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SHORT COMMUNICATION

Skeletal muscle ferritin abundance is tightly related to plasma ferritin concentration in adults with obesity

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Abstract

Obesity is associated with complex perturbations to whole-body and tissue iron homeostasis. Recent evidence suggests a potentially important influence of iron storage in skeletal muscle on whole-body iron homeostasis, but this association is not clearly resolved. The primary aim of this study was to assess the relationship between whole-body and skeletal muscle iron stores by measuring the abundance of the key iron storage (ferritin) and import (transferrin receptor) proteins in skeletal muscle, as well as markers of whole-body iron homeostasis in men ($n = 19$) and women ($n = 43$) with obesity. Plasma ferritin concentration (a marker of whole-body iron stores) was highly correlated with muscle ferritin abundance ($r = 0.77$, $P = 2 \times 10^{-13}$) and negatively associated with muscle transferrin receptor abundance ($r = -0.76$, $P = 1 \times 10^{-12}$). These relationships persisted when accounting for sex, age, BMI and plasma C-reactive protein concentration. In parallel with higher whole-body iron stores in our male *versus* female participants, men had 2.2-fold higher muscle ferritin abundance ($P = 1 \times 10^{-4}$) compared with women. In accordance with lower muscle iron storage, women had 2.7-fold higher transferrin receptor abundance ($P = 7 \times 10^{-10}$) compared with men. We conclude that muscle iron storage and import proteins are tightly and independently related to plasma ferritin concentration in adults with obesity, suggesting that skeletal muscle may be an underappreciated iron store.

KEYWORDS
iron, obesity, skeletal muscle

1 | INTRODUCTION

Iron regulation is critical to human health – iron is required for the function of numerous proteins in circulation and tissues yet toxic when it accumulates in excess (Ganz, 2013). Maintaining systemic iron homeostasis requires the coordinated interplay of iron absorption from the gastrointestinal tract, transport in circulation, incorporation into iron-containing proteins, and storage/release from tissues (Ganz, 2013). Unfortunately, iron homeostasis is often compromised in adults with obesity, which can lead to adverse haematological (e.g. iron-deficiency anaemia) and metabolic disorders (e.g. iron overload-mediated insulin resistance; Aigner, Feldman, & Datz, 2014; Cepeda-Lopez, Melse-Boonstra, Zimmermann, & Herter-Aeberli, 2015; Yanoff et al., 2007). Regulation of iron homeostasis is dependent, in part, on

effective storage in tissues, yet factors underlying the regulation of iron storage in different tissues in adults with obesity are not well understood.

In addition to the iron incorporated into functional proteins, iron is also stored in tissues, bound to ferritin, providing an essential pool of iron that can be released when whole-body iron status is low (Torti & Torti, 2002; Walters, Miller, & Worwood, 1973). However, excess tissue iron storage is associated with adverse consequences, largely driven by iron-mediated oxidative damage and consequent cellular dysfunction (Puntarulo, 2005). The liver is traditionally viewed as the key iron storage depot (Ganz, 2013), and evidence indicates a strong link between elevated whole-body iron stores, high hepatic iron content and liver dysfunction in adults with obesity (Dongiovanni, Fracanzani, Fargion, & Valenti, 2011). Although skeletal muscle is not traditionally viewed as an important iron storage site, a recent study reported elevated markers of iron storage in skeletal muscle from adults with obesity (Moreno-Navarrete et al., 2016). However, the regulation of skeletal muscle iron storage in obesity has not been well described, and it is even unknown if skeletal muscle iron stores are related to whole-body iron stores.

The primary aim of this study was to examine relationships between whole-body iron stores and the abundance of the key skeletal muscle iron storage (ferritin) and import (transferrin receptor) proteins in adults with obesity. We hypothesized that plasma ferritin concentration, a marker of whole-body iron stores (Walters et al., 1973; Worwood, 2007), would be positively correlated with muscle ferritin protein abundance and negatively correlated with muscle transferrin receptor abundance. Further, we hypothesized that, in concert with other known sex differences in systemic and hepatic iron homeostasis (Ganz & Nemeth, 2012), men with obesity would have higher muscle ferritin protein abundance and lower muscle transferrin receptor protein abundance compared with women with obesity. Recognizing that inflammation can influence plasma ferritin concentrations, we examined potential relationships between plasma C-reactive protein concentration, plasma ferritin concentration and skeletal muscle iron proteins.

2 | METHODS

This study involved analyses from samples collected as part of two investigations into metabolic regulation in humans with obesity (NCT02717832 and NCT02706093). Samples collected from all participants who completed the baseline clinical study visit for one of these two protocols between June 2016 and October 2019 were analysed for the present study, with the exception of one individual who was excluded due to a recent blood donation. In total, we studied 62 physically inactive young adults with obesity (BMI 30–40 kg m^{-2}; age 18–44; 19 men, 43 women). All participants were non-smokers, weight stable for >6 months, and had no history of cardiometabolic disease. All women were premenopausal. We recently published a paper examining the effect 12 weeks of exercise training on insulin sensitivity and the baseline samples from 30 of these same

New Findings

- **What is the central question of this study?**
  Obesity is associated with complex perturbations to iron homeostasis: is plasma ferritin concentration (a biomarker of whole-body iron stores) related to the abundance of ferritin (the key tissue iron storage protein) in skeletal muscle in adults with obesity?

- **What is the main finding and its importance?**
  Plasma ferritin concentration was tightly correlated with the abundance of ferritin in skeletal muscle, and this relationship persisted when accounting for sex, age, body mass index and plasma C-reactive protein concentration. Our findings suggest that skeletal muscle may be an important iron store.

participants were included in that study (Ryan et al., 2020). However, no iron-related outcomes were included in this previous publication, and the findings presented here are novel. The study was conducted according to the standards in the latest version of the *Declaration of Helsinki*. All protocols were approved by the University of Michigan Institutional Review Board (HUM00111275 and HUM00106883) and all participants provided written, informed consent.

2.1 | Experimental procedures

In the morning after an overnight fast, a venous blood sample was collected, and a muscle biopsy sample was obtained from the vastus lateralis. Upon collection, the muscle tissue was quickly dissected free of any adipose or connective tissue, rinsed with saline, blotted dry, flash frozen in liquid nitrogen and then stored at −80°C until further analysis. On a separate day, body composition was determined using dual-energy X-ray absorptiometry (Lunar DPX, GE Healthcare, Milwaukee, WI, USA) and subjects completed an MRI scan for liver R2* determination (an index of liver iron content; Labranche et al., 2018).

2.2 | Analytical procedures

2.2.1 | Circulating markers of iron status and inflammation

Plasma concentrations of ferritin (S-22, Ramco Laboratories, Inc., Stafford, TX, USA), hepcidin (a hormone regulating iron homeostasis; ICE-004, Intrinsic LifeSciences, La Jolla, CA, USA), high-sensitivity C-reactive protein (CR120C, Cal Biotech, El Cajon, CA, USA), and IL-6 (HS600C, R&D Systems, Minneapolis, MN, USA) were assessed using enzyme-linked immunosorbent assays. Haemoglobin concentration

and haematocrit were assessed by the University of Michigan Pathology Laboratory (Sysmex XN9000).

2.2.2 | Muscle ferritin and transferrin receptor abundance

Skeletal muscle ferritin and transferrin receptor protein abundance were assessed using immunoblotting. A small piece (~25 mg) of muscle tissue was homogenized with 2 × 5 mm steel beads (TissueLyser II, Qiagen, Germantown, MD, USA) in ice cold radio immunoprecipitation assay buffer (no. 9806, Cell Signaling Technology, Danvers, MA, USA) supplemented with protease and phosphatase inhibitors, rotated at 50 rpm for 1 h at 4°C, and then centrifuged at 15,000 g for 15 min at 4°C. The supernatant was collected, and protein concentration was determined using a bicinchoncic acid assay. Fifteen micrograms of protein were separated onto handcast 10% gels via SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes and stained with Memcode total protein stain (no. 24580, Thermo Fisher Scientific, Waltham, MA, USA) – the total protein stain signal intensity in each lane was quantified to normalize protein expression (Moritz, 2017). Membranes were blocked in 5% BSA in Tris-buffered saline–Tween 20 for 2 h and incubated with appropriate primary antibodies overnight at 4°C. Primary antibodies for ferritin (ab75973, RRID:AB_1310222) and transferrin receptor (ab84036, RRID:AB_10673794) were obtained from Abcam (Cambridge, MA, USA). This primary antibody for ferritin recognizes both the heavy and light chain of ferritin. Membranes were then incubated with appropriate secondary antibody for 90 min, developed using enhanced chemiluminescence, and imaged. To reduce gel-to-gel variability, an internal standard sample (composite skeletal muscle lysate from 8 obese individuals) was loaded on each gel and protein expression was also normalized to this lane.

2.2.3 | Liver magnetic resonance imaging

Liver R2* is an magnetic resonance imaging (MRI)-derived parameter that is directly proportional to liver iron content (Labranche et al., 2018; Wood et al., 2005). MRI scans were conducted using a 3 tesla clinical MRI system (Philips Healthcare, Best, Netherlands). A multi-echo Dixon product sequence was performed with a breath-hold lasting ≥16 s. Nominal acquisition conditions of this fast-field-echo sequence were: 77 axial 3D slices; six echos (echo time (TE) = 0.96 ms + n × 0.7 ms, n = 0, 1, 5); repetition time (TR) = 6.2 ms; flip angle = 3 deg; acquisition matrix = 160 (R/L) by 160 (A/P); parallel imaging SENSE = 2; and field of view = 400 mm. Images were converted to 3D Meta-Image format files using in-house Matlab (The Mathworks, Natick, MA, USA) routines and the T2* meta-image file was examined and quantified using 3D Slicer ver4.6.2 (https://www.slicer.org). All analyses were completed by a single trained investigator who was blinded with respect to all participant characteristics. Large regions of interest were manually defined on three 5 mm axial slices (each separated by 10 mm) through the right lobe of the liver; large vessels were avoided. Hepatic T2* was quantified as the average T2* value within the three slices. The coefficient of variation for T2* for the three slices within each scan averaged 5.1%. Hepatic R2* was calculated as 1/T2* and expressed in Hz.

2.3 | Statistical analysis

Data were checked for normality, and non-normally distributed variables were log-transformed prior to statistical analysis. The primary statistical analyses involved simple and multiple linear regression analyses to determine the relationship between plasma ferritin concentration and muscle protein abundances. We compared men and women using an independent samples Student's t test. P-values <0.05 were considered statistically significant.

3 | RESULTS

Across all participants, we found a strong positive correlation between plasma ferritin concentration and muscle ferritin abundance (r = 0.77, P = 2 × 10^{-13}; Figure 1a). Using multiple regression analysis, plasma ferritin concentration was independently related to muscle ferritin abundance ($r_{partial}$ = 0.70, P = 8 × 10^{-10}), whereas sex, age, BMI and plasma CRP concentration were not (Table 1). Although inflammation can increase plasma ferritin concentrations, there was no positive correlation between plasma ferritin concentration and either plasma CRP or IL-6 concentration (Figure 1b,d). As expected, there was no significant correlation between plasma ferritin concentration and BMI (Figure 1f).

Consistent with its positive association with muscle ferritin abundance, plasma ferritin concentration was negatively correlated with muscle transferrin receptor abundance (r = −0.76, P = 1 × 10^{-12}, Figure 1c). Multiple regression analysis revealed that plasma ferritin concentration was independently related to muscle transferrin receptor abundance ($r_{partial}$ = −0.54, P = 1 × 10^{-5}). Muscle transferrin receptor abundance was also independently related to sex ($r_{partial}$ = −0.33, P = 0.01) but not to age, BMI, or plasma CRP concentration (Table 1).

As expected, men had significantly higher plasma ferritin and hepcidin concentrations, as well as higher liver R2* (index of liver iron content; Table 2). Interestingly, in parallel with higher whole-body iron stores, men also had 2.2-fold higher muscle ferritin abundance (P = 1 × 10^{-4}) compared with women (Table 2). As expected based on their lower muscle ferritin abundance, women had 2.7-fold higher muscle transferrin receptor abundance (P = 7 × 10^{-10}) compared with men.

4 | DISCUSSION

Our novel findings indicate that plasma ferritin concentration, a marker of whole-body iron stores, is tightly and independently associated with skeletal muscle ferritin and transferrin receptor abundance in adults

FIGURE 1 Relationship between plasma ferritin concentration and skeletal muscle iron protein abundance in adults with obesity. The relationship between plasma ferritin concentration and (a) skeletal muscle ferritin abundance, (b) plasma C-reactive protein concentration (CRP), (c) skeletal muscle transferrin receptor (Tfrc) abundance, (d) plasma IL-6 concentration, and (f) body mass index. (e) Representative images of the ferritin protein band, Tfrc protein band and the Memcode total protein stain from a female (F) and male (M) participant. Men are indicated by black diamonds and women by open circles. The regression line is indicated in black, with the 95% confidence intervals represented by dotted lines. *$P \leq 1 \times 10^{-12}$
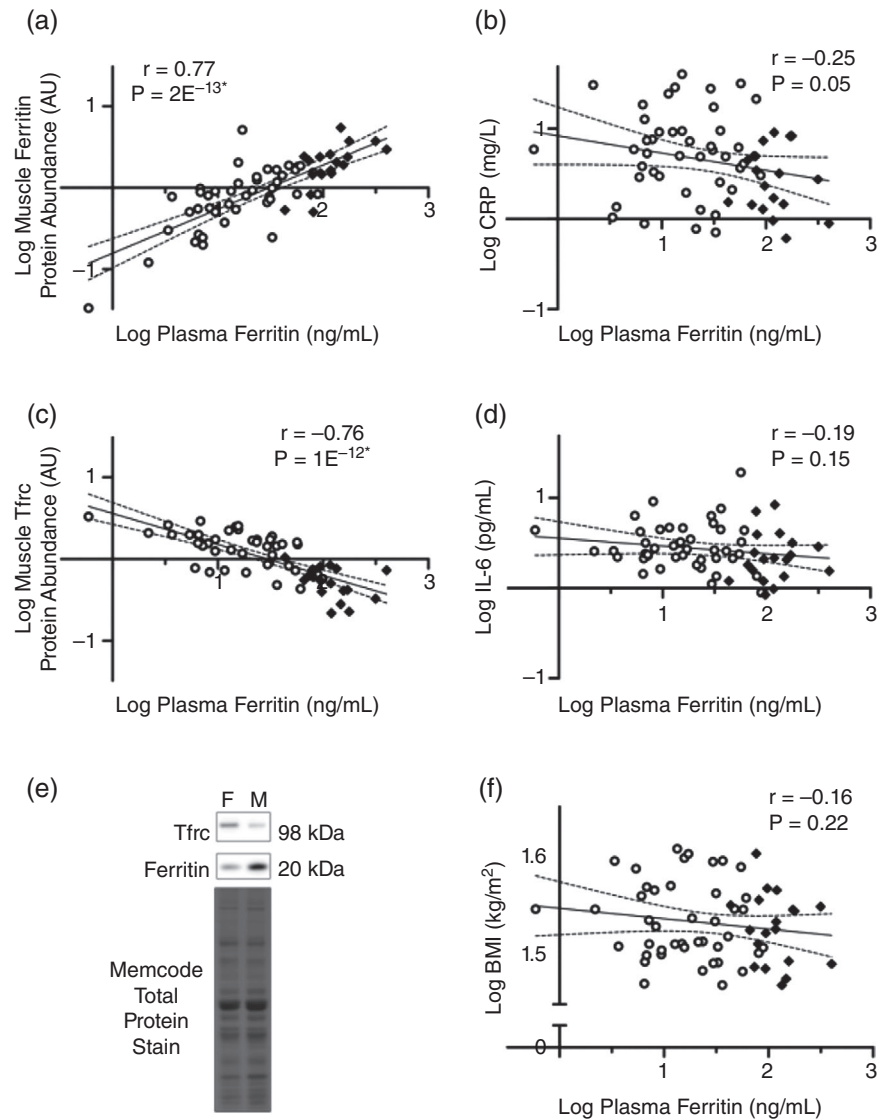
with obesity. We interpret this to suggest coordinated regulation of muscle and whole-body iron stores, such that whole-body iron stores play a very important role in determining the magnitude of iron storage in skeletal muscle. Additionally, our data reveal large sex differences in these muscle iron proteins, which appear to be driven by sex differences in whole-body iron stores. These findings have important implications for the understanding of tissue and systemic iron homeostasis in adults with obesity.

A mobilizable pool of iron stored in tissues is critical for providing iron for erythropoiesis – dietary iron absorption is relatively inefficient, and inadequate iron storage can result in iron-deficiency anaemia (Worwood, 2007; Zimmermann & Hurrell, 2007). On the other hand, excessive tissue iron storage is associated with organ toxicity due to iron-mediated oxidative damage (Puntarulo, 2005). The liver is traditionally viewed as the primary iron storage site (Ganz, 2013), and a role for muscle as an iron store is not widely recognized. Although iron concentration in skeletal muscle is much lower than that found in the liver, the large muscle mass in humans results in comparable total iron content among these tissues (Torrance, Charlton, Schmaman, Lynch, &

Bothwell, 1968). The strong correlations we observed between plasma ferritin concentration and muscle ferritin and transferrin receptor abundance suggest that alterations in whole-body iron status are accompanied by changes in muscle iron storage, supporting a previously underappreciated role for skeletal muscle in the regulation of iron homeostasis.

The regulation of iron homeostasis in obesity is complex, as evidenced by iron deficiency in many obese adults and iron overload in others (Aigner et al., 2014). These opposing iron-related disorders are associated with different health complications in obesity. Whereas iron deficiency leads to anaemia (Zimmermann & Hurrell, 2007), high body iron stores have been associated with metabolic dysfunction and the development of type 2 diabetes (Jehn, Clark, & Guallar, 2004; Jiang et al., 2004). The mechanisms linking iron and metabolic dysfunction are incompletely understood (Fernández-Real, McClain, & Manco, 2015), but some pre-clinical evidence indicates that excess muscle iron impairs peripheral insulin signalling (Cui et al., 2019). Although whole-body iron stores vary markedly among adults with obesity (Aigner et al., 2014), it was reported that markers of iron content in skeletal muscle

TABLE 1 Multiple linear regression analysis of factors influencing muscle iron proteins in adults with obesity

Variable	Partial r	P
Model 1		
Plasma [Ferritin]	0.70	$8 \times 10^{-10}*$
Sex	−0.19	0.16
Plasma [CRP]	−0.10	0.44
Age	0.16	0.24
BMI	0.05	0.73
Model 2		
Plasma [Ferritin]	−0.54	$1 \times 10^{-5}*$
Sex	−0.33	0.01*
Plasma [CRP]	0.001	0.99
Age	−0.12	0.38
BMI	−0.22	0.10

Model 1: muscle ferritin protein abundance; adjusted $R^2 = 0.59$; $P = 9 \times 10^{-11}$. Model 2: muscle transferrin receptor protein abundance; adjusted $R^2 = 0.61$; $P = 3 \times 10^{-11}$. *$P < 0.05$. BMI, body mass index; CRP, C-reactive protein.

TABLE 2 Subject characteristics and iron-related parameters in men and women with obesity

	Men (n = 19)	Women (n = 43)	P
Age (years)	31 ± 6	30 ± 7	0.87
Body mass index (kg m^{-2})	34 ± 3	34 ± 3	0.46
Body fat (%)	37 ± 3	46 ± 4	$2 \times 10^{-12}*$
Plasma [Ferritin] (ng ml^{-1})	135 ± 88	24 ± 21	$3 \times 10^{-14}*$
Plasma [Hepcidin] (ng ml^{-1})	45 ± 25	23 ± 15	$7 \times 10^{-5}*$
Haemoglobin concentration (g dl^{-1})[a]	14.6 ± 0.7	12.4 ± 1.3	$1 \times 10^{-6}*$
Haematocrit (%)[a]	43 ± 3	38 ± 3	$1 \times 10^{-5}*$
Plasma [CRP] (mg dl^{-1})	0.4 ± 0.3	1.0 ± 1.0	0.01*
Plasma [IL-6] (pg ml^{-1})	2.7 ± 2.0	3.6 ± 3	0.09
Liver R2* (Hz)[b]	58 ± 10	43 ± 9	$7 \times 10^{-6}*$
Muscle ferritin protein abundance (AU)	2.2 ± 1.2	1.0 ± 0.8	$1 \times 10^{-4}*$
Muscle Tfrc protein abundance (AU)	0.6 ± 0.2	1.5 ± 0.7	$7 \times 10^{-10}*$

Data are presented as means ± SD. [a]Haemoglobin concentration and haematocrit were not obtained from 6 men and 11 women so $n = 13$ men and $n = 32$ women for these parameters. [b]MRI scans were not obtained on 2 men and 8 women so liver R2* sample sizes were $n = 17$ men and $n = 35$ women. *$P \leq 0.01$. BMI, body mass index; CRP: C-reactive protein; Tfrc, transferrin receptor.

correlated with BMI, suggestive of increased muscle iron storage in obesity (Moreno-Navarrete et al., 2016). Our findings suggest that whole-body iron stores are a key determinant of muscle iron storage among adults with obesity. Because we did not study normal-weight individuals in this study, we cannot conclude whether this phenomenon contributes to differences in iron homeostasis often reported between normal-weight and obese groups (Aigner et al., 2014). While we do not discount the possibility that other obesity-related factors may also affect muscle iron storage, the strong and independent correlation we found between plasma ferritin concentration and muscle ferritin abundance suggests that the impact of other obesity-related factors may be secondary to whole-body iron stores. Based on our findings, we contend that studies comparing muscle iron storage between groups should consider the influence of whole-body iron status. Further studies are warranted to determine if the tight relationship we observed between plasma ferritin concentration and skeletal muscle ferritin abundance in individuals with BMI 30–40 kg m^{-2} extends across a larger range of BMI.

Our data are the first to our knowledge to reveal sex differences in the abundance of the key iron storage and import proteins in human skeletal muscle. Sex differences in whole-body iron stores are well-established, and the large sex difference in muscle iron protein abundance we observed fits well with the notion of coordinated regulation of whole-body and muscle iron storage. There was no difference in BMI between the men and women in our study but, as expected, women had significantly higher body fat percentage. It is possible that elevated adiposity may partially contribute to lower iron storage in women. However, menstrual blood loss has been identified as a primary factor driving sex differences in whole-body iron storage (Worwood, 2007).

4.1 | Experimental considerations

The aim of this study was to assess the relationship between whole-body and skeletal muscle iron stores. We did not assess factors responsible for differences in whole-body iron stores between individuals (e.g. dietary iron intake, dietary iron absorption, menstrual blood losses, etc.). Additionally, we did not control the timing of testing relative to the menstrual phase, but plasma ferritin concentration and other circulating iron biomarkers remain relatively stable across the menstrual cycle (Belza, Henriksen, Ersbøll, Thilsted, & Tetens, 2005; Puolakka, 1980). Therefore, it is unlikely that standardizing testing relative to the menstrual phase would have altered our findings. It is important to recognize that inflammation can influence plasma ferritin concentration, which can impact the interpretation of plasma ferritin concentration as a biomarker of total body iron stores (Worwood, 2007). There is strong evidence that acute infection and chronic inflammatory diseases result in increases in plasma ferritin concentration independent of iron stores (Worwood, 2007). Obesity is often accompanied by low-grade inflammation, and the plasma CRP and IL-6 concentrations in our participants with obesity reflect the expected elevated levels of inflammation (i.e. compared to plasma CRP

and IL-6 values reported in normal-weight populations; Aronson et al., 2004; Kern, Ranganathan, Li, Wood, & Ranganathan, 2001). While we cannot discount a potential small influence of inflammation on plasma ferritin concentrations in our study, multiple pieces of evidence suggest that our key findings are not driven by a confounding influence of inflammation on the interpretation of plasma ferritin concentration. First, there were no positive associations between plasma ferritin and either plasma CRP or IL-6 concentrations in our participants, whether we looked across all participants or within men/women. Despite more than two-fold higher mean plasma CRP concentrations in the women, mean plasma ferritin concentration was more than five-fold higher in men. Finally, our novel finding that plasma ferritin concentration is tightly correlated with muscle ferritin abundance – independent of plasma CRP concentration – supports a link between plasma ferritin concentration and tissue iron stores.

5 | CONCLUSION

In conclusion, our findings indicate that muscle iron storage and import proteins are tightly and independently correlated with plasma ferritin concentration in adults with obesity. We contend that this relationship suggests coordinated regulation of muscle and whole-body iron stores, such that the magnitude of muscle iron storage may be determined, at least in part, by whole-body iron stores. We also reveal large sex differences in these iron-related proteins in skeletal muscle, which appear to be driven by the sex differences in whole-body iron stores. Together, our findings suggest that skeletal muscle may be an important iron store and highlight the need for further investigation into the functional implications of muscle iron storage in humans.

COMPETING INTERESTS

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

B.J.R. and J.F.H. contributed to the conception and design of the work. All authors contributed to the acquisition, analysis, or interpretation of data for the work and to drafting of the work or revising it critically for important intellectual content. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All individuals designated as authors qualify for authorship, and all those who qualify for authorship are listed.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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