The Impact of Nutrition and Training Practices on Iron and Bone Status in Athletes

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The Impact of Nutrition and Training Practices on Iron and Bone Status in Athletes

Submitted by

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Declaration of Authorship and Sources

This thesis contains no material that has been extracted in whole or in part from a thesis that I have submitted towards the award of any other degree or diploma in any other tertiary institution.

No other person's work has been used without due acknowledgment in the main text of the thesis.

All research procedures reported in the thesis received the approval of the relevant Ethics/Safety Committees (where required).

Nikita Catherine Fensham **11 August 2023**

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List of Publications Related to Thesis

1. **Fensham N.C.**, McKay A.K.A, Burke L.M. (2023). Bone turnover markers and bone mineral density values in a convenience sample of elite endurance athletes. In preparation for *Bone*.

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2. **Fensham N.C.**, Heikura I.A., McKay A.K.A, Tee N., Ackerman K.E., Burke L.M. (2022). Short-term carbohydrate restriction impairs bone formation at rest and during prolonged exercise to a greater degree than low energy availability. *J Bone Miner Res*. 37(10):1915-25. doi: 10.1002/jbmr.4658

3. **Fensham, N. C.**, Govus, A. D., Peeling, P., Burke, L. M., & McKay, A. K. A. (2023). Factors influencing the hepcidin response to exercise: an individual participant data meta-analysis. *Sports Med*. doi: 10.1007/s40279-023-01874-5 [online ahead of print]

4. **Fensham N.C.**, McKay A.K.A., Tee N., Lundy B., Anderson B., Morabito A., Ross M.L.R., Burke, L.M. (2022). Sequential submaximal training in elite male rowers does not result in amplified increases in interleukin-6 or hepcidin. *Int J Sport Nutr Exerc Metab*. 32(3):177-85. doi: 10.1123/ijsnem.2021-0263

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IL-6 (A) and hepcidin-25 (B) concentrations in the calcium and control groups across the two exercise sessions. Data are presented as estimated means with standard error. * significant difference to baseline (p<.05). ** significant difference to 3h post-EX1 (p<.05). ns: no significant difference between bracketed timepoints (p>.05). # significant main effect of trial (p<.05).

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Abstract

Athletes utilise various training and nutrition approaches to optimise training adaptation and competition performance. An important consideration is the potential impact of these practices on athlete health, and the subsequent effects on availability for training and competition and their longevity in the sport. Indeed, regular physical activity and mechanical loading is well-known to have significant benefits for bone health. However, excessive training load combined with insufficient recovery, including inadequate macro- and micronutrient support, places an athlete at increased risk for bone stress injuries. In particular, iron deficiency is also highly prevalent in athletes, especially in those with high training loads, and has been associated with an increased risk of low bone mineral density (BMD) in clinical populations. Considering the prevalence of and possible associations between bone stress injuries and iron deficiency, the studies comprising this thesis aimed to explore the impact of common training and nutrition practices on bone and iron metabolism in athletes.

Study 1 (described in Chapter 3): Bone mineral density and bone turnover marker values in a convenience sample of elite endurance athletes

Study 1 aimed to (1) examine BMD site discordance within and between sports, (2) investigate athlete bone turnover marker (BTM) ranges, and (3) explore possible links between bone and iron metabolism. A cross-sectional analysis of baseline data from athletes who participated in various studies within our research group over the period November 2015-January 2022 was conducted. Specifically, studies with baseline dual-energy x-ray absorptiometry (DXA) and where iron and bone markers were measured via venous blood sample collection prior to and following exercise were included. Significant discordance was shown between and within sports with lumbar spine BMD of rowers being higher than all other sports ($p < 0.0001$) and higher than the proximal femur site within rowers ($p = 0.002$). By contrast, racewalkers and runners had higher proximal femur than lumbar spine BMD ($p \leq 0.001$). Age significantly contributed to the variance in both fasting carboxyterminal telopeptide (CTX) and procollagen-1 N-terminal peptide (P1NP) with higher concentrations observed in those younger than 25 years old compared with older athletes ($p \leq 0.001$). In addition to age, assay method and resting interleukin-6 (IL-6) further explained fasting CTX concentrations (marginal $R^2 = 0.49$). Post-exercise IL-6, however, was not significant in explaining post-exercise CTX at 1 h ($p = 0.1$). Finally, significant medium-strong correlations were present between fasting CTX and resting ferritin and hepcidin ($p < 0.001$).

Study 2 (described in Chapter 4): Short-term carbohydrate restriction impairs bone formation at rest and across prolonged exercise to a greater degree than low energy availability

Study 2 aimed to investigate the differential impact of energy and carbohydrate manipulation on BTMs at rest and across exercise, recognising the role of nutrition in bone adaptation to frequent mechanical loading. In a parallel group design, 28 elite racewalkers completed two 6-day phases. In the Baseline phase, all athletes adhered to a high carbohydrate/high energy availability diet (CON). During the Adaptation phase, athletes were allocated to one of three dietary groups: CON, low carbohydrate/high fat with high energy availability (LCHF), or low energy availability (LEA). At the end of each phase, a 25 km racewalk was completed, with venous blood taken fasted, pre-exercise, and 0, 1, 3 h post-exercise to measure CTX, P1NP, and osteocalcin (carboxylated, gla-OC; undercarboxylated, glu-OC). Following Adaptation, LCHF showed decreased fasted P1NP (~26%; p<.0001, d=3.6), gla-OC (~22%; p=.01, d=1.8), and glu-OC (~41%; p=.004, d=2.1), which were all significantly different to CON (p<.01), whereas LEA demonstrated significant, but smaller, reductions in fasted P1NP (~14%; p=.02, d=1.7) and glu-OC (~24%; p=.049, d=1.4). Both LCHF (p=.008, d=1.9) and LEA (p=.01, d=1.7) had significantly higher CTX pre- to 3 h post-exercise but only LCHF showed lower P1NP concentrations (p<.0001, d=3.2). All markers remained unchanged from Baseline in CON.

Study 3 (described in Chapter 5): Factors influencing the hepcidin response to exercise: an individual participant data meta-analysis

Study 3 aimed to expand on the broader literature findings showing similar responses of bone and iron markers across exercise and in response to certain dietary interventions. Here, the aim was to amalgamate multiple small studies to investigate how both athlete and exercise session characteristics influence IL-6 and hepcidin concentrations through an individual participant data meta-analysis. Following a systematic review of the literature, a one-stage meta-analysis with mixed-effects linear regression, using a stepwise approach to select the best-fit model, was employed. Results demonstrated that exercise is associated with a 1.5- to 2.5-fold increase in hepcidin concentrations, with pre-exercise hepcidin concentration accounting for ~44% of the variance in 3 h post-exercise hepcidin concentration. Although collectively accounting for only a further ~3% of the variance, absolute 3 h post-exercise hepcidin concentrations appear higher in males with lower cardiorespiratory fitness and higher pre-exercise ferritin levels. On the other hand, a greater magnitude of change between the pre- and 3 h post-exercise hepcidin concentration was largely attributable to exercise duration (~44% variance) with a much smaller

contribution from VO_2max, pre-exercise ferritin, sex, and post-exercise IL-6 (~6% combined). Although females tended to have a lower absolute 3 h post-exercise hepcidin concentration (1.4 nmol.L^{-1}, [95% CI -2.6, -0.3], p=0.02) and 30% less change (95% CI [-54.4, -5.1], p=0.02) than males, with different explanatory variables being significant between sexes, sample size discrepancies and individual study design biases preclude definitive conclusions.

Study 4 (described in Chapter 6): Sequential submaximal training in elite male rowers does not result in amplified increases in interleukin-6 or hepcidin

Study 4 addressed the lack of investigation of the effects of repeated training bouts completed in close succession on the IL-6 and hepcidin responses to exercise. Although this study design was set up to investigate the effect of calcium supplementation on CTX concentrations across the same period, it provided a unique opportunity to explore iron-calcium reciprocity and determine whether an intervention (i.e., calcium prior to exercise) designed to limit the impact of exercise on bone may result in an adverse effect on another body system. In a randomised, crossover design, 16 elite male rowers completed two trials, a week apart, with either high (1000 mg) or low (<50 mg) calcium pre-exercise meals. Each trial involved two, submaximal 90 min rowing ergometer sessions, 2.5 h apart, with venous blood sampled at baseline, pre-exercise, and 0, 1, 2 and 3 h after each session. Peak elevations in IL-6 (~7.5-fold, p<.0001) and hepcidin (~3-fold, p<.0001) concentrations relative to baseline were seen at 2 and 3 h after the first session (EX1) respectively. Following the second session (EX2), concentrations of both IL-6 and hepcidin remained elevated above baseline, exhibiting a plateau rather than an additive increase (2 h post-EX1 vs 2 h post-EX2, p=1.00). Pre-exercise calcium resulted in a slightly greater elevation in hepcidin across all timepoints compared to control (p=.0005), however no effect on IL-6 was evident (p=.27).

Summary and future directions

The research studies included in this thesis aimed to explore the impact of certain nutrition and training practices on bone and iron status in athletes and the potential interplay between the two systems. In summary, our findings demonstrate that: (1) Fasting CTX and P1NP concentrations decrease with age and, although limited by the lack of general population data, may be higher in athletes, (2) Resting IL-6 concentrations may contribute to fasting CTX concentrations, (3) Duration of exercise contributes significantly to post-exercise IL-6 concentrations and the magnitude of change in hepcidin concentrations from pre- to 3 h post-exercise, (4) Twice-daily training with a short recovery results in an elevated plateau in IL-6 and hepcidin concentrations, when supported by adequate energy and CHO consumption, (5) Short-term adherence to a

ketogenic diet may be more detrimental to at-rest and across-exercise bone formation than the same period of LEA, yet bone turnover is maintained with a high EA/high CHO diet, (6) Significant BMD measurement site discrepancies exist between and within sports, raising the issue of the impact of mechanical loading on detecting at-risk athletes and the potential need for additional tools (e.g., BTMs) and protocols. Outcomes from this thesis support current recommendations that athletes should aim to achieve adequate energy and CHO availability to minimise potential detriments to bone turnover balance, at least in the short term. Furthermore, this work supports previous recommendations of morning, prior to or within 30 min following the first exercise session, iron consumption in order to maximise absorption. Invitations to expand the findings of this work extend to establishing a robust database of normative (and even sports-specific) values for athlete BTM and areal BMD ranges and employing longitudinal studies to assess the impact of BTM and iron status perturbations on BMD and bone architecture.

List of Abbreviations

$1,25[OH]_2D$	1,25-dihydroxyvitamin D
25[OH]D	25-hydroxy-vitamin D
ACSM	American College of Sports Medicine
AIC	Akaike Information Criterion
AR	Androgen receptor
AUC	Area under the curve
BIC	Bayesian Information Criterion
BM	Body mass
BMC	Bone mineral content
BMD	Bone mineral density
BTM	Bone turnover marker
CHO	Carbohydrate
CT	Computed tomography
CTX	Carboxyterminal telopeptide
CV	Coefficient of variation
DMT-1	Divalent metal transporter 1
DXA	Dual-energy x-ray absorptiometry
EA	Energy availability
EEE	Exercise energy expenditure
EHMC	Exercise hypogonadal male condition
EPO	Erythropoietin
ER	Estrogen receptor
FFM	Fat free mass
FGF-23	Fibroblast growth factor 23
gla-OC	Carboxylated osteocalcin
glu-OC	Undercarboxylated osteocalcin
Hb	Haemoglobin
Hct	Haematocrit
HR-pQCT	High resolution peripheral quantitative computed tomography
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin-6
IOC	International Olympic Committee

LCHF	Low carbohydrate, high fat
LEA	Low energy availability
LH	Luteinizing hormone
MRI	Magnetic resonance imaging
NEAT	Non-exercise activity thermogenesis
NTX	N-terminal telopeptide
OC	Osteocalcin
OPG	Osteoprotegenin
P1CP	Procollagen-1 C-terminal propeptide
P1NP	Procollagen-1 N-terminal propeptide
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
RANKL	Receptor activator of NF-kB ligand
REDs	Relative Energy Deficiency in Sport
RMR	Resting metabolic rate
T3	Triiodothyronine
TEF	Thermic effect of food
Triad	Female and Male Athlete Triad
VO_2max	Maximal oxygen uptake
WHO	World Health Organization

Introduction

"If we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health."

~ Hippocrates

In elite athletes, certain training and nutrition practices augment performance goals but may not be optimal for long-term health outcomes. Indeed, manipulation of both energy and carbohydrate availability, either through changes in energy intake or exercise energy expenditure, is commonly undertaken to enhance training adaptation and/or improve body composition. However, the impact on athletes' health outcomes and, in turn, their availability for training and competition, is an important consideration. Failure to consider the health consequences of performance goals is to the detriment of both the athlete's wellbeing and their longevity in the sport.

Recognising that athletes engage in various training and nutritional practices that prioritize training adaptation and performance, investigating the health impact, specifically on bone and iron metabolism, was the focus of this body of work. Bone stress injuries account for a significant proportion of all musculoskeletal injuries in athletes, and result in significant time lost in training and competition. In addition to training load and underlying bone status, nutritional deficiencies are a notable risk factor for these injuries. Iron deficiency is highly prevalent in athletes and may be more prevalent in those with high training loads and/or insufficient dietary intake. Further, data in clinical populations suggest a link between iron deficiency and low bone mineral density. Considering the prevalence of, and possible associations between, bone stress injuries and iron deficiency, the studies comprising this thesis aimed to explore the impact of common training and nutrition practices on bone and iron metabolism in athletes.

The studies are outlined in Chapters 3, 4, 5, and 6. Chapter 7 will discuss the novel findings of this research and potential future directions.

Chapter 3 (***study 1***): Bone mineral density and bone turnover marker values in a convenience sample of elite endurance athletes

Chapter 4 (***study 2***): Short-term carbohydrate restriction impairs bone formation at rest and across prolonged exercise to a greater degree than low energy availability

Chapter 5 (***study 3***): Factors influencing the hepcidin response to exercise: an individual participant data meta-analysis

Chapter 6 (***study 4***): Sequential submaximal training in elite male rowers does not result in amplified increases in interleukin-6 or hepcidin

1 Chapter 1: Review of the literature

Exercise is widely accepted and promoted as a lifestyle intervention to improve health and longevity (O'Keefe et al., 2020; Paffenbarger et al., 1986). But, in elite athletes, who sit on the far-right of the reverse J-shaped curve (O'Keefe et al., 2020), there is a fine balance between performing the training loads required to be competitive while maintaining health. It may be argued that at these levels of competition, the pursuit of performance outcomes may outweigh the health benefits of exercise. For practitioners caring for athletes, recognizing circumstances which may put an athlete at increased risk for illness and injury, both short- and long-term, is a key responsibility. Here, managing training loads and ensuring adequate recovery becomes paramount. Dietary manipulation has become an increasingly common strategy to address various sport-specific performance constraints. Meanwhile, research into the health impact of these strategies lags practice. In this review, the current knowledge of the impact of exercise and common nutrition practices on bone and iron indices will be outlined.

1.1 Bone health in athletes

1.1.1 Epidemiology of bone stress injuries

Bone stress injuries, including fractures and reactions, account for a substantial proportion of musculoskeletal injuries in athletes and result in significant time losses from training and competition (Bennell et al., 1996b). Up to 5.7 bone stress injuries occur per 100,000 athlete exposures in collegiate-level athletes (Rizzone et al., 2017) with athletics, gymnastics, and rowing athletes incurring the highest rates of bone stress injuries (Ruddick et al., 2019). While the predominant site of injury depends on the loading pattern of the sport, the foot and lower leg seem to be the most common injury site across sports (Ruddick et al., 2019). Although the precise pathophysiology of bone stress fractures is unknown, theoretically, an increase in the magnitude and/or rate of bone loading with insufficient remodelling time, leads to the accumulation of damage (Warden et al., 2006). The precipitating cause is often multifactorial, attributed to factors such as repetitive load (Rizzone et al., 2017; Ruddick et al., 2019; Warden et al., 2006), change in training surface (Milgrom et al., 2003), insufficient adaptation time in a training program (Warden et al., 2006), biomechanical patterns (O'Leary et al., 2021), and bone geometry (O'Leary et al., 2021). Increasingly recognized is the role of low energy availability states on bone health (Hutson et al., 2021), influencing bone mineral density, microarchitecture (Ackerman et al., 2011), and remodelling (Ihle & Loucks, 2004). Inadequate intakes of macronutrients and micronutrients, interactively or independently of energy availability, may also affect bone health (Sale & Elliott-Sale, 2019).

1.1.2 Bone composition

Bone tissue provides multiple functions, including movement, mineral and acid-base balance, haematopoiesis, and growth factor and cytokine reserve (Clarke, 2008), which are critical for both health and athletic performance. Bone is composed of minerals (50-70%), organic matrix (20-40%), water (5-10%), and lipids (<3%) (Song, 2017). The matrix is comprised mostly of type 1 collagen (95%), upon which calcium and phosphate-rich hydroxyapatite crystals are deposited (Song, 2017). This arrangement confers both flexibility as well as rigidity to bone (Song, 2017). Non-collagenous proteins represent about 2% of the bone matrix and regulate collagen fibre formation and mineralization (Hoenig et al., 2022). Some of these proteins, such as osteocalcin (OC) may play an endocrine role as well (Moser & Van Der Eerden, 2019). Bone tissue is organized into both cortical (compact) and trabecular (cancellous) bone in different

ratios, depending on the site (Clarke, 2008). Cortical bone is made up of osteons with a central Haversian canal for blood vessels and nerves, whereas trabecular bone is composed of a network of trabeculae (Hoenig et al., 2022). Osteons (Figure 1.1) are composed of concentric lamellae, a pattern with collagen fibrils in alternating directions, which confers strength (Clarke, 2008) and prevents propagation of fracture lines (Hoenig et al., 2022). The cortical periosteum exhibits higher formation than resorption (Clarke, 2008). Meanwhile, the endosteum is subject to the opposite, likely as a secondary outcome of biomechanical forces or bone-marrow cytokine exposure (Clarke, 2008). These processes result in the widening of the bone marrow compartment and thinning of the cortex with aging (Clarke, 2008).

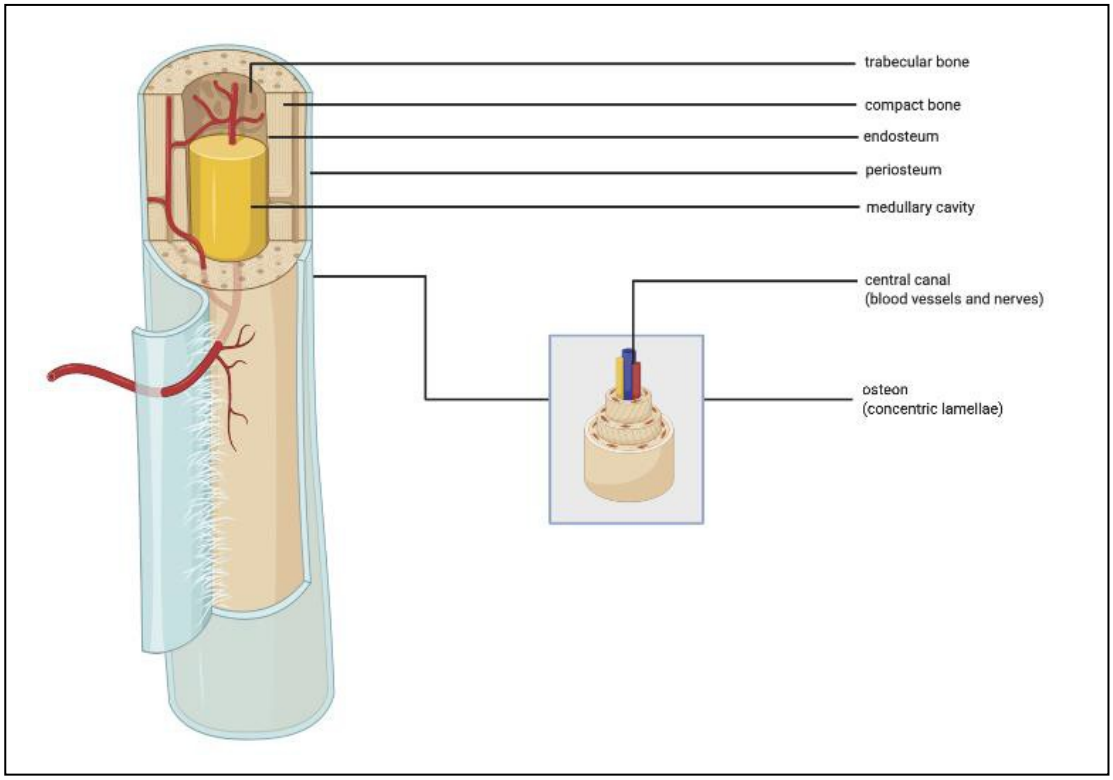

Figure 1.1: Macroscopic structure of bone tissue *(created with biorender.com)*

1.1.3 Bone adaptation to exercise

Extensive reviews (Hoenig et al., 2022) of the work of others in bone metabolism outline our understanding of bone adaptation to exercise. Bone adapts to applied load, enabling growth by modelling and repair by remodelling (Hoenig et al., 2022). During childhood, skeletal growth occurs via modelling, a spatially independent process of adding and removing bone tissue to augment bone size and strength (Hoenig et al., 2022). Although an imperfect and incomplete

determinant of bone strength, bone mineral density (BMD), as measured by dual energy x-ray absorptiometry (DXA), peaks around 16-21 years at the hip and up to 40 years at the lumbar spine (Berger et al., 2010). A 10% increase in peak bone mass may delay the onset of osteoporosis by up to 13 years (Hernandez et al., 2003). The benefit of mechanical loading on BMD is well-recognized with physical activity during childhood and adolescence resulting in a greater cortical area and thickness (Hoenig et al., 2022), leading to a reduced fracture incidence in adulthood (Fredericson et al., 2005; Karlsson & Rosengren, 2020; Rudolph et al., 2021). High- and odd-impact sports have been shown to confer the most benefit to BMD, not only at the loaded sites but also at unloaded axial and peripheral sites, whereas low- or non-impact sports may confer no benefit at all (Nevill et al., 2004; Tenforde & Fredericson, 2011). Multiple cross-sectional studies demonstrate the relevance of sport type in BMD accrual at loaded sites. Runners typically demonstrate increased BMD at impact sites, such as the femoral neck (Ackerman et al., 2011; McCormack et al., 2019; Tam et al., 2018), calcanei (Fredericson et al., 2007), and tibia (Smock et al., 2009), yet reductions at the lumbar spine (Scofield & Hecht, 2012; Tam et al., 2018). Ballet dancers have an increased BMD at the femoral neck and trochanter, yet reductions at the radius (Wewege & Ward, 2018). On the other hand, non-weight bearing athletes show very little benefit over non-athletes, with some suffering from reduced BMD secondary to large periods of time without load or with low energy and/or nutrient availability. Jockeys have consistently reduced total body and lumbar spine density, with similar or reduced BMD at the femoral neck to controls (Dolan et al., 2012a; Dolan et al., 2012b). Similarly, cyclists demonstrate reductions at both lumbar spine and femoral neck (Nagle & Brooks, 2011) and swimmers display no significant benefit over controls, even in the upper limbs (Bellver et al., 2019; Miller et al., 2020). Compared to endurance athletes, power athletes have greater BMD of the upper limb and lumbar spine (Bennell et al., 1997), and wrestlers and judoists have higher whole-body BMD (Sagayama et al., 2020). Longitudinal studies echo these findings: female gymnasts showed greater percentage increases in BMD at the lumbar spine and femoral neck compared to runners, swimmers, and non-athletes over 8 months, independent of menstrual status (Taaffe et al., 1997). Meanwhile, male badminton players displayed increases at all sites over 12 years compared to both ice hockey players and controls, especially at the humerus, with ice hockey players accruing greater density at the femoral neck than controls (Tervo et al., 2010).

However, with repetitive loading, which is usually more frequent and forceful in athletes than the general population, microdamage accumulates, resulting in reduced stiffness and elasticity and apoptosis of osteocytes (Hoenig et al., 2022). This triggers remodelling to increase fracture

resistance (Hoenig et al., 2022). Remodelling is a coupled process occurring throughout life, with osteoclasts and osteoblasts acting sequentially in a coordinated fashion to preserve mechanical strength and calcium-phosphate homeostasis (Clarke, 2008; Hoenig et al., 2022). These temporary structures of osteoclasts and osteoblasts are referred to as basic multicellular units (Hoenig et al., 2022). The remodelling process takes place at these discrete locations with resorption followed by formation in an orderly fashion; this contrasts with bone modelling at the endosteal and periosteal surfaces which results in a change in shape or size of the bone (Schini et al., 2022). The remodelling process (Figure 1.2) comprises four phases involving both osteoblasts and osteoclasts: activation, resorption, reversal, and formation (Clarke, 2008). Recruitment and activation of mononuclear precursors from the circulation and subsequent fusion to form preosteoclasts results in binding to the bone matrix forming annular resorption compartments (Clarke, 2008). Resorption, regulated by the ratio of receptor activator of NF-kB ligand (RANKL) to osteoprotegenin (OPG), interleukin-1, interleukin-6 (IL-6), macrophage colony stimulating factor, parathyroid hormone (PTH), vitamin D3 ($1,25[OH]_2D$), and calcitonin, results in osteoclastic secretion of hydrogen ions to mobilize the matrix (Clarke, 2008). Tartrate-resistant acid phosphatase, cathepsin K, matrix metalloproteinase 9, and gelatinase digest the matrix and form lacunae (Clarke, 2008). Following apoptosis of the multinucleated osteoclasts after 2-4 weeks, and possibly secondary to a rise in transforming growth factor beta (TGF-β) induced RANKL inhibition, the process of reversal begins; resorption cavities become sites of formation as monocytes, osteocytes, and pre-osteoblasts are recruited (Clarke, 2008). Formation, requiring 3-6 months, involves matrix synthesis and mineral regulation by osteoblasts. Osteoblasts eventually undergo apoptosis, or become osteocytes, where their primary function is mechanosensation and transduction of stress signals into biological activity (Clarke, 2008). Where there is an imbalance between microdamage formation and resolution, the risk of bone stress injury is heightened significantly (Hoenig et al., 2022). Notably, the transition time between the phases of resorption (~4 weeks) and formation (~3 months) creates a local porosity and a potentially increased vulnerability to a given load; combined with rapid training load progression and insufficient recovery during this transition, the risk of a bone stress injury may be amplified (Hoenig et al., 2022).

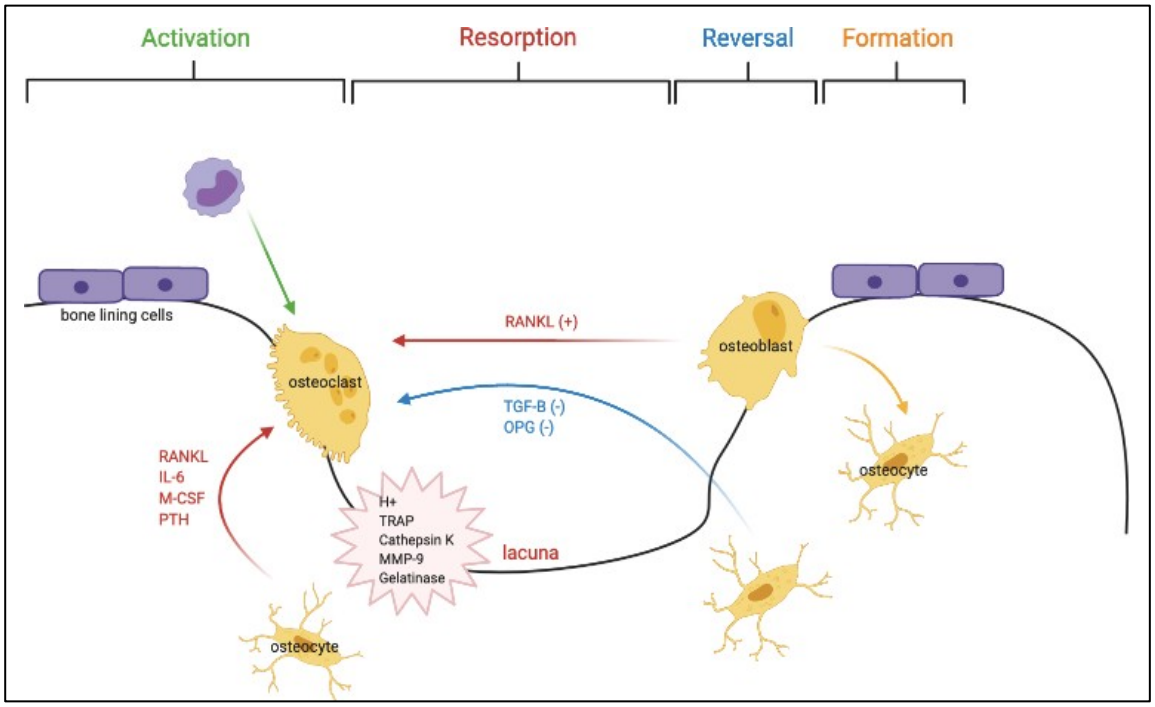

Figure 1.2: Bone remodelling process. RANKL: receptor activator of NF-kB ligand; IL-6: interleukin-6; M-CSF: macrophage colony stimulating factor; PTH: parathyroid hormone; TGF-B: transforming growth factor beta; OPG: osteoprotegenin; H+: hydrogen ion; TRAP: tartrate resistant acid phosphatase; MMP-9: matrix metalloproteinase 9 *(created with biorender.com)*

1.1.4 Measurement of bone strength

Bone strength is represented as a composite of both quantity and quality (Viguet-Carrin et al., 2006), where the former may be represented by areal BMD as measured by DXA, and the latter may include both mineral-collagen homeostasis and bone architecture/geometry (Link & Heilmeier, 2016).

1.1.4.1 Bone mineral density

The measurement of areal BMD by DXA is a common metric used as a proxy for bone strength, although density is only one component of this. Nevertheless, it provides useful information regarding the risk of fracture, with a relative risk of 1.6-2.6-fold with each standard deviation decrease in age-adjusted BMD (Dimai, 2017; Marshall et al., 1996). The World Health Organization (WHO) diagnosis of osteoporosis, which is defined as "a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture", is based on areal BMD measurements as a proxy for bone strength (NIH Consensus Development Panel on Osteoporosis Prevention, 2001).

DXA is an imaging technique using an x-ray source below the patient to emit photons at two different energy levels specific to soft tissue and cortical bone; the attenuations of these emissions are detected above the patient to form a planar image (Krugh & Langaker, 2020). The total pixels are used to calculate a value for BMD, and bone mineral content (BMC) can be quantified by multiplication by projected area (Njeh et al., 1999). The effective dose of radiation (accounting for type of radiation and tissue irradiated) is 3.6 μSv (4.6 μSv including ovaries) for a total body scan (Lewis et al., 1994), 13 μSv for an adult spine, and 9 μSv for an adult hip (Damilakis et al., 2010). This is considered relatively low when compared to the daily background radiation of 7 μSv (Njeh et al., 1999).

Site-specific DXA measurements of BMD at the posterior-anterior spine (lumbar vertebrae 1-4) and hip (total proximal femur, femoral neck, or trochanter), or one-third radius of the non-dominant arm (if spine and hip measurements are not feasible) are used for diagnostic purposes (Leib et al., 2004) with these sites being the most common osteoporotic fracture sites (Golob & Laya, 2015; Liu et al., 2019). In postmenopausal women, this is based on a T-score, which refers to the standard deviation from the mean BMD of a young healthy white reference population, with the lowest value of the sites measured used for diagnosis: (Leib et al., 2004; 2001)

- Osteoporosis: T-score less than or equal to -2.5, between -1.0 and -2.5 and increased fracture risk via the FRAX© assessment tool or a fragility fracture, or presence of a fragility fracture regardless of T-score
- Osteopenia or low bone mass: T-score between -1.0 and -2.5
- Normal: T-score -1.0 or above (Camacho et al., 2020)

These definitions are not to be used in premenopausal women, men under 50 years of age, or children (Leib et al., 2004). Z-scores, or the number of standard deviations from age and sex-matched controls, are recommended for these populations, with a categorization of “low bone density for chronological age” for a z-score less than -2.0 (Camacho et al., 2020; 2004; 2001). In addition, it is suggested that different cut-offs should apply to athletes given their increased BMD relative to the general population (~5-15%) and, therefore, the American College of Sports Medicine (ACSM) endorses the term “low BMD” for a z-score between -1.0 and -2.0, and “osteoporosis” as a z-score less than -2.0, both in conjunction with secondary clinical risk factors (e.g., nutritional deficiencies, hypoestrogenism, stress fractures) (Nattiv et al., 2007).

Relying solely on areal BMD measurements of the hip and lumbar spine to assess bone status in athletes may be problematic. Indeed, due to the differential effects of sport-specific loading patterns on regional BMD sites, there may be increased risk of discordance between these sites. In postmenopausal populations, studies of T-score discordance in classification between hip and spine, denote 'minor' as being one WHO diagnostic class apart and 'major' as being osteoporotic versus normal; such studies show a prevalence of 4% and 40% respectively on average (Moayyeri et al., 2005; Mounach et al., 2009; Woodson, 2000). A further review reported that 4 out of 10 patients will be exhibit discordance between sites (El Maghraoui et al., 2007). Reasons for this may be technical (e.g., positioning, or technician or machine error) or physiological (e.g., differing rates of bone loss or effects of mechanical strain) (Woodson, 2000). Rates of discordance in athletes have not yet been evaluated. Furthermore, sport-specific loading patterns may place a region that is not traditionally measured at increased risk of bone stress injuries; for example, ribs in rowers (Lundy et al., 2022b), foot and lower leg in hockey and netball (Ruddick et al., 2019). Finally, although in older adults lower areal BMD is a risk factor for fracture (Johnell et al., 2005), the relationship between areal BMD and fracture in young adults is weaker (Ferrari et al., 2012; Frolich et al., 2020; Writing Group for the ISCD Position Development Conference, 2004). In fact, factors such as low hormone (Rudolph et al., 2021) and energy availability states (Heikura et al., 2018b) place athletes at increased risk of stress fractures, despite BMD values similar to study controls. With BMD at traditional sites being a less reliable marker of bone strength or fracture risk in athletes, there may be a role for alternative or additional markers of bone status in athletes (e.g., bone biomarkers, architecture imaging).

1.1.4.2 Bone turnover markers

Markers of bone resorption (e.g., C-terminal telopeptide of type 1 collagen; CTX) and formation (e.g., procollagen-1 N-terminal propeptide; P1NP) reflect the metabolic activity of bone tissue, with evidence of potential predictive value in changes to areal (Verroken et al., 2018) and volumetric (Pye et al., 2017) BMD. Collagen is secreted as procollagen, which is assembled into glycine-proline-hydroxyproline triple helix fibrils, via processes that include the cleavage of amino/N- and carboxyl/C-propeptide flanks to form telopeptides and stabilization through the formation of enzymatic-facilitated crosslinks (Viguet-Carrin et al., 2006). Therefore, during synthesis of collagen, the N- and C-terminal ends of procollagen are released and can be detected in serum as P1NP and C-terminal propeptide (P1CP) respectively (Song, 2017). During cathepsin K-mediated matrix dissolution, the telopeptides from the N- (NTX)

and C-terminals (i.e., CTX) are released and can be detected in either urine or serum (Song, 2017). In this thesis, measured serum CTX refers to isomerised β-CTX-I. Sclerostin, produced by osteocytes and an antagonist of Wnt/β-catenin osteoblastic differentiation, is an emerging marker of bone metabolism, although inconsistency in results and research infancy limits its use to date (Delgado-Calle et al., 2017). Additional markers of formation are bone alkaline phosphatase, produced by osteoblasts during matrix maturation, and OC, which is the second most abundant protein in bone, also secreted by osteoblasts. However, OC may be a marker of overall bone metabolism, rather than just formation, due to the release of matrix-embedded OC during resorption (Ivaska et al., 2004). Osteocalcin has further been shown to have endocrinological properties, involved in energy metabolism, muscle hypertrophy, and male fertility (Karsenty & Mera, 2018; Moser & Van Der Eerden, 2019; Zoch et al., 2016).

The International Osteoporosis Federation and International Federation of Clinical Chemistry currently recommend the use of P1NP and CTX as markers of formation and resorption, respectively, in monitoring osteoporosis treatment and fracture risk (Williams & Sapra, 2020). This is supported by a meta-analysis of 10 studies, which showed a significant, yet moderate association with fracture risk (gradient of risk ~ 1.2 for CTX and P1NP) unadjusted for BMD (Johansson et al., 2014). Although this was recently updated to adjust for BMD and clinical confounders, the significant associations were found to be maintained (Tian et al., 2019). However, their use in clinical settings has been complicated by inadequate control of pre-analytical variability (Szulc et al., 2017) and heterogeneity of assays (Cavalier et al., 2021; Cavalier et al., 2019). This has resulted in difficulty in interpretation, as well as a lack of consistency in the markers used in research (Vasikaran et al., 2011). Current best practice in managing osteoporotic populations involves the concomitant measurement of bone turnover markers (BTMs), with rapid response, and BMD, with slower change (Williams & Sapra, 2020). The utility of BTMs in non-osteoporotic populations is limited by the lack of normative age-related reference values (Michelsen et al., 2013) and poor knowledge of causes of variation. Although the evidence is considered weak, meta-analyses suggest that blood concentrations of CTX peak within 2 h of the cessation of a single exercise bout, with larger increases occurring with longer durations and greater amounts of work (Dolan et al., 2022). Interestingly, this may not occur with resistance or high-impact exercise, which is considered less osteolytic (Dolan et al., 2022). In contrast, bone formation markers change quite transiently and minimally following a single exercise session (Dolan et al., 2022). Given the current low quality of evidence (Dolan et al., 2022) and the potential additional effects of acute nutritional manipulation on BTMs (Heikura et al., 2019), further robust investigation of sources of pre-

analytical variability is essential before these markers can be used in athlete populations. Whether BTMs have potential as a surrogate marker of bone health or in assessing fracture risk remains to be explored.

The terminology used in the literature referring to markers of bone formation and resorption often varies between authors/research groups and continues to evolve. While the medical community, particularly those in osteoporosis research and clinical practice, still use the term 'bone turnover markers' (Vasikaran et al., 2024), a few in the sports community have transitioned from this same term (Sale et al., 2015) to using 'bone biomarkers' (Dolan et al., 2022), 'bone (re)modelling markers' (Dolan et al., 2020), or simply 'markers of bone resorption and formation'(Dolan et al., 2020). It might be argued that the term 'bone turnover' implies a coupling of bone resorption and formation at a particular site and, therefore, this term is inaccurate when reporting circulating concentrations of these markers, where it is impossible to pinpoint the site from which they are being produced. However, the term 'bone turnover markers' has been defined as "biochemical products measured usually in blood or urine that reflect the metabolic activity of bone" (Vasikaran et al., 2011), implying that the term encompasses 'bone metabolism'. Similarly, 'bone (re)modelling markers' "represent processes involved in the formation or resorption of bone" and "provide information about dynamic bone activity" (Dolan et al., 2020). As a result, and due to peer reviewer preferences, this thesis may interchange between these terms which essentially refer to the same process; that is, the resorption and formation markers of bone, without implying that this is localized to a certain site. Furthermore, the terms 'bone health' and 'bone status' may be used in this thesis as an all-encompassing term for the condition of the bone at a particular time, recognizing that it is a dynamic structure and comprises mineral content, architecture/geometry, and tissue properties.

1.1.4.3 Bone architecture and geometry

The major limitations of DXA are the areal nature of measurements, resulting in BMD underestimations in small individuals and overestimations in tall individuals, as well as the inability to differentiate bone compartments (Link & Kazakia, 2020). Although not currently standard of care in clinical practice, more advanced imaging techniques to measure bone quality, particularly bone microarchitecture, have been developed. These include quantitative ultrasound, peripheral quantitative computed tomography (pQCT), high-resolution pQCT (HR-pQCT), multi-detector CT, and magnetic resonance imaging (MRI) (Link & Kazakia, 2020). The particular advantages of HR-pQCT are its ability to measure volumetric BMD, to assess

cortical and trabecular bone characteristics separately, and to evaluate bone strength via finite element analysis (FEA), all with a relatively low radiation dose (~3 μSv) (Nishiyama & Shane, 2013). Unfortunately, this tool remains largely limited to research settings for the moment and is currently confined to scanning peripheral sites, mainly the distal tibia and radius (Link & Kazakia, 2020). Multi-detector CT offers the ability to scan more central sites and provide similar information with the disadvantage of substantially higher radiation exposure (Link & Kazakia, 2020). MRI, with the advantage of no radiation exposure, is limited by susceptibility to motion artifacts and morphological discrepancy deficiencies (Link & Kazakia, 2020).

Nevertheless, these tools show potential in improved evaluation of bone health characteristics and fracture risk. Using HR-pQCT, higher cortical thickness and lower trabecular separation has been shown in weight-bearing athletes (Schipilow et al., 2013), yet amenorrheic athletes demonstrate the opposite with lower cortical thickness and higher trabecular separation (Ackerman et al., 2011). While HR-pQCT measures have been shown to predict fracture independent of areal BMD in postmenopausal women (Sornay-Rendu et al., 2017) and in older men (Langsetmo et al., 2018), similar to DXA, data on athletes are scarce. The only study that has analysed this to our knowledge found that, although athletes with bone stress injuries had reduced areal BMD on DXA measurements and impaired cortical bone area and thickness on radial/tibial HR-pQCT, there was no association to the anatomic fracture sites (Stürznickel et al., 2022). So, while these technologies are promising and insightful, bone health is multifactorial, highlighting that a composite assessment using history, imaging, and BTMs is likely the best approach. Indeed, there seems to be a discrepancy in the predictive and monitoring value of these tools in older, osteoporotic populations as opposed to athletes. Certainly, the frequency and pattern of loading combined with specific nutritional needs in athletes may require adjustment of population-based cut-off values used in both research and practice to evaluate bone health and fracture risk.

1.1.5 Bone response to nutritional manipulation

1.1.5.1 Background to energy availability

Bone characteristics are determined by a complex range of interacting factors including genetics, mechanical loading, the hormonal environment, and nutritional status (Hoenig et al., 2022). Among the nutritional factors of interest in athletes, low energy availability (LEA) has gained major attention since it can attenuate the positive effects of bone loading and contribute

to the risk of injuries (Barrack et al., 2014; Heikura et al., 2018b). Manipulation of caloric intake relative to energy expended is a central tenet to weight loss. In athletes, sport-specific performance goals, such as achieving body composition "ideals", making weight, or improving power-to-weight ratios, may be the stimuli for engaging in an intentional energy deficit (Burke et al., 2018a; Loucks, 2004). Alternatively, a lack of knowledge around the required energy intake to support large training loads, financial constraints that impede achieving such intake, or energy requirements that exceed the absorptive capacity of the gut may lead to unintentional energy deficits (Burke et al., 2018a; Loucks, 2004). Regardless of the underpinning cause, exposure to LEA is a common experience for athletes and, while potentially unavoidable and even beneficial in the short-term, may result in downstream adverse health outcomes (Mountjoy et al., 2018).

Energy availability (EA) is defined as energy intake minus *exercise* energy expenditure, normalized to fat free mass (FFM), and refers to the energy remaining as an input to all physiological systems after exercise has been accounted for (Loucks et al., 2011). This contrasts with energy balance which uses *total* energy expenditure, including the thermic effect of food (TEF), non-exercise activity thermogenesis (NEAT), and resting metabolic rate (RMR) (Loucks et al., 2011). The use of EA is preferred to energy balance due to its intuitive capture of adequacy of energy intake for health and function, as well as changes that occur to total energy expenditure if there is a decline in RMR as a homeostatic compensation to an energy deficit (Lieberman et al., 2018). LEA is the underpinning cause of both the Female and Male Athlete Triad (Triad) (De Souza et al., 2014; Fredericson et al., 2021; Nattiv et al., 2021; Nattiv et al., 2007) and Relative Energy Deficiency in Sport (REDs) (Mountjoy et al., 2018; Mountjoy et al., 2023), which highlight the disturbances in body systems that occur as a result of exposure to LEA.

The seminal papers of bone health in female athletes, published by Professor Barbara Drinkwater in the 1980's, linked amenorrhea to decreased BMD (Drinkwater et al., 1984) and the improvement of the latter with the resumption of menses (Drinkwater et al., 1986). In 1992, the term 'Female Athlete Triad' was coined to describe the cluster of disordered eating, amenorrhea, and osteoporosis secondary to hypoestrogenism seen in female athletes (Yeager et al., 1993). The first position stand on the Female Athlete Triad was published in 1997 (Otis et al., 1997) and subsequently revised in 2007 (Nattiv et al., 2007) to reflect the spectrum of pathology in terms of bone health and menstrual cycle disturbances, but also to review the branch of 'disordered eating' within the concept of the broader term of 'low energy

availability'. With increasing recognition that the physiological disturbances related to LEA extended beyond that of bone and reproduction and that these were not exclusive to female athletes, an International Olympic Committee (IOC) expert panel introduced the broader term, REDs, to cover a wider range of impairments of health and performance (Mountjoy et al., 2018; Mountjoy et al., 2014; Mountjoy et al., 2023). The positioning of REDs has evolved over the past decade with Consensus statements being released by the Working Group in 2014 (Mountjoy et al., 2014), 2018 (Mountjoy et al., 2018), and 2023 (Mountjoy et al., 2023). The most recent statement defines REDs as follows: "a syndrome of impaired physiological and/or psychological functioning experienced by female and male athletes that is caused by exposure to problematic (prolonged/severe) low energy availability. The detrimental outcomes include, but are not limited to, decreases in energy metabolism, reproductive function, musculoskeletal health, immunity, glycogen synthesis, and cardiovascular and haematological health, which can all individually and synergistically lead to impaired well-being, increased injury risk, and decreased sports performance" (Mountjoy et al., 2023). It is further noted that the Female Athlete Triad was expanded in 2021 to recognize a companion problem in male athletes in which LEA is associated with reproductive dysfunction and impaired bone health (Fredericson et al., 2021; Nattiv et al., 2021).

The original positioning of REDs recognized a threshold of EA reduction at which the risk of reproductive system and bone impairment was most likely (Mountjoy et al., 2018; Mountjoy et al., 2014). A threshold of 30 kilocalories per kilogram of fat free mass (kcal.kg^{-1} FFM), roughly equivalent to the amount required to maintain RMR, was suggested as the point at which luteinizing hormone (LH) pulsatility was disrupted and low triiodothyronine (T3) syndrome was induced in women; this concept was derived from short-term (4-5 day) tightly controlled experiments on previously sedentary women, conducted by Professor Anne Loucks (Loucks & Heath, 1994b; Loucks & Thuma, 2003). Loucks further demonstrated that these disruptions were chiefly dependent on a mismatch between energy intake and exercise expenditure rather than the stress of exercise itself (Loucks & Heath, 1994a; Loucks et al., 1998). However, even these studies identified the absence of a universal threshold for disruption for all body systems in all individuals; indeed, the reproductive suppression disappeared after 14 years of gynaecological age (Loucks, 2006), indicating a difference in responsiveness to LEA according to reproductive axis maturation.

The application of an absolute threshold has also been contested by data from longer term studies, noting that short-term experimental trials may overlook the detection of subclinical

menstrual disturbances (De Souza et al., 2019). Indeed, some authors have proposed a dose-response continuum in the relationship between EA and menstrual disturbances, including those of both ovulation and menses occurrence (De Souza et al., 2019). In both cross-sectional studies and randomized controlled trials over a 3-month period, these researchers noted that the frequency and severity of menstrual abnormalities increased with decreasing energy availability (Lieberman et al., 2018; Reed et al., 2015; Williams et al., 2015). Although an EA of around 45 $kcal.kg^{-1}$ FFM per day has been postulated to support optimal physiological function in exercising women (De Souza et al., 2019; Loucks et al., 2011), there is still little certainty on how the dose of LEA affects different body systems or even the best way to assess EA (Burke et al., 2018b).

In men, LEA seems to be better tolerated than in women, although the research in this population is scant. Here, there is debate as to whether the reduced testosterone seen in male athletes is secondary to LH disruption as in female athletes or whether it is an adaptation to training and, even then, whether it is clinically undesirable. With the growing evidence of menstrual disorders in female athletes in the late 1970s and early 1980s, reports arose of reduced LH pulse frequency and amplitude (MacConnie et al., 1986) and blunted testosterone levels (Wheeler et al., 1984) in male runners in comparison to controls. Following a series of studies showing lower resting testosterone (25-50%) secondary to endurance training with non-significant effects on LH pulsatility (Hackney et al., 1997; Hackney et al., 1988; McColl et al., 1989; Wheeler et al., 1991), the term 'exercise hypogonadal male condition' (EHMC) was coined (Hackney & Hackney, 2005). This aimed to describe a state of adaptation rather than pathology due to the lack of translation into health and performance decrements, distinguishing it from the Triad or REDs (Hackney, 2020). The changes in testosterone and translation to reproductive outcomes have been equivocal: whereas some have demonstrated changes, albeit still in physiological range, to semen quality and sperm characteristics (Arce et al., 1993; De Souza et al., 1994), others have shown no disruption (Ayers et al., 1985). Early studies failed to find a definitive correlation between testosterone and BMD in male athletes (Bennell et al., 1996a).

Very few controlled studies have been conducted in men to elucidate the EA continuum along which physiological disruption occurs comparative to women. Recently, research in a large, international cohort of male athletes identified low sex drive as the most effective screening symptom for LEA (Lundy et al., 2022c). Interestingly, Hooper et al. (Hooper et al., 2017) demonstrated that male runners with clinically low testosterone had a significantly lower EA

than controls (27.2±12.7 vs 45.4±18.2 kcal.kg^{-1} FFM), approximating the typical region of EA previously suggested for low T3 and LH pulsatility disruption in women. In contrast, a repeated measures randomized controlled trial in exercising men that manipulated EA (15 vs 40 kcal.kg^{-1} FFM) over 4 days, either through exercise or diet, found no significant differences in T3, testosterone, or insulin-like growth factor 1 (IGF-1) (Koehler et al., 2016). Similarly, another randomized crossover trial over 5 days, with EA conditions of 15 and 45 kcal.kg^{-1} FFM, showed no significant differences in BTMs or metabolic hormones in male participants, which was in contrast to the female participants in the same study where such disruptions were observed (Papageorgiou et al., 2017). In summary, high-quality research in male athletes is significantly lacking. While it appears that male reproductive systems may be less sensitive to reductions in EA than female reproductive systems, there is insufficient evidence in this population to confirm this hypothesis. Certainly, the visibility of reproductive consequences may be less objective than in women (i.e., menses) and, unfortunately, may result in underdiagnosis and progression of undesirable health consequences.

1.1.5.2 Impact of low energy availability on bone

Indeed, there is evidence of a greater risk of bone injuries, and lost training days, in both male and female athletes who exhibit higher scores related to Female Athlete Triad or REDs risk factors (Barrack et al., 2014; Hayashi et al., 2018a; Heikura et al., 2018b; Kraus et al., 2019; McCormack et al., 2019; Ruddick et al., 2019). Amenorrheic athletes demonstrate significantly lower lumbar spine BMD (Ackerman et al., 2011; Drinkwater et al., 1984; Micklesfield et al., 1995), radius trabecular and tibia cortical density (Ackerman et al., 2011), increased lifetime fracture risk (Ackerman et al., 2015), lower stiffness and failure load (Ackerman et al., 2015), and reduced markers of bone formation (Zanker & Swaine, 1998) than eumenorrheic counterparts. In trials implementing specific energy availability cut-offs, markers of formation are reduced but resorption indices seem to require lower EA. Ihle and Loucks (Ihle & Loucks, 2004) showed that a 5-day diet-exercise intervention in sedentary women resulted in a linear decrease in P1CP below 30 kcal.kg^{-1} LBM, an abrupt drop in OC between 20 and 30 kcal.kg^{-1} LBM, and an increase in NTX below 10 kcal.kg^{-1} LBM. Similarly, Papageorgiou et al. (Papageorgiou et al., 2018b) showed that, in active women, dietary-induced EA of 15 kcal.kg^{-1} LBM vs 45 kcal.kg^{-1} LBM resulted in lower P1NP after 3 days with no significant change in CTX. However, no significant change in either marker was noted when LEA was induced by exercise. While data in men are scant, no change in either P1NP or CTX concentrations after 5 days of an EA of 15 kcal.kg^{-1} LBM was observed in male participants, which was in contrast

to the lower P1NP and higher CTX observed in the female participants (Papageorgiou et al., 2017). The differential effects of EA on bone formation and resorption markers as well as between sexes is curious. Also, given the differing impacts of LEA on the hypothalamic-pituitary-gonadal axis in women and men, the indirect effect of hormonal disruptions deserves consideration.

It is well-recognized that declining sex steroid levels are associated with osteoporosis in both women and men (Khosla et al., 2008; Riggs et al., 2002). Seminal studies in postmenopausal women published in the 1940s (Albright et al., 1941) and castrated men in 1989 (Stěpán et al., 1989) established the basis for an association between gonadal hormones and bone. However, the disparate sex steroid actions on bone metabolism may partly explain the discrepant findings seen with respect to bone turnover and density effects in male versus female athletes subject to LEA. Indeed, estrogen receptor-alpha (ER-α), -beta (ER-β), and androgen receptor (AR) are present in trabecular bone but only ER-α is present in cortical bone, suggesting that the action of estrogen is necessary for cortical bone remodelling (Khosla & Monroe, 2018). In females, high estrogen suppresses remodelling in both trabecular and cortical bone whereas, in males, estrogen is sufficient to suppress cortical remodelling, but high testosterone is required to prevent trabecular bone loss (Khosla & Monroe, 2018). These compartment-specific actions of sex steroids and the predominance of cortical bone in humans (Khosla & Monroe, 2018) highlight the proportionate importance of estrogen in BMD. In fact, a study in male collegiate wrestlers, runners and golfers found that oestradiol levels, not testosterone, were predictive of BMD (Ackerman et al., 2012). Both animal (Pacifici, 2012) and human studies (Eghbali-Fatourechi et al., 2003) have demonstrated an increase in RANKL expression in the setting of estrogen deficiency, leading to osteoclastic resorption. Nonetheless, the effects of EA may also be independent of estrogen in that bone formation and resorption is unperturbed in an energy replete state, regardless of estrogen (De Souza et al., 2008). Further, other hormones affected by LEA (e.g., cortisol (Mancini et al., 2004), IGF-1 (Chihara & Sugimoto, 1997)) have been shown to affect osteoblast and osteoclast activity. Finally, while current evidence suggests EA affects bone turnover, the accompanying reductions in macro- and micronutrients make it difficult to isolate the disruptions to energy per se; no study to date has compared EA to direct manipulation of macronutrient intake/availability.

1.1.5.3 Macronutrient influences on bone

Carbohydrate (CHO) availability refers to the availability of endogenous (muscle and liver glycogen) and exogenous CHO (consumed around exercise) required to sustain the demands of an exercise session (Impey et al., 2018). Here, total dietary CHO intake, including timing of intake around exercise sessions, can be seen to maximize glycogen content, with exogenous CHO supply to achieve muscle and central nervous system needs (= high carbohydrate availability). Meanwhile, CHO intakes associated with suboptimal glycogen content and lack of exogenous CHO supply is considered low CHO availability (Impey et al., 2018). Although low CHO availability may be accidentally or purposefully pursued around exercise sessions to promote enhanced training adaptation (Bartlett et al., 2015), there is renewed interest in downstream effects on other body systems.

Generally, circulating concentrations of CTX are attenuated following glucose, fat, or protein for ~ 2 h, independent of time of day, returning to baseline concentrations after 6-9 h (Bjarnason et al., 2002). Interestingly, intravenous glucose has less of an effect than oral intake, implicating gastrointestinal incretins in the response (Bjarnason et al., 2002). On the other hand, CTX increases in response to exercise, regardless of fed-state (Scott et al., 2012), with a recent meta-analysis reporting that concentrations peak within 2 h of exercise cessation with larger increases evident following longer duration exercise and greater work done (Dolan et al., 2022). In contrast, P1NP does not seem to be acutely affected by feeding (Bergmann et al., 2019) or exercise (Dolan et al., 2022). Given that CTX appears to increase in response to exercise but is attenuated by nutrient ingestion, the potential importance of fuelling around exercise to limit bone resorption is highlighted. Indeed, de Sousa et al. (de Sousa et al., 2014) reported a reduced CTX level at 80 min following a high intensity running session performed 8 days after an 'overload program' when supported with CHO (1 $g.kg^{-1}$ maltodextrin) compared to placebo. Similarly, Sale and colleagues demonstrated an attenuation of the post-exercise P1NP and CTX responses when CHO was consumed prior to and during a 2 h run at 70% VO_2max (Sale et al., 2015). Hammond et al. (Hammond et al., 2019) further extended these findings by investigating whether it was the provision of energy itself or specifically CHO that influenced the acute exercise-induced bone turnover response. Here, the authors compared the effect of high CHO (12 $g.kg^{-1}$ CHO, 60 $kcal.kg^{-1}$ FFM), low CHO (3 $g.kg^{-1}$ CHO, 60 $kcal.kg^{-1}$ FFM), and energy restricted (3 $g.kg^{-1}$ CHO, 20 $kcal.kg^{-1}$ FFM) conditions around two high-intensity exercise sessions (85% VO_2peak), separated by 3 hours, on bone turnover markers for up to 17 h following the second session. While no difference was observed between trials for P1NP, CTX

was suppressed for up to 3 h following the afternoon exercise session in the high CHO group compared to both the low CHO and energy restricted conditions. Taken together, these findings might suggest that energy specifically from CHO is necessary to limit bone resorption around exercise but that P1NP is less variable acutely to exercise and nutrition.

Although CTX responses to CHO availability have been demonstrated in terms of acute changes (de Sousa et al., 2014; Hammond et al., 2019; Sale et al., 2015; Townsend et al., 2017), there have been few investigations of longer-term changes in CHO availability on bone metabolism in athletes. Here, animal studies and studies in drug-resistant epileptic children suggest that ketogenic low-CHO high-fat (LCHF) may be detrimental to bone health (Best & Hsu, 2023). Recently, Heikura and colleagues (Heikura et al., 2019) reported the effects of a 3.5-week exposure to a LCHF diet on bone markers in elite endurance athletes. Compared to pre-intervention responses and to responses in a cohort who followed a diet providing high CHO availability, the LCHF diet was associated with an increase in fasting and across-exercise concentrations of CTX, while there was a concomitant decrease in blood concentrations of P1NP and OC. These data suggest that prolonged exposure to low CHO availability may increase bone resorption and reduce bone formation, warranting further investigation. Of further interest in this study, a 3-day period of restoration of CHO availability returned the CTX response to exercise to baseline values but failed to restore the exercise-response of P1NP and OC concentrations.

Of interest, the same group (Heikura et al., 2019) demonstrated increased post-exercise IL-6 concentrations in athletes following the LCHF diet (McKay et al., 2019a). Similarly, associations between post-exercise CTX and IL-6 have been demonstrated in studies investigating acute CHO consumption around exercise sessions (Hammond et al., 2019; Sale et al., 2015). Interleukin-6 is known to be released from muscle in response to exercise (Starkie et al., 2000; Steensberg et al., 2000), particularly under conditions of low muscle glycogen content (Keller et al., 2001; Steensberg et al., 2001). In fact, larger increases in IL-6 are seen with prolonged duration of exercise (Fischer, 2006), a factor which similarly drives larger increases in CTX (Dolan et al., 2022),. Research in *in vitro*, animal models, and clinical populations indicate that IL-6 plays a role in both bone formation and resorption (Sims, 2021). However, as limited research has been undertaken in athletic populations, it is unknown whether this relationship demonstrates causation or merely a temporal association. Whether the outcome is different depending on the source of IL-6 (i.e., immune cell or muscle cell) (Hennigar et al., 2017), the presence of other cofactors (e.g., PTH) (Grey et al., 1999; Grey et

al., 1996; Insogna et al., 2002), and the mechanical stimulus applied (Dolan et al., 2022) remains to be explored. Of additional interest is the role of increased IL-6 in limiting iron availability through the activity of the hormone hepcidin (Nemeth et al., 2004a). Indeed, certain micronutrients, such as calcium, vitamin D, and iron are essential to bone health. In athletes, the risk of micronutrient deficiency increases as training load increases, secondary to inadequate replenishment via dietary intake and/or reduced absorption from the gut (Peeling et al., 2023).

1.1.5.4 Micronutrient influences on bone

Calcium and vitamin D

Calcium plays a vital role in various processes in the body including muscle contraction, nerve transmission, and cellular signalling, with 99% stored in bone mineral (Song, 2017). Homeostatic regulation of blood calcium concentrations is achieved by PTH and 1,25-dihydroxyvitamin D (1,25$[OH]_2$D) (Song, 2017). The release of PTH from the parathyroid glands is stimulated by a reduction in free blood calcium concentration, which triggers osteoclast-mediated resorption, and renal calcium reabsorption, as well as transcription of 1-alpha-hydroxylase and suppression of 24-hydroxylase to increase 1,25$[OH]_2$D production in the kidney (Owens et al., 2018; Song, 2017). Cholecalciferol, derived from dietary sources or UVB radiation, is hydroxylated in the liver to 25-hydroxy-vitamin D (25[OH]D) and further in the kidney to the active form 1,25$[OH]_2$D. This then binds tissue receptors, forming a heterodimer with retinoid X receptor to activate or repress genes through vitamin D response elements (Owens et al., 2018). Calcium transport machinery is upregulated in the intestine to increase calcium absorption (Song, 2017; Wongdee et al., 2019), and RANKL (Kim et al., 2006) and fibroblast growth factor 23 (FGF-23) (Shimada et al., 2004) are induced in bone.

There is some evidence to suggest that 25[OH]D levels less than 40 $ng.ml^{-1}$ (Miller et al., 2016) or calcium intake less than 1500 $mg.d^{-1}$ (Nieves et al., 2010) are associated with increased stress fracture incidence. An individual's vitamin D status is assessed by serum concentrations of 25[OH]D, rather than 1,25$[OH]_2$D, due to its long half-life of 21-30 days (Owens et al., 2018). Supplementation over 8 weeks (2000 mg calcium and 800 IU vitamin D), even in the absence of measurement of serum levels, has been shown to lower this incidence by 20% in military recruits compared to the control group receiving placebo (Lappe et al., 2008). During exercise, both PTH and CTX increase secondary to a decrease in serum ionized calcium (Kohrt et al., 2018) (Barry et al., 2011; Sherk et al., 2017). Although the origin of the drop in serum ionized

calcium was originally attributed to dermal calcium losses (i.e., sweat calcium content) (Kohrt et al., 2019), the cause is presently unknown and likely to be multifactorial. Intravenous administration of calcium as well as consumption of a dairy-based meal or supplement prior to endurance activity attenuates the increase in PTH and CTX measured 30-120 min post-exercise (Barry et al., 2011; Guillemant et al., 2004; Haakonssen et al., 2015; Kohrt et al., 2018). Although the long-term translation of these acute changes has not been investigated, this nutrition strategy is of interest for its potential to limit bone resorption in athletes with high training loads, particularly in sports involving primarily non-weight bearing exercise.

Findings with respect to vitamin D status and BMD outcomes have been inconsistently reported, particularly in athletes and among different ethnicities (Allison et al., 2015; Hannan et al., 2008). These inconsistencies may be due to polymorphisms in the binding protein; indeed, bioavailable (loosely bound and free) vitamin D may be a more useful and accurate marker of vitamin D status (Allison et al., 2018; Owens et al., 2018), despite the current reliance on 25[OH]D measurements in guidelines and research. The Endocrine Society clinical practice guideline recommends maintaining 25[OH]D blood levels above 30 $ng.ml^{-1}$ (defined as sufficient) for the general population (Holick et al., 2011). A recent meta-analysis found the prevalence of 25[OH]D < 20 $ng.ml^{-1}$ (= deficient) in adult and adolescent elite athletes to be 30% and 39%, respectively (Harju et al., 2022). Currently, supplementation or equivalent sunlight exposure of 2000-4000 $IU.d^{-1}$ for 25[OH]D levels below 75 $nmol.L^{-1}$ or free concentrations less than 2 $ng.ml^{-1}$ is suggested in athletic populations (Owens et al., 2018). No ergogenic effect above 75 $nmol.L^{-1}$ (30 $ng.ml^{-1}$) has been found in athletes, and there is no consensus on the optimal concentration for skeletal health (Owens et al., 2018).

Iron

Animal models, and cross-sectional data in non-athletic populations, including postmenopausal women and patients with thalassemia or hereditary hemochromatosis, demonstrate an association between ferritin status and bone mineral density or turnover (Balogh et al., 2018; Chon et al., 2014; Jandl et al., 2020; Kim et al., 2012; Lu et al., 2020; Toxqui et al., 2014; Toxqui & Vaquero, 2015). Iron is known to be involved in various enzymatic reactions in the body. In bone, this includes involvement in hydroxylation of procollagen in collagen synthesis (Toxqui & Vaquero, 2015; Viguet-Carrin et al., 2006) and in the cytochrome P450 system in vitamin D metabolism (Toxqui & Vaquero, 2015). Furthermore, interactions between vitamin D and hepcidin concentrations have been observed *in vitro* (Zughaier et al., 2014) and in human trials of healthy subjects (Bacchetta et al., 2014; Smith et al., 2017), kidney disease patients

(Moran-Lev et al., 2019), and paediatric inflammatory bowel disease patients (Smith et al., 2017).

In iron overload conditions, iron increases osteoclast activity and inhibits osteoblast function (Balogh et al., 2018) and bone marrow stem-cell differentiation (Balogh et al., 2016). In athletes, however, iron deficiency is more prevalent (Nabhan et al., 2020). In iron deficiency, even with ample oxygen, hypoxia-inducible factor 1 alpha is stabilized (Siegert et al., 2015), stimulating erythropoietin (EPO) production in the kidney and upregulating FGF-23 production in osteoblasts (Wheeler & Clinkenbeard, 2019). This results in reduced $1,25[OH]_2D$, reduced intestinal absorption of calcium and phosphate, and renal reabsorption of phosphate (Wheeler & Clinkenbeard, 2019). Of interest, in mice, improved calcium absorption has been demonstrated following hepcidin administration (Kraidith et al., 2016). Hepcidin, the major regulator of iron availability in circulation, is of particular significance in athletes as it is hypothesized to contribute to the prevalence of iron deficiency in this population (Sim et al., 2019). More specifically, it peaks 3-6 h post-exercise (Peeling et al., 2009b), which might present a window during which high calcium foods or supplementation may be better absorbed. Finally, although the mechanism is uncertain, intestinal luminal iron is known to inhibit calcium absorption (Wongdee et al., 2019), requiring consideration when advising supplementation or dietary changes to correct deficiency.

These nutrient interactions and the effect on bone require further investigation. Athletes represent a unique physiological model for research where targeting nutrient timing around exercise may translate into improved bone health outcomes. The occurrence of iron deficiency in athletes is often accompanied by LEA (Petkus et al., 2017), both of which impact bone health. The potential of IL-6 as a common link between bone and iron metabolism is currently exploratory but presents an attractive hypothesis (Figure 1.3). With these links in mind, the effects of exercise and nutrition on iron metabolism are of pertinence.

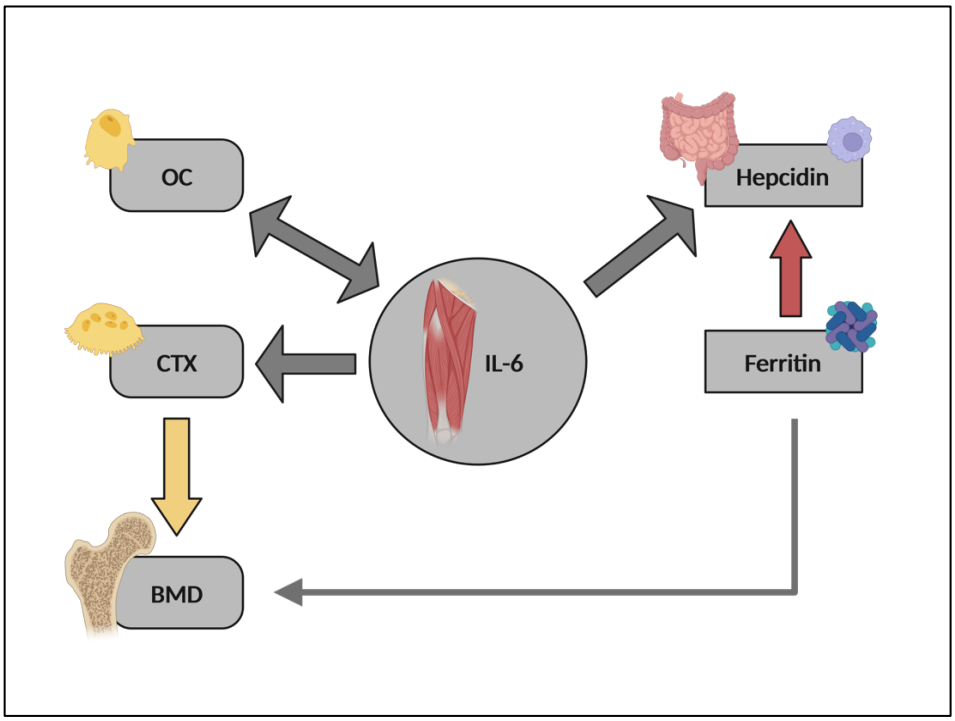

Figure 1.3: Proposed interactions between bone, muscle, and iron metabolism. BMD = bone mineral density; CTX = carboxy-terminal telopeptide; IL-6 = interleukin-6; OC = osteocalcin *(created in biorender.com)*

1.2 Iron metabolism in athletes

1.2.1 Epidemiology

Iron deficiency can occur both with and without anaemia, as well as with or without symptoms (Pasricha et al., 2021). Common symptoms include fatigue, poor concentration, dizziness, and headache (Pasricha et al., 2021), with clear evidence of impaired aerobic performance seen with anaemia (Nielsen & Nachtigall, 1998) and even hemodynamic instability occurring in severe cases (Pasricha et al., 2021). The prevalence of iron deficiency in athletes (commonly a serum ferritin < 35 $\mu g.L^{-1}$) is estimated at ~52% in women and ~15% in men, compared to ~49-57% and ~6-9% in non-athletic women and men, respectively (Nabhan et al., 2020). However, several study design issues (e.g., retrospective analyses, timing and circumstances of blood collection, ongoing treatment) suggest that the true prevalence in athletes may, in fact, be higher. Exercise-associated losses through sweat, haematuria, foot strike haemolysis, and gastrointestinal micro ischemia, as well as dietary insufficiency (e.g., LEA (Petkus et al., 2017)) contribute to the increased risk of iron deficiency (Peeling et al., 2008). Increasingly

recognized, however, is the role of exercise-induced increases in the body's master iron regulatory hormone, hepcidin (Peeling, 2010; Peeling et al., 2008; Sim et al., 2019).

1.2.2 Physiology

Iron plays several important roles in body systems that underpin the health and performance of athletes. Elemental iron exists in 3 main oxidation states in the biology: ferrous (2+), ferric (3+) and ferryl (4+) (Beard, 2001). Conversion between states serves to transfer electrons as well as participate in binding of ligands, such as oxygen, nitrogen, and sulphur (Beard, 2001). Iron is primarily involved in reactions of oxidation-reduction, electron transfer for energy metabolism, and oxygen transport (Beard, 2001). The four major classes of iron-containing proteins are hemeproteins (haemoglobin, myoglobin, cytochromes), iron-sulfur enzymes, other iron proteins (ferritin, transferrin, hemosiderin), and other iron-containing enzymes containing neither heme nor sulphur (Beard, 2001).

Haemoglobin (Hb), found in red blood cells, is comprised of two pairs of identical subunits (alpha-2 and beta-2), each of which is a helix containing a heme protein with a porphyrin ring surrounding a ferrous iron that is able to reversibly bind oxygen (Beard, 2001). The affinity to haemoglobin for oxygen is influenced by pH, partial pressure of carbon dioxide (pCO_2), phosphates, and temperature; decreased pH or higher pCO_2 in arterial blood results in increased offloading of oxygen to tissues (Beard, 2001). Myoglobin is a single-chain hemeprotein present in the cytoplasm of tissues, which facilitates the diffusion of oxygen from red blood cells into mitochondria (Beard, 2001). Finally, the cytochrome family and other enzymes involved in electron transfer require iron to function in energy metabolism (Beard, 2001).

Total body stores of iron account for ~3 g in men and ~2.5 g in women (Daher & Karim, 2017). The daily recommended dietary allowance, of which only 5-18% (depending on source and nutrient co-ingestion) is absorbed (Hurrell & Egli, 2010), is 8 mg in adult men and 18 mg in menstruating women (27 mg in pregnancy) (Institute of Medicine Panel on Micronutrients, 2001). Daily losses through the urogenital tract, faeces, and skin are typically ~1-2 mg per day, with greater amounts in premenopausal women, likely as a result of menstruation (Beard, 2001). As free radicals from excess iron are toxic and precluded from excretion due to iron's low solubility (Beard, 2001), various mechanisms operate to tightly control absorption and recycling as a means of homeostatic regulation (Beard, 2001; Daher & Karim, 2017).

Dietary iron in ferric form is converted to ferrous form at the apical border of the enterocyte by the enzyme ferrireductase, after which it is transported through the divalent metal transporter 1 (DMT-1) into the cell (Daher & Karim, 2017). From there, it is either stored as ferritin or transported into the bloodstream via ferroportin at the basal end of the cell (Daher & Karim, 2017). The ferrous form is first converted into the ferric form by hephaestin on the basal membrane, and subsequently bound to transferrin for transport in the bloodstream. Alternatively, it binds directly to ceruloplasmin in the bloodstream and is then converted into the ferric form to allow transport by transferrin (Briguglio et al., 2020). Meanwhile, the heme form of iron seems to be passively and more efficiently absorbed via heme carrier protein (Briguglio et al., 2020; Daher & Karim, 2017). At the target tissue, transferrin, usually 25-50% saturated, binds to its receptor to allow for uptake via endocytosis, and subsequent use or storage (Beard, 2001; Briguglio et al., 2020). Iron is predominantly stored as ferritin in the liver (60%) and the remainder in the muscle tissue and reticuloendothelial system (Beard, 2001).

The major regulator of iron flux is hepcidin, a peptide produced by hepatocytes (Nemeth et al., 2004b). Its synthesis is regulated by hepcidin encoding gene (HAMP1) expression via multiple pathways: (1) the bone morphogenetic protein SMAD (BMP/SMAD) pathway in response to serum (Ramos et al., 2011) or liver iron (Feng et al., 2012), (2) the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway in response to IL-6 (Wrighting & Andrews, 2006), or (3) stress erythropoiesis (Kautz et al., 2014). These pathways function to upregulate hepcidin synthesis in response to increased hepatic or serum iron and increased IL-6 levels, whereas decreased hepatic or serum iron, and increased erythropoietin downregulate hepcidin synthesis (Daher & Karim, 2017). Hepcidin controls iron efflux from enterocytes and macrophages by binding to ferroportin (Nemeth et al., 2004b) and enterocyte absorption by binding to DMT-1 (Brasse-Lagnel et al., 2011) causing internalization and degradation of these transporters.

1.2.3 Measurement

While the effect of iron deficiency on health and performance is well-known, the definition of iron deficiency is inconsistent across different populations. In the general population, iron status is classified as 1) iron repletion, 2) low iron stores, 3) absolute iron deficiency (with or without anaemia), and 4) functional iron deficiency (with or without absolute iron deficiency) (Pasricha et al., 2021) (Table 1.1.). Ferritin concentration, in either serum or plasma, is the primary marker used for diagnosis of iron deficiency with a threshold of < 15 $\mu g.L^{-1}$ being specific and < 30

$\mu g.L^{-1}$ being highly likely when coupled with a suggestive history (Pasricha et al., 2021). However, as ferritin is an acute-phase reactant, it can be elevated in the setting of inflammation, such as acute infection or autoimmune conditions (Pasricha et al., 2021). Here, a threshold of $< 70\ \mu g.L^{-1}$ is indicative of iron deficiency in adults and children older than 5 years (WHO, 2020). The measurement of hepcidin concentrations is an emerging tool for differentiating between absolute and functional iron deficiency, and for identifying the need for supplementation, the likelihood of responding, and the appropriate route of administration (Pasricha et al., 2021).

Table 1.1: Categories of iron status in the general population and associated markers

	Iron repletion	Low iron stores	Absolute iron deficiency without anaemia	Absolute iron deficiency with anaemia	Functional iron deficiency	Functional iron deficiency with absolute iron deficiency
Haemoglobin	N	N	N or L-N	L	L	L
Mean cell volume and mean cell haemoglobin concentration	N	N	N or L	L	L	L
Ferritin ($\mu g.L^{-1}$)	>30-60	15-30	<15-30	<15-30	N or H	<70-100
Transferrin saturation (%)	>20	>20	<20	<15	<20	<20
Reticulocyte haemoglobin content	N	N	L	L	L	L
Soluble transferrin receptor	N	N	H	H	N	N or H
Hepcidin	N	L-N	L	L	H relative to transferrin saturation	N or L
Bone marrow iron	N	D or A	A	A	D	A

From: Pasricha SR, Tye-Din J, Muckenthaler MU, Swinkels DW. Iron deficiency. Lancet. 2021;397(10270):233-48.

Note: A = absent, D = detectable, H = high, L = low, N = normal

In athletes, different thresholds for diagnosing iron deficiency are suggested, with the lower limit of normal based on iron absorption studies using ^{59}Fe (Nielsen & Nachtigall, 1998). The

following classification has been suggested for athletes (Clénin et al., 2015; Peeling et al., 2007; Sim et al., 2019):

- Stage 1—iron deficiency:
    - Iron stores in the bone marrow, liver and spleen are depleted, but haematopoiesis is maintained and Hb, mean corpuscular volume, and mean cellular Hb are normal
    - Ferritin < 35 $\mu g.L^{-1}$, Hb > 115 $g.L^{-1}$, transferrin saturation > 16%
- Stage 2—iron-deficient non-anaemia:
    - Impaired erythropoiesis secondary to dwindling iron supply to the bone marrow, with red cells appearing hypochromic and microcytic but Hb is still normal
    - Ferritin < 20 $\mu g.L^{-1}$, Hb > 115 $g.L^{-1}$, transferrin saturation < 16%
- Stage 3—iron-deficient anaemia:
    - Hb concentration affected resulting in anaemia
    - Ferritin < 12 $\mu g.L^{-1}$, Hb < 115 $g.L^{-1}$, transferrin saturation < 16%, soluble transferrin receptor < 2.5 $mg.dL^{-1}$

At a minimum, the classification of iron status in athletes requires the measurement of serum ferritin, Hb, and transferrin saturation (Sim et al., 2019). Ferritin is an acute phase reactant and can be highly variable depending on the timing, duration, and intensity of preceding activity (Clénin et al., 2015). For this reason, blood collection procedures should be standardized between tests, with consideration given to the time of day, hydration state, prior activity levels, and any signs and symptoms of illness (Sim et al., 2019). It is recommended that assessment be conducted 2-3 times a year in athletes, or more frequently if history and blood results indicate (Sim et al., 2019). Testing should be conducted first thing in the morning either seated or lying supine (McKay et al., 2022a). High-intensity exercise should be avoided in the 24 h before, and eccentric exercise avoided 2-3 days prior (McKay et al., 2022a). Athletes should arrive in a well-hydrated state (urine specific gravity < 1.025), with no signs or symptoms of illness or infection (McKay et al., 2022a). While menstrual cycle phase influences remain equivocal for the moment, this should be recorded and/or standardized as far as possible (McKay et al., 2022a).

Importantly, due to activity-induced limitations of iron status interpretation in athletic populations, the measurement of multiple parameters is preferable, including soluble transferrin receptor, Hb mass, and C-reactive protein (Sim et al., 2019). While useful, ferritin does not reflect the functional iron stores present in the iron-containing proteins (Malczewska et al.,

2001). Soluble transferrin receptor (sTfR), the truncated form of the cellular receptor that is upregulated in response to a deficient cellular iron pool, has been proposed as a promising marker, due to its non-response to exercise and inflammation, and its reflection of the functional iron pool (Clénin et al., 2015; Malczewska et al., 2001). Serum iron has a morning peak which is twice that of the evening, has high interindividual variability, is lowered in acute phase reactions, and raised secondary to haemolysis; thus, it is considered obsolete in isolation and only of use to calculate transferrin saturation (Clénin et al., 2015). Where practical, Hb is best assessed as mass, rather than concentrations, to account for the effects of increased plasma volume in athletes (Clénin et al., 2015). Hepcidin, although recognized as the primary regulator, has not yet been employed in routine use (Clénin et al., 2015) but, with calibration and standardization, may be an increasingly available clinical marker to guide both diagnosis and supplementation (Pasricha et al., 2021).

1.2.4 Sex differences

It has been suggested that premenopausal women have an increased requirement for iron in comparison to men, primarily because of menstrual blood loss (Harvey et al., 2005). A recent study showed that eumenorrheic exercising women had a greater prevalence of iron deficiency (ferritin < 15 ug.L^{-1}) than their amenorrheic counterparts, but this was not associated with self-reported menses (intensity or duration) or with significant differences in serum iron, haemoglobin, or dietary intake between groups (Petkus et al., 2019). Whether this is because of menstrual blood losses or menstrual cycle hormone concentrations has not yet been elucidated. Indeed, characterization of menstrual cycle status and phase has not been adequately reported or controlled for in studies involving female participants (Elliott-Sale et al., 2021). *In vitro* and animal model data suggest that estrogen suppresses hepcidin synthesis (Hou et al., 2012; Yang et al., 2012) and inhibits IL-6 gene expression (Pottratz et al., 1994). *In vivo* studies have shown a decrease in hepcidin concentrations in response to endogenous estrogen stimulation using *in vitro* fertilization pre-treatment protocols (Lehtihet et al., 2016). On the other hand, progesterone has been shown to increase hepcidin synthesis *in vitro* and *in vivo* (Li et al., 2016). The hypothesis is that hepcidin is lowest during the early follicular phase, when both estrogen and progesterone are low, allowing for increased iron availability in circulation to compensate for menstrual blood losses (Badenhorst et al., 2022). However, human studies are not conclusive. While some authors report no difference in hepcidin or ferritin between the early follicular (low estrogen and progesterone) and mid-luteal (moderate estrogen and high progesterone) phases (Zheng et al., 2021), others report lower transferrin saturation and serum

iron in the early follicular versus mid-luteal phase (Alfaro-Magallanes et al., 2023); these differences may relate to the ferritin status of the participants. Currently, however, there is insufficient evidence to conclude how menstrual cycle patterns influence iron status, at baseline and post-exercise, and whether hormone concentrations or blood loss itself is largely responsible. Furthermore, the occurrence of, and mechanisms for, differences in the exercise-induced hepcidin response between male and female athletes are unknown.

Testosterone is well-known to have significant erythropoietic and anabolic effects, which underpin the prohibition of exogenous supplementation in sport (Hartgens & Kuipers, 2004; Wood & Stanton, 2012). Testosterone appears to act through both erythropoietin (EPO) stimulation (Shahani et al., 2009) and EPO-independent hepcidin suppression to induce erythropoiesis (Bachman et al., 2010; Coviello et al., 2008). In hypogonadotropic hypogonadal men, testosterone supplementation resulted in hepcidin suppression and an increase in ferroportin, in addition to an increase in EPO (Dhindsa et al., 2016). In contrast, men receiving androgen deprivation therapy for prostate cancer exhibited reductions in Hb and Hct with an increase in hepcidin, but no change in EPO (Gagliano-Jucá et al., 2018). Follow up animal studies have confirmed the role of hepcidin in mediating testosterone's effects but suggest that hepcidin's role may be more significant when iron availability is constrained e.g., chronic inflammation, iron deficiency (Guo et al., 2020). This may be particularly relevant for athletes undergoing intense training with an increased risk of LEA, repeated elevations of IL-6, and generally lower-range testosterone levels. Recently, a study was performed in healthy men subject to an energy deficit (55% of total daily energy expenditure) in which 4 weeks of testosterone supplementation was received (Hennigar et al., 2020a). Here, the decrease in Hb and Hct was attenuated with associated reductions in hepcidin and ferritin and an increase in EPO, when compared to those receiving placebo (Hennigar et al., 2020a). In summary, testosterone facilitates iron availability and incorporation into red blood cells through EPO stimulation, hepcidin suppression, and ferroportin upregulation. In situations of limited iron availability, the role of hepcidin in mediating testosterone's effects may be even more significant. In athletes, particularly those who may exhibit lower-range testosterone levels, the risk of iron deficiency is heightened by the lack of hepcidin suppression, which may be further exacerbated by states of LEA and dietary iron insufficiency.

1.2.5 Effect of exercise on iron regulation

An increased risk of iron deficiency in athletes compared to the sedentary population is largely attributed to a variety of exercise-specific iron losses and iron metabolism regulatory processes. Traditionally, gastrointestinal micro-ischemia (Peters et al., 2001) was assumed to be the predominant mechanism of increased losses, with sweat (Waller & Haymes, 1996) and haematuria (Jones & Newhouse, 1997) contributing negligible amounts (Clénin et al., 2015). Foot strike haemolysis (Telford et al., 2003) is another mechanism of iron loss, although subsequent salvage by macrophages is associated with preservation of the liberated iron (Clénin et al., 2015). However, the identification of hepcidin as a key regulatory hormone (Nemeth et al., 2004b) has focused significant attention on its associated post-exercise increases (Peeling et al., 2009b; Roecker et al., 2005) as a contributor to the increased prevalence of iron deficiency in athletes.

Following a single bout of exercise, hepcidin concentrations typically peak after 3-6 h (Peeling et al., 2009b). Of note, this is preceded by an immediate post-exercise increase in the pleiotropic cytokine IL-6 (Peeling et al., 2009b). In healthy humans at rest, injection of recombinant human IL-6 was demonstrated to result in a 7.5-fold increase in urinary hepcidin (Nemeth et al., 2004a). Similarly, injection of lipopolysaccharide induced a peak hepcidin response 3 h following the IL-6 peak concentration (Kemna et al., 2005). This hepcidin response aims to sequestrate iron in the setting of inflammation/infection, reducing the iron availability in circulation for presumed microbes (Ganz, 2003). Notably, the post-exercise increase in hepcidin is positively associated with both immediate post-exercise IL-6 as well as baseline ferritin status (Peeling et al., 2017; Peeling et al., 2014) supporting the role of hepcidin in regulating circulating iron availability in response to body stores (Nemeth et al., 2004b).

In the setting of exercise, post-exercise increases in IL-6 are derived from the muscle as opposed to immune cells (Pedersen et al., 2000; Pedersen et al., 2001; Starkie et al., 2001b; Steensberg et al., 2000) or adipose tissue (Lyngsø et al., 2002), with possible contributions from brain (Nybo et al., 2002) and peritendon (Langberg et al., 2002). Interleukin-6 release is purported to serve to increase liver glucose output (Febbraio et al., 2004), increase glucose transporter 4 (GLUT4) translocation and insulin-stimulated glucose uptake (Carey et al., 2006), and increase lipolysis and free fatty acid availability (van Hall et al., 2003). Therefore, the magnitude of increase is larger with longer duration exercise (Fischer, 2006) and in glycogen depleted states (Febbraio et al., 2003; Keller et al., 2001; Steensberg et al., 2001). The first study to compare the effect of exercise as opposed to a resting state on both IL-6 and hepcidin was conducted by Peeling and colleagues (Peeling et al., 2009b). Here, blood and urine samples were taken pre-

trial, and 0 h-, 3 h-, 6 h-, and 24 h-post trial during either 60-min running or rest (Peeling et al., 2009b). Interleukin-6 was 6.9-times greater at 0 h post- than pre-exercise with urinary hepcidin being 1.7-3.1 times greater at 3-24 h post than pre- or 0 h post-exercise (Peeling et al., 2009b). Furthermore, there was no difference between the running and rest trial hepcidin concentrations at the 6 h post run time point (Peeling et al., 2009b), suggestive of the diurnal pattern of hepcidin (Troutt et al., 2012). In fact, at rest, a 2- to 6-fold increase in hepcidin has been demonstrated to occur between 06h00 and 15h00 (Kemna et al., 2007; McCormick et al., 2019). Moreover, augmentation with exercise during the afternoon seems to amplify already higher hepcidin concentrations (McCormick et al., 2019). The pattern of change in hepcidin concentrations following repeated exercise sessions with short recovery is currently unknown, however.

Subsequent studies have investigated the effect on post-exercise hepcidin responses of different exercise durations (Newlin et al., 2012), modalities (Sim et al., 2013), and intensities (Peeling et al., 2009c; Sim et al., 2013), training surfaces (Peeling et al., 2009c), oxygen availability (Badenhorst et al., 2014; Goto et al., 2018; Govus et al., 2014), heat (Hayashi et al., 2020; McKay et al., 2021a; Zheng et al., 2021), menstrual cycle (Barba-Moreno et al., 2022; Zheng et al., 2021), oral contraception (Alfaro-Magallanes et al., 2021; Sim et al., 2015), and nutritional interventions (Badenhorst et al., 2015a; Badenhorst et al., 2016; Dahlquist et al., 2017; Díaz et al., 2015; Hayashi et al., 2018b; McKay et al., 2020; McKay et al., 2021b; McKay et al., 2021c; McKay et al., 2019a; McKay et al., 2019b; Robson-Ansley et al., 2011; Sim et al., 2012). As each of these studies has employed a single intervention and largely controlled for other potentially influential variables, the relative contribution of these variables to the hepcidin response is currently unknown.

1.2.5.1 Impact of exercise characteristics

Several studies have explored the effect of manipulating single exercise characteristics. Newlin and colleagues compared a 60-min and 120-min exercise bout at the same intensity (65% VO_2max) on hepcidin responses in women, finding greater increases with the longer exercise duration (Newlin et al., 2012). Both Sim and colleagues and Peeling and colleagues investigated the effect of altering intensity (65% vs 85% vVO_2peak and 75-80% vs 90-95% vVO_2peak, respectively) in well-trained male athletes, finding a greater post-exercise IL-6 with the higher intensity running trial (Peeling et al., 2009c; Sim et al., 2013). However, this failed to translate into a difference in 3 h hepcidin concentrations between intensities. In the same study, Sim and colleagues compared the modalities of running and cycling. Similarly, no difference between

modalities was found for either immediately post-exercise IL-6 or 3 h post-exercise hepcidin concentrations (Sim et al., 2013). Of note, the trials conducted by Sim and colleagues and Peeling and colleagues were all < 60 min in duration. Therefore, as an exercise session is a combination of duration, intensity, and mode, the interaction between these exercise characteristics needs to be considered. Indeed, ascertaining the most contributory characteristic might enable improved tailoring of iron consumption when absorption is more likely to occur.

1.2.5.2 Impact of multiple training sessions

The only study to our knowledge that has investigated the effect of multiple training sessions on the hepcidin response to exercise is that of Peeling and colleagues (Peeling et al., 2009a). Here, participants completed a continuous 10-km run (70% vVO_2peak) and a 10 x 1 km interval run (90% vVO_2peak) 12 h later. When compared to the trial involving a single interval run session only, no cumulative effect on either IL-6 or hepcidin responses was observed. However, this scenario where sessions are separated by 12 h may not represent the training practices of certain athletes. In sports like triathlon or rowing, 2-3 prolonged workouts may be undertaken daily, with sessions commencing within the range of peak hepcidin (3-6 h) from the previous session. Indeed, Ronsen and colleagues have previously shown significantly more pronounced elevations in IL-6 (69%) following a second cycling session of 65 min, compared to the first session, after only a 3 h recovery period (Ronsen et al., 2002). It is plausible that this may translate into amplified increases in hepcidin, particularly if the second session extends later into the day. Should this occur, these athletes may be at increased risk for iron deficiency with shorter windows of opportunity for intestinal iron absorption. Additionally, with higher training loads, these athletes may be more at risk of low EA, further exacerbating the downstream effect on body systems and the potential development of anaemia.

1.2.5.3 Impact of carbohydrate availability on iron regulation

Both dietary and training session manipulation to alter carbohydrate availability is commonly practiced in pursuit of metabolic adaptation (Heikura et al., 2018a). The presence of adequate muscle glycogen or exogenous glucose attenuates the release of IL-6 from the muscle. Numerous studies have shown that CHO ingestion during exercise, rather than immediately before (Lancaster et al., 2003) or afterward (Badenhorst et al., 2015b; Miles et al., 2007), attenuates the post-exercise IL-6 increase. This finding is most apparent in sessions lasting longer than 90 min (Febbraio et al., 2003; Nieman et al., 2005; Nieman et al., 1998; Robson-

Ansley et al., 2009b; Robson-Ansley et al., 2011; Sale et al., 2015; Scharhag et al., 2006) compared to sessions less than 90 min (Sim et al., 2012; Timmons et al., 2004). Indeed, IL-6 mRNA is not decreased in the presence of adequate glycogen (Febbraio et al., 2003; Starkie et al., 2001a) where the stimulus to increase glucose and free fatty acid availability and uptake is not required. While it appears that post-exercise hepcidin responses mirror IL-6 responses to carbohydrate (Pasiakos et al., 2016; Sim et al., 2012), this may be dependent on starting glycogen status. If insufficient CHO has been consumed to replenish muscle glycogen in the days leading up to an exercise bout lasting > 90 min, as illustrated in the case of keto-adapted athletes, CHO consumption before (2 $g.kg^{-1}$ BM) and during (~70 $g.h^{-1}$) exercise fails to attenuate post-exercise IL-6 (McKay et al., 2019b). Interestingly, neither a short (4 day) period of low CHO availability nor subsequent pre- and during exercise CHO restoration affected the hepcidin response to > 90 min exercise (McKay et al., 2019b). In contrast, however, following a glycogen depleting task the day prior, performing a 60 min cycle at 65% maximal mean power, as opposed to a 60 min run at 55% vVO_2, was shown to result in a substantial increase in hepcidin with no difference in IL-6 (McKay et al., 2020). Therefore, while IL-6 has an influence on the hepcidin response to exercise, other factors (e.g., baseline ferritin, exercise intensity, prior days' nutrition) certainly deserve consideration as well.

Longer term trials investigating chronic CHO manipulation in athletes are scarce. Recently, McKay and colleagues investigated the effects of a 3-week intervention in elite racewalkers consuming either a high CHO or a ketogenic LCHF diet on iron metabolism across a ~2 h long racewalk (McKay et al., 2019a). Compared to the high CHO group, the LCHF group demonstrated a greater IL-6 response immediately post-exercise, attributed by the authors to likely reduced glycogen levels (McKay et al., 2019a). A trivial increase in the 3 h post-exercise hepcidin response was seen following the LCHF intervention, yet this was attenuated in the high CHO group (McKay et al., 2019a). However, the LCHF group had higher resting ferritin levels (McKay et al., 2019a). Subsequently proposed in a follow-up study by this group investigating the effect of acute CHO restoration (McKay et al., 2019b), this likely played a more dominant role in the observed higher hepcidin response following the intervention than did the change in IL-6.

1.2.5.4 Impact of energy availability on iron metabolism

Few studies have examined the impact of low energy availability on iron metabolism. Here, it is difficult to differentiate whether the effects are directly due to energy status, the associated

reduction in CHO availability, or the reduction in dietary iron intake. In both low energy and low CHO availability, low muscle glycogen may be driving the resultant effects on IL-6 concentrations. However, inconsistent results suggest that additional mechanisms may be at play.

Pasiakos et al. showed no significant difference in post-training IL-6 or hepcidin concentrations between groups of soldiers subjected to either an energy-matched or ~55% energy deficit over a 4-day ski march (Pasiakos et al., 2016). However, a significant negative association between hepcidin concentrations and energy balance was found (Pasiakos et al., 2016). On the other hand, Hammond et al. reported greater elevations in immediate and 3 h post-run IL-6 concentrations in a group consuming a low energy diet (~20 kcal.kg^{-1} FFM), yet similar muscle glycogen concentrations to a group following a low CHO diet (~59 kcal.kg^{-1} FFM) (Hammond et al., 2019). Additionally, despite similar muscle glycogen concentrations immediately post-exercise followed by significantly different values at 3 h between the high- and low-CHO groups (both ~58-59 kcal.kg^{-1} FFM), there was no significant difference in IL-6 concentrations at either time points (Hammond et al., 2019). Potential explanations for these discrepant results include a threshold effect of muscle glycogen on muscle IL-6 release, or a direct effect of CHO intake on IL-6 release.

More recently, Ishibashi and colleagues compared a 3-day LEA diet (~20 kcal.kg^{-1} FFM) with a normal EA diet (~45 kcal.kg^{-1} FFM) in male runners, showing greater immediately post-exercise IL-6 concentrations and associated reduced resting muscle glycogen levels in the former trial, but no difference between groups in 3 h post-exercise hepcidin concentrations (Ishibashi et al., 2020). Although the area under the curve for hepcidin over the full trial period was significantly larger in LEA with a large effect size of d=1.25, the ferritin concentrations being significantly greater on day 4 in the LEA group are a notable confounding factor. A similar cross over study design was employed by Hennigar and colleagues investigating the effect of energy balance and energy deficit (45%) conditions during a 72 h military training protocol (Hennigar et al., 2020b). Here, fasting hepcidin was greater on day 3 in the energy deficit trial compared with the energy balance trial, with concomitant reduction in muscle glycogen yet no difference in fasting IL-6 concentrations (Hennigar et al., 2020b). Interestingly, iron absorption, measured via stable iron isotope consumption, was reduced in both trials, albeit by a greater magnitude in the energy deficit trial. This suggests that additional exercise-induced intestinal changes may contribute to impaired iron absorption in addition to the effects of hepcidin. Both the Ishibashi et al. (Ishibashi et al., 2020) and Hennigar et al. (Hennigar et al.,

2020b) studies lead to the observation that the acute-phase response and IL-6 independent gluconeogenic stimuli may work together in upregulating hepcidin during physical activity, particularly when energy stores are depleted. Since the energy-deficient conditions in both these studies also necessitated a proportionate reduction in CHO availability, it is difficult to distinguish or attribute the source of the associated effects, emphasizing the need for a trial employing a direct comparison of these conditions.

In summary, the hepcidin response to exercise is affected by multiple characteristics involving both the athlete and the exercise session, the interaction of which has not been carefully investigated. Furthermore, the IL-6 and hepcidin response to repeated bouts of exercise over the day where both energy and macronutrient availability considerations are involved deserves attention. Elucidation of such factors will enable practitioners to evaluate the risk of iron deficiency for each individual athlete, and tailor nutrition and training strategies accordingly to meet the demand.

1.3 Summary and future direction

Despite the well-known benefits of exercise, specific scenarios that occur in sport are now recognized as potentially harmful to health and performance. The Female Athlete Triad raised awareness of the potential reproductive and bone health impacts of disordered eating in female athletes (Nattiv et al., 2007). However, recognition that men were also affected and that the health consequences extended beyond bone led to the broader REDs (Mountjoy et al., 2018; Mountjoy et al., 2014; Mountjoy et al., 2023; Tenforde et al., 2016). Iron deficiency has been proposed as both a marker of energy deficiency and a potentiator of the metabolic, reproductive, and bone consequences (Petkus et al., 2017). This hypothesis is strengthened by evidence that exercise promotes the release of hepcidin (Peeling et al., 2009b), which may be exacerbated through larger increases of IL-6 in a glycogen depleted state (Steensberg et al., 2001), such as LEA or repeated training bouts (Ronsen et al., 2002).

While the bone health consequences of LEA have been explored in sedentary and exercising women (Barrack et al., 2014; De Souza et al., 2008; Heikura et al., 2018b; Ihle & Loucks, 2004; Papageorgiou et al., 2017; Papageorgiou et al., 2018b; Tenforde et al., 2018), little is known about elite athletes and men. Furthermore, because a reduction in EA is usually associated with a concomitant reduction in CHO, it is difficult to discern the extent to which each plays a role in disturbing iron and bone metabolism. Chronic adherence to ketogenic LCHF diets has been

shown in animal models (Simm et al., 2017; Williams et al., 2019; Wu et al., 2019; Wu et al., 2017; Zengin et al., 2016) and epileptic patients (Bergqvist et al., 2008; Draaisma et al., 2019) to have possible detrimental effects on bone, with preliminary evidence of unfavourable bone turnover in elite athletes (Heikura et al., 2019). These observations merit further enquiry due to the interest in both acute and chronic manipulation of CHO availability for training adaptation and performance goals.

The interconnectedness of energy, macro- and micronutrient availability, and bone and iron metabolism outlined in this review form the basis of the investigations undertaken for this thesis. In Chapter 3, a cross-sectional analysis exploring the association between markers of bone and iron status, under standardized nutrition conditions, will provide insight into the potential interactions between these systems. Chapter 4 an interventional study will investigate the relative impact of energy and CHO in the bone turnover response at rest and across exercise. Chapters 5 and 6 take a further look into iron metabolism with its implications on bone health. Chapter 5 employs a systematic review and meta-analysis analysing factors influencing the IL-6 and hepcidin response to exercise. Finally, Chapter 6 extends these findings to look at the impact of performing exercise in close succession where the IL-6 and hepcidin response could be amplified.

2 Chapter 2: Methodology

As per Australian Catholic University guidelines, a methodology chapter describing the methods used in each study in full is included. However, each chapter includes its own methods section as written for the respective journal for which the manuscript was prepared.

2.1 Study 1

Bone turnover markers and bone mineral density values in a convenience sample of elite endurance athletes

2.1.1 Study design

This study is a cross-sectional study using baseline data from athletes who participated in various intervention studies conducted by this research group between November 2015-January 2022. November 2015 was selected as the start date as it represented the commencement of a series of studies by the research group involving elite athletes, where a standardised protocol for blood collection and metric analysis was used. This provided a convenience sample where data mining could be instituted with confidence in the circumstances under which the data were captured. Specifically, studies in which iron and bone markers were measured via venous blood sample collection prior to and following exercise were included. Data sets [149 male (27.3±4.8 years) and 17 female (26.1±3.9 years)] from high performance athletes involved in racewalking, running, rowing, and triathlon were utilized (Table 3.1), with the number of participants contributing to each analysis varying according to the study design and completeness of data capture.

2.1.2 Ethics approval

Ethics approval for the conduct of the individual studies was granted by either the Ethics Committee of the Australian Institute of Sport (AIS: 20150802, 20161201, 20170401, 20171203, 20181203, 20200905) or the Australian Catholic University (ACU: 2020-238HC, 2021-36HC). Ethics approval for the retrospective analysis of data from individual studies already completed was granted by the Australian Institute of Sport Ethics Committee (ref: 20200401). Where studies were still due to take place, explicit consent for use of data in this project was provided by athletes.

2.1.3 Data extraction

Athlete characteristics (age, sex, habitual sport, VO_{2max}, body composition, and bone mineral density), exercise session characteristics (modality, duration, intensity as a percentage of VO_{2max}), and results (resting ferritin, resting and immediately post-exercise IL-6, resting and 3 h post-exercise hepcidin, fasting/pre-exercise/1 h post-exercise CTX and P1NP) were extracted from the baseline phase/control arm of 10 research-embedded training camps. The athletes were typically Tier 4 (Elite) competitors based on the McKay system for standardizing athlete calibre and training status (McKay et al., 2022b), although some individuals met Tier 3 (well-trained) and Tier 5 (world class) status. All bone turnover markers were measured following at least 24 h of a standardized nutrition protocol as previously described (Fensham et al., 2022a; Fensham et al., 2022b; Lundy et al., 2022a; McKay et al., 2023; McKay et al., 2020; McKay et al., 2021b; McKay et al., 2021c; Mirtschin et al., 2018).

2.1.4 Bone mineral density measurement

Dual energy x-ray absorptiometry (DXA) of the lumbar spine and proximal femur was performed at the time of the individual studies. While all scans were performed using the same machine type (Lunar iDXA), 11 (6.8%) of these were performed on a machine based at the Australian Catholic University (Fitzroy, VIC, Australia) with the remainder performed on a machine at the Australian Institute of Sport (Bruce, ACT, Australia). The scan images were re-analysed (enCORE version 18; GE Healthcare, Chicago, IL, USA) for this study by one author (NF), and the body composition scans were re-analysed using best practice protocols (Australian Institute of Sport, 2023). Two femur and one lumbar spine scans were unable to be reanalysed due to initial positioning errors and a large intervertebral BMD z-score discrepancy, respectively. A further 3 BMD and 1 body composition scan data were unavailable, and 12 body composition scans were excluded from analyses as the subject was too tall for the machine. The reference database used for z-score calculations was the Australia Combined Geelong/Lunar, matched for age, sex, and ethnicity. Due to the lack of a z-score reference database for those under the age of 20, 9 scans were excluded from analyses using BMD. Bone mineral density was classified as either "normal" or "low" (lumbar spine or proximal femur z-score ≤ -1 (Nattiv et al., 2007)). Two athletes had a normal z-score at one site but did not have a scan of the other site and were excluded from classification. Further, any discordance between sites (i.e., z-score ≤ -1 at one site but > -1 at the other site) was noted. For 8 athletes, 2 of which were classified "low", L2-4 BMD and z-scores was used to evaluate discordance, due to positioning errors with image capture

precluding the use of L1-4. Total n for body composition was 135 males and 16 females (Table 3.2) and for BMD 136 males and 16 females (Table 3.3B).

2.1.5 Blood analysis

Due to the retrospective cross-sectional nature of this study, blood analysis was not performed as a batch. Rather, the results and corresponding coefficients of variation (CV; where applicable) specific to the analysis performed at the time of the individual study were extracted. In addition, the method used was noted to account for the uncertainty around assay harmonization (Bhattoa et al., 2021; Cavalier et al., 2021; Cavalier et al., 2019). Details of the assay used, and the CV obtained in each analysis are included are provided in Table 2.1.

2.1.6 Statistical analysis

Statistical analysis was executed in R version 4.2.0 (R Core Team, Vienna, Austria) with significance set at $p<0.05$. Age was categorized in two ways: 1) < 20, 20-24, 25-29, 30-34, 35+; 2) < 24, ≥ 25 years old. Ferritin values > 350 $ug \cdot L^{-1}$ (n=5), indicating hyperferritinemia, were removed and the remaining values coded according to the following range categories: <30, 30-50, 50-100, >100 $ug \cdot L^{-1}$. These groups were chosen based on The Royal College of Pathologists Australia definition of iron deficiency (Iron Studies Standardised Reporting Protocol Working Group, 2021), in combination with athlete (Peeling et al., 2014) and isotope fractional iron absorption studies (Galetti et al., 2021). All BMD values were included in descriptive data to maximize sport representation within normative values. However, when the time between DXA assessment and collection of blood variables was > 12 months, these data were not analysed together, and similarly for body composition data and blood variables separated by > 1 month. Participant age and VO_2max among sports were compared with one-way analysis of variance (ANOVA) and Welch's, respectively, using the 'car' package, after checking normality with the Shapiro-Wilk test and homogeneity of variance with the Fligner-Killeen test ('stats' package).

Correlations between 1) fasting CTX/P1NP and age, 2) fasting CTX/P1NP and lumbar spine/proximal femur BMD, 3) fasting CTX/P1NP and resting IL-6/hepcidin/ferritin, 4) post-exercise IL-6 and 1 h post-exercise CTX/P1NP, 5) resting ferritin and lumbar spine/proximal femur BMD, 6) total/lean/fat mass and total/spine/femur BMD, 7) lean mass and post-exercise IL-6, and 8) bone mineral content and fasting CTX/P1NP were calculated using Kendall's Tau correlation ('stats' package; ≤ ± 0.19 = weak, ± 0.20-0.29 = medium, ≥ ± 0.35 = strong (Botsch, 2011)).

Within sport and between site absolute BMD and z-score differences were compared using linear mixed models with sport and measurement site as fixed terms and subject as a random effect ('lme4' package); sex was not included due to non-significance and lack of representation in 2 of 4 sports. Discordance between sites as a proportion of "low" BMD classifications was calculated.

Generalized additive modelling, with subject as a smooth term and random effect, was used to compare 1) fasting CTX/P1NP values between ferritin groups, 2) fasting CTX/P1NP between BMD classifications, 3) resting hepcidin/ferritin between BMD classifications, 4) total/lean/fat mass and bone mineral content between sport and sex, and 5) the significance of sport when controlling for total and lean mass.

Finally, mixed-effects linear regression using the 'nlme' package was employed to construct the following models:

1. Fasting CTX or P1NP as the outcome variable with age (or age category), sex (2 levels: male and female), American College of Sports Medicine (ACSM) BMD classification (Nattiv et al., 2007) (2 levels: normal or low), assay method (3 levels: Cobas [Roche Diagnostics, Germany], iSYS [Immunodiagnostics Systems, UK], ELISA [Novatein Biosciences, USA]), resting IL-6 and ferritin/hepcidin concentrations as fixed effects in a random intercept per subject model. Despite fasting CTX and resting iron markers being ~2 h apart in most studies, it must be noted that no significant change in iron markers occurs between fasting and fed (i.e., resting) (Fensham et al., 2022b). Ferritin or hepcidin with and without BMD classification were tested for significance in separate models initially – as none proved significant, stepwise regression was performed from the largest number of subject data. Nesting subject within study did not significantly improve the model yet culminated in the same variables for best fit.
2. CTX or P1NP at 1 h post-exercise as the outcome variable with cantered pre-exercise CTX or P1NP, respectively, as a covariate to account for aggregation bias and assay differences, in a random slope and random intercept per study model. Age; sex (2 levels: male and female); post-exercise IL-6; exercise modality (2 levels: racewalking and cycling); exercise duration; exercise relative intensity; and ACSM BMD classification (2 levels: normal or low) were included as fixed effects.

These models included a heterogenous residual variance structure fit per study to estimate the within-study variance. Stepwise regression was performed using the 'stepAIC' function from the 'MASS' package; further reduced models were also constructed via elimination of variables

sequentially according to their contribution to AIC. All models were fit using maximum likelihood estimation and compared using both AIC and BIC, and the model with the lowest AIC/BIC values was chosen as the final model. This final model was refitted with restricted maximum likelihood estimation and model diagnostics verified via visual inspection of residual and QQ plots. Marginal and conditional R^2 values for the final models were calculated as a proportion of the model explained by the fixed effects alone (marginal R^2), or by fixed and random effects combined relative to the model's total variance (conditional R^2), using the 'MuMIn' package. Where significant differences were detected, post-hoc tests were performed with Tukey's Honestly Significant Difference adjustment. Significant parameters identified from the fasting models were used as subgroups in calculating reference ranges (central 95%).

Table 2.1: Method of analysis and corresponding study coefficient of variation at time of analysis for each marker						
		CTX	**P1NP**	**IL-6**	**Ferritin**	**Hepcidin**
Reference						
(Heikura et al., 2019; McKay et al., 2019a)	**Assay**	IDS-iSYS	IDS-iSYS	ELISA	Roche Cobas	WCX-TF MS
	n	28	28	53	52	52
	Study CV (%)	6.2	4.6	5.9		
(McKay et al., 2020)	**Assay**	ELISA*	ELISA*	ELISA	Roche Cobas	WCX-TF MS
	n	11	11	11	11	11
	Study CV (%)	<10	<10	5.7		
(McKay et al., 2021b)	**Assay**			ELISA	Roche Cobas	WCX-TF MS
	n			20	21	21
	Study CV (%)			4.9		
(Fensham et al., 2022b; Lundy et al., 2022a)	**Assay**	Roche Cobas		ELISA	Roche Cobas	IDxi ELISA
	n	16		16	15	15
	Study CV (%)	4.2		3.4		4.9
(Fensham et al., 2022a; McKay et al., 2021c)	**Assay**	Roche Cobas	Roche Cobas	ELISA	Roche Cobas	IDxi ELISA
	n	24	24	28	28	28
	Study CV (%)	4.2	3.0	3.0		5.9
(McKay et al., 2023)	**Assay**	Roche Cobas		ELISA	Roche Cobas	
	n	9		10	10	
	Study CV (%)	4.2		6.0		

Note: CV = coefficient of variation, CTX = carboxy-terminal telopeptide, ELISA = enzyme-linked immunosorbent assay, IDS = Immunodiagnostic Systems, IL-6 = interleukin-6, P1NP = procollagen-1 N-terminal propeptide. N represents number of available data points with resting concentrations of respective analytes. *unpublished data analysed by an external laboratory with strict protocols ensuring CV values of less than 10%

2.2 Study 2

Short-term carbohydrate restriction impairs bone formation at rest and during prolonged exercise to a greater degree than low energy availability

2.2.1 Participants

Twenty-eight elite male racewalkers, eligible for participation in either national or international competition, were recruited for this study via convenience sampling. Based on previous work (Heikura et al., 2019) investigating BTM responses to either a high carbohydrate or ketogenic diet, it was estimated that including 5-10 participants per group (osteocalcin: *d*=2.00; CTX: *d*=1.52; P1NP: *d*=1.27) was appropriate to detect statistical significance with an alpha of 0.05 and power of 0.8 (GPower version 3.1.9.6). Screening of hormonal and metabolic health was conducted prior to the start of the study – no exclusions were required on the basis of these results. No athletes had a known medical condition or were taking medication or supplements during the study, and recent fractures within the past 3 months were ruled out. All athletes completed the study. Athlete characteristics are presented in Table 4.1. Athletes took part in one of two separate training camps held in January 2019 in Canberra, Australia (n=20) and January 2021 in Melbourne, Australia (n=8). Written informed consent was obtained following explanation of the risks and requirements of the study. The study conformed to the standards required by the Declaration of Helsinki. Ethics approval was obtained from the ethics committees of the Australian Institute of Sport (2019; ref: 20181203) and the Australian Catholic University (2021; ref: 2020-238HC).

2.2.2 Study protocol

This study was a parallel group design. Each training camp comprised of two, 6-day phases (Figure 4.1). During phase 1 (Baseline), all athletes adhered to a high carbohydrate (~65% of total energy intake), high energy availability (> 40kcal.kg^{-1} FFM.d^{-1}) control (CON) diet. For phase 2 (Adaptation), athletes were assigned to one of three diets: high carbohydrate/high energy availability (CON, n=10), low carbohydrate/high fat/high energy availability (LCHF, n=8), or low energy availability (LEA, n=10). Due to the inability to blind participants to the diet, allocations to dietary interventions were based on athlete preference whilst matching for individual characteristics (age, 20 km personal best time, training status). During both phases, a structured training plan was followed to ensure similar training volume and intensity among groups (McKay et al., 2021c). On the final day of each phase, a 25 km racewalking protocol was performed, where venous blood samples were taken to measure BTMs.

2.2.3 Baseline bone mineral density

Hip and lumbar spine (L1-4) BMD were measured at baseline via dual-energy x-ray absorptiometry (DXA) with Lunar iDXA machines (version 16; GE Healthcare, Australia). Measurements were conducted by experienced practitioners certified in clinical bone densitometry. As body composition measurements were performed simultaneously, best practice protocols were followed (Nana et al., 2015).

2.2.4 Dietary intervention

A brief description of dietary control is provided here; full details are specified elsewhere (manuscript in preparation). All meals were formulated and compliance with intake was monitored by a team of accredited sports dietitians, chefs, and nutritionists. Meals were devised using FoodWorks Professional Edition 9 (Xyris Software, Brisbane, Australia). The CON diet targeted an energy availability of ~40 kcal.kg^{-1} FFM.d^{-1}, comprised of 65% carbohydrate, 15% protein, and 20% fat. The LCHF group was set the same energy availability target, but with low carbohydrate (< 50 g.d^{-1}), moderate protein (2.2 g.kg^{-1}.d^{-1}) and high fat [remainder (~80%) of target energy] intakes. The LEA group had a target energy availability of ~15 kcal.kg FFM.d^{-1}, with a similar macronutrient composition as CON (60% of energy from carbohydrate, 25% of energy from protein, 15% of energy from fat). Calcium in the pre-exercise meal, the other dietary characteristic known to acutely affect markers of bone turnover (Lundy et al., 2022a), was minimised (< 50 mg) in each of standardized meals consumed prior to the long walk test protocol.

Energy availability was calculated as the difference between energy intake and exercise energy expenditure, normalized to fat free mass (measured via DXA). Exercise energy expenditure was estimated from a 4-stage incremental economy test, using collected respiratory gases inputted into the Weir equation (Weir, 1949). Resting metabolic rate was measured directly and subtracted from these values, which were then converted to prospective caloric estimates per kilometre. Cross-training sessions were accounted for in metabolic equivalents. Total exercise energy expenditure was predicted by multiplying these values by the planned training sessions, and energy intake requirements calculated accordingly to achieve target energy availability. Training session completion was monitored twice daily and meals adjusted as necessary. Non-exercise activity thermogenesis was not considered significant in this context. As a fortunate consequence of this research camp environment, on-site researchers were able to monitor and confirm that athletes undertook very little activity outside of the prescribed training times.

2.2.5 Test protocol

On the last day of each dietary phase, a 25 km racewalk was performed, combining both field and laboratory components. One athlete completed only 19 km as he was a junior athlete (18 years old). Athletes arrived in the morning following an overnight fast. An intravenous catheter was inserted and the first blood sample was drawn (±06h30). Athletes were then provided with a standardized breakfast, consisting of 2 $g.kg^{-1}$ body mass of carbohydrate for all groups in the Baseline phase. The same breakfast was provided to the CON group in the Adaptation phase, whereas the LCHF group received an isocaloric high-fat (~80%) option and the LEA group consumed a meal containing 1 $g.kg^{-1}$ body mass carbohydrate. A pre-exercise blood sample was taken (±08h30) 15 min prior to the onset of the racewalk, which commenced 2 h after breakfast. Kilometres 1, 7, 13, 19, and 25 were performed on a treadmill at a pace equivalent to ~75% of the athlete's VO_2max and the remaining kilometres were performed at a consistent, self-nominated pace on a flat, outdoor, road circuit. Carbohydrate gels were consumed following each treadmill bout, totalling ~60 $g.h^{-1}$ for all groups during Baseline and CON during Adaptation. The LEA group consumed the equivalent of 30 $g.h^{-1}$ during Adaptation and the LCHF group ingested isocaloric (to CON) high fat snacks. Water was consumed *ad libitum*. Venous cannulas were flushed with ~3 ml saline after each treadmill bout. Environmental temperature and relative humidity were recorded at 30 min intervals with a final individualized value averaged across the exercise bout. Upon completion of the racewalk protocol, another venous blood sample was collected (±10h30). At 30 min post-exercise, athletes received a standardized recovery shake (1.5 $g \cdot kg^{-1}$ body mass carbohydrate and 0.3 $g \cdot kg^{-1}$ body mass protein for all groups at Baseline, or an isocaloric high-fat low-carbohydrate option for LCHF and 0.75 $g.kg^{-1}$ body mass carbohydrate for LEA at Adaptation). A further blood sample was collected at 1 h post-exercise (±11h30) after which lunch was provided in accordance with trial phase and dietary allocation. A final blood sample was collected at 3 h post-exercise (±13h30).

2.2.6 Blood analysis

Blood samples were taken at rest (fasted), pre-exercise, and immediately, 1 h, and 3 h post-exercise. Samples were collected into BD Vacutainer SST II tubes (East Rutherford, NJ, USA), which were left to clot at room temperature for 30 min prior to being centrifuged at 1500g at 4°C for 10 min. Serum was aliquoted into 1 ml Eppendorf tubes and frozen at -80°C for batch analysis. Concentrations of beta-isomerized carboxy-terminal telopeptide (CTX), procollagen-1 N-terminal peptide (P1NP), carboxylated osteocalcin (gla-OC), and undercarboxylated osteocalcin (glu-OC) were measured from each sample. CTX and P1NP concentrations were

assessed by electrochemiluminescence immunoassay (Cobas e411, Roche Diagnostics, Basel, Switzerland). Carboxylated and undercarboxylated osteocalcin measurements were performed using enzyme immunoassay (EIA) kits (Takara Bio inc., Shiga, Japan) analysed on a FLUROstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany). Calculated coefficients of variation were 4.2% (CTX), 3.0% (P1NP), 10.7% (glu-OC), and 3.9% (gla-OC).

2.2.7 Statistical analysis

Statistical analysis was performed using R Studio (v1.4.1106, R Core Team, 2021) with significance set at $p<.05$. Athlete characteristics, differences in nutrient intake, and energy availability between groups and phases were analysed with a two-way analysis of variance (ANOVA; parametric) after verifying normality with the Shapiro-Wilk test. Where normality was violated (age only), the Kruskal-Wallis test (non-parametric) was used for between-group comparisons. Bone turnover markers were analysed in three ways: 1) using the change in fasted values from Baseline to Adaptation, 2) assessing absolute concentrations across time, and 3) by calculating area under the concentration-time curve (AUC) for each participant (pre-exercise to 3 h post-exercise), using the PKSolver add-in in Microsoft Excel (v16.48), prior to further analysis in R. Linear mixed-effect models were estimated for all 3 analyses with restricted maximum likelihood through the 'lme4' package in R. As applicable, fixed effects included Diet, Phase, and Timepoint with random effects of subject and heat index, each nested within study, to account for inter-individual variation and camp timing. Normality was assessed through quantile-quantile plots – characteristic departures were not detected. Homoscedasticity was tested with the Fligner-Killeen test. Statistical significance of fixed effects was determined using Type II Wald tests with Kenward-Roger approximation. Post-hoc analysis for significant effects was performed using Tukey's Honestly Significant Difference test. Where unequal variance was detected between groups, a Welch's ANOVA and post-hoc Dunnett's test was applied. Cohen's *d* effect sizes were computed using R package 'emmeans' with values of 0.2, 0.5, and 0.8 as small, medium, and large effects, respectively.

2.3 Study 3

Factors influencing the hepcidin response to exercise: an individual participant data meta-analysis

This systematic review and individual participant data meta-analysis followed the PRISMA-IPD (Preferred Reporting Items for Systematic Reviews and Meta-Analyses of Individual Participant Data) guidelines (Stewart et al., 2015). Ethics approval was granted by the Australian Catholic University Human Research Ethics Committee (2021-223N). The full protocol was registered prior to data extraction on the PROSPERO international prospective register of systematic reviews (ID: CRD42021293423).

2.3.1 Search strategy and selection process

An electronic systematic search of the literature was conducted in Pubmed, SPORTDiscus, and OpenGrey. A date of publication restriction was not applied. A combination of the following keywords and subject headings was applied: 'hepcidin' AND 'exercise' OR 'athlete'. The initial search identified 256 records (Fig. 5.1), of which 53 were detected as duplicates and, therefore, discarded. A further 147 records were excluded as irrelevant after title and abstract screening. Two authors (NF and AM) independently reviewed the full-text articles, and conflicts were resolved by discussion and consensus. Searches were exclusive to original papers with human participants, published in English, without date restrictions and current to November 2021. Twenty-nine articles were deemed ineligible, leaving 27 studies for possible inclusion in the meta-analysis (see Fig. 5.1). Inspection of relevant review paper reference lists revealed no further articles unidentified by the systematic search.

2.3.2 Eligibility criteria

Studies including healthy adults performing exercise where pre-exercise, immediately post-exercise, and 3 h post-exercise inflammatory and iron markers were analysed were considered eligible for this meta-analysis. The following eligibility criteria were applied:

- Population: adults (aged 18-40 years) of both sexes who were healthy, not pregnant/ breastfeeding, and not taking any medication (including the oral contraceptive pill) were included.
- Intervention: exercise with duration, relative intensity, and mode specified

- Outcomes: pre- and 3 h post-exercise hepcidin (urinary or serum)
- Study design: parallel or crossover trials accepted
- Comparators: only the control arm of the trial was included
- Time: studies investigating the acute response to exercise (pre- to 3 h post-exercise) were included.
- Other:
    - Ferritin as a marker of iron status had to be measured at baseline prior to the exercise trial
    - Pre- and immediately post-exercise IL-6 had to be measured

2.3.3 Data collection and management

Original individual participant data were requested from the corresponding authors of the 27 eligible trials (Table 5.1; see also Online Resource 1). Of these, it was uncertain whether ferritin was measured for two studies, and therefore, it was decided to enquire directly with the authors prior to exclusion. A standardized email with full details of the registered protocol was sent to the authors to request data contribution to the meta-analysis. After signing a letter of agreement, authors provided their de-identified data in an individualized spreadsheet template. Data for one condition only (control) was requested for crossover trials, and pre-intervention/baseline data for parallel trials. All raw data were saved in a secure online password-protected folder unique to each corresponding author. Reasons for missing data and queries about specific values and measurement units were confirmed via email to the author providing the data. Two authors, representing six studies did not respond to the data request. We did not include the aggregate data from these studies in the meta-analysis due to our inability to confirm data integrity. One further study was excluded due to insufficient data points, and a final study was excluded following notification from the author of a retrospective assessment of unsatisfactory measurement outcomes. These exclusions resulted in a final dataset comprising 17 studies (see Fig. 5.1).

2.3.4 Data items

Collected variables of each de-identified participant included: age; sex; VO_2max (and corresponding power (W) or speed (km/h)); pre-exercise nutritional state (fed or fasted); exercise mode, duration, and intensity; pre-exercise ferritin, IL-6, and hepcidin; immediate post-exercise IL-6; and 3 h post-exercise hepcidin. Where available, data were cross-checked with the published protocol. For interval-based protocols, active recovery minutes were

included in the total duration of the exercise session; conversely, only exercise time was used if there was complete rest (i.e., no active component) between intervals. Similarly, for intensity, a weighted average of %VO_2max of interval time plus %VO_2max of active recovery time was calculated. Where intensity was given as a percentage of heart rate maximum, this was converted to %VO_2max via established methods (Swain et al., 1994). The collected data were then combined into a single spreadsheet where continuous variables were converted into common SI units of measurement and categorical variables were assigned a numeric code. Further information requested from authors to assess data integrity and risk of bias included, where applicable: method of recruitment, randomization procedures, trial order allocation, extent of blinding, reasons for missing/ incomplete data, and method of menstrual cycle phase confirmation.

2.3.5 Individual participant data integrity

Data were checked for completeness with any missing data queried with authors. Where values seemed implausible, units of measurement were verified with the authors and any risk of error queried.

2.3.6 Risk of bias assessment within and across studies

Risk of bias assessment was guided by the Cochrane Risk of Bias Tool using the domains of selection, performance, attrition, reporting, and other (Higgins et al., 2022). Each study was assessed individually according to these domains and assigned a designation of low, high, or unclear risk of bias for each one (Online Resource 2). An overall impression of risk of bias across studies was summarized from these assessments.

2.3.7 Specification of outcomes and effect measures

The primary outcome of interest was the concentration of hepcidin at 3 h post-exercise and the change from pre-exercise values. Continuous explanatory variables were VO_2max, pre-exercise ferritin, post-exercise IL-6, exercise duration, and relative exercise intensity. Categorical explanatory variables were sex, pre-exercise nutritional state, and exercise modality. Data regarding menstrual cycle phase were collected but not included due to the lack of documentation/verification. Of the 7 studies including females, 5 studies made no attempt to record or verify the menstrual phase (Burden et al., 2015; Govus et al., 2014; McCormick et al., 2019; McKay et al., 2020; McKay et al., 2019a), one made some attempt (questionnaire, menses onset) (Newlin et al., 2012), and only one used a 3-step method (Elliott-Sale et al.,

2020) to establish phases (menses onset, urinary luteinizing hormone, and serum 17β-oestradiol and progesterone) (Zheng et al., 2021).

Based on the results of the primary analysis, secondary analyses were performed as follows:

1. Post-exercise IL-6 was designated as an outcome variable with similar explanatory variables.
2. Data were further split by sex and re-analysed with post-exercise hepcidin and change in hepcidin as outcome variables.

2.3.8 Synthesis methods

A one-stage meta-analysis approach was employed with mixed-effects linear regression using the 'nlme' package (Pinheiro et al., 2022) in R version 4.2.0 (R Core Team, Vienna, Austria). As three studies used urine (rather than serum) for hepcidin analysis (Online Resource 3), and it is not possible to convert between urine and serum hepcidin, statistical analyses were performed separately on serum and urine studies. Accordingly, the following 4 models were constructed as described below:

1. Post-exercise serum hepcidin was modelled as an outcome variable with centred pre-exercise hepcidin as a covariate included to account for aggregation bias, in both a stratified intercept per study and random intercept per study model.
2. Post-exercise urinary hepcidin was analysed using a stratified intercept per study model only.
3. The change in serum hepcidin was modelled following log transformation of both pre- and post-exercise hepcidin to improve model fit, with study used as a fixed effect and a random intercept.
4. Post-exercise IL-6 was analysed with a random intercept model only, with centred pre-exercise values used as a covariate; both pre- and post-exercise values were log-transformed prior to pre-exercise centring to improve model fit.

All models included a heterogenous residual variance structure fit per study to estimate the within-study variance. Sex (2 levels: male, female); pre-exercise nutritional state (2 levels: fasted, fed); pre-exercise ferritin; exercise modality (3 levels: running, cycling, racewalking), exercise duration; relative exercise intensity; participant VO_2max; and post-exercise IL-6 (for the hepcidin model) were included as fixed effects (Online Resource 4). Pre-exercise nutritional

state and modality were excluded from all above models (except the random intercept post-exercise hepcidin model) due to convergence errors.

The ‘stepAIC’ function from the ‘MASS’ package (Venables & Ripley, 2002) was used in the first instance to perform stepwise regression from the full model including all plausible explanatory variables. This result was then checked manually using p-values as a guide, excluding non-significant (p>0.05, confidence interval crossing zero) variables; further reduced models were also constructed via elimination of variables sequentially according to their contribution to the Akaike Information Criterion (AIC) value. All models were fit using maximum likelihood estimation and compared using both AIC and Bayesian Information Criterion (BIC) values, and the model with the lowest AIC/BIC values was chosen as the final model. Where it was unclear between AIC’s, preference was given to the model with the lower BIC which penalizes more explanatory variables, yielding a more parsimonious model. Similarly, where BIC’s were equivalent, the model with the lower AIC was chosen. This final model was refitted with restricted maximum likelihood estimation and model diagnostics verified via visual inspection of residual and QQ plots. Fixed effect parameter estimates and 95% confidence intervals were obtained via the ‘sjPlot’ package and back-transformed from the logarithmic scale for interpretation. Marginal and conditional R^2 values for the final models were calculated as a proportion of the model explained by the fixed effects alone (marginal R^2), or by fixed and random effects combined relative to the model’s total variance (conditional R^2) (Nakagawa & Schielzeth, 2013). Post-hoc testing, where applicable, was conducted via the ‘emmeans’ package (Lenth, 2022) with Tukey’s Honestly Significant Difference adjustment. Finally, predictor effects for the change in hepcidin per study were used to construct a forest plot using the ‘forestploter’ package (Dayimu, 2022). The full code can be found in Online Resource 5 of the supplementary material to this thesis.

2.4 Study 4

Sequential submaximal training in elite male rowers does not result in amplified increases in interleukin-6 or hepcidin

2.4.1 Participants

Eighteen elite male rowers from the Rowing Australia National Training Centre, in preparation for potential Olympic representation, were recruited. One participant with lactose intolerance was excluded due to his inability to complete one of the dietary arms, while another was unable to complete the required training load due to injury. Two athletes with known mild hemochromatosis were included since their ferritin values were within range of the other athletes, and not receiving treatment due to their ferritin being <300 $\mu g.L^{-1}$ (Kowdley et al., 2019). Since the removal of their data did not alter the interpretation of the results, they have been retained in the current dataset. Another with previously diagnosed hypothyroidism was deemed eligible, due to the presentation of normal thyroid function tests at the time of the study. None of the final cohort of 16 participants, characterized in Table 6.1, were iron deficient or anaemic [serum ferritin <35 $\mu g.L^{-1}$ and Hb <115 $g.L^{-1}$; (Sim et al., 2019)] or taking oral iron supplements. Written informed consent was obtained from each athlete prior to study commencement. This study conformed to the standards set by the Declaration of Helsinki. Ethics approval was obtained from the Australian Institute of Sport Ethics Committee (ref: 20200905).

2.4.2 Experimental overview

In a randomized, double-blinded, crossover design, athletes completed two trials, one week apart, involving either a high (CAL) or low calcium (CON; control) intervention (see Figure 6.1). All trials were performed at the same training centre to ensure comparable environmental conditions. On trial mornings, athletes arrived at the laboratory (06h00-07h00) in a fasted, rested state, and a blood sample was collected from a cannula placed in a forearm vein. Athletes then consumed either a low (<50 mg) or high calcium (1000 mg) standardized breakfast (t = 0 min) with athletes and the researchers involved with data collection or data entry remaining blinded to the treatment order. After 115 min, a pre-exercise blood sample was drawn and 5 min later (t = 120 min) the first exercise session (EX1) commenced. This session involved 3 x 30 min sets, with a 5 min break between sets. Immediately post-EX1, blood was sampled, and at t = 250 min (30 min post EX1 and 120 min prior to EX2) the same low or high calcium meal was consumed according to group allocation. Blood samples were drawn 1 and 2 h post-EX1

(t = 280 and 340 min), and at t = 370 min, a repetition (EX2) of the earlier session was undertaken, noting a recovery period of 150 min between exercise bouts. Blood was collected at the break between the first and second sets of EX2 (t = 400 min, equating to 3 h post-EX1). Blood was collected on completion of EX2 (t = 470 min), prior to the consumption of a recovery meal, with further samples at 1, 2, and 3 h post-EX2 (t = 530, 590, and 650 min).

The majority of exercise sessions were completed on a rowing ergometer (Concept 2, Morrisville, Vermont, USA). In select cases, where repetitive loading would have caused injury risk, a Wattbike Pro cycling ergometer (Wattbike Ltd., Nottingham, UK) was substituted in some exercise sets. This supports real-world practice and was replicated in both trials. Session intensity was prescribed at 90-100% of T1 power (Watts), with targets established from an incremental exercise protocol, starting at 150 W and increasing by 15 W every 6 min. The power at which capillary blood lactate reached 2 mmol.L^{-1} was designated the athlete's T1 power value. Mean power was recorded for each effort, as was heart rate (HR, beats per min; Wahoo Tickr X, Wahoo Fitness, Atlanta, USA) and subjective rating of perceived exertion (RPE) according to the Modified Borg Scale (6-20).

2.4.3 Dietary intake

A standardized diet and training prescription was followed for 24 h prior to each trial. Trial day intake (see Figure 6.1) was also standardized and matched for energy, carbohydrate, protein and iron content as closely as possible. The intervention for the larger study in which this project was embedded involved manipulation of calcium content of meals consumed 2 h pre-exercise, with the intention of manipulating serum ionic calcium concentrations prior to the onset of exercise. Water was provided *ab libitum* during the sessions and recorded accordingly. A CHO-rich gel (Science in Sport PLC, London, UK) was consumed (~30 g CHO) during each 5 min break between exercise sets. To maintain real-life practice, athletes were permitted to consume coffee prior to exercise and were able to request more food in the recovery period between exercise sessions in addition to the pre-exercise meal. This was done in accordance with the dietary treatment, recorded, and repeated in the second trial. Nutrient composition of diets was calculated using a computerized dietary analysis package (Nutritics Ltd., Dublin, Ireland).

2.4.4 Blood analysis

During each trial, 10 venous blood samples were collected into either 6- or 8-ml serum separator tubes (BD Vacutainer, Melbourne, Australia). Blood samples were taken at rest (fasted), pre-exercise (2 h post breakfast), and immediately, 1 h, 2 h, and 3 h post each exercise session

(Figure 6.1). Samples were left to clot for 30 min before being centrifuged at 1500 G for 10 min at 4°C. Serum was aliquoted into 1.5 ml cryotubes and frozen at -80°C until batch analysis was performed. Ferritin analysis was performed on 6 samples per trial (baseline, pre session, 1 h, 2 h and 3 h post each session) using a COBAS Integra 400 automated biochemistry analyser (Roche Diagnostics, Rotkreuz, Switzerland). Concentrations of IL-6 were determined for all 10 trial samples using a commercially available ELISA (Quantikine HS; R&D Systems, Minneapolis, MN, USA) on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany). Measurement of hepcidin-25 was conducted on all trial samples using Intrinsic Hepcidin IDxi ELISA Kit (Intrinsic LifeSciences LLC, CA, USA) according to the manufacturer's instructions. The coefficient of variation was 3.4% and 4.9% for IL-6 and hepcidin-25 concentrations respectively. Additionally, whole blood was used to determine haemoglobin and haematocrit with the point-of-care i-STAT device (Abbott Point of Care Inc., Princeton, NJ, USA).

2.4.5 Statistical analysis

Pre-trial and trial day intake, as well as baseline haemoglobin and iron markers were compared with paired sample t-tests or the Wilcoxon test. Training variables (power, heart rate, and RPE) were compared with a general linear mixed model using R package 'lme4', with Trial (calcium vs control) and Session (first vs second) as fixed variables and Subject Identification and Week as random effects. Hepcidin, IL-6, and ferritin response data were also analysed with a general linear mixed model, with Timepoint and Trial as fixed effects, and Subject Identification and Week as random effects, to account for interindividual variation and the crossover design respectively. Furthermore, baseline ferritin was included as a covariate in the hepcidin model to account for the recognized influence on its response (Peeling et al., 2017; Peeling et al., 2014). All models were estimated using restricted maximum likelihood. Visual inspection of the residual plots did not reveal obvious deviations from homoscedasticity or normality. P-values were obtained using Type II Wald F tests with Kenward–Roger degrees of freedom, as employed in the R package 'car'. Where applicable, post-hoc Tukey's honestly significant difference was applied to identify where differences between timepoints existed. Significance was set at $p<.05$. Effect size (partial eta-squared) was calculated with R package 'effectsize'. With values of 0.01, 0.06, and 0.14 classified as small, medium, and large effect respectively.

3 Chapter 3

Bone turnover markers and bone mineral density values in a convenience sample of elite endurance athletes

Publication statement:

This chapter is comprised of the following paper prepared for *Bone*.

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3.1 Abstract

Areal bone mineral density (BMD) is a risk factor for fracture in older adults but is less sensitive and specific for predicting fracture risk in younger populations such as competitive athletes. Sport-specific loading patterns contribute to an increased range in BMD measurements derived from dual energy x-ray absorptiometry (DXA) in athletes. Bone turnover markers (BTMs) provide an additional insight into bone status but their utility in young adults is limited by the lack of reference data and, in the case of athletes, the acute increases in the bone resorption marker carboxy-terminal telopeptide (CTX), following exercise. Some evidence suggests an association of CTX with interleukin-6 (IL-6), an immune marker with links to iron regulation, creating intriguing hypotheses surrounding system crosstalk. This study (1) examined BMD site discordance in athletes within and between sports, (2) investigated athlete BTM ranges, and (3) explored possible links between bone and iron metabolism. We conducted a cross-sectional analysis of baseline data from athletes who participated in various studies within our research group over the period November 2015-January 2022. Specifically, studies which conducted a baseline DXA scan for body composition and BMD and where iron and bone markers were measured via venous blood sample collection prior to and following exercise were included. Significant discordance was shown between and within sports with lumbar spine BMD of rowers being higher than all other sports ($p < 0.0001$) and higher than the proximal femur site within rowers ($p = 0.002$). Contrarily, racewalkers and runners had higher proximal femur than lumbar spine BMD ($p \leq 0.001$). Age significantly contributed to the variance in both fasting CTX and P1NP with higher concentrations observed in athletes younger than 25 years old as opposed to those older ($p \leq 0.001$). In addition to age, assay method and resting IL-6 concentrations further explained fasting CTX concentrations (marginal $R2 = 0.49$). Post-exercise IL-6, however, was not significant in explaining post-exercise CTX at 1 h post-exercise ($p = 0.1$). Finally, significant medium-strong correlations were present between fasting CTX and resting ferritin and hepcidin ($p < 0.001$). BMD discordance at traditional sites with potentially higher fasting BTM values in athletes, suggests that protocols for assessment of bone status and fracture risk in athletes may require review. An athlete-specific database of BMD and BTM reference values would enable earlier identification and investigation of athletes at risk of bone stress injuries.

Keywords: bone turnover markers, bone mineral density, athlete, reference values, iron, IL-6

3.2 Introduction

Bone stress fractures account for 0.15-20% of all musculoskeletal injuries in competitive and recreational athletes (Bennell et al., 1996b; Ruddick et al., 2019; Snyder et al., 2006). Although the precise pathophysiology of bone stress fractures is unknown, theoretically, an increase in the magnitude and/or rate of bone loading with insufficient remodelling time, leads to the accumulation of damage (Warden et al., 2006). Intrinsic factors, such as bone geometry (O'Leary et al., 2021), low energy and nutrient availability (Hutson et al., 2021), and biomechanics (O'Leary et al., 2021), combined with extrinsic factors, including repetitive loading (Rizzone et al., 2017; Ruddick et al., 2019; Warden et al., 2006), training surface (Milgrom et al., 2003), and insufficient adaptation time in a training program (Warden et al., 2006), contribute to the risk of bone stress injuries.

In older adults, lower areal bone mineral density (BMD) is a risk factor for fracture, with each standard deviation decrease in hip BMD increasing the risk of any fracture by approximately 1.4 and 1.66 at 65 years and 80 years of age, respectively, in both men and women (Johnell et al., 2005). Dual-energy x-ray absorptiometry (DXA) is the current 'gold standard' method of assessing areal BMD, estimating this from a two-dimensional image. However, its failure to measure important contributors to bone strength, such as cortical and trabecular geometry and microarchitecture (Bouxsein & Seeman, 2009) mean that it is neither specific nor sensitive for osteoporotic fracture risk (Kanis et al., 2005). Indeed, a weaker relationship between areal BMD and fracture is evident in young adults (Ferrari et al., 2012; Frolich et al., 2020; Writing Group for the ISCD Position Development Conference, 2004), highlighting the difficulty in relying solely on this metric without consideration of microarchitecture, secondary causes, clinical risk factors, or level of physical activity (Ferrari et al., 2012; Frolich et al., 2020; Writing Group for the ISCD Position Development Conference, 2004). In fact, amenorrheic (Rudolph et al., 2021) and low energy availability states (Heikura et al., 2018b) place athletes at increased risk of stress fractures, despite BMD values similar to study controls. The International Society of Clinical Densitometry (ISCD) suggests a z-score cut-off of ≤ -2 for "below the expected range for age" in premenopausal women and men younger than 50 years old (Writing Group for the ISCD Position Development Conference, 2004). However, this guideline is thought to be inappropriate for certain athletes, in whom a higher BMD secondary to mechanical loading is expected (Tenforde & Fredericson, 2011). As such, the traditional cut-off may reflect an already reduced BMD (Jonvik et al., 2022). Therefore, it has been proposed that further investigation of secondary causes and risk of injury should be carried out at a BMD z-score of < -1 (Jonvik

et al., 2022; Nattiv et al., 2007) or even < 0 in high-impact sports (Jonvik et al., 2022). Nevertheless, considering the limitations of BMD in predicting fracture risk in younger populations, the possibility of discordance between measured sites in athletes within and between sports, and the relatively slow change in BMD impeding early identification of at-risk states, reliance on BMD alone as a measure of bone quality in athletes is insufficient.

Bone resorption (e.g. C-terminal telopeptide of type 1 collagen; CTX) and formation (e.g. procollagen-1 N-terminal propeptide; P1NP) markers reflect the metabolic activity of bone tissue, with evidence of potential predictive value in changes to areal (Verroken et al., 2018) and volumetric (Pye et al., 2017) BMD, as well as a significant, yet moderate association to fracture risk (gradient of risk ~ 1.2 for CTX and P1NP) (Johansson et al., 2014). Furthermore, they may have value in detecting secondary causes of accelerated bone loss (such as steroid therapy or hypogonadism) and monitoring response to treatments (Vasikaran et al., 2011). However, their use in clinical settings has been complicated by inadequate control of pre-analytical variability (Szulc et al., 2017) and heterogeneity of assays (Cavalier et al., 2021; Cavalier et al., 2019), resulting in difficulty in interpretation, as well as the lack of consistency in the markers used in research (Vasikaran et al., 2011). Moreover, their utility in non-osteoporotic populations is limited by the lack of normative reference values by age (Michelsen et al., 2013) and knowledge of causes of variation, which becomes particularly relevant when considering athletes and the effects of exercise on these markers (Dolan et al., 2022).

The beneficial effects of exercise throughout the lifespan on bone are well-recognized (Santos et al., 2017), with athletes in high- and odd-impact sports in particular demonstrating enhanced BMD and bone geometry compared to non-athletic counterparts (Tenforde & Fredericson, 2011). Independent of sport-type, greater lean body mass is positively associated with BMD (Camhi & Katzmarzyk, 2012), via increased mechanical strain conferring adaptation (Bass et al., 2005). However, some exercise modes also acutely increase a marker of bone resorption (CTX) (Dolan et al., 2022), which may potentially be detrimental when this occurs in excess of formation. Of interest, previous studies have demonstrated possible correlations between CTX and the cytokine/myokine interleukin-6 (IL-6) (Sale et al., 2015), suggesting that exercise-related changes in IL-6 may be driving similar change in bone metabolism. Indeed, studies which have employed low carbohydrate dietary interventions have shown similar post-exercise patterns in both markers (Fensham et al., 2022a; Heikura et al., 2019; McKay et al., 2021c; McKay et al., 2019a). *In vitro* and animal models indicate that IL-6 plays a dichotomous role in bone (Sims, 2021), yet evidence for a definitive role in humans is less clear, requiring further

exploration. A further potential link between IL-6 and bone relates to its role in iron metabolism. Interleukin-6 contributes to the post-exercise increase in the regulatory hormone hepcidin (Peeling et al., 2009b), which inhibits enteral iron absorption and macrophage iron release (Nemeth et al., 2004b), placing athletes at risk of developing iron deficiency. In turn, iron deficiency may predispose an individual to reduced bone strength, through its role in hydroxylation of procollagen and vitamin D metabolism (Toxqui & Vaquero, 2015). Therefore, a greater understanding of the impact of these exercise-related changes in biomarkers on bone metabolism may lead to better strategies around nutrition and load modification to achieve performance outcomes while limiting detrimental long-term health effects.

Repetitive bone stress injuries represent a significant impediment to achieving consistent training and performance goals, rendering it important to identify any predisposing risk factors at an early stage to avoid potential long-term adverse health effects. Our research group has conducted a series of investigations of different diet and/or exercise interventions in high performance athletes, which have accumulated data sets describing exercise characteristics as well as markers of both bone and iron status at rest and in response to exercise. Given the controlled conditions and standardized protocols under which these data were collected, they provide a valuable opportunity to contribute normative data to the literature and perform an exploratory analysis on the possible relationship between variables. The first aim of this study was to explore the need for athlete-specific bone formation and resorption markers and BMD ranges by characterizing baseline bone turnover marker ranges measured under standardized conditions and classify bone mineral density as well as site discordance in this population. Further, we aimed to identify any associations between the dynamic bone turnover markers and the static BMD values as measures of bone status, as well as associations between body composition and bone measures. Finally, we aimed to explore the association between iron and bone status in athletes and the possible contribution of IL-6, ferritin, and hepcidin to bone turnover.

3.3 Methods

3.3.1 Study design

This study is a cross-sectional study using baseline data from athletes who participated in various intervention studies conducted by this research group between November 2015-January 2022. November 2015 was selected as the start date as it represented the commencement of a series of studies by the research group involving elite athletes, where a standardised protocol

for blood collection and metric analysis was used. This provided a convenience sample where data mining could be instituted with confidence in the circumstances under which the data were captured. Specifically, studies which conducted a baseline DXA scan for body composition and BMD and where iron and bone markers were measured via venous blood sample collection prior to and following exercise were included. Data sets [149 male (27.3±4.8 years) and 17 female (26.1±3.9 years)] from high performance athletes involved in racewalking, running, rowing, and triathlon were utilized (Table 3.1), with the number of participants contributing to each analysis varying according to the study design and completeness of data capture.

Table 3.1: Participant characteristics across sports included in the analysis

Habitual sport	Racewalking	Running	Rowing	Triathlon
Male/Female	99/10	30/0	16/0	4/7
Age at time of study	27.6±4.6 (18-37)	27.3±6.2 (19-44)	25.9±3.4 (20-32)	24.4±3.0 (20-29)
VO_2max	62.6±5.8 (51.0-74.2)	66.5±3.5 (61.0-74.0)*	Unavailable	69.4±6.7 (57.7-77.7)*
Exercise mode for testing	Racewalking	Running	Rowing/ cycling ergometer	Cycling

Note: values presented as mean ± standard deviation (range). No significant difference between sports for age. *significantly higher than racewalking (p<.05)

3.3.2 Ethics approval

Ethics approval for the conduct of the individual studies was granted by either the Ethics Committee of the Australian Institute of Sport (AIS: 20150802, 20161201, 20170401, 20171203, 20181203, 20200905) or the Australian Catholic University (ACU: 2020-238HC, 2021-36HC). Ethics approval for the retrospective analysis of data from individual studies already completed was granted by the Australian Institute of Sport Ethics Committee (ref: 20200401). Where studies were still due to take place, explicit consent for use of data in this project was provided by athletes.

3.3.3 Data extraction

Athlete characteristics (age, sex, habitual sport, VO_{2max}, body composition, and bone mineral density), exercise session characteristics (modality, duration, intensity as a percentage of VO_{2max}), and results (resting ferritin, resting and immediately post-exercise IL-6, resting and 3 h post-exercise hepcidin, fasting/pre-exercise/1 h post-exercise CTX and P1NP) were extracted from the baseline phase/control arm of 10 research-embedded training camps. The athletes were typically Tier 4 (Elite) competitors based on the McKay system for standardizing athlete calibre

and training status (McKay et al., 2022b), although some individuals met Tier 3 (highly-trained) and Tier 5 (world class) status. All bone turnover markers were measured following at least 24 h of a standardized nutrition protocol as previously described (Fensham et al., 2022a; Fensham et al., 2022b; Lundy et al., 2022a; McKay et al., 2023; McKay et al., 2020; McKay et al., 2021b; McKay et al., 2021c; Mirtschin et al., 2018).

3.3.4 Bone mineral density and body composition measurement

Dual energy x-ray absorptiometry (DXA) was performed at the time of the individual studies, measuring body composition and lumbar spine and total proximal femur BMD. While all scans were performed using the same machine type (Lunar iDXA), 11 (6.8%) of these scans were performed on a machine based at the Australian Catholic University (Fitzroy, VIC, Australia) with the remainder performed on a machine at the Australian Institute of Sport (Bruce, ACT, Australia). The BMD scan images were reanalysed (enCORE version 18; GE Healthcare, Chicago, IL, USA) for this study by one author (NF), and the body composition scans were reanalysed using best practice protocols (Australian Institute of Sport, 2023). Two femur BMD and two body composition scans were unable to be reanalysed due to initial positioning errors and one lumbar spine scan was excluded due to a large intervertebral BMD z-score discrepancy. A further 3 BMD and 1 body composition scan data were unavailable, and 12 body composition scans were excluded from analyses as the subject was too tall for the machine. The reference database used for z-score calculations was the Australia Combined Geelong/Lunar, matched for age, sex, and ethnicity. Due to the lack of a z-score reference database for those under the age of 20, 9 scans were excluded from analyses using BMD. Bone mineral density was classified as either “normal” or “low” (lumbar spine or total proximal femur z-score ≤ -1 (Nattiv et al., 2007)). Further, any discordance between sites (i.e., z-score ≤ -1 at one site but > -1 at the other site) was noted. Two athletes had a normal z-score at one site but did not have a scan of the other site and were excluded from classification. For 8 athletes, 2 of which were classified “low”, L2-4 BMD and z-scores was used to evaluate discordance, due to positioning errors with image capture precluding the use of L1-4. Total n for body composition was 135 males and 16 females (Table 3.2) and for BMD 136 males and 16 females (Table 3.3B).

3.3.5 Blood analysis

Due to the retrospective cross-sectional nature of this study, blood analysis was not performed as a batch. Rather, the results and corresponding coefficients of variation (CV; where applicable) specific to the analysis performed at the time of the individual study were extracted.

In addition, the method used was noted to account for the uncertainty around assay harmonization (Bhattoa et al., 2021; Cavalier et al., 2021; Cavalier et al., 2019).

3.3.6 Statistical analysis

Statistical analysis was executed in R version 4.2.0 (R Core Team, Vienna, Austria) with significance set at $p<0.05$. Age was categorized in two ways: (1) using multiple data bins [< 20, 20-24, 25-29, 30-34, 35+] and (2) using a binary approach [< and ≥ 25 years old]. Ferritin values > 350 $ug{\cdot}L^{-1}$ (n=5), indicating hyperferritinemia, were removed and the remaining values coded according to the following range categories: <30, 30-50, 50-100, >100 $ug{\cdot}L^{-1}$. These groups were chosen based on The Royal College of Pathologists Australia definition of iron deficiency (Iron Studies Standardised Reporting Protocol Working Group, 2021), in combination with athlete (Peeling et al., 2014) and isotope fractional iron absorption studies (Galetti et al., 2021). All BMD values were included to maximize sport representation within normative values. However, when the time between BMD assessment and collection of blood variables was > 12 months, these data were not analysed together, and similarly for body composition data and blood variables separated by > 1 month. Participant age and VO_2max among sports were compared with one-way analysis of variance (ANOVA) and Welch's, respectively, after checking normality with the Shapiro-Wilk test and homogeneity of variance with the Fligner-Killeen test.

Correlations between relevant iron, bone, and body composition measures were calculated using Kendall's Tau correlation (≤ ± 0.19 = weak, ± 0.20-0.29 = medium, ≥ ± 0.35 = strong (Botsch, 2011)). Within sport and between site absolute BMD and z-score differences were compared using linear mixed models with sport and measurement site as interacted fixed terms, and subject as a random effect; sex was not included due to non-significance and lack of representation in 2 of 4 sports. Generalized additive modelling with subject as a smooth term and random effect was used to compare 1) fasting CTX/P1NP values between ferritin groups, 2) fasting CTX/P1NP between BMD classifications, 3) resting hepcidin/ferritin between BMD classifications, and 4) total/lean/fat mass and bone mineral content between sport and sex, and 5) the significance of sport when controlling for total and lean mass.

Finally, mixed-effects linear regression was employed to construct the following models:

Fasting CTX or P1NP as the outcome variable with age; sex; American College of Sports Medicine (ACSM) BMD classification (Nattiv et al., 2007); assay method; resting IL-6; and ferritin/hepcidin concentrations as fixed effects in a random intercept per subject model. Ferritin or hepcidin with and without BMD classification were tested for significance in separate models

initially – as none proved significant, stepwise regression was performed from the largest number of subject data.

CTX or P1NP at 1 h post-exercise as the outcome variable with centred pre-exercise CTX or P1NP, respectively; age; sex; post-exercise IL-6; exercise modality; exercise duration; exercise relative intensity; and BMD classification (Nattiv et al., 2007) included as fixed effects, in a random slope and random intercept per study model.

These models included a heterogenous residual variance structure fit per study to estimate the within-study variance. Stepwise regression was performed via elimination of variables sequentially according to their contribution to AIC. All models were fit using maximum likelihood estimation; the model with the lowest AIC/BIC value was chosen as the final model. This final model was refitted with restricted maximum likelihood estimation. Marginal and conditional R^2 values for the final models were calculated for the fixed and combined fixed-random effects, respectively. Where significant differences were detected, post-hoc tests were performed with Tukey's Honestly Significant Difference adjustment. Significant parameters identified from the fasting models were used as subgroups in calculating reference ranges (central 95%).

3.4 Results

3.4.1 Body composition, bone mineral density, and site discordance

Male rowers had higher total, lean, and fat mass, and bone mineral content than all other athletes (p<0.0001) with bone mineral content being lowest in triathletes (Table 3.2; p<0.0001). Additionally, total mass, lean mass, and bone mineral content were significantly higher in male racewalkers and triathletes than their female counterparts, with significantly higher fat mass in female than male racewalkers (p≤0.0001).

The estimated mean of absolute BMD and z-scores per sport is provided in Table 3.3A. No significant differences between sports were found for total proximal femur z-scores; however, lumbar spine z-scores were significantly higher in rowers compared to all other sports (Fig. 3.1; p<0.0001). When total and lean mass were controlled for, rowers still had significantly higher lumbar spine BMD (p=0.02) and z-scores (p=0.01), with no differences noted between sports at the proximal femur (p > 0.1). In both racewalking and running, participant total proximal femur z-scores were significantly higher than lumbar spine z-scores (p≤0.001), whereas the opposite was true in rowing (p=0.002). Those whose primary sport was triathlon showed no significant difference between lumbar spine and total proximal femur z-scores (p=0.92). In

those classified as having “low” BMD (n=45; 29.6%), the majority of these involved a lumbar spine site measurement z-score being ≤ -1: 58% in racewalkers, 71% in runners, and 71% in triathletes. None of the rowing athletes were classified as “low” (Table 3.3B).

3.4.2 Relationships between body composition, bone, and iron status

Significant, strong correlations were identified between total and lean mass, and total BMD (τ_b = 0.38 and 0.36, $p < 0.001$); no correlation between total fat mass and total BMD was evident ($p = 0.20$). Significant medium correlations were present between total mass and both lumbar spine BMD and z-scores (both $\tau_b = 0.31$, $p < 0.001$), as well as between lean mass and both lumbar spine BMD and z-scores (both $\tau_b = 0.28$, $p < 0.001$). Weak significant correlations were found between lumbar spine BMD or z-scores and fat mass (both $\tau_b = 0.19$, $p = 0.001$). Only weak correlations existed between total mass and both total proximal femur BMD and z-scores (both $\tau_b = 0.13$, $p = 0.04$), with no significant correlation between lean mass ($p = 0.09$) or fat mass ($p > 0.1$) and total proximal femur BMD or z-scores. No significant correlation was evident between post-exercise IL-6 and lean mass, or CTX or P1NP and bone mineral content ($p > 0.1$).

Significant, medium-strong correlations were identified between fasting CTX and both resting ferritin ($\tau_b = 0.17$, $p = 0.02$) and hepcidin ($\tau_b = 0.36$, $p < .001$) concentrations. However, there were no significant differences in CTX concentrations between ferritin stratification groups ($p = 0.17$). No correlation was found between the iron markers and P1NP ($p > 0.05$). Further, no correlations were found between CTX, P1NP, or ferritin concentrations and absolute BMD or z-scores. No significant differences between BMD classification groups were found for CTX, P1NP, or ferritin concentrations.

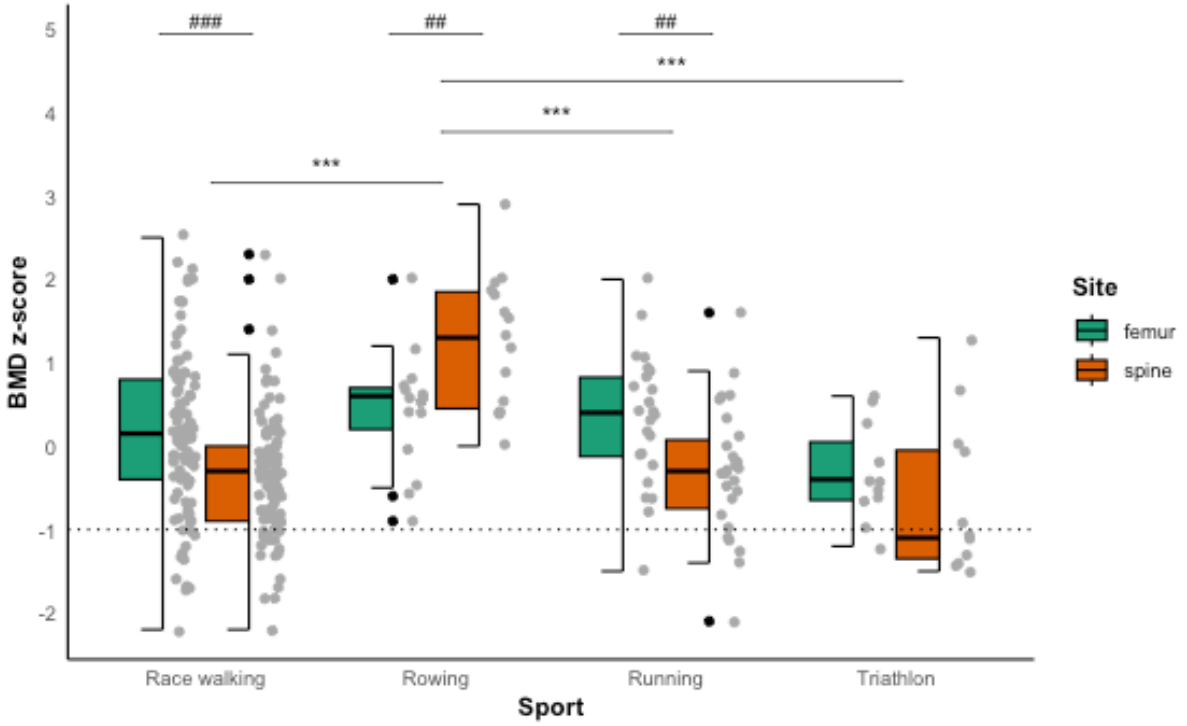

Figure 3.1: Within- and across-sport bone mineral density z-scores by measurement site. Dotted line represents classification cut-off used for 'low bone mineral density' (Jonvik et al., 2022; Nattiv et al., 2007). Grey dots represent subject data points with the black dots as outliers of the boxplot. #significantly higher lumbar spine z-score than all other sports (p<0.001) *significantly different between sites within sport (p<0.001) **significantly different between sites within sport (p<0.01)

Table 3.2: Body composition measures among represented sports								
	Male athletes				Female athletes			
	Total mass (kg)	Total lean mass (kg)	Total fat mass (kg)	Total bone mineral content (kg)	Total mass (kg)	Total lean mass (kg)	Total fat mass (kg)	Total bone mineral content (kg)
Racewalking (M=98/F=10)	66.4 (9.6)*#	56.0 (8.7)*#	7.9 (2.5)#	2.9 (0.4)*#$	53.4 (3.7)	42.4 (3.4)$	8.9 (3.7)*	2.2 (0.3)$
Running (M=28)	67.9 (10.5)#	55.8 (7.9)#	7.7 (3.0)#	3.0 (0.4)#$				
Rowing (M=5)	92.0 (1.6)	75.5 (3.4)	11.9 (1.9)	4.0 (0.2)				
Triathlon (M=4/F=6)	63.0 (3.1)*#	53.8 (2.6)*#	7.5 (1.3)#	2.6 (0.2)*#	56.1 (5.6)	45.1 (1.5)	8.5 (2.8)	2.4 (0.4)
	TBLH mass (kg)	TBLH lean mass (kg)	TBLH fat mass (kg)	TBLH bone mineral content (kg)				
Racewalking (M=1)	76.4	64.3	9.1	3.0				
Running (M=1)	65.5	55.2	7.6	3.1				
Rowing (M=10)	90.7 (7.2)	76.7 (4.8)	11.8 (4.2)	3.5 (0.3)				
Note: values represent median (interquartile range). M = number of male athletes, F = number of female athletes. *p*-values derived from GAM models. *significantly higher than other sex within sport #significantly lower than rowing $significantly different to triathlon ($p<0.05$) TBLH = total body less head (athlete too tall for scan bed). These athletes were not compared due to n.								

Table 3.3A: Bone mineral density and z-scores among represented sports				
	Lumbar spine absolute BMD ($g.cm^{-2}$)	Lumbar spine z-score	Proximal femur absolute BMD ($g.cm^{-2)}$	Proximal femur z-score
Racewalking	1.18±0.01*# (1.15, 1.20)	-0.36±0.11*# (-0.58, -0.15)	1.11±0.01 (1.09, 1.14)	0.15±0.10 (-0.06, 0.36)
Running	1.18±0.02# (1.14, 1.22)	-0.34±0.17*# (-0.67, -0.01)	1.14±0.02 (1.10, 1.21)	0.32±0.17 (-0.02, 0.66)
Rowing	1.37±0.03* (1.31, 1.42)	1.25±0.22* (0.82, 1.69)	1.13±0.02 (1.09, 1.18)	0.43±0.22 (-0.01, 0.87)
Triathlon	1.13±0.03# (1.07, 1.20)	-0.62±0.26 # (-1.13, -0.10)	1.05±0.03 (0.98, 1.11)	-0.33±0.26 (-0.84, 0.19)
Note: values represent estimated mean ± standard error (95% CI). *significantly different to femur within sport #significantly different to rowing (p<0.05)				

Table 3.3B: Bone mineral density classification and discordance between sites among represented sports						
	Normal	Low	Discordance between sites within low classification			
			Spine=Femur	Spine<Femur	Spine>Femur	Unclassified
Racewalking	62/8 (69.3%)	30/1 (30.7%)	2/0 (6.5%)	17/1 (58.1%)	9/0 (29.0%)	2/0 (6.5%)
Running	18/0 (72.0%)	7/0 (28.0%)		5/0 (71.4%)		2/0 (28.6%)
Rowing	15/0 (100%)					
Triathlon	1/3 (36.4%)	3/4 (63.6%)	0/1 (14.3%)	2/3 (71.4%)	1/0 (14.3%)	
Note: Absolute numbers ordered as n=Male/Female (% of total n; or % of total 'low' for discordance). 1 runner and 1 racewalker had spine z-scores < -2 and another racewalker had a femur z-score < -2. Unclassified = z-score < -1 at one site but other BMD site missing.						

3.4.3 Fasting bone turnover marker values

The final model for fasting CTX included 85 data points, and 62 for fasting P1NP. For CTX, the best model included age (continuous; Fig. 3.2), assay method, and resting IL-6 concentration (marginal R^2 = 0.49, conditional R^2 = 0.94). Age alone resulted in the best fit model for P1NP (marginal R^2 = 0.26, conditional R^2 = 0.80; Fig. 3.3). The difference between the marginal and conditional R^2 values (~0.50 or ~50%) can be attributed to the random effect of subject (i.e., interindividual variability). Subsequent categorization of age into 5 groups, revealed groups younger than (<20 vs 20-24) and older than 25 (25-29 vs 30-34 vs 35+) were similar to each other ($p > 0.05$). While not statistically significant for CTX on either side of this range (20-24 > 30-34, $p = 0.08$; and less than 20 > 30-34 and 35+, $p = 0.06$), P1NP values were statistically significantly higher in the younger athletes (20-24 > 30-34, p = 0.04 and 20-24 > 25-30, p = 0.03). In keeping with existing reference value literature (Vasikaran et al., 2014) and for improved interpretation, 2 levels were used as a covariate, albeit resulting in a slight reduction in explained variance for CTX (marginal R^2 CTX = 0.42; P1NP = 0.31). Post-hoc analysis demonstrated significantly higher CTX concentrations in the 24 years and younger age category (estimated mean ± standard error 775±44 $pg.mL^{-1}$; 95% CI [671, 879]) than the 25 years and older age category (494±36 $pg.mL^{-1}$; 95% CI [408, 581]; $p = 0.001$). Although the two automated assays were not significantly different (p=0.06), the ELISA method appeared to produce lower CTX values (Cobas vs ELISA: estimated mean difference 501±79 $pg.mL^{-1}$, p<0.0001; iSYS vs ELISA: 416±81 $pg.mL^{-1}$, p=0.003). Although there was no significant difference between assays for P1NP when age was accounted for, similar to CTX, concentrations were higher in the younger age category (109.0±5.7 $ug.L^{-1}$; 95% CI [97.7, 120.4]) than those older than 25 (72.8±4.0 $ug.L^{-1}$; 95% CI [63.8, 81.7]; $p = 0.0004$). The central 95% distribution by sex, age, and assay, calculated from raw values, is represented in Table 3.4A and 3.4B. Finally, resting IL-6 proved to be significant (p= 0.01) in the CTX model only (age continuous), with a 1 $pg{\cdot}mL^{-1}$ increase in IL-6 resulting in a 55.8 $pg{\cdot}mL^{-1}$ (95% CI [18.0, 93.8]) increase in CTX. Of note, neither ferritin concentrations nor BMD classification were significant when controlling for the other covariates.

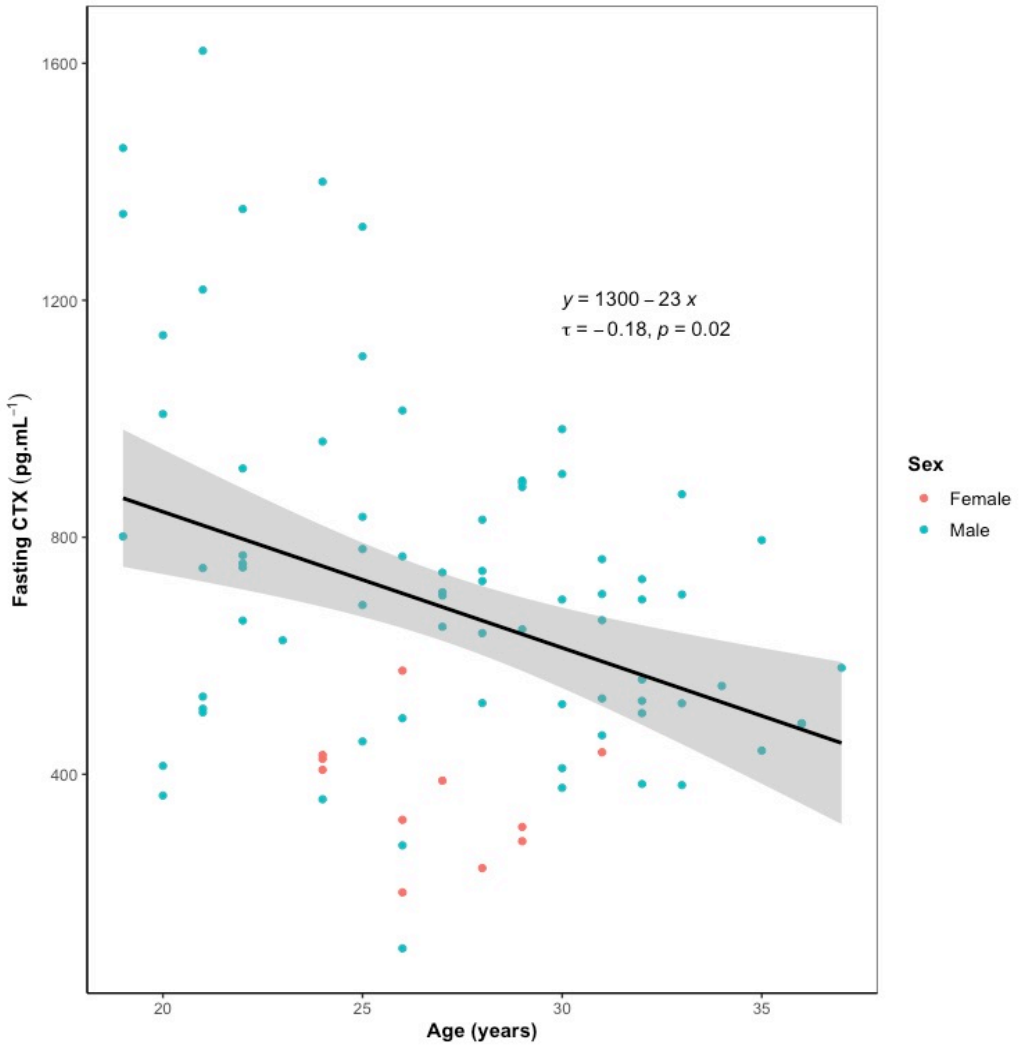

Figure 3.2: Fasting carboxy-terminal telopeptide (CTX) concentrations by age with linear trend line (black) and 95% confidence interval (grey shading), colored by sex. The equation represents that of the linear trend line and the correlation coefficient is Kendall's Tau (τ) with the respective p-value.

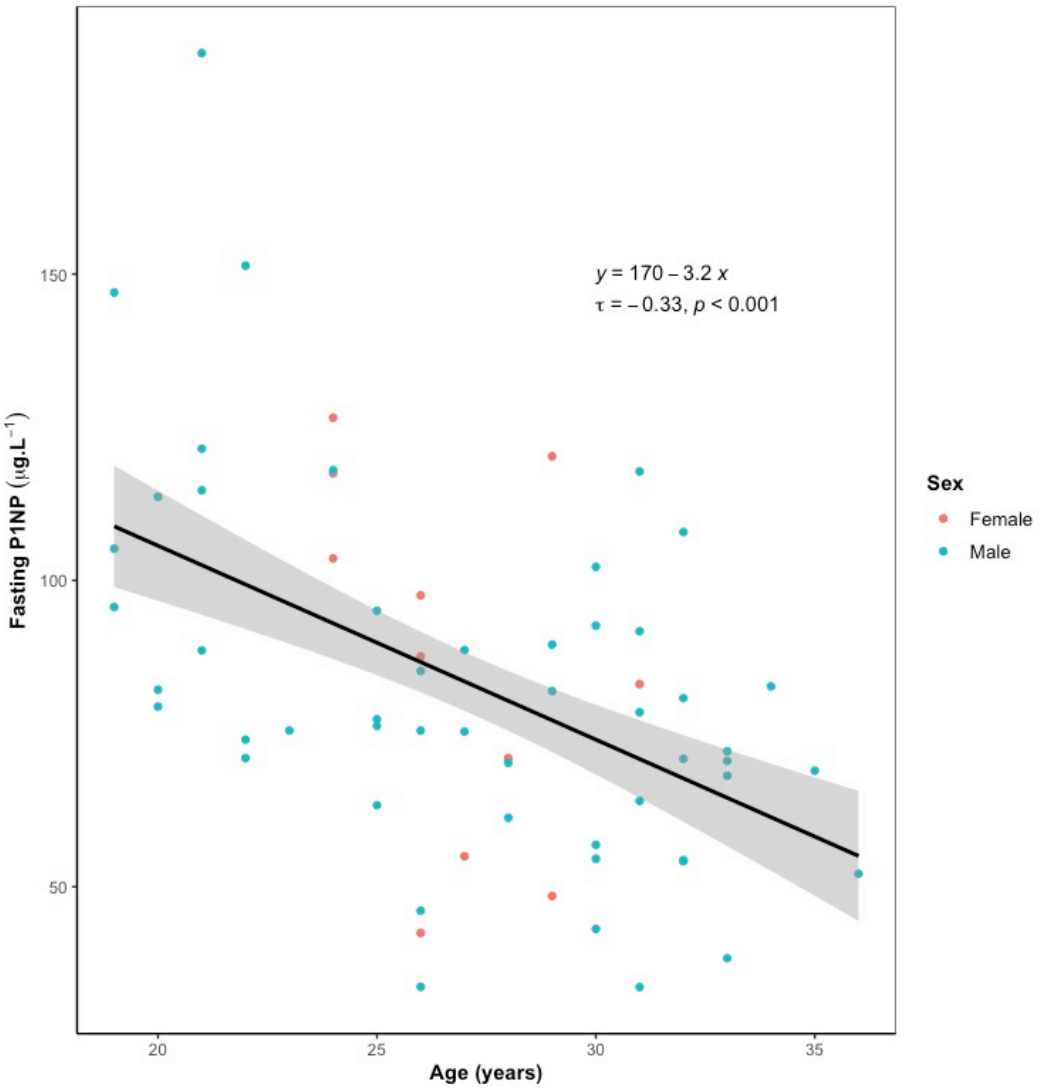

Figure 3.3: Fasting procollagen-1 N-terminal propeptide (P1NP) concentrations by age with linear trend line (black) and 95% confidence interval (grey shading), colored by sex. The equation represents that of the linear trend line and the correlation coefficient is Kendall's Tau (τ) with the respective p-value.

Table 3.4A: Central 95% distribution of carboxy-terminal telopeptide by age, sex, and analysis method										
	Male athletes				Female athletes				Combined	
	<24	Ref. range	25+	Ref. range	<24	Ref. range	25+	Ref. range	<24	25+
Roche Cobas	219.46-1656.90 (17)	400-900	317.66-1112.23 (37)	100-600	No data	150-800	No data	150-800 (100-700 for 30-39)	219.46-1656.90 (17)	317.66-1112.23 (37)
IDS-iSYS	184.25-1756.69 (5)	Uncertain	190.40-964.54 (19)	110-830	No data	Uncertain	182.93-661.18 (4)	50-570	589.82-1351.12 (5)	169.79-931.09 (23)
Novatein ELISA	280.68-578.54 (3)	Uncertain	106.16 (1)	Uncertain	396.13-447.98 (3)	Uncertain	152.97-385.28 (4)	Uncertain	329.87-521.80 (6)	59.44-413.63 (5)
Note: calculated as mean±2*standard deviation, n in brackets. Reference values from: (Michelsen et al., 2013; Vasikaran et al., 2014) ELISA = enzyme-linked immunosorbent assay, IDS = Immunodiagnostics Systems										

Table 3.4B: Central 95% distribution of procollagen-1 N-terminal propeptide by age, sex, and analysis method										
	Male athletes				Female athletes				Combined	
	<24	Ref. range	25+	Ref. range	<24	Ref. range	25+	Ref. range	<24	25+
Roche Cobas	46.81-156.18 (8)	15-115	31.05-109.26 (20)	15-80	No data	15-90	No data	15-90	46.81-156.18 (8)	31.05-109.26 (20)
IDS-iSYS	45.75-167.89 (5)	Uncertain	37.37-113.67 (19)	27.3-95.9	No data	Uncertain	29.18-107.87 (4)	19.3-76.3	45.75-167.89 (5)	36.47-112.14 (23)
Novatein ELISA	17.45-235.30 (3)		33.67 (1)		92.74-139.03 (3)		29.18-107.87 (4)		49.77-192.49 (6)	15.61-150.03 (5)
Note: calculated as mean±2*standard deviation, n in brackets. Reference values from: (Michelsen et al., 2013; Vasikaran et al., 2014) ELISA = enzyme-linked immunosorbent assay, IDS = Immunodiagnostics Systems										

3.4.4 Contributors to the bone turnover marker response to exercise

Concentrations of CTX at 1 h post-exercise were largely explained by pre-exercise CTX concentrations (marginal R^2 = 0.59), indicating minimal change within the first hour post-exercise. Prior to adjustment with covariates, a 1.0 $pg{\cdot}mL^{-1}$ increase in pre-exercise concentrations resulted in a 0.93 $pg{\cdot}mL^{-1}$ (95% CI [0.66, 1.21]) increase in 1 h post-exercise concentrations ($p < 0.001$). Following stepwise regression, the additional covariates of age, relative exercise intensity, and post-exercise IL-6 resulted in the best-fit model (marginal R^2 = 0.72), encompassing 34 data points. However, IL-6 was not significant ($p = 0.1$) and did not improve the BIC value; it was removed from the final model. A 10% increase in relative exercise intensity increased 1 h post-exercise CTX by 64.8 $pg{\cdot}mL^{-1}$ (95% CI [15.6, 113.9]; $p = 0.01$) whereas being 1 year older reduced 1 h post-exercise CTX by 11.4 $pg.mL^{-1}$ (95% CI [-19.0, -3.8]; $p = 0.005$).

Similarly, pre-exercise P1NP accounted for the majority of the variance in 1 h post-exercise P1NP (marginal $R^2 = 0.50$), indicating minimal change within an hour post-exercise: a 1.0 $ug.L^{-1}$ increase in pre-exercise concentrations resulted in an 1.08 $ug.L^{-1}$ (95% CI [0.80, 1.36]) increase in 1 h post-exercise concentrations ($p < 0.001$). Despite stepwise regression resulting in a model (marginal $R^2 = 0.80$) including the additional covariates of exercise duration, relative exercise intensity, and post-exercise IL-6, only duration was significant ($p < 0.001$). After removing the non-significant variables, the BIC of the model remained the same, being equivalent to that of the unadjusted model, and the confidence interval of the coefficient of exercise duration approached zero (95% CI [-1.40, -0.18], $p = 0.01$).

3.5 Discussion

The central aim of this study was to investigate characteristics of bone turnover markers and bone mineral density in high performance athletes representing several sports. Discordance of BMD z-scores between- and within- sports highlights both the influence of loading patterns on bone and the existence of other factors (e.g., reproductive hormone status, nutritional status) in determining BMD in athletic populations. Our results suggest that athletes tend towards higher fasting concentrations of CTX and P1NP than the published reference ranges for non-athlete individuals of the same age. Both CTX and P1NP fasting concentrations tended to decrease with age, although there was a large interindividual variability. Sex and BMD status did not affect the concentrations significantly, although sample size and z-score cut-off values chosen, respectively, limit the implication of this result. Interestingly, resting IL-6 was associated with

fasting CTX concentrations, but post-exercise IL-6 failed to explain 1 h post-exercise CTX, where relative exercise intensity and age were the main parameters of influence.

Some research groups have previously advocated for using a BMD z-score cut-off of -1 for the bone health of competitive athletes to account for their increased bone loading and susceptibility to fractures (Jonvik et al., 2022; Nattiv et al., 2007). However, there are limitations to the use of absolute BMD classifications across all athletes due to the sport-specific impacts on BMD status at a specific site. Indeed, there were significant differences within sports at the BMD sites typically measured in the general population, reflecting the unique loading patterns in these athletes. For example, rowers had considerably higher lumbar spine BMD and z-scores than those at the femur, whereas racewalkers and runners exhibited the opposite. On the other hand, there was no difference between sites in triathletes. Although the triathletes z-scores at both sites were not statistically different from racewalkers and runners, it appears that the z-scores of the spine and femur in the triathlete group were, on average, lower than all other sports. This discordance between BMD z-scores at these sites across sports might be explained by the different frequency and patterns of bone loading within the athlete's training and competition practices (Bennell et al., 1997; Duncan et al., 2002). However, disruptions to metabolic and reproductive hormones commonly associated with low energy availability (Mountjoy et al., 2018), which may occur according to the physical demands and perceived/real pressure around body composition specific to the sport (Heikura et al., 2018b; Lundy et al., 2022a), may also affect bone characteristics. It must be noted that the limitation of convenience sampling and the retrospective study design is the lack of data available regarding their past history of LEA or previous fractures (stress or traumatic), precluding the conclusion that the data represented here is representative of all athletes of the sports from which they were drawn. The significant associations between total/lean body mass and total/spine BMD, highest in the rowers, point to the importance of maintaining muscle mass and strength for bone health, despite performing non-weight bearing activities. Indeed, sport-type remained notably important for spine BMD, when body composition variables were controlled for, yet this was not the case for proximal femur BMD. The variability in BMD between sports raises the question of whether protocols (e.g., the use of traditional or additional sites (Lundy et al., 2022b)), and classification of BMD status, should be tailored to the loading pattern of the sport, with particular focus on the areas of both high- and low-load. Indeed, further research is warranted in defining and quantifying high risk sites and corresponding 'optimal' BMD in different sports.

Nevertheless, the increased risk of bone stress injuries in athletes despite 'normal' BMD (Carbuhn et al., 2022; Heikura et al., 2018b), highlights the limitation of relying solely on BMD as a measure of stress fracture risk and the potential of BTMs to add predictive value (Vasikaran et al., 2011). Notably, BMD failed to contribute to the variance in BTM concentrations and there was no difference in BTM concentrations between those classified as having normal as opposed to low BMD. Bone turnover marker changes occur in considerably less time than BMD changes, which lends support to their use in monitoring osteoporosis treatment response (Vasikaran et al., 2011), but their clinical relevance in young and/or non-osteoporotic populations is constrained by a lack of normative data (Callegari et al., 2017). Indeed, a paucity of data exists in both sexes less than 25 years old (Vasikaran et al., 2014). Here, we report intervals for athletes of an age range 19-37 years. Of note, a large proportion (~50%) of the variance in both markers in our analysis could be attributed to interindividual variability, despite our strict pre-analytical control. Given the evidence of genetic contributions to bone turnover rates (Roshandel et al., 2010), a large and robust data set is required to establish accurate reference ranges. Nevertheless, in the current study, age contributed significantly to the variance in both fasting CTX and P1NP values as well as the post-exercise CTX response. Many of the studies quantifying BTM reference ranges have been performed in post-menopausal and osteoporotic populations, with a number in premenopausal women in their third or fourth decade of life (de Papp et al., 2007; Eastell et al., 2012; Glover et al., 2009). The majority of our data was obtained from highly trained to elite male athletes, making it difficult to draw conclusions about potential sex differences in this age group or how our female data compares to the published literature. Indeed, the number of female athletes represented in this convenience dataset is low, making this study more applicable to male athletes. Still, while we acknowledge the limitation of retrospective data mining analyses not being representative of all athletes, robust data on bone health in young men are lacking, making our data set a small contribution to expanding our understanding of how these markers might differ in athletes and how they can be used. Future studies in both sexes are most certainly required to verify our results, especially in the younger age range where even very little general population data exists.

Exercise training has been shown to increase markers of both bone resorption [pyridoniline (Davidović Cvetko et al., 2022), CTX (Evans et al., 2008)] and bone formation [osteocalcin (Davidović Cvetko et al., 2022; Evans et al., 2008), P1NP, bone alkaline phosphatase (Evans et al., 2008)] but the application of these relative increases over the long-term on bone status remains speculative for the moment. For example, athletes in high- or odd-impact sports have higher BMD and superior bone geometry at loaded sites (Tenforde & Fredericson, 2011)

without apparent changes in bone resorption activity (Dolan et al., 2022), which suggests that exercise-associated increases in CTX could be potentially detrimental to long-term bone status. Previous studies comparing athletes to controls have primarily used osteocalcin as a marker, or less specific urinary biomarkers (Maïmoun & Sultan, 2011), limiting our interpretation of the findings of 'increased turnover'. The 95% confidence interval of fasting BTMs in our study had a greater upper limit than the 'general population' references reported in an Australian consensus paper (Vasikaran et al., 2014). However, we note several limitations of the data in this summary report, including the reliance on a single large study (covering ages 20-97 years) (Jenkins et al., 2013). Here, the Roche cobas analyser was employed (Jenkins et al., 2013). Although the large sample size of the Jenkins et al. (2013) study is noted, limitations of the data include the failure to screen participants for existing bone or metabolic diseases or medication, as well as variable lengths of time over which samples had been stored. Similar values (CTX: ~800 $pg{\cdot}mL^{-1}$; P1NP: ~90 $ug{\cdot}L^{-1}$) to the Jenkins et al. (2013) study were obtained in a German study, the only one identified that used the IDS-iSYS assay (Michelsen et al., 2013), but timing of blood sampling and fasting status was not controlled. A more robustly conducted Indian study (Pal et al., 2021) using the Roche cobas analyser reported a higher upper limit for their reference range for men 20-39 years old, similar to that seen in our study (CTX: >1000 $pg.mL^{-1}$; P1NP: >120 $ug.L^{-1}$). The translation of relative changes in BTM into long-term bone structure and function in athletes requires longitudinal studies. However, because the mechanical stimulus on bone far exceeds that of the general population and is associated with a significant risk of stress fractures, establishing whether BTM characteristics are clinically relevant and have any potential use in monitoring bone status and secondary risk factors in athletes is of interest.

Methodological variables for consideration include the control of pre-analytical variables such as age, sex, recent fracture, comorbidities/drugs, and sample acquisition timing, as well as the specific analyte and assay (Vasikaran et al., 2011). In our study, baseline samples were taken in the morning [prior to 10:30 am (Szulc et al., 2017)] following an overnight fast. We further accounted for some themes (e.g., assay, sex, age) within the statistical analysis of our data. Indeed, we found significant differences in fasting BTM concentrations between those younger than and older than 25 years old. The age at which peak bone mass is achieved differs by sex and skeletal site (Berger et al., 2010), and may be explained by differing bone turnover rates. Measured longitudinally by areal BMD via DXA, lumbar spine peak bone mass occurs between 33-40 years in women and 19-33 years in men, whereas hip peak bone mass occurs earlier between 16-19 years in women and 19-21 years in men (Berger et al., 2010). A smaller study

suggested an increase in bone mineral content until 25 years of age, together with higher concentrations of BTMs that plateaued thereafter (Walsh et al., 2009). Similarly, the previously discussed studies of CTX and P1NP reference ranges found decreasing concentrations of both BTM with increasing age in those younger than 40 years (Jenkins et al., 2013; Michelsen et al., 2013; Pal et al., 2021). This highlights the need for age-matching intervention groups when planning future studies investigating BTMs in athletes and may suggest that a tighter age range may be required.

However, analytical variability requires consideration due to the use of different techniques over the course of our studies. While the ELISA CTX values were lower than the other two methods in our study, we failed to observe significant differences between the automated assays. Recently, a multicentre study investigating differences between assays in an older population (66.1±11.7 years) reported close agreement between the automated assays for P1NP ('total P1NP' [Roche Cobas] and 'intact P1NP' [IDS-iSYS]) (Cavalier et al., 2019); we were unable to find any publications reporting on the comparison to the ELISA. Conversely, the same group found significant variation between all three assays for CTX, attributed to the instability of the analyte and long-term storage and, specifically with the manual ELISA, dependency on the operator (Cavalier et al., 2021). Although results obtained from each method may be reliable and useful for comparing interventions, when attempting to establish reference ranges for a population, ideally harmonization of assays should be achieved. Achieving harmonization would involve obtaining samples under strict pre-analytical conditions and distribution of samples between commercial, as well as a higher-order, assays (Vasikaran et al., 2011). Subsequent regression would allow for correction factors between assays to be obtained, minimising differences between assay results (Vasikaran et al., 2011). Ideally, in future, laboratories adhere to a reference standard assay, similar to how CTX and P1NP have become reference analytes.

One of the aims of this study was to investigate the possible links between iron and bone status. Exercise results in a peak in CTX within 2 h, which returns to baseline thereafter, and very little change observed in P1NP (Dolan et al., 2022). Similarly, IL-6 also responds to exercise with an abrupt increase immediately post-exercise (Ostrowski et al., 1998a; Ostrowski et al., 1998b; Peeling et al., 2009b). Temporal associations between IL-6 and CTX have previously been noted in exercise studies (Sale et al., 2015), but whether these markers merely respond similarly to the same exercise bout characteristics or whether IL-6 acts as a stimulus for subsequent bone resorption is uncertain. Indeed, previous research *in vitro*, in animal models, and in clinical

populations demonstrates a role for this cytokine in both bone resorption and formation (Sims, 2021). In our analysis, while resting IL-6 explained some of the variance in fasting CTX concentrations, post-exercise IL-6 was not significant in the post-exercise CTX response. This may be due to the different sources of IL-6 at rest and following exercise. In fact, while IL-6 at rest is a marker of inflammation, sourced from immune cells and adipocytes, post-exercise IL-6 is largely derived from the muscle itself, with greater increases occurring following glycogen depletion (Hennigar et al., 2017). Of interest, we did not find a significant association between lean body mass and post-exercise IL-6, which may have been expected given that the source of IL-6 here is the contracting muscle with larger increases occurring with modes of exercise recruiting larger muscle groups (Fischer, 2006). This lack of association in our analysis might suggest a more influential role for muscle glycogen, assumed to be replete in our athletes on standardized diets. Additionally, the contribution of IL-6 to fasting concentrations of CTX, but not P1NP, in our analysis may suggest a greater role of the cytokine in stimulating osteoclastogenesis at rest. Conversely, the failure of post-exercise IL-6 to explain variance in post-exercise CTX or P1NP suggests that either other covariates (e.g., total work done (Dolan et al., 2022)) play a greater role or that the effects are not observed in the immediate period post-exercise. Indeed, as very little change in P1NP is seen acutely (Dolan et al., 2022), the effects of potential exercise-induced increases in soluble IL-6 receptor (Leggate et al., 2010; Robson-Ansley et al., 2009a), a proposed requirement for bone formation signalling (Sims, 2021), may not be appreciated in such short-term analyses. Over the long-term, the combination of endocrine, metabolic, and mechanical stimuli likely interacts to produce a resultant bone phenotype in athletes, emphasizing that a combination of metrics (e.g., bone turnover markers, IL-6, BMD, bone geometry), rather than a single measure, should be monitored.

The association between ferritin/hepcidin and CTX concentrations in our study is intriguing and might reflect the shared link to IL-6 (Peeling et al., 2009b; Sims, 2021). However, we failed to find any further suggestion that ferritin/hepcidin contributed to CTX variance or that differences in ferritin/hepcidin concentrations differed between BMD classification groups. Previous research in healthy individuals has demonstrated negative associations between ferritin and both BMD (Kim et al., 2012; Kim et al., 2020; Lu et al., 2020) and bone resorption (N-terminal telopeptide; NTX) (Toxqui et al., 2014). Impaired bone architecture has been demonstrated in hemochromatosis (Jandl et al., 2020) and thalassemic (Baldini et al., 2014) patients where iron overload is prevalent, as well as in iron deficient rats (Medeiros et al., 2004). Interestingly, hepcidin may play a preventative role and is a potential therapeutic agent for osteoporosis with in vivo experiments demonstrating its action reducing iron accumulation,

reactive oxygen species production, and PGC-1β expression, and thereby suppressing osteoclast function and limiting bone loss (Zhang et al., 2021). The lack of association of ferritin with BMD in our analysis could potentially indicate the protective effect of mechanical loading on bone in athletes as opposed to the general population. Alternatively, the relatively acute changes in ferritin status in athletes as opposed to the long-term change that could be seen in BMD are not appreciated in this cross-sectional study design. Nevertheless, frequent increases in hepcidin associated with exercise (Peeling et al., 2009a) serve to prevent iron overload in athletes, which in turn may be protective for bone, provided that nutrient needs are adequately met through the diet.

3.6 Conclusion

The limitation of using BMD as a lone assessment of bone health in athletes is clear with site-specific loading patterns, discordance between sites and across sports, and the prevalence of bone stress injuries occurring independently of BMD. Further expansion of an athlete-specific database for CTX and P1NP concentrations, with robust pre-analytical control and harmonization of assays, has the potential to provide insight into how BTMs could be used in the shorter term to evaluate bone strength and fracture risk. Our study is limited by a relatively small sample size, especially in the female cohort, as well as the cross-sectional study design, which precludes definitive conclusions from being drawn. However, the dietary standardization and controlled sampling time and conditions strengthens the analysis by attenuating pre-analytical sources of variability. The links between iron and bone indices are intriguing and further research in both clinical and athletic populations could lead to nutritional or pharmacological interventions that limit bone loss. Extension of this study is required with a larger sample size and longitudinal monitoring to ascertain the clinical application of the combined use of bone turnover markers and BMD in monitoring bone health in athletes.

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Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

Nikita Fensham: Conceptualization; Methodology; Investigation; Data curation; Formal analysis; Project administration; Resources; Software; Visualization; Writing - original draft; Writing - review & editing. **Alannah McKay:** Conceptualization; Methodology; Investigation; Data curation; Project administration; Resources; Software; Validation; Supervision; Writing - review & editing. **Louise Burke:** Conceptualization; Methodology; Investigation; Data curation; Project administration; Resources; Supervision; Writing - review & editing.

Availability of data

As the data included in this study comprises both published and unpublished data by several different authors, data will be made available on request.

Interlinking chapter

Site discordance in BMD between and within sports, along with the weak relationship between areal BMD and fracture risk in young adults (Ferrari et al., 2012; Frolich et al., 2020; Writing Group for the ISCD Position Development Conference, 2004), suggests that additional markers of bone strength may be required in athletic populations. Although the value of using BTMs has been demonstrated in situations of secondary causes of bone loss as well as in the monitoring of osteoporosis treatment (Vasikaran et al., 2011), appropriate reference ranges and causes of variability have yet to be established in athlete populations. While previous studies have demonstrated effects of exercise (Dolan et al., 2022) and dietary manipulations (Dolan et al., 2020) on the short-term response of BTMs, it has been difficult to ascertain the degree to which each of these stimuli play a role. In athletes, where dietary manipulation is common practice for achieving performance goals, and where frequent mechanical loading predisposes the bone to stress injuries (Warden et al., 2006), it is important to determine whether popular dietary approaches support bone health. The potential influence of IL-6 release on CTX responses raises the hypothesis that strategies previously demonstrated to affect IL-6 responses (i.e., LEA (Ishibashi et al., 2020), LCHF (McKay et al., 2019a)) around exercise may similarly affect bone. Therefore, the following study aimed to investigate the differential impact of energy and carbohydrate manipulation on BTMs at rest and across exercise.

4 Chapter 4

Short-term carbohydrate restriction impairs bone formation at rest and during prolonged exercise to a greater degree than low energy availability

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4.1 Abstract

Bone stress injuries are common in athletes, resulting in time lost from training and competition. Diets that are low in energy availability have been associated with increased circulating bone resorption and reduced bone formation markers, particularly in response to prolonged exercise. However, studies have not separated the effects of low energy availability *per se* from the associated reduction in carbohydrate availability. The current study aimed to compare the effects of these two restricted states directly. In a parallel group design, 28 elite racewalkers completed two 6-day phases. In the Baseline phase, all athletes adhered to a high carbohydrate/high energy availability diet (CON). During the Adaptation phase, athletes were allocated to one of three dietary groups: CON, low carbohydrate/high fat with high energy availability (LCHF), or low energy availability (LEA). At the end of each phase, a 25 km racewalk was completed, with venous blood taken fasted, pre-exercise, and 0, 1, 3 h post-exercise to measure carboxyterminal telopeptide (CTX), procollagen-1 N-terminal peptide (P1NP), and osteocalcin (carboxylated, gla-OC; undercarboxylated, glu-OC). Following Adaptation, LCHF showed decreased fasted P1NP (~26%; $p<.0001$, $d=3.6$), gla-OC (~22%; $p=.01$, $d=1.8$), and glu-OC (~41%; $p=.004$, $d=2.1$), which were all significantly different to CON ($p<.01$), whereas LEA demonstrated significant, but smaller, reductions in fasted P1NP (~14%; $p=.02$, $d=1.7$) and glu-OC (~24%; $p=.049$, $d=1.4$). Both LCHF ($p=.008$, $d=1.9$) and LEA ($p=.01$, $d=1.7$) had significantly higher CTX pre- to 3 h post-exercise but only LCHF showed lower P1NP concentrations ($p<.0001$, $d=3.2$). All markers remained unchanged from Baseline in CON. Short-term carbohydrate restriction appears to result in reduced bone formation markers at rest and during exercise with further exercise-related increases in a marker of bone resorption. Bone formation markers during exercise seem to be maintained with LEA although resorption increased. In contrast, nutritional support with adequate energy and carbohydrate appears to reduce unfavourable bone turnover responses to exercise in elite endurance athletes.

Keywords: bone modelling and remodelling, biochemical markers of bone turnover, bone-muscle interactions, exercise, nutrition

4.2 Introduction

Minimizing time lost due to injury is an important consideration in elite athletes (Feddermann-Demont et al., 2014) who need to be regularly available for training and competition. Bone stress injuries (reactions and fractures) account for ~20% of annual injuries in competitive track and field athletes (Bennell et al., 1996b). Data from the National Collegiate Athletic Association indicates an injury rate of 4.6-7.2 stress fractures per 100,000 athlete exposures in male track athletes and 11.6-22.3 per 100,000 in females – these rates being amongst the highest, alongside cross-country and women's gymnastics (Rizzone et al., 2017). While participation in weight-bearing sport has been associated with increased bone density at loaded sites (Tenforde & Fredericson, 2011), this benefit may be attenuated in low reproductive hormone or nutritional status (Tenforde et al., 2018). Although multiple factors are often at play, inadequate nutritional support is a key consideration for bone health (Sale & Elliott-Sale, 2019). Previous studies have identified a role for both overall energy availability (Ihle & Loucks, 2004; Murphy et al., 2021) and carbohydrate availability (de Sousa et al., 2014; Heikura et al., 2019; Sale et al., 2015) in the bone turnover response to exercise. However, as energy restriction involves a relative reduction in carbohydrate intake, it has been difficult to ascertain whether the effect on bone is due to inadequate energy or lack of carbohydrate.

Assessment of the short-term impact of nutritional or pharmacological interventions relies on the measurement of bone turnover markers (BTMs), as opposed to the longer-term impact on bone mineral density (BMD) (Wu et al., 2021). The procollagen-1 N-terminal peptide (P1NP) released during collagen synthesis and the C-terminal telopeptide (CTX) during matrix dissolution represent markers of bone formation and resorption, respectively (Song, 2017), and are the most commonly used clinical markers (Wu et al., 2021). Whereas carboxylated osteocalcin (gla-OC) is involved in matrix mineralization (Moser & Van Der Eerden, 2019), there is increasing recognition of an endocrine role for its undercarboxylated form (glu-OC) in enhancing glucose and fatty acid uptake and catabolism during exercise (Mera et al., 2016). The current utility of BTMs lies in short-term monitoring of the response to antiresorptive and anabolic therapies in the management of osteoporosis, allowing for more frequent clinical decision-making than sole reliance on annual BMD measurements (Wu et al., 2021). Significant associations between BTM changes and future fracture risk have been observed, independent of BMD (Tian et al., 2019). Collectively, these BTMs reflect the bone remodelling, a measure of bone quality which, along with BMD, significantly contributes to bone strength (Wu et al., 2021). However, it must be noted that these markers are unable to indicate the status of the

processes occurring at a specific site. Additionally, the utility of BTMs in both non-osteoporotic individuals and with respect to non-pharmacological interventions is under-explored.

Energy availability is defined as the amount of energy remaining for physiological system function after accounting for the energy expended in purposeful exercise, expressed relative to fat-free mass (FFM) (Loucks et al., 2011). Low energy availability may result from intentional energy restriction and/or an unintentional failure to meet high energy expenditure demands (Loucks, 2004). Meeting energy requirements with adequate energy and nutrient intake should be a priority for the majority of the season, but athletes may periodically engage in short periods of low energy availability to achieve body composition goals, make a specific weight class, improve economy, or increase power/weight ratios (Burke et al., 2018a). Although there is no absolute threshold at which various body systems are simultaneously affected, previous short-term (5 day) controlled experiments in healthy females have shown suppression of bone formation at, and below, an energy availability of 30 $kcal.kg^{-1}$ $LBM.d^{-1}$, with increased bone resorption at 10 $kcal.kg^{-1}$ $LBM.d^{-1}$ (Ihle & Loucks, 2004). Studies in males are limited, but it has been suggested that men can sustain lower energy availability without significant physiological disruption (De Souza et al., 2019). Indeed, Papageorgiou and colleagues demonstrated that following a 5 day intervention, women exhibited increased bone resorption and reduced bone formation at an energy availability of 15 $kcal.kg^{-1}$ $LBM.d^{-1}$, yet no significant effect was found in men (Papageorgiou et al., 2017). With this in mind, our study focused exclusively on males, expanding on the limited literature base in this population with respect to the impact of energy manipulation on bone metabolism.

In recent years, there has been an increased interest in using low carbohydrate/high fat diets to improve endurance exercise capacity through a shift towards primary use of fatty acids and ketones as a fuel source (Volek et al., 2015). However, this has been shown to reduce capacity for sustained high-intensity race performance in competitive athletes due to an impairment of exercise economy from differences in the stoichiometry of energy production from fat versus carbohydrate oxidation (Burke et al., 2020). Further, 3.5 weeks of a ketogenic (< 50 $g.d^{-1}$ of carbohydrate) diet in elite racewalkers was observed to increase bone resorption (CTX) and decrease bone formation (P1NP) markers at both rest and across exercise (Heikura et al., 2019). While there are no published long-term athlete studies, evidence from both animal models (Bielohuby et al., 2010) and children with intractable epilepsy being treated with a ketogenic diet (Simm et al., 2017) suggests that there may be a detrimental effect of the ketogenic diet on bone health, potentially due to carbohydrate restriction. A second interest in this diet is its

ability to separate the effects of low carbohydrate availability from energy availability, allowing for further inquiry into the influences of macronutrients on health and performance, independent of overall energy intake. To our knowledge, only one previous study, involving a one-day intervention, has compared energy and carbohydrate availability directly in relation to exercise (Hammond et al., 2019). Here, the comparator low carbohydrate/high fat diet was not ketogenic in nature, making it difficult to disentangle the energy and carbohydrate effects. Our study aims to expand upon this through the manipulation of macronutrient targets.

As adjustment of both energy and macronutrient intake is common practice in athletes and may be used as a short-term strategy to achieve body composition or weight goals prior to competition (Burke et al., 2018a), determining the health and performance effects of these diet practices is of interest. Accordingly, our aim of this study was to determine whether overall EA or CHO availability exerted greater effects on bone metabolic responses to exercise.

4.3 Methods

4.3.1 Participants

Twenty-eight elite male racewalkers, eligible for participation in either national or international competition, were recruited for this study via convenience sampling. Based on previous work (Heikura et al., 2019) investigating BTM responses to either a high carbohydrate or ketogenic diet, it was estimated that including 5-10 participants per group (osteocalcin: d=2.00; CTX: d=1.52; P1NP: d=1.27) was appropriate to detect statistical significance with an alpha of 0.05 and power of 0.8 (GPower version 3.1.9.6). Screening of hormonal and metabolic health was conducted prior to the start of the study – no exclusions were required on the basis of these results. No athletes had a known medical condition or were taking medication or supplements during the study, and recent fractures within the past 3 months were ruled out. All athletes completed the study. Athlete characteristics are presented in Table 4.1. Athletes took part in one of two separate training camps held in January 2019 in Canberra, Australia (n=20) and January 2021 in Melbourne, Australia (n=8). Written informed consent was obtained following explanation of the risks and requirements of the study. The study conformed to the standards required by the Declaration of Helsinki. Ethics approval was obtained from the ethics committees of the Australian Institute of Sport (2019; ref: 20181203) and the Australian Catholic University (2021; ref: 2020-238HC).

Table 4.1: Baseline characteristics of athletes allocated to each diet group				
		CON (n=10)	**LCHF (n=8)**	**LEA (n=10)**
Age	years	27 (21-33)	28 (25-29)	30 (28-33)
Body mass	kg	66.6±6.2	66.2±7.7	67.8±5.4
Fat free mass	kg	58.7±5.5	58.6±7.9	58.3±4.9
VO_{2max}	$ml.kg^{-1}.min^{-1}$	63.2±3.6	67.5±5.7	62.1±6.1
BMD spine (L1-4)	$g.cm^{-2}$	1.19±0.07	1.11±0.10	1.24±0.13[a]
	Z-score	0.02±0.49	-0.58±0.65	0.36±0.97[a]
BMD total hip	$g.cm^{-2}$	1.17±0.09	1.09±0.14	1.10±0.13
	Z-score	0.73±0.66	0.18±0.94	0.26±1.05
Data are presented as mean ± standard deviation (except for age which is presented as median (interquartile range). CON=high energy/high carbohydrate, LCHF=low carbohydrate/high fat, LEA=low energy availability. [a] indicates significantly higher than LCHF (p<.05)				

4.3.2 Study protocol

This study was a parallel group design. Each training camp comprised of two, 6-day phases (Figure 4.1). During phase 1 (Baseline), all athletes adhered to a high carbohydrate (~65% of total energy intake), high energy availability (> $40kcal.kg^{-1}$ $FFM.d^{-1}$) control (CON) diet. For phase 2 (Adaptation), athletes were assigned to one of three diets: high carbohydrate/high energy availability (CON, n=10), low carbohydrate/high fat/high energy availability (LCHF, n=8), or low energy availability (LEA, n=10). Due to the inability to blind participants to the diet, allocations to dietary interventions were based on athlete preference whilst matching for individual characteristics (age, 20 km personal best time, training status). During both phases, a structured training plan was followed to ensure similar training volume and intensity among groups (McKay et al., 2021c). On the final day of each phase, a 25 km racewalking protocol was performed, where venous blood samples were taken to measure BTMs.

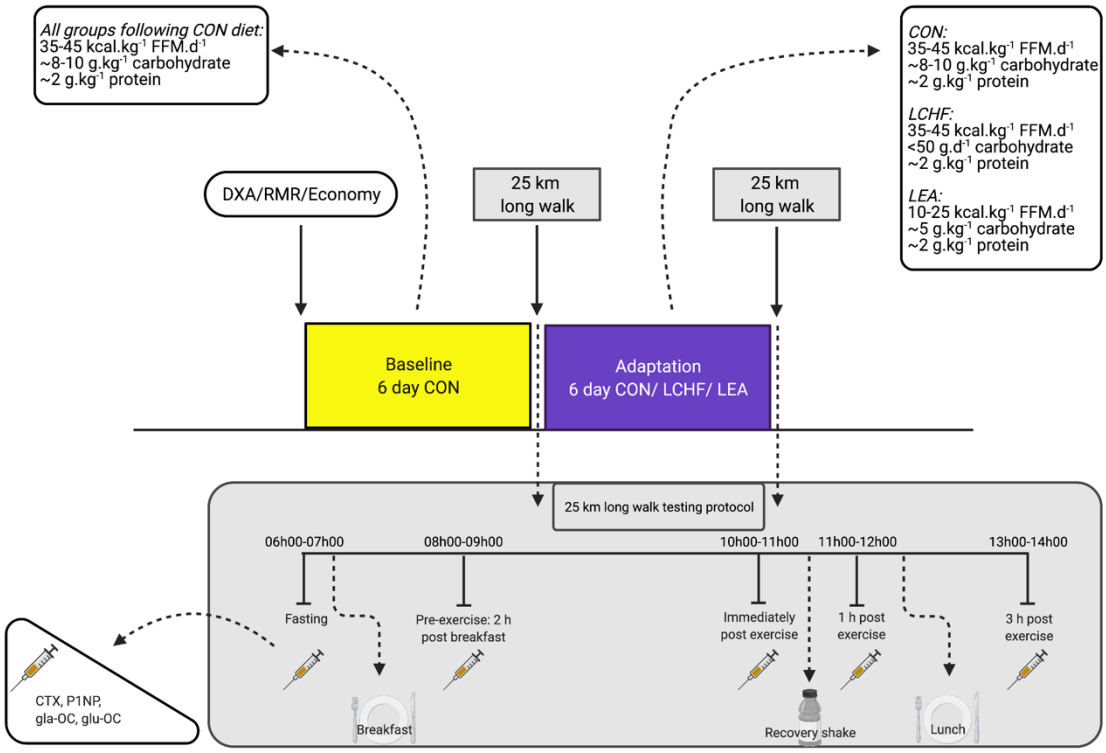

Figure 4.1: Overview of experimental protocol undertaken by elite racewalking participants, including the dietary interventions and the exercise test with timing of blood sampling. CON = high energy/high carbohydrate, LCHF = low carbohydrate/high fat, LEA = low energy availability, FFM = fat-free mass, DXA = dual energy x-ray absorptiometry, RMR = resting metabolic rate, CTX = carboxy-terminal telopeptide, P1NP = procollagen-1 N-terminal peptide, gla-OC = carboxylated osteocalcin, glu-OC = undercarboxylated osteocalcin. (Created with biorender.com)

4.3.3 Baseline bone mineral density

Hip and lumbar spine (L1-4) BMD were measured at baseline via dual-energy x-ray absorptiometry (DXA) with Lunar iDXA machines (version 16; GE Healthcare, Australia). Measurements were conducted by experienced practitioners certified in clinical bone densitometry. As body composition measurements were performed simultaneously, best practice protocols were followed (Nana et al., 2015).

4.3.4 Dietary intervention

A brief description of dietary control is provided here; full details are specified elsewhere (manuscript in preparation). All meals were formulated and compliance with intake was monitored by a team of accredited sports dietitians, chefs, and nutritionists. Meals were devised using FoodWorks Professional Edition 9 (Xyris Software, Brisbane, Australia). The CON diet targeted an energy availability of ~40 kcal.kg^{-1} FFM.d^{-1}, comprised of 65% carbohydrate, 15% protein, and 20% fat. The LCHF group was set the same energy availability target, but with low

carbohydrate (< 50 $g.d^{-1}$), moderate protein (2.2 $g.kg^{-1}.d^{-1}$) and high fat [remainder (~80%) of target energy] intakes. The LEA group had a target energy availability of ~15 kcal.kg $FFM.d^{-1}$, with a similar macronutrient composition as CON (60% of energy from carbohydrate, 25% of energy from protein, 15% of energy from fat). Calcium in the pre-exercise meal, the other dietary characteristic known to acutely affect markers of bone turnover (Lundy et al., in review), was minimised (< 50 mg) in each of standardized meals consumed prior to the long walk test protocol.

Energy availability was calculated as the difference between energy intake and exercise energy expenditure, normalized to fat free mass (measured via DXA). Exercise energy expenditure was estimated from a 4-stage incremental economy test, using collected respiratory gases inputted into the Weir equation (Weir, 1949). Resting metabolic rate was measured directly and subtracted from these values, which were then converted to prospective caloric estimates per kilometre. Cross-training sessions were accounted for in metabolic equivalents. Total exercise energy expenditure was predicted by multiplying these values by the planned training sessions, and energy intake requirements calculated accordingly to achieve target energy availability. Training session completion was monitored twice daily and meals adjusted as necessary. Non-exercise activity thermogenesis was not considered significant in this context. As a fortunate consequence of this research camp environment, on-site researchers were able to monitor and confirm that athletes undertook very little activity outside of the prescribed training times.

4.3.5 Test protocol

On the last day of each dietary phase, a 25 km racewalk was performed, combining both field and laboratory components. One athlete completed only 19 km as he was a junior athlete (18 years old). Athletes arrived in the morning following an overnight fast. An intravenous catheter was inserted and the first blood sample was drawn (±06h30). Athletes were then provided with a standardized breakfast, consisting of 2 $g.kg^{-1}$ body mass of carbohydrate for all groups in the Baseline phase. The same breakfast was provided to the CON group in the Adaptation phase, whereas the LCHF group received an isocaloric high-fat (~80%) option and the LEA group consumed a meal containing 1 $g.kg^{-1}$ body mass carbohydrate. A pre-exercise blood sample was taken (±08h30) 15 min prior to the onset of the racewalk, which commenced 2 h after breakfast. Kilometres 1, 7, 13, 19, and 25 were performed on a treadmill at a pace equivalent to ~75% of the athlete's VO_2max and the remaining kilometres were performed at a consistent, self-nominated pace on a flat, outdoor, road circuit. Carbohydrate gels were consumed following each treadmill bout, totalling ~60 $g.h^{-1}$ for all groups during Baseline and CON during

Adaptation. The LEA group consumed the equivalent of 30 $g.h^{-1}$ during Adaptation and the LCHF group ingested isocaloric (to CON) high fat snacks. Water was consumed *ad libitum*. Venous cannulas were flushed with ~3 ml saline after each treadmill bout. Environmental temperature and relative humidity were recorded at 30 min intervals with a final individualized value averaged across the exercise bout. Upon completion of the racewalk protocol, another venous blood sample was collected (±10h30). At 30 min post-exercise, athletes received a standardized recovery shake (1.5 $g \cdot kg^{-1}$ body mass carbohydrate and 0.3 $g \cdot kg^{-1}$ body mass protein for all groups at Baseline, or an isocaloric high-fat low-carbohydrate option for LCHF and 0.75 $g.kg^{-1}$ body mass carbohydrate for LEA at Adaptation). A further blood sample was collected at 1 h post-exercise (±11h30) after which lunch was provided in accordance with trial phase and dietary allocation. A final blood sample was collected at 3 h post-exercise (±13h30).

4.3.6 Blood analysis

Blood samples were taken at rest (fasted), pre-exercise, and immediately, 1 h, and 3 h post-exercise. Samples were collected into BD Vacutainer SST II tubes (East Rutherford, NJ, USA), which were left to clot at room temperature for 30 min prior to being centrifuged at 1500g at 4°C for 10 min. Serum was aliquoted into 1 ml Eppendorf tubes and frozen at -80°C for batch analysis. Concentrations of beta-isomerized carboxy-terminal telopeptide (CTX), procollagen-1 N-terminal peptide (P1NP), carboxylated osteocalcin (gla-OC), and undercarboxylated osteocalcin (glu-OC) were measured from each sample. CTX and P1NP concentrations were assessed by electrochemiluminescence immunoassay (Cobas e411, Roche Diagnostics, Basel, Switzerland). Carboxylated and undercarboxylated osteocalcin measurements were performed using enzyme immunoassay (EIA) kits (Takara Bio inc., Shiga, Japan) analysed on a FLUROstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany). Calculated coefficients of variation were 4.2% (CTX), 3.0% (P1NP), 10.7% (glu-OC), and 3.9% (gla-OC).

4.3.7 Statistical analysis

Statistical analysis was performed using R Studio (v1.4.1106, R Core Team, 2021) with significance set at $p<.05$. Athlete characteristics, differences in nutrient intake, and energy availability between groups and phases were analysed with a two-way analysis of variance (ANOVA; parametric) after verifying normality with the Shapiro-Wilk test. Where normality was violated (age only), the Kruskal-Wallis test (non-parametric) was used for between-group comparisons. Bone turnover markers were analysed in three ways: 1) using the change in fasted values from Baseline to Adaptation, 2) assessing absolute concentrations across time, and 3) by calculating area under the concentration-time curve (AUC) for each participant (pre-exercise to

3 h post-exercise), using the PKSolver add-in in Microsoft Excel (v16.48), prior to further analysis in R. Linear mixed-effect models were estimated for all 3 analyses with restricted maximum likelihood through the 'lme4' package in R. As applicable, fixed effects included Diet, Phase, and Timepoint with random effects of subject and heat index, each nested within study, to account for inter-individual variation and camp timing. Normality was assessed through quantile-quantile plots – characteristic departures were not detected. Homoscedasticity was tested with the Fligner-Killeen test. Statistical significance of fixed effects was determined using Type II Wald tests with Kenward-Roger approximation Post-hoc analysis for significant effects was performed using Tukey's Honestly Significant Difference test. Where unequal variance was detected between groups, a Welch's ANOVA and post-hoc Dunnett's test was applied. Cohen's *d* effect sizes were computed using R package 'emmeans' with values of 0.2, 0.5, and 0.8 as small, medium, and large effects, respectively.

4.4 Results

4.4.1 Participant characteristics

Baseline characteristics are presented as mean ± standard deviation (or median and interquartile range for age) in Table 4.1. The athletes were well-matched for age, body mass, fat-free mass, and VO_{2max} and, since all were male, sex was eliminated as a source of pre-analytical variability. Here, we note the first quartile for age being lower in the CON group than the other two groups; more specifically, one athlete was 18 years old. On aggregate, statistically significantly lower spine BMD (p=.04) and z-scores (p=.03) were noted in the LCHF group compared to the LEA group only, yet there was no difference between groups for hip BMD or z-scores. Individual data indicated z-scores $-2 < z < -1$ for two LEA athletes' hip BMD and two LCHF athletes' spine BMD; hence, the practical application to the effect on BTMs is questionable.

4.4.2 Dietary analysis

There was no difference in energy intake, energy availability, macro- or micronutrient intake between the 3 diets during the Baseline phase (p>.05; Table 4.2). In keeping with the study design, during the Adaptation phase, energy intake and energy availability were significantly lower in the LEA group than both CON and LCHF (p<.001), with fat intake greatest in the LCHF group (p<.001). Carbohydrate intake was significantly reduced in the LCHF and LEA groups (p<.001) during Adaptation; however, the percentage of total energy intake was largely maintained (~60 %) in the LEA group.

Table 4.2: Dietary intake for each diet group during both baseline and adaptation phases							
		Baseline			**Adaptation**		
		CON (n=10)	**LCHF (n=8)**	**LEA (n=10)**	**CON (n=10)**	**LCHF (n=8)**	**LEA (n=10)**
Energy intake	$kcal.d^{-1}$	3824±623	3843±462	3727±335	3970±537	3730±411	2335±238 [a,b,c]
	$kcal.kg^{-1}$	58±6	59±4	55±2	61±5	57±3	35±3 [a,b,c]
Energy availability	$kcal.kg^{-1}$ $FFM.d^{-1}$	40±3	41±1	41±4	41±4	41±2	15±2 [a,b,c]
Carbohydrate	$g.d^{-1}$	613±102	616±76	599±53	639±87	36±6 [a,b]	338±33 [a,b,c]
	$g.kg^{-1}.d^{-1}$	9.4±1.0	9.5±0.6	8.9±0.4	9.8±0.8	0.5±0.1 [a,b]	5.0±0.4 [a,b,c]
Protein	$g.d^{-1}$	144±22	144±17	141±14	148±21	145±16	141±14
	$g.kg^{-1}.d^{-1}$	2.2±0.2	2.2±0.1	2.1±0.1	2.3±0.2	2.2±0.0	2.1±0.1
Fat	$g.d^{-1}$	83±15	84±11	79±9	84±13	330±39 [a,b]	40±7 [a,b,c]
	$g.kg^{-1}.d^{-1}$	1.3±0.2	1.3±0.1	1.2±0.1	1.3±0.2	5.1±0.4 [a,b]	0.6±0.1 [a,b,c]
Data are presented as mean ± standard deviation. CON=high energy/high carbohydrate, LCHF=low carbohydrate/high fat, LEA=low energy availability. [a] indicates significant difference to baseline (p<.05), [b] indicates significant difference to CON (p<.05), [c] indicates significant difference to LCHF (p<.05)							

4.4.3 Percent change in fasting concentrations

From Baseline to Adaptation (Figure 4.2), all groups showed an increase (p<.001) in fasted CTX concentrations with no significant difference between groups (~8-16%; p=.60). The LCHF group showed decreased fasting P1NP (-26±4%; p<.0001, d=3.6), gla-OC (-22±7%; p=0.01, d=1.8), and glu-OC (-41±8%; p=.004, d=2.1), with these percent changes all significantly different to CON (p<.01). The LEA group also demonstrated significant, although smaller, reductions in P1NP (-14±3%; p=.02, d=1.7) and glu-OC (-24±7%; p=.049, d=1.4) with a non-significant reduction in gla-OC (-7±6%; p=.81, d=.56); P1NP and gla-OC percent changes were significantly lower than CON (p<.001 and p=.03, respectively). In contrast, the CON group showed an increase in P1NP (7±3%; p=.76, d=.65) and gla-OC (16±6 %; p=.25, d=1.1) and an unchanged glu-OC (3±8 %; p=1.00, d=.12). No differences between LCHF and LEA were found for these percent changes (p>.05).

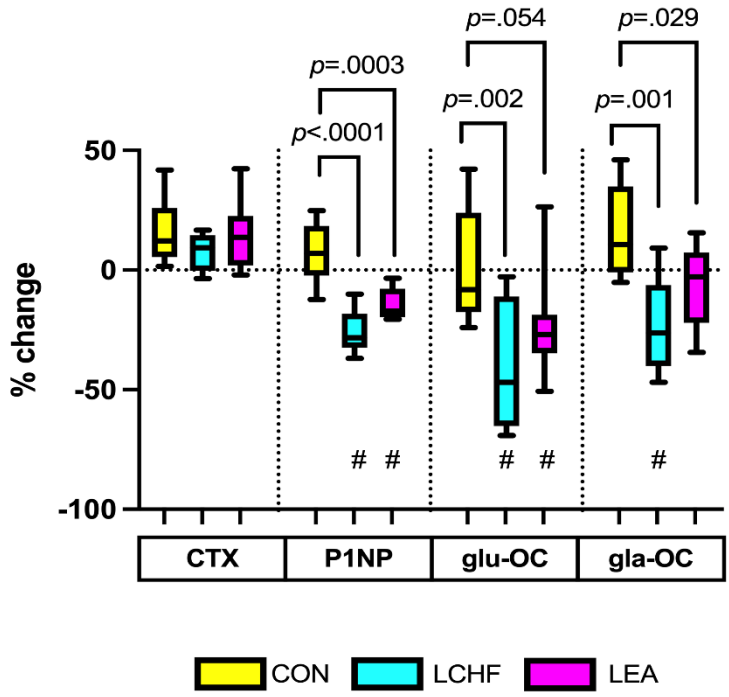

Figure 4.2: Percent change in fasted concentrations of bone turnover markers from Baseline to Adaptation. CON = high energy/high carbohydrate, LCHF = low carbohydrate/high fat, LEA = low energy availability, CTX = carboxy-terminal telopeptide, P1NP = procollagen-1 N-terminal peptide, gla-OC = carboxylated osteocalcin, glu-OC = undercarboxylated osteocalcin. Raw data presented in boxplot as median, upper and lower quartiles, and minimum and maximum. p-values on figure represent comparisons to CON (between group); # represents significant change within group from Baseline to Adaptation with p-values in text.

4.4.4 Changes across time and exercise

Peak CTX concentrations occurred in the fasted state (p<.05) for all groups, decreasing by ~45% prior to the onset of exercise, signifying a clear effect from consuming a meal (Figures 4.3A and 4.3B). At 1 h post-exercise, CTX concentrations were greater than pre-exercise for all groups (~20%; p<.001), demonstrating a possible acute response to exercise. Both LCHF (p=.0001, d=1.4) and LEA (p=.02, d=.91) demonstrated elevated CTX overall following Adaptation, with CON remaining similar to Baseline (p=.67, d=.42).

A meal effect, yet of small magnitude, was also suggested to occur with P1NP (Figures 4.3C and 4.3D) and gla-OC (Figures 4.3G and 4.3H) with pre-exercise concentrations being ~8% lower than fasted for both markers (p<.0001, d=1.0; and p=.01, d=.62 respectively). The subsequent influence of exercise was evidenced by an increase in both markers immediately post-exercise compared to pre-exercise values (p<.0001; P1NP: d=2.5, gla-OC: d=.86). Similarly, immediately post-exercise glu-OC concentrations were higher than pre-exercise for all groups in the Adaptation phase (Figure 3F; p=.01, d=.97) and those at 3 h post-exercise in both phases (Figures 3.3E and 3.3F; p<.05, Baseline: d=.89, Adaptation: d=1.4).

4.4.5 Exercise-associated area under the curve

Both LCHF (p=.008, d=1.9) and LEA (p=.01, d=1.7) groups had significantly higher CTX AUC values following Adaptation (28.0% and 30.9% increase respectively; Figure 4.4A) but only the LCHF group showed significantly reduced (21.1%; p<.0001, d=3.2) P1NP AUC values (Figure 4.4B). Although both the LCHF (p=.003, d=2.1) and LEA (p=.01, d=1.7) groups had lower exercise-associated glu-OC AUC after Adaptation (29.2% and 19.8% reduction respectively; Figure 4.4C), only the LCHF group also had lower (23.2%) gla-OC (Figure 4.4D; p=.001, d=2.4). Exercise-associated AUC remained unchanged for all markers for CON (p>.10).

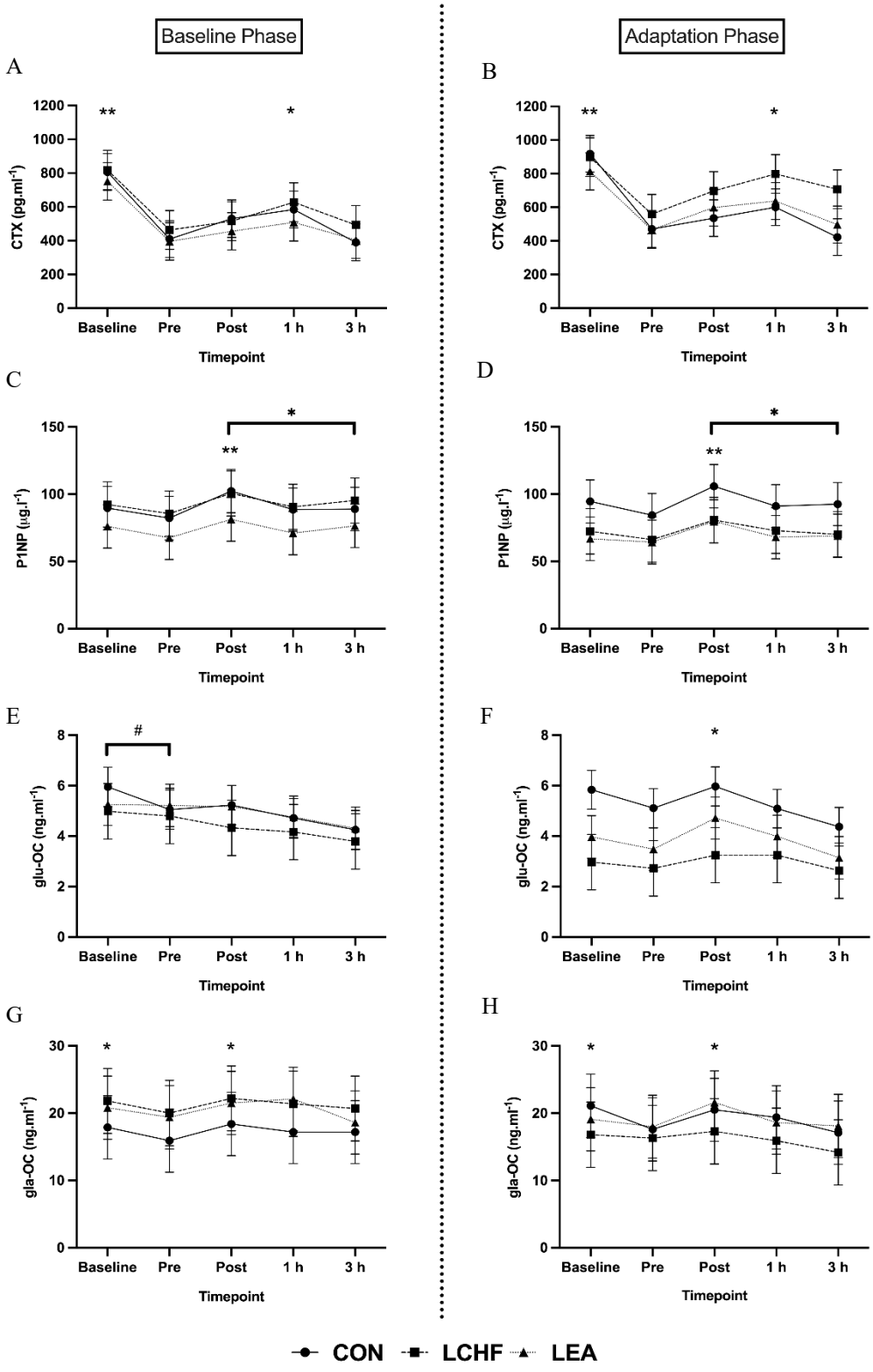

Figure 4.3: Concentration of bone turnover markers for each diet group in each phase across time. CON = high energy/high carbohydrate, LCHF = low carbohydrate/high fat, LEA = low energy availability, CTX = carboxy-terminal telopeptide, P1NP = procollagen-1 N-terminal peptide, gla-OC = carboxylated osteocalcin, glu-OC = undercarboxylated osteocalcin. * time effect: significantly higher than pre-exercise in all groups (p<.05). ** time effect: significantly higher than all other timepoints in all groups (p<.05). # time by trial effect: significantly higher than Adaptation at specific timepoint (p<.05).

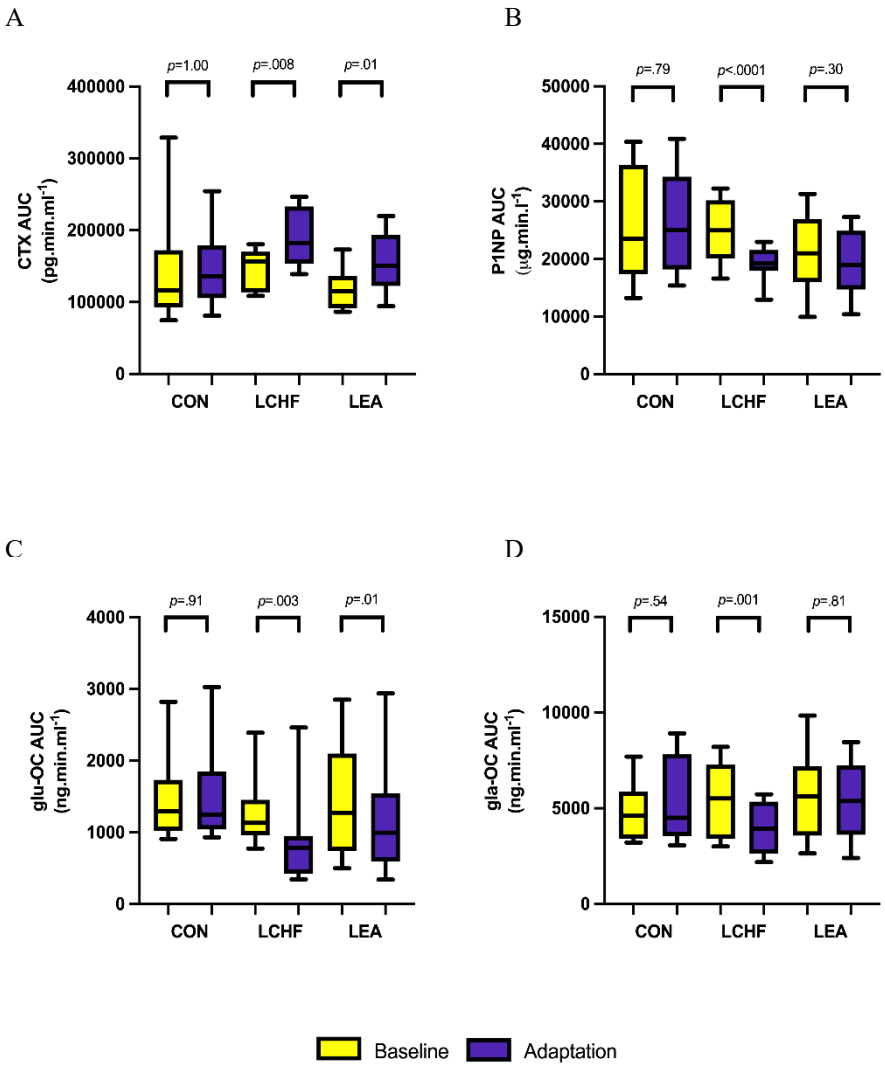

Figure 4.4: Exercise-related area under the curve concentrations of bone turnover markers (pre-exercise to 3 h post-exercise) for each diet group in each phase. CON = high energy/high carbohydrate, LCHF = low carbohydrate/high fat, LEA = low energy availability, CTX = carboxy-terminal telopeptide, P1NP = procollagen-1 N-terminal peptide, gla-OC = carboxylated osteocalcin, glu-OC = undercarboxylated osteocalcin. Raw data presented in boxplot as median, upper and lower quartiles, and minimum and maximum. *p*-values represent difference from Baseline to Adaptation.

4.5 Discussion

This is the first study to compare the effect of short-term (6 day) adherence to high energy availability/high carbohydrate (CON), high energy availability/ketogenic (LCHF), and low energy availability (LEA) diets on markers of bone resorption and formation around a prolonged bout of exercise. Our main findings indicate that, in comparison to undertaking exercise with high energy/high carbohydrate dietary support, a marker of bone resorption was increased when exercise was undertaken following either the LEA or LCHF ketogenic diet. In contrast, bone formation markers during exercise were negatively affected by the LCHF ketogenic diet only. In addition, fasted concentrations of P1NP and glu-OC declined in both LEA and LCHF, yet CTX was similar between groups. Therefore, this study suggests that carbohydrate may be key for maintaining bone formation during prolonged exercise, but that both overall energy and carbohydrate are necessary to support bone formation at rest and limit exercise-related bone resorption.

Fasted P1NP and both forms of osteocalcin declined to a greater degree in the LCHF group than in LEA, without significant differences in CTX in either group compared to the high energy availability/high carbohydrate diet. Although data on healthy young males need to be expanded, there is evidence of an intra-individual weekly biological variation of ~9% for CTX, ~7% for P1NP (Wang et al., 2020), and ~10% for osteocalcin (Panteghini & Pagani, 1995). Comparing these values with the magnitude of changes seen in our study and, noting the additional factors of tight dietary and activity control as well as well-matched groups, it is plausible that a true effect was observed. Our results align with the findings of a previous study of low energy availability in well-trained young male distance runners, in which energy balanced (~40 $kcal.kg^{-1}$ $FFM.d^{-1}$) and restricted (50% of balanced condition) diets were compared over a 5-day period. Here the authors reported a ~15% reduction in fasting P1NP concentrations in the energy-restricted group without significant change in urine N-terminal telopeptide (NTX) or serum osteocalcin (Zanker & Swaine, 2000). Murphy and colleagues demonstrated a reduction in P1NP (15-25%, compared to 14% in the current study) and up to 6% increase in CTX following 5 d adherence to low energy availability (~15 $kcal.kg^{-1}$ $FFM.d^{-1}$) in recreationally active males performing concurrent cycling (Murphy et al., 2021). In contrast, Papageorgiou and colleagues showed no difference in P1NP nor CTX in the recreationally active young male cohort of their study, which compared low energy availability (~15 $kcal.kg^{-1}$ $LBM.d^{-1}$) and control (~45 $kcal.kg^{-1}$ $LBM.d^{-1}$) diets over 5 d of running (Papageorgiou et al., 2017). Further studies are required to confirm these discrepant results of

low energy availability in males on fasted bone resorption markers and related magnitude, but it appears that bone formation markers may be more consistently affected and to a similar magnitude over a short-term period.

The novel aspect of this study was the inclusion of a high energy LCHF group against which to compare the relative effects on bone. Though a longer intervention period than the current study, our group has previously reported (Heikura et al., 2019) that 3.5 weeks of a LCHF diet in elite racewalkers resulted in a ~22% increase in fasted CTX concentrations, a ~14% decline in P1NP, and a ~25% decline in total OC. In contrast, in the current study, our LCHF group exhibited smaller increases in fasted CTX (~8 %) but greater reductions in P1NP (~26%) and osteocalcin (gla-OC ~22%, glu-OC ~41%). As the participants were similar demographically, it could be speculated that these differences are attributable to the different lengths of the diet intervention. Nevertheless, the current study may suggest that the short-term low carbohydrate availability suppresses bone formation markers at rest to a greater extent than both high energy availability/high carbohydrate and low energy availability diets, whilst exerting a smaller effect on bone resorption markers. The translation of circulating biomarkers to structural bone changes requires further research, as currently there is no evidence to indicate site-specific remodelling or for the prediction of fracture risk in non-osteoporotic populations.

It is further noted that both LCHF and LEA groups demonstrated lower fasted glu-OC following Adaptation than the CON group. It is increasingly recognized that glu-OC may play an important role in energy metabolism, where lower glu-OC has been associated with impaired glucose metabolism, increased adiposity, and lower testosterone (Zoch et al., 2016). While it should be noted that these studies, from animal models and cross-sectional studies of humans, require further support from well-controlled intervention studies, restriction of carbohydrate and energy intake while expending substantial energy via high intensity exercise may lead to unfavourable metabolic adaptations.

Our study not only explores the effect of short-term energy and macronutrient manipulation on fasted concentrations of BTMs but also the response to feeding. Pre-exercise concentrations were lower than fasted concentrations for CTX, P1NP, and gla-OC, although the magnitude was substantially larger for CTX (~45% versus ~8%). The effect of feeding was explicitly examined by Scott and colleagues (Scott et al., 2012) where comparisons were made between undertaking exercise fasted or 2 h following breakfast (2.3 MJ, 60% carbohydrate, 32% fat, 8% protein). Here, CTX was lower in the fed condition from 1 h post-meal until 1 h after a 60 min

run. In contrast, P1NP was unaffected by time or food intake prior to exercise, with OC declining prior to exercise regardless of food consumption. Similarly, Bjarnason and colleagues demonstrated a 50 % reduction in CTX over 2 h following an oral glucose tolerance test as opposed to fasting, yet no response in osteocalcin was observed (Bjarnason et al., 2002). In a similar population of racewalkers to those in the current study, we previously showed that CTX decreased 2 h following breakfast as well, but this was not seen for P1NP or osteocalcin (Heikura et al., 2019). Although the meal effect is clear for CTX, it is less so for the other markers of bone turnover. These findings underline the importance of standardized procedures when comparing BTMs between each other and across time, especially when monitoring the effects of a nutritional intervention or pharmacological therapy.

Regardless of diet or phase, exercise seemed to have an effect on markers of bone turnover, with both P1NP and CTX increasing over exercise, then declining either to, or below, fasted levels by 3 h. However, we note that, without a resting control, the acute effect of exercise on these markers is inconclusive. This response over time needs to be viewed against the background of circadian variation in these markers, which, although heavily influenced by feeding (Bjarnason et al., 2002), typically follows a peak in the early morning hours (~02h00-05h00) and a nadir in the afternoon (~12h00-16h00) (Hannon & Eastell, 2000). In our study, the exercise bout occurred between 08h00 and 11h00 with blood sampling and feeding on either side as described. Thus, whilst circadian variability could have influenced our results, some insight can be gleaned from the between-group comparisons. In the LCHF group there was an increase in CTX and a decrease in P1NP from pre-exercise to 3 h post-exercise. In contrast, the higher carbohydrate intake in the other groups resulted in maintenance of bone formation, and marginally offsetting the increased bone resorption from short-term reduced energy availability. The acute effects of carbohydrate consumption around exercise have been shown previously. Sale and colleagues (Sale et al., 2015) compared an 8% carbohydrate solution to placebo consumed by physically active men before, during and immediately post a 120 min treadmill run at 70% VO_{2max}, where the carbohydrate group demonstrated lower CTX and P1NP concentrations up to 2 h following exercise. The similar findings in the current study now extend this evidence of acute carbohydrate provision on bone turnover to periods of short-term (~1 week) carbohydrate manipulation. It must be noted that, although the majority of circulating CTX and P1NP is derived from bone, the possibility of derivation from other type 1 collagen sources, such as tendons and cartilage, remains (Vasikaran et al., 2011). Nevertheless, taken together with the lower bone-specific gla-OC in the LCHF group, a role for carbohydrate in supporting bone formation is suggested.

Of further interest is the crosstalk between bone and other systems, and the influence of exercise and nutrition thereon. In the same study by Sale and colleagues, carbohydrate provision resulted in lower interleukin-6 (IL-6) concentrations up to 2 h after exercise, with a strong correlation between post-exercise IL-6 and CTX (Sale et al., 2015). Interleukin-6 is released from the muscle during exercise and serves to increase glucose availability, particularly in situations of low glycogen (Hennigar et al., 2017). This myokine has also been shown to be involved in a feedforward loop with osteocalcin, which itself may increase uptake and catabolism of fatty acids and glucose in the muscle (Mera et al., 2016), creating a crosstalk between muscle and bone. Higher IL-6 concentrations were evident following a short-term LCHF diet compared to LEA or CON, with these data reported elsewhere (McKay et al., 2021c); however, no significant correlations between post-exercise IL-6 and BTMs were apparent. *In vitro* studies have shown that IL-6 increases the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) and decreases osteoprotegerin (OPG) expression in osteoblasts, resulting in increased bone resorption (CTX) and production of glu-OC (Mera et al., 2016). Whilst we observed a decrease in glu-OC following Adaptation in both the LCHF and LEA groups, both had significant increases in CTX across exercise, with LCHF also exhibiting a decline in P1NP and gla-OC. Therefore, in support of the higher IL-6 concentrations (McKay et al., 2021c), short-term LCHF appears to result in greater exercise-related bone resorption than an isoenergetic high carbohydrate diet or a low energy availability diet.

To our knowledge, the only other study to compare the separate effects of energy and carbohydrate availability in relation to exercise is by Hammond and colleagues(Hammond et al., 2019). Following a morning high intensity interval training (HIIT) run, participants consumed one of three diets in a randomized crossover order: isocaloric (60 $kcal.kg^{-1}$ FFM) high carbohydrate (12 $g.kg^{-1}$ BM) or non-ketogenic low-carbohydrate high-fat (3 $g.kg^{-1}$ BM), or energy and carbohydrate restricted (20 $kcal.kg^{-1}$ FFM, 3 $g.kg^{-1}$). Three-and-a-half hours later, they performed a second HIIT run and then continued the diets for a further 17 hours. Both conditions where lower carbohydrate was consumed, regardless of energy availability, showed increased CTX concentrations prior to and up to 3 h after the afternoon HIIT, with no effect of the diet on P1NP. Here, the authors concluded that carbohydrate may have a more important (energy independent) influence on limiting bone resorption during exercise. In contrast, our study suggests that carbohydrate may be important for maintaining bone formation but both adequate energy and carbohydrate are needed to limit exercise-related bone resorption. Differences in intervention period lengths, dietary composition (both carbohydrate and energy

availability targets), participant characteristics, and study design features may account for the slight difference in results.

The strengths of this study lie in the tight dietary and activity control as well as the closely matched groups in terms of baseline characteristics. We acknowledge the small sample size as a limitation yet highlight the corresponding small pool of elite athletes in the population from which it is feasible to draw upon at any given time (McKay et al., 2022b). We also note the inclusion of younger athletes in the CON group who may have had higher bone turnover. However, whilst increased turnover may be more likely for males younger than 20 years old, there seems to be less variability between age groups thereafter (Shao et al., 2020). With the majority aged in their mid-late 20s, as indicated by the interquartile ranges, and the aforementioned strengths of control in this study, we expect that this contribution to preanalytical variability has been somewhat moderated. Furthermore, the statistically significant difference in spine BMD between the LEA and LCHF groups could have influenced BTM concentrations. Yet, with little evidence demonstrating a correlation between BTMs and BMD in young males (possibly due to different site accrual rates (Szulc et al., 2001)) and the individuals' distribution between groups, we are hesitant of the clinical relevance. Here we emphasize the related and final limitation of our study in extrapolating circulating BTM changes to infer implications to bone structure or function over the longer term in this population with the currently available evidence.

4.6 Conclusion

Short-term carbohydrate restriction appears to result in reduced circulating markers of bone formation at rest and during exercise with a further exercise-related increase in a bone resorption marker. Although short-term LEA seemed to be better tolerated with relatively unchanged bone formation markers across exercise, the marker of bone resorption was still increased. Both carbohydrate and energy restriction may further impair energy metabolism through the reduced endocrine action of osteocalcin. In contrast, the provision of a diet with adequate energy and carbohydrate to support training in elite athletes appears to prevent the unfavourable imbalance between bone resorption and formation markers and may improve energy metabolism. We acknowledge the short-term nature of this study and the limitations of translating these findings to longer term outcomes. Nevertheless, as bone turnover markers provide insight into bone quality (Wu et al., 2021), may predict fracture risk in some populations (Tian et al., 2019), and offer a more accessible and shorter-term monitoring tool

than DXA (Wu et al., 2021), they are a useful tool to evaluate responses to nutritional or pharmacological interventions, and deserve further attention for use in athletic populations. Athletes may frequently engage in short-term periods of dietary manipulation throughout the season, and the potential long-term impact of accumulating these short, periodic cycles on bone health is of interest. Although the effects on future bone strength warrants further investigation, the current study supports the notion that a short-term low energy diet may be better tolerated than a ketogenic diet, provided that adequate protein and carbohydrate is sustained.

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Authorship: The study was designed by LMB, IAH, AKA, NT, and NF. Data were collected and analysed by LMB, AKA, IAH, NT, and NF, and interpreted by LMB, AKA, IAH, NT, KA, and NF. All authors contributed to drafting the manuscript or revising it critically for important intellectual content. All authors approved the final version of the submitted manuscript.

Conflicts of interest: The authors and funding agents do not have any conflicts of interest.

Interlinking chapter

A key outcome from Chapter 4 is that carbohydrate availability plays a key role in supporting bone formation and limiting bone resorption, but that adequate energy is also required to maintain bone formation. Additional research is still required to evaluate whether these changes in circulating markers of bone turnover translate into changes in bone strength and fracture risk in the long-term. Furthermore, similar changes in other markers associated with these dietary changes confirm the importance of carbohydrate availability in modulating the exercise response. In a companion paper, there were greater exercise-induced responses in both IL-6 and hepcidin in response to the LCHF diet, potentially translating into reduced iron stores if continued over the long-term (McKay et al., 2021c). With Chapter 3 suggesting a possible contribution of IL-6 to CTX concentrations, Chapter 4 showing similar responses of these markers to the ketogenic diet, and evidence in clinical populations for associations between iron deficiency and low BMD, the next Chapter aimed to explore the effect of exercise on iron metabolism on a larger scale.

Although several studies have investigated the effect of dietary and exercise session interventions on the hepcidin response to exercise, the small sample sizes of these studies limit the generalizability of the findings. Indeed, as for bone, the reliability of the marker measured to ascertain the health status of an athlete is based on accurate knowledge of factors that may cause acute fluctuations in that marker. Furthermore, knowledge of these factors is important in understanding the wider health effects of interventions designed to improve performance. In Chapter 5, we aimed to investigate how characteristics of both athlete and exercise session influence post-exercise IL-6 and hepcidin concentrations through an individual participant data meta-analysis.

5 Chapter 5

Factors influencing the hepcidin response to exercise: an individual participant data meta-analysis

Publication statement:

This chapter is comprised of the following paper online ahead of publication in *Sports Medicine*.

Fensham, N. C., Govus, A. D., Peeling, P., Burke, L. M., & McKay, A. K. A. (2023). Factors influencing the hepcidin response to exercise: an individual participant data meta-analysis. *Sports Med*. doi: 10.1007/s40279-023-01874-5 [online ahead of print]

5.1 Abstract

Background: Hepcidin, the master iron regulatory hormone, has been shown to peak 3-6 h post-exercise, and is likely a major contributor to the prevalence of iron deficiency in athletes. Although multiple studies have investigated the hepcidin response to exercise, small sample sizes preclude the generalizability of current research findings. Objective: The aim of this individual participant data meta-analysis was to identify key factors influencing the hepcidin-exercise response. Methods: Following a systematic review of the literature, a one-stage meta-analysis with mixed-effects linear regression, using a stepwise approach to select the best-fit model, was employed. Results: We show that exercise is associated with a 1.5- to 2.5-fold increase in hepcidin concentrations, with pre-exercise hepcidin concentration accounting for ~44% of the variance in 3 h post-exercise hepcidin concentration. Although collectively accounting for only a further ~3% of the variance, absolute 3 h post-exercise hepcidin concentrations appear higher in males with lower cardiorespiratory fitness and higher pre-exercise ferritin levels. On the other hand, a greater magnitude of change between the pre- and 3 h post-exercise hepcidin concentration was largely attributable to exercise duration (~44% variance) with a much smaller contribution from VO_2max, pre-exercise ferritin, sex, and post-exercise IL-6 (~6% combined). Although females tended to have a lower absolute 3 h post-exercise hepcidin concentration (1.4 nmol.L^{-1}, [95% CI -2.6, -0.3], p=0.02) and 30% less change (95% CI [-54.4, -5.1], p=0.02) than males, with different explanatory variables being significant between sexes, sample size discrepancies and individual study design biases preclude definitive conclusions. Conclusion: Our analysis reveals the complex interplay of characteristics of both athlete and exercise session in the hepcidin response to exercise and highlights the need for further investigation into unaccounted-for mediating factors.

Key points

- Models derived from currently available data suggest that hepcidin concentrations are doubled by exercise, with a 1.5- to 2.5-fold increase in values at 3 h post-exercise compared to pre-exercise hepcidin.
- Ferritin remains a useful and practical marker of iron stores and, along with small contributions from sex and cardiorespiratory fitness, further modifies post-exercise hepcidin concentrations.
- When evaluating baseline iron status, sport and exercise researchers and practitioners should control for the influence of the previous day's exercise, an athlete's habitual diet, and sex/menstrual cycle phase. When advising athletes on iron consumption around exercise, the time of day of exercise sessions, as well as exercise duration should be considered.

5.2 Introduction

The prevalence of iron deficiency in athletes (as commonly determined by a serum ferritin < 35 $\mu g.L^{-1}$) is estimated at ~52% in females and ~15% in males, compared to ~49-57% and ~6-9% in non-athletic females and males, respectively (Nabhan et al., 2020). However, considering the retrospective nature of these epidemiological analyses, the lack of standardization of blood collection, and the role of ferritin as an acute-phase reactant, it is likely that the true prevalence in athletes may, in fact, be higher. Several factors seem to contribute to suboptimal iron status in athletes, including increased exercise-associated losses via sweat, haematuria, foot strike haemolysis, and gastrointestinal micro ischemia (see (Peeling et al., 2008) for review). Further, dietary practices low in bioavailable iron (e.g., vegan or vegetarian) (Haider et al., 2018) or energy availability (Petkus et al., 2017) may also compound this problem. Increasingly recognized, however, is the role of exercise-induced increases in the body's master iron regulatory hormone, hepcidin (Peeling, 2010; Peeling et al., 2008; Sim et al., 2019).

Hepcidin concentrations, which peak 3-6 h after exercise (Peeling et al., 2009b), transiently decrease macrophage iron efflux and intestinal iron absorption (Nemeth et al., 2004b), potentially increasing an athletes risk of developing an iron deficiency. Interestingly, recent reports have attributed ~77% of the variance in hepcidin concentration measured 3 h after exercise to an athlete's baseline ferritin and iron concentration, exercise duration, and post-exercise interleukin-6 (IL-6) concentration (Peeling et al., 2017). Furthermore, 50% of the post-exercise IL-6 response was explained by the effect of exercise duration alone, with other factors such as exercise intensity and modality moderating this response (Fischer, 2006). While IL-6 is secreted from multiple cell types, including leukocytes, adipocytes, and myocytes (Hennigar et al., 2017), its stimulation of hepcidin potentially serves to limit iron availability to circulating microbes in a presumed situation of inflammation and infection; a response that is transiently mimicked by exercise. In the context of a high ferritin concentration, hepcidin acts to curtail further iron storage and potential toxicity, whereas hepcidin suppression facilitates increased iron availability when stores are insufficient. Various studies have investigated the impact of different interventions or stimuli on the hepcidin response to exercise, including nutrition (Badenhorst et al., 2015a; Badenhorst et al., 2016; Dahlquist et al., 2017; Díaz et al., 2015; Ishibashi et al., 2020; McKay et al., 2020; McKay et al., 2021b; McKay et al., 2021c; McKay et al., 2019a; Sim et al., 2012), temperature (Hayashi et al., 2020; McKay et al., 2021a; Zheng et al., 2021), oxygen availability (Badenhorst et al., 2014; Goto et al., 2018; Govus et al., 2014), exercise timing (McCormick et al., 2019; Peeling et al., 2009a), menstrual phase (Barba-

Moreno et al., 2022; Zheng et al., 2021), and exercise characteristics (Goto et al., 2020; Newlin et al., 2012; Peeling et al., 2009c; Sim et al., 2013). Remarkably consistent from these studies, however, is the time-course of the post-exercise responses of IL-6 and hepcidin, which are characterised by a transient rise in IL-6 immediately after exercise, followed by an increase in plasma hepcidin concentration, peaking at 3-6 h after the exercise stimulus.

Research in sport and exercise science is usually conducted with small sample sizes, due to the relatively low proportion of athletes in the wider population (McKay et al., 2022b), their limited time availability, the logistical and ethical challenges of collecting numerous venous blood draws, and restrictions on their training and diet. This sample size issue (typically <30 athletes) reduces the capacity of a study to accurately estimate the population-level effect in athletes, limiting the generalizability of research findings. Therefore, this study aims to meta-analyse individual participant data from multiple studies to determine the athlete and exercise characteristics that significantly influence the post-exercise hepcidin response. By strengthening our understanding of the factors that moderate the hepcidin and IL-6 responses to exercise, we provide valuable insights to inform future research and tailor clinical practice guidelines.

5.3 Methods

This systematic review and individual participant data meta-analysis followed the PRISMA-IPD (Preferred Reporting Items for Systematic Reviews and Meta-Analyses of Individual Participant Data) guidelines (Stewart et al., 2015). Ethics approval was granted by the Australian Catholic University Human Research Ethics Committee (2021-223N). The full protocol was registered prior to data extraction on the PROSPERO international prospective register of systematic reviews (ID: CRD42021293423).

5.3.1 Search strategy and selection process

An electronic systematic search of the literature was conducted in Pubmed, SPORTDiscus, and OpenGrey. A date of publication restriction was not applied. A combination of the following keywords and subject headings was applied: ‘hepcidin’ AND ‘exercise’ OR ‘athlete’. The initial search identified 256 records (Fig. 5.1), of which 53 were detected as duplicates and, therefore, discarded. A further 147 records were excluded as irrelevant after title and abstract screening. Two authors (NF and AM) independently reviewed the full-text articles, and conflicts were resolved by discussion and consensus. Searches were exclusive to original papers

with human participants, published in English, without date restrictions and current to November 2021. Twenty-nine articles were deemed ineligible, leaving 27 studies for possible inclusion in the meta-analysis (see Fig. 5.1). Inspection of relevant review paper reference lists revealed no further articles unidentified by the systematic search.

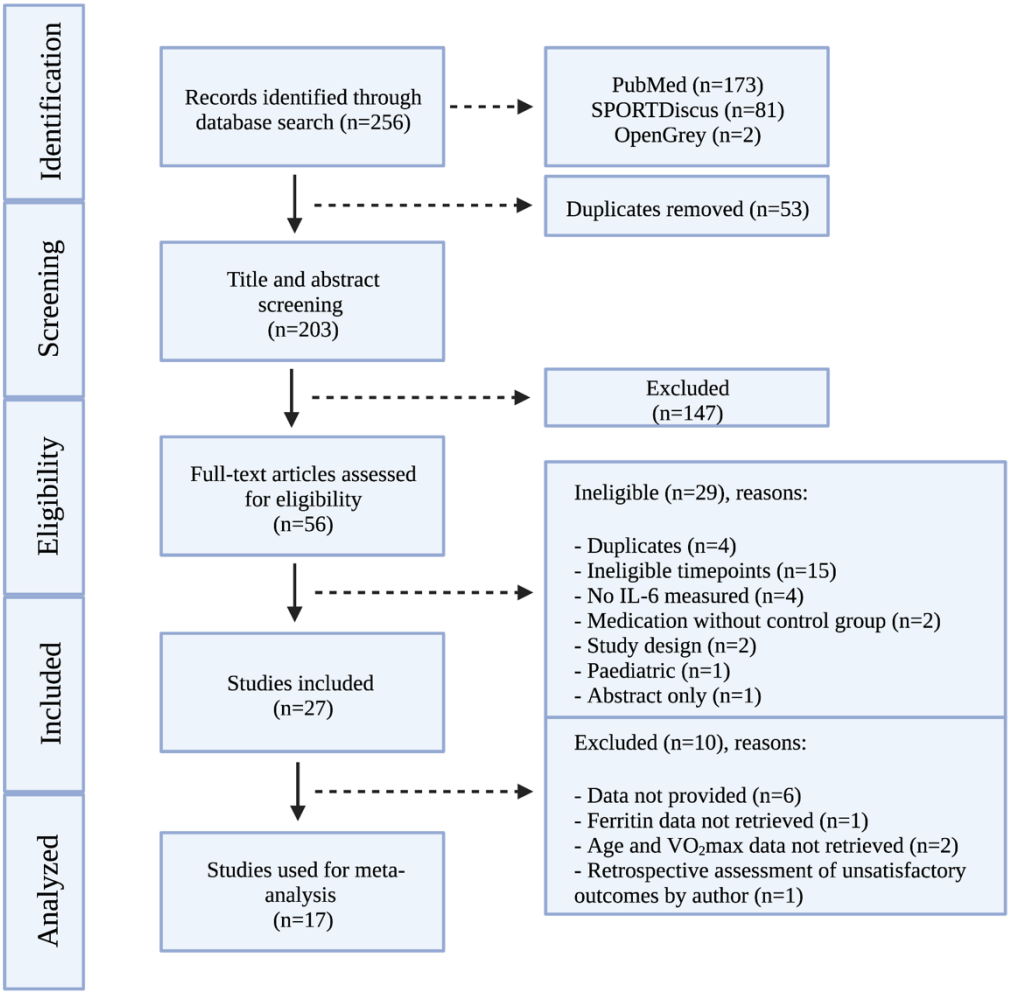

Figure 5.1: PRISMA flow chart of studies included in the individual participant data meta-analysis. (IL-6 = interleukin-6, n = number of studies, VO_2max = maximal oxygen consumption)

5.3.2 Eligibility criteria

Studies including healthy adults performing exercise where pre-exercise, immediately post-exercise, and 3 h post-exercise inflammatory and iron markers were analysed were considered eligible for this meta-analysis. The following eligibility criteria were applied:

- Population: adults (aged 18-40 years) of both sexes who were healthy, not pregnant/ breastfeeding, and not taking any medication (including the oral contraceptive pill) were included.
- Intervention: exercise with duration, relative intensity, and mode specified
- Outcomes: pre- and 3 h post-exercise hepcidin (urinary or serum)
- Study design: parallel or crossover trials accepted
- Comparators: only the control arm of the trial was included
- Time: studies investigating the acute response to exercise (pre- to 3 h post-exercise) were included.
- Other:
    - Ferritin as a marker of iron status had to be measured at baseline prior to the exercise trial
    - Pre- and immediately post-exercise IL-6 had to be measured

5.3.3 Data collection and management

Original individual participant data were requested from the corresponding authors of the 27 eligible trials (Table 5.1; see also Online Resource 1). Of these, it was uncertain whether ferritin was measured for two studies, and therefore, it was decided to enquire directly with the authors prior to exclusion. A standardized email with full details of the registered protocol was sent to the authors to request data contribution to the meta-analysis. After signing a letter of agreement, authors provided their de-identified data in an individualized spreadsheet template. Data for one condition only (control) was requested for crossover trials, and pre-intervention/baseline data for parallel trials. All raw data were saved in a secure online password-protected folder unique to each corresponding author. Reasons for missing data and queries about specific values and measurement units were confirmed via email to the author providing the data. Two authors, representing six studies did not respond to the data request. We did not include the aggregate data from these studies in the meta-analysis due to our inability to confirm data integrity. One further study was excluded due to insufficient data points, and a final study was excluded following notification from the author of a retrospective assessment of unsatisfactory measurement outcomes. These exclusions resulted in a final dataset comprising 17 studies (see Fig. 5.1).

Table 5.1: Summary of the studies retrieved from the systematic review, and those included/excluded from the individual participant data meta-analysis

	Study	Year	Country in which the study was conducted	Study design	Population description	Modality	Intervention/comparison	Data set requested
Urinary hepcidin studies included	Peeling et al. (Peeling et al., 2009b)	2009	Australia	Randomized, counterbalanced, crossover	Moderately trained endurance runners	Running	60-min run vs 60-min rest	Run trial
	Peeling et al. (Peeling et al., 2009c)	2009	Australia	Randomized, counterbalanced, crossover	Highly trained male endurance athletes	Running	Ground surface (grass vs road) and intensity (continuous and interval)	Interval (INT) trial
	Peeling et al. (Peeling et al., 2009a)	2009	Australia	Randomized, counterbalanced, crossover	Highly trained male triathletes and endurance runners	Running	One-session (T1) vs two-session (T2; separated by 12 h) training	T1 trial
Serum hepcidin studies included	Sim et al. (Sim et al., 2012)	2012	Australia	Randomized, crossover	Well-trained male endurance runners and triathletes	Running	3ml.kg^{-1} 6% carbohydrate solution (or placebo) consumed every 20 min throughout run trial	Placebo (PLA) condition
	Newlin et al. (Newlin et al., 2012)	2012	United States	Randomized, counterbalanced, crossover	Female active runners	Running	60-min vs 120-min in early follicular phase	120-min trial
	Sim et al. (Sim et al., 2013)	2013	Australia	Randomized, counterbalanced, crossover	Well-trained male triathletes	Running and cycling	Modality (cycling vs running) and intensity (low 65% vs high 85% VO_2peak power or velocity)	High-intensity cycling (H-C) condition
	Badenhorst et al. (Badenhorst et al., 2014)	2014	Australia	Repeated measures, counterbalanced, crossover	Well-trained male endurance runners and/or triathletes	Running	Simulated post-exercise altitude (HYP: ~2900m, FiO_2=0.1513) exposure vs normoxia (NORM: FiO_2=0.2093) for 3 h	Normoxia (NORM) trial

	Govus et al. (Govus et al., 2014)	2014	Australia	Randomized, counterbalanced, crossover	Moderately trained endurance runners or triathletes	Running	Exercise in either normoxic (FiO_2=0.2093) or hypoxic (FiO_2=0.1450) conditions	Normoxia (NORM) trial
	Burden et al. (Burden et al., 2015)	2015	United Kingdom	Double-blind, randomized, placebo-controlled	National and international standard endurance runners from the London Marathon endurance performance group at St Mary's University, London	Running	Intravenous iron injection (500mg) vs placebo (0.9% saline)	Placebo group
	Badenhorst et al. (Badenhorst et al., 2015a)	2015	Australia	Randomized, counterbalanced, crossover	Well-trained male recreational endurance runners and triathletes	Running	24 h high-carbohydrate (HCHO; 10 $g.kg^{-1}$) vs low-carbohydrate (LCHO; 3 $g.kg^{-1}$) diet following glycogen depleting task	High-carbohydrate (HCHO) trial
	McCormick et al. (McCormick et al., 2019)	2019	Australia	Randomized, repeated measures, crossover	Endurance-trained runners	Running	Morning (AM) vs afternoon (PM) exercise response	Morning (AM) exercise condition
	McKay at al. (McKay et al., 2019a)	2019	Australia	Parallel groups. Dietary allocation by matching for characteristics (sex, age, training status, and personal best times).	Internationally competitive racewalking athletes	Racewalking	High-carbohydrate (HCHO; 8-8.5 $g.kg^{-1}$) vs periodized carbohydrate (PCHO; 8-8.5 $g.kg^{-1}$) vs low-carbohydrate high-fat (<50 $g.d^{-1}$) for 3 weeks	Baseline phase data
	McKay et al. (McKay et al., 2020)	2020	Australia	Randomized, crossover	Triathletes with > 18 months elite training history	Cycling and running	High-carbohydrate (HIGH; 6-8 $g.kg^{-1}$) vs alternate day sleep-low (LOW; 0 $g.kg^{-1}$) post-high intensity training (HIT; day 1 and 3) and prior to a low intensity session (LIT; day 2 and 4). Both cycling (C) and running (R)	High carbohydrate (HIGH) trial, low intensity (LIT) cycling

	McKay et al. (McKay et al., 2021b)	2021	Australia	Parallel groups. Dietary allocation based on preference while matching for characteristics (age, body mass, VO_2max, and training history).	International-level male racewalkers	Racewalking	High-carbohydrate (MAX; 10 $g.kg^{-1}$) vs moderate-carbohydrate (CON; 6 $g.kg^{-1}$) for 2-weeks in 2 phases (Baseline and Adapt)	Baseline phase
	McKay et al. (McKay et al., 2021c)	2021	Australia	Parallel groups. Dietary allocation based on preference while matching for characteristics (age, 20km personal best time, training status, and load).	Elite male racewalkers	Racewalking	High-carbohydrate high energy availability (CON; 40 $kcal.kg^{-1}$ $FFM.d^{-1}$, 65% carbohydrate) vs low-carbohydrate high-fat (40 $kcal.kg^{-1}$ $FFM.d^{-1}$, <50g carbohydrate) vs low energy availability (15 $kcal.kg^{-1}$ $FFM.d^{-1}$, 65% carbohydrate). Two 6-day phases (Baseline and Adaptation)	Baseline phase
	McKay et al. (McKay et al., 2021a)	2021	Australia	Repeated measures, crossover	Moderately trained males	Running	Impact of heat stress with matching of absolute (speed) and relative (heart rate; HR) conditions: COOL (18°C/50% relative humidity, 80% HRmax) vs HOT-HR (35°C/50%, 80% HRmax) vs HOT-PACE (35°C/50%, at speed of COOL). Separated by 72 h.	COOL condition
	Zheng et al. (Zheng et al., 2021)	2021	New Zealand	Randomized, counterbalanced, crossover	Healthy eumenorrheic females	Cycling	Early follicular phase (EFP) vs mid-luteal phase (ML), and moderate (MOD; 20°C) vs warm (WARM; 32°C) conditions	Early follicular phase (EFP) and moderate temperature (MOD) condition
Studies excluded from	Badenhorst et al. (Badenhorst et al., 2015b)	2015	Australia	Repeated measures, counterbalanced, crossover	Well-trained male endurance runners and triathletes	Running	Early (immediate and 2 h post-exercise) vs delayed (2 h and 4 h post-exercise) carbohydrate (1.2 $g.kg^{-1}$, 12 $g.kg^{-1}$ 10%) feeding	Early carbohydrate (ECHO) feeding

	Díaz et al. (Díaz et al., 2015)	2015	Spain	Longitudinal	Well-trained male triathletes and marathon runners	Running	Response to exercise following 28 day supplementation with Vitamin C (500 $mg.d^{-1}$) and E (400 $IU.d^{-1}$)	Pre-supplementation values
	Badenhorst et al. (Badenhorst et al., 2016)	2016	Australia	Randomized, counterbalanced, crossover	Endurance-trained male athletes	Running	7 d high-carbohydrate (HCHO; 8 $g.kg^{-1}$) vs low-carbohydrate (LCHO; 3 $g.kg^{-1}$) diet on day 7 exercise response	High-carbohydrate (HCHO) trial day 1
	Dahlquist et al. (Dahlquist et al., 2017)	2017	Canada	Randomized, placebo-controlled, single-blinded, triple crossover	Highly trained male cyclists (Performance Level 4 and 5)	Cycling	Post-exercise drink (550 ml) effects: non-caloric placebo (PLA) vs carbohydrate (75 g) and protein-rich (25 g) with additional vitamin D3 (5000 IU) and K2 (1000 mcg) (VPRO) or without (PRO)	Placebo (PLA) condition
	Hayashi et al. (Hayashi et al., 2018b)	2018	Japan	Randomized, crossover	Untrained females	Cycling	Pre-exercise meal consumption (FED; 509 kcal, 83% CHO, 8% PRO, 9% fat) vs fasted (CON)	Fasted (CON) condition
	Goto et al. (Goto et al., 2018)	2018	Japan	Randomized, crossover	Trained (5d/week 3h/d) track-and-field sprinters	Cycling	Exercise performed under hypoxic (HYPO; FiO_2 14.5%) or normoxic (NOR; FiO_2 20.9%) conditions. Separated by 1 week	Normoxia (NOR) trial
	Barba-Moreno et al. (Barba-Moreno et al., 2022)	2020	Spain	Observational and randomized controlled	Healthy endurance-trained females	Running	Early follicular phase (EFP) vs mid-follicular phase (MFP) vs luteal phase (LP) response to exercise bout	Early follicular phase (EFP) condition
	Hayashi et al. (Hayashi et al., 2020)	2020	Japan	Randomized, crossover	Healthy and active males	Cycling	Heat and hypoxia (HHYP; 32°C, FiO_2 14.5%) vs hypoxia (HYP; 23°C, FiO_2 14.5%) vs normoxia (NOR; 23°C, FiO_2 20.9%) during exercise	Normoxia (NOR) trial
	Ishibashi et al. (Ishibashi et al., 2020)	2020	Japan	Randomized, crossover	Well-trained male long-distance runners	Running	Neutral energy availability (NEA; >45 $kcal.kg^{-1}$ $FFM.d^{-1}$) vs low energy availability (LEA; <20 $kcal.kg^{-1}$ $FFM.d^{-1}$)	Neutral energy availability (NEA) condition

							over 4 days. Separated by 1 week.	
	Goto et al. (Goto et al., 2020)	2020	Japan	Randomized, crossover	Recreationally trained males	Resistance vs cycling	Resistance exercise (RE) vs endurance exercise (END) vs rest (REST) trial. Separated by 1 week.	Endurance exercise (END) trial
Note: d = days, FFM = fat free mass, FiO_2 = fraction of inspired oxygen, $g.kg^{-1}$ = grams per kilogram, h = hours, $IU.d^{-1}$ = international units per day, km = kilometres, $mg.d^{-1}$ = milligrams per day, min = minutes, $ml.kg^{-1}$ = millilitres per kilogram								

5.3.4 Data items

Collected variables of each de-identified participant included: age; sex; VO_2max (and corresponding power (W) or speed ($km.h^{-1}$)); pre-exercise nutritional state (fed or fasted); exercise mode, duration, and intensity; pre-exercise ferritin, IL-6, and hepcidin; immediate post-exercise IL-6; and 3 h post-exercise hepcidin. Where available, data were cross-checked with the published protocol. For interval-based protocols, active recovery minutes were included in the total duration of the exercise session; conversely, only exercise time was used if there was complete rest (i.e., no active component) between intervals. Similarly, for intensity, a weighted average of %VO_2max of interval time plus %VO_2max of active recovery time was calculated. Where intensity was given as a percentage of heart rate maximum, this was converted to %VO_2max via established methods (Swain et al., 1994). The collected data were then combined into a single spreadsheet where continuous variables were converted into common SI units of measurement and categorical variables were assigned a numeric code. Further information requested from authors to assess data integrity and risk of bias included, where applicable: method of recruitment, randomization procedures, trial order allocation, extent of blinding, reasons for missing/ incomplete data, and method of menstrual cycle phase confirmation.

5.3.5 Individual participant data integrity

Data were checked for completeness with any missing data queried with authors. Where values seemed implausible, units of measurement were verified with the authors and any risk of error queried.

5.3.6 Risk of bias assessment within and across studies

Risk of bias assessment was guided by the Cochrane Risk of Bias Tool using the domains of selection, performance, attrition, reporting, and other (Higgins et al., 2022). Each study was assessed individually according to these domains and assigned a designation of low, high, or unclear risk of bias for each one (Online Resource 2). An overall impression of risk of bias across studies was summarized from these assessments.

5.3.7 Specification of outcomes and effect measures

The primary outcome of interest was the concentration of hepcidin at 3 h post-exercise and the change from pre-exercise values. Continuous explanatory variables were VO_2max, pre-exercise ferritin, post-exercise IL-6, exercise duration, and relative exercise intensity. Categorical explanatory variables were sex, pre-exercise nutritional state, and exercise modality. Data

regarding menstrual cycle phase were collected but not included due to the lack of documentation/verification. Of the 7 studies including females, 5 studies made no attempt to record or verify the menstrual phase (Burden et al., 2015; Govus et al., 2014; McCormick et al., 2019; McKay et al., 2020; McKay et al., 2019a), one made some attempt (questionnaire, menses onset) (Newlin et al., 2012), and only one used a 3-step method (Elliott-Sale et al., 2020) to establish phases (menses onset, urinary luteinizing hormone, and serum 17β-oestradiol and progesterone) (Zheng et al., 2021).

Based on the results of the primary analysis, secondary analyses were performed as follows:

1. Post-exercise IL-6 was designated as an outcome variable with similar explanatory variables.
2. Data were further split by sex and re-analysed with post-exercise hepcidin and change in hepcidin as outcome variables.

5.3.8 Synthesis methods

A one-stage meta-analysis approach was employed with mixed-effects linear regression using the 'nlme' package (Pinheiro et al., 2022) in R version 4.2.0 (R Core Team, Vienna, Austria). As three studies used urine (rather than serum) for hepcidin analysis (Online Resource 3), and it is not possible to convert between urine and serum hepcidin, statistical analyses were performed separately on serum and urine studies. Accordingly, the following 4 models were constructed as described below:

1. Post-exercise serum hepcidin was modelled as an outcome variable with centred pre-exercise hepcidin as a covariate included to account for aggregation bias, in both a stratified intercept per study and random intercept per study model.
2. Post-exercise urinary hepcidin was analysed using a stratified intercept per study model only.
3. The change in serum hepcidin was modelled following log transformation of both pre- and post-exercise hepcidin to improve model fit, with study used as a fixed effect and a random intercept.
4. Post-exercise IL-6 was analysed with a random intercept model only, with centred pre-exercise values used as a covariate; both pre- and post-exercise values were log-transformed prior to pre-exercise centring to improve model fit.

All models included a heterogenous residual variance structure fit per study to estimate the within-study variance. Sex (2 levels: male, female); pre-exercise nutritional state (2 levels: fasted, fed); pre-exercise ferritin; exercise modality (3 levels: running, cycling, racewalking), exercise duration; relative exercise intensity; participant VO_2max; and post-exercise IL-6 (for

the hepcidin model) were included as fixed effects (Online Resource 4). Pre-exercise nutritional state and modality were excluded from all above models (except the random intercept post-exercise hepcidin model) due to convergence errors.

The 'stepAIC' function from the 'MASS' package (Venables & Ripley, 2002) was used in the first instance to perform stepwise regression from the full model including all plausible explanatory variables. This result was then checked manually using p-values as a guide, excluding non-significant (p>0.05, confidence interval crossing zero) variables; further reduced models were also constructed via elimination of variables sequentially according to their contribution to the Akaike Information Criterion (AIC) value. All models were fit using maximum likelihood estimation and compared using both AIC and Bayesian Information Criterion (BIC) values, and the model with the lowest AIC/BIC values was chosen as the final model. Where it was unclear between AIC's, preference was given to the model with the lower BIC which penalizes more explanatory variables, yielding a more parsimonious model. Similarly, where BIC's were equivalent, the model with the lower AIC was chosen. This final model was refitted with restricted maximum likelihood estimation and model diagnostics verified via visual inspection of residual and QQ plots. Fixed effect parameter estimates and 95% confidence intervals were obtained via the 'sjPlot' package and back-transformed from the logarithmic scale for interpretation. Marginal and conditional R^2 values for the final models were calculated as a proportion of the model explained by the fixed effects alone (marginal R^2), or by fixed and random effects combined relative to the model's total variance (conditional R^2) (Nakagawa & Schielzeth, 2013). Post-hoc testing, where applicable, was conducted via the 'emmeans' package (Lenth, 2022) with Tukey's Honestly Significant Difference adjustment. Finally, predictor effects for the change in hepcidin per study were used to construct a forest plot using the 'forestploter' package (Dayimu, 2022). The full code can be found in Online Resource 5.

5.4 Results

5.4.1 Study selection and individual participant data obtained

Of the 27 studies identified as eligible for inclusion, data were not obtained for six studies and a further four studies were excluded due to incomplete/uncertain data (Fig. 5.1). Following removal of participants with missing data points, 17 studies comprising 229 participants (176 males, 53 females) were included, with a subset of 14 studies comprising 202 participants (153 males, 49 females) in the serum hepcidin analysis (Fig. 5.2).

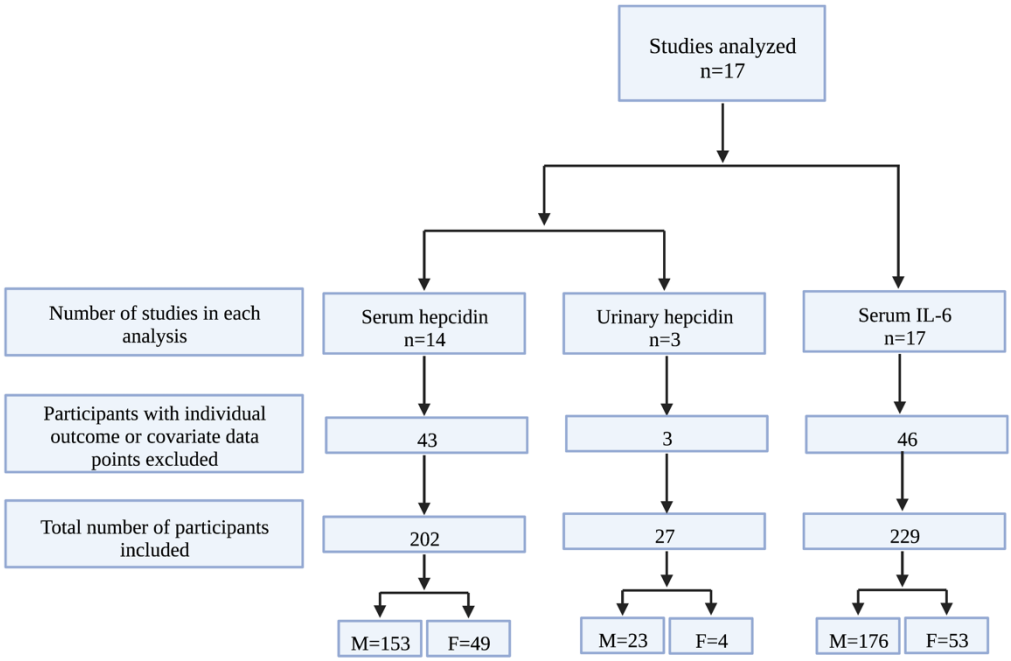

Figure 5.2: Number of studies and participants included in each statistical analysis.

5.4.2 Data integrity

Two participants in one study (Badenhorst et al., 2014) were younger than 18 years, and were therefore, excluded from the analysis. Participants with missing values (outcome or covariate) were removed before model building (n = 46; Fig. 5.2). Summary statistics were calculated for the outcome variables across all studies, with close agreement to the published values or figures.

5.4.3 Risk of bias within studies

Because most researchers employed convenience sampling of their target population and many interventions could not be blinded (e.g., nutrition-related), no studies were excluded on these parameters. As we collected objective information on the exercise bout itself (duration, relative intensity), any potential influence of non-blinding was factored into the analysis; therefore, a low bias risk is considered. Similarly, as the outcome data could not be plausibly influenced by the lack of blinding (objective measurement), studies were classified as a low risk of bias. Data

were checked for plausibility and completeness with any queries directed to and confirmed with the author providing the data. Uncertainty about quality of the hepcidin data was suggested by one author; this study was excluded.

5.4.4 Results of syntheses

5.4.4.1 Estimate of serum hepcidin concentrations at 3 h post-exercise

Both the stratified by study and random intercept methods resulted in a best-fit model with pre-exercise hepcidin, pre-exercise ferritin, VO_2max, and sex as explanatory variables. Both were an improvement upon the unadjusted model (i.e., which included pre-hepcidin only). Parameter estimates and confidence intervals for each model are presented in Online Resources 6 (stratified) and 7 (random). As the stratified model is less generalizable, only the random intercept model will be discussed further (Table 5.2). Without covariate adjustment, 1.0 nmol.L^{-1} higher pre-exercise hepcidin was associated with a 2.2 nmol.L^{-1} (95% CI [1.7, 2.7], $p < 0.001$) higher 3 h post-exercise hepcidin concentration (Fig. 5.3), with the model fixed effects explaining 44% of the total variance (marginal R^2=0.44, conditional R^2=0.96; Online Resource 7: Table 2). When adjusting for pre-exercise ferritin, VO_2max and sex, a 1.0 nmol.L^{-1} increase in pre-exercise hepcidin concentration was associated with a 2.0 nmol.L^{-1} (95% CI [1.5, 2.6], $p < 0.001$) higher post-exercise hepcidin concentration (marginal R^2=0.47 conditional R^2=0.98; Online Resource 7: Table 3). Conversely, a 1 mL.kg^{-1}.min^{-1} increase in VO_2max was associated with a 0.1 nmol.L^{-1} (95% CI: [-0.2, -0.1]) lower hepcidin concentration 3 h post-exercise. Finally, females had on average a 1.4 nmol.L^{-1} (95% CI: [-2.6, -0.3], $p = 0.02$) lower hepcidin concentration 3 h post-exercise than males.

Table 5.2: Post-exercise serum hepcidin model parameters with adjustment for final covariates			
	Outcome: **3 h post-exercise serum hepcidin**		
Predictors	**Estimates**	**95% CI**	***p***
(Intercept)	14.21	9.24 – 19.17	**<0.001**
Pre-exercise hepcidin (centred)	2.04	1.49 – 2.59	**<0.001**
VO_2max	-0.11	-0.17 – -0.05	**<0.001**
Pre-exercise ferritin	0.02	0.01 – 0.03	**<0.001**
Sex [Female]	-1.41	-2.56 – -0.25	**0.017**
Random Effects			
σ^2	1.64		
$\tau_{00\ StudyID}$	34.32		
$\tau_{11\ StudyID.Pre\text{-}exercise\ hepcidin}$	0.68		
$\rho_{01\ StudyID}$	0.43		
ICC	0.96		
$N_{StudyID}$	14		
Observations	202		
Marginal R^2 / Conditional R^2	0.472 / 0.979		
Note: final model fitted with restricted maximum likelihood. σ^2 = within-group (residual) variance; τ_{00} = between-group random intercept variance; τ_{11} = between-group random slope variance; ρ_{01} = random intercept-slope correlation; CI = confidence interval; ICC = intraclass correlation coefficient; VO_2max = maximal oxygen consumption			

Table 5.3: Change in hepcidin model parameters, adjusted for final covariates			
	Outcome: **ln(post-exercise hepcidin)-ln(pre-exercise hepcidin)**		
Predictors	**Estimates**	**95% CI**	***p***
(Intercept)	1.1092	0.2171 – 2.0013	**0.015**
Pre-exercise ferritin	0.0018	0.0006 – 0.0031	**0.005**
Post-exercise IL-6	0.0145	0.0012 – 0.0278	**0.033**
VO_2max	-0.0161	-0.0268 – -0.0055	**0.003**
Sex [Female]	-0.2976	-0.5439 – -0.0513	**0.018**
Exercise duration	0.0086	0.0024 – 0.0149	**0.007**
Random Effects			
σ^2	0.09		
$\tau_{00\ StudyID}$	0.15		
ICC	0.62		
$N_{StudyID}$	14		
Observations	202		
Marginal R^2 / Conditional R^2	0.505 / 0.812		
Note: model fitted with restricted maximum likelihood. As outcome variable is the difference of natural logs, parameters are interpreted as a percentage in-text. σ^2 = within-group (residual) variance; τ_{00} = between-group random intercept variance; CI = confidence interval; ICC = intraclass correlation coefficient; VO_2max = maximal oxygen consumption			

5.4.4.2 Estimate of the change in serum hepcidin from baseline

A forest plot depicting the magnitude of the change in hepcidin from baseline to 3 h post-exercise stratified by study is presented in Fig. 5.4. Adding the covariates of VO_2max, pre-exercise ferritin, sex, and post-exercise IL-6 improved model fit compared to the null model including study ID as the only explanatory variable (Online Resource 8: Table 1).

When studies were pooled (Online Resource 8: Table 5), the null (no covariate) model estimated that hepcidin changed on average by 88.3% (95% CI [57.1, 119.4], $p < 0.001$) from pre- to 3 h post-exercise (Online Resource 8: Table 2). The final model including VO_2max, duration, pre-exercise ferritin, sex, and post-exercise IL-6, explained ~50% of the total variance (Table 5.3). The variable accounting for the most variance was exercise duration, with a marginal R^2 of 0.44. In comparison, ferritin and IL-6 explained 3% and 9%, respectively, when included as the only covariate in the model. When holding all other variables constant, the change in hepcidin from pre- to 3 h post-exercise was 30% less in females compared to males (95% CI [-54.4, -5.1], p=0.02).

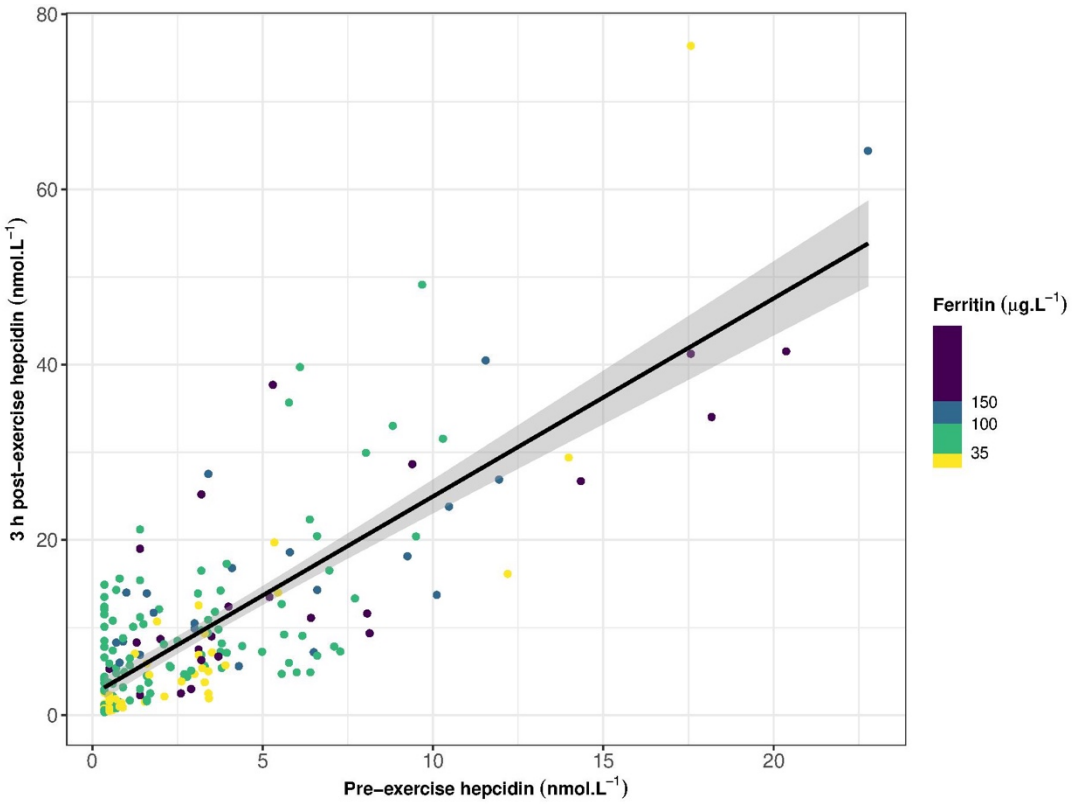

Figure 5.3: The relationship between pre- and 3 h post-exercise hepcidin concentrations, colour-coded by ferritin concentration (pooled values). The black line represents the linear trend, and the grey-shaded area represents the 95% confidence intervals. Coloured shading indicates participants' serum ferritin concentration (µg.L^{-1}).

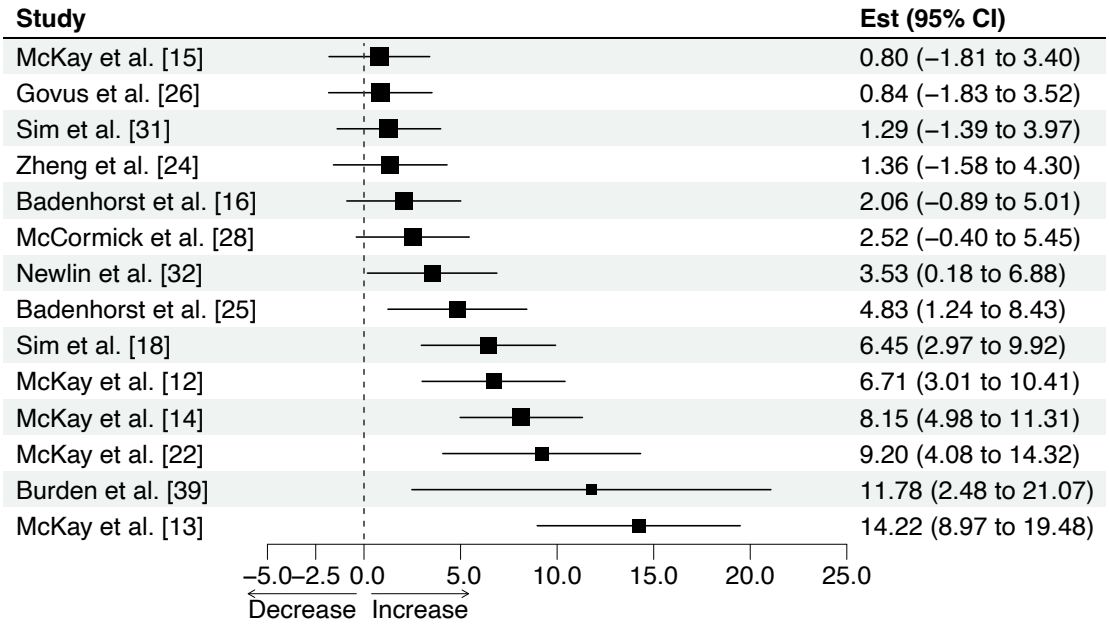

Figure 5.4: Forest plot depicting the change in serum hepcidin (nmol.L^{-1}) with the point estimate (Est.) and the 95% confidence interval (CI) per study

5.4.4.3 Sub-analysis: sex differences in absolute 3h post-exercise hepcidin concentration

Absolute post-exercise hepcidin concentration was higher (p=0.02) in males (mean estimate: 8.6 nmol.L^{-1}, 95% CI [5.1, 12.1]) compared to females (mean estimate: 7.2 nmol.L^{-1}, 95% CI [3.6, 10.8], p=0.02), when the covariates of pre-exercise hepcidin, ferritin, and VO_2max were held constant. Both male and female models supported the large contribution of pre-exercise hepcidin, similar to the combined-sex model, with marginal R^2 values of 0.33 and 0.47 respectively. However, the additional covariates that improved model fit were different between sexes (Online Resource 7). In males, the covariates of VO_2max and ferritin further improved model fit (all p<0.05; marginal R^2 = 0.46, conditional R^2 = 0.98; Online Resource 7: Table 6). Conversely, in females, while exercise duration contributed most to the variance (marginal R^2 = 0.52), it was not significant when added to pre-exercise hepcidin as the only additional covariate (p=0.21; Online Resource 7: Table 7). Similarly, while ferritin was also not significant as the only additional covariate (p=0.07), and did not significantly improve model fit, the best model incorporated a combination of both ferritin and exercise duration (marginal R^2=0.51, conditional R^2=0.96; Online Resource 7: Table 9). Both in the best model and in the model controlling for all covariates, ferritin was significant (p=0.03 and p=0.01, respectively).

However, almost a quarter of the female data were derived from a single study investigating exercise duration (Newlin et al., 2012) (Online Resource 1) and, therefore, this result should be interpreted with caution. For both sexes, post-exercise hepcidin was approximately 1.5-2.0 times higher than the pre-exercise value (males: 2.1, 95% CI [1.5, 2.7]; females: 1.4, 95% CI [0.7, 2.1]). The contribution of ferritin was similar between sexes, with each 10 μg.L^{-1} associated with an additional ~0.2 nmol.L^{-1} to post-exercise hepcidin (males: 0.1 nmol.L^{-1}, 95% CI [0.04, 0.2]; females: 0.2 nmol.L^{-1}, 95% CI [0.03, 0.5]).

5.4.4.4 Urinary hepcidin concentrations at 3 h post-exercise

The final stratified model is presented in Online Resource 9. Without adjustment for additional covariates, pre-exercise urinary hepcidin accounted for ~28% of the variance in the post-exercise value. Exercise duration was the only variable to improve the model (marginal R^2=0.48; p=0.04), with each 10 min increase in duration adding 2.3 nmol.L^{-1} to the post-exercise urinary hepcidin concentration (95% CI [0.2, 4.5]).

5.4.4.5 Post-exercise serum IL-6

Covariates of pre-exercise IL-6, sex, VO_2max, exercise duration, and relative exercise intensity produced a model with a marginal R^2 of 0.69 and a conditional R^2 of 0.92 (Online Resource 10: Table 2). Pre-exercise IL-6 alone contributed ~10% of the variance (p<.001), with each 1.0 pg.mL^{-1} adding 0.5 pg.mL^{-1} (95% CI [0.3, 0.7]) to the post-exercise IL-6 value. Exercise duration (Fig. 5.5) alone accounted for 56% of the variance in post-exercise IL-6. Once again, sex differences were evident with females having a ~33% lower post-exercise IL-6 concentration than males (95% CI [-43.4, -20.5]).

5.4.5 Risk of bias across studies

We were unable to obtain individual data for six studies from two authors. We were reluctant to use the aggregate data in our meta-analysis due to the potential of introducing bias when the integrity of the data and completeness could not be verified. However, we acknowledge that data from these studies, involving 48 males and 10 females, could influence our results. We also note the underrepresentation of female (n = 53) participants relative to males (n = 176) in our analysis, and the lack of consideration (or inadequate confirmation) for menstrual cycle phase (5 out of 7 studies made no attempt to confirm phase). In addition, these data are biased towards developed Western nations, with a large proportion derived from Australia (14 of 17 studies). Reporting on ethnicity was not requested by the research team when contacting authors, and therefore, not accounted for in the current analysis. Further, although we attempted

to flag and exclude data that may have suffered from erroneous measurement, and accounted for study variance through weight-adjustment, the possibility of measurement bias cannot be excluded. Finally, this analysis is biased towards endurance activities, namely running, cycling, and racewalking, with fewer studies investigating the influence of resistance exercise and intermittent sports.

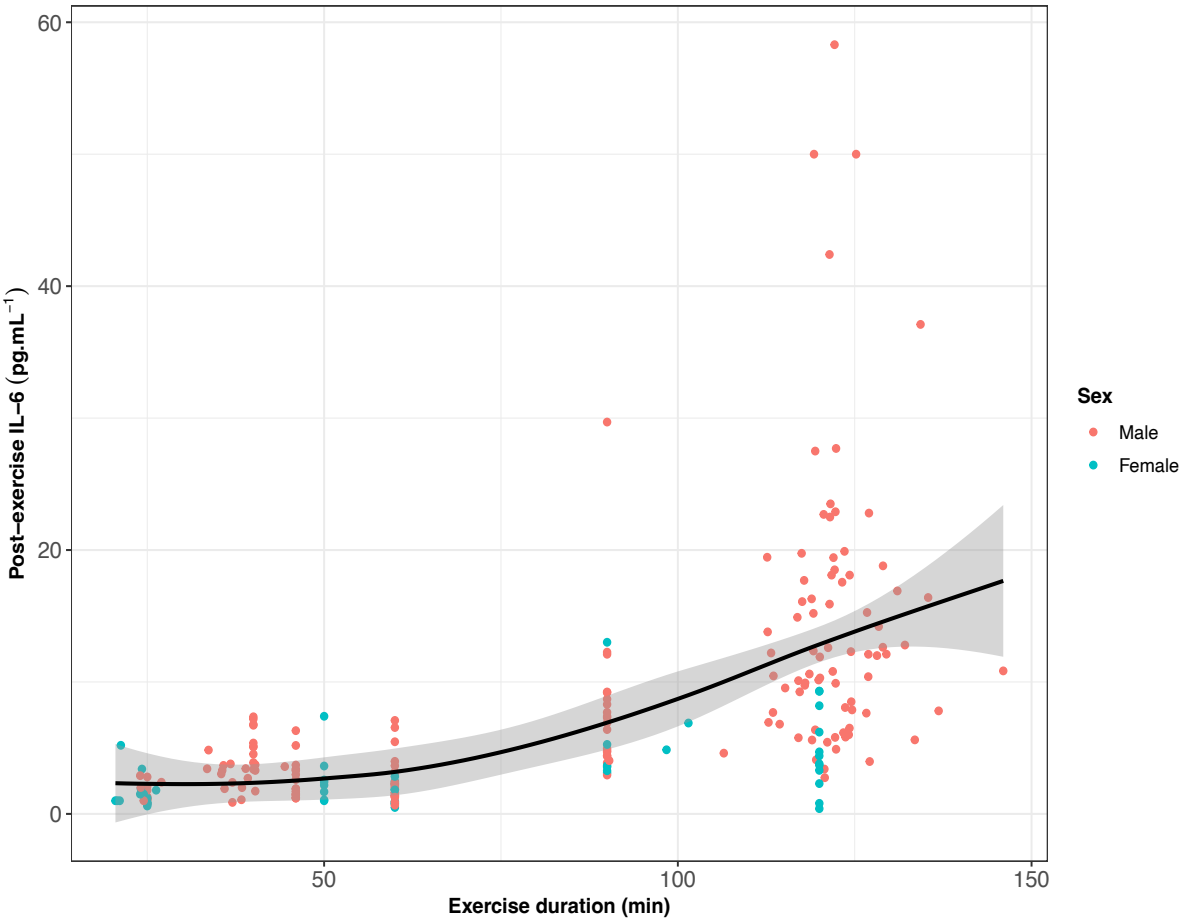

Figure 5.5: The relationship between exercise duration and post-exercise IL-6 concentrations, colour-coded by sex. The black line represents the fitted values with a 'loess' smoother and the grey-shaded area represents the 95% confidence intervals.

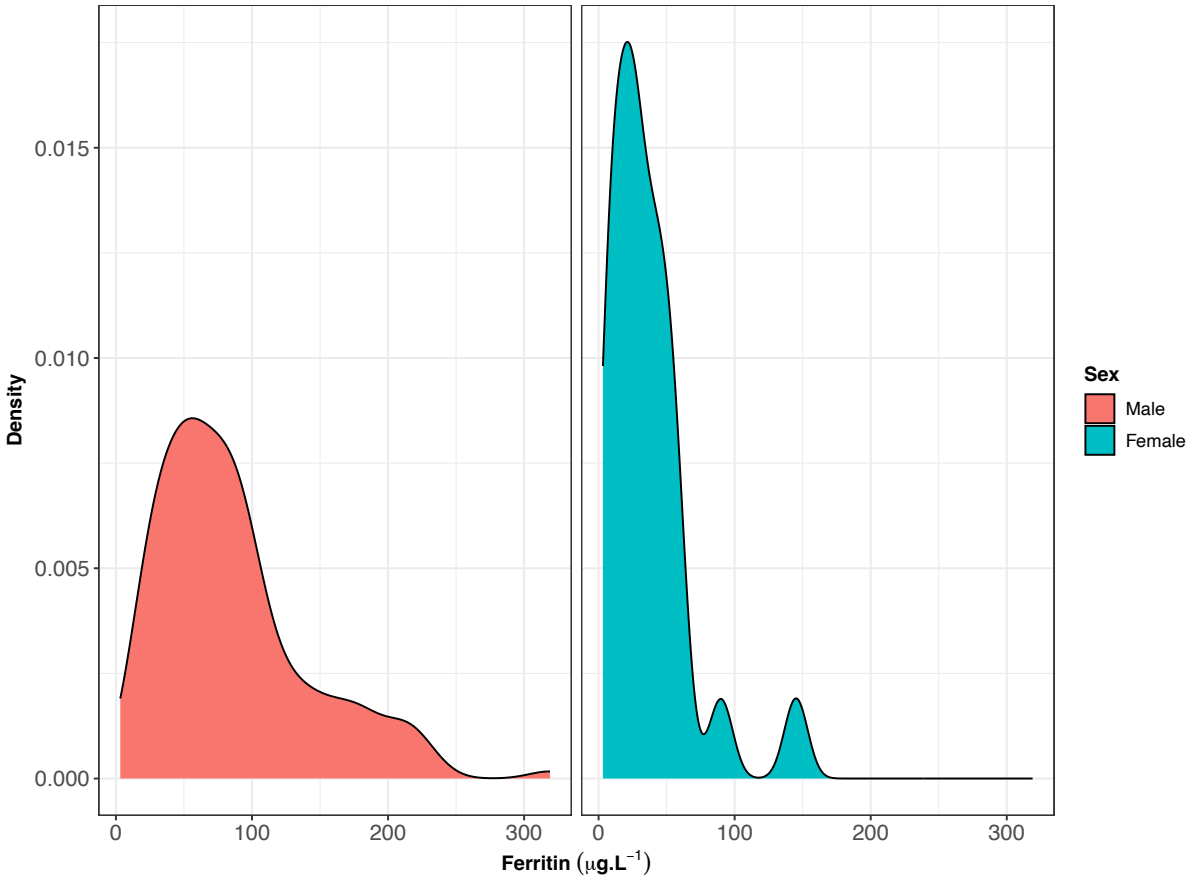

Figure 5.6: Distribution of pre-exercise ferritin concentrations of all participants by sex, pooled for each study included in the meta-analysis.

5.5 Discussion

This meta-analysis of individual participant data reveals that exercise is associated with a 1.5- to 2.5-fold increase in hepcidin concentrations, with absolute values at 3 h post-exercise being approximately double that of pre-exercise hepcidin values, both with and without adjustment for additional covariates. Nevertheless, a larger magnitude of absolute change in the hepcidin concentration can be attributed to the collective contribution of higher pre-exercise ferritin, lower VO_2max, male sex, longer duration, and higher post-exercise IL-6. Moreover, exercise duration played a dominant role in determining the post-exercise IL-6 concentration, with longer exercise bouts resulting in a larger response. However, while sex differences were apparent in the magnitude of both hepcidin and IL-6 responses to exercise, study design limitations (i.e., a low sample of females taken predominately (25%) from only one study analysed) preclude definitive conclusions to be drawn on the impact of sex.

5.5.1 The influence of ferritin and pre-exercise hepcidin

Following on from the work of Peeling and colleagues (Peeling et al., 2014), who demonstrated a strong influence of baseline ferritin status on the post-exercise hepcidin response, many researchers have since incorporated this variable into their analysis (Fensham et al., 2022b; McKay et al., 2021b; McKay et al., 2021c; McKay et al., 2019a; Peeling et al., 2017). In our analysis, while ferritin helped to explain the magnitude of the 3 h post-exercise hepcidin response that was not accounted for by pre-exercise hepcidin concentration alone, its contribution appears smaller than hypothesized. Ferritin likely has a greater influence on the post-exercise hepcidin concentration via a threshold effect, with hepcidin concentrations being suppressed at low serum ferritin concentrations to promote intestinal iron uptake (Galetti et al., 2021). In the current study, the lower median pre-exercise ferritin values in females (~30 $\mu g.L^{-1}$) likely accounted for the failure of this variable to significantly explain the post-exercise hepcidin concentration in comparison to males who had higher pre-exercise serum ferritin values (~75 $\mu g.L^{-1}$; Fig. 5.6). In support of our observations, Peeling and colleagues observed an attenuation in urinary hepcidin in 3 participants with serum ferritin < 35 $\mu g.L^{-1}$ (Peeling et al., 2009b), with a follow-up study showing a non-significant increase in the post-exercise serum hepcidin response in participants with a serum ferritin < 30 $\mu g.L^{-1}$ (Peeling et al., 2014). More recent analysis has established clinically useful thresholds in both serum ferritin and hepcidin concentrations with respect to iron absorption at rest: Galetti and colleagues (Galetti et al., 2021) used a generalized additive model to derive a ferritin threshold of < 50 $\mu g.L^{-1}$ and resting hepcidin of < 3 $nmol.L^{-1}$ at which iron absorption was increased in women aged 18-50 years (serum ferritin: 33.7 ± 27.1 $\mu g.L^{-1}$; haemoglobin 133 ± 9 $g.L^{-1}$). A strong correlation (r = 0.50) between baseline ferritin and pre-exercise hepcidin has previously been reported (Galetti et al., 2021; Peeling et al., 2014) but our analysis suggests that their use in predicting the 3 h post-exercise hepcidin response is different: given the fairly consistent doubling factor between pre- and 3 h post-exercise hepcidin, pre-exercise hepcidin may be a more useful measurement for this purpose, especially when ferritin stores are low. Where resources are limited, measuring pre-exercise hepcidin provides insight into an athlete's baseline iron status while also being a predictive tool for the potential post-exercise iron absorption capability. Together, however, both pre-exercise hepcidin and ferritin likely provide important insight into systemic iron metabolism and, therefore, both should be measured where resources allow.

5.5.2 Sex differences

Sex was found to be a significant factor in both the IL-6 and hepcidin responses to exercise, when all other covariates were held constant. However, several limitations preclude us from

making definitive conclusions about this outcome. When each sex was analysed separately, only pre-exercise hepcidin was a consistent contributor to explain the post-exercise hepcidin response, with other contributing variables differing between the sexes. Such an outcome was likely a result of the sample size discrepancy between sexes resulting in lower observed power to confidently detect significant contributing variables. Nevertheless, in males, pre-exercise ferritin and VO_2max were additive and subtractive covariates, respectively; in females, exercise duration and pre-exercise ferritin were the most contributive variables. However, it must be noted that ~25% of the female cohort was derived from a single study, which specifically set out to investigate the effect of exercise duration on the post-exercise hepcidin response (Newlin et al., 2012). Accordingly, given the key variable of manipulation in the primary study of female participants was the greatest contributor to explain post-exercise hepcidin response in our model, this result is interpreted with caution. Of note, most females in our meta-analysis presented with sub-optimal pre-exercise ferritin values (median ~30 $\mu g.L^{-1}$; see Fig. 5.6), which may have contributed to a blunted post-exercise hepcidin response in this cohort. Interestingly, despite iron deficiency being more prevalent in female athletes (Nabhan et al., 2020), the literature focusing on post-exercise hepcidin is skewed towards observations on male athletes, with male participants outnumbering females by 3:1 at the time of our review. This suggests that there is likely a gap in the literature investigating the underlying mechanism of iron deficiency in female athletes. Under-representation (Costello et al., 2014; Cowley et al., 2021) of female participants in sports science research is well-known but is particularly frustrating when applied to areas in which female athletes may have greater risks or differences.

5.5.3 The female athlete

Quantitatively, female athletes contribute fewer data to our knowledge of sports science, with a recent audit reporting only 34% of participants from over 5000 publications were female (Cowley et al., 2021). Furthermore, the quality of information gained from female-specific or female-inclusive studies is limited by the failure to standardize or report on menstrual cycle phase or status (Smith et al., 2022); factors that may contribute to the heterogeneity of findings (Elliott-Sale et al., 2021). Indeed, in our review of the literature for this investigation, most studies did not provide any documentation of the menstrual status/phase of their female participants, while some used protocols with limited utility (e.g., a questionnaire related to menses onset). In fact, only one of the studies included in this meta-analysis measured 17β-oestradiol and progesterone to account for phase of the menstrual cycle (Zheng et al., 2021). Measurement of female sex steroid hormones may be particularly relevant to iron-related research, with sex hormone concentrations possibly affecting hepcidin activity (Badenhorst et

al., 2021). Clearly, the influence of menstrual cycle on iron metabolism requires further research, which is focused on improved definitions and characterization of the menstrual cycle in line with best practice guidelines (Elliott-Sale et al., 2021). However, given the lack of information provided by the studies included within our meta-analysis, we are unable to conclude whether menstrual cycle phase explains the sex differences observed.

5.5.4 Exercise duration and diurnal variation

In the current analysis, exercise duration was a predominant factor influencing both the magnitude of change in hepcidin and the post-exercise IL-6 concentration. However, the well documented influence of diurnal variation on IL-6 and hepcidin concentrations should also be considered as a potential confounder/contributor to these findings. A 2- to 6-fold rise in hepcidin occurs between 06h00 and 15h00 (Kemna et al., 2005), potentially resulting in a higher absolute hepcidin concentration measured at 3 h post-exercise in some of the included studies. Since longer duration exercise bouts and their recovery phase are more likely to finish later in the day, some diurnal effect may have contributed to the resultant significance of exercise duration in our model. Diurnal variation has also been reported for IL-6 (Nilsonne et al., 2016) but, given that most of the exercise trials in this meta-analysis would have been complete by midday (maximum duration ~ 2 h), this is unlikely to have reflected in the *immediate* post-exercise IL-6 values. More likely, the significance of duration here relates to the depletion of muscle glycogen as exercise progresses (Keller et al., 2001), with the relationship between exercise duration and IL-6 following an approximately exponential trajectory (Fischer, 2006) (Fig. 5.5).

5.5.5 Effects of nutritional state and exogenous supplementation

In contrast to the hepcidin response, pre-exercise IL-6 accounted for a small proportion of the post-exercise IL-6 value in our analysis, emphasizing the different sources of cytokine release. For instance, the majority of post-exercise IL-6 is derived from the muscle itself, serving to stimulate fatty acid and glucose availability to fuel exercise (Hennigar et al., 2017). Conversely, at rest, most of the circulating IL-6 is derived from white adipose tissue and leukocytes (Toft et al., 2011). Of note, the post-exercise muscle-derived IL-6 response may be attenuated by carbohydrate consumption during exercise (Nieman et al., 1998), which would be dependent on the amount consumed, exercise duration, and degree of muscle glycogen depletion (Keller et al., 2001). Although information regarding nutritional state (i.e., exercise performed in a fed or fasted state) was sought from the authors, we were unable to factor it into our analysis due to model complexity, limiting our ability to definitively discern the probability of the different

sources of IL-6 in this analysis. A further, related limitation here is the different approaches used by authors in standardizing diet and exercise in the 24 h prior to the onset of their trials (Online Resource 11). While some researchers relied on retrospective methods such as dietary recall and replication, other researchers specifically prescribed macronutrient targets and abstinence from exercise. Diet and exercise in the previous 12-24 h affects post-exercise IL-6 (Badenhorst et al., 2015a) and pre- (Badenhorst et al., 2015a) and post-exercise hepcidin concentrations (McKay et al., 2020). Further, control of iron supplementation was not always explicitly detailed by authors. As acute iron intake would affect baseline hepcidin concentrations, this may bias absolute concentrations in this analysis; however, this also lends support to the inclusion of pre-exercise hepcidin values in analyses to account for this variability and inconsistency. Depending on the research question, and especially when iron absorption is being measured directly, control or standardization of diet (including supplements) and exercise is prudent (McKay et al., 2022a); alternatively, this information should be explicitly documented.

5.5.6 The potential influence of training status

The effect of participant VO_2max on the hepcidin and IL-6 response to exercise has not been specifically investigated previously. By combining data from male and female participants with a variety of training statuses, our results showed that participants with a higher VO_2max had an attenuated increase in both IL-6 and hepcidin compared to individuals with a lower VO_2max, albeit to a small degree. The use of VO_2max as a proxy for training status is, however, imperfect and is not a sole predictor of performance across intensities (Podlogar et al., 2022). Furthermore, differences in measurement methods (Schoffelen et al., 2019) and modality used (Basset & Boulay, 2000) limit the accuracy of a specific individual's training status classification or characterization of their underlying physiology. Nevertheless, with training, an enhanced ability to oxidize fat at a specific intensity results in a reduced reliance on glycogen (Brooks & Mercier, 1994), which may attenuate increases in IL-6 (Hennigar et al., 2017). Indeed, this has been previously suggested in work examining the effect of a 3 week intensive training and dietary intervention study where athletes who performed at a lower percentage of their improved VO_2max (Burke et al., 2017) also had a lower post-exercise IL-6 (McKay et al., 2019a). Thus, a reduced IL-6 stimulus may account for the lower post-exercise hepcidin concentration. However, a specific study design that controls for the VO_2max measurement method in participants across a range of performance capacities would be needed to verify our observations and subsequent mechanism speculations.

5.5.7 Limitations of hepcidin alone as a predictor of iron absorption

While hepcidin is the master iron regulator, there is demonstrably a complex interplay of characteristics of the athlete and exercise session that influence the direction and magnitude of its response to an exercise stimulus. It is important to note that hepcidin concentrations are only a surrogate marker of iron absorption; there are many other factors that play a role in regulating intestinal iron absorption and iron efflux from iron storage sites that were not considered in the current study. In fact, Roe and colleagues reported that plasma hepcidin in the range of 0.1-7.8 nmol.L^{-1} explained only 36% of the variance in iron absorption (Roe et al., 2009). Therefore, the interaction between the absolute concentration of hepcidin and the additional factors influencing gut absorption (i.e., exercise-induced intestinal changes, co-consumption of nutrients) are likely important considerations for both future research and clinical practice.

5.6 Conclusions and Implications

Models derived from currently available data suggest that hepcidin concentrations are doubled by exercise, with a 1.5- to 2.5-fold increase in values at 3 h post-exercise compared to pre-exercise hepcidin. Nevertheless, there are a multitude of influential factors, some of which are not adequately accounted for in the available studies. For example, baseline ferritin levels might only become a significant contributor to this increase when iron stores are above a certain threshold (Peeling et al., 2014). Exponential increases in IL-6 became most notable after an exercise duration of 2 h, and both variables impact upon the change in hepcidin from pre- to post-exercise. We have also shown that improved cardiorespiratory fitness may attenuate both the IL-6 and hepcidin response to exercise. Although sex may influence the post-exercise hepcidin response, the contribution here was small, likely reflecting differences in sample size and sex representation across studies, which impede conclusive remarks. Whether or not male versus female reproductive hormones and/or menstrual cycle phase play a role in the sex differences observed remains unknown due to the lack of documentation by authors of the included studies. Importantly, the findings of this analysis need to be confirmed in athletic populations from a broader range of ethnicities and countries.

Ferritin remains a useful and practical marker of iron stores and, in addition to measurement of baseline pre-exercise hepcidin concentrations, is useful in forecasting post-exercise hepcidin concentrations. Sport and exercise practitioners should consider the time of day and duration of exercise sessions when advising athletes on iron consumption around exercise. The influence of the previous day's exercise and an athlete's habitual diet should be controlled as far as

practically possible for clinical diagnostic testing purposes and, for the moment, it would be prudent for practitioners to assess a female's iron status in the same menstrual cycle phase. In future iron metabolism research involving females, menstrual cycle phase should be characterized (where applicable) and sex steroid hormone levels should be documented to improve our understanding of sex differences in iron metabolism, enabling us to better tailor our advice accordingly.

Declarations

Ethics approval

Ethics approval was granted by the Australian Catholic University Human Research Ethics Committee (2021-223N).

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Acknowledgement

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Conflict of interest

The authors have no conflicts of interest to declare. Although an intellectual interest bias has informed the motivation for conducting this meta-analysis, with some of the authors having published extensively in this area and whose papers are included, these authors declare that this has not impacted the results.

Author contributions

NF and AM were responsible for conceiving the idea for the review and meta-analysis and study selection. NF conducted data retrieval from individual authors and subsequent data consolidation. Statistical analysis was performed by NF and AG. The first draft of the manuscript was written by NF. All authors contributed to data interpretation and reviewing the manuscript. All authors read and approved the final manuscript.

Availability of data

The datasets generated during and/or analysed during the current study are not publicly available as they were provided by individual study authors after signed agreement. Data may

be able to be provided upon request from the authors pending approval by the Australian Catholic University's Human Research Ethics Committee.

Consent to publish

A letter of agreement, signed by the authors of the individual studies prior to data transfer, referred to our registered protocol in which the dissemination/publication plans were detailed.

Supplementary files

Online Resource 1: Summary of studies retrieved through the systematic search, and those included/excluded from the individual participant data meta-analysis

Online Resource 2: Risk of bias assessment

Online Resource 3: Method utilized for analysis of hepcidin, interleukin-6, and ferritin concentrations in each study

Online Resource 4: Code dictionary and reference values

Online Resource 5: R code for all analyses and graphical plots

Online Resource 6: 3 h post-exercise serum hepcidin model parameter comparison (stratified intercept)

Online Resource 7: 3 h post-exercise serum hepcidin model parameter comparison (random intercept)

Online Resource 8: Change in serum hepcidin model parameter comparison

Online Resource 9: 3 h post-exercise urinary hepcidin model parameter comparison (stratified intercept)

Online Resource 10: Post-exercise serum interleukin-6 model parameter comparison (random intercept)

Online Resource 11: Diet and exercise control prior to each study's trial

Interlinking chapter

Studies show that a large proportion of the magnitude of exercise-associated increases in both IL-6 and hepcidin can be attributed to the duration of the exercise session. However, only single exercise bouts were available for inclusion in this meta-analysis. Surprisingly, no studies had been performed looking at twice-daily training; a strategy commonly utilized in many high-performance sports. Furthermore, consideration should be given to the possible interactions between diurnally induced elevations and the second exercise session of the day. In such a situation, athletes may be at risk of sustained or amplified increases in hepcidin; these may both contribute to iron deficiency and require consideration in the timing of supplementation to manage it. Additionally, more frequent mechanical loading combined with repeated elevations in IL-6 and PTH around each exercise session, as well as reduced opportunity for energy, carbohydrate, and micronutrient intake and/or absorption from the gut, may predispose these athletes to an increased risk of bone stress injuries. As part of a wider research initiative, Chapter 6 sought to determine whether performing twice-daily training resulted in a cumulative effect on concentrations of both IL-6 and hepcidin. Opportunistically, this study design was set up for investigation of the effect of calcium supplementation on CTX concentrations across the same period (Lundy et al., 2022a). Given iron-calcium reciprocity had been suggested in animal models, this presented a unique opportunity to explore this interaction and determine whether an intervention (i.e., calcium prior to exercise) designed to limit the impact of exercise on bone may result in an adverse effect on another body system.

6 Chapter 6

Sequential submaximal training in elite male rowers does not result in amplified increases in interleukin-6 or hepcidin

Publication statement:

This chapter is comprised of the following paper published in the *International Journal of Sports Nutrition and Exercise Metabolism.*

Fensham N.C., McKay A.K.A., Tee N., Lundy B., Anderson B., Morabito A., Ross M.L.R., Burke, L.M. Sequential submaximal training in elite male rowers does not result in amplified increases in interleukin-6 or hepcidin. Int J Sport Nutr Exerc Metab. 2022;32(3):177-85. doi: 10.1123/ijsnem.2021-0263

6.1 Abstract

Previous research investigating single bouts of exercise have identified baseline iron status and circulating concentrations of interleukin-6 (IL-6) as contributors to the magnitude of post-exercise hepcidin increase. The current study examined the effects of repeated training bouts in close succession on IL-6 and hepcidin responses. In a randomized, crossover design, 16 elite male rowers completed two trials, a week apart, with either high (1000 mg) or low (<50 mg) calcium pre-exercise meals. Each trial involved two, submaximal 90 min rowing ergometer sessions, 2.5 h apart, with venous blood sampled at baseline, pre-exercise, and 0, 1, 2 and 3 h after each session. Peak elevations in IL-6 (~7.5-fold, $p<.0001$) and hepcidin (~3-fold, $p<.0001$) concentrations relative to baseline were seen at 2 and 3 h after the first session (EX1) respectively. Following the second session (EX2), concentrations of both IL-6 and hepcidin remained elevated above baseline, exhibiting a plateau rather than an additive increase (2 h post-EX1 vs 2 h post-EX2, $p=1.00$). Pre-exercise calcium resulted in a slightly greater elevation in hepcidin across all timepoints compared to control ($p=.0005$), however no effect on IL-6 was evident ($p=.27$). Performing multiple submaximal training sessions in close succession with adequate nutritional support does not result in an amplified increase in IL-6 or hepcidin concentrations following the second session in male elite rowers. Although effects of calcium intake require further investigation, athletes should continue to prioritize iron consumption around morning exercise prior to exercise-induced hepcidin elevations to maximize absorption.

Keywords: iron, endurance exercise, nutrient timing, hepcidin, athlete

6.2 Introduction

Endurance athletes are at risk of iron deficiency (Sim et al., 2019), which can impair performance due to the role of iron in key metabolic processes, including energy production and oxygen transport (Beard, 2001). Contributors to this risk include exercise-induced iron losses from gastrointestinal micro-ischemia, sweat, haemolysis, and haematuria (Peeling et al., 2008). However, reduced bioavailability of dietary iron and iron recycling are also involved (Sim et al., 2019) with the hormone hepcidin playing a major role in these processes. Hepcidin regulates iron flux by binding to the ferroportin transporter to block intestinal absorption of iron and its release from macrophages (Nemeth et al., 2004b). Its synthesis in hepatocytes is upregulated by a range of factors including increased hepatic or serum iron, hypoxia, and inflammation (Ganz & Nemeth, 2012). Indeed, an increase in circulating interleukin-6 (IL-6) concentrations provides a potent stimulus for hepcidin expression, with peak hepcidin concentrations occurring ~3-6 h following the IL-6 peak (Peeling et al., 2009b). During endurance exercise, skeletal muscle acts as an endocrine organ, with IL-6 release increasing liver glucose output, muscle glucose uptake and free fatty acid availability (Fischer, 2006). This response is affected by prevailing carbohydrate (CHO) availability: exaggerated in glycogen depleted states and attenuated when CHO is ingested during prolonged exercise (Fischer, 2006). The downstream increase in hepcidin creates a post-exercise window in which impairment of dietary iron absorption and macrophage iron recycling may contribute to iron depletion in athletic populations.

Previous work has focused on IL-6 and hepcidin responses to a single bout of exercise (McKay et al., 2020; Peeling et al., 2017; Sim et al., 2012). To our knowledge, the consequence of repeated exercise bouts has only been examined in a single study involving two running sessions separated by 12 h and overnight sleep (Peeling et al., 2009a); here, no cumulative effect was found. However, elite athletes frequently undertake two or more training sessions within a day, with subsequent sessions occurring within the 3-6 h window of the hepcidin peak of the previous bout. This may increase the risk of iron deficiency by extending the period of impaired iron absorption over the day, particularly if there is an amplified hepcidin response to the second session due to reduced CHO availability. A larger investigation conducted by our group of the effect of pre-exercise calcium ingestion on both parathyroid hormone (PTH) and bone turnover markers over successive exercise bouts (Lundy et al. in review) presented an opportunity to examine iron regulation in this circumstance as well. Furthermore, this protocol enabled examination of possible crosstalk between bone and iron metabolism. Both iron overload and

deficiency have been shown to impair bone homeostasis (Toxqui & Vaquero, 2015), and IL-6 has been implicated in increased bone resorption in the presence of PTH (Sims, 2021). Thus, the primary aim of this study was to examine the response of IL-6 and hepcidin to twice daily training in elite endurance athletes, with an opportunistic aim of determining whether pre-exercise calcium intake would interact with these effects via its downstream effects on bone turnover (Haakonssen et al., 2015).

6.3 Methods

6.3.1 Participants

Eighteen elite male rowers from the Rowing Australia National Training Centre, in preparation for potential Olympic representation, were recruited. One participant with lactose intolerance was excluded due to his inability to complete one of the dietary arms, while another was unable to complete the required training load due to injury. Two athletes with known mild hemochromatosis were included since their ferritin values were within range of the other athletes, and not receiving treatment due to their ferritin being <300 $\mu g.L^{-1}$ (Kowdley et al., 2019). Since the removal of their data did not alter the interpretation of the results, they have been retained in the current dataset. Another with previously diagnosed hypothyroidism was deemed eligible, due to the presentation of normal thyroid function tests at the time of the study. None of the final cohort of 16 participants, characterized in Table 6.1, were iron deficient or anaemic [serum ferritin <35 $\mu g.L^{-1}$ and Hb <115 $g.L^{-1}$; (Sim et al., 2019)] or taking oral iron supplements. Written informed consent was obtained from each athlete prior to study commencement. This study conformed to the standards set by the Declaration of Helsinki. Ethics approval was obtained from the Australian Institute of Sport Ethics Committee (ref: 20200905).

6.3.2 Experimental overview

In a randomized, double-blinded, crossover design, athletes completed two trials, one week apart, involving either a high (CAL) or low calcium (CON; control) intervention (see Figure 6.1). All trials were performed at the same training centre to ensure comparable environmental conditions. On trial mornings, athletes arrived at the laboratory (06h00-07h00) in a fasted, rested state, and a blood sample was collected from a cannula placed in a forearm vein. Athletes then consumed either a low (< 50 mg) or high calcium (1000 mg) standardized breakfast (t = 0 min) with athletes and the researchers involved with data collection or data entry remaining blinded to the treatment order. After 115 min, a pre-exercise blood sample was drawn and 5

min later (t = 120 min) the first exercise session (EX1) commenced. This session involved 3 x 30 min sets, with a 5 min break between sets. Immediately post-EX1, blood was sampled, and at t = 250 min (30 min post EX1 and 120 min prior to EX2) the same low or high calcium meal was consumed according to group allocation. Blood samples were drawn 1 and 2 h post-EX1 (t = 280 and 340 min), and at t = 370 min, a repetition (EX2) of the earlier session was undertaken, noting a recovery period of 150 min between exercise bouts. Blood was collected at the break between the first and second sets of EX2 (t = 400 min, equating to 3 h post-EX1). Blood was collected on completion of EX2 (t = 470 min), prior to the consumption of a recovery meal, with further samples at 1, 2, and 3 h post-EX2 (t = 530, 590, and 650 min).

Table 6.1: Athlete characteristics and resting iron status of low calcium (CON) and high calcium (CAL) trials

		Trial		***p***
		CON	**CAL**	
Athlete characteristics	**Age (years)**	26.4 ± 3.4		-
	T1 power target (W)	257 ± 13		-
	Pre-trial body mass (kg)	96.5 ± 5.5	96.6 ± 5.9	.62
Resting iron status	**Haemoglobin (g.L^{-1})**	168 ± 11	164 ± 12	.07
	Iron (µmol.L-1)	27.4 ± 8.6	26.0 ± 8.3	.52
	Ferritin (µg.L^{-1})	159 ± 90	155 ± 98	.68
	Transferrin (g.L^{-1})	2.8 ± 0.3	2.6 ± 0.3	.06
	Transferrin saturation (%)	39 ± 14	39 ± 15	.90
	Ionized calcium (mmol.L^{-1})	1.27 ± 0.03	1.26 ± 0.03	.37

Note: values are presented as mean ± standard deviation.

The majority of exercise sessions were completed on a rowing ergometer (Concept 2, Morrisville, Vermont, USA). In select cases, where repetitive loading would have caused injury risk, a Wattbike Pro cycling ergometer (Wattbike Ltd., Nottingham, UK) was substituted in some exercise sets. This supports real-world practice and was replicated in both trials. Session intensity was prescribed at 90-100% of T1 power (Watts), with targets established from an incremental exercise protocol, starting at 150 W and increasing by 15 W every 6 min. The power at which capillary blood lactate reached 2 mmol/l was designated the athlete's T1 power value. Mean power was recorded for each effort, as was heart rate (HR, beats per min; Wahoo Tickr X, Wahoo Fitness, Atlanta, USA) and subjective rating of perceived exertion (RPE) according to the Modified Borg Scale (6-20).

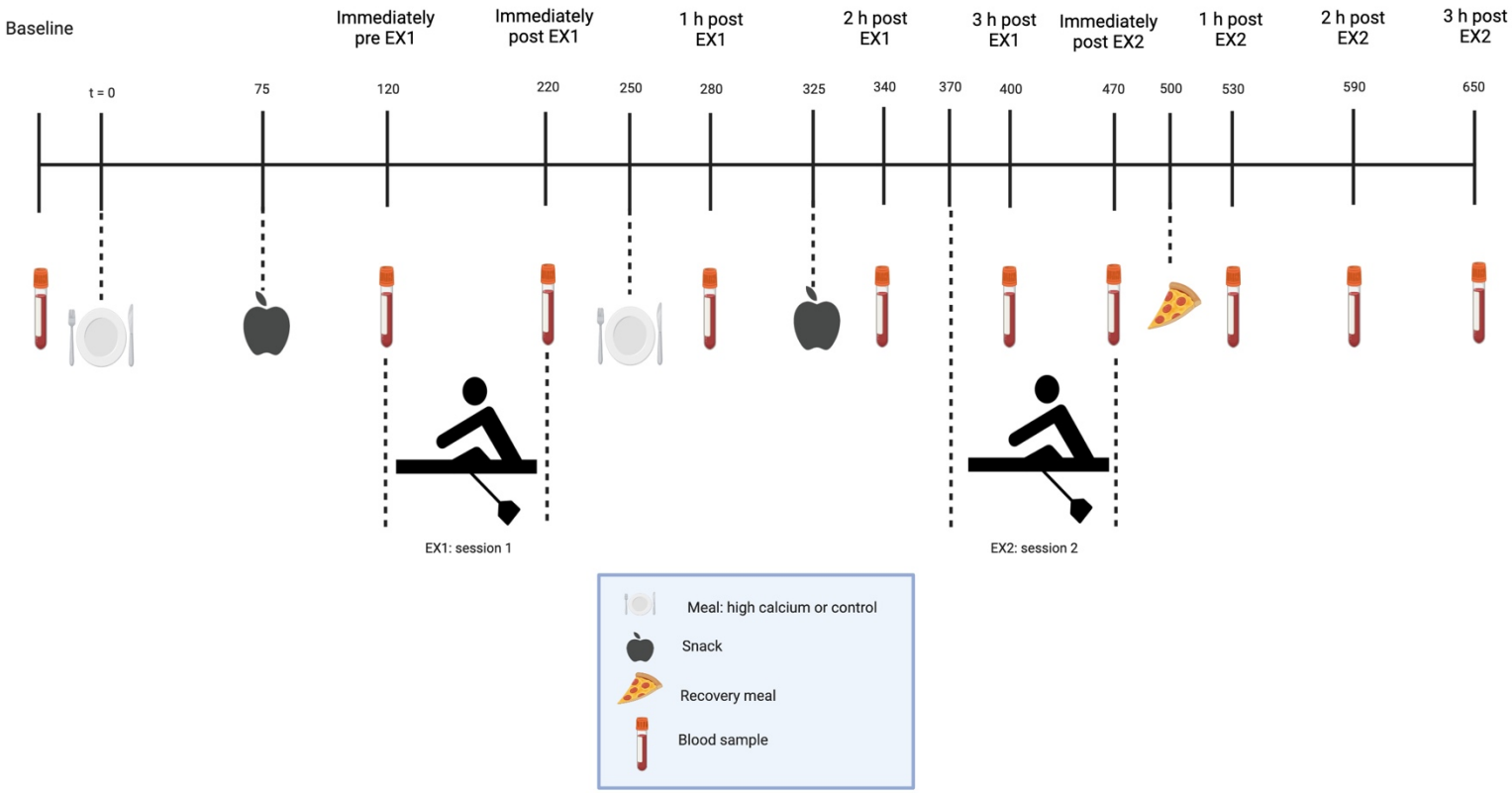

Figure 6.1: Overview of experimental protocol, including timing of meals, exercise sessions, and blood sampling (created with Biorender.com).

6.3.3 Dietary intake

A standardized diet and training prescription was followed for 24 h prior to each trial. Trial day intake (see Figure 5.1) was also standardized and matched for energy, carbohydrate, protein, and iron content as closely as possible. The intervention for the larger study in which this project was embedded involved manipulation of calcium content of meals consumed 2 h pre-exercise, with the intention of manipulating serum ionic calcium concentrations prior to the onset of exercise. Water was provided *ab libitum* during the sessions and recorded accordingly. A CHO-rich gel (Science in Sport PLC, London, UK) was consumed (~30 g CHO) during each 5 min break between exercise sets. To maintain real-life practice, athletes were permitted to consume coffee prior to exercise and were able to request more food in the recovery period between exercise sessions in addition to the pre-exercise meal. This was done in accordance with the dietary treatment, recorded, and repeated in the second trial. Nutrient composition of diets was calculated using a computerized dietary analysis package (Nutritics Ltd., Dublin, Ireland).

6.3.4 Blood analysis

During each trial, 10 venous blood samples were collected into either 6- or 8-ml serum separator tubes (BD Vacutainer, Melbourne, Australia). Blood samples were taken at rest (fasted), pre-exercise (2 h post breakfast), and immediately, 1 h, 2 h, and 3 h post each exercise session (Figure 6.1). Samples were left to clot for 30 min before being centrifuged at 1500 G for 10 min at 4^{o}C. Serum was aliquoted into 1.5 ml cryotubes and frozen at -80^{o}C until batch analysis was performed. Ferritin analysis was performed on 6 samples per trial (baseline, pre session, 1 h, 2 h and 3 h post each session) using a COBAS Integra 400 automated biochemistry analyser (Roche Diagnostics, Rotkreuz, Switzerland). Concentrations of IL-6 were determined for all 10 trial samples using a commercially available ELISA (Quantikine HS; R&D Systems, Minneapolis, MN, USA) on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany). Measurement of hepcidin-25 was conducted on all trial samples using Intrinsic Hepcidin IDxi ELISA Kit (Intrinsic LifeSciences LLC, CA, USA) according to the manufacturer's instructions. The coefficient of variation was 3.4% and 4.9% for IL-6 and hepcidin-25 concentrations respectively. Additionally, whole blood was used to determine haemoglobin and haematocrit with the point-of-care i-STAT device (Abbott Point of Care Inc., Princeton, NJ, USA).

6.3.5 Statistical analysis

Pre-trial and trial day intake, as well as baseline haemoglobin and iron markers were compared with paired sample t-tests or the Wilcoxon test. Training variables (power, heart rate, and RPE)

were compared with a general linear mixed model using R package 'lme4', with Trial (calcium vs control) and Session (first vs second) as fixed variables and Subject Identification and Week as random effects. Hepcidin, IL-6, and ferritin response data were also analysed with a general linear mixed model, with Timepoint and Trial as fixed effects, and Subject Identification and Week as random effects, to account for interindividual variation and the crossover design respectively. Furthermore, baseline ferritin was included as a covariate in the hepcidin model to account for the recognised influence on its response (Peeling et al., 2017; Peeling et al., 2014). All models were estimated using restricted maximum likelihood. Visual inspection of the residual plots did not reveal obvious deviations from homoscedasticity or normality. P-values were obtained using Type II Wald F tests with Kenward–Roger degrees of freedom, as employed in the R package 'car'. Where applicable, post-hoc Tukey's honestly significant difference was applied to identify where differences between timepoints existed. Significance was set at $p<.05$. Effect size (partial eta-squared) was calculated with R package 'effectsize'. With values of 0.01, 0.06, and 0.14 classified as small, medium, and large effect respectively.

6.4 Results

6.4.1 Dietary intake

Dietary intake during the 24 h pre-trial standardization period was well-matched, with energy intakes of 21983 ± 5500 vs 21662 ± 7300 kJ (p=.82); CHO intakes of 6.8 ± 1.5 vs 7.2 ± 1.7 g.kg^{-1} body mass (p=.048) and iron intakes of 21.9 ± 7.2 vs 23.4 ± 7.6 mg (p=.09). Trial day analysis revealed no differences in intakes of CHO and iron, but greater intakes of energy, protein, and fat in the calcium trial (Table 6.2). Although statistically significant, these differences, which likely occurred as a result of the foods chosen to manipulate calcium content of the pre-trial meals, are small in practical terms (~10 kJ.kg^{-1}, 0.3 g.kg^{-1} protein, 0.1 g.kg^{-1} fat). The desired differences in calcium intakes were achieved.

6.4.2 Successive exercise bouts

Characteristics of EX1 and EX2 are summarized in Table 6.3. There were no significant differences between trials or sessions for power or heart rate. While there were no differences in RPE between trials, EX2 was perceived as marginally more strenuous than EX1 (see Table 5.3).

Table 6.2: Energy and nutrient intake of athletes on days of low calcium (CON) and high calcium (CAL) trials

	Trial		*p*
	CON	**CAL**	
Energy (kJ)	19293 ± 2100	20345 ± 1600	<.001*
- kJ.kg^{-1}	200 ± 23	211 ± 22	.001*
Protein (g)	193 ± 31	220 ± 22	.002*
- g.kg^{-1}	2.0 ± 0.3	2.3 ± 0.3	.003*
Fat (g)	151 ± 16	163 ± 14	.003*
- g.kg^{-1}	1.6 ± 0.2	1.7 ± 0.2	.004*
Carbohydrate (g)	614 ± 69	615 ± 69	.89
- g.kg^{-1}	6.4 ± 0.8	6.4 ± 0.8	.98
Calcium (mg)	224 ± 160	2319 ± 160	< .001*
Iron (mg)	4.8 ± 1.6	4.9 ± 2.0	.88
Water intake (ml)	658 ± 290	730 ± 300	.34

Note: values are presented as mean ± standard deviation. * significant difference between trials (p<.05)

Table 6.3: Characteristics of successive exercise bouts undertaken on days of low calcium (CON) and high calcium (CAL) trials

	Session	**Trial**		***p***		
		CON	**CAL**	**Trial**	**Session**	**Interaction**
Actual power (W)	**Combined**	260 ± 5	255 ± 5	.27	.35	.12
	EX1	259 ± 6	260 ± 6			
	EX2	261 ± 6	250 ± 6			
Actual power as a percentage of prescribed T1 power (%)	**Combined**	101.2 ± 1.6	99.5 ± 1.6	.27	.33	.11
	EX1	100.7 ± 1.9	101.5 ± 1.9			
	EX2	101.7 ± 1.9	97.4 ± 1.9			
HR (bpm)	**Combined**	144 ± 4	144 ± 4	.81	.39	.12
	EX1	141 ± 5	145 ± 5			
	EX2	148 ± 5	143 ± 5			
RPE (6 – 20)	**Combined**	11.7 ± 0.4	11.4 ± 0.4	.13	.002*	.32
	EX1	11.4 ± 0.4	11.0 ± 0.4			
	EX2	11.9 ± 0.4	11.8 ± 0.4			

Note: values are presented as mean estimate ± standard error. p-values represent comparison between trials (CAL vs CON), between sessions (EX1 vs EX2), and interaction between trial and session. EX1 = first session; EX2 = second session; HR = heart rate; RPE = rating of perceived exertion. * significant difference between sessions (p<.05).

6.4.3 Iron regulatory response

Fasting values for haemoglobin, serum iron, transferrin, transferrin saturation and ionized calcium were similar on Trial morning (Table 6.1). There were no differences in serum ferritin (Figure 6.2) between trials, $F(1,156)=0.34$, $p=.56$, $\eta_p^2=.002$, or across timepoints, $F(5,156)=1.21$, $p=.31$, $\eta_p^2=.04$, and no interaction between variables, $F(5,156)=1.12$, $p=.35$, $\eta_p^2=.03$.

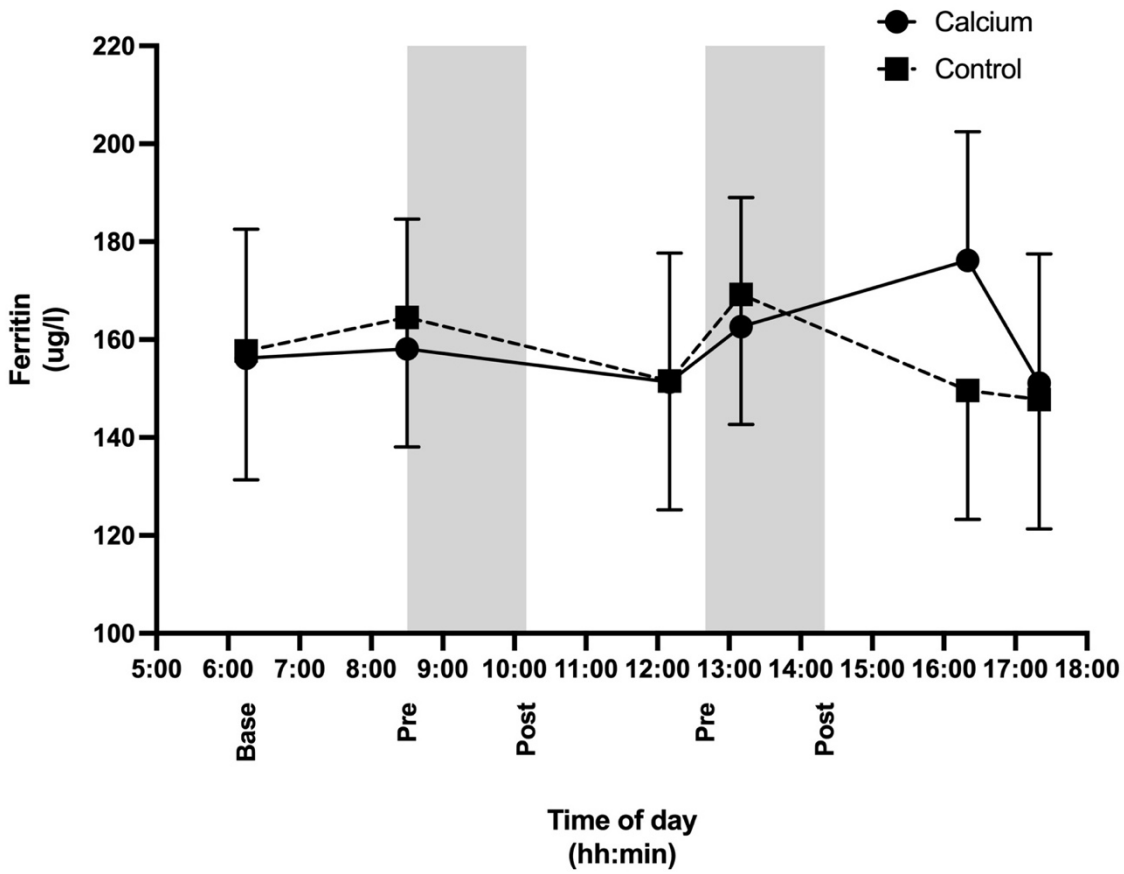

Figure 6.2: Ferritin concentrations in the calcium and control groups across the two exercise sessions. Data are presented as estimated means with standard error.

No differences were present between trials for IL-6, $F(1,271)=1.21$, $p=.27$, $\eta_p^2=.004$, (Figure 6.3A). IL-6 increased in response to exercise, $F(9,272)=8.02$, $p<.0001$, $\eta_p^2=.21$, but there was no interaction with trial, $F(9,271)=1.63$, $p=.11$, $\eta_p^2=.05$. Specifically, IL-6 increased in response to EX1, with a ~5-fold increase in concentrations relative to baseline at 1 h post-EX1 ($p=.048$) and a peak at 2 h post EX1 (~7.5-fold increase, $p<.0001$). At 3 h post-EX1, IL-6 concentrations were not statistically different to baseline ($p=.81$). Following EX2, IL-6 reached similar peak concentrations but, in contrast to the decrease seen post-EX1, concentrations plateaued with no mean difference between 1 h, 2 h, and 3 h post-EX2 ($p=1.00$).

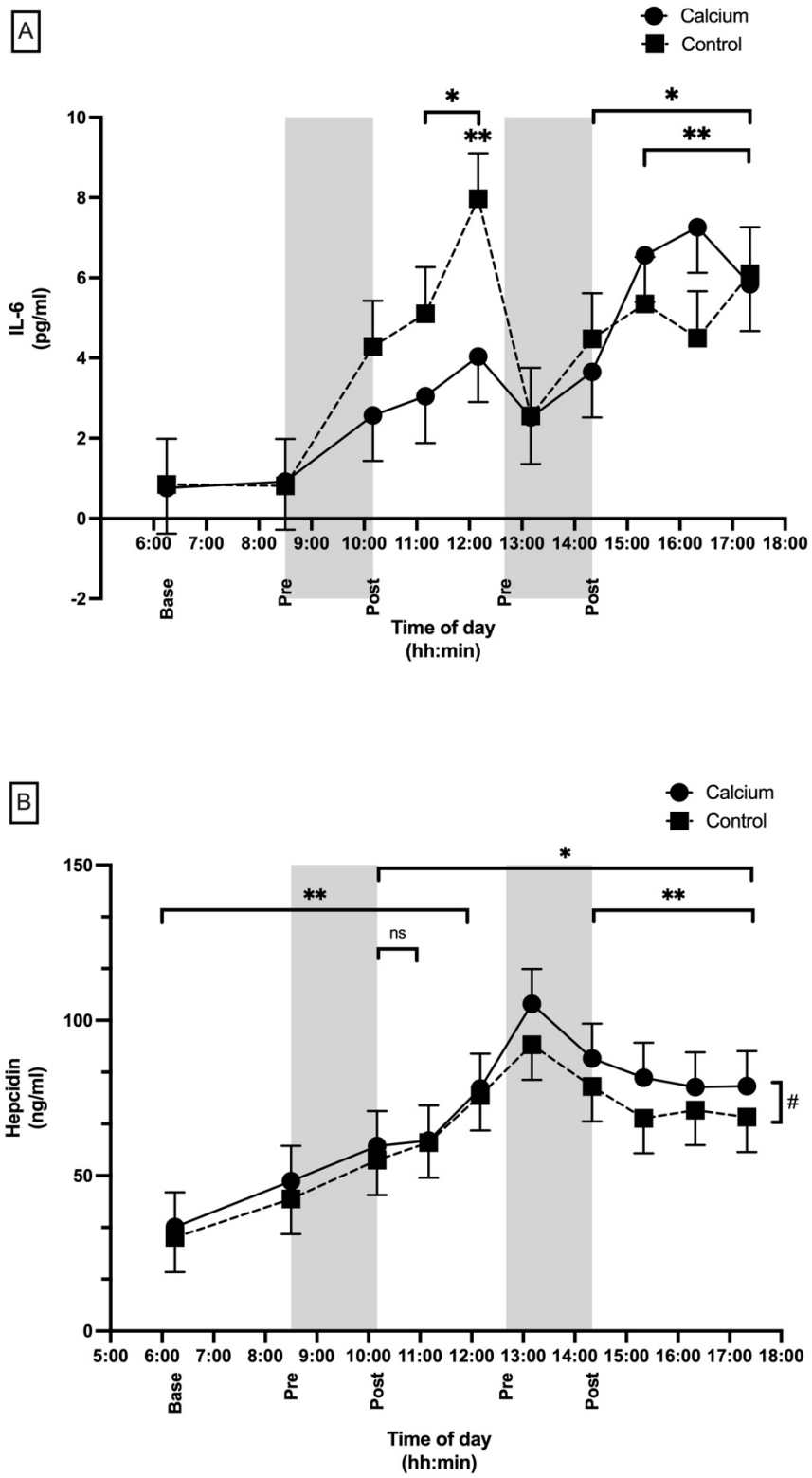

Figure 6.3: IL-6 (A) and hepcidin-25 (B) concentrations in the calcium and control groups across the two exercise sessions. Data are presented as estimated means with standard error. * significant difference to baseline (p<.05). ** significant difference to 3h post-EX1 (p<.05). ns: no significant difference between bracketed timepoints (p>.05). # significant main effect of trial (p<.05).

There were both time, $F(9,270)=39.89$, $p<.0001$, $\eta_p^2=.57$, and trial effects, $F(1,270)=12.43$, $p=.0005$, $\eta_p^2=.04$, for hepcidin concentrations, but no interaction between factors, $F(9,270)=0.50$, $p=.88$, $\eta_p^2=.02$. Trial effects show that calcium ingestion resulted in small, consistently higher (~10.7%) hepcidin concentrations. Baseline ferritin had a significant positive association with the hepcidin response to exercise ($p=.0007$, $\eta_p^2=.17$). Hepcidin concentrations were greater than baseline at all timepoints following the commencement of EX1 ($p<.0001$; Figure 6.3B), with a peak (~3.1-fold increase) occurring at 3 h post-EX1 ($p<.02$). After the second exercise bout, the elevated hepcidin concentrations remained at a plateau, with no difference between values 1 h, 2 h, and 3 h post-EX2 ($p=1.00$) and the lattermost value being ~2.3-fold greater than baseline.

6.5 Discussion

This is the first study to examine the time-course of changes in blood concentrations of IL-6 and hepcidin across two consecutive and proximate training sessions. Main findings were: 1. Although the first exercise session was associated with the expected increase in IL-6 and hepcidin concentrations, a successive exercise bout, commenced before the return of hepcidin to baseline concentrations, was associated with a hepcidin plateau rather than an amplified increase. 2. Baseline ferritin concentrations continue to play a role in the hepcidin response to successive bouts of exercise, and 3. An interaction between calcium metabolism and the exercise-induced hepcidin response is possible, since a high calcium pre-exercise meal was associated with a small but significantly greater increase in post-exercise hepcidin concentrations than a low calcium control trial. These outcomes are relevant to considerations around nutrient timing for elite athletes, who commonly perform two or more training sessions each day.

Endurance exercise is associated with an immediate post-exercise surge in blood concentrations of IL-6, followed by a hepcidin peak 3-6 h later (Peeling et al., 2009b). This upregulation of hepcidin occurs in response to the inflammatory role of IL-6 and serves to resist microbial infection by limiting iron availability (Nemeth et al., 2004b). Previous studies in both cycling (Starkie et al., 2000) and running (Starkie et al., 2001b) have established skeletal muscle, rather than circulating monocytes, as the predominant source of IL-6 following exercise. The magnitude and time-course of the post-exercise increase in IL-6 depends on intensity, duration, and mode of exercise, as well as pre-exercise muscle glycogen content (Febbraio & Pedersen, 2002). With regard to CHO availability, Steensberg and colleagues (Steensberg et al., 2001)

showed a net IL-6 release within 1 h of concentric exercise in a glycogen-deplete state, which was delayed until 2 h in the glycogen-replete leg. At the end of exercise (~4-5 h), there was no difference in net IL-6 release between legs, coinciding with a similar level of muscle glycogen content. The current study adds to our body of knowledge from both physiological and real-world aspects by investigating the effect of repeated bouts of exercise, completed with a short recovery interval (2.5 h). This reflects common practice in elite rowing with median weekly volumes of ~20 h and up to 20 sessions, including up to ~5 each of ergometer and stationary cycling (Tran et al., 2015). Although muscle biopsies were not performed in our study to verify muscle glycogen concentrations, CHO was consumed by participants the day prior to and day of each trial (~7 $g.kg^{-1}$ body mass), as well as during (~60 $g.h^{-1}$) and between (~3$g.kg^{-1}$) exercise bouts in accordance with current sports nutrition guidelines (Thomas et al., 2016). We observed a substantial increase in IL-6 after the first exercise bout, peaking after 2 h of recovery, with a similar increase in response to the second session. The lack of difference in the peak IL-6 response between training sessions may suggest that muscle glycogen content had minimal influence on the response to multiple exercise sessions undertaken in close succession. It is plausible that when nutritional support before and during training maintains CHO availability, potential elevations in inflammatory responses can be attenuated. Other factors such as the training status of the athletes and the relative intensity of the training sessions, which interact with CHO utilization and availability (Nieman et al., 1998; Starkie et al., 2000), may also have contributed to the magnitude of the IL-6 response. The relative contributions of differences in athlete physiology and exercise bout characteristics across studies requires further investigation.

We note the sudden and rapid decrease in IL-6 at the 30 min mark of the second exercise session. This may be an artefact of sampling during exercise due to the redistribution of blood to muscle. Indeed, other studies which have sampled IL-6 during exercise (Fischer et al., 2004; Ostrowski et al., 1998a) have reported smaller increases in IL-6 than when muscle contraction has ceased and blood has recirculated. Attention is also drawn to recovery after the second session, where IL-6 remained persistently elevated at similar peak concentrations to that reached after the first session. Here, consideration must be given to diurnal influences on IL-6, with studies suggesting a morning trough at 08h00-09h00 and evening peak at ~20h00 (Nilsonne et al., 2016). A recent investigation involving endurance running, of similar duration and occurring at a similar time of day as the first exercise session in our study, found IL-6 increased immediately following exercise, and remained above baseline after ~9-10 h post-exercise (McCormick et al., 2019). Meanwhile, when the running session was delayed to the

afternoon, IL-6 had already risen above morning concentrations prior to the onset of exercise (McCormick et al., 2019). However, in contrast to the findings by McCormick and colleagues, where late afternoon (16h00) running amplified the diurnal effect, we did not observe an additive effect of the second session. This may be explained by the earlier timing of the second session in our study (12h40-14h10) or the effect of different modes of exercise (running versus rowing) on IL-6 responses. Indeed, running has been shown to elicit a larger response than cycling (Nieman et al., 1998), which may be due to the amount of muscle mass recruited or the weightbearing nature of exercise. How this compares to rowing, which also involves significant whole-body muscle recruitment but is non-weightbearing, is unknown.

An additional explanation for the plateau in IL-6 concentrations after the second session is the possibility of immune-cell derived IL-6 production. In response to microtrauma to skeletal muscle during exercise, infiltrating immune cells release a variety of cytokines to initiate the healing process in the post-exercise recovery period (Hennigar et al., 2017). Thus, immune-cell derived IL-6 may remain elevated at a lower magnitude for a longer duration during recovery than that derived acutely from muscular contraction (Hennigar et al., 2017). Among the few studies of rowing exercise models, an investigation of a single session showed minimal change in number and activity of monocytes and no change in IL-6 up to 1.5 h post-exercise (Henson et al., 2000). Following two 90-min cycling bouts at 60% VO_{2max}, separated by 3 h, Li & Gleeson (2005) showed higher monocytes and IL-6 concentrations following the second session, both of which were attenuated by CHO ingestion. Whether performing a subsequent rowing session elicits greater muscle damage and immune-cell IL-6 release is unknown, and without surrogate markers of inflammation or leukocyte activity, the likely source of the measured IL-6 in our study remains speculative.

In the current study, hepcidin concentrations peaked at 3 h post-exercise, with the magnitude of increase being strongly associated with baseline ferritin status. This is in line with previous research (Peeling et al., 2017; Peeling et al., 2014) and supports the role of hepcidin in regulating circulating iron availability in response to body stores (Nemeth et al., 2004b). This is the first study to take hourly measurements of hepcidin concentrations during the post-exercise period in an athletic population, providing a more comprehensive understanding of the time course of the hepcidin rise. Previous studies reporting hepcidin measurements immediately and 3 h post-exercise (Peeling et al., 2009b) (Newlin et al., 2012) may have led to the assumption that a somewhat linear increase occurs between these two timepoints. Our study now provides further insight into the pattern of post-exercise increase to the ~3 h peak. Contrary

to our expectations, however, superimposing further exercise during the period of the anticipated hepcidin response to the first rowing session did not further amplify the initial peak. In the absence of exercise, a 2- to 6-fold rise in hepcidin has been observed from 06h00 to 15h00 (Kemna et al., 2007; McCormick et al., 2019), with the suggested pattern being an initial sharp increase followed by a plateau and slow return to baseline after 21h00. McCormick and colleagues (2019) measured hepcidin at the same four timepoints over a day intercepted with either a morning (06h30) or afternoon (16h00) run for 90 min at 65% $VO_{2max.}$ Here, it was demonstrated that the hepcidin increase following the afternoon run was additional to the diurnally increased pre-exercise concentration. The observed plateau in our hepcidin results is curious given that diurnal rhythms would predict a further increase, but this may relate to the earlier timing of our second session (12h40-14h00).

A limitation of our study includes the lack of control conditions involving a rest or single training session to establish the independent effects of each exercise session and diurnal rhythms. The lack of a compounding effect on IL-6 by the second exercise provides another potential explanation for the plateaued hepcidin response. However, although IL-6 contributes to the hepcidin response, its individual correlation is small in comparison to other factors, such as baseline iron status (Peeling et al., 2017). Indeed, the interaction between superior training status and relatively modest glycogen depletion during low intensity exercise may result in an uncoupling of the correlation between IL-6 and hepcidin (McKay et al., 2019b). Therefore, protocols involving differences in exercise intensity, duration, glycogen status or training status (Fischer, 2006) may elicit a larger IL-6 response to successive exercise bouts, potentially amplifying the downstream effects on hepcidin. We recognize that although hepcidin levels are used as a surrogate for reduced iron absorption, other factors are likely at play. Indeed, in one study of male participants, circulating hepcidin concentrations (albeit in ranges lower than those seen in our study), accounted for only 36% of the variation in iron absorption (Roe et al., 2009). Furthermore, greater absorption of iron from a standardized meal was observed in endurance runners when it was consumed 30 min following morning exercise, compared to afternoon exercise or in the morning in the absence of exercise (McCormick et al., 2019). For the moment, however, in relation to timing the intake of iron supplements or iron-rich meals to coincide with lower hepcidin levels, our study, showing lowest concentrations up to 1 h post-exercise, continues to support intake in the morning, and/or immediately adjacent to strenuous exercise.

The opportunistic element of this study, investigating an interaction between pre-exercise calcium intake and exercise-associated iron regulation, revealed a potential increase in the post-exercise hepcidin response. Here, we note that our study was embedded within a separate investigation of the effects of pre-exercise dietary calcium on bone metabolism during and after repeated sessions of non-weight bearing exercise (Lundy et al. in review). However, pre-existing data support our decision to examine the iron regulatory responses to the calcium-supported and placebo exercise conditions separately. Iron-calcium reciprocity has been demonstrated in thalassemic mice, where hepcidin seems to restore calcium absorption (Kraidith et al., 2016). Further, in relation to bone, it has been shown that hepcidin increases intracellular calcium in *in vitro* osteoblasts (Li et al., 2012; Xu et al., 2011), with hepcidin knock-out mice exhibiting bone loss (Li et al., 2020). Iron is also necessary for collagen synthesis and Vitamin D activation (Toxqui & Vaquero, 2015). Therefore, it appears possible that calcium ingestion may influence hepcidin concentrations, with potential downstream effects for both bone and iron status. We note the small magnitude of difference in hepcidin concentrations between trials and the unknown translation to changes in iron absorption. Future research should investigate the magnitude and mechanistic underpinnings of this relationship and its applicability to athletic populations.

In summary, our study shows that, in elite male rowers, performing successive sessions of submaximal exercise within close proximity does not result in an amplified effect on IL-6 and hepcidin responses, at least when sports nutrition practices achieve guidelines for CHO support. However, permutations to exercise protocols, training times, and time between training sessions may result in a different outcome and should be explored. These findings should guide the timing of intake of iron supplements or iron-rich meals around exercise sessions to support iron status in athletes. Furthermore, interaction with calcium intake might need further consideration, especially if there are reasons to promote high calcium intakes prior to prolonged exercise sessions for bone health (Haakonssen et al., 2015). It is already recognized that co-ingestion of iron and calcium reduces effective absorption of iron from the gut in the short-term (Lönnerdal, 2010). However, there is a need to examine additional interactions between iron-calcium reciprocity in exercise settings. Nevertheless, based on outcomes of the current study, we suggest athletes should consider prioritizing iron consumption adjacent to morning exercise to maximize absorption, prior to exercise-induced hepcidin elevations.

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Authorship: The study was designed by LMB, AKA, BL, and NF. Data were collected by LMB, AKA, NT, BL, BA, AM, and MR, and analysed by LMB, AKA, NT, and NF; data interpretation and manuscript preparation were undertaken by LMB, AKA, and NF. All authors approved the final version of the paper.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

7 Chapter 7: Discussion and conclusions

The primary focus for an athlete is improving performance and, indeed, exercise and nutrition science research has been aligned with this objective. Various strategies, including manipulation of macronutrient intake and timing to enhance training adaptations, energy restriction to alter body composition, and the use of sports foods and supplements as ergogenic aids, have garnered much attention in the literature (Thomas et al., 2016). However, the health benefits and detriments of training and nutrition strategies have been, until quite recently, given much less attention by athletes, coaching staff, practitioners, and researchers. Failure to take heed of the effect of illness and injury on missed training and competition (Feddermann-Demont et al., 2014) makes the focus on performance alone paradoxical. Although such occurrences may be inevitable with high training loads, sound nutritional strategies can reduce the risk of, and accelerate recovery from, illness and injury (Close et al., 2019).

The prevalence of bone stress injuries (Snyder et al., 2006), low BMD (Scofield & Hecht, 2012), and iron deficiency (Nabhan et al., 2020) is reported to be higher in endurance athletes than the general population, with both high training load and suboptimal nutrition playing a role in increasing the risk of these conditions (Heikura et al., 2018b; McKay et al., 2021c; Mountjoy et al., 2018; Petkus et al., 2017). The separate and overlapping effects of exercise and nutrition on bone and iron metabolism, and a central link between the two in IL-6, was further investigated in this thesis. Collectively, the findings of these studies, together with companion study support, suggest that exercise-induced increases in markers of bone resorption and iron absorption/release inhibition can be increased by exercise scenarios involving low energy and CHO availability, and attenuated through provision of adequate amounts and timing of energy and CHO. Where exercise duration is prolonged, nutritional support considerations, including both macro- and micronutrient supplementation strategies, become more significant.

7.1 Novel findings of this thesis

Various studies have investigated the BTM response to a single bout of exercise (Dolan et al., 2022). However, the magnitude of response has been small, transient, and highly variable (Dolan et al., 2022). Indeed, the variability is exacerbated by the lack of standardization of timing of blood sampling in relation to exercise and time of day, and nutritional and exercise practices at the time of and the days prior to testing (Dolan et al., 2022). Furthermore, although

these results are interesting to compare within and between intervention groups within a study, the absolute BTM values currently have uncertain significance (Callegari et al., 2017). The lack of standardisation of assays used across research studies, producing disparate results, adds to the uncertainty of information provided by these markers (Bhattoa et al., 2021; Cavalier et al., 2021; Cavalier et al., 2019). While both CTX and P1NP are the recommended markers for monitoring response to osteoporosis treatment (Wu et al., 2021) and may predict fracture risk in older adults (Johansson et al., 2014; Vasikaran et al., 2011), a gap in the literature exists exploring the clinical usefulness of BTMs in a younger population. Indeed, there are limited data on the normal reference ranges appropriate in young adults (Vasikaran et al., 2014), and none published in athletes. The net effect of coupling the anabolic effect of increased mechanical loading with potentially catabolic exercise-induced metabolic changes (Wherry et al., 2022), and its translation to resting bone turnover rates is unknown.

Therefore, one of the aims of ***Study 1*** was to characterize baseline CTX and P1NP concentrations in an elite athlete cohort by sex, age group, and assay. Using existing data collected as part of individual studies conducted by this research group, in which standardised nutrition was implemented for the 24 h prior to testing and samples were taken in the morning in a fasted state, the central 95% distribution was calculated. Female athletes were significantly under-represented at just n = 11 for each of CTX and P1NP, in contrast to 85 male data sets for CTX and 56 data sets for P1NP. Further analysis revealed significantly higher CTX and P1NP values in those 25 years and older, with the represented age range being 19-37 years, possibly explained by the age at which peak bone mass is achieved (Berger et al., 2010). While sex did not appear significant, the large discrepancy in sample size between male and female athletes cautions further interpretation of this result. Our values seemed higher than those of the general population of the same age (Vasikaran et al., 2014). However, a major limitation of such a comparison was the lack of reference data in the general population, and the existence of significant flaws in the single large study upon which these were based (Jenkins et al., 2013). Of note, there were significant differences between results produced by automated versus manual assays for CTX but not for P1NP, a finding largely echoed by consensus groups (Cavalier et al., 2021; Cavalier et al., 2019). Nevertheless, despite our high degree of control over pre-analytical variability, a large proportion of the variance (~50%) could be attributed to interindividual variability. While we recognize that our sample size in this study is relatively small and cannot necessarily be used as a reference, it does emphasize the need for large-scale multicentre collaborations between sporting organizations to establish normative values for athletic populations. A major strength of this study was the standardisation of the timing of

blood samples relative to prior feeding practices, minimising pre-analytical variability. Indeed, future research protocols should require stipulation of prior day feeding and exercise, timing of blood sampling, sample storage times, and particular automated assay use. Furthermore, when conducting intervention studies, age-matching should be an important consideration and, perhaps, a tighter range of within-study inclusion criteria is required. Establishing reference ranges for BTMs in athletes will enable improved interpretation of individual values: multicentre, cross-sectional studies with standardized pre-testing protocols might facilitate this. Exclusion of athletes with a history of conditions known to significantly alter bone (re)modelling, such as eating disorders/REDs, endocrine disorders, bone tumours, and certain long-term medications (e.g. steroids) may be required to establish "normal" ranges, while regression analysis could be utilized to establish the differences between age, sex, and sport. Furthermore, investigation of the clinical outcomes, such as bone stress injuries or long-term bone strength changes, of having biomarker results outside, compared to inside, the reference range is needed.

A further aim of ***Study 1*** was to characterize BMD status across athletes from different sports and explore the effect of mechanical loading on the prevalence of site discordances. While the diagnosis of low BMD for age is recommended to be a z-score cut off of ≤ -2 via measurement of areal BMD at the lumbar spine or proximal femur (Writing Group for the ISCD Position Development Conference, 2004), the appropriateness of this cut-off in athletes is debated. Indeed, due to the benefit of exercise to BMD (Tenforde & Fredericson, 2011), it is suggested that a BMD z-score of < -1 (Jonvik et al., 2022; Nattiv et al., 2007) or even < 0 in high-impact sports (Jonvik et al., 2022) might raise concern in athletes. However, the relationship between BMD alone and fracture risk in younger populations is weak (Ferrari et al., 2012; Frolich et al., 2020; Writing Group for the ISCD Position Development Conference, 2004) with bone stress injuries in athletes occurring despite "normal" (z-score > -2) BMD or despite BMD equal to that of athletes with a significantly lower prevalence of stress injuries (Carbuhn et al., 2022; Heikura et al., 2018b; Rudolph et al., 2021). Therefore, the measurement and classification of BMD status in athletes may require different standards and additional risk factor considerations. ***Study 1*** demonstrated the notable impact of site-specific mechanical loading with rowers' lumbar spine z-scores being significantly higher than racewalkers, runners, and triathletes, and both racewalkers and runners having significantly higher total femur than lumbar spine z-scores. Interestingly, despite the majority of triathletes being classified as having low BMD, there was no significant difference between their total femur and lumbar spine z-scores, suggestive of the variable loading nature of the sport. With these discrepancies in z-scores

between sites and occurrence of bone stress injuries at sites that are heavily loaded, yet not measured (e.g., rib stress fractures in rowers (Lundy et al., 2022b; McDonnell et al., 2011)), there is further opportunity to tailor BMD measurement and classification according to sport. Cross-sectional studies examining traditional BMD site discordances across a range of sports are needed to establish norms, with further longitudinal studies necessary to establish whether raising the z-score cut-off translates into earlier detection of secondary risk factors and reductions in bone stress injuries. Combining serial tracking of fasting BTM concentrations with BMD and microarchitecture analysis and bone stress injury incidence has potential to provide insight into how BTMs could be used in a predictive capacity.

The last aim of ***Study 1*** was to explore the interaction between bone and iron status. Although impaired bone architecture has been demonstrated in clinical populations with iron overload (Baldini et al., 2014; Jandl et al., 2020) as well as in iron deficient rats (Medeiros et al., 2004), the lack of association between ferritin and BMD in our athletes suggests either a protective effect of mechanical loading on bone or that, because most of the athletes were not iron deficient or overloaded, iron status was not a contributory variable. Nevertheless, the effect of iron status on BMD is probably better appreciated through a longitudinal study design, monitoring ferritin concentrations over a longer period, in keeping with the slower change in BMD. Interestingly, IL-6, involved in both bone turnover (Sims, 2021) and hepcidin stimulation (Nemeth et al., 2004a; Peeling et al., 2009b; Peeling et al., 2017), explained some of the variance in fasting CTX concentrations. This did not hold true for post-exercise IL-6 and post-exercise CTX concentrations. Both CTX (Dolan et al., 2022) and IL-6 (Peeling et al., 2009b) increase following exercise, with temporal associations previously noted (Sale et al., 2015). One explanation for this discrepancy is the differing sources of IL-6 with resting IL-6 predominantly released from immune cells, as opposed to post-exercise IL-6 from muscle cells. Alternatively, additional variables influencing both IL-6 and CTX concentrations post-exercise, such as exercise duration and intensity, may play a more dominant role. Teasing out the effect of IL-6 on bone turnover in the presence of exercise is challenging, but the contribution of IL-6 to CTX at rest in the current study signals that nutrition and training practices that have been shown to raise IL-6 potentially translate into increased bone resorption. Indeed, work by this research group has demonstrated increased CTX concentrations following a 3-week ketogenic LCHF diet (Heikura et al., 2019). This was accompanied by higher post-exercise IL-6 concentrations in the LCHF group than athletes following a high CHO diet (McKay et al., 2019a). Together, these results might suggest a role for carbohydrate independent of total energy on IL-6 responses and CTX responses. While LEA has been shown to have a negative impact on bone

in both the short- and long-term (Papageorgiou et al., 2018a), it is unknown whether this is as a result of the necessary reduction in carbohydrate. Therefore ***Study 2*** aimed to further investigate the changes in BTMs occurring following short-term manipulation of either carbohydrate or energy.

Study 2 implemented strict dietary control and similarly matched training load over two 6-day phases in a parallel group design, allowing for both within- and between-group comparisons. Here, we demonstrated that, despite a matched EA to the high CHO group (CON), the LCHF diet resulted in higher bone resorption and lower bone formation markers across a ~2 h exercise bout. In contrast, the LEA group demonstrated higher bone resorption only. The group that continued to follow a CON diet throughout the study maintained similar bone turnover rates. A companion study looking at iron metabolism utilising the same protocol demonstrated a greater IL-6 response to exercise following the LCHF diet only (McKay et al., 2021c). Although muscle glycogen was not measured in this study, it was postulated to be a contributory factor, in contrast to the LEA and CON groups who consumed enough CHO to remain above a certain (currently unknown) glycogen threshold. While IL-6 might stimulate bone resorption (Sims, 2021), as we observed higher CTX concentrations in both LCHF and LEA groups, suggesting additional exercise-induced mechanisms at play. Indeed, in ***Study 1***, post-exercise IL-6 did not prove significant in explaining post-exercise CTX concentrations. Of note, athletes consumed energy and macro-nutrients before, during, and after the exercise bout according to their diet allocation. As previous research has demonstrated an attenuation of CTX with feeding (Bjarnason et al., 2002) and during-exercise CHO consumption (Sale et al., 2015), this may account for the differences in the AUC between groups and phases, with the CON group receiving double the amount of carbohydrate than the LEA group and the LCHF group receiving high fat options prior to and during the exercise session.

Although the clinical significance of exercise-induced changes in BTMs remains uncertain, especially considering that markers are typically only measured up to a few hours post-exercise, we also note that the percent change in fasting bone formation marker concentrations was reduced in both the LCHF and LEA groups but not in the CON group. As these percent changes (~26% and ~14%, respectively) were larger than the weekly biological variation that might be expected with P1NP (~7%) (Panteghini & Pagani, 1995) and given the strict dietary and training control in our study, it is likely that this was a true effect. Evidence suggests a role for IGF-1 in bone formation (Lindsey et al., 2018) with studies examining LEA scenarios (Loucks & Thuma, 2003; Papageorgiou et al., 2018b) and the ketogenic diet (Clemmons, 2006; Coopmans et al.,

2020) demonstrating a reduction in this hormone, which may explain our observations of reductions in P1NP in both LCHF and LEA groups. Repeat studies are required to confirm our findings but it appears that the ketogenic diet may be more detrimental to skeletal health than a short-term low energy diet. Even so, a high EA/high CHO diet maintains bone turnover most optimally. The nature of an elite athlete's training and competition cycle does require dietary flexibility, however, with periodisation of macronutrients and energy being necessary to achieve sport-specific competition requirements. Therefore, the translation of these periodic, short-term dietary changes into clinical outcomes, namely bone stress injury rates and long-term BMD and architecture changes, should be the focus of future research in this area.

Both ***Study 1*** and ***Study 2*** highlighted the potential influence of IL-6 on bone metabolism and the similarities in response to exercise and diet. As IL-6 is known to stimulate hepcidin (Nemeth et al., 2004a; Peeling et al., 2009b; Peeling et al., 2017), we were equally interested in other factors that influence the magnitude of change in hepcidin concentrations. This led to the development of ***Study 3*** which employed a systematic review and individual participant data meta-analysis to elucidate the most influential factors on post-exercise hepcidin and IL-6. Although multiple studies have demonstrated an increase in IL-6 concentrations immediately post-exercise followed by a rise in hepcidin around 3 h later (Badenhorst et al., 2015a; Badenhorst et al., 2016; Badenhorst et al., 2014; Barba-Moreno et al., 2022; Dahlquist et al., 2017; Díaz et al., 2015; Goto et al., 2018; Goto et al., 2020; Govus et al., 2014; Hayashi et al., 2020; Ishibashi et al., 2020; McCormick et al., 2019; McKay et al., 2020; McKay et al., 2021a; McKay et al., 2021b; McKay et al., 2021c; McKay et al., 2019a; Newlin et al., 2012; Peeling et al., 2009a, 2009c; Sim et al., 2013; Sim et al., 2012; Zheng et al., 2021), the contribution of various athlete and exercise bout characteristics to each response was unknown. Following individual participant data retrieval from the study authors, we employed a one-stage approach with stepwise regression to ascertain the factors resulting in the best model fit. We found that the greatest magnitude of change between pre- and 3 h post-exercise hepcidin was largely attributed to exercise duration (~44%) with VO_2max, pre-exercise ferritin, sex, and post-exercise IL-6 accounting for only ~6% of the variance combined. Furthermore, exercise duration also accounted for ~56% of the variance in post-exercise IL-6. As hepcidin follows a diurnal pattern (Kemna et al., 2005), this may have contributed to the higher concentrations measured with longer duration exercise sessions, as the 3 h sampling time point would have occurred later into the day. However, as the measurement of IL-6 was immediately post-exercise, the effect of exercise duration was likely secondary to depletion of muscle glycogen (Fischer, 2006; Keller et al., 2001). Here, we note a limitation of our analysis in being unable

to factor in whether exercise was undertaken in a fed or fasted state and the effect of differing approaches to dietary standardisation in the 24 h prior to the trials. Diet and exercise in the previous 12-24 h affects post-exercise IL-6 (Badenhorst et al., 2015a) and pre- (Badenhorst et al., 2015a) and post-exercise hepcidin concentrations (McKay et al., 2020). Further, although partially accounted for by the inclusion of pre-exercise hepcidin in our models, control of iron supplementation was not always explicitly detailed by authors. Depending on the research question, and especially when iron absorption is being measured directly, control or standardization of diet (including supplements) and exercise is prudent (McKay et al., 2022a).

Although a small contribution, we note the relatively lower post-exercise IL-6 and 3 h post-exercise hepcidin concentrations in female as opposed to male participants. When analysed separately for hepcidin concentrations, VO_2max and ferritin were of significance in male participants and exercise duration in female participants. The contribution of baseline ferritin to post-exercise hepcidin concentrations has been previously demonstrated in male participants (Fensham et al., 2022b; McKay et al., 2021b; McKay et al., 2021c; McKay et al., 2019a; Peeling et al., 2017; Peeling et al., 2014) and is certainly the likely reason for the lack of contribution of ferritin to the female participants' hepcidin concentrations at sub-optimal values (median ~30 $\mu g.L^{-1}$ vs ~75 $\mu g.L^{-1}$ in male participants). Of the female data, 25% were from a single study that explicitly looked at the effect of exercise duration (~120 min), cautioning our further interpretation of this model. Nevertheless, it is interesting to note that, despite a quarter of the female participants completing a relatively long period of exercise (~120 min) that might be expected to reduce muscle glycogen significantly, the post-exercise IL-6 values were generally lower than the male participants completing the same duration. This may be due to greater muscle glycogen utilization in men (~25%) during moderate intensity running than women (Tarnopolsky et al., 1990). Furthermore, within women, muscle glycogen may be inversely related to estrogen with ~25% less utilized during the mid-luteal as opposed to the early follicular phase (Devries et al., 2006; Hackney, 1999). Here, we note that most studies did not provide any documentation of the menstrual cycle status or phase for their female participants, while some used protocols with limited utility (e.g., a questionnaire related to menses onset). In fact, only one of the studies included in this meta-analysis measured 17β-oestradiol and progesterone to account for phase of the menstrual cycle (Zheng et al., 2021). While not the focus of this work, the influence of menstrual cycle and hormonal contraception use on inflammatory and iron markers requires further research, focused on improved characterization in line with best practice guidelines (Elliott-Sale et al., 2021). While recruitment of a larger muscle mass in men than women might also be a plausible explanation for higher IL-6

concentrations seen in the male participants (Fischer, 2006), interestingly, results from ***Study 1***, demonstrating no association between post-exercise IL-6 and lean mass, do not support this hypothesis.

The influence of exercise duration on both IL-6 and hepcidin concentrations combined with the realisation that all the available studies for our meta-analysis examined a single exercise bout led to the development of ***Study 4***. Here, we aimed to characterise the effect of twice-daily training on IL-6 and hepcidin concentrations. In such circumstances, athletes may need to complete two exercise bouts within close succession; this means that the second bout is undertaken within the 3-6 h hepcidin peak from the first session and with incomplete repletion of muscle glycogen content. ***Study 4*** was undertaken in elite rowers for whom 2-3 training sessions each day is common practice, and involved a protocol of two submaximal 90-min exercise sessions, performed mainly on the rowing ergometer, each separated by 2.5 h. Although hypothesized to result in a cumulative effect, the second session did not amplify the IL-6 or hepcidin response above that seen following the first exercise session. Indeed, concentrations of both markers remained elevated, where concentrations at 2 h following the second exercise session were similar to those at 2 h after the first session. Although we did not measure muscle glycogen, the provision of adequate energy and CHO before, during, and after each session may have limited the hypothesized further increase in IL-6 after the second session. Furthermore, while previous research comparing hepcidin responses to morning and afternoon sessions has attributed the larger response following the afternoon session (16h00) to diurnal variation (McCormick et al., 2019), the relatively earlier timing of our second session (12h40-14h10) may explain the lack of an additive effect seen in our study. However, due to the lack of a non-exercise control, the contribution of diurnal variation in hepcidin responses is speculative. Indeed, persistent elevations in hepcidin and IL-6 may have implications for long-term iron and bone status, precluding iron absorption for extended periods of time and potentially increasing bone resorption. Unfortunately, further blood samples were not taken beyond 3 h following the second session and therefore the pattern thereafter and continuing into the next day is unknown. In studies employing shorter exercise bouts (60-75 min) interspersed by ~3 h rest, IL-6 had returned to baseline by 4 h (Ronsen et al., 2002) or the next day (Hammond et al., 2019), and similarly for CTX (Scott et al., 2013). Based upon our findings, athletes performing two exercise sessions in a day should continue to prioritise iron consumption around exercise in the morning or up to 1 h post-exercise. Pending additional research, meeting energy and CHO needs according to sports nutrition guidelines (Thomas et al., 2016) is recommended to at least limit the theoretical possibility of an amplified IL-6 and

hepcidin response, where iron availability is a concern. Future studies should include non-exercise and single session control arms to ascertain the additional effect of the second exercise session separate from the effect of diurnal variation. Measurements of IL-6 and hepcidin on the day following a double-training day are also necessary to discern the impact of this type of training on subsequent day iron regulation. Further value may be derived through the manipulation of energy and macronutrient proportions leading up to, the day of, and/or the days following a double-training day to emulate free living conditions of these athletes and the potential impact of these fluctuations on long-term iron status.

This study was undertaken as part of a larger project looking at the effect of pre-exercise calcium supplementation on BTMs (Lundy et al., 2022a). ***Study 1*** showed that resting IL-6 contributed to fasting CTX concentrations and so ***Study 4*** allowed for further interrogation of these relationships as a secondary, unpublished pattern analysis (Figure 7.1). Given that calcium supplementation would suppress CTX independent of IL-6 (Barry et al., 2011; Guillemant et al., 2004), it was an opportunity to observe how this might decouple IL-6 and CTX responses to exercise. Although pre-exercise calcium (~1000mg) attenuated CTX concentrations during exercise and recovery (Lundy et al., 2022a), it is interesting to note that the general pattern in the CTX response to each exercise session was similar, regardless of the trial condition. Pre-exercise calcium consumption resulted in a similar IL-6 response to the placebo condition yet, in both trials, CTX and IL-6 perturbations appeared synchronized up to ~1 h post exercise. Of note, athletes consumed a CHO-rich meal 30 min following each exercise session; such feedings have previously been demonstrated to suppress CTX during recovery (Hammond et al., 2019; Townsend et al., 2017) but with no appreciable impact on IL-6 (Hammond et al., 2019). Despite the strict dietary and training session control, IL-6 fluctuations were variable both within and between individuals. In fact, training results in a reduction in baseline, but not post-exercise, concentrations of IL-6 (Fischer, 2006) and twin studies report moderate to high heritability in IL-6 (15-61%) (Sas et al., 2012; Wörns et al., 2006). The contributions of VO_2max (trained status) and pre-exercise IL-6 to post-exercise IL-6 concentrations were demonstrated in ***Study 3*** and could account for some of the variability in post-exercise IL-6 responses seen in ***Study 4***. With genetic considerations being relevant to IL-6 (~15-61% heritability) (Sas et al., 2012; Wörns et al., 2006), CTX (Roshandel et al., 2010), and BMD (~45-80% heritability) (Duncan et al., 2003; Krall & Dawson-Hughes, 1993; Videman et al., 2007), crossover study designs are likely to provide greater accuracy. Where this is not feasible, sex and age effects on bone and iron markers (***Study 1 and Study 3***, (Amaral et al., 2015)) emphasise the need for group matching in intervention studies. Nevertheless, a large proportion

of the variance is still environmentally determined, leaving a gap for manipulation of nutrition and training to exert influence on both iron and bone metabolism. Importantly, the long-term implications of these interventions and the associated perturbations in these markers should be the focus of future work in this area.

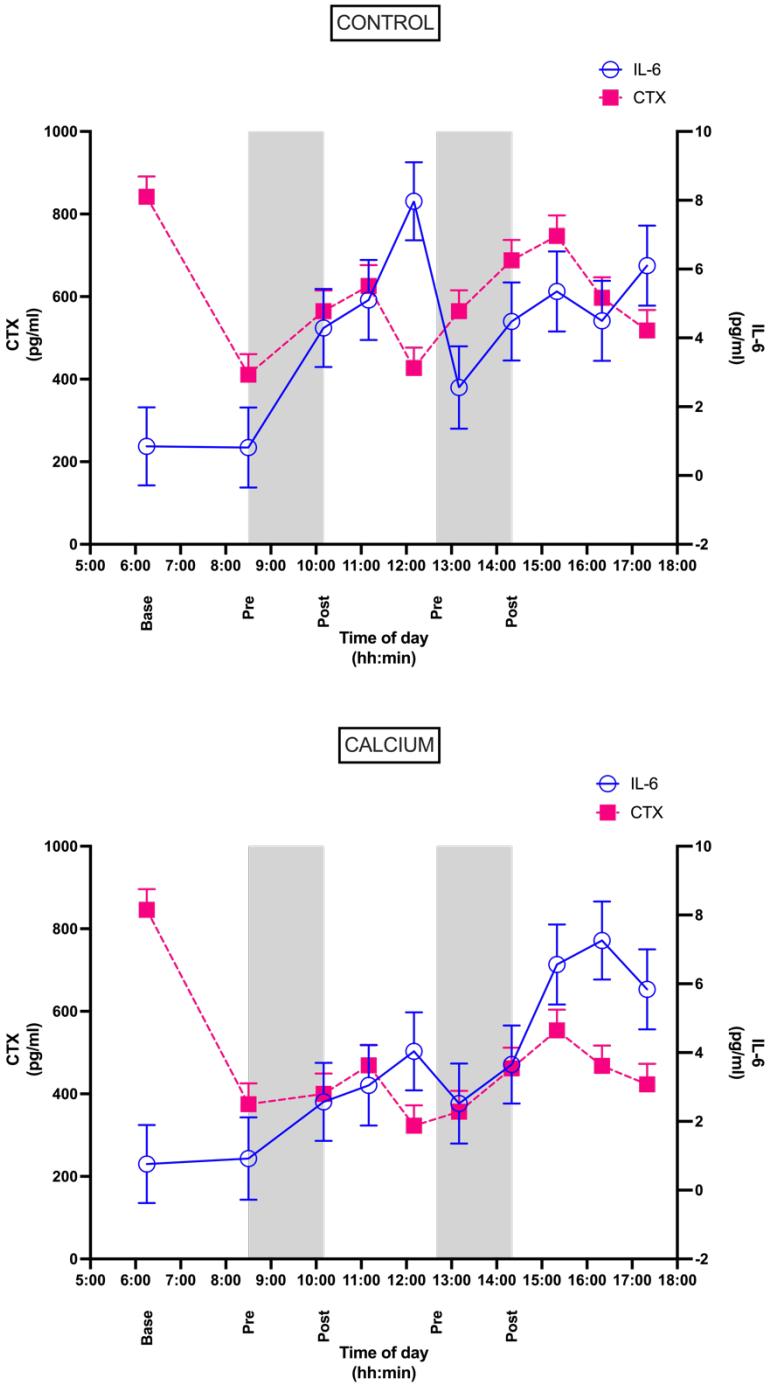

Figure 7.1: Carboxy-terminal telopeptide (CTX) and interleukin-6 (IL-6) concentrations across two 90-min exercise sessions under both control and pre-exercise calcium conditions (unpublished)

7.2 Future research directions

The studies comprising this thesis have addressed several disparate, yet curiously linked, questions that existed in the literature regarding the effect of nutrition and training practices on both iron and bone metabolism. The theme of crosstalk between systems weaved a continuous thread among the studies. In applied research, mechanistic answers are difficult to reach and the multitude of influential factors on a specific outcome is ever present. Recognising this interplay, there is a need for more research in free-living individuals, over longer periods of time to identify the clinical tools with the most utility in improving athlete care.

Several studies have looked at the BTM response to an exercise bout (Dolan et al., 2022), recovery intervals between exercise (Scott et al., 2013), and nutrition both acutely (Hammond et al., 2019; Sale et al., 2015; Scott et al., 2012; Townsend et al., 2017) and over the short-term (Heikura et al., 2019; Ihle & Loucks, 2004; Papageorgiou et al., 2017; Papageorgiou et al., 2018b; Zanker & Swaine, 2000). However, both the *interpretation of the absolute values* and the *translation of these changes into clinical outcomes* remains a significant gap in the literature. While ***Study 1*** of this thesis attempted to compare baseline BTM values of an athlete cohort to those of the general population, stark limitations were apparent. Not only was there limited reference data in younger populations (Vasikaran et al., 2014) but attempts to control pre-analytical variability in this reference data were lacking (Jenkins et al., 2013). Therefore, further research with strict pre-analytical control and assay standardization in younger populations is required, in the first instance, and in athletes, in the second, to elucidate whether BTM values might differ as a result of increased mechanical load. Establishing a reference range will enable improved interpretation of the perturbations elicited by different training and nutrition practices.

Expanding this knowledge requires longer term follow up of the clinical consequences of these relatively acute fluctuations in BTMs. Although cross-sectional analysis reveals the effect of chronic energy deficient states (Ackerman et al., 2015; Ackerman et al., 2019; Ackerman et al., 2011) and ketogenic diets (Bergqvist et al., 2008; Best & Hsu, 2023; Bielohuby et al., 2010; Simm et al., 2017) on bone health, it is still unclear whether *periodic, acute dietary interventions*, combined with high training loads, have deleterious consequences on long-term bone health. While ***Study 2*** of this thesis suggests that short-term energy restriction may be better tolerated than a short-term ketogenic diet, especially when comparing changes to fasting bone formation markers, several questions remain. The *role and mechanism of carbohydrate in*

bone metabolism between sexes warrants further investigation, both separate from and concurrent with mechanical loading. Recognizing that bone resorption is necessary for microfracture repair and bone remodelling (Hoenig et al., 2022), the *balance between circulating resorption and formation markers* that represents homeostasis, as opposed to alerting to pathology, is of interest.

Impact loading has immense benefits for BMD status (Tenforde & Fredericson, 2011) yet concerns around training and dietary practices employed by athletes that may counteract this are pervasive. Suggestions that a higher z-score cut-off for athletic populations is needed as an earlier alert to an already compromised BMD status have been raised (Jonvik et al., 2022; Nattiv et al., 2007), yet there is little literature to support a separate guideline currently. Further, the prevalence of fractures in some sports at sites not traditionally measured (Lundy et al., 2022b) might suggest that measurement protocols require revision. In ***Study 1***, the discrepancy between sites of athletes in different sports highlighted both the loading pattern and the potential effect of secondary risk factors (e.g., LEA) in specific sports. *Establishing BMD norms for different sports*, together with the bone stress injury patterns and markers of REDs, might provide a better evidence base to support the use of a higher z-score threshold in athletes. Improved interpretation of BMD and BTM changes in paediatric populations would also enable earlier detection and intervention of secondary risk factors impairing skeletal growth during the crucial years of bone acquisition. Furthermore, whether whole-body BMD, frequently measured as part of a body composition analysis, may serve as a red flag to carry out diagnostic lumbar spine and proximal femur measurements is a potentially intriguing concept to investigate. Finally, determining whether combining periodic BTM measurements with longitudinal bone stress injury incidence, as well as beginning and end of study DXA and HR-pQCT, would provide valuable insight into how BTMs might be used as a clinical tool to alert for secondary risk factors and predict future BMD and microarchitecture changes.

Similarly, the *effect on iron status on bone health* in athletes requires a longitudinal investigation. ***Studies 1 and 3*** of this thesis suggested a role for IL-6 in fasting CTX and post-exercise hepcidin, respectively. ***Study 4***, and the companion study (Lundy et al., 2022a), demonstrated a similar time-course response of both IL-6 and CTX to exercise, especially within the first hour of each session. However, ferritin concentrations were not associated with BMD status in ***Study 1***, potentially attributed to the fact that most athletes had sufficient iron stores and/or that the cross-sectional study design was unable to capture the different time periods over which ferritin and BMD change. Improved insight into how iron status changes

according to training load and whether insufficiency is related to injury incidence and bone health, independent of training load or EA, is required. Future studies might achieve this through serial tracking of iron status over a year with DXA and HR-pQCT measurements at the start and end, intermittent dietary analysis, and continuous training load quantification.

Finally, the *effect of training load and EA on iron and bone metabolism in subsequent days/weeks* is of interest. ***Study 4*** showed that, in a setting of adequate energy and CHO provision according to training session and individual demands, IL-6 and hepcidin concentrations reach a plateau up to 3 h following the second session. However, little is known about the subsequent fluctuations leading into the next day. While previous studies employing shorter sessions (60-75 min) separated by either ~3 h (Hammond et al., 2019; Ronsen et al., 2002) or 12 h (Peeling et al., 2009a) have demonstrated a return to baseline IL-6 concentrations by 4-17 h post exercise, and hepcidin by 12 h (Peeling et al., 2009a), this has not been investigated in response to longer sessions, such as those used in our study, or for the response to subsequent day training. In times of high training load, this may predispose an athlete to increased bone resorption and reduction of iron stores, which may be further exacerbated if inadequate dietary intake occurs. Further, the protracted effects of these potential perturbations on iron and bone metabolism when EA and CHO availability is restored is unknown. Indeed, previous studies looking at the effect of restoring CHO availability over 3 days, following a ~3-week ketogenic diet in elite male racewalkers, demonstrated a failure of such restoration to attenuate post-exercise IL-6 (McKay et al., 2019b) and exercise-related P1NP (Heikura et al., 2019) in response to a single exercise bout. Additionally, a 12-month trial providing ~350 $kcal.d^{-1}$ increase in energy intake (20-40% above total daily energy expenditure) to female participants with amenorrhea/oligomenorrhea resulted in no change in areal BMD or estrogen concentrations, despite resumption of or increasing frequency of menses (64% of cohort vs 19% of control) (De Souza et al., 2021; De Souza et al., 2022). Further research is required to ascertain the time period over which adaptation of bone and iron metabolism occurs to a specific diet, how this is reflected in blood markers and imaging, and whether this is modulated by sport type.

7.3 Conclusion

The research studies comprising this thesis aimed to explore the impact of certain nutrition and training practices on bone and iron status in athletes and the potential interplay between the two systems. In summary, our findings demonstrate that:

(1) Fasting CTX and P1NP concentrations decrease with age and, although limited by the lack of general population data, may be higher in athletes
(2) Resting IL-6 concentrations may contribute to fasting CTX concentrations
(3) Duration of exercise contributes significantly to post-exercise IL-6 concentrations and the magnitude of change in hepcidin concentrations from pre- to 3 h post-exercise
(4) Twice-daily training with a short recovery results in an elevated plateau in IL-6 and hepcidin concentrations, even when supported by adequate energy and CHO consumption
(5) Short-term adherence to a ketogenic diet may be more detrimental to at-rest and across-exercise bone formation than the same period of LEA, yet bone turnover is maintained with a high EA/high CHO diet
(6) Significant between and within sport BMD measurement site discrepancies exist, raising the issue of the impact of mechanical loading on detecting at-risk athletes and the potential need for additional tools (e.g., BTMs) and protocols.

Outcomes from this thesis support current recommendations that athletes should aim to achieve adequate energy and CHO availability to minimise potential detriments to bone turnover balance, at least in the short term. Furthermore, this work supports previous recommendations of morning intake of iron, or intake prior to or within 30 min following the first exercise session, in order to maximise iron absorption. Invitations to expand the findings of this work extend to establishing a robust database of athlete BTM and areal BMD ranges and employing longitudinal studies to assess the impact of BTM and iron status perturbations on BMD and bone architecture.

7.4 Reflections on the PhD experience

My goal to pursue a career around sports medicine was born in my second year of medical school. Without the sound advice of my dear friend, Richard Burman, and the accomplished Professor Ross Tucker, I would have quit medical school to pursue sports science. Finishing medical school and continuing on to work in the public healthcare system in South Africa were some of the most challenging, yet rewarding, years of my life so far. During the Performance Nutrition diploma that I undertook in my community service year, mostly out of a selfish pursuit to improve my own nutrition approach, I was made aware of the incredible expertise of Professors John Hawley and Louise Burke. In June 2019, I took a chance and emailed John to enquire about a possible PhD opportunity. To my absolute surprise, he replied, and a few days and Skype calls later, I was applying for the PhD. In October 2019, I received an offer and a

scholarship to boot! I couldn't believe my luck. Almost simultaneously, I received an offer of acceptance to MPhil Sports Medicine course run by Assoc. Prof. Jeroen Swart at UCT. After speaking to Jeroen, I was encouraged to pursue the PhD – although there was certainly apprehension and doubt about leaving clinical medicine and taking a "non-traditional" path in my career. On the 3rd of January 2020, I arrived in Australia. Severe bushfires in Canberra saw us hastily leaving to Melbourne for the relocated Supernova study. January was a whirlwind month – new country, new people, research camp, finding a place to live. February saw the end of the research camp and the start of a new journey in my rental in Port Melbourne. But in quick succession, my Dad landed up in ICU with a pulmonary embolism and the COVID pandemic locked us inside. I'd be lying if I said that 2020 was a happy year. I enveloped myself in reading the literature and writing an (overly) comprehensive literature review. Confined to my 5 km radius, knowing nobody, my daily outing was a takeaway coffee.

In 2021, life improved dramatically. Relocating to the Gold Coast and then Sydney, where I had better support and found a new perspective was a game-changer. I grew as a person (physically as well, thank goodness) and as a neophyte researcher. Sitting here writing this at the end, with a complete thesis, I am emotional reflecting on what I have overcome and achieved over the past three and a half years. I was not prepared for the mountain that I would need to climb, in a new country, doing something completely outside of my clinical comfort zone, alone – but I did it. A lot had to change with the challenges presented by COVID regulations and, notoriously, I judge myself more harshly than others, disappointed that I didn't achieve more during my PhD time. But the skills I have learnt, the people I have met, the places I have seen, and the introspection I have done, have framed the aspirations I have for the future. I am immensely grateful for this journey, and I hope that my future career as a clinician-researcher, and the passion I have for it, benefits others in ways that I can't yet imagine.

8 References

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9 Chapter 9: Research Portfolio Appendix

9.1 Statements of Contribution

Fensham N.C., McKay A.K.A, Burke L.M. (2023). Bone turnover markers and bone mineral density values in an elite athlete cohort: what is 'normal'? In preparation for *Bone*.

Contribution statement: NF contributed to the conception and design, assembly of data, data analysis and interpretation, drafting, revising, and approval of the final manuscript. AM contributed to the conception and design, assembly of data, data analysis and interpretation, revising, and approval of the final manuscript. LB contributed to the conception and design, data analysis and interpretation, revising, and approval of the final manuscript.

Approximate percentage contributions: N.C. Fensham 70%; A.K.A. McKay 15%; L.M. Burke 15%

I acknowledge that my contribution to the above paper is above 50%

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As principal supervisor of this project, I certify that the above contributions are true and correct:

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Fensham N.C., Heikura I.A., McKay A.K.A, Tee N., Ackerman K.E., Burke L.M. (2022). Short-term carbohydrate restriction impairs bone formation at rest and during prolonged exercise to a greater degree than low energy availability. *J Bone Miner Res*. 37(10):1915-25.

Contribution statement: NF contributed to the conception and design, collection and assembly of data, data analysis and interpretation, drafting, revising and approval of the final manuscript. IH contributed to the conception and design, collection and assembly of data, data analysis and interpretation, drafting, revising and approval of the final manuscript. AM contributed to the conception and design, collection and assembly of data, data analysis and interpretation, and revising and approval of the final manuscript. NT contributed to the conception and design, collection of data, data analysis and interpretation, and revising and approval of the final manuscript. KA contributed to data interpretation and revising and approval of the final manuscript. LB contributed to the conception and design, collection and assembly of data, data analysis and interpretation, and revising and approval of the final manuscript.

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9.2 Appendices to Publications

The supplementary files to Chapter 5 of this thesis can be accessed at:
https://figshare.com/s/ac093950756743c13b47
This is a **private link** for use by the examiners of this thesis only.

These files are supplementary to the following publication online ahead of print in *Sports Medicine.*

Fensham, N. C., Govus, A. D., Peeling, P., Burke, L. M., & McKay, A. K. A. (2023). Factors influencing the hepcidin response to exercise: an individual participant data meta-analysis. *Sports Med*. doi: 10.1007/s40279-023-01874-5 [online ahead of print]

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