detectable by using the transferrin receptor ratio and that this ratio is a more sensitive
marker in the detection of the different states of iron depletion (Wians et al 2001;
Suominen et al 1998;). Hastka et al demonstrated the usefulness of integrating ferritin
and the transferrin receptor to conceptualizing progressive stages of iron deficiency in a
population of anemic and non-anemic, male and female patients. Our results also
demonstrate the presence of the progressive stages but in recreational female runners.

Especially in endurance and elite athletes, research has shown that it is fairly
common to see some level of iron deficiency (Malczewska et al 2001; Mercer et al 2005;
Schumacher et al 2002) but neither iron deficiency nor iron status in general have been
assessed in detail in recreational athletes. The results of our study show the high
prevalence of iron deficiency that also exists in recreational female athletes, with the
transferrin receptor ratio identifying 42\% of the runners as iron deficient. It also
indicates the necessity of taking into account at least two indices of iron status
simultaneously, not only ferritin, which has previously been suggested by others (Cook et al 2003; Hinton et al 2007).

As with all tests that yield values on a continuous scale but aim to provide a three stage diagnostic classification, cut-offs must balance both sensitivity and specificity (Zweig and Campbell 1993). This research shows that the ranges specified by ferritin levels in three stages of iron depletion do not correlate significantly with three levels of the transferrin receptor ratio. The determination of optimal cut-offs is based on the intended clinical use of the test and the costs associated with false positive (inadequate specificity) and false negative (inadequate sensitivity) results (Remaley et al. 1999). In non-anemic female recreational runners, costs of a false positive diagnosis of iron deficiency might include unnecessary iron supplementation and even endoscopic investigation. Additionally, taking iron if you are not deficient is unnecessary, and may be dangerous. About 1 in 250 people of Northern European descent have a disease where they absorb and store too much iron, hemochromatosis, which can be deadly. Costs of a false negative classification might include subsequent development of anemia in untreated patients and failure to detect underlying nutritional disorders. In this population, the transferrin receptor ratio greater than 3.6 is the determining marker to classify subjects into their true iron status and incorporate both functional iron and available iron stores (Suominen et al 1998, Sinclair, 2005.).

The soluble transferrin receptor has been explored as an indicator of iron status in other populations with more complex scenarios and disease states. It has been proposed as a potential marker of functional iron and iron bioavailability for erythropoiesis in
chronic kidney disease and also for identifying coexisting iron deficiency in patients with concomitant anemia of chronic disease, and has been proposed as an alternative to ferritin for discriminating between iron deficiency and anemia of chronic disease (Malyszko et al 2007).

The average daily intake of iron did not show a significant correlation to ferritin but was statistically significant when correlated with the sTfR ratio ($p < .05$). When we modeled the correlation with Vitamin C we did find the correlation, while it wasn’t significant, the relationship between iron intake and both ferritin and the sTfR ratio improved. This increase in correlation relationship has been found in similar results in the literature. Beck et al conducted a study in a population of healthy women with low iron stores and found that addition of ascorbic acid to breakfast improved iron status (ferritin increased significantly as well as a significant decrease in soluble transferrin receptor) compared to a group consuming breakfast without a detectable amount of ascorbic acid and not showing any change in iron status (Beck et al 2010). Vitamin C consumed with iron helps maintain iron in ferrous form for better absorption and neutralizes the inhibitory effects of other nutrients. Phytates and tannins are two inhibitors of iron absorption: phytates are in all plants and whole grain products and tannins, most commonly found in fruits and beans. Sharma et al (1995) showed that supplementation of 500 mg of vitamin C in a population of vegetarian runners improved iron status within two months. This research supports our findings that the relationship strengthened between iron status and average iron intake in the diet when vitamin C was added into the correlation.
Limitations

Our study should be interpreted in the context of its strengths and limitations. We recruited a large sample of female, premenopausal, recreational runners; thus, our findings may be generalized to this population. A limitation of this study is the enhancers and inhibitors in the diet that affect iron absorption could be even more thoroughly investigated. One such example is with polyphenols and their role in iron absorption. Polyphenols are ring structures that contain more than one hydroxyl group. They are commonly found as tannic acid, gallic acid present in tea, coffee, red wine, spinach and spices such as cinnamon. These hydroxyl groups bind iron and make it unavailable for absorption (Zijp, Korver, and Tijburg 2000). The inhibitory effect of these polyphenols on iron absorption has been widely studied. Layrisse et al examined iron absorption from controlled breakfast meals that included specific doses of tea and coffee. The results showed that a dose-dependent administration of tea and espresso coffee indicated a 50% decrease in iron absorption (Layrisse et al. 2000). This suggests that the consumption of tea and coffee is one of the important determinants of iron absorption. In our particular investigation subjects were not controlled with tea and coffee intake.

Hepcidin as a marker of iron status was not included in this study. The specificity of hepcidin concentration as an index of iron status reflects the physiology of hepcidin itself. Iron deficiency and erythropoiesis are each associated with suppressed hepatic hepcidin release, facilitating increased intestinal iron absorption and release from macrophage stores through intact membrane channel ferroportin on enterocytes and macrophages (Young et al. 2009). Reduced hepcidin is an essential part of the
physiological response to an iron deficit. Since ferritin, sTfR and hepcidin (a signal that increased iron is needed) each reflect different aspects of iron metabolism, combined evaluation of these indices may provide complementary clinical information. A low hepcidin concentration may predict efficient intestinal absorption of supplemental iron, potentially identifying patients who would benefit most from oral iron therapy (Young et al 2009). Auersperger et al (2012) conducted a study with female long distance runners looking at long term endurance exercise, hepcidin, inflammatory parameters and iron status. He concluded that over long term exercise hepcidin and sTfR were affected with no positive relation to inflammation.

Another consideration for potential limitation is the use of contraceptive pills. Typically, women reduce their risk of iron deficiency by taking birth control pills which results in less blood loss during their menses. In contrast, women who are not taking a birth control pill may suffer from a higher amount of blood loss therefore a greater depletion of iron. This is a contributing factor to a woman’s overall iron status. In this study we did collect information regarding contraceptive pills however, women were not excluded for not being on a birth control pill.

Reference ranges for the measurement of sTfR are non-existent as there are several commercial assays available, and the technique is not standardized (Skikne et al, 1998). This leads to confusion in the transferability of results from one study to another, both nationally and internationally; however this problem is not unique in reference to diagnostic testing. There must be increased pressure to establish international guidelines
for the calibration of sTfR, first highlighted by Skikne in 1998, as well as the transferrin receptor ratio. Currently, there is no National Quality Assessment Scheme so it remains a challenge to compare across different laboratory analyses with different populations, which is necessary if sTfR and the transferrin receptor ratio are to be utilized on a wider basis.

In a systematic review of the sTfR, measurements appear to have high sensitivity, but suffer from low specificity when it comes to differentiating iron deficiency from other causes of increased erythropoiesis (e.g. hemolytic states such as ineffective erythropoiesis from B12/folate deficiency and thalassaemia) (Rodenberg and Gustafson 2007). This can be corrected by incorporating ferritin in the calculated index of sTfR/logFerr.

It is well known that reliance upon hemoglobin and hematocrit or ferritin to indicate poor iron status is problematic since these may occur too far into iron deficiency or, as in the case of ferritin, are unable to differentiate iron status from inflammation. The strong correlation between various levels of ferritin and sTfR or its ratio provides more sensitivity, which others have found also. The uniqueness is that this work demonstrates this phenomenon in recreational female athletes who represent a much larger population who can potentially be impacted by therapy.

Conclusions

Iron depletion in athletes is ergolytic. It reduces VO2 max, impairs muscle function, increases glycogen use, increases muscle fatigability, and inhibits
improvements from training, even when hemoglobin levels may still be within the normal clinical range. It also causes muscle and bone degeneration that may permanently impair performance (Beard and Tobin, 2000; Brownlie et al, 2004; Clark SF, 2008). Few data exists on the effect of physical exercise and sTfR levels. This is important in the assessment of iron status in training athletes and especially important in female athletes who experience additional physiological effects which alter their iron status. Clinical indices exist for understanding iron status levels prior to becoming the extremely low iron status of anemia but they were not explored relative to recreational female runners. This study demonstrated the prevalence of low iron status found in this population and showed that additional biochemical markers of iron stores are necessary to fully evaluate iron status. The classification stages of ferritin levels are significant in this population but in analyzing the soluble transferrin receptor we concluded that there are two significant categories of low iron status which can be distinguished by measuring both ferritin and the soluble transferrin receptor in this population.

Recommendations

The results of this study show that there are a significant percentage of women who classify as iron deficient without anemia or are in a state of mild iron deficiency at which that low level of iron could be affecting their performance and energy levels. Additional research is necessary to evaluate how much their performance is being affected by evaluating VO2 max and other measurements of speed or endurance. Further research is also needed on the dietary intake of this population to more completely understand the significance of intake on iron absorption levels.
Chapter 5

Biochemical Markers of Iron Status in Recreational Female Runners

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There are over 12 million recreational runners in the United States who, by definition, run at least 100 days out of every year (Running USA 2007). Although running offers extensive benefits, it can be harmful if one fuels or trains improperly. Female runners have unique challenges due to anatomical, biomechanical, hormonal, nutritional and functional factors causing poor performance, repetitive stress, and acute injuries. Research regarding recreational female athletes remains limited (Ireland, 2004), and conclusions are generally extrapolated from studies of collegiate, Norwegian elite, and high school athletes (DiSantolo 2008; Sundgot-Borgen J, 1998; Rowland T, 2012). Although the study of elite athletes can shed light on the health of female runners, recreational running potentially across the lifespan may have long-term health impacts yet to be studied in women (Prather, 2005).

Regular exercise has been shown to deplete iron stores, and several studies have reported that female athletes are more prone than male athletes to develop an iron deficiency. There are multiple contributing factors to overall iron status including training volume and diet intake, and due to the additional factor of the menstrual cycle, females are at higher risk of iron deficiency. The prevalence of iron deficiency in active female adolescents has been reported to be as high as 46% in and reported at 25%-35% in adult female athletes (Constantini et al 2000; Malczewska et al 2001).

The assessment of iron status is typically evaluated by hemoglobin concentration but the measurement of ferritin has been reported as a more specific marker of iron status. In the sports health care environment, it is generally agreed that low serum ferritin levels
are frequently found in athletes, yet there is no agreement as to the significance of these findings, i.e., whether it represents true iron deficiency or a physiological response to training (Eichner 1992 and Sherman 1990). Because ferritin is an acute phase protein whose levels alter with various conditions and inflammation, increases in ferritin may mask an actual iron deficiency. There is a debate regarding the ferritin cutoff for iron deficiency in athletes due to conflicting results about the effect of iron deficiency without anemia on performance (Eichner, 2000; Hinton et al., 2000; Brownlie et al., 2004; Rodenberg and Gustafson, 2007). The clinical definition of significant iron deficiency in athletes is the level of iron deficit that will negatively affect exercise performance. Due to lack of consistency between studies, however, there is currently no quantitative component to the definition (Rowland 2012).

Although there have been a few iron studies focused on elite athletes, none have been conducted to focus specifically on the recreational female runner.

In the assessment of iron status, liver derived serum ferritin, is recognized as the most commonly used index for the body’s available iron stores, however, it may be an inappropriate measurement for iron status in athletes. Physical activity is accompanied by an inflammation-like state in the joints and muscles which may induce a rise in plasma ferritin persisting for a few days following strenuous exercise. Therefore, false elevations may be observed in athletic populations and iron deficiency may go unrecognized. Additionally, serum ferritin is an indicator of iron stores rather than functional iron so a more direct indicator of functional iron would enhance assessment of iron status.
Recent work with iron deficiency anemia provides evidence that the soluble transferrin receptor (sTfR) may be a more sensitive marker for evaluating iron status because it is not an acute phase protein like ferritin. sTfR has a low biological variability and remains quite stable after exercise or a brief training period (Malczewska et al 2000). The sTfR is a transmembrane protein present in all cell types. Enhanced synthesis of the transferrin receptor represents the initial cellular response to a declining iron supply. The transferrin receptor ratio (TfR ratio) defined as the ratio of serum transferrin receptor to log serum ferritin has been shown to be useful in identifying functional iron depletion in athletes whose serum ferritin concentrations are marginal but do not fall below the threshold for iron depletion (Malczewska et al 2000). This ratio would also detect false ferritin elevation from exercise because it incorporates the relationship to the sTfR levels. The appropriate clinical strategies for screening athletes for iron deficiency remain undetermined, but the sTfR and the TfR ratio may prove to be the optimum identifier of low iron status in recreational athletes.

Low iron results in decreased energy efficiency and impaired endurance in runners. While physical activity uses up iron in the body, food intake is able to replenish iron in the body. Diet is critical to health and performance. Only a few studies have been conducted with recreational female runners and none have evaluated iron status and looked at the relationship it has with iron intake. A more comprehensive evaluation of iron status and dietary intake from a population of recreational female runners is needed to understand and explain the prevalence of iron depletion and other potential iron status abnormalities.
The objectives of this study were to describe and evaluate the serum iron levels in recreational female runners using multiple measures including ferritin, hemoglobin, hematocrit, and soluble transferrin receptor ratio levels.

We hypothesize that the soluble transferrin receptor-log ferritin ratio will accurately predict iron deficiency without anemia as well as correlate with dietary iron and thus will enhance our ability to more precisely identify those with low iron status in this population.

Methods

The athletes in this study were initially 125 female recreational runners 18 years of age and older. Additional inclusion criteria to be classified as a recreational runner required that participants had run at least 15 miles per week (33 kilometers) for the past 6 weeks which was established by using a prescreening form completed by participants during the consent process. Runners who were pregnant, had known thyroid or adrenal abnormalities, any known metabolic disorder affecting bone metabolism, or those with regular corticosteroid usage were excluded from the study. The exclusion criteria were developed from a larger parent study. This study was approved by the Institutional Review Board at The Ohio State University (2009H0177).

Runners were provided instructions for and completed a three day dietary record for two week days and one weekend day. Diet records were analyzed using a computer program (ESHA Food Processor professional grade software) that provided data for total energy, carbohydrate, fat, protein, and total iron. Anthropometric assessments including
body mass (kg) and height (cm) were obtained on a standard physician scale with height rod. Venous blood samples were drawn from each participant (antecubital venipuncture, three evacuated tubes) after an 8 hour fast and between the hours of 6-9am to document hematocrit and hemoglobin biochemical markers. Complete data was collected from a total of 116 runners. This evaluation of iron status only included premenopausal women (n=101) where the blood sample could be tested for ferritin and sTfR (n=93).

Enzyme-linked immunosorbent assay method, ELISA

Serum samples were recovered through centrifugation, stored at -80°C, and later assayed for: serum ferritin and soluble transferrin receptor. Serum ferritin concentration was measured using a solid-phase enzyme-linked immunosorbent assay method (ELISA) (ALPCO Diagnostics, Salem, New Hampshire). Soluble transferrin receptor was assayed with an immunoassay (Ramco-TfR, Ramco Laboratories, Houston, Texas). Serum control samples (Lyphochek, Bio-Rad) were used along with the kit set of standards for the calibration curves. The ELISA system utilizes one rabbit anti-ferritin antibody for the solid phase (micro titer wells) immobilization and a mouse monoclonal anti-ferritin antibody in the antibody-enzyme conjugate system. The 96 well plate was coated with the solid phase antibody and to each well, 100µL of each participants’ serum was added. The samples reacted simultaneously with the antibodies, resulting in the ferritin molecules being sandwiched between the solid phase and the enzyme-linked antibodies. The color intensity of the samples was measured spectrophotometrically at 450 nm. The concentration of ferritin is directly proportional to the color intensity of the samples. All
blood assays were performed in duplicate, the results being expressed as means. The soluble transferrin receptor assay was also standardized. To minimize errors, all samples from the same subject were assayed on the same day.

The distributions of ferritin and sTfR concentrations were not normal, so the values were subjected to logarithmic transformation before data processing. Thus, the soluble transferrin receptor index (sTfR/log(Ferr)) was calculated as defined by Punnonen et al (1997): serum transferrin receptor concentration divided by logarithm (base 10) of ferritin concentration and multiplied by 100.

Statistical Analysis

The collected data was analyzed using SPSS version 20.0 (IBM© Chicago, IL, 2012). Descriptive statistics were utilized to determine the characteristics of the runners including age, height, weight, years running and miles run per week. Descriptive methods were also used to describe the runners’ dietary intake through total calories, protein, fat, iron and type of iron (heme vs. non heme). Iron status classification was evaluated using an analysis of variance (ANOVA) to analyze the difference in soluble transferrin receptor, the means between each class of iron status and to assess significant relationships with the clinical ferritin level categories as the variable of interest analyzed with the soluble transferrin receptor and the soluble transferrin receptor/log ferritin ratio as the dependent variables. Linear and logarithmic correlation analyses between ferritin and the soluble transferrin receptor were carried out by Pearson’s product moment correlation coefficients to determine the strength and significance of their relationship.
The level of statistical significance was set \textit{a priori} at $\alpha = 0.05$ ($P<0.05$ is significant). A linear regression model was also made to evaluate the relationship between daily intake of iron and ferritin levels.

Results and Discussion

The 93 women runners were $64.5 \pm 2.6$ inches tall and weighed $130.6 \pm 19.0$ pounds (Table 1). The runners ranged in age from 18.4 to 51.5 years (mean age = $34.1 \pm 8.4$ years) and reported running $23.4 \pm 10.3$ miles per week for an average of $10.3 \pm 8.1$ years. It is easy to notice the variability in the group in terms of running status. The values of the hematological indices, hemoglobin ($13.02 \pm 1.00$ g/dL), and hematocrit ($38.21 \pm 2.84$ %) were within normal limits with the exception of 12 runners in whom the hemoglobin level was found to be below the clinical level of 12 g/dL (Table 2). Levels of ferritin (mean $17.22 \pm 6.15$ ng/mL) below the normal low limit (in reference to Suominen et al) were found in 63 recreational runners while values outside the normal ranges (as defined by the sTfR manufacturer kit) for the soluble transferrin receptor (mean $4.29 \pm 2.15$ ng/mL) were only found in a total of 30 runners. Consequently, 39 recreational runners had elevated transferrin receptor index values (mean $3.68 \pm 2.03$) when using a defined value from previous literature.

It is important to note that the clinical normal range for ferritin has been determined from a population of healthy, non-anemic adult women. The clinical values for the soluble transferrin receptor come solely from the manufacturer kit, and there has yet to be detailed literature on the normal ranges for a female athlete population and,
more specifically, a population of female recreational runners. This is also true for the
normal value given for the transferrin receptor index.

A linear regression model controlling for cortisol indicated that cortisol does not
contribute materially to the correlation relationship between ferritin and the soluble
transferrin receptor. A negative relationship exists between sTfR and ferritin ($r = \rho
<0.001$) as well as between ferritin and the soluble transferrin ratio ($r= \rho<0.001$) (Table
3).

In any group of training endurance athletes, 1 out of every 3 or 4 females can be
expected to satisfy criteria for iron deficiency without anemia (Fallon 2008). Other
groups have evaluated anemia and iron status in elite runners, but ours is the first study
evaluating an array of iron status markers in recreational runners. Our data indicate that
by utilizing these additional status markers, we can make a distinction between iron
deficiency with anemia, severe iron deficiency without anemia, and mild iron deficiency
without anemia. Biochemical criteria have been used by others to classify individuals as
having iron deficiency with anemia, iron deficiency without anemia, and iron depletion
(Suominen et al 1998).

Malczewska et al. (2000) and Schumacher et al. (2002) provided evidence that
sTfR is a valuable marker in the diagnosis of iron deficiency. They demonstrated that the
sTfR levels increase when iron stores diminish, and that the changes following acute
exercise remain stable. In agreement with both studies, we found that there was a
statistically significant negative relationship between the ratio of soluble transferrin
receptor to log ferritin and ferritin.
Establishing reliable reference values for ferritin and sTfR to study subclinical stages of low iron has been a challenge (Schumacher et al. 2000; Rodenberg and Gustafson 2007). Currently, there are strict concepts of predictable reference values, and reference limits have been designed to distinguish between non-anemic patients and patients with iron deficiency anemia. The reference values have also been generalized to healthy adults rather than being specific for males and females. The measurement of serum ferritin in combination with the sTfR will allow researchers to determine the prevalence of low iron without anemia in a population that had not previously been evaluated.

Conclusions

Iron depletion in athletes is serious. It reduces VO2 max, impairs muscle function, increases glycogen use, increases muscle fatigability, and inhibits improvements from training, even though hemoglobin levels may still be within the normal clinical range. The prevalence of iron deficiency is high in recreational female runners compared to the general population of women, 42% and 12-16% respectively (Eichner R, 2010). Adult women who run at least 15 miles per week should be evaluated for iron deficiency and depletion of iron stores using the more precise tests of sTfR and TRI to reduce negative effects decreased iron has on training and performance and to prevent low iron leading to severe anemia. Healthcare professionals should be aware of the prevalence of low iron in this population and take preventative measures through assessment, diagnosis, and education of athletes on dietary iron intake and the body’s iron requirements.
ORAL SCRIPT

Hello, my name is ______ and I am here today to ask your consideration for participation in a research project through the Ohio State University Labs in Life at COSI. We are looking for female runners, ages 18 and up, who train at least 15 miles per week and have been running for at least 6 weeks. Participants may not be pregnant or have any known adrenal or thyroid abnormalities. Our research will look at your diet, running history, menstrual history, and bone density. Participants will be asked to give an estimated 2-3 hours total for the research. You will complete an online questionnaire to show us how you feel about your diet and body attitudes, keep a three day food record and visit the COSI Labs in Life for the laboratory visit. During the laboratory visit, bone mineral density and blood samples will be taken. Please know that the iDXA machine used to measure bone mineral density emits a small amount of radiation. Although this radiation is not considered significant, it is not advised that pregnant women participate for risk of harming the fetus so you will be screened for pregnancy (urine dipstick) prior to participation. There are a number of blood hormones and parameters we wish to look at which will require we take a small blood sample which will be one needle stick with 3 small tubes of blood (about 40 ml total). If you are interested in participating, please e-mail one of us or call on the phone to schedule the lab visit. We will ask you to sign an informed consent at that time.

Are there any questions?

Jackie Buell (614)437-4474 buell.7@osu.edu

General lab number (614)437-4472
APPENDIX B: Recruitment Ad

ATTENTION RUNNERS

If you are a female who runs at least 15 miles a week for the last 6 weeks, LABS in Life @ COSI wants you!

We are conducting a study on nutritional habits and attitudes, menstrual status and bone density on female runners ages 18 and over.

As part of the study, you will

• fill out an informed consent and HIPAA form (10 minutes)
• fill out an anonymous questionnaire (about an hour of your time)
• keep track of three days of eating so we can analyze it (about 30 minutes of your time)
• have a visit to the Labs in Life at COSI for a bone density test as well as a blood sample (about 40 minutes all total).

If you are interested in our study,

please call 614 437 4474

or e-mail us to see if you qualify!
APPENDIX C: Consent Form

The Ohio State University Consent to Participate in Research

Study Title: Female Athlete Triad in Recreational Endurance Runners
Principal Investigator: Buell, Jackie

Sponsor:

- **This is a consent form for research participation.** It contains important information about this study and what to expect if you decide to participate. Please consider the information carefully. Feel free to discuss the study with your friends and family and to ask questions before making your decision whether or not to participate.

- **Your participation is voluntary.** You may refuse to participate in this study. If you decide to take part in the study, you may leave the study at any time. No matter what decision you make, there will be no penalty to you and you will not lose any of your usual benefits. Your decision will not affect your future relationship with The Ohio State University. If you are a student or employee at Ohio State, your decision will not affect your grades or employment status.

- **You may or may not benefit as a result of participating in this study.** Also, as explained below, your participation may result in unintended or harmful effects for you that may be minor or may be serious depending on the nature of the research.

- **You will be provided with any new information that develops during the study that may affect your decision whether or not to continue to participate.** If you decide to participate, you will be asked to sign this form and will receive a copy of the form. You are being asked to consider participating in this study for the reasons explained below.
1. Why is this study being done?

This study will measure the bone mass in adult recreational female runners alongside the diet attitudes and habits, and hormonal markers including menstrual cycle history. Prior studies show that eating attitudes that lead to not eating enough energy to fuel your activity level may be harmful to the bone mass. This study includes questionnaires that screen for disordered eating to include eating disorders and depression to see if there are relationships with bone mass.

2. How many people will take part in this study?

We are seeking 150 runners for this study.

3. What will happen if I take part in this study?

This study will be conducted in the new Labs in Life at COSI. The Lab is a working research laboratory with glass walls so that the viewing public can watch research in action. If you schedule a visit to the lab during COSI open hours, the COSI guests will be able to watch your participation. If you prefer not to have anyone watching, we can schedule you outside of COSI open hours. It will never be the case that anyone outside the lab can see your results well enough to understand them in terms of your confidentiality.

If you choose to participate, we will have you complete an on-line questionnaire, a three day food and activity record, and a laboratory visit. You may complete the on-line questionnaire as well as the food and activity record prior to your visit, and should have received e-mail instructions for doing so. If you have not completed the questionnaire prior to your visit, you will be asked to complete it at the Laboratory visit. The laboratory visit will include a urinary screen for pregnancy, then the iDXA scans as well as a venous blood draw (about 6-7 teaspoons) from the front of arm to look substances in the blood that may relate to bone health.

4. How long will I be in the study?

This study will only include about an hour of your time prior to the lab visit (30 minutes for questionnaire, 30 minutes for 3 day food and activity record), then a lab visit that will last about 45 minutes but we suggest we each schedule this for an hour in case we need the extra time. You will not be obligated to participate in any other laboratory studies unless you choose to do so.

5. Can I stop being in the study?

You may leave the study at any time. If you decide to stop participating in the study, there will be no penalty to you, and you will not lose any benefits to which you are otherwise entitled. Your decision will not affect your future relationship with The Ohio State University.
6. **What risks, side effects or discomforts can I expect from being in the study?**

This research will take place in the Labs in Life at COSI science museum in a glass-walled laboratory. If you are uncomfortable being viewed while participating, please request an appointment outside of COSI working hours.

If you take part in this research, you will have a set of iDXA scans. An iDXA scan takes two low energy x-rays and passes them through your body while you lay still on your back on the scanner to determine how much bone and tissue is there. The scans you will have include a lean body mass measurement (total body scan), a slow mode hip scan, slow mode lumbar scan, and regular forearm scan. These tests involve a small amount of radiation. To give you an idea about how much radiation you will get, we will make a comparison with an every-day situation. Everyone receives a small amount of unavoidable radiation each year. Some of this radiation comes from space and some from naturally-occurring radioactive forms of water and minerals. This research gives your body the equivalent of about 2 extra days’ worth of this natural radiation. The radiation dose we have discussed is what you will receive from this study only and does not include any exposure you may have received or will receive from other tests.

The study also asks to collect a small sample of venous blood from you. Anytime blood is taken from a vein in the arm, there is a risk of bruising at the site as well as a risk for infection. The research team will take the steps to prevent these side effects but their risk does exist. We intend to test your blood for a number of hormones thought to be related to nutrition and bone health (hydration, hematocrit and hemoglobin, ferritin, soluble transferrin receptor, pre-albumin, thyroid stimulating hormone, free thyroxin, cortisol, parathyroid hormone and vitamin D). By measuring these levels in your blood, there is always the risk for identifying unknown health or medical conditions. The lab will gladly share your study values with you at your request once the tests are finished. Should there be reason for concern for your medical safety, we will contact you via telephone and ask that you follow up with your primary care physician. Any costs incurred by follow up evaluations will be your responsibility. In the absence of a primary care doctor, Kelsey Logan is the physician on this study group and her office can be reached at: (614)366-9324, and again, costs incurred are your responsibility.

The questionnaires used in this study are combined into an on-line survey. We will ask questions about your menstrual cycle, running history and mileage, a depression screening to include a question about suicidal ideology, an eating disorder screen to look for restrictive attitudes, a tendency to diet form and some questions about body image, then calcium consumption. If there is clinical concern for your safety and well being from these tools, Dr David Dagg of the research team will call you. You can contact him (614-937-3996) at any point in the process with your concerns about your psychological health as well. Please realize that any person with suicidal ideas and thoughts should seek the help of a professional for diagnosis and treatment. Subjects who need to seek
help must do so at their own cost, the study does not have funding for this sort of treatment.

7. **What benefits can I expect from being in the study?**
   You will learn your bone mass and body composition from your iDXA scans. You are also welcome to see the results of your three day diet and activity analysis, your blood values or your questionnaire screens.

8. **What other choices do I have if I do not take part in the study?**
   You may choose not to participate without penalty or loss of benefits to which you are otherwise entitled.

9. **Will my study-related information be kept confidential?**
   Efforts will be made to keep your study-related information confidential. However, there may be circumstances where this information must be released. For example, personal information regarding your participation in this study may be disclosed if required by state law. Also, your records may be reviewed by the following groups (as applicable to the research):
   - Office for Human Research Protections or other federal, state, or international regulatory agencies;
   - U.S. Food and Drug Administration;
   - The Ohio State University Institutional Review Board or Office of Responsible Research Practices;
   
   If the study involves the use of your protected health information, you may also be asked to sign a separate Health Insurance Portability and Accountability Act (HIPAA) research authorization form.

10. **What are the costs of taking part in this study?**
   It will not cost you to participate in the study.

   However, if you are found to have low bone mass, the cost of physician visits and treatment will be incurred by you (your insurance) and not the research study. Similarly, if the results of the depression screen reveal major depressive symptoms or suicidal ideology, the cost of formal evaluation and treatment will be incurred to you (your insurance) and not the research study. One of the questionnaires will also screen for eating disorders. Any cost incurred for formal evaluation and treatment will be the responsibility of the subject/patient.
11. Will I be paid for taking part in this study?

No, this study does not include any pay to the subjects. The LABS in Life @ COSI will pay the cost of your parking the day of your laboratory visit.

12. What happens if I am injured because I took part in this study?

If you suffer an injury from participating in this study, you should notify the researcher (Jackie Buell, 614.437.4474), study physician (Kelsey Logan, 614-366-9324) or study psychologist (David Dagg, 614-937-3996) immediately, who will determine if you should obtain medical treatment at The Ohio State University Medical Center.

The cost for this treatment will be billed to you or your medical or hospital insurance. Neither COSI nor the Ohio State University has no funds set aside for the payment of health care expenses for this study.

13. What are my rights if I take part in this study?

If you choose to participate in the study, you may discontinue participation at any time without penalty or loss of benefits. By signing this form, you do not give up any personal legal rights you may have as a participant in this study.

You will be provided with any new information that develops during the course of the research that may affect your decision whether or not to continue participation in the study.

You may refuse to participate in this study without penalty or loss of benefits to which you are otherwise entitled.

An Institutional Review Board responsible for human subjects research at The Ohio State University reviewed this research project and found it to be acceptable, according to applicable state and federal regulations and University policies designed to protect the rights and welfare of participants in research.
14. Who can answer my questions about the study?

For questions, concerns, or complaints about the study you may contact Jackie Buell, the study PI at 614.437.4474.

For questions about your rights as a participant in this study or to discuss other study-related concerns or complaints with someone who is not part of the research team, you may contact Ms. Sandra Meadows in the Office of Responsible Research Practices at 1-800-678-6251.

If you are injured as a result of participating in this study or for questions about a study-related injury, you may contact

the study PI, Jackie Buell, at 614.437-4474 or e-mail her at buell.7@osu.edu
the study physician, Kelsey Logan, at 614-366-9324
the study psychologist, David Dagg at 614-937-3996

Please include your telephone contact and e-mail address so that the investigators may contact you if the need arises from your results.

Telephone number_______________________________________

E-mail address___________________________________________


**Signing the consent form**

I have read (or someone has read to me) this form and I am aware that I am being asked to participate in a research study. I have had the opportunity to ask questions and have had them answered to my satisfaction. I voluntarily agree to participate in this study.

I am not giving up any legal rights by signing this form. I will be given a copy of this form.

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**Investigator/Research Staff**

I have explained the research to the participant or his/her representative before requesting the signature(s) above. There are no blanks in this document. A copy of this form has been given to the participant or his/her representative.

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APPENDIX D: ELISA Protocol

**Ferritin ELISA**
For the quantitative determination of ferritin in human serum

Catalog Number: 25 – FERHU-E01
Size: 96 wells
Version: 112509 – ALPCO September

For “In Vitro Diagnostic” use within the United States of America. This product is for “Research Use Only” outside of the United States of America.
INTENDED USE

This Ferritin ELISA is intended for the quantitative determination of ferritin in human serum. This assay is to be used in the diagnosis of diseases affecting iron metabolism. 

INTRODUCTION

One of the most prevalent disorders of man is the dietary deficiency of iron and the resulting anemia. Therefore, the assays of iron, total iron binding capacity, and other assessments of iron compounds in the body are clinically significant. Iron-storage compounds in the body include hemoglobin, hemosiderin, myoglobin, and the cytochromes. In most tissues, ferritin is a major iron-storage protein. Human ferritin has a molecular weight of approximately 450,000 daltons, and consists of a protein shell around an iron core; each molecule of ferritin may contain as many as 4,000 iron atoms. Under normal conditions, this may represent 25% of the total iron found in the body. In addition, ferritin can be found in several isomers.

High concentrations of ferritin are found in the cytoplasm of the reticuloendothelial system, the liver, spleen, and the bone marrow. Methods previously used to measure iron in such tissues are invasive, cause patient trauma, and lack adequate sensitivity. The measurement of ferritin in serum is useful in determining changes in body iron storage, and is non-invasive with relatively little patient discomfort. Serum ferritin levels can be measured routinely and are particularly useful in the early detection of iron-deficiency anemia in apparently healthy people.

Serum ferritin measurements are also clinically significant in the monitoring of the iron status of pregnant women, blood donors, and renal dialysis patients. High ferritin levels may indicate iron overload without apparent liver damage, as may be noted in the early stages of idiopathic hemochromatosis. Ferritin levels in serum have also been used to evaluate clinical conditions not related to iron storage, including inflammation, chronic liver disease, and malignancy.

This Ferritin Enzyme Immunoassay provides a rapid, sensitive, and reliable assay. The antibodies developed for the test will determine a minimal concentration of human ferritin of 5.0 ng/mL. There is minimal cross-reactivity with human serum albumin, alpha-fetoprotein, human hemoglobin, human transferrin, and ferric chloride.

PRINCIPLE OF THE ASSAY

This Ferritin Quantitative Test is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes rabbit anti-ferritin for the solid phase (microtiter wells) immobilization and mouse monoclonal anti-ferritin in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in the ferritin molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 45 minute incubation at room temperature, the wells are washed with water to remove unbound labeled antibodies. A solution of 3,3',5,5'-
Tetramethylbenzidine (TMB) is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of 1N HCl, and the resulting yellow color is measured spectrophotometrically at 450 nm. The concentration of ferritin is directly proportional to the color intensity of the test sample.

**REAGENTS AND MATERIALS PROVIDED**

1. *Antibody-Coated Wells (1 plate, 96 wells)*  
   Microtiter wells coated with rabbit anti-ferritin.

2. *Enzyme Conjugate Reagent (13 mL)*  
   Contains mouse monoclonal anti-ferritin conjugated to horseradish peroxidase.

3. *Reference Standard Set (0.5 mL/vial)*  
   Contains 0, 15, 80, 250, 500, and 1,000 ng/mL human liver or spleen ferritin in bovine serum with preservatives. 0.5 mL each, liquid, ready to use.

4. *TMB Reagent (One-Step) (1 bottle, 11 mL)*  
   Contains 3, 3’, 5, 5’ tetramethylbenzidine (TMB) stabilized in buffer solution.

5. *Stop Solution (1N HCl) (1 bottle, 11 mL)*  
   Contains diluted hydrochloric acid.
MATERIALS REQUIRED BUT NOT PROVIDED
1. Distilled or deionized water
2. Precision pipettes: 0.02, 0.05, 0.1, 0.2 and 1 mL
3. Disposable pipette tips
4. Microtiter well reader capable of reading absorbance at 450nm.
5. Vortex mixer, or equivalent
6. Absorbent paper
7. Graph paper
8. Quality control material

WARNINGS AND PRECAUTIONS
1. **CAUTION**: This kit contains human material. The source material used for manufacture of this kit tested negative for HBsAg, HIV 1/2 and HCV by FDA-approved methods. However, no method can completely assure absence of these agents. Therefore, all human blood products, including serum samples, should be considered potentially infectious. Handling and disposal should be as defined by an appropriate national biohazard safety guideline or regulation, where it exists.

2. Do not use reagents after expiration date and do not mix or use components from kits with different lot numbers.

3. Do not use the reagent when it becomes cloudy or contamination is suspected.

4. Do not use the reagent if the vial is damaged.

5. Replace caps on reagents immediately. Do not switch caps.

6. Each well can be used only once.

7. Do not pipette reagents by mouth.

8. Solutions containing additives or preservatives, such as sodium azide, should not be used in the enzyme reaction.

9. Avoid contact with 1N HCl. It may cause skin irritation and burns. If contact occurs, wash with copious amounts of water and seek medical attention if irritation persists.

10. For in vitro diagnostic use.

STORAGE CONDITIONS
1. Store the unopened kit at 2-8°C upon receipt and when it is not in use, until the expiration shown on the kit label. Refer to the package label for the expiration date.

2. The opened and used reagents are stable until the expiration date if stored properly at 2-8°C.

3. Keep microtiter plate in a sealed bag with desiccant to minimize exposure to damp air.

SPECIMEN COLLECTION AND PREPARATION
1. Serum should be prepared from a whole blood specimen obtained by acceptable medical techniques. This kit is for use with serum samples without
additives only. Avoid grossly hemolytic (bright red), lipemic (milky), or turbid samples.

2. Specimens should be capped and may be stored for up to 48 hours at 2-8°C. Specimens held for a longer time should be frozen only once at -20°C prior to assay. Thawed samples should be inverted several times prior to testing.

3. Specimens with expected values greater than 1,000 ng/ml (e.g. dialysis patients) should be diluted with Zero Standard prior to assaying. A 1:10 initial dilution is recommended.

**INSTRUMENTATION**

A microtiter well reader with a bandwidth of 10 nm or less and an optical density range of 0 to 2 OD or greater at 450 nm wavelength is acceptable for absorbance measurement.

Page 3 of 8
REAGENT PREPARATION
1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
3. Samples with expected values greater than 1000 ng/mL should be diluted with Zero Standard prior to assaying. A 1:10 initial dilution is recommended.

ASSAY PROCEDURE
1. Secure the desired number of coated wells in the holder.
2. Dispense 20μL of standards, samples, and controls (not included) into appropriate wells.
3. Dispense 100μL of Enzyme Conjugate Reagent into each well.
4. Gently mix for 30 seconds. It is very important to have complete mixing.
5. Incubate at room temperature (18-25°C) for 45 minutes.
6. Remove the incubation mixture by flicking well contents into a suitable waste container.
7. Rinse the wells 5 times with distilled or deionized water. (Please do not use tap water.)
8. Strike the wells sharply on absorbent paper to remove residual water droplets.
9. Dispense 100 μL TMB Reagent into each well. Gently mix for 5 seconds.
10. Incubate at room temperature, in the dark, for 20 minutes.
11. Stop the reaction by adding 100μL of Stop Solution (1N HCl) into each well.
12. Gently mix for 5 seconds.
13. Read OD at 450nm with a microtiter well reader within 15 minutes.

CALCULATION OF RESULTS
1. Calculate the mean absorbance value (OD450) for each set of reference standards, controls and samples.
2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/mL on graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of ferritin in ng/mL from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.
4. Any diluted samples must be further converted by the appropriate dilution factor.

PROCEDURAL NOTES
1. Manual Pipetting: It is recommended that no more than 32 wells be used for each assay run. Pipetting of all standards, samples, and controls should be completed within 3 minutes.
2. Automated Pipetting: A full plate of 96 wells may be used in each assay run. However, it is recommended that pipetting of all standards, samples, and controls be completed within 3 minutes.

3. All standards, samples, and controls should be run in duplicate concurrently so that all conditions of testing are the same.

4. It is recommended that the wells be read within 15 minutes following addition of Stop Solution.

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**EXAMPLE OF STANDARD CURVE**

1. Results of a typical standard run of the assay are shown below. Ferritin (ng/mL) Absorbance (450 nm)

<table>
<thead>
<tr>
<th>Ferritin (ng/mL)</th>
<th>Absorbance (450 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.074</td>
</tr>
<tr>
<td>15</td>
<td>0.150</td>
</tr>
<tr>
<td>80</td>
<td>0.362</td>
</tr>
<tr>
<td>250</td>
<td>1.017</td>
</tr>
<tr>
<td>500</td>
<td>1.699</td>
</tr>
<tr>
<td>1000</td>
<td>2.728</td>
</tr>
</tbody>
</table>
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