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Chronic Adherence to a Ketogenic Diet Modifies Iron Metabolism in Elite Athletes

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Abstract

Purpose: The short-term restriction of carbohydrate (CHO) can potentially influence iron regulation via modification of post-exercise interleukin-6 (IL-6) and hepcidin levels. This study examined the impact of a chronic ketogenic low CHO-high fat (LCHF) diet on iron status and iron-regulatory markers in elite athletes. **Methods**: International-level race walkers (n=50) were allocated to one of three dietary interventions; i) a high CHO diet (HCHO; n=16), ii) periodized CHO availability (PCHO; n=17) or iii) a LCHF diet (n=17) while completing a periodized training program for 3 weeks. A 19-25 km race walking test protocol was completed at baseline and following adaptation, and changes in serum ferritin, IL-6 and hepcidin concentrations were measured. Results from HCHO and PCHO were combined into one group (CHO; n=33) for analysis. **Results**: The decrease in serum ferritin across the intervention period was substantially greater in the CHO group (37%) compared to the LCHF (23%) group (p=0.021). After dietary intervention, the post-exercise increase in IL-6 was greater in LCHF (13.6-fold increase; 95% CI 7.1-21.4), than athletes adhering to a CHO-rich diet (7.6-fold increase; 5.5-10.2; p=0.033). While no significant differences occurred between diets, confidence intervals indicate 3 h post-exercise hepcidin concentrations were lower after dietary intervention compared to baseline in CHO (β=-4.3; -6.6, -2.0), with no differences evident in LCHF. **Conclusion**: Athletes who adhered to a CHO-rich diet experienced favorable changes to the post-exercise IL-6 and hepcidin response, relative to the LCHF group. Lower serum ferritin after 3 weeks of additional dietary CHO might reflect a larger more adaptive hematological response to training.

Keywords: hepcidin, inflammation, LCHF, carbohydrate, race walkers.

Introduction

It is well established that performance in endurance sports (>90 min of moderate-high intensity exercise) is enhanced by strategies that increase the muscles' endogenous (1) and exogenous (2) carbohydrate (CHO) utilization. For this reason, the traditional dietary guidelines for endurance athletes have encouraged a diet providing sustained high CHO availability (HCHO) to support the quality of daily training, promote recovery and prepare optimally for competition (3). More recently, investigation of the cellular responses to training and nutrition interactions has identified that exercising under conditions of low CHO availability amplifies the cascade of signaling events that underpin metabolic adaptation. Furthermore, low CHO availability can delay the restoration of muscle glycogen, thus extending the period of enhanced cellular signaling (4). This work has enabled sports scientists and coaches to exploit the specificity of nutritional support for each workout, by integrating strategies of "train high" and "train low" into the athlete's training program, both in an applied context (5) and in laboratory investigations (6). Of note, the deliberate periodization of CHO availability during a training block can enhance training responses, transferring to superior performance in sub-elite endurance athletes (6). However similar evidence is not yet available in elite competitors (7). An alternate, but somewhat contentious approach to nutrition for endurance athletes involves adopting a ketogenic low CHO-high fat (LCHF) diet (8). Chronic adaptation to a LCHF diet alters the muscle to better utilize the body's largest fuel source (fat) during exercise, rather than the limited glycogen stores, thereby achieving a sustained exposure to high plasma levels of ketone bodies (8). While this scenario may be suitable for exercise conducted at moderate intensities (60-70% of aerobic capacity), it may impair, rather than enhance, performance of endurance events performed at higher intensities (7).

To date, interest in these different approaches to nutrition strategies for fuelling endurance exercise has focused on sports performance, with little consideration to the effect on the athlete's health. However, factors such as the inflammatory response to exercise can be influenced by an athlete's glycogen status, with concentrations of the inflammatory cytokine interleukin-6 (IL-6) highest when exercise is undertaken with low muscle glycogen stores (9). An increased IL-6 response signals the up-regulation of hepatic glucose release to maintain blood glucose concentrations and sustain exercise intensity (9). However, IL-6 is also a key regulator of exercise-induced hepcidin activity (10), with peak levels of this hormone reported at 3-6 h post-exercise (11). Hepcidin is the body's primary iron-regulatory hormone, reacting in a homeostatic manner to keep iron stores within a healthy physiological range (12). Hepcidin governs the movement of iron through the only known iron export transporter, ferroportin. An increase in hepcidin levels internalizes ferroportin, consequently limiting the amount of iron that can be absorbed from the diet and recycled by macrophages (12). In response to exercise, inflammatory signals up-regulate hepcidin activity, which likely results in a transient disruption to iron absorption. Over time, such a response may lower an athlete's iron stores. Serum ferritin, a key marker of iron status, is an acute phase reactant that also increases in response to inflammation (13). Therefore, a decrease in iron status may be difficult to detect during periods of inflammation, and caution should be taken assessing iron status solely via serum ferritin assessment (14).

Given the link between increases in IL-6 and hepcidin activity, it is possible that manipulating CHO availability around exercise could influence post-exercise iron metabolism. For instance, consuming a low CHO diet for 24 h following a glycogen-depleting exercise task increases post-

exercise IL-6 and hepcidin levels more than a HCHO diet (15), however the longer term physiological implications of such a strategy are unknown. Therefore, periodically or chronically restricting CHO availability over several weeks, particularly during blocks of intensified training stress, may have undesirable implications for an athlete's iron status. This is an important consideration, since adequate iron stores are required for optimal adaptation to training, and low iron stores may contribute to poor health outcomes and impair athletic performance (16). Therefore, the aim of this study was to examine the inflammatory and hepcidin response in elite athletes, following three different approaches to CHO availability during a 3-week block of intensified training.

Methods

Participants

Internationally competitive race walking athletes participated in this study (Table 1). All athletes met the International Association of Athletics Federations (IAAF) walk standard for a World Championship or Olympics, in either the 20 or 50 km event. This investigation was the culmination of three separate training camps that occurred in November 2015 (n=11 athletes), January 2016 (n=19) and January 2017 (n=28). Of note, 11 athletes participated in two of the three camps, and three athletes completed all three. During this study, four athletes were unable to complete the required training due to injury or illness, and another four athletes had unusually high serum ferritin levels (>300 μg/L) and were excluded from the analysis. Therefore, a total of 50 athlete data sets, representing 37 athletes (31 male and 6 female), from three separate but identically conducted training camps were available for analysis. The studies providing these data sets were approved by the Ethics Committee of the Australian Institute of Sport (AIS)

(approval: 20150802, 20161201). Written informed consent was obtained from all athletes prior to study commencement.

Experimental Overview

The study protocols involved in these training camps have been described previously (7) and outcomes from three separate training camps have been combined to facilitate more comprehensive analysis. During this investigation, male and female athletes completed 3 weeks of intensified training and testing while adhering to one of three dietary interventions: (i) a HCHO diet (n=16); (ii) periodized CHO availability (PCHO) (n=17) or (iii) a LCHF diet (n=17). Athletes were allocated into their dietary group by matching key characteristics (e.g., sex, age, training status and personal best times) and accounting for dietary preference/non-acceptance of a diet. All camps were conducted at the AIS with athletes living on-site, undertaking all meals and performing all training sessions under rigorous supervision. This process ensured a high level of dietary compliance throughout the study. As part of each training camp, athletes completed a baseline (Baseline) and post-intervention (Adapt) testing session to evaluate the impact of each diet on the iron-regulatory response to exercise (See Figure 1).

Following the Baseline testing session, athletes commenced their allocated dietary intervention and prescribed training programs for 3 weeks. All diets were designed by accredited sports dietitians and prepared by professional chefs, and matched for total energy intake and an energy availability of ~40 kcal·kg fat free mass·day^{-1}. The HCHO intervention consisted of a daily intake of 8.0 g·kg^{-1} (females) and 8.5 g·kg^{-1} (males) body mass of CHO, with all training sessions performed with high CHO availability (17). The PCHO intervention consisted of the same daily

CHO intake as HCHO diet, with differences attributed to the timing of CHO intake. Here, CHO was strategically periodized around key training sessions, achieved by a combination of overnight fasting and delayed post-session refueling (4, 6). Finally, the LCHF intervention emulated a ketogenic diet, comprising 80% fat and restricted CHO intake to <50 $g \cdot day^{-1}$ (8, 18). Full details of each dietary intervention are presented in Table 2. The methodology used to achieve strict dietary control have been detailed previously (19).

While adhering to their allocated dietary interventions, all athletes completed the same 3 week block of periodized training that included race walking, resistance training and cross-training (running, swimming or cycling). Individual training load was prescribed throughout the study to accommodate training history and event preparation. Six key race walking training sessions each week were compulsory and monitored by the research team. All remaining sessions were self-recorded by athletes in a computerized daily training log.

During this investigation, athletes completed a standardized exercise session, involving a hybrid laboratory and field race walking protocol at Baseline and Adapt. For these sessions, athletes arrived to the laboratory at a standardized time, in an overnight fasted and rested state. While the subject was resting quietly in a supine position, an indwelling cannula was inserted into an antecubital vein and a venous blood sample collected (fasted). Cannulas were flushed with a 3 ml saline solution every ~30 min throughout the trial to keep the vein patent. Athletes then received a standardized breakfast providing 2 $g \cdot kg^{-1}$ body mass CHO. The exception here occurred during the Adapt test of the LCHF group, who consumed a high fat isocaloric meal in accordance with

their dietary guidelines. Athletes then provided a pre-exercise venous blood sample, which occurred either 105 min (camp 3) or 120 min (camp 1 and 2) post-breakfast.

At 2 h post-meal, athletes commenced a combined laboratory and field race walking protocol, which involved an evenly paced 19 and 25 km race walking session for female and male athletes, respectively. During this effort, lasting approximately 2 h, kilometers 0-1, 12-13 and 24-25 (camp 1 and 2) or kilometers 0-1, 6-7, 12-13, 18-19 and 24-25 (camp 3) were completed on a motorized treadmill. Athletes walked at their target speed (10-11 $km \cdot h^{-1}$ for females, 12-13 $km \cdot h^{-1}$ for males), as established previously during a graded exercise test (7). This speed corresponded approximately to the individual's 50 km race walk pace and an exercise intensity of ~75% VO_{2max}. The remaining distance was completed outdoors on a flat 5 km circuit with athletes unimpeded by vehicle or pedestrian traffic. Immediately post-exercise, a third venous blood sample was drawn. Given the long protocol duration, a standardized recovery drink, in accordance with the athlete's dietary allocation (0 or 1.5 $g \cdot kg^{-1}$ body mass CHO), was provided at 30 min post-exercise to avoid hunger. Athletes abstained from any further food intake until a final blood sample was collected 3 h post-exercise.

For trials completed with high CHO availability, athletes were provided with a CHO solution prior to, and at regular intervals throughout the exercise protocol. During exercise, this regimen resulted in a total CHO intake of ~60 $g \cdot h^{-1}$ (camp 1 and 2) or 70 $g \cdot h^{-1}$ (camp 3). The exception here occurred during the Adapt test, when the LCHF group had their CHO drinks substituted with a non-caloric electrolyte drink and consumed high-fat snacks to match the total energy

consumed during the CHO-fuelled tests. Unrestricted water was provided to all athletes on-course, with intakes recorded during the first trial and replicated during the Adapt trial.

Blood Analysis

For each participant, four blood samples per testing session were collected into 4 ml serum separator tubes (BD Vacutainer, Australia). Subsequently, samples were left to clot for 30 min before centrifugation at 2200 G for 10 min. Serum was divided into 1 ml cyrotubes and stored at -80^{o}C until batch analysis could be conducted. When athletes were fasted, an additional 2 ml K_3EDTA tube was collected and whole blood was immediately analyzed for hemoglobin and hematocrit by fluorescent flow cytometry on a XN-L 550 analyzer (Sysmex Corporation, Kobe, Japan). Serum iron and serum ferritin analysis were made on the fasted serum sample via a COBAS Integra 400 automated biochemistry analyzer (Roche Diagnostics, Switzerland). Concentrations of IL-6 were analyzed in duplicate on serum samples collected immediately pre- and post-exercise using a commercially available ELISA (Quantikine HS, R&D Systems, Minneapolis, USA) on a BMG Labtech FLUOstar OPTIMA plate reader. The coefficient of variation (CV) for IL-6 determination was 5.9%.

Hepcidin-25 measurements were performed on serum obtained when athletes were fasted and at 3 h post-exercise (www.hepcidinanalysis.com, Nijmegen, The Netherlands) using a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS) (20, 21). An internal standard (synthetic hepcidin-24; custom-made Peptide International Inc.) was used for quantification (20, 21). Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionization TOF MS platform (Bruker Daltonics). Serum hepcidin-25

concentrations were expressed as nmol/L (nM). The lower limit of detection of this method is 0.5 nM. When values were below this limit, the lower limit of detection divided by the square root of 2 was used (22). The median reference level of serum hepcidin-25 (Dutch population) is 4.5 nM for men, 2.0 nM for pre-menopausal women, and 4.9 nM for post-menopausal women (www.hepcidinanalysis.com). The reference levels for the WCX-TOF MS method are derived from those obtained by an validated ELISA method (23), based on the regression line between the ELISA and WCX-TOF MS results obtained from patients without hepcidin isoforms (20).

Dietary Iron Analysis

To estimate dietary iron intake, week one of each dietary intervention (which was constructed around a 7-day menu), for a 64 kg male, was analyzed using dietary analysis software (Food Works 8 Professional program; Xyris Software Australia Pty Ltd, Australia). Foods were categorized into heme (red meat, poultry and fish) and non-heme iron sources (breads/cereals/grains, eggs, vegetables, fruits, nuts/seeds and dairy) to determine the contribution of different foods to overall iron intake. Comprehensive breakdown of energy and macronutrients consumed as a part of each diet have been presented in Table 2 and conformed closely to the study goals (19).

Statistical Analysis

As no substantial differences were evident between HCHO and PCHO for any haematological variable and the same total CHO content was consumed daily, athletes adhering to these two diets were pooled into one group (CHO) for analysis. Differences in dietary iron intake between groups were analyzed using t-tests. Blood variables were analyzed with a General Linear Mixed

Model using the R package lme4 (24) to accommodate the unbalanced design and the repeated measurements (25). A random intercept for both athlete and camp was included to adjust for baseline levels and inter-individual homogeneity, as well as different conditions between camps. All models were estimated using Restricted Maximum Likelihood. Visual inspections of residual plots did not reveal any obvious deviations from homoscedasticity or normality. P-values were obtained using Type II Wald F tests with Kenward-Roger degrees of freedom as implemented in the R package car (26). Results are reported as mean estimates with a 95% confidence interval (CI). Initial models included all possible interactions but non-significant interactions were dropped from the model for ease of interpretation. In the case of hepcidin-25, serum ferritin was included as a covariate in the model to account for the known influence of serum ferritin on the 3 h post-exercise hepcidin response (27). In addition, effect sizes were calculated based on classical Cohen's *d* while accounting for study design by using the square root of the sum of all variance components in the denominator (28). The magnitude of change is presented as an x-fold change. To construct a 95% confidence interval around the fold change, a bootstrapped confidence interval based on 10,000 replications was calculated using the R package boot (29).

Results

All athletes adhered to their allocated diets and the desired differences in dietary fat and CHO consumption were achieved (7, 19). Dietary iron intakes are summarized in Table 3. Substantial differences in iron intake between diets were evident (t=-1.92, p=0.05), with the CHO-rich diets providing a greater daily iron intake (17.8 ± 4.5 $mg \cdot day^{-1}$) as compared to LCHF (13.7 ± 2.1 $mg \cdot day^{-1}$). Heme iron sources, including red meat, poultry and fish contributed a similar absolute proportion of iron to all diets (CHO 1.7 ± 0.6 $mg \cdot day^{-1}$, LCHF 1.8 ± 1.2 $mg \cdot day^{-1}$; t=0.80,

p=0.90), however, non-heme iron provision differed substantially between diets (CHO 16.1 ± 4.4 $mg \cdot day^{-1}$, LCHF 11.9 ± 1.6 $mg \cdot day^{-1}$; t=-2.26, p=0.03). The CHO groups consumed a large proportion of iron from foods such as breads, cereals and grains (49%) compared to the LCHF group (7%), whose major iron source was from nuts and seeds (LCHF 26%; CHO 1%).

Substantial differences in iron stores were evident after 3 weeks of training and dietary intervention (see Table 4). The decrease in resting serum ferritin from Baseline to Adapt was significantly different between dietary treatments (F(1,59)=5.63, p=0.021), with a small and moderate 23% and 37% decrease evident after 3 weeks of dietary manipulation in the LCHF and CHO groups, respectively. Reductions to resting serum iron were also present in the Adapt trial in both groups (F(1,60)=14.78, p<0.001), however, the magnitude was similar between dietary interventions (F(1,60)=0.03, p=0.854), with small decreases evident in both the CHO (16%) and LCHF (20%) groups. The increase in hemoglobin (F(1,60)=16.1, p<0.001) and hematocrit (F(1,59)=13.1, p<0.001) was greater in LCHF during the Adapt trial.

A substantial increase in IL-6 occurred across the ~2 h exercise bout during both Baseline and Adapt trials (F(1,150)=343.14, p<0.001), with an attenuated post-exercise IL-6 response observed after 3 weeks of training and dietary intervention in both groups compared to Baseline (F(1,150)=4.36, p=0.038) (see Figure 2A). Furthermore, significant differences were evident between dietary interventions (F(1,75)=4.75, p=0.033), with a greater post-exercise IL-6 response in LCHF during the Adapt trial (13.6-fold increase; 95% CI: 7.1-21.4), when compared to CHO (7.6-fold increase; 5.5-10.2).

Hepcidin-25 consistently increased from pre- to 3 h post-exercise during both Baseline and Adapt trials ($F(1,157)=178.18$, $p<0.001$) (see Figure 2B), with the increase greater at Baseline than after 3 weeks of training and dietary intervention in both groups ($F(1,157)=11.34$, $p<0.001$). While no significant differences between dietary interventions were evident ($F(1,157)=2.55$, $p=0.112$), confidence intervals indicate 3 h post-exercise hepcidin-25 concentrations were lower in the Adapt trial (4.8-fold increase; 2.4-8.5) compared to Baseline (7.1-fold increase; 4.2-11.5) in CHO (β=4.3, -6.6 -2.0). In contrast, only trivial differences in the 3 h post-exercise hepcidin response were evident between Baseline (4.3-fold increase; 2.0-8.3) and Adapt trials (6.3-fold increase; 3.5-10.4) for LCHF. Finally, serum ferritin was a significant variable in the hepcidin model ($F(1,54)=57.46$, $p<0.001$), demonstrating that higher pre-exercise values were consistently associated with a larger hepcidin response 3 h post-exercise.

Discussion

This study is the first to examine issues of iron metabolism in athletes adhering to a LCHF diet. The iron content of a ketogenic LCHF diet was lower than that of CHO-rich menus, resulting from the removal of grains and cereals (including fortified manufactured foods) that typically contribute a substantial source of non-heme iron to Western diets. Furthermore, 3 weeks of adaptation to a ketogenic LCHF diet by elite race walkers resulted in a greater post-exercise IL-6 and hepcidin response compared to athletes consuming a CHO-rich diet. However despite favorable changes to iron-regulatory markers and an increased dietary iron intake, the greatest decrement to serum ferritin occurred in athletes consuming a CHO-rich diet.

In the current study, elite race walkers had a substantially greater IL-6 response to a standardized 2 h bout of exercise after 3 weeks of adherence to a LCHF diet than a similar group of athletes who consumed a CHO-rich diet. It has been well documented that low muscle glycogen status is associated with increased IL-6 production from contracting skeletal muscle (9, 30). In these situations, IL-6 up-regulates hepatic glucose production in a homeostatic-like manner to maintain blood glucose concentrations throughout prolonged exercise (9). While muscle glycogen stores were not directly measured in the current study, we assume that after 3 weeks of strict CHO restriction, glycogen stores were likely at least partially depleted. Therefore, we suggest that the likely greater decrement to muscle glycogen stores resulting from adherence to a LCHF diet was the signal for an increased post-exercise IL-6 response, in an attempt to sustain blood glucose concentrations, thus providing a fuel source to sustain exercise intensity.

The role of IL-6 however, extends beyond that of hepatic glucose regulation, also being a key regulator of hepcidin activity (10), particularly during the acute post-exercise recovery period (16). Given the greater post-exercise increase in IL-6 levels evident after adaptation to the LCHF diet, it seems logical that 3 weeks of strict CHO restriction might also amplify the hepcidin-25 response to exercise. However the influence of dietary manipulation on hepcidin activity was less conclusive, with a modest attenuation of hepcidin-25 levels after 3 h of recovery from a standardized exercise session in the group who had consumed a CHO-rich diet, while no clear change was evident in the LCHF group between Baseline and Adapt. Alterations to hepcidin activity are physiologically important, since increases in this hormone leads to the internalization of ferroportin, inhibiting iron absorption from the gut and recycling from macrophages (12). The attenuated IL-6 response from consumption of a CHO-rich diet may have positively influenced

hepcidin activity, resulting in favorable alterations in iron regulation during the post-exercise period.

Factors other than inflammation are also known to influence hepcidin concentrations (31), with an athlete's resting serum ferritin status a major determinant of the 3 h post-exercise hepcidin response (27). Athletes who are iron-depleted (serum ferritin <30 μg/L) exhibit a suppressed post-exercise hepcidin response compared to athletes with either sub-optimal (serum ferritin 30-50 μg/L) or healthy (serum ferritin >50 μg/L) iron stores (27). Such a response is interpreted to be a homeostatic outcome to encourage iron absorption and transport when stores are low overriding the inflammatory stimulus for an increase in hepcidin post-exercise, thereby encouraging the absorption of iron from the gut. In the current study, the LCHF group had substantially greater resting serum ferritin levels during the Adapt trial than the CHO group, which in part, may explain the greater hepcidin response evident in this group. Furthermore, this pattern of response is supported by data showing that resting serum ferritin was a significant variable in our hepcidin-25 modeling approach, with higher serum ferritin values associated with a greater hepcidin-25 response. However, it should be noted that athletes in a previous study (27) with a range of serum ferritin values similar to the present cohort (30-100 μg/L) produced a similar post-exercise hepcidin response. Therefore, it is likely that the differences in hepcidin expression evident here occurred as a function of both an altered post-exercise IL-6 response induced by dietary manipulation, and via disparities in serum ferritin stores between groups after 3 weeks of dietary intervention.

Another consideration is the type and total iron content present in the dietary treatments investigated. Absolute heme iron intake was similar between all diets, given the deliberate attempt to match protein intake across dietary conditions. It is well-known that the heme form of dietary iron shows superior bioavailability, with absorption rates of approximately 5-35% from a single meal compared with 2-20% from non-heme sources (32). Differences in total iron intake between groups came from a greater proportion of non-heme iron sources in the CHO group. These foods included breakfast cereals fortified with iron, breads, and whole grains, all of which are CHO-rich foods eliminated from the LCHF diet. While nuts and seeds contributed non-heme iron to the LCHF diet, quantities were not substantial enough to match intakes from the CHO-rich diets. Finally, all diets provided iron intakes that were well above the recommended daily intake (RDI) of 8 $mg \cdot day^{-1}$ for males (33). However given the large decrements in iron stores across the 3 week training period in all dietary treatments, the adequacy of the iron RDI for elite athletes during periods of intensified training is an area for further exploration by clinicians and dietitians.

Adequate iron stores are crucial in allowing athletes to adapt appropriately to training stimuli, which ultimately, may influence athletic performance (34). The large reduction to serum ferritin concentrations is not an uncommon finding following prolonged periods of strenuous (35) or specialized (e.g. altitude exposure) training (36). This effect may be attributed to incorporation of iron into new tissues and cells induced by adaptation to training, as well as exercise-associated avenues of iron loss, including hemolysis, hematuria, gastrointestinal bleeding and the iron content of sweat (16). The decrease in serum ferritin across the intervention period was substantially greater in the CHO group (37%) than the LCHF group (23%). This outcome is

difficult to explain, as with the greater iron content of the CHO-rich diets and the more favorable inflammatory and hepcidin responses to exercise, we expected athletes would have maintained better iron status over the training camp in comparison to the LCHF group.

There are several possible explanations for the divergent findings in iron status. First, we postulate that a reduction in serum ferritin represents a positive change in functional iron status (i.e. a movement of stores towards new tissue synthesis or iron-related processes) rather than a simple drain of iron stores. The CHO group may have experienced greater adaptation to training, and hence required a greater draw on their available iron stores to support adaptive processes, including increased erythropoiesis, DNA synthesis and iron-dependent oxidative enzyme production (37). Although this is a self-limiting scenario (if there are insufficient iron stores to contribute to the increased stimulus for iron-requiring activities), there are other situations in which a successful response to an increase in training stress involves the net use, rather than preservation of iron stores. For example, a positive outcome of altitude training (increase in hemoglobin mass) is associated with a reduction in ferritin concentrations (38). Thus the greater reduction to serum ferritin in athletes adhering to CHO-rich diets in the current study may represent an adaptive response to the training performed. Indeed, we noted that the performance trials associated with this study showed a substantially greater improvement in 10 km race performance with CHO-supported training (HCHO: +6.6%, PCHO: +5.4%) than the LCHF group (-1.6%) (7). Despite this suggestion, an increase to hemoglobin and hematocrit was evident in LCHF during Adapt. Such an outcome does not appear to support the prospect of greater adaptation in CHO, however, the biological variation of these markers should be considered. Within- and between-subject coefficient of variation for both of these markers is

estimated to be ~2.8% and ~6.5%, respectively (39), which is attributed to the measurement being highly dependent on plasma volume changes (40), and therefore not appropriate for determining training adaptation. As such, the small changes to hemoglobin and hematocrit seen here are unlikely to be clinically meaningful, and for both markers, fall within the biological variation of the tests. Therefore, without directly quantifying changes in adaptation via an assessment of hemoglobin mass (as opposed to concentration), these conclusions remain speculative, and further research is required.

Secondly, it is known that ferritin is an acute phase protein, elevated in response to inflammation as part of a physiological cascade to restore homeostasis (13). As such, during periods of inflammation serum ferritin may not accurately represent iron status (14). Given a schedule involving twice daily sessions and prolonged training sessions (30-40 km) at least weekly, it is possible that the resting serum ferritin concentrations in the LCHF group were artificially inflated during the Adapt trial as a result of low-level chronic inflammation. Specific testing of this theory is required to support the disassociation of serum ferritin concentrations from iron status in this scenario, and future studies should incorporate a more comprehensive panel of inflammatory markers, including C-reactive protein, IL-10, IL-1ra and tumor necrosis factor-α.

Conclusions

In summary, alterations to the iron regulatory response to exercise were evident after 3 weeks of adherence to a LCHF diet. While elite athletes who chronically trained and acutely exercised with high CHO availability experienced an attenuated post-exercise IL-6 response, and a (relative to LCHF) attenuation of the 3 h post-exercise hepcidin expression, those who were

adapted to a LCHF diet and exercised with low CHO availability did not present with the same outcomes. Furthermore, the LCHF diet was associated with a lower total intake of dietary iron given the removal of CHO-rich foods that typically provide a substantial contribution of non-heme iron in the Western Diet. Lower serum ferritin after 3 weeks of training with high CHO intake might reflect a larger, more adaptive hematological response to training, however further investigation is required before a full understanding of the effect of a ketogenic diet on iron status and iron metabolism can be achieved. Athletes adhering to a LCHF diet should routinely monitor their iron status in combination with markers of inflammation, to determine changes to iron availability. In athletes with an increased risk of iron deficiency, including those with sub-optimal ferritin stores, endurance competitors, females and vegetarians, iron supplementation may be appropriate to offset the low dietary iron and maintain adequate iron stores.

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References

1. Hawley JA, Schabort EJ, Noakes TD, Dennis SC. Carbohydrate-loading and exercise performance. *Sports Med*. 1997;24(2):73-81.

2. Stellingwerff T, Cox GR. Systematic review: carbohydrate supplementation on exercise performance or capacity of varying durations. *Appl Physiol Nutr Metab*. 2014;39(9):998-1011.

3. Coyle EF. Timing and method of increased carbohydrate intake to cope with heavy training, competition and recovery. *J Sports Sci*. 1991;9(1):29-52.

4. Bartlett JD, Hawley JA, Morton JP. Carbohydrate availability and exercise training adaptation: too much of a good thing? *Eur J Sport Sci*. 2015;15(1):3-12.

5. Stellingwerff T. Contemporary nutrition approaches to optimize elite marathon performance. *Int J Sports Physiol Perform*. 2013;8(5):573-8.

6. Marquet L-A, Brisswalter J, Louis J et al. Enhanced endurance performance by periodization of CHO intake: "sleep low" strategy. *Med Sci Sports Exerc*. 2016;48(4):663-72.

7. Burke LM, Ross ML, Garvican-Lewis LA et al. Low carbohydrate, high fat diet impairs exercise economy and negates the performance benefit from intensified training in elite race walkers. *J Physiol*. 2017;595(9):2785-807.

8. Volek JS, Noakes T, Phinney SD. Rethinking fat as a fuel for endurance exercise. *Eur J Sport Sci*. 2015;15(1):13-20.

9. Steensberg A, Febbraio MA, Osada T et al. Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. *J Physiol*. 2001;537(2):633-9.

10. Nemeth E, Rivera S, Gabayan V et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest*. 2004;113(9):1271-6.

11. Peeling P, Dawson B, Goodman C et al. Effects of exercise on hepcidin response and iron metabolism during recovery. *Int J Sport Nutr Exerc Metab*. 2009;19(6):583-97.

12. Nemeth E, Tuttle MS, Powelson J et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science*. 2004;306(5704):2090-3.

13. Streetz K, Wüstefeld T, Klein C, Manns M, Trautwein C. Mediators of inflammation and acute phase response in the liver. *Cell Mol Biol (Noisy-le-Grand)*. 2001;47(4):661-73.

14. Dignass A, Farrag K, Stein J. Limitations of serum ferritin in diagnosing iron deficiency in inflammatory conditions. *Int J Chron Dis*. 2018; Article ID 9394060, https://doi.org/10.1155/2018/9394060.

15. Badenhorst CE, Dawson B, Cox GR, Laarakkers CM, Swinkels DW, Peeling P. Acute dietary carbohydrate manipulation and the subsequent inflammatory and hepcidin responses to exercise. *Eur J Appl Physiol*. 2015;115(12):2521-30.

16. Peeling P, Dawson B, Goodman C, Landers G, Trinder D. Athletic induced iron deficiency: new insights into the role of inflammation, cytokines and hormones. *Eur J Appl Physiol*. 2008;103(4):381-91.

17. Coyle EF, Coggan AR, Hemmert M, Ivy JL. Muscle glycogen utilization during prolonged strenuous exercise when fed carbohydrate. *J Appl Physiol.* 1986;61(1):165-72.

18. Phinney SD, Bistrian BR, Evans W, Gervino E, Blackburn G. The human metabolic response to chronic ketosis without caloric restriction: preservation of submaximal exercise capability with reduced carbohydrate oxidation. *Metabolism*. 1983;32(8):769-76.

19. Mirtschin JG, Forbes SF, Cato LE et al. Organization of dietary control for nutrition-training intervention involving periodized carbohydrate availability and ketogenic low CHO high fat diet. *Int J Sport Nutr Exerc Metab*. 2018; 28(5):480-9.

20. Kroot JJ, Laarakkers CM, Geurts-Moespot AJ et al. Immunochemical and mass-spectrometry–based serum hepcidin assays for iron metabolism disorders. *Clin Chem* 2010;56(10):1570-9.

21. Swinkels DW, Girelli D, Laarakkers C et al. Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. *PLoS One*. 2008;3(7):e2706.

22. Croghan C, Egeghy P. Methods of dealing with values below the limit of detection using SAS. In: *Southern SAS User Group*; 2003: 22-24.

23. Galesloot TE, Vermeulen SH, Geurts-Moespot AJ et al. Serum hepcidin: reference ranges and biochemical correlates in the general population. *Blood*. 2011;117(25):e218-e25.

24. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: 2017. Available from: http://www.R-project.org.

25. Jennrich RI, Schluchter MD. Unbalanced repeated-measures models with structured covariance matrices. *Biometrics*. 1986;42(4):805-820.

26. Fox J, Wrisberg S. *An R Companion to Applied Regression*, 2nd ed. Sage Publications: Thousand Oaks; 2011.

27. Peeling P, Sim M, Badenhorst CE et al. Iron status and the acute post-exercise hepcidin response in athletes. *PLoS One.* 2014;9(3):e93002.

28. Cohen J. Stati*stical Power Analysis for the Behavioural Sciences.* L. Erlbaum Associates; 1988.

29. Canty A, Ripley B. Package 'boot' [Internet]. 2017. Avaiable from: http://cran.rproject.org/web/packages/ boot/index.html.

30. Hennigar SR, McClung JP, Pasiakos SM. Nutritional interventions and the IL-6 response to exercise. *FASEB J.* 2017;31(9):3719-28.

31. Peeling P, McKay AK, Pyne DB et al. Factors influencing the post-exercise hepcidin-25 response in elite athletes. *Eur J Appl Physiol.* 2017;117(6):1233-9.

32. Beard J, Tobin B. Iron status and exercise. *Am J Clin Nutr*. 2000;72(2):594s-7s.

33. Trumbo P, Yates AA, Schlicker S, Poos M. Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *J Am Diet Assoc*. 2001;101(3):294-301.

34. Zhu YI, Haas JD. Altered metabolic response of iron-depleted nonanemic women during a 15-km time trial. *J Appl Physiol.* 1998;84(5):1768-75.

35. Auersperger I, Škof B, Leskošek B, Knap B, Jerin A, Lainscak M. Exercise-induced changes in iron status and hepcidin response in female runners. *PloS One*. 2013;8(3):e58090.

36. Govus AD, Garvican-Lewis LA, Abbiss CR, Peeling P, Gore CJ. Pre-altitude serum ferritin levels and daily oral iron supplement dose mediate iron parameter and hemoglobin mass responses to altitude exposure. *PLoS One*. 2015;10(8):e0135120.

37. Beard JL. Iron biology in immune function, muscle metabolism and neuronal functioning. *J Nutr*. 2001;131(2):568S-80S.

38. Wehrlin JP, Zuest P, Hallén J, Marti B. Live high-train low for 24 days increases hemoglobin mass and red cell volume in elite endurance athletes. *J App Physiol*. 2006;100(6):1938-1945.

39. Coşkun A, Carobene A, Kilercik M et al. Within-subject and between-subject biological variation estimates of 21 hematological parameters in 30 healthy subjects. *Clin* Chem Lab Med. 2018;56(8):1309-18.

40. Eichner ER. Sports anemia, iron supplements, and blood doping. *Med Sci Sports Exerc*. 1992;24(9 Suppl):S315-8.

Figure 1. Schematic diagram of the study design, detailing dietary interventions and testing protocols followed.

Figure 2. (A) Interleukin-6 (IL-6) concentrations pre- and post-exercise and (B) hepcidin-25 concentrations pre- and 3 h post-exercise in the carbohydrate (CHO) and low carbohydrate high fat (LCHF) groups at Baseline and Adapt. Data presented as raw means ± standard deviation. Hepcidin-25 analysis was performed on fasted samples and IL-6 analysis performed on fed samples. * Indicates a significant increase from pre-exercise. # Indicates a significant difference between diets.

FIGURE LEGENDS

Figure 1

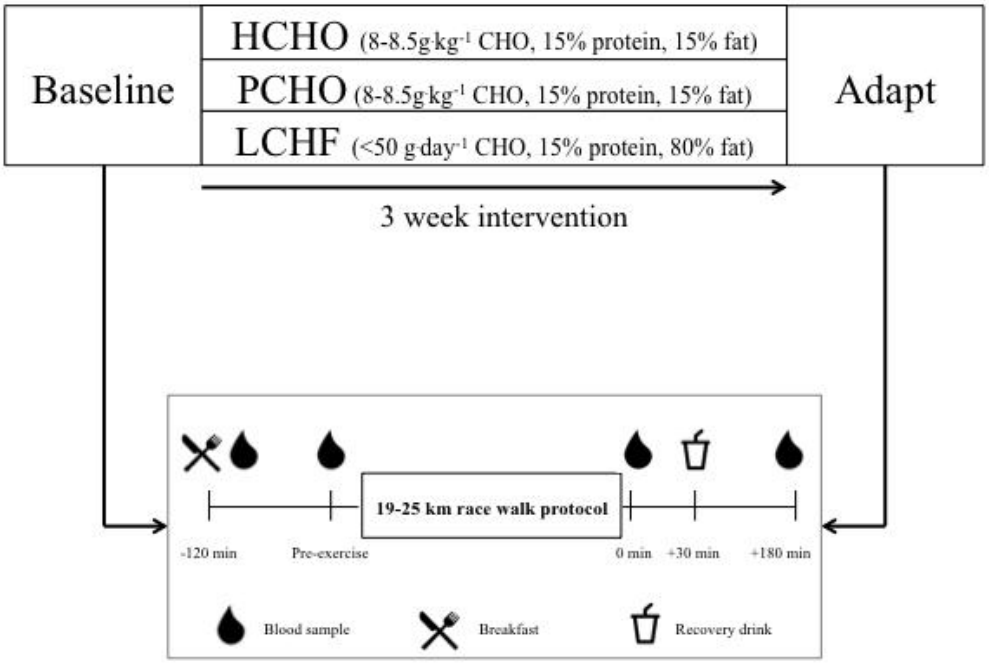

Figure 2

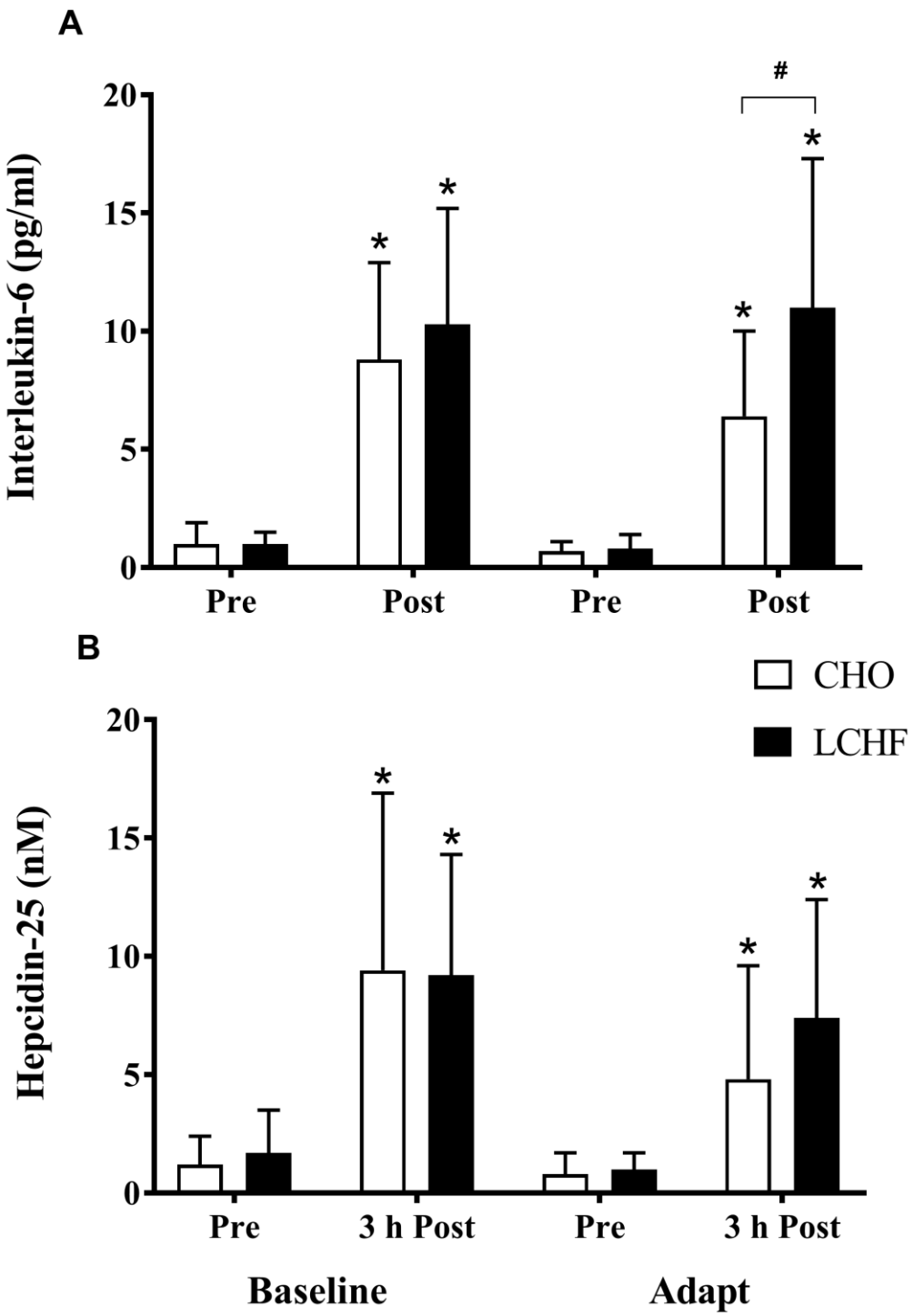

Table 1. Characteristics of athletes adhering to the high carbohydrate diet and periodized carbohydrate diet (combined and presented as CHO) and the low carbohydrate high fat diet (LCHF). Data presented as mean ± standard deviation.

	CHO		**LCHF**	
	Males (n=28)	*Females* (n=5)	*Males* (n=16)	*Female* (n=1)
Age (y)	26.1 ± 3.8	27.2 ± 3.4	28.6 ± 3.6	30.7
Body mass (kg)	66.6 ± 7.4	56.4 ± 3.9	67.7 ± 6.7	51.5
VO_{2max} ($ml \cdot kg \cdot min^{-1}$)	61.8 ± 5.8	56.5 ± 4.1	65.1 ± 4.9	53.6
10 km personal best (min:sec)	40:59 ± 1:14	44:52 ± 0:21	40:40 ± 1:10	42:43
20 km personal best (min:sec)	83:37 ± 3:31	92:08 ± 1:35	82:52 ± 2:32	90:20

Table 2. Mean daily intake of athletes adhering to the high carbohydrate diet and periodized carbohydrate diet (combined and presented as CHO) and a low carbohydrate high fat diet (LCHF). Data presented as mean ± standard deviation.

	CHO	**LCHF**
Carbohydrate (g)	541 ± 69	35 ± 5
Fat (g)	77 ± 12	317 ± 46
Protein (g)	138 ± 21	142 ± 20
Total energy intake (kJ)	14,579 ± 1,987	15,101 ± 2,164

Table 3. Mean daily dietary iron intake from week 1 of the high carbohydrate diet and periodized carbohydrate diet (combined and presented as CHO) and low carbohydrate high fat diet (LCHF) for a 64 kg male. Data presented as mean ± SD. * indicates a significant difference between CHO and LCHF.

	Mean Daily Iron Intake ($mg \cdot day^{-1}$)	
	CHO	**LCHF**
Total	17.8 ± 4.5	13.7 ± 2.1*
Breakfast	4.4 ± 2.2	3.0 ± 2.1
Lunch	4.3 ± 1.9	2.6 ± 1.4
Dinner	5.4 ± 2.2	5.2 ± 1.0
Snacks	3.7 ± 1.5	2.9 ± 2.0
Red Meat	1.2 ± 0.9	1.4 ± 1.5
Poultry	0.3 ± 0.3	0.3 ± 0.3
Fish	0.2 ± 0.6	0.2 ± 0.5
Breads/Cereals/ Grains	8.8 ± 5.3	0.9 ± 1.0 *
Eggs	1.0 ± 1.0	1.5 ± 1.5
Nuts/Seeds	0.2 ± 0.4	3.6 ± 2.4 *
Vegetables	2.0 ± 0.9	2.6 ± 1.0
Fruits	2.2 ± 1.1	0.2 ± 0.3 *
Dairy	0.3 ± 0.2	0.9 ± 0.7
Other	1.6 ± 1.7	2.2 ± 1.3

Table 4. Hematology data at Baseline and Adapt for the carbohydrate (CHO) and low carbohydrate high fat (LCHF) groups. Data presented as estimated marginal mean ± standard error [95% confidence interval]. * Indicates a significant difference from baseline ($p<0.05$). † Indicates a significant difference to CHO ($p<0.05$).

		CHO		LCHF	
		Baseline	Adapt	Baseline	Adapt
Serum Iron (umol/L)	Pre	22.7 ± 2.5 [17.8-27.6]	19.1 ± 2.5* [14.2-24.0]	21.2 ± 2.7 [15.9-26.5]	17.3 ± 2.7* [12.0-22.5]
Serum Ferritin (ug/L)	Pre	90.6 ± 7.9 [74.8-106.3]	57.5 ± 7.9* [41.7-73.2]	85.6 ± 8.8 [68.1-103.0]	68.9 ± 8.8*† [51.4-86.4]
Hemoglobin (g/dL)	Pre	14.4 ± 0.2 [14.1-14.8]	14.1 ± 0.2 [13.7-14.4]	14.4 ± 0.2 [13.9-14.8]	14.8 ± 0.2*† [14.4-15.2]
Hematocrit (%)	Pre	41.1 ± 0.5 [40.2-42.0]	40.4 ± 0.5 [39.5-41.3]	40.6 ± 0.5 [39.5-41.7]	41.9 ± 0.5*† [40.8-43.0]
IL-6 (pg/ml)	Pre	1.0 ± 0.6 [-0.1-2.2]	0.7 ± 0.6 [-0.4-1.9]	0.8 ± 0.8 [-0.8-2.5]	0.6 ± 0.8 [-1.0-2.2]
	Post	8.8 ± 0.6 [7.6-9.9]	6.3 ± 0.6 [5.2-7.5]	10.2 ± 0.8 [8.6-11.8]	10.8 ± 0.8† [9.2-12.5]
Hepcidin-25 (nM)	Pre	0.4 ± 0.6 [-0.9-1.7]	1.8 ± 0.6 [0.6-3.1]	1.1 ± 0.9 [-0.6-2.9]	1.3 ± 0.9 [-0.4-3.1]
	3 h Post	8.7 ± 0.6 [7.4-9.9]	5.8 ± 0.4 [4.5-7.1]	8.6 ± 0.9 [6.9-10.3]	7.7 ± 0.9 [6.0-9.4]

All sample analysis was performed on fasted samples, except IL-6 analysis that was performed on fed samples.