OBESITY, IRON AND YOUNG WOMEN:

STUDIES INVESTIGATING THE NUTRITIONAL IMPLICATIONS OF EXCESS ADIPOSITY ON IRON STATUS IN YOUNG WOMEN

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STUDENT DECLARATION

I, Hoi Lun Cheng, hereby declare that this thesis is my own work and does not, to the best of my knowledge, contain material from any other source unless due acknowledgement is made. This thesis was completed under the guidelines set out by The University of Sydney's Faculty of Health Sciences, and has not been submitted to any other university or institution as a part or a whole requirement for any higher degree.

For works that are either published, in press or currently under review and contain multiple authors, I declare that I was the principal researcher of all manuscripts included in this thesis.

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LIST OF ABBREVIATIONS

AGB	Adjustable gastric banding
AI	Anaemia of inflammation
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BMI	Body mass index
BMP-SMAD	Bone morphogenetic protein-sons of mothers against decapentaplegic signalling pathway
BPD	Biliopancreatic diversion
C	Cytosine allele of the *TMPRSS6* rs855791 polymorphism
CI	Confidence interval
CRF	Chronic renal failure
CRP	C-reactive protein
CSS	Cross-sectional study
DNA	Deoxyribonucleic acid
DS	Duodenal switch
DXA	Dual-energy x-ray absorptiometry
EAR	Estimated average requirement
ELISA	Enzyme-linked immunosorbent assay
FPN	Ferroportin
GBP	Gastric bypass
HAMP	Hepcidin gene
Hb	Haemoglobin
HFE	Hereditary haemochromatosis protein
HIF	Hypoxia-inducible factor
HJV	Hemojuvelin gene
HPHI	Higher-protein, higher-haem iron diet

IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IQR	Interquartile range
JAK/STAT-3	Janus kinase/signal transducer and activator of transcription-3
LPLI	Lower-protein, lower-haem iron diet
MANOVA	Multivariate analysis of variance
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MetS	Metabolic syndrome
mRNA	Messenger ribonucleic acid
NASH	Non-alcoholic steatohepatitis
NHANES	National Health and Nutrition Examination Survey
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
RDI	Recommended dietary intake
RYGBP	Roux-en-Y gastric bypass
SD	Standard deviation
SE	Standard error
SG	Sleeve gastrectomy
SLC40A1	Ferroportin gene
SNP	Single nucleotide polymorphism
sTfR	Soluble transferrin receptor
sTfR-F	Soluble transferrin receptor-ferritin index
T	Thymine allele of the *TMPRSS6* rs855791 polymorphism
TfR	Transferrin receptor
TIBC	Total iron binding capacity
TMPRSS6	Transmembrane protease, serine 6 (matriptase-2 gene)
TNF-α	Tumour necrosis factor-alpha

Tsat	Transferrin saturation
UCR	Urea/creatinine ratio
VLED	Very low energy diets
WHO	World Health Organisation

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ABSTRACT

Young women (18-30 years) are at high risk of weight gain and obesity. In Australia, young women are gaining weight more rapidly than other age and gender groups, including young men of similar age. Lifestyle changes such as moving away from the family home, cohabitation with friends or a partner, and for some young women, marriage and pregnancy increase the risk of weight gain.

Recent evidence supports a link between obesity and disrupted iron metabolism, with the iron regulatory hormone hepcidin proposed as a key mediator. Chronic inflammation associated with obesity is believed to elevate circulating hepcidin concentration, which in turn, leads to reduced functional iron status. With iron deficiency already common in women of reproductive age, obesity-related iron disturbances may further contribute to iron inadequacy. Apart from fatigue, suboptimal iron status may lead to other reproductive, cognitive and mental health problems, some of which may also influence the capacity for young women to engage in and be successful with weight management. Energy-restricted diets used for weight management may further exacerbate this issue by providing insufficient dietary iron for women during this life stage.

Despite growing literature on obesity, inflammation and iron metabolism, few dedicated studies have examined the influence of excess adiposity, weight loss, dietary iron intake and genetics on iron status in young overweight and obese women. Moreover, research utilising biochemical markers such as soluble transferrin receptor (sTfR), sTfR-ferritin index, and hepcidin that are appropriate for individuals with obesity and underlying inflammation is scarce. Hence, this thesis aimed to add to the current body of topic-related research by: (1) systematically reviewing the evidence on the relationship between obesity, inflammation and iron status in adults; (2) examining the prevalence of iron deficiency, severity of obesity-related iron disturbances and hepcidin concentration in young overweight and obese women; (3) assessing the impact of weight loss and manipulating protein and haem iron intake on iron status during a weight management intervention;

and (4) investigating the potential influence of the common transmembrane protease, serine 6 (*TMPRSS6*) rs855791 polymorphism on hepcidin and body iron responses to altered protein and haem iron intake during energy restriction.

A systematic search of the literature found 31 studies that measured iron status in obese individuals, 10 of which reported data from non-obese comparison groups. Findings from the systematic review indicated that obese individuals, particularly those with a body mass index (BMI) >35 kg/m^2, exhibit biochemical alterations that are consistent with an elevated level of inflammation. These alterations include higher haemoglobin and serum ferritin concentration, and lower transferrin saturation. A small number of studies showed a tendency for greater sTfR and hepcidin levels in the obese although research is required to substantiate this data which has only been reported in a limited number of studies.

A cross-sectional study was conducted as a part of this thesis to examine links between excess adiposity, inflammation and iron status in a sample of young healthy overweight and obese women. In the 114 participants recruited to this study, mean age and BMI were 22.3 years and 33.7 kg/m^2 respectively. Mean sTfR was within the normal range [1.61 (SD: 0.44) mg/l]. Median hepcidin concentration was low [6.40 (IQR: 7.85) ng/ml], and median C-reactive protein (CRP) indicated mildly elevated levels of inflammation [3.58 (IQR: 5.81) mg/l]. BMI was negatively associated with serum iron (coefficient=-0.379; p=0.008) and transferrin saturation (coefficient=-0.588; p=0.009), but positively associated with CRP (coefficient=0.127; p<0.001) in multivariate modelling. Hypoferritinaemia (serum ferritin <15.0 µg/l) was found in 17% of participants, most of whom also had hepcidin concentrations <5 ng/ml suggesting the presence of simple iron deficiency rather than inflammatory-related disturbances.

A subset (n=36) of these participants later completed a 12-month randomised controlled trial comparing two iso-energetically restricted (5600 kJ/day) diets with contrasting macronutrient and haem iron content on iron status. The higher-protein, higher-haem iron diet (HPHI) provided 32%

protein and 12.2 mg iron daily, while the lower-protein, lower-haem iron diet (LPLI) provided 20% and 9.9 mg respectively. Following 12 months of intervention, participants on the HPHI diet exhibited higher ferritin (HPHI: 52.0; LPLI: 39.0 µg/l; p=0.021) and lower sTfR-ferritin index (HPHI: 0.89; LPLI: 1.05; p=0.024) although median concentrations remained within normal range for both diet groups. Improvements in iron status accompanied successful losses of ≥10% initial weight, which was more apparent in LPLI participants who showed greater serum iron (HPHI: 13.5; LPLI: 20.0 µmol/l; p=0.002), transferrin saturation (HPHI: 19.4%; LPLI: 29.8%; p=0.001) and lower sTfR (HPHI: 1.92; LPLI: 1.24 mg/l; p=0.034) at 12 months. This was likely to be a reflection of either stronger compliance to the dietary prescription or the potential influence of iron regulatory changes unrelated to significant hepcidin reduction.

A final exploratory study was undertaken to examine the potential influence of the common *TMPRSS6* rs855791 genetic polymorphism on iron status at baseline and following 12 months of energy restriction using the HPHI and LPLI diets. A total of 76 and 27 participants from the cross-sectional and longitudinal weight loss studies were included in this analysis respectively. In the cross-sectional cohort, the iron-lowering thymine (T) allele was significantly associated with lower serum iron (coefficient=-3.116; p=0.047) and higher hepcidin (coefficient=0.607; p=0.023) at baseline. After completing 12 months of intervention using the HPHI diet, T hetero- and homozygotes had lower serum iron (p=0.028) and transferrin saturation (p=0.043) than individuals who were homozygous for the cytosine (C) allele. Intriguingly, an equivalent trend was not observed in those following the LPLI diet, suggesting that rs855791-associated iron variability may be dependent on dietary haem iron and/or protein intake. Furthermore, rs855791 was not associated with serum ferritin (HPHI: p=0.89; LPLI: p=0.91), although a significant diet × time effect (p=0.026) was observed.

Outcomes from this series of studies highlight simple iron deficiency as a major nutritional issue in healthy overweight and obese young women. Suboptimal iron status is particularly problematic in this population as young women frequently engage in unhealthy and restrictive dietary practices

that limit iron intake, and menstrual iron losses accompanying polycystic ovary syndrome can be higher in overweight and obesity. Although nutrient-rich energy-restricted diets used in the 12-month longitudinal study allowed reasonable maintenance of iron homeostasis in most young women, greater protein and haem iron intake may be useful in those who have higher physiological requirements for iron. Weight loss *per se* may also be beneficial towards normalising the mild obesity-related iron disturbances observed although the mechanism for this is currently unclear. Evaluation of potential genetic influences revealed that the *TMPRSS6* rs855791 polymorphism can significantly influence individual iron variability. However, this significant genetic impact appears to diminish with limited protein and haem iron intake, with dietary factors playing a more important role in determining storage and functional iron status.

This thesis supports the use of serum ferritin as an indicator of iron deficiency in young overweight and obese women not burdened with obesity-related comorbidities. However, measurement of additional markers such as sTfR, sTfR-ferritin index and CRP should be considered in those with class II obesity or above (BMI >35 kg/m^2) as inflammatory-related iron disruptions may be more pronounced. As simple iron deficiency is clearly a problem in this population with dieting potentially further reducing nutrient intake, performance of initial iron deficiency screening in young women who seek weight management advice is justified. For those identified to be at greatest risk of suboptimal iron status during weight management, higher-protein and haem iron diets can be recommended for superior maintenance of iron homeostasis. Contrary to expectations, the levels of inflammation and hepcidin were lower in this cohort than in previous studies of the obese. This was likely a result of the absence of obesity-related comorbidities. Hence, the impact of inflammatory-related iron disturbances was not able to be thoroughly examined in this thesis. Future research in larger, more severely obese cohorts evaluating the influence of comorbid conditions such as cardiovascular disease, metabolic syndrome and type 2 diabetes is needed to clarify the relative contribution of excess adiposity to inflammatory and iron disturbances.

PUBLICATIONS ARISING FROM THIS THESIS

Published manuscripts:

1. Cheng HL, Bryant, C, Cook R, O'Connor H, Rooney K, Steinbeck K. The relationship between obesity and hypoferraemia in adults: a systematic review. *Obes Rev* 2012; 13: 150-61
2. Cheng HL, Bryant C, Rooney K, Steinbeck K, Griffin H, Petocz P, O'Connor H. Iron, hepcidin and inflammatory status of young healthy overweight and obese women in Australia. *PLoS ONE* 2013; 8: e68675. doi:10.1371/journal.pone
3. Cheng HL, Griffin H, Bryant C, O'Connor H, Rooney K, Steinbeck K. Impact of diet and weight loss on iron and zinc status in overweight and obese young women. *Asia Pac J Clin Nutr* 2013; 22: 574-82

Conference presentations – oral:

4. Cheng HL, Bryant C, O'Connor H, Rooney K, Steinbeck K. Iron status of overweight and obese young women presenting for weight management. *Obes Res Clin Pract* 2011; 5: S17

Conference presentations – poster:

5. Cheng HL, O'Connor H, Rooney K, Steinbeck K. Hormonal contraceptives use was a stronger predictor of iron status than weight, inflammation or the *TMPRSS6* rs855791 genotype in overweight and obese young women. *Obes Facts* 2013; 6: S134
6. Cheng HL, Bryant C, O'Connor H, Rooney K, Steinbeck K. Iron status, hypoferraemia and inflammation in overweight and obese young women. *Obes Facts* 2012; 5: S102
7. Cheng HL, Bryant C, O'Connor H, Rooney K, Steinbeck K. Impact of diet and weight loss on iron status in overweight and obese young women. *Obes Facts* 2012; 5: S200

OTHER PUBLICATIONS DURING CANDIDATURE

Published manuscripts:

1. O'Connor H, Munas Z, Griffin H, Rooney K, Cheng HL, Steinbeck K. Nutritional adequacy of energy restricted diets for young obese women. *Asia Pac J Clin Nutr* 2011; 20: 206-11
2. Griffin H, Cheng HL, O'Connor H, Rooney K, Petocz P, Steinbeck K. Higher protein diet for weight management in young overweight women: a 12-month randomized controlled trial. *Diabetes Obes Metab* 2013; 15: 572-5

Manuscripts in press:

3. Cheng HL, Kay M, Parker H, Cook R, Orr R, O'Connor H. Anthropometric characteristics of junior representative rugby league players in Australia. *J Sci Med Sport* doi: http://dx.doi.org/doi:10.1016/j.jsams.2013.07.020 (in press)

Conference presentations – oral:

4. Cheng HL, Orr R, Parker P, Kay M, Cook R, Peng Cox E, O'Connor H. Anthropometric characteristics of junior representative rugby league players in Australia. *J Sci Med Sport* 2012; 15: S16

Conference presentations – poster:

5. Griffin HJ, O'Connor HT, Rooney KB, Steinbeck KS, Cheng HL, Petocz P. Comparison of higher-protein and higher-carbohydrate diets in overweight and obese young women. *Obes Rev* 2010; 11: 823

CHAPTER 1.

Introduction

INTRODUCTION

Obesity and iron deficiency are both individually major disease burdens around the world.[1, 2] Young women are particularly vulnerable to weight gain and iron deficiency.[3-6] Factors associated with weight gain in young women include moving away from the family home, reduced physical activity and high consumption of takeaway foods.[3, 4] Regular menstrual iron loss increases the risk of iron deficiency in this age and gender group.[7] Obesity and iron deficiency have generally been regarded as independent disease burdens, although an increasing amount of research over the past decade indicates that these conditions can interact, resulting in a greater iron deficiency risk in those who are overweight and obese.[8-10]

The connection between obesity and reduced functional iron status was first documented in a cohort of adolescents by Wenzel *et al* in 1962.[11] A year later, Seltzer & Mayer reported similar outcomes from a study comparing iron status between obese and non-obese adolescents.[12] Both studies found an association between obesity and hypoferraemia (reduced serum iron concentration). In comparison to normal weight controls, obese adolescents were found to have significantly lower percent saturation of iron and higher unsaturated iron binding capacity as indicators of iron deficiency.[12] Low physical activity levels and decreased muscle myoglobin concentration were proposed as potential causes, and were the first of various hypotheses that attempted to explain the iron disruptions observed in obese populations.[8, 13, 14]

Micronutrient deficiencies often exist side-by-side with excessive energy intake in overweight and obese individuals.[15] This has typically been attributed to poor diet quality secondary to frequent consumption of energy dense nutrient-poor foods.[15] In the case of iron deficiency, other factors such as increased body surface area, expanded plasma volume, and menorrhagia accompanying polycystic ovary syndrome (PCOS) have been proposed as potential contributors to obesity-related hypoferraemia.[8, 9, 14] The unremarkable nature of these factors meant that the connection between obesity and disturbed iron metabolism was probably overlooked for many years.

Since the discovery of the key iron regulatory hormone hepcidin in 2000,[16] a new wave of interest has illuminated on another potential mechanism underpinning obesity-related iron disruption. Notably, a study in 2007 by Yanoff *et al* demonstrated significant associations between obesity, inflammation, hypoferraemia and elevated serum ferritin.[8] Hepcidin, which inhibits gut iron absorption and induces sequestration of body iron stores, was proposed as a key mediator of the iron disturbances observed in this study.[8] The clinical implications of these findings were important, as it demonstrated that using routine biochemical indices to assess iron status (such as serum iron and ferritin) was unsuitable for obese individuals with underlying inflammation. It also emphasised the relevance of utilising alternative non-acute phase reactant iron markers, such as soluble transferrin receptor (sTfR), in this population. Subsequent studies dedicated to the topic have found similar disruptions to iron metabolism,[9, 10, 17, 18] with more recent research also reporting higher serum hepcidin in the obese.[9, 13, 19]

In addition to obesity-related iron investigations, another area of research that has undergone rapid expansion since 2000 is the genetics of hepcidin and iron status. A growing number of single nucleotide polymorphisms (SNP) are being identified as moderators of hepcidin and iron marker concentration,[20-23] with some resulting in extreme iron-related pathologies.[24, 25] The rs855791 polymorphism in the transmembrane protease, serine 6 (*TMPRSS6*) gene is one of the more common SNPs that have been reported to be significantly associated with iron status in the general population.[20, 26-28] Individuals with the iron-lowering allele of this SNP exhibit lower concentrations of some markers such as haemoglobin and serum iron,[21, 26] and a higher concentration of serum hepcidin.[29] Hence, the risk of iron deficiency may potentially be greater in individuals with this genetic predisposition for lower iron status.[20]

Despite young women being especially vulnerable to developing obesity and iron deficiency,[3, 6] few studies have utilised appropriate biomarkers, including sTfR and hepcidin, to examine the impact of excess weight or weight loss on iron status in this population. In the handful of dedicated studies that have done so, issues related to study design have limited the generalisability of

outcomes to the wider population of young, healthy overweight and obese women.[9, 30, 31] Some of these issues include the selection of a non-representative sample such as mildly overweight military recruits,[30] significant confounding from obesity-related comorbidities, and the use of bariatric surgery as the primary method of weight loss.[9, 31] As food and energy restriction, which is a more common strategy for weight loss in young women, can compromise nutrient intake particularly for iron,[32] the importance of appropriately assessing iron alterations during energy restriction and weight loss is particularly highlighted. Furthermore, the potential impact of enhancing protein and haem iron intake, as well as the influence of SNP rs855791 on iron status of young women during energy restriction are currently unexplored areas of research which warrant clinical investigation.

THESIS AIMS

The primary objective of this thesis was to address the paucity of research assessing relationships between obesity, inflammation and iron status in young healthy women. This thesis contains a series of studies in this target population that examine the associations between excess weight, weight loss, dietary protein and haem iron intake, as well as genetics on iron and hepcidin status. The specific aims of each study are to:

1. systematically review the evidence on the relationship between obesity, inflammation and iron status in adults;
2. examine the prevalence of iron deficiency, severity of obesity-related iron disturbances, and hepcidin concentration in a community sample of young, healthy overweight and obese Australian women;
3. assess the impact of two energy-restricted diets with contrasting protein and haem iron content, and weight loss on iron status during a clinical weight management trial; and
4. investigate the influence of the common *TMPRSS6* rs855791 polymorphism on iron and hepcidin status, as well as physiological iron responses to altered protein and haem iron intake during energy restriction.

SPECIFIC HYPOTHESES

1. Obese populations would present with higher levels of serum ferritin, sTfR and hepcidin, reflecting poorer functional iron status and greater sequestration of body iron stores when compared to non-obese control groups.
2. Young, healthy overweight and obese Australian women would have significant inflammatory-mediated iron disturbances such as increased serum ferritin, sTfR and hepcidin. These disturbances would contribute to a higher iron deficiency prevalence than when the routine definition of hypoferritinaemia is used alone. Furthermore, increasing severity of obesity would also be significantly associated with a greater level of inflammation and a poorer iron profile.
3. Higher intake of protein and haem iron during energy restriction would facilitate better maintenance of iron status than conventional diets containing less of these nutrients. Additionally, weight loss induced by these diets would lead to biochemical alterations that reflect lower inflammation and inflammatory-mediated iron disruptions.
4. The iron-lowering allele of the *TMPRSS6* SNP rs855791 would be associated with both a poorer baseline iron profile and a greater decrement in iron status during dietary energy restriction. Individuals carrying the iron-lowering allele of rs855791 and who are prescribed a lower-protein and haem iron diet would exhibit the least favourable iron profile.

SIGNIFICANCE OF THIS RESEARCH

In young women, excess weight can potentially exacerbate the risk of iron deficiency, a condition which is already prevalent in this population. Outcomes from this series of studies will provide a greater insight into the biochemical alterations that mediate this risk in overweight and obese young women. This project will extend the current body of topic-related literature into (1) evaluating the potential reversibility of obesity-mediated iron disruptions with weight loss; (2) assessing the benefit of enhanced protein and haem iron intake during dietary energy restriction on maintaining iron status and (3) exploring the nutrigenetic impact of SNP rs855791 on iron alterations during planned weight reduction. These outcomes will potentially guide the design of optimal patient care plans in clinical practice for maintaining iron homeostasis in healthy overweight and obese women, particularly while undergoing supervised weight management to treat existing and/or prevent obesity-related comorbidities later in life.

THESIS OUTLINE

Chapter 2 reviews the background literature on obesity, inflammation, hepcidin and iron status. It discusses the clinical implications of obesity-related iron disruptions on assessing iron status, and the health of young overweight and obese women. Furthermore, this chapter explores the current evidence on how weight loss, dietary energy restriction and genetics interact with iron and inflammatory status in this population.

Chapter 3 reports a systematic review of current literature related to obesity-related iron disruption. It compares iron and inflammatory markers between obese and non-obese control groups across different studies in order to clarify the association between obesity, iron and inflammatory status in adult populations.

Chapter 4 reports findings from a cross-sectional study conducted in young, healthy overweight and obese women. This study examines the prevalence of iron deficiency and discusses the contribution of inflammation and hepcidin to iron disturbances and deficiency observed in this population.

Chapter 5 presents the iron-related outcomes of a 12-month randomised controlled trial conducted in overweight and obese young women. This trial compares the efficacy of two energy-restricted diets contrasting in macronutrient and haem iron content on weight loss and iron status. It also investigates whether a higher magnitude of weight loss induces greater improvements in iron status by comparing iron alterations between participants who successfully lost <5% and ≥10% of initial weight.

Chapter 6 observes baseline iron status and iron alterations in a subset of participants from the cross-sectional study and clinical trial who were genotyped for the *TMPRSS6* SNP rs855791. This study examines the association between rs855791 genotype and various iron traits, and explores the potential influence of this SNP on iron response to the two diets reported in Chapter 5.

Chapter 7 summarises the key findings from the systematic review, as well as the cross-sectional, longitudinal intervention and genetic studies. This chapter also provides the nutritional implications and limitations of this thesis, as well as future directions for research.

CHAPTER 2.

Background literature

LITERATURE REVIEW

Overweight and obesity as an independent health issue in young women

Over the past few decades, obesity has rapidly become an epidemic affecting a large proportion of the global population. In Australia, findings from the 2007-08 National Health Survey estimated that almost two-thirds (61%) of adults are classified as either overweight or obese.[33] This issue is particularly problematic in young women as rates of overweight and obesity in those aged between 18-24 years have increased rapidly from 26% to 35% within the past 20 years.[5] Research of global obesity trends also indicates an increased risk for further weight gain in those who are already classified as overweight. Between 1980 to 2000 there has been a 5.4 point shift in body mass index (BMI) from 29.7 to 37.7 kg/m^2 at the 95th percentile for Australian women between the ages of 18-30 years.[3] The speed and magnitude of this shift was by far the greatest in Australia when compared to age-matched women in the United States and the United Kingdom.[3] Furthermore, the absence of an equivalent trend in young Australian men highlights the vulnerability of young women to weight gain.[3]

The greater risk of weight gain in this age and gender group has been attributed to factors related to major lifestyle change such as moving away from the family home, cohabitation and pregnancy.[34-37] These changes in addition to behavioural factors such as frequent consumption of takeaway foods,[4] reduced inclination to be physically active,[34] and binge eating and drinking tendencies have all been linked with weight gain in young women.[38] With the rising incidence of obesity, young overweight and obese women are exposed to the risks of developing obesity-related comorbidities and lifestyle diseases, both immediately and later in life.

Health effects of overweight and obesity

Obesity is well established as a risk factor for many common lifestyle diseases such as cardiovascular disease, type 2 diabetes, osteoarthritis and various types of cancer.[39] In 2003, high body mass was responsible for 8% of the total burden of disease and injury in Australia,[40] with the overall cost to the Australian society and government estimated at $58.2 billion in 2008.[33]

In young women, overweight and obesity is associated with other health risks specific to this demographic. Body image is an important part of self-esteem in this population,[41] and young overweight and obese women have been reported to be at increased risk of mental health problems. The odds ratios of developing depressive symptoms in overweight (odds ratio: 1.2) and obese (odds ratio: 1.3) women aged 18-27 years when compared to those in the healthy weight range have been shown to be higher in The Australian Longitudinal Study on Women's Health.[42] Furthermore, overweight and obesity in this population may adversely affect reproductive health. The link between obesity and polycystic ovary syndrome (PCOS) is well known,[43] and there is increasing evidence to suggest that excess weight can impact deleteriously on other fertility and obstetric outcomes.[44-46] The risk of obesity and metabolic disease in the offspring of obese mothers is also known to be elevated.[44-48] Hence, the early treatment and prevention of excess weight gain are particularly relevant for this population.

Obesity and inflammation

Obesity-related chronic disease often develops later in life. However, prior to manifestation of overt disease outcomes, excess adiposity is known to induce chronic systemic inflammation,[49, 50] a condition which is believed to directly influence progression of disease precursors such as insulin resistance, impaired glucose tolerance and atherosclerosis.[51, 52] Circulating levels of proinflammatory cytokines and acute phase proteins such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and C-reactive protein (CRP) have all been reported to be higher in the obese when compared to non-obese comparison groups,[53-55] with BMI also known to be positively correlated with inflammatory status.[49, 56, 57] The location of body fat deposits play an important role in influencing level of inflammation.[49] IL-6 production has been shown to be three-fold higher in abdominal compared to subcutaneous adipocytes *in vitro*,[58] whereas *in vivo* measurement of inflammation in bariatric surgery patients indicated 50% greater IL-6 production in visceral versus subcutaneous fat deposits.[59] Portal vein IL-6 (which is primarily derived from visceral fat) was also directly correlated to CRP, demonstrating the significant impact of abdominal fat on whole body inflammation.[59]

In human adults, the majority of excess fat is stored in the body as white adipose tissue.[49] Previously, white adipose tissue was believed to be a metabolically inert fat depot, whereas it is now recognised as a highly active endocrine organ.[49] Adipose tissue is known to secrete a repertoire of bioactive proteins including adipokines and proinflammatory cytokines which contribute significantly to obesity-related inflammation.[60] Some of these bioactive proteins include but are not limited to: leptin, resistin, monocyte chemoattractant protein-1 (MCP-1), and the abovementioned cytokines TNF-α and IL-6.[59] Despite extensive research over the past 60 years, the question of why excess adiposity triggers an inflammatory response remains poorly understood.[61] Current opinion proposes several mutually inclusive explanations including macrophage infiltration, adipose tissue hypoxia and endoplasmic reticulum stress.[61] Macrophage infiltration may arise as a result of local cellular hypoxia to facilitate remodelling and angiogenesis of adipose tissue.[62, 63] In murine *ex vivo* studies, infiltrated macrophages were shown to account for approximately 50% and the majority of all adipose-derived IL-6 and TNF-α respectively.[64, 65] The high demand for structural and functional proteins for maintaining massively hypertrophied adipocytes is also thought to be a considerable burden on the endoplasmic reticulum (a major site of protein synthesis) and thus, contributes to the greater level of inflammation in obesity.[66]

Obesity, inflammation and micronutrients

In addition to the increase in chronic disease risk, obesity is also associated with the disruption of micronutrient metabolism.[67] Abnormal levels of certain vitamins and minerals (e.g. vitamins A, D, folic acid, calcium, iron and zinc) in obesity have been extensively reviewed,[68, 69] with some nutrients reported to be directly linked with inflammation.[68] The transient drop in micronutrient concentration with inflammation can be explained partly as an evolutionary adaptation in host defence. In acute infection, a normal immune response induces nutrient depletion due to the activation of antioxidative processes and immune cell proliferation.[70] Micronutrient storage pools are also redistributed away from the circulatory system to limit their bioavailability to invading pathogens.[71-73] Specifically, the sequestration of iron in reticuloendothelial macrophages occurs with acute infection (see later: *Regulation of hepcidin*).[74] While obesity does not constitute an

infectious state, obesity-related inflammation appears to cause a similar functional iron deficiency,[75] although the question of why this occurs remains to be elucidated.

Iron deficiency as an independent health issue in young women

Iron deficiency with or without anaemia is a major global health issue.[76] Of all the nutrient deficiency syndromes, iron deficiency is the most common, affecting a large number of individuals across both industrialised and non-industrialised nations.[76] Although no accurate figures exist for its global prevalence, the World Health Organisation (WHO) estimates that most preschool children and pregnant women in non-industrialised countries and at least 30-40% of the population in industrialised countries are iron deficient.[76] Of those affected by this nutrient deficiency, a significant proportion include women of reproductive age due to regular menstrual iron loss.[7] In 2000, an Australian population-based study estimated that over 20% of women between 25-50 years were either iron deficient or had marginally low iron status.[77] Furthermore, the WHO also estimates an anaemia prevalence of 12.4% and 15% in pregnant and non-pregnant Australian women respectively.[78] As women of reproductive age have such a high physiological demand for iron, it is not surprising that the Australian recommended dietary intakes (RDI) for iron are the greatest in this age and gender group (Table 2.1).[79]

Table 2.1. Comparison of Australian nutrient reference values for pregnant and non-pregnant women aged 19-30 years, postmenopausal women aged >50 years and men aged >18 years

	Nutrient reference values	
Population	**Estimated average requirement (EAR)**	**Recommended dietary intake (RDI)**
Women		
Premenopausal (19-30 years)	8 mg/day	18 mg/day
Pregnant (19-30 years)	22 mg/day	27 mg/day
Postmenopausal (>50 years)	5 mg/day	8 mg/day
Men (>18 years)	6 mg/day	8 mg/day

Health effects of iron deficiency

Iron deficiency impacts adversely on health across all age groups. It can influence growth, cognitive development and behaviour in children, and reduced physical performance, work productivity and immune function in both children and adults.[76, 78] Recent literature also indicates a possible effect of iron deficiency on impaired cognitive performance and increased depressive symptoms in adults.[80-82]

Similar to overweight and obesity, iron deficiency is an important issue in women of reproductive age as there are additional health implications specific to this population. Maternal iron deficiency (especially iron deficiency anaemia) during pregnancy can adversely affect the health of both the mother and the child. Negative health outcomes to the mother include increased risk of pre-term delivery,[83, 84] post-partum depression and poorer mother-to-infant interaction,[85, 86] whereas greater susceptibility to fat accumulation, metabolic syndrome and cardiovascular dysregulation and compromised cognitive development have been reported in offspring.[87-89] These adverse health effects emphasise the importance of regular monitoring and maintenance of iron homeostasis in women of reproductive age.

Clinical assessment of iron status

Biochemical markers such as haemoglobin, serum iron, transferrin saturation, total iron binding capacity (TIBC) and serum ferritin are routinely used to assess the iron status of individuals in clinical settings.[90, 91] Deviation of these biomarkers from their reference range provides clinicians with an indication of iron abnormalities (Table 2.2). In the case of simple iron deficiency, onset is characterised by depletion of storage iron resulting in reduced serum ferritin (stage I iron deficiency). As the condition progresses to complete exhaustion of iron stores, iron deficient erythropoiesis occurs as iron supply to erythropoietic cells decrease which is reflected by a reduction in serum iron and transferrin saturation, and a rise in TIBC (stage II iron deficiency). With continued negative iron balance, the condition eventually progresses into iron deficiency

anaemia as senescent erythrocytes are no longer adequately replaced owing to low iron supply (stage III iron deficiency).[92-96]

Table 2.2. Typical reference ranges for routine biochemical markers of iron status

Biochemical marker	Reference range[a]
Haemoglobin (g/l)	120- 150
Serum iron (µmol/l)	10 - 30
Transferrin saturation (%)	15 - 50
Total iron binding capacity (µmol/l)	42 - 80
Serum ferritin (µg/l)	15 - 150

[a]Reference ranges based on premenopausal women over 18 years of age (South Western Sydney Local Health District Pathology Service, Sydney, Australia)

Routine iron indices are useful in most cases although external factors such as diurnal variation (serum iron, transferrin saturation and TIBC can exhibit changes throughout the day) limit accuracy and validity of iron status assessment.[76] Similarly, studies have observed that inflammation can decrease serum iron and transferrin saturation and elevate serum ferritin.[76] The anaemia of inflammation (AI) or anaemia of chronic disease is an example of where inflammatory status can significantly affect the concentration of routine iron biomarkers. Anaemia of inflammation is a common condition in individuals with chronic disease, characterised by low haemoglobin and serum iron concentration despite the presence of sufficient body iron stores.[97, 98] In clinical practice, AI can easily be misinterpreted as iron deficiency anaemia, especially if inflammatory status is not assessed, due to the similar biochemical presentation of the two conditions (Table 2.3).[90] Obesity itself has recently been suggested to affect iron metabolism via inflammation.[8, 18] Hence, the use of multiple indices that include alternative markers such as soluble transferrin receptor (sTfR), sTfR-ferritin index (sTfR-F) and hepcidin are required to more accurately assess iron status of individuals with underlying inflammation (see later: *Clinical implications of obesity and iron deficiency*).[76]

Table 2.3. Comparison of biochemical alterations with iron deficiency and anaemia of inflammation

Biochemical marker	Iron deficiency	Anaemia of inflammation
Haemoglobin	normal to ↓	↓
Serum iron	↓	↓
Transferrin saturation	↓	normal to ↓
Serum ferritin	↓	normal to ↑
sTfR	↑	normal
sTfR-ferritin index	↑	↓
CRP	normal to ↓	↑

CRP, C-reactive protein; sTfR, soluble transferrin receptor

Adapted from Wians et al. *Am J Clin Pathol* 2001; 115: 112-8 and Beguin. *Clin Chim Acta* 2003; 329: 9-22

Hepcidin: a link between obesity and iron deficiency

Role of hepcidin in iron metabolism

Hepcidin is a 25-amino acid peptide hormone first isolated from plasma and urine in 2000 and 2001 respectively.[16, 99] Since its discovery, extensive research has been undertaken to elucidate its function and mechanism of action in the human body.[98]

Hepcidin is synthesised primarily by hepatocytes and is a key regulator of iron status. It induces degradation of the iron exporter ferroportin from the cell membrane in three primary locations: duodenal enterocytes, hepatocytes and reticuloendothelial macrophages (Figure 2.1).[97] The presence of ferroportin at these physiological sites is crucial as: (1) duodenal enterocytes are responsible for delivering iron from the gut; (2) the liver is a major iron storage depot; and (3) the reticuloendothelial system is responsible for recycling iron from senescent erythrocytes.[97, 100-103] Consequently, a reduction in the membrane density of ferroportin results in diminished dietary iron absorption and sequestration of hepatic and reticuloendothelial iron stores from the systemic circulation.[97] This subsequently leads to hypoferraemia as the amount of bioavailable iron becomes limited.[97, 104]

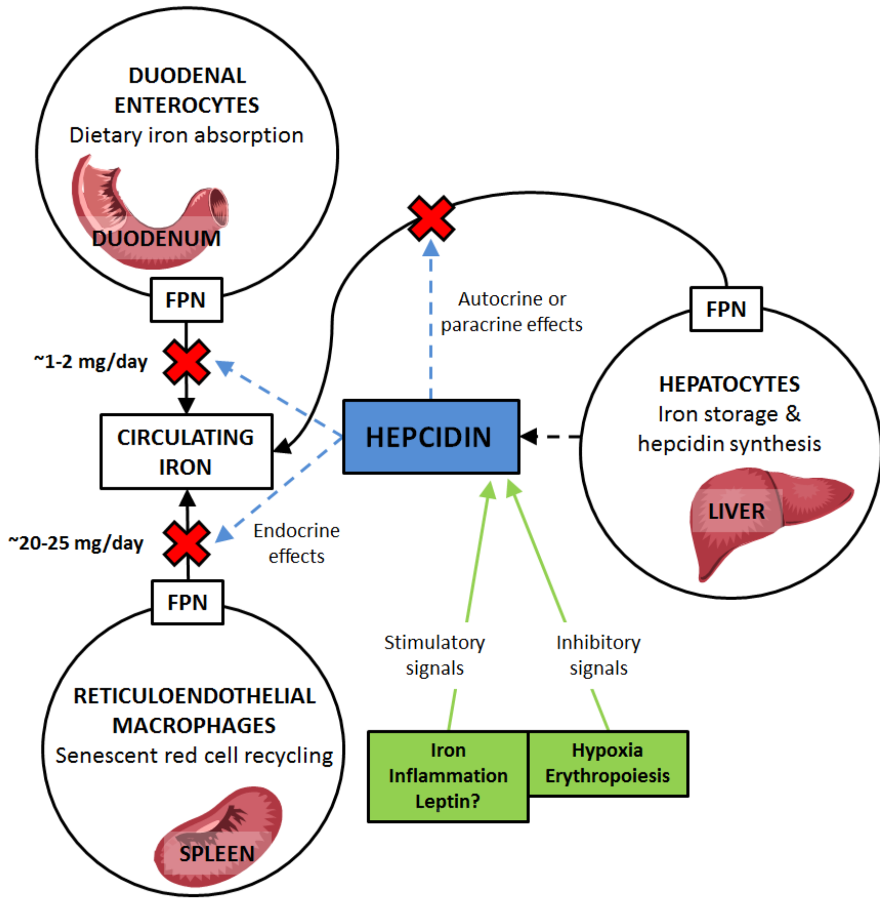

Figure 2.1. Main target sites and regulators of hepcidin [Red crosses denote hepcidin-mediated inhibition of cellular iron export via ferroportin (FPN), which consequently lead to the blocking of intracellular iron efflux from hepatocytes, duodenal enterocytes and reticuloendothelial macrophages into the hepatic portal and systemic circulation; hepcidin itself is primarily regulated by a number of signals including iron, inflammation, hypoxia, erythropoiesis and possibly leptin; figure adapted from Collins et al. *J Nutr* 2008; 138: 2284-8 and Ganz. *Best Pract Res Cl Ha* 2005; 18: 171-82]

The importance of hepcidin for iron homeostasis is particularly evident in individuals with hepcidin-related pathologies. Complete ablation of hepcidin synthesis leads to iron overload secondary to inadequate attenuation of dietary iron uptake.[100, 105] On the contrary, hepcidin overproduction results in microcytic iron-refractory iron deficiency anaemia that is unresponsive to regular oral iron therapy (see later: *Genetic factors affecting iron status*).[106, 107] Research into these

pathological disorders has paved the way for further investigations into the biological and genetic mechanisms involved in regulating hepcidin production.

Regulation of hepcidin

The current body of research has identified several biological pathways responsible for regulating hepatic hepcidin production: (1) iron feedback regulation; (2) requirement for iron based on hypoxia and erythropoietic demands; and (3) regulation by inflammation.

In iron feedback regulation, hepcidin is produced to limit unnecessary iron uptake when body iron stores are abundant.[97] *In vitro* evidence indicates that hepatocytes possess the ability to sense and respond to the presence or absence of iron.[98] The iron-sensing mechanism appears to be related to the protein complex formed by interactions between transferrin receptors 1 and 2 and the hereditary haemochromatosis (HFE) protein on the hepatocyte membrane. When transferrin-bound iron (holotransferrin) attaches to this protein complex, downstream bone morphogenetic protein-sons of mothers against decapentaplegic (BMP-SMAD) and/or mitogen-activated protein kinase (MAPK) signalling occurs to initiate hepcidin expression.[74]

In addition to iron-driven feedback regulation, hepcidin is also regulated by oxygen demand and erythropoiesis. Under conditions of low oxygen (e.g. anaemia or hypoxia), hepcidin production is suppressed to allow for increased iron absorption and mobilisation to facilitate erythrocyte production.[100, 108] Beta-thalassemia (major and intermedia) resulting in increased but ineffective erythropoiesis is a classic example of iron overload caused by inappropriate erythropoiesis-driven hepcidin suppression.[109] Based on earlier research, oxygen-sensing hypoxia-inducible factors (HIF) were believed to play a crucial role in feedback regulation,[97, 110] although more recent evidence indicates a greater involvement of erythroid precursors from the bone marrow (bone marrow being the site of erythropoiesis).[98] At present, much is still unknown about the specific iron sensing and signalling mechanisms that control hepcidin production. Proteins involved in the crosstalk between different iron processing organs also remain largely unexplored.

Aside from the abovementioned homeostatic pathways, inflammation is also known to modify hepatic hepcidin production via Janus kinase/signal transducer and activator of transcription-3 (JAK/STAT-3) signalling.[97, 103] As mentioned previously, the inflammatory-mediated increase in circulating hepcidin and subsequent iron sequestration is believed to be related to host defence (see earlier: *Obesity, inflammation and micronutrients*).[97, 102] Evidence from animal and human studies show increased susceptibility to infection in iron-overloaded individuals,[100, 111] with *in vitro* research demonstrating a direct stimulatory effect of IL-6 and other proinflammatory cytokines such as interleukin-1β (IL-1β) on hepcidin expression.[112-114] Hepcidin is also believed to be the primary mediator of reduced functional iron status under non-infectious inflammatory conditions such as obesity and anaemia of inflammation (AI) (see later: *Hepcidin in anaemia of inflammation and obesity: the similarities and differences*).[8, 115]

Although yet to be widely established as a regulator of hepcidin synthesis, a 2007 study reported a stimulatory effect of leptin on hepcidin expression. Exposure of human hepatoma cells to leptin *in vitro* was demonstrated to induce significant elevation of hepcidin expression via JAK/STAT-3 signalling.[116] This data corroborates previous research where a significant correlation between serum leptin and hepcidin in obese children was observed,[19, 117] and indicates the possibility that an alternative stimulus to inflammation for explaining hepcidin elevation in obesity.

Hepcidin in anaemia of inflammation and obesity: the similarities and differences

Inflammation, hypoferraemia and increased serum ferritin concentration are observed in both AI and obesity. Therefore, earlier research proposed that the underlying biological mechanisms behind obesity-related iron disruption may be similar to AI.[8, 19] However, with the growing number of studies reporting serum hepcidin in obese populations, considerable differences have been found to distinguish the two separate conditions.

The first of these differences relate to the magnitude of hepcidin elevation. In clinical populations with multiple myeloma and chronic renal failure, studies have reported extremely elevated levels of

hepcidin when compared to healthy controls.[118, 119] In contrast, while serum hepcidin in morbidly obese women was observed to be nine-fold higher than non-obese age and haemoglobin-matched controls,[9] this concentration remained markedly below the levels reported in individuals with chronic disease (Table 2.4). Measurement of hepcidin in overweight and obese children also found a mean concentration that was only 40-50% higher than normal weight controls.[13, 19]

Table 2.4. Comparison of adult serum hepcidin concentration between populations of healthy obese and chronically ill individuals

Population	Serum hepcidin (ng/ml)		
	Ganz *et al* (2008)[a,b]	Małyszko *et al* (2005)[c]	Tussing-Humphreys *et al* (2010a)[a]
Healthy (weight non-discriminatory)	65.0	53.3	-
Healthy (BMI <27 kg/m^2)	-	-	9.70
Obese (BMI >37 kg/m^2)	-	-	88.0
Inflammation (CRP >100 mg/l)	550.0	-	-
Multiple myeloma	300.0	-	-
CRF (not requiring dialysis)	250.0	-	-
CRF (haemodialysis)	-	205.7	-

BMI, body mass index; CRF, chronic renal failure CRP; C-reactive protein

[a]Results reported as medians

[b]Results estimated from graphical presentation using a ruler

[c]Results reported as means

As a result of this disparity in hepcidin elevation, true iron status is also different between AI and obesity. Iron sequestration in AI only causes a functional deficiency as iron stores are redistributed away from the systemic circulation into hepatic and reticuloendothelial cells.[97, 120] However, liver biopsies obtained from obese bariatric surgery patients indicate the presence of minimal hepatic iron stores reflective of a truly iron depleted state.[9, 121] As reticuloendothelial macrophages process a considerably larger amount of iron compared to duodenal enterocytes (20-25 mg vs. 1-2 mg per day respectively; Figure 2.1), Tussing-Humphreys *et al* (2010a) hypothesised that moderate hepcidin elevation with obesity may be sufficient to inhibit dietary iron absorption while allowing some mobilisation of iron from storage sites. This continual usage of body iron stores with minimal

dietary iron replenishment presumably leads to negative iron balance, which over a prolonged period of time, may result in true iron deficiency.[9]

In addition to the abovementioned dissimilarities, there is some, but inconsistent evidence to suggest that the physiological site of hepcidin production may be different between obese individuals and those with AI. Serum hepcidin is primarily secreted by the liver, but in 2006 higher hepcidin protein and mRNA were detected in adipose tissue samples obtained from obese women (compared to normal weight controls) which suggests a role for adipose tissue in hepcidin synthesis.[121] Although hepcidin expression occurred at a much smaller scale to hepatocytes, the sheer size of adipose tissue deposits was thought to contribute substantially to hepcidin concentrations in the obese.[121] However, contradicting results have recently emerged where analysis of the abdominal subcutaneous adipose tissue blood supply in lean and obese individuals showed zero net release of hepcidin.[122]

Clinical implications of obesity and iron deficiency

Problems associated with the use of routine iron studies

The interaction between obesity and iron metabolism is an important issue with significant clinical implications, one of which relates to the accuracy of routine iron biomarkers in overweight and obese individuals. Since obesity is recognised as a proinflammatory condition, the reliability of routine clinical iron indices is questionable as inflammatory-mediated disruptions to serum iron, transferrin saturation and serum ferritin can complicate detection of iron abnormalities.[8, 76, 115] In clinical settings where hypoferritinaemia (low ferritin) is often used as the primary indicator of iron deficiency, overestimation of true iron status can occur in individuals with obesity-related inflammation. While supplementary analysis of acute phase proteins and proinflammatory cytokines such as CPR, α-1-acid glycoprotein, alpha-1-antichymotrypsin, IL-6 and TNF-α can provide an indication of inflammatory status,[96] a number of alternative iron biomarkers have been shown to be important for gauging iron status in those with underlying inflammation.

In recent years, there has been increasing utilisation of an alternative iron marker soluble transferrin receptor (sTfR) in obesity research. Soluble transferrin receptor is a useful indicator of iron deficiency under inflammatory conditions as it is not influenced by infection or inflammation, and it does not vary with age, gender or pregnancy.[76] Soluble transferrin receptor concentrations however, can become elevated in haematological disorders (those involving altered erythropoietic activity) such as haemolytic anaemia, β-thalassemia and sickle cell anaemia.[76, 123] Hence, care must be taken to eliminate the presence of such conditions before sTfR can be employed as a marker of iron status.

Some studies have also explored the potential of using sTfR to ferritin ratios.[94, 124] In particular, the sTfR-ferritin index (sTfR-F) (calculated by dividing sTfR by log ferritin) has been reported to be especially valuable for assessing iron status under both inflammatory and non-inflammatory conditions.[125] The sTfR-ferritin index is useful for distinguishing true iron deficiency from AI whereby a ratio of <0.8-1.0 with concurrent anaemia is indicative of AI, and a ratio of >1.5-2.0 the presence of true iron deficiency.[125, 126] Unfortunately, neither sTfR or sTfR-F are frequently used in routine clinical practice which may be attributed to the cost associated with its measurement, as well as the lack of standardised reference ranges and cut-offs.[2]

Nutrient adequacy of energy-restricted diet plans for obese individuals

During dietary weight management, restriction of food intake is essential for achieving negative energy balance. Nutrient intake during periods of energy restriction can be compromised, especially if the dietary intervention is poorly designed.[127] Body image dissatisfaction in young women can often drive fad dieting and adoption of unhealthy weight loss strategies such as elimination of core food groups, which increase the risk of nutrient deficiency.[128-130] Of all nutrients, the dietary requirement for iron (EAR: 8 mg/day; RDI: 18 mg/day) is one of the most difficult to meet in women of reproductive age.[131] Furthermore, a 2010 study demonstrated that meeting recommended iron intakes can be even more challenging during periods of energy restriction.[32] Dietary modelling of a 6000 kJ/day higher-protein (33% daily energy) meal plan that

included red meat three times a week was only able to meet 81% of the RDI (Appendix D1).[32] In fact, manipulation of this meal plan to incorporate red meat on all days of the week still did not satisfy the recommended requirement for iron (95% of the RDI). Therefore, monitoring and addressing iron deficiency is crucial in overweight and obese young women as iron deficiency risk may be further exacerbated by suboptimal dietary iron intake.

Efficacy of weight management programmes in obese individuals

Many lifestyle and behavioural weight management interventions rely primarily on food intake restriction and an increase in physical activity, both of which require substantial levels of self-control and motivation. As iron deficiency is known to influence fatigue, exercise capacity and psychological health,[6, 76, 132-134] compliance to prescribed diet and exercise regimens may be adversely affected by iron deficiency. To date however, the psychological impact of low iron status on motivation and compliance to weight management interventions has not been explored.

Impact of weight loss on iron status

As evidence suggests a significant inverse association between obesity and iron status, it would be logical to hypothesise that weight loss can bring about beneficial changes to iron status in overweight or obese individuals. The impact of weight reduction on iron status has been examined in a number of studies, the majority of which evaluated the impact of bariatric surgery-induced weight loss on overall nutritional status. Intriguingly, iron alterations reported after bariatric surgery have been inconsistent despite massive weight and/or inflammatory reduction reported across most studies.[135-142]

Aside from bariatric surgery-based research, outcomes from earlier dietary weight loss studies have been equally conflicting. Two studies adopting rigorous energy restriction using very low energy diets (VLED) observed an acute decrease in circulating iron concentration.[143, 144] This drop was reported to occur immediately following commencement of energy restriction irrespective of iron intake and supplementation,[143, 144] although iron recovery was superior in the supplemented

groups.[144] On the other hand, no decline in serum iron and transferrin saturation was observed with VLED-induced weight loss in children (mean age was 12.6 years).[145] Most studies involving moderately restrictive diets demonstrated good maintenance of iron status following implementation of a hypocaloric diet for six to 24 weeks,[145-147] although one study did report reduced iron status following 15 weeks of 50% energy restriction.[80] Of all of the abovementioned dietary energy restriction studies, only one measured sTfR (which increased with a VLED intervention),[144] and none reported hepcidin as all except one predated the discovery of the hormone.

Since 2010, a small number of weight loss studies incorporating serum hepcidin have emerged. One study monitored sTfR and hepcidin status in a cohort of obese premenopausal women undergoing bariatric surgery,[31] while two examined hepcidin alterations in children and adults following dietary energy restriction.[117, 148] Two of these studies showed some improvements in serum hepcidin,[31, 117] while the other reported a significant increase following 12 weeks of weight loss.[148] Tussing-Humphreys *et al* (2010b) also showed a decrease in sTfR six months following bariatric surgery.[31] However, issues relating to study design and the target population have limited the generalisability of these findings to the healthy overweight and obese population where dietary weight management practices are commonly utilised. Some of these issues include the recruitment of a paediatric sample,[117] and confounding effects of obesity surgery on decreased gastric acid secretion which is required for conversion of molecular iron to absorbable ferrous iron, decreased surface area for nutrient absorption and reduced tolerance to red meat.[149]

Genetic factors affecting iron status

Apart from major environmental factors that influence iron status, genetics is also an important determinant of individual iron variation.[150] The past decade has seen a rise in the number of animal and human studies investigating genetic influences on hepcidin expression and iron status. Studies examining hepcidin-linked mutations have focused on a number of key genes such as: *HAMP* (hepcidin gene); *HFE* (hereditary haemochromatosis protein gene); *HJV* (hemojuvelin gene); *TfR2*

(transferrin receptor 2 gene); *SLC40A1* (ferroportin gene); and *TMPRSS6* (matriptase-2 gene) that underpin many iron-related pathologies.[151-154] The excess or shortage of hepcidin that result from genetic mutations can phenotypically manifest as iron overload or deficiency disorders.[150]

The majority of genetic research since the discovery of hepcidin has been dedicated to studying iron overload disorders.[150] Various mutations in the coding and non-coding regions of the *HAMP*, *HFE*, *HJV* and *TfR2* genes have been linked with blunted hepcidin production leading to various types of hereditary haemochromatosis. For example, the R56X nonsense mutation of *HAMP* (rs104894695) and the G320V missense mutation of *HJV* (rs74315323) were identified in individuals with early-onset juvenile haemochromatosis.[155, 156] Furthermore, pathogenic variants of the *TfR2* (Y250X; rs80338880) and *HFE* (C282Y; rs1800562) genes are known to cause adult-onset hereditary haemochromatosis,[24, 157] the latter of which is very common and accounts for >95% of all hereditary haemochromatosis cases in European populations.[24, 158]

Iron overload can very occasionally occur in individuals without defective hepcidin secretory mechanisms. Gain-of-function mutations have been identified in the ferroportin gene (*SLC40A1*) whereby individuals lack the ability to respond appropriately to the presence of hepcidin, essentially causing hepcidin resistance.[159, 160] The C326S mutation has been associated with the most severe iron overload phenotype of all currently identified ferroportin mutations, and has been found to inhibit hepcidin-ferroportin binding completely.[159] The resulting uncontrolled ferroportin-mediated flux of iron across duodenal enterocytes leads to hyperabsorption of dietary iron and inappropriately high iron levels.[161]

In contrast to iron overload disorders, variants in the *TMPRSS6* gene have been found to cause hepcidin over-expression and iron deficiency.[25, 151, 162] In mice, a chemically-induced *TMPRSS6* mutation dubbed the 'mask' phenotype has been shown to bring about iron deficiency anaemia characterised by low circulating and splenic iron together with other symptoms such as alopecia.[162] Point mutations in the *TMPRSS6* locus have been identified in humans as the cause for iron

refractory iron deficiency anaemia unresponsive to oral iron administration.[151, 163-165] Mechanistically, the *TMPRSS6* gene encodes a membrane serine protease called matriptase-2. Based on earlier *in vitro* research, the pathway by which matriptase-2 regulates hepcidin expression is believed to be related to its role in hemojuvelin degradation.[101, 166] As hemojuvelin is a co-receptor to BMP-SMAD mediated hepcidin expression,[167] matriptase-2 deficiency presumably leads to accumulation of hemojuvelin on hepatocyte membranes and aberrant downstream signalling for hepcidin production.[106] I*n vivo* research however, has yielded contradictory outcomes as a 2011 study analysing liver homogenates from *TMPRSS6* knockout mice unexpectedly observed a reduced level of hemojuvelin when compared to wild-type mice.[168] Evidently, the impact of *TMPRSS6*/matriptase-2 on body iron metabolism remains to be clarified.

The impact of genotype on iron variability in the general population has also been examined in a number of genome-wide association,[20, 21, 26, 169-174] and smaller genetic studies.[22, 23, 28, 153, 175-178] Among them, substantial attention has been paid to single nucleotide polymorphisms (SNP) in the *TMPRSS6* locus of healthy individuals. The synonymous rs4820268 SNP is one example of a common genetic variant that is strongly associated with serum iron in individuals of European and Indian ancestry.[21] Another common polymorphism in linkage disequilibrium with rs4820268 is the SNP rs855791 (involving a non-synonymous alanine to valine change). SNP rs855791 has been significantly correlated to several erythrocyte and iron traits such as haemoglobin, serum iron, transferrin saturation, ferritin and sTfR.[20, 26-28, 172, 175-177] The comparability of results across European, Australian, North American and Chinese populations demonstrates the significant impact of this particular SNP on individuals with different ethnic backgrounds.

Although the associations between rs855791 and haematological and iron traits have been reported extensively, little research has examined its influence on human serum hepcidin levels or iron responses to altered energy and haem iron intake. The lack of research on rs855791-hepcidin associations is surprising given that hepcidin is theorised to be a major link between *TMPRSS6* genotype and iron status. At present, only two studies (both of which report results from a single

common sample) have measured serum hepcidin in a population genotyped for rs855791.[29, 175] The outcomes of these studies suggest that individuals homozygous for the alanine-coding allele are able to more effectively inhibit hepcidin production (and thus increase serum iron and transferrin saturation) than individuals homozygous for the valine-coding allele.[29] Moreover, these iron variations could only partly be explained by hepcidin, suggesting the existence of novel hepcidin-independent pathways for iron regulation via *TMPRSS6*/matriptase-2.[175] Clearly, despite the rapid growth of research related to the genetics and mechanisms of iron regulation over the past decade, there is much that remains unknown about the determinants of iron variability between individuals.

SUMMARY

Obesity and iron deficiency are both major public health issues around the world with profound health implications, particularly for young women. In the past five years, a growing amount of evidence supports a link between these two disease burdens which may be mediated by inflammation and the key iron regulatory hormone hepcidin. Since the discovery of hepcidin in 2000, extensive research has contributed to a rapid increase in our understanding of the hormone and its physiological function in the human body. However, much about the mechanisms and genetics of hepcidin regulation remains to be elucidated.

As inflammation and hepcidin are the common denominators in AI and obesity-related hypoferraemia, earlier studies suggested that iron disruptions in the two conditions may occur in a similar fashion. However, newer research incorporating the measurement of serum hepcidin in the obese indicates otherwise. Nevertheless in clinical practice, both AI and obesity-related inflammation can lead to inaccurate estimation of iron status, especially if routine biomarkers such as serum ferritin are used. Hence, it is important that alternative markers such as sTfR, sTfR-F and hepcidin are assessed simultaneously when underlying inflammation is suspected.

Young women are a population already vulnerable to weight gain and iron deficiency, with recent evidence in premenopausal obese women also indicating true iron deficiency secondary to mildly elevated hepcidin levels. The risk of iron inadequacy may be further exacerbated by dieting (which is common practice in overweight and obese young women), low protein and haem iron intake, and a genetic predisposition for lower iron status resulting from the *TMPRSS6* SNP rs855791. Despite this, dedicated research on obesity-related iron disruption in young women is limited, which emphasises the importance of extending the current body of topic-related literature focussed on this population.

CHAPTER 3.

The relationship between obesity and hypoferraemia in adults:

a systematic review

PUBLICATION STATEMENT

The work presented in this chapter is an updated version of the following publication:

Cheng HL, Bryant C, Cook R, O'Connor H, Rooney K, Steinbeck K. The relationship between obesity and hypoferraemia in adults: a systematic review. *Obes Rev* 2012; 13: 150-61 (Appendix D2)

As co-authors of the paper 'The relationship between obesity and hypoferraemia in adults: a systematic review' we confirm that Hoi Lun Cheng has made the following contributions:

- Conception and design of the research – determined the inclusion/exclusion criteria and research databases for conducting the initial search (with guidance from all co-authors)
- Systematic search of the literature and selection of eligible studies – conducted the initial search and selected all eligible studies (with assistance from RC)
- Statistical analysis and interpretation of the findings – extracted all relevant data (with assistance from RC), conducted all biochemical unit conversions, generated all forest plots and interpreted most of the findings presented (with guidance from all co-authors)
- Writing the paper and critical appraisal of content – primarily responsible for drafting and editing of the manuscript following peer review (with guidance from all co-authors)

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ABSTRACT

A growing number of studies suggest a potential link between obesity and altered iron metabolism. The purpose of this systematic review was to examine existing literature on iron status in obese populations. A comprehensive literature search was conducted. Included studies recruited participants ≥18 years with a body mass index ≥30 kg/m^2 and provided descriptive statistics for haemoglobin or ferritin at a minimum. There were 31 studies meeting all eligibility criteria, of these 12 examined iron status in free-living obese individuals and 19 reported baseline iron status of bariatric surgery candidates. Non-obese comparison groups were employed by 10 (32%) manuscripts. In these, seven obese groups reported higher mean haemoglobin concentration; six reported significantly higher ferritin concentration; and four significantly lower transferrin saturation. Due to insufficient data, it was not possible to make conclusions regarding mean differences for soluble transferrin receptor (sTfR) and hepcidin which were reported in only five and two studies respectively. Existing evidence suggests a tendency for higher haemoglobin and ferritin concentration and lower transferrin saturation in obesity. Alteration of iron biomarkers in obese populations may be a result of obesity-related inflammation and/or related comorbidities. Further research incorporating measurement of inflammatory cytokines, sTfR and hepcidin is required to confirm the impact of obesity on iron status.

Keywords: obesity, iron deficiency, inflammation, hepcidin

INTRODUCTION

Obesity and iron deficiency are independently both major disease burdens affecting significant proportions of the global population. A connection between obesity and iron deficiency was first suggested in 1962 when a low mean serum iron concentration was reported in an obese adolescent cohort by Wenzel *et al* and subsequently in 1963 by Seltzer & Mayer.[11, 12] The association was apparently initially overlooked and attributed to physical inactivity, poor dietary intake and higher iron requirements from larger blood volume and body surface area in the obese.[8, 12, 14] Many later studies also reported low iron status in obese cohorts but the association was again largely ignored until the discovery of hepcidin, an iron regulatory hormone involved in controlling gastrointestinal iron absorption and circulation. A study by Yanoff *et al* (2006) demonstrating an association between obesity, the inflammatory marker C-reactive protein (CRP) and iron deficiency reignited scientific enquiry.[8, 97] A number of recent manuscripts support earlier findings of an association between obesity and iron deficiency in both adults,[10, 18] as well as children and adolescents.[13, 19, 179]

Clinically, iron deficiency may further add to the burden of obesity and complicate weight management. Iron deficiency has been associated with fatigue, depression and reduced exercise capacity and this may reduce the efficacy of behavioural weight management programmes which aim to increase physical activity and improve motivation and psychological well-being.[6, 132-134] Restricted energy diets used for weight management may also be low in iron, particularly for women during reproductive years where requirements are higher.[32] Treatment of iron deficiency including improved dietary iron intake and/or iron supplementation may not be efficacious in obesity, as obesity-related inflammation may alter iron absorption and circulation, a situation similar to the anaemia of chronic disease.[57, 115] Unfortunately, the independent effect of iron deficiency on weight management and the treatment of hypoferraemia in obesity are yet to be adequately evaluated.

The objective of this paper was to systematically review publications relevant to the relationship between obesity and iron status. Importantly, we aimed to identify specific markers in iron metabolism that may be differentially affected by obesity, which may inform further hypothesis driven research.

METHODS

Eligibility criteria

Participants

Studies recruiting non-pregnant or lactating participants of either gender, ≥18 years with a body mass index (BMI) ≥30 kg/m^2 were considered. Where age and BMI ranges were not provided, studies were accepted if the mean age and BMI were ≥18 years and ≥30 kg/m^2 respectively. In studies failing to report mean BMI, only those involving bariatric surgery candidates exhibiting overt obesity were accepted. To minimise the influence of potential confounders on iron metabolism, studies were rejected if participants had undergone previous bariatric surgery, or if they were conducted in countries undergoing nutrition transition where iron deficiency secondary to gastrointestinal parasitic infection may exist.

Outcome measures

To be included, studies were required to report descriptive statistics for haemoglobin or serum/plasma ferritin at a minimum. Due to lack of standardisation in iron deficiency definitions, studies reporting only prevalence data without descriptive statistics for each iron biomarker were excluded as variable haematological cut points for iron deficiency made data incomparable between studies.

Literature search

The search was performed using the electronic databases All EBM Reviews Multifile (via OvidSP), AMED (via OvidSP), APAIS-Health (via Informit Online), CINAHL (via EBSCO), EMBASE, MEDLINE (via OvidSP), OLDMEDLINE (via OvidSP), PRE-MEDLINE (via OvidSP), PsychINFO (via OvidSP), PubMed, SCOPUS, SPORTDiscus (via EBSCO), Web of Science (via ISI Web of Knowledge) from the earliest record to July 2013.

The search strategy used for MEDLINE and subsequently adapted for other electronic databases combined terms covering areas of obesity and iron deficiency (Appendix A1). Each electronic database search did not include language restrictions, and the search terms used included: *anemi* (where * was used for truncation); anaemi*, iron deficien*; iron deplet*; obes*, overweight, abdominal obesity, morbid obesity.*

Study selection

After elimination by title and/or abstract and removal of duplicates, the search results were screened by one reviewer (HLC) against participant recruitment criteria. Manuscripts matching the participant recruitment criteria were retrieved and independently evaluated by two reviewers (HLC and RC). Abstracts containing all information required for eligibility were included. In cases where abstracts and journal articles contained insufficient information or information presented in alternate formats, attempts were made to contact authors for further information. Reference lists of all eligible studies were handsearched for additional articles. To minimise publication bias, attempts to contact authors of eligible articles were made to obtain any relevant unpublished data. In multiple articles containing similar authorship and suspected of utilising duplicated or overlapping participant groups, only the most relevant study was included in this systematic review.

Data extraction

Data related to study and participant characteristics (age, BMI or any indication of weight in bariatric surgery studies where BMI was unavailable), mean and standard deviation (SD) of haematological markers (haemoglobin and/or ferritin concentration) were extracted independently by two reviewers (HLC and RC). The same protocol was applied in the extraction of additional haematological or inflammatory markers including transferrin saturation, soluble transferrin receptor (sTfR), serum hepcidin, CRP and corresponding data for non-obese control groups with BMI between 18.5-29.9 kg/m^2 when available. In longitudinal and intervention studies, only baseline data was extracted. In studies where data were stratified or grouped into separate

categories (e.g. BMI strata or treatment groups), all relevant categories were extracted. Control group strata were pooled for mean differences analyses except in studies where adjusted mean differences between obese groups and normal weight controls (with BMI between 18.5-24.9 kg/m^2) were reported in order to maintain confounder adjustment.

In studies where haematological markers were presented in units outside of the conventional system, conversion to conventional units was performed for ease of comparison.[18, 180-182] Means were estimated from median and range,[183] and SD were calculated from 95% confidence intervals and standard errors.[184] Where mean and SD could not be estimated using mathematical formulae, data was extracted as reported in the manuscript. In studies providing individual data points or data in graphical form, mean and SD were calculated or measured using a ruler independently by two reviewers (HLC and RC).

Assessment of methodological quality

To identify potential sources of bias, study quality was assessed independently by two reviewers (HLC and RC) using the following quality checklist adapted from items often used for assessment of epidemiological studies[185, 186]: (1) was the study population sufficiently described (age, gender and menopausal status)?; (2) were the sampling procedures sufficiently described (sampling frame (e.g. electoral register, convenience sample) and how was sample size determined)?; (3) was the participant exclusion criteria appropriate (e.g. did studies exclude participants based on abnormal haematological values)?; (4) were outcomes (anthropometric and haematological) measured using appropriate methods?; and (5) were important confounders accounted for through exclusion, data separation or statistical adjustment (gender, age/menopausal status, ethnicity, dietary iron intake, oral contraceptive use, blood donation, iron treatment, alcohol consumption, menorrhagia or amenorrhoea, haemorrhage or gastrointestinal bleeding, acute infection, liver disease or haemochromatosis, inflammatory disorders, chronic disease)?

Several assumptions were made in the quality assessment process due to variation in descriptions of study design and participant characteristics. Studies that conducted a complete physical examination or similar were assumed to have measured (not self-reported) anthropometric values, and studies that described participants as generally healthy, non-users of most medications or similar were assumed to be free of chronic diseases.

Summary measures and synthesis of results

Important study characteristics including study design, participant characteristics (gender and menopausal status), sample sizes, outcome variables and covariates used in statistical adjustment were summarised and presented in tabulated form.

Data for each outcome variable (haemoglobin, ferritin, transferrin saturation, sTfR, hepcidin and CRP) were also summarised and presented in tabulated form. Data from non-bariatric surgery studies were tabulated separately from bariatric surgery studies to minimise potential bias from comorbidities related to severe obesity. Forest plots were generated to evaluate mean differences and 95% confidence intervals in haemoglobin concentration, ferritin concentration and transferrin saturation between obese and non-obese groups in studies with appropriate data. Data from bariatric surgery studies was identified in the forest plots where appropriate.

Meta-analyses of mean differences were not performed due to substantial methodological heterogeneity in participant selection criteria, adjustment of important confounders and laboratory methodologies between studies.

RESULTS

Study selection and characteristics

A flowchart summarising the number of manuscripts identified and subsequently included or excluded is presented in Figure 3.1. The electronic database search yielded 13944 manuscripts. Following screening, elimination of ineligible manuscripts, removal of duplicates and handsearching, a total of 27 full manuscripts and four abstracts describing 31 studies were included in this review.

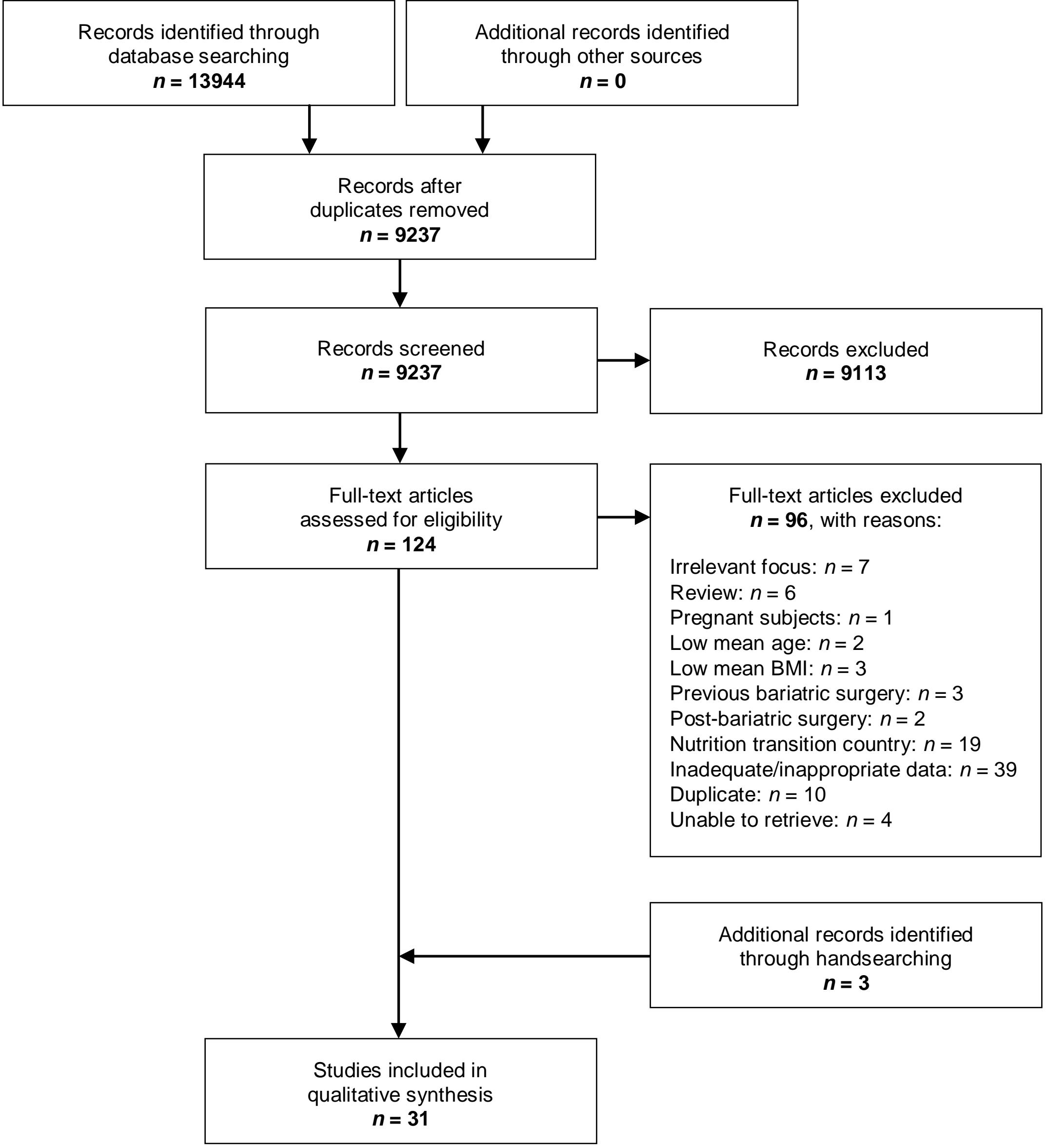

Figure 3.1. Flowchart for identification and inclusion of relevant studies

Table 3.1 and Table 3.2 summarise important characteristics extracted from each included study, separated according to the non-bariatric or bariatric surgery focus. Of the included studies, less than half (*n*=12; 39%) examined iron status of free-living obese populations.[8, 10, 17, 77, 80, 143, 148, 187-191] Majority of included manuscripts (*n*=19; 61%) were studies investigating the nutritional status of severely obese bariatric surgery candidates with reported pre-surgery data.[9, 121, 135, 137-140, 142, 192-202] Of the 12 non-bariatric surgery studies included, seven were cross-sectional, two were case-control, and three were longitudinal intervention studies. Of the 19 bariatric surgery studies, three were cross-sectional, eleven were prospective, three were retrospective and two were randomised controlled trials. Just over a third (*n*=10) of all studies contained non-obese control groups.

Table 3.1. Relevant characteristics of included non-bariatric surgery studies

Author (Country)	**Study design**	**Gender**	**Menopausal status**	**Participants**	**Obese group outcomes**	**Control group outcomes**	**Covariates included in statistical adjustment**
Ahmed *et al*, 2008 (Australia)	Cross-sectional	Both	Both	Male (Normal): *n* = 219 Female <50 y (Normal): *n* = 219 Female ≥50 y (Normal): *n* = 197 Male (Overweight): *n* = 315 Female <50 y (Overweight): *n* = 101 Female ≥50 y (Overweight): *n* = 170 Male (Obese): *n* = 149 Female <50 y (Obese): *n* = 107 Female ≥50 y (Obese): *n* = 132	Age, BMI, ferritin	Age, BMI, ferritin	None reported
Anna *et al*, 2009 (USA) [Abstract]	Cross-sectional	Male	N/A	BMI <25: *n* = 694 BMI 25.1 – 30: *n* = 855 BMI 30.1 – 35: *n* = 452 BMI >35: *n* = 241	Age, BMI, Hb	Age, BMI, Hb	None reported
Ausk & Ioannou, 2008 (USA)	Cross-sectional	Both	Both	BMI <25: *n* = 6059 BMI 25 – 30: *n* = 5108 BMI 30 – 35: *n* = 2366 BMI 35 – 40 : *n* = 850 BMI ≥40: *n* = 465	Age, BMI, Hb, ferritin, Tsat	Age, BMI, Hb, ferritin, Tsat	Mean differences adjusted for: gender, menopausal status, ethnicity, age, educational attainment, alcohol intake, smoking, dietary iron intake, blood donation
Beard *et al*, 1997 (USA)	Intervention	Female	Not reported	VLED 1758 kJ/day: *n* = 14 VLED 2763 kJ/day: *n* = 17 VLED 3349 kJ/day: *n* = 12	Age, BMI, ferritin, Tsat	N/A	None reported
Fricker *et al*, 1990 (France)	Cross-sectional	Female	Premenopausal	Obese: *n* = 20 Non-obese: *n* = 20	Age, BMI, Hb, ferritin, Tsat	Age, BMI, Hb, ferritin, Tsat	None reported

Author (Country)	Study design	Gender	Menopausal status	Participants	Obese group outcomes	Control group outcomes	Covariates included in statistical adjustment
Hamalainen *et al*, 2012 (Finland)	Case-control	Female	Not available	MetS: n = 170	Age, BMI, Hb, ferritin, sTfR, CRP	N/A	Age, smoking, physical activity, inflammation
Kretsch *et al*, 1998 (USA)	Intervention	Female	Premenopausal	n = 14	Age, BMI, Hb, ferritin, Tsat	N/A	None reported
Lecube *et al*, 2006 (Spain)	Case-control	Female	Postmenopausal	Obese: n = 50 Non-obese: n = 50	Age, BMI, Hb, ferritin, Tsat, sTfR	Age, BMI, Hb, ferritin, Tsat, sTfR	None reported
Micozzi *et al*, 1989 (USA)	Cross-sectional	Female	Not reported	n = 8234	Age, BMI, Hb, Tsat	Age, BMI, Hb, Tsat	Age
Murer *et al*, 2012 (Switzerland) [Abstract]	Intervention	Both (assumed)	Not reported	n = 32	Age, BMI, ferritin, sTfR, hepcidin, CRP	N/A	None reported
Świtoniak & Król, 1992 (Poland)	Cross-sectional	Female	Not reported	Normal weight: n = 69 Overweight: n = 85 Obese: n = 62	Age, BMI, Hb, Tsat	Age, BMI, Hb, Tsat	None reported
Yanoff *et al*, 2007 (USA)	Cross-sectional	Both	Not reported	Obese: n = 234 Non-obese: n = 172	Age, BMI, Hb, ferritin, Tsat, sTfR, CRP	Age, BMI, Hb, ferritin, Tsat, sTfR, CRP	Gender, ethnicity, age, socioeconomic status

BMI, body mass index; CRP, C-reactive protein; Hb, haemoglobin; MetS, metabolic syndrome; N/A, not applicable; sTfR, soluble transferrin receptor; Tsat, transferrin saturation; VLED, very-low-energy diet

Table 3.2. Relevant characteristics of included bariatric surgery studies

Author (Country)	Study design	Gender	Menopausal status	Participants	Obese group outcomes	Control group outcomes	Covariates included in statistical adjustment
Aasheim *et al*, 2009 (Sweden, Norway)	Randomised controlled trial	Both	Not reported	GBP group: *n* = 31 DS group: *n* = 29	Age, BMI, Hb, CRP	N/A	None reported
Amaral *et al*, 1984 (USA)	Prospective	Both	Not reported	Male: *n* = 18 Female: *n* = 132	Age, per cent ideal body weight, Hb	N/A	None reported
Anty *et al*, 2008 (France)	Prospective	Female	Both	*n* = 178	Age, BMI, Hb, ferritin, Tsat	N/A	None reported
Bekri *et al*, 2006 (France)	Cross-sectional	Both	Not reported	Lean controls: *n* = 9 Obese: *n* = 8 Obese with diabetes: *n* = 7 Obese with NASH: *n* = 10	Age, BMI, Hb, ferritin, Tsat, CRP	Age, BMI, CRP	None reported
Belgaumkar *et al*, 2011 (UK) [Abstract]	Prospective	Both	Not reported	*n* = 19	Age, BMI, Hb, ferritin	N/A	None reported
Brolin *et al*, 1998 (USA)	Randomised controlled trial	Female	Premenopausal	Placebo group: *n* = 27 Intervention group: *n* = 29	Age, weight, Hb, ferritin, Tsat	N/A	None reported
Cooper *et al*, 1999 (Australia)	Prospective	Both	Not reported	*n* = 17 (in biochemical analysis)	Age, BMI, Hb, ferritin	N/A	None reported
Coupaye *et al*, 2009 (France)	Prospective	Both	Not reported	AGB group: *n* = 21 GBP group: *n* = 49	Age, BMI, Hb, ferritin, Tsat, CRP	N/A	None reported
de Luis *et al*, 2013 (Spain)	Prospective	Female	Not reported	BMI 35-40: *n* = 11 BMI 40-45: *n* = 28 BMI 45-50: *n* = 40 BMI >50: *n* = 36	Age, BMI, Hb, ferritin	N/A	None reported

Author (Country)	Study design	Gender	Menopausal status	Participants	Obese group outcomes	Control group outcomes	Covariates included in statistical adjustment
Ernst *et al*, 2009 (Switzerland)	Prospective	Both	Not reported	$n = 232$	Age, BMI, Hb, ferritin	N/A	None reported
Gasteyger *et al*, 2006 (Switzerland)	Prospective	Female	Premenopausal	$n = 36$	Age, BMI, Hb, ferritin	N/A	None reported
Ortiz Espejo *et al*, 2011 (Spain)	Retrospective	Both	Not reported	$n = 35$	Age, ferritin	N/A	None reported
Palomar *et al*, 2005 (Spain)	Prospective	Both	Both	$n = 35$	Age, BMI, Hb, ferritin, Tsat	N/A	None reported
Payne & DeWind, 1969 (USA)	Prospective	Both	Not reported	$n = 80$	Age, weight, Hb	N/A	None reported
Skroubis *et al*, 2006 (Greece)	Retrospective	Both	Both	RYGBP group: $n = 79$ BPD group: $n = 95$	Age, BMI, Hb, ferritin	N/A	None reported
Smith *et al*, 2013 (UK) [Abstract]	Cross-sectional	Both	Not reported	Normoglycaemic: $n = 216$ Pre-diabetic: $n = 237$ Diabetic: $n = 223$	Age, BMI, ferritin, Tsat	N/A	None reported
Thurnheer *et al*, 2012 (Switzerland)	Prospective	Both	Not reported	Male: $n = 98$ Female: $n = 257$	Age, BMI, Hb, ferritin	N/A	None reported
Toh *et al*, 2009 (Australia)	Retrospective	Both	Not reported	RYGBP group: $n = 103$ SG group: $n = 46$	Age, BMI, Hb, ferritin	N/A	None reported
Tussing-Humphreys *et al*, 2009 (USA)	Cross-sectional	Female	Premenopausal	$n = 20$	Age, BMI, Hb, ferritin, Tsat, sTfR, hepcidin, CRP	Age, BMI, Hb, ferritin, Tsat, sTfR, hepcidin, CRP	Ethnicity

AGB, adjustable gastric banding; BMI, body mass index; BPD, biliopancreatic diversion; CRP, C-reactive protein; DS, duodenal switch; GBP, gastric bypass; Hb, haemoglobin; N/A, not applicable; NASH, non-alcoholic steatohepatitis; RYGBP, Roux-en-Y gastric bypass; SG, sleeve gastrectomy; sTfR, soluble transferrin receptor; Tsat, transferrin saturation

Participant gender and menopausal status varied across studies with 18 mixed gender, 12 female and one male only study. Of the 12 studies examining iron indices in female only participants, five focused specifically on premenopausal and one on postmenopausal women. The studies by Ahmed *et al* and Anty *et al* utilised the age cut-off of 50 years to differentiate menopausal status in female participants, but evidence to confirm menstruation status was not provided.[77, 137]

Concentrations of haemoglobin and ferritin in addition to transferrin saturation were the most frequently reported haematological markers. Few studies reported sTfR (*n*=5) or serum hepcidin (*n*=2).[8, 9, 17, 148, 189]

Most of the extracted data were presented as mean and SD although some studies reported means or medians in combination with either range, 95% confidence interval or interquartile range.

Comparison of laboratory methodologies used in the analysis of haemoglobin concentration showed mostly automated methods of measurement. Ferritin analysis was comparatively more varied, although automated immunoassays were the most common method of analysis. Of the five studies examining sTfR, two utilised immunoassays[8, 9] one used particle-enhanced nephelometry,[17] and another study used a particle enhanced immunoturbidimetric assay.[189] In the two studies reporting hepcidin, Tussing-Humphreys *et al* utilised a hepcidin specific immunoassay,[9] whereas the method employed by Murer *et al* was not reported.[148] However, a previous study by the same research team suggests that a combined weak cation exchange chromatography and time-of-flight mass spectrometry method may have been used.[13]

Methodological quality

A descriptive assessment of methodological quality is presented in Appendix A2. Overall study quality was moderate with most studies (*n*=25) unable to meet at least three of the five important quality items (Appendix A2). Non-bariatric surgery studies were generally of higher quality with 30% of studies (*n*=3) meeting at least three quality items. Most bariatric surgery studies (with the

exception of Tussing-Humphreys *et al*[9]) were poorer quality [with only 5% of studies (*n*=1) meeting at least three quality items].

Main quality related issues for all studies were linked to both study and outcome level bias. Some confounders were frequently accounted for (e.g. gender, age/menopausal status, ethnicity), but other important confounders (e.g. acute infection, amenorrhoea/menorrhagia, oral contraceptive use, iron treatment/supplementation, obesity-related comorbidities) were often not considered even in studies where iron status was a primary outcome. The study by Ausk & Ioannou based on data from The National Health and Nutrition Examination Survey III (NHANES III) contained one of the most comprehensive confounder adjustment lists, but still failed to account for oral contraceptives use and iron treatment/supplementation.[10] A few (*n*=4) studies excluded participants with anaemia or abnormally high or low ferritin which likely biased results.[17, 77, 80, 190] In addition, some studies (*n*=2) used ferritin as the sole biomarker for assessment of iron status with few utilising the more recently discovered markers sTfR and hepcidin. This may have resulted in potential underestimation of iron deficiency due to elevation of serum ferritin through obesity-related chronic inflammation.[57, 77, 141, 203]

Results of individual studies

Summaries of the data extracted for each non-bariatric or bariatric surgery study are presented in Appendices A3 to A6. Haemoglobin and transferrin saturation showed higher consistency with lower SD when compared to ferritin concentration which was highly variable (large SD) even in studies with large sample size and multiple confounder adjustment.[10] Too few studies used sTfR or hepcidin for robust conclusions to be drawn.

Forest plots were used to illustrate the mean differences in concentration of haemoglobin and ferritin as well as transferrin saturation between obese and non-obese control groups. There were 10 obese groups from seven included studies reporting data for haemoglobin.[8-10, 17, 187, 188, 191] Of these, seven groups tended to have higher (with four showing significantly higher) haemoglobin

concentrations in the obese when compared to non-obese controls (Figure 3.2). Larger studies and those in women all showed higher haemoglobin concentration in obese groups with the exception of the study by Yanoff *et al*[8] The two remaining obese groups from Anna *et al* showing similar and non-significantly lower haemoglobin concentration were those with all male participants.[187]

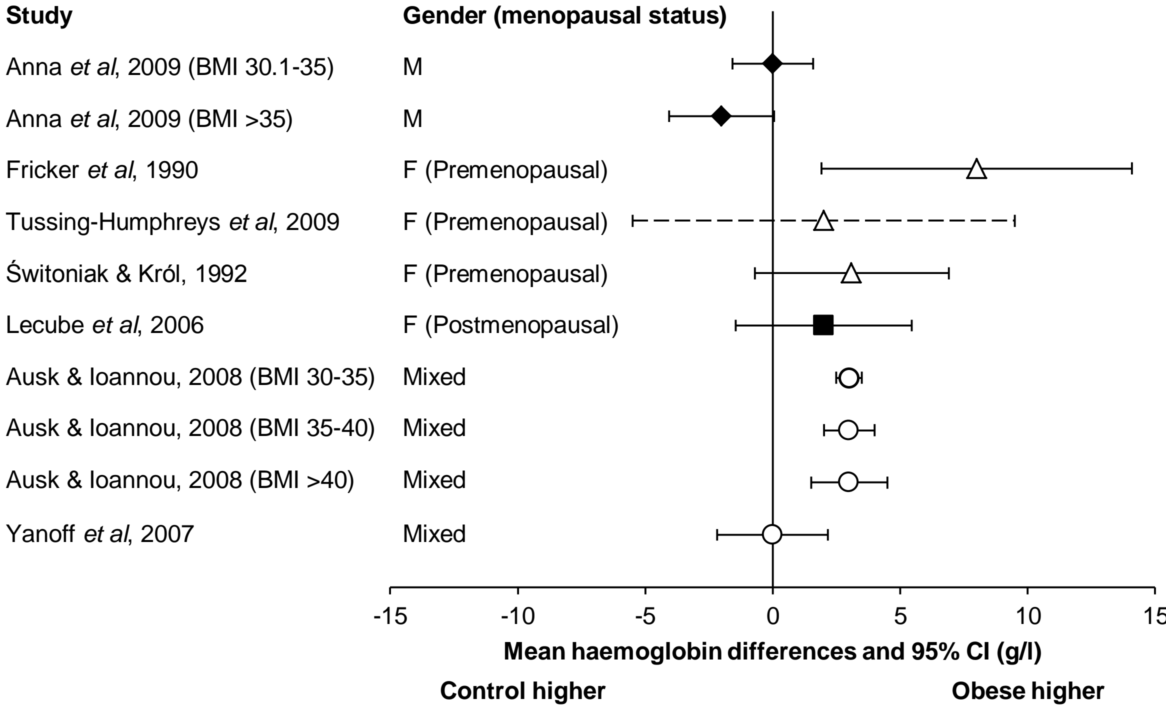

Figure 3.2. Summary of mean haemoglobin differences between obese and non-obese groups with 95% confidence intervals (CI) arranged according to gender and menopausal status (Dashed line denotes results from a bariatric surgery study; BMI, body mass index; M, male; F, female)

All except one of the obese groups from six studies reported higher ferritin concentrations compared to non-obese controls (Figure 3.3).[8-10, 17, 77, 188] In three groups this difference did not reach statistical significance (two of which were male or postmenopausal female). The only group reporting slightly lower ferritin concentration in the obese was also a postmenopausal female group.[77] NHANES III data found a marked increase in ferritin concentration in higher (35-40 kg/m^2 and > 40 kg/m^2) compared to lower (30-35 kg/m^2) BMI strata in the obese.[10]

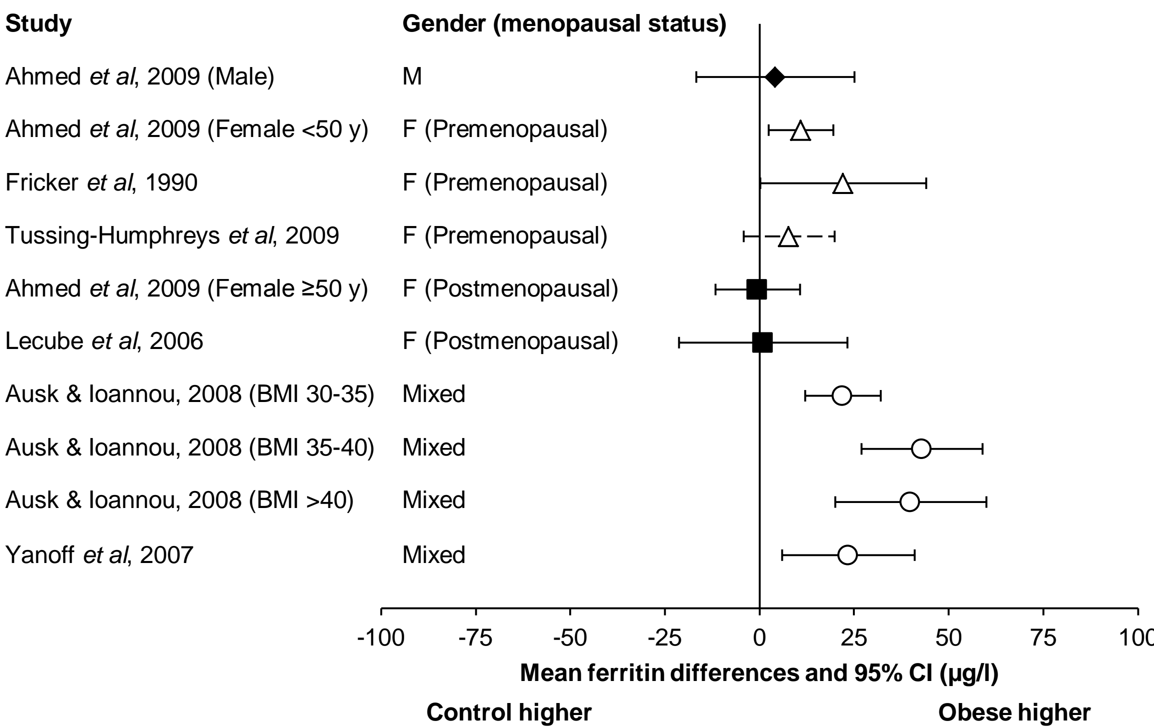

Figure 3.3. Summary of mean ferritin differences between obese and non-obese groups with 95% confidence intervals (CI) arranged according to gender and menopausal status (Dashed line denotes results from a bariatric surgery study; BMI, body mass index; M, male; F, female)

A trend for lower transferrin saturation in seven of eight obese groups was also observed (Figure 3.4).[8-10, 17, 188, 191] Of the eight groups, four showed significantly lower transferrin saturation levels in the obese compared to the corresponding control groups. Observation of the NHANES III data showed a stepwise decrease in transferrin saturation with increasing levels of obesity (Figure 3.4).[10]

Mean differences in sTfR, hepcidin and CRP between obese and controls were not computed as few studies reported these biomarkers. When reported, sTfR was slightly and hepcidin and CRP markedly elevated in the obese (Appendices A5 and A6).[8, 9, 17, 121]

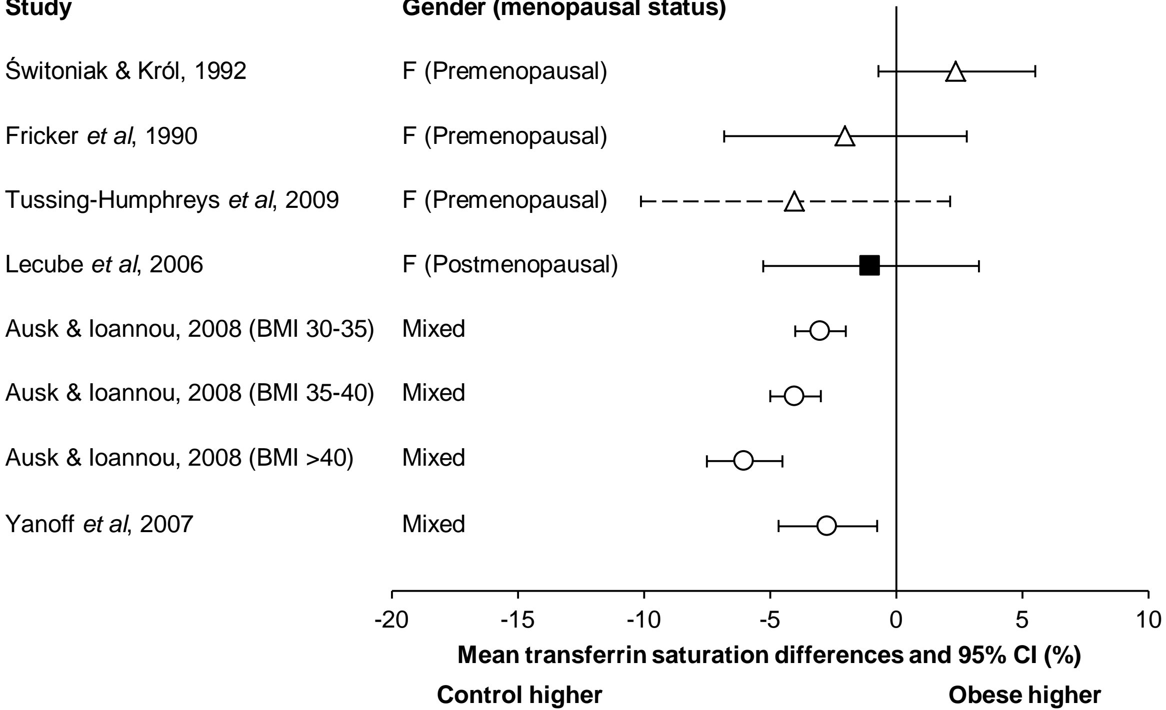

Figure 3.4. Summary of mean transferrin saturation differences between obese and non-obese groups with 95% confidence intervals (CI) arranged according to gender and menopausal status (Dashed line denotes results from a bariatric surgery study; BMI, body mass index; M, male; F, female)

DISCUSSION

To our knowledge, this is the first manuscript to systematically review studies reporting iron status in obese cohorts. The obese tended to have higher haemoglobin and ferritin concentrations and lower transferrin saturation compared to the non-obese. The higher ferritin and lower transferrin saturation is consistent with the mechanism of obesity-related inflammation.[204] However, assessment of other biomarkers such as sTfR (which is elevated in iron deficiency) and hepcidin (which is elevated in inflammatory states) in concert with clearly defined obesity populations are required to clarify the causal relationship between obesity and disturbed iron metabolism. Future investigations in different BMI and waist strata with care to measure and adjust for known confounders (e.g. gender, menopausal status, dietary treatment/supplementation) are also required.

Traditionally, routine iron biomarkers such as haemoglobin, transferrin saturation and ferritin have been used to gauge iron status in individuals. However, in the presence of inflammation, the typical presentation of iron status is altered which can complicate detection of abnormalities in the clinical setting. Additional to routine iron studies, sTfR is useful in inflammatory states as unlike ferritin, it is not an acute-phase reactant.[8] Other measurements such as the sTfR-ferritin index have also been reported to be valuable indicators of iron status in this circumstance.[94, 205]

Recent interest in iron metabolism in chronic inflammatory states such as obesity has focused on hepcidin. Hepcidin is a 25 amino acid peptide hormone synthesised primarily by the liver and is a key regulator of iron homeostasis. It induces the internalisation and degradation of ferroportin in duodenal enterocytes, hepatocytes and macrophages which result in the inhibition of gastro-intestinal iron absorption and release of iron stores from the liver and reticuloendothelial system.[97, 206] This review identified only two studies measuring serum hepcidin in adults, one of which showed it to be higher in the obese.[9] Similar results were also reported in a paediatric population where mean serum hepcidin concentrations were higher in obese compared to non-obese children irrespective of dietary iron intake.[13]

The mechanisms resulting in increased circulating hepcidin concentration in obesity remain to be fully explained. There are considerable data demonstrating the proinflammatory cytokine interleukin-6 (IL-6) as a stimulator of hepcidin expression through signalling and activation of signal transducer and activator of transcription-3 (STAT-3) which binds to the promoter region of the hepcidin gene (*HAMP*) inducing mRNA transcription.[101, 103, 153] Increases in inflammatory cytokines including IL-6 have been well documented in obesity and may contribute to significant increases in systemic hepcidin concentrations.[56, 57, 207] There is also *in vitro* evidence suggesting the adipokine leptin (which is known to be markedly increased in obesity) may also stimulate hepcidin expression through the STAT-3 pathway.[116] Furthermore, hepcidin mRNA has been shown to be expressed in adipose tissue which may also significantly contribute to elevated local and systemic concentrations in obese individuals with large adipose deposits. However, studies examining hepcidin mRNA expression in hepatic vs. adipose tissue have provided conflicting results.[9, 121]

Studies included in this review show a trend towards higher haemoglobin concentrations in some obese groups. There are comorbidities more common in the obese which may raise haemoglobin and explain the trend illustrated in Figure 3.2. Obese individuals are at greater risk of chronic tissue hypoxia secondary to obstructive sleep apnoea and other obesity-related respiratory conditions which may lead to polycythaemia.[208, 209] This rise is of course dependent on adequate iron availability, and the higher haemoglobin concentrations observed in the obese suggest that adequate iron is available for erythropoiesis.

Higher ferritin concentrations were observed in all but one of the obese cohorts (Figure 3.3). Chronic inflammation associated with obesity is known to elevate this marker and may explain the higher mean ferritin concentrations observed in the obese groups.[57, 94, 203] The rise in ferritin from the BMI strata of 35-40 kg/m^2 onwards reported in the NHANES III dataset may reflect the impact of obesity-related inflammation on ferritin concentration.[10] The inflammatory cytokine CRP was most frequently reported in the included studies and levels are consistent with obesity-related inflammation.[57] However, additional studies designed to specifically investigate the association

between obesity-related inflammation and iron biomarkers such as ferritin are needed to confirm this relationship, and to determine how body fat distribution and degree of obesity may alter ferritin concentration.

Lower transferrin saturation indicates decreased circulating protein bound iron.[103] In obesity, lower transferrin saturation may be due to impaired iron absorption or decreased release from the reticuloendothelium – both of which fit with the inflammatory hepcidin hypothesis.[97] Decreased transferrin saturation coupled with increased ferritin is suggestive of an inflammatory state. The progressive decline with increasing obesity suggests a biological link and may support the hypothesis that there is the underlying abnormality of iron absorption and iron storage release characteristic of the anaemia of inflammation.[115]

Participants undergoing bariatric surgery tend to be more obese and have a higher frequency and severity of obesity-related comorbidities.[210] Iron status in this population may therefore not accurately reflect what occurs in less severely obese cohorts. Due to the lack of studies with control groups and heterogeneity across all studies, it is difficult to conclude what effect the degree of obesity and comorbidity had on markers of iron status. However, results from pre-bariatric and healthy obese cohorts appear to be similar. Although not assessed by this review there are numerous reasons why the obese could also have simple iron deficiency. This may result from inadequate micronutrient intake, particularly in bariatric surgery patients and those with poor dietary habits or as a result of reduced energy diets in which iron is typically a limiting nutrient.[32] Menorrhagia associated with polycystic ovary syndrome and gastro-oesophageal reflux with oesophagitis are also two potential causes of iron deficiency that are more prevalent in the obese population.[211, 212] Furthermore, chronic negative iron balance secondary to mild hepcidin elevation and partial mobilisation of iron stores has also been proposed to increase the risk of simple iron deficiency in the obese.

Particular challenges surround the detection of abnormalities in iron status. Conventional reference intervals quoted for ferritin, transferrin saturation, sTfR and other biomarkers are based on reference groups defined by the lack of anaemia. Patients with subclinical abnormalities in iron status without anaemia may be included in these reference groups, leading to a widening of reference intervals and decreased sensitivity of the biomarkers.[94] Similar challenges also exist in the definition of hypoferraemia in obesity. Currently, the degree to which obesity-related inflammation alters iron metabolism remains unclear. Inflammatory elevation of ferritin leads to reduction in its sensitivity as an indicator of iron status,[205] and some iron replacement studies have suggested alternate ferritin cut points for identifying disturbances in iron status in the presence of inflammation but further validation is required, particularly in obese populations.[205, 213]

At present, there are insufficient well-designed studies with non-obese control groups comparing ferritin, sTfR, hepcidin and CRP simultaneously to offer firm evidence about the abnormalities of iron metabolism in the obese. There is a need for more dedicated studies employing control groups and wider use of additional iron biomarkers in future research in order to more accurately compare iron and inflammatory status between obese and non-obese populations. This study also revealed a large heterogeneity in gender and menopausal status between studies which may have biased results. Other sources of possible bias stem from the small, non-random, convenience samples recruited in the majority of studies (particularly bariatric surgery studies) that may not accurately reflect iron status of the wider population and the lack of adjustment for important confounders (e.g. acute infection, oral contraceptive use, iron treatment/supplementation). Despite this, most of the smaller studies were consistent with results of the NHANES III data.[10]

In conclusion, this review highlights (1) the considerable challenge in identifying abnormalities of iron metabolism in the obese, (2) the need for further research, particularly well-designed topic-focused studies with appropriate adjustment of important confounders, wider use of novel iron biomarkers and inflammatory markers such as sTfR, hepcidin and CRP, in addition to existing routine iron markers in future studies, and (3) the need for validated markers or algorithms able to

differentiate between disruptions in true or functional iron status secondary to obesity-related inflammation. Despite the limitations of heterogeneous methodologies and populations in the studies examined, this systematic review supports evidence for obesity associated disturbances in iron metabolism, the mechanisms for which are currently undefined.

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CONFLICT OF INTEREST

This systematic review was supported by Meat and Livestock Australia. The authors declare that Meat and Livestock Australia had no influence on any part of the review process including the conclusions drawn.

CHAPTER 4.

Iron, hepcidin and inflammatory status of overweight and obese young women

PUBLICATION STATEMENT

The work presented in this chapter is largely based on the following publication:

Cheng HL, Bryant CE, Rooney KB, Steinbeck KS, Griffin HJ, Petocz P, O'Connor HT. Iron, hepcidin and inflammatory status of young healthy overweight and obese women in Australia. *PLoS ONE* 2013; 8(7): e68675. doi:10.1371/journal.pone (Appendix D3)

As co-authors of the paper 'Iron, hepcidin and inflammatory status of young healthy overweight and obese women in Australia' we confirm that Hoi Lun Cheng has made the following contributions:

- Conception and design of the research – selected appropriate biochemical markers for analysis (with guidance from all co-authors)
- Data collection and biochemical analysis – recruited and collected data from the final 54% of participants, and conducted all biochemical analyses involving ELISA
- Statistical analysis and interpretation of the findings – completed all statistical analyses (with guidance from PP) and interpreted most of the findings presented (with guidance from all co-authors)
- Writing the paper and critical appraisal of content – primarily responsible for drafting and editing the manuscript following peer review (with guidance from all co-authors)

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ABSTRACT

Background & Aims: Evidence suggests obesity-related inflammation alters iron metabolism potentially increasing the risk of iron deficiency. This cross-sectional study aimed to investigate iron, hepcidin and inflammatory status in young, healthy overweight and obese women. **Methods:** 114 young (18-25 years), healthy comorbidity-free women with a body mass index (BMI) ≥27.5 kg/m^2 were recruited. Biochemical data were analysed using mean ± standard deviation or median (interquartile range) and multivariate modelling. Biochemical markers were also stratified according to varying degrees of overweight and obesity. **Results:** Anaemia (haemoglobin <120 g/l) and iron deficiency (serum ferritin <15.0 µg/l) were prevalent in 10% and 17% of participants respectively. Mean/median soluble transferrin receptor was 1.61 ± 0.44 mg/l; hepcidin 6.40 (7.85) ng/ml and C-reactive protein (CRP) 3.58 (5.81) mg/l. Multivariate modelling showed that BMI was a significant predictor of serum iron (coefficient=-0.379; standard error=0.139; p=0.008), transferrin saturation (coefficient=-0.588; standard error=0.222; p=0.009) and CRP (coefficient=0.127; standard error=0.024; p<0.001).Stratification of participants according to BMI showed those with ≥35.0 kg/m^2 had significantly higher CRP (p<0.001) than those in lower BMI categories. **Conclusions:** Increasing obesity was associated with minor disturbances in iron metabolism. However, overall outcomes indicated simple iron deficiency (hypoferritinaemia) was the primary iron-related abnormality with no apparent contribution of inflammation or hepcidin, even in those with BMI >35.0 kg/m^2. This indicates that obesity alone may not be sufficient to induce clinically significant disturbances to iron metabolism as previously described. This may be attributed to the lack of comorbidity in this cohort.

Keywords: iron, hepcidin, inflammation, iron deficiency, obesity, overweight

INTRODUCTION

Obesity is regarded as a proinflammatory condition characterised by the presence of chronic, low grade systemic inflammation.[8, 10] In the past decade, a growing number of studies have suggested that obesity-related inflammation can lead to an iron handling defect similar to anaemia of inflammation (AI),[10] with hepcidin proposed as a key mediator.[8, 9, 13] Our recent systematic review investigating the impact of obesity on iron status also reported elevated BMI to be associated with increased serum ferritin and reduced transferrin saturation.[214] However the question of whether these alterations are due to inflammation-mediated functional deficiency or true iron deficiency remains unclear due to lack of data for soluble transferrin receptor (sTfR) and hepcidin.[214]

At a population level, premenopausal women are at higher risk of iron deficiency. In Australia, it has been estimated that over 20% of women between 25-50 years are either iron deficient or have marginally low iron status.[77] In addition to regular menstrual iron loss,[7] low iron intake (which is often reported in this group) and restrictive dietary practices for weight loss in overweight women can also increase the risk of iron deficiency.[32, 215] Overweight and obese young women may therefore have a combination of age, dietary and inflammatory-associated factors perturbing iron status.

Currently, only a handful of dedicated studies on obesity-related iron deficiency have focused on adolescent girls or young women.[9, 30, 216] One US study analysed serum hepcidin and reported it to be significantly higher in bariatric surgery candidates compared to non-obese women.[9] Severe obesity however, is frequently associated with comorbidities (e.g. obstructive sleep apnoea, gastro-oesophageal reflux and fatty liver disease) that influence haematological markers or increase blood loss,[214, 217, 218] and findings from this study may not be applicable to the wider population of less severely obese young women who are not burdened with comorbid conditions. Furthermore, other studies performed in more representative populations have been limited by the absence of hepcidin measurement, with mixed outcomes further complicating interpretation.

This study aimed to clarify the relationship between obesity, inflammation and iron status and identify the prevalence and nature of iron deficiency in a cohort of young healthy overweight and obese women by using a broad range of iron markers including sTfR and hepcidin.

METHODS

Ethics statement

This study was registered with the Australian New Zealand Clinical Trials Registry (ID: ACTRN12613000072718) and approved by the Ethics Review Committee of the Sydney South West Area Health Service and the University of Sydney Ethics Committee. Signed, written informed consent was obtained from all participants (the participant consent form used is presented in Appendix B3). Ethics approval was obtained for the referral of participants to their family physician upon detection of abnormal pathology results and review by the study medical officer (KSS).

Study design

This cross-sectional study reports anthropometric and biochemical characteristics of a community sample of young overweight and obese women (the study protocol and information for participants are presented in Appendices B1 and B2). Participants included in this study responded to one of two advertisements: one for recruitment into this cross-sectional study and the other for recruitment into a related 12-month clinical weight loss trial (where a similar but more stringent eligibility criteria was enforced).[219] This cross-sectional study reports the baseline characteristics of volunteers who responded to either advertisement and met the eligibility criteria outlined in this manuscript. A subset of these participants also met the recruitment criteria for entry into the weight loss trial and results from this trial have been published elsewhere (Appendices D4 and D5).[219]

Participants

Young women aged between 18 to 25 years with a measured BMI ≥27.5 kg/m^2 were recruited. Exclusion criteria included: self-report of significant medical conditions [e.g. diabetes mellitus, disorders of the liver (including haemochromatosis) and kidney, autoimmune or metabolic diseases and malignancy] or use of medications that may influence weight, iron or inflammatory status; pregnancy or lactation; vegetarianism; zinc supplementation; smoking; and previous bariatric surgery.

Volunteers reporting iron supplementation or blood donation within three months prior to recruitment were asked to participate only following a three-month washout period. A two-week washout period was required prior to recruitment for self-reported vitamin, fish oil or mineral (other than iron or zinc) supplementation.

Data collection

Participants attended two appointments at the obesity clinic of a university teaching hospital in Sydney, Australia. The first visit involved anthropometric assessment and collection of medical and socio-demographic history including self-reported use of contraceptive medication and history of polycystic ovary syndrome (PCOS). Participants reported to the second visit following a 12-hour overnight fast for collection of a morning venous blood sample. Those reporting current or recent (week prior) acute infection had blood collection rescheduled to avoid infection-related inflammation.

Height (to the nearest 0.1 cm) and weight (to the nearest 0.1 kg) were measured without shoes using a wall-mounted stadiometer (Hyssna Limfog AB, Hyssna, Sweden) and an electronic digital platform scale (Teraoka Seiko, Tokyo, Japan) respectively. Waist circumference was measured using a metal retractable tape (Lufkin W606PM; Cooper Industries, Sparks, USA) to the nearest 0.1 cm and according to international guidelines.[220] In participants who were subsequently recruited to the weight loss trial, baseline body composition was also measured by dual-energy x-ray absorptiometry (DXA) (GE Healthcare, Chalfont St Giles, UK). Abdominal (android) fat was calculated according to the manufacturer's instructions by taking the lower boundary at the pelvis cut; upper boundary above the pelvis cut by 20% of the distance between the pelvis and neck cuts; and lateral boundaries at the arm cuts (Figure 4.1).

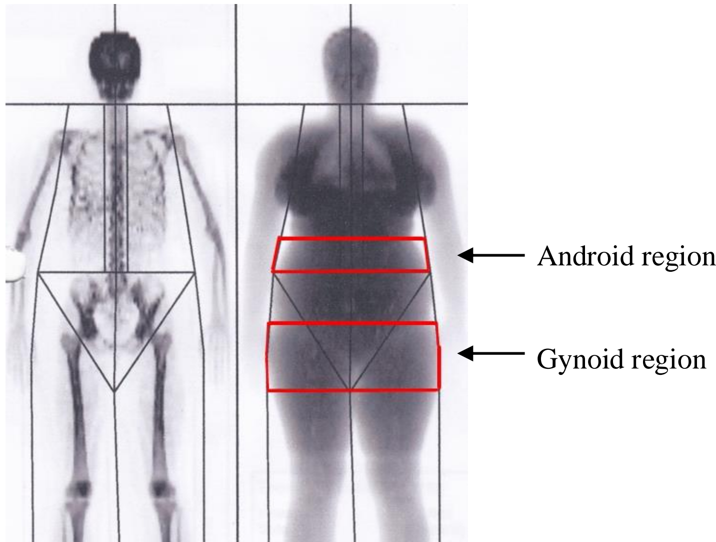

Figure 4.1. Regions of android (abdominal) and gynoid fat as measured by dual-energy x-ray absorptiometry (DXA)

Biochemical analysis

Haemoglobin, serum iron, transferrin saturation and serum ferritin were analysed at a nationally accredited commercial diagnostic pathology laboratory. Stored frozen plasma was used to analyse sTfR, hepcidin and C-reactive protein (CRP). Haemoglobin was measured using the Sysmex Instrument (Roche Diagnostics Australia, Sydney, Australia). Serum iron, transferrin saturation and serum ferritin were measured using the Roche Modular E170 Immunoassay Analyser (Roche Diagnostics Australia, Sydney, Australia). The reference ranges used were 120-165 g/l for haemoglobin; 10.0-30.0 µmol/l for serum iron; 12.0-45.0% for transferrin saturation; and 15.0-165.0 µg/l for serum ferritin. Plasma sTfR and CRP were analysed using commercial ELISA kits (R&D Systems, Minneapolis, USA). Reference ranges provided were 0.74-2.39 mg/l for sTfR; and 0.11-4.52 mg/l for CRP. A conversion factor was applied to transform sTfR values from nmol/l to mg/l.[181] The sTfR-ferritin index (sTfR-F) was used to identify participants with iron depletion (sTfR-F 1.80-2.20), iron deficient erythropoiesis (sTfR-F >2.20), and AI (sTfR-F <1.00 with concurrent haemoglobin <120 g/l).[94, 125] Clinically elevated CRP was defined as >10.00 mg/l.[53] Plasma hepcidin was measured by an on-line extraction coupled to liquid chromatography-tandem mass spectrometry method using the Xevo TQ MS (Waters Corporation, Milford, USA).[221] Inter-

assay accuracy was 95% with a coefficient of variation of 8.2%. Assay sensitivity was 2.00 ng/ml, and values below the detectable range were defined as 1.00 ng/ml. A reference range of 1.92-32.40 ng/ml was used.[222, 223]

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 19 for Windows (IBM Corporation, Armonk, USA). Data are presented as percentages, mean ± standard deviation (SD) or median (interquartile range).

The relationship between obesity, iron and inflammatory status was assessed using two multivariate multiple regression models via MANOVA. Pearson's correlations between BMI and waist circumference as well as between BMI and the DXA variables (total body fat percentage, absolute fat mass and abdominal fat percentage) were assessed prior to running the models. The first regression model included BMI (as a representative anthropometric variable) and potential confounders [use of contraceptive medication, ethnicity (coded dichotomously as European or non-European) and PCOS] as predictors for the response variables: haemoglobin, serum iron, transferrin saturation, serum ferritin, sTfR, sTfR-F, hepcidin and CRP. As DXA measurement was only conducted in a subset of participants, body composition was excluded from initial multivariate analysis to maximise the number of participants included in the first regression model. A second multivariate model including BMI, total body fat percentage and the aforementioned confounder variables was subsequently produced to evaluate the impact of body composition on the biochemical markers. Significance was set at 0.01 for the MANOVA models to protect against type 1 error with regression coefficients and standard errors (SE) also reported.

Secondary analyses were carried out to investigate biochemical differences between varying degrees of overweight and obesity. This was done by stratifying BMI into three categories and comparing biochemical markers using one way ANOVA and Bonferroni-adjusted post hoc tests. For the regression models and secondary analyses, variables were assessed for normality with natural log transformations performed on the serum ferritin, sTfR-F, hepcidin and CRP variables. Significance for the post hoc tests was set at 0.05.

RESULTS

A total of 114 overweight and obese women were recruited to this cross-sectional study. A summary of participant characteristics is presented in Table 4.1. Mean age and BMI were 22.3 ± 2.3 years and 33.7 ± 4.4 kg/m^2 respectively. Overall use of hormonal contraceptive medication was 32% (*n*=36) [oral contraceptive pill (*n*=30), contraceptive implant (*n*=4), depot injection (*n*=1) or patch (*n*=1)]. PCOS was self-reported in 12% (*n*=15) of participants. DXA body composition information was obtained from a subset (*n*=69) of participants (Table 4.1).

Table 4.1. Summary of participant characteristics

Participant characteristic (*n*=114)	**Mean ± SD or percentage**
Age (years)	22.3 ± 2.3
Ethnicity (%)	
European	65.8%
Asian	12.3%
African	3.5%
South American	4.4%
Other	14.0%
Contraceptive medication (%)	31.6%
PCOS (%)	12.4%
Weight (kg)	94.6 ± 13.3
BMI (kg/m^2)	33.7 ± 4.4
Waist circumference (cm)	93.8 ± 10.0
Total body fat (%)[a]	49.8 ± 4.5
Absolute fat mass (kg)[a]	44.5 ± 7.6
Abdominal fat (%)[a]	55.7 ± 4.3

PCOS, polycystic ovary syndrome; SD, standard deviation
[a]Body composition data obtained from 69 participants. Two participants were precluded from body composition analysis as the measurement equipment did not accommodate body weights of above 130 kg

A summary of participant iron, hepcidin and inflammatory status is presented in Table 4.2. Anaemia (haemoglobin <120 g/l) was detected in 10%; (*n*=11) of participants with none having AI (sTfR-F <1.00). Iron deficiency anaemia was identified in seven of the anaemic participants who simultaneously had serum ferritin and/or transferrin saturation below normal range. The degree of anaemia was mild (107-114 g/l) in the remaining four participants, three of which had borderline to

low mean corpuscular volume. Microcytosis with normal iron parameters raises the possibility of thalassemia trait. Pathology results outside of the reference range and which were deemed of potential clinical significance by the study medical officer (KSS) were forwarded to the participant's family physician with their consent.

Table 4.2. Summary of participant biochemistry

Biochemical marker	Mean ± SD or Median (IQR)	Prevalence of abnormality
Hb (g/l)	131 ± 8	
Hb <120 g/l		9.6%
Serum iron (µmol/l)	15.3 ± 6.8	
Serum iron <10.0 µmol/l		21.9%
Tsat (%)	22.5 ± 10.6	
Tsat <12.0%		14.0%
Serum ferritin (µg/l)	34.0 (37.0)	
Serum ferritin <15.0 µg/l		16.7%
sTfR (mg/l)	1.61 ± 0.44	
sTfR >2.39 mg/l		6.1%
sTfR-F	1.02 (0.65)	
sTfR-F 1.80-2.20		6.1%
sTfR-F >2.20		7.0%
sTfR-F <1.00 + Hb <115 g/l		0.0%
Hepcidin (ng/ml)	6.40 (7.85)	
Hepcidin >32.40 ng/ml		0.9%
CRP (mg/l)	3.58 (5.81)	
CRP >10.00 mg/l		14.0%

CRP, C-reactive protein; Hb, haemoglobin; IQR, interquartile range; SD, standard deviation; sTfR, soluble transferrin receptor; sTfR-F, soluble transferrin receptor-ferritin index; Tsat, transferrin saturation

Iron deficiency without anaemia (serum ferritin <15.0 µg/l) was present in just under 17% (*n*=19) of participants. As serum ferritin has been reported to be elevated in obesity,[214] sTfR and sTfR-F were also used to assess iron status. By using elevated sTfR (>2.39 mg/l), 6% of participants were identified as iron deficient. Alternatively, when sTfR-F >1.80 was used, iron deficiency was present in 13% of the cohort. Individuals identified as iron deficient by these alternative methods were also identified by hypoferritinaemia alone.

Median hepcidin was 6.40 ng/ml with only one participant presenting with hepcidin (37.40 ng/ml) above the reference range (in this participant, all other biochemical measures of iron and inflammatory status were normal). Hepcidin concentration was below 5.00 ng/ml in 80% of participants with hypoferritinaemia. Median CRP (3.58 mg/l) was within the assay specified normal range. Clinically elevated CRP (>10.00 mg/l) was observed in 14% of participants.

Multivariate multiple linear regression models were employed to assess the relationship between obesity, iron and inflammatory status. BMI and total body fat percentage were selected as representative predictors for the anthropometric and body composition measures respectively due to strong correlations between BMI and waist circumference (Pearson's r=0.839) and between BMI and each DXA variable (all Pearson's r>0.737). In the initial regression model (Table 4.3), BMI was a significant predictor of serum iron (coefficient=-0.379; SE=0.139; p=0.008), transferrin saturation (coefficient=-0.588; SE=0.222; p=0.009) and CRP (coefficient=0.127; SE=0.024; p<0.001). Following the inclusion of total body fat percentage as an additional predictor, the association between BMI and CRP remained (coefficient=0.149; SE=0.042; p=0.001), with no significant relationship observed between the DXA and iron variables. Use of contraceptive medication was a significant confounder of iron but not hepcidin status in both regression models. Its confounding effect on the first model is shown in Table 4.3.

Table 4.3. Results from the first multivariate model used to assess the associations between BMI, inflammation, iron and hepcidin status

Predictor variable	Wilks' lambda *p* value	Response variable *p* value							
		Hb	Serum iron	Tsat	Serum ferritin[a]	sTfR	sTfR-F[a]	Hepcidin[a]	CRP[a]
BMI	<0.001	0.907	0.008	0.009	0.039	0.833	0.264	0.318	<0.001
Contraception	<0.001	0.600	<0.001	0.006	0.134	0.002	0.007	0.147	<0.001
Ethnicity	0.026	0.124	0.417	0.161	0.553	0.011	0.196	0.723	0.914
PCOS	0.682	0.361	0.957	0.759	0.335	0.602	0.361	0.412	0.288
R^2		0.032	0.181	0.140	0.083	0.148	0.108	0.043	0.347

BMI, body mass index; CRP, C-reactive protein; Hb, haemoglobin; PCOS, polycystic ovary syndrome; sTfR, soluble transferrin receptor; sTfR-F, soluble transferrin receptor-ferritin index; Tsat, transferrin saturation

[a]Natural log transformation performed on the serum ferritin, sTfR-F, hepcidin and CRP variables

Secondary analyses of biochemical data stratified according to World Health Organization BMI classifications were also performed to examine iron, hepcidin and inflammatory status with increasing degrees of overweight and obesity (Table 4.4).[1] Classes II (35.0-39.9 kg/m^2) and III (≥40.0 kg/m^2) obesity were combined into a single category (defined as ≥35.0 kg/m^2) as the number of participants with higher levels of obesity were small, and this BMI threshold was reported to be associated with substantial iron disturbances in our recent systematic review.[214] Serum iron (p=0.029), transferrin saturation (p=0.042), ferritin (p=0.021) and CRP (p<0.001) were significantly different between BMI categories, with CRP showing a significant increment between categories two and three (p<0.001) in the post hoc analysis (Table 4.4).

Table 4.4. Biochemical differences between varying categories of overweight and obesity

BMI category	BMI (kg/m^2)	*n*	Age (years)	Biochemical marker							
				Hb (g/l)	Serum iron (µmol/l)	Tsat (%)	Serum ferritin[a] (µg/l)	sTfR (mg/l)	sTfR-F[a]	Hepcidin[a] (ng/ml)	CRP[a] (mg/l)
1	27.5-29.9	28	22.0 ± 2.1	132 ± 8	17.2 ± 6.3	25.9 ± 11.3	31.0 (34.0)	1.64 ± 0.47	1.03 (0.68)	5.25 (8.28)	1.62 (4.15)
2	30.0-34.9	48	21.7 ± 2.3	130 ± 10	16.0 ± 7.4	22.9 ± 10.9	30.5 (31.0)	1.61 ± 0.49	1.02 (0.80)	6.30 (7.70)	1.62 (4.15)
3	≥35.0	38	23.1 ± 2.3	130 ± 8	13.0 ± 6.0[b]	19.3 ± 9.1[b]	46.0 (49.0)[b]	1.58 ± 0.35	0.99 (0.43)	9.20 (9.78)	6.24 (8.17)[b,c]

Mean ± SD or median (interquartile range)

BMI, body mass index; CRP, C-reactive protein; Hb, haemoglobin; sTfR, soluble transferrin receptor; sTfR-F, soluble transferrin receptor-ferritin index; Tsat, transferrin saturation

[a]Natural log transformation performed on the serum ferritin, sTfR-F, hepcidin and CRP variables

[b]Biochemical concentration significantly different from BMI category 1 in Bonferroni-adjusted post hoc analysis ($p<0.05$)

[c]Biochemical concentration significantly different from BMI category 2 in Bonferroni-adjusted post hoc analysis ($p<0.05$)

DISCUSSION

This study describes iron status in a cohort of healthy overweight and obese young women by using a broad range of iron biomarkers. Unlike studies in older or more severely obese populations, selection of this cohort allowed for examination of obesity, inflammation and iron handling associations independent of potential confounding from significant comorbidity. Young overweight women are of specific interest as they are at risk of iron deficiency.[32] As suboptimal iron status even in the absence of anaemia is reported to adversely affect physical performance, mental health and cognitive function,[42, 76, 81] success with dietary and behavioural weight management programmes may be reduced secondary to the effects of iron deficiency.[214] Adverse effects from maternal iron deficiency anaemia on birth outcomes,[76] offspring cognitive development,[76] and increase risk for post-partum depression also highlight the importance of iron adequacy in this population.[85]

Results from this study indicate the presence of obesity-related inflammation, and mild iron disruptions such as reduced serum iron and transferrin saturation with increasing BMI. In line with data from the US National Health and Nutrition Examination Survey III,[10] no cases of AI were identified in our participants. Contrary to our expectations however, simple iron deficiency (hypoferritinaemia) was the most common iron-related abnormality (17%).

Median hepcidin in this cohort was well below the concentration reported in morbidly obese bariatric surgery candidates in the US (88.02 ng/ml).[9] Our hepcidin data was in fact, consistent with the non-obese (BMI <27.0 kg/m^2) control group of this US study as well as levels reported in healthy, non-anaemic premenopausal female blood donors.[9, 224] The lowest hepcidin concentrations were observed in those with the lowest iron stores. This is consistent with normal suppression of hepatic hepcidin secretion in the iron depleted state to allow for greater iron absorption and mobilisation.[74] It is likely that low iron intake was a major contributing factor to the high prevalence of simple iron deficiency observed,[32] although this cannot be confirmed as dietary iron intake was not assessed. Nevertheless, there was no apparent contribution of obesity-related

inflammation to the prevalence of iron deficiency observed, as evidenced by the modest rate (14%) of clinically elevated CRP, low median hepcidin and failure of sTfR and sTfR-F to detect a greater percentage of participants with iron deficiency.

Iron but not hepcidin status of young female military recruits has been reported previously.[30] This study concluded that a critical level of adiposity may be required for significant inflammation, hepcidin elevation and iron disturbances to occur which was partially supported by this study.[30] In our subset of participants with a higher degree of obesity (BMI >35.0 kg/m^2), mean hepcidin concentration remained in the lower normal range despite significantly greater CRP compared to those who were less obese. Given the healthy state of our cohort, this result indicates that a greater level of comorbidity (which is more often seen in older and severely obese individuals) may also be required to elicit substantial hepcidin elevation.

This study is primarily limited by the absence of dietary iron intake assessment. As baseline data collection procedures were primarily intended for screening and recruitment into the weight loss trial, assessment of dietary intake was only conducted upon commencement of the weight loss intervention. Iron intake variation however, is not likely to have affected the main finding of this study which was that obesity-related inflammation in the absence of comorbidity did not elicit significant hepcidin-mediated iron disruptions. Furthermore, body composition was only measured in a subset of participants and this may have compromised statistical significance of regression outcomes between the DXA and iron variables.

This study adds to the body of literature on excess weight, iron and inflammatory status in young women inclusive of the markers sTfR, sTfR-F and hepcidin. Results support a positive relationship between obesity and inflammation, with mild disruptions to some iron markers such as serum iron and transferrin saturation. However, simple iron deficiency as reflected by low ferritin remains the major iron-related abnormality in this cohort. There was no apparent contribution of inflammation and hepcidin to iron deficiency prevalence, which may be attributed to the absence of comorbidity

in this sample. As true iron deficiency has been shown to compromise reproductive and mental health, cognitive function and general wellbeing, this study highlights the importance of addressing simple iron deficiency in healthy overweight and obese young women.

ACKNOWLEDGEMENTS

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CHAPTER 5.

Impact of diet and weight loss on iron status in overweight and obese young women

PUBLICATION STATEMENT

The work presented in this chapter is largely based on the following publication:

Cheng HL, Griffin HJ, Bryant CE, Rooney KB, Steinbeck KS, O'Connor HT. Impact of diet and weight loss on iron and zinc status in overweight and obese young women. *Asia Pac J Clin Nutr* 2013; 22: 574-82 (Appendix D4)

As co-authors of the paper 'Impact of diet and weight loss on iron and zinc status in overweight and obese young women' we confirm that Hoi Lun Cheng has made the following contributions:

- Conception and design of the research – selected appropriate biochemical markers for analysis (with guidance from all co-authors)
- Data collection and biochemical analysis – recruited, collected data and conducted dietetic consultations in the final 27% of participants in the randomised controlled trial, as well as conducting all biochemical analyses involving ELISA
- Statistical analysis and interpretation of the findings – completed all statistical analyses and interpreted most of the findings presented (with guidance from all co-authors)
- Writing the paper and critical appraisal of content – primarily responsible for drafting and editing the manuscript following peer review (with guidance from all co-authors)

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ABSTRACT

Young women are at risk of iron deficiency with evidence suggesting obesity-related inflammation may exacerbate this risk. This study assessed iron and inflammatory status during a 12-month weight loss trial in young overweight (18-25 years; BMI ≥27.5kg/m^2) women randomised to a higher-protein, higher-haem iron (HPHI; 32% protein; 12.2 mg/day iron) or lower-protein, lower-haem iron (LPLI; 20%; 9.9 mg/day respectively) diet. Data: mean ± SD or median (range). In completers (HPHI: *n*=21; LPLI: *n*=15) HPHI participants showed higher ferritin [52.0 (7.0-139) vs. 39.0 (6.0-71.0) µg/l; *p*=0.021] and lower soluble transferrin receptor-ferritin index (sTfR-F) [0.89 (0.57-1.61) vs. 1.05 (0.44-3.40); *p*=0.024] although mean concentrations remained within normal range in both diets. C-reactive protein (CRP) [HPHI: 3.54 (0.08-13.26); LPLI: 4.63 (0.18-27.31) mg/l] and hepcidin [HPHI: 5.70 (1.00-25.60); LPLI: 8.25 (2.20-26.50) ng/ml] were not substantially elevated at baseline, and no between-diet differences were observed for CRP at the longitudinal time points. Compared to those with <5% weight loss, HPHI participants losing ≥10% initial weight showed lower sTfR-F [0.76 (0.40) vs. 1.03 (0.92); *p*=0.019] at six months. Impact of ≥10% weight loss on iron was more apparent in LPLI participants who exhibited greater serum iron (20.0 ± 12.7 vs. 13.5 ± 6.3 µmol/l; *p*=0.002), transferrin saturation (29.8 ± 20.3% vs. 19.4 ± 9.1%; *p*=0.001) and lower sTfR (1.24 ± 0.52 vs. 1.92 ± 0.68 mg/l; *p*=0.034) at 12 months. This study shows that normal iron status can be maintained during 12 months of energy restriction. In the absence of elevated baseline inflammation and hepcidin, a more favourable iron profile in those with ≥10% weight loss may reflect stronger compliance or the potential influence of iron regulatory mechanisms unrelated to significant hepcidin reduction.

Keywords: obesity, weight loss, young adult, iron

INTRODUCTION

Young women are at an increased risk of weight gain and obesity.[3] At this life stage, factors such as moving away from home, cohabitation and pregnancy are all associated with weight gain.[32, 34] In addition to the deleterious effects on metabolic and reproductive health,[39, 48] obesity is also linked with abnormal micronutrient metabolism, particularly for calcium, iron, zinc, vitamins A, D and folic acid.[68, 69, 225] These micronutrient disturbances may contribute to the aetiology of obesity, as supported by studies showing leptin suppression in zinc deficiency,[68, 226] or arise from excess adiposity such as the hypoferraemia of obesity.[9]

Obesity-related alterations to micronutrient concentration are of particular concern in young women as: (1) requirement for nutrients such as iron are higher in this population;[32] (2) obesity-related inflammation may contribute to reduced micronutrient status,[214, 227] although this was not observed for iron in our previous study conducted in a cohort of young women (Chapter 4);[228] and (3) unhealthy eating habits can compromise nutrient adequacy.[229] This was demonstrated in our aforementioned study of young overweight and obese women where despite minimal inflammatory- and hepcidin-mediated disturbances to iron metabolism, simple iron deficiency remained a prevalent (17%) health issue in this group.[228] Dietary iron intake, particularly haem iron, is also often reported to be low in young women,[230-232] and reduced food consumption during weight reduction can limit micronutrient intake (even when nutritionally balanced diets are used).[32] As iron has been reported as a limiting nutrient in energy-restricted diets for young women,[32] requirement for iron may be more easily met using animal-based, higher-protein meal plans that are nutrient dense and provide iron in the most bioavailable form.[32]

Current literature examining micronutrient (particularly iron) alteration during medium to longer term (minimum six months) diet-induced weight loss is limited. Many studies focus on bariatric surgery-induced weight loss which can affect nutrient absorption.[149] Some report favourable changes to iron and inflammatory status,[31, 137, 233] although the impact of different degrees of weight loss on these biomarkers is unknown. With reduced iron and elevated inflammatory status all

correlated to increasing BMI,[68] it is reasonable to assume that a larger reduction in excess weight will result in greater improvement of these markers.

The objective of this study was to compare the effect of two energy-restricted diets with contrasting protein and haem iron content as well as differing degrees of weight loss (<5% vs. ≥10%) induced by these diets on iron and inflammatory status in young overweight and obese women. We hypothesise that participants who lose ≥10% of initial weight with either diet will have superior iron status and a lower level of inflammation than those on the same diet but who were less successful (<5%) with weight loss. Additionally, amongst the successful participants, those who lose ≥10% of weight via the higher-protein, higher-haem iron diet would show a more favourable iron profile compared to participants on a diet containing lower protein and haem iron content.

METHODS

Study design

This study examined iron and inflammatory status in young overweight women who completed a 12-month single blind randomised controlled trial comparing the efficacy of two iso-energetically restricted diets (5600 kJ/day) differing in macronutrient and haem iron content (HPHI: higher-protein, higher-haem iron diet; LPLI: lower-protein, lower-haem iron diet) on weight loss (the study protocol and information for participants are presented in Appendices B1 and B4).[219] Due to the popularity of higher-protein diets, participants were blinded to the macronutrient distribution of the HPHI and LPLI diets to ensure a similar level of perceived therapeutic efficacy. This was achieved by informing participants that the two diets differed only in the type of meat prescribed (i.e. red or white). All participants were provided with an identical behaviour modification programme and a standard exercise prescription based on national activity guidelines.[234, 235] Participants attended counselling sessions weekly up to three months, fortnightly from three to six months and monthly from six to 12 months.

Ethics

This study was registered with the Australian New Zealand Clinical Trials Registry (ID: ACTRN12609000307202). All procedures were in accordance with the ethical standards of the Sydney South West Area Health Service Ethics Review Committee and the Human Research Ethics Committee of The University of Sydney. Signed and informed consent was obtained from all participants (the participant consent form used is presented in Appendix B5).

Participants

Healthy women aged 18-25 years with a measured BMI ≥27.5 kg/m^2 were recruited. Exclusion criteria were applied to minimise confounding on weight loss outcomes.[219] Since iron status was an important outcome of interest and animal protein was included in both diets, volunteers were ineligible if they had any haematological disorders, were anaemic (haemoglobin <120 g/l) or vegetarian. As iron deficiency without anaemia (serum ferritin <15.0 μg/l) is prevalent in this

population, iron deficient volunteers were advised to take the same ferrous fumarate supplement (10 mg elemental iron per day, five days a week) for three months and randomised upon normalisation of iron status and discontinuation of iron supplementation. Self-reported use of contraceptive medication was recorded due to its confounding effect on iron status.[236] Recruited participants were required to cease all dietary supplements and blood donation for the entire duration of the trial. Volunteers reporting recent (week prior) acute infection had venepuncture (at baseline, six and 12 months) rescheduled to avoid infection-related inflammation.

Dietary intervention, compliance and estimated dietary iron intake

The HPHI diet provided 32% protein, 41% carbohydrate, 25% fat and 12.2 mg iron per day, whereas the LPLI diet provided 20% protein, 58% carbohydrate, 21% fat and 9.90 mg iron. Haem iron, estimated as 40% of total iron from meat, poultry, and fish,[237] was calculated at 1.90 and 0.40 mg/day for the HPHI and LPLI diets respectively. Both diet plans met the Australian estimated average requirement (EAR), but not the recommended dietary intake (RDI) for iron (HPHI: 68%; LPLI: 55%).[79] Further details on the dietary prescriptions are presented in Appendices B6 and B7. A comparison of the nutrient composition of the two diets is shown in Appendix B8, and the seven-day diet models used to do so are presented in Appendices B9 and B10.

To monitor recent protein intake and compliance to the protein prescription, 24-hour urine samples were collected for measurement of urea/creatinine ratio (UCR).[238] As studies have reported the need for at least 12 days of dietary data to estimate iron intake confidently,[239] average iron intake was estimated using three-day food records collected at the end of the first, second, third, sixth and twelfth months totalling 15 days for trial completers. To maximise accuracy, training on how to record food intake using household measures was provided by a dietitian. Kitchen scales were also given to each participant. When required, recorded food portions were verified with the assistance of a visual aid.[240] Nutrient analysis was performed using FoodWorks Version 6.0.25175 (Xyris Software, Brisbane, Australia).

Anthropometry

Height (nearest 0.1 cm) and weight (nearest 0.1 kg; measured at every visit) were recorded using a wall-mounted stadiometer (Hyssna Limfog AB, Hyssna, Sweden) and digital platform scale (Teraoka Seiko, Tokyo, Japan) respectively. Waist circumference was measured at baseline, three, six and 12 months according to international guidelines.[220]

Biochemical analysis

Fasting morning venous blood samples were collected at baseline, six and 12 months. Haemoglobin, serum iron, transferrin saturation and serum ferritin were analysed at a nationally accredited commercial diagnostic laboratory. Reference ranges were 120-165 g/l for haemoglobin; 10.0-30.0 µmol/l for serum iron; 12.0-45.0% for transferrin saturation; and 15.0-165.0 µg/l for ferritin. Plasma was stored for analysis of soluble transferrin receptor (sTfR) and C-reactive protein (CRP) using commercial ELISA kits (R&D Systems, Minneapolis, USA). Median inter-assay coefficients of variation reported in the kits were 5.70% and 6.60% for sTfR and CRP respectively. Reference ranges were 0.74-2.39 mg/l for sTfR and 0.110-4.52 mg/l for CRP, with clinically elevated CRP defined as >10.0 mg/l.[53] sTfR results were converted from nmol/l to mg/l.[181] The sTfR-ferritin index (sTfR-F), described as a useful indicator of iron status in inflammation was calculated.[94] Plasma hepcidin was measured only at baseline using an on-line extraction coupled to liquid chromatography-tandem mass spectrometry method with the Xevo TQ MS (Waters Corporation, Milford, USA).[221] Inter-assay accuracy was 95.0% with an 8.20% coefficient of variation. Assay sensitivity was 2.00 ng/ml with values below the detectable range defined as 1.00 ng/ml. A reference range of 1.92-32.4 ng/ml was used.[222, 223]

Statistical analysis

Statistical analysis was performed using PASW Statistics 18 for Windows (IBM Corporation, Armonk, USA). As weight loss was the primary outcome of this trial, sample size was calculated on the basis of detecting a weight difference between the diet groups. Based on a significance level of 0.05 and 80% power, 28 participants were required in each diet to detect a 5 kg weight

difference. With attrition estimated at approximately 20%,[241] a recruitment goal of 70 participants was established.

In this study, data were analysed only for participants who completed to six and 12 months to determine true biochemical alterations associated with the dietary intervention which were not based on statistical assumptions used for treating missing data. Participants were categorised into non-responder (<5% loss of initial weight) and responder (≥10% loss of initial weight) groups within each diet to examine the influence of weight loss success on the biochemical markers. These cut-offs were selected as they have previously been used to reflect weight loss success,[242] and 10% weight reduction is generally associated with important improvements in chronic disease risk factors.[243] Categorising participants according to these cut-offs also allowed for a clear distinction between the two degrees of weight loss.

Variables were assessed for normality with natural log transformations performed on the serum ferritin, sTfR-F, CRP and hepcidin variables. Baseline age, weight, BMI and the dietary compliance measures (UCR and dietary iron intake) were compared between the two diets using unpaired t-tests. Paired t-tests were used to assess anthropometric changes (from baseline) within each diet. Pearson's tests were used to correlate dietary iron intake against serum ferritin concentration. Repeated measures ANOVA (adjusted for baseline BMI) was used to compare anthropometric changes between the diets. Biochemical differences between the diets and responder groups at baseline and the longitudinal time points (where the baseline value of the dependent variable was included as an additional covariate) were assessed using ANCOVA. All ANCOVA analyses were adjusted for baseline BMI and contraceptive medication, whereas ANCOVA tests comparing serum iron, transferrin saturation and ferritin concentrations were also adjusted for lnCRP (to account for potential inflammatory differences). Significance was set to $p<0.05$, with data presented as a percentage, mean ± SD or median (range).

RESULTS

A total of 71 participants (HPHI: *n*=36; LPLI: *n*=35) were recruited to the trial with 44 (HPHI: *n*=24; LPLI: *n*=20) completing to six and 36 (HPHI: *n*=21; LPLI: *n*=15) completing to 12 months. Reasons for high attrition in this trial have been described (Appendices D4 and D5).[219, 244] Results reported in this study refer to six- and 12-month completers only. All baseline characteristics were similar between diets with the exception of higher BMI in HPHI participants (Table 5.1). Use of contraceptive medication was reported by 36%. At recruitment (prior to pre-trial iron supplementation), iron deficiency (ferritin <15.0 µg/l) was identified in six participants (17%; HPHI: *n*=4; LPLI: *n*=2). All participants presented with baseline hepcidin concentration within normal range and clinically elevated CRP (>10.0 mg/l) was observed in five (HPHI: *n*=1; LPLI: *n*=4) participants. As baseline hepcidin was unremarkable indicating minimal likelihood of substantial hepcidin-mediated iron disturbances, measurement of this marker at six and 12 months was not pursued. Mean values for all biomarkers were within normal range throughout the trial.

Table 5.1. Comparison of participant age, anthropometric and biochemical characteristics between diets at baseline

Baseline characteristic	**6-month completers at baseline**			**12-month completers at baseline**		
	HPHI diet (*n*=24)	**LPLI diet (*n*=20)**	***p* value**	**HPHI diet (*n*=21)**	**LPLI diet (*n*=15)**	***p* value**
Age (years)	22.4 ± 2.3	21.8 ± 2.2	0.339	22.4 ± 2.3	22.1 ± 2.1	0.678
Weight (kg)	95.8 ± 9.1	92.9 ± 11.7	0.368	96.2 ± 8.9	92.5 ± 11.6	0.291
Ethnicity (%)						
European	83.3	75.0		81.0	86.7	
Asian	4.17	10.0		4.76	6.67	
African	0.00	5.00		0.00	6.67	
South American	4.17	10.0		4.76	0.00	
Other	28.33	0.00		9.52	0.00	
BMI (kg/m^2)	34.3 ± 3.5	32.2 ± 3.6	0.063	34.6 ± 3.4	32.2 ± 3.6	0.047
Hb (g/l)	131 ± 8	130 ± 9	0.797	132 ± 8	130 ± 10	0.456
Serum iron (µmol/l)	13.5 ± 6.2	16.3 ± 7.2	0.344	14.0 ± 6.2	15.5 ± 6.2	0.749
Tsat (%)	19.5 ± 8.5	24.7 ± 12.2	0.147	20.5 ± 8.4	23.9 ± 11.4	0.393
Serum ferritin (µg/l)[a]	33.0 (89.0)	37.0 (185.0)	0.286	34.0 (80.0)	35.0 (82.0)	0.985
<15.0 µg/l (%)	16.7	10.0		9.52	13.3	
sTfR (mg/l)	1.56 ± 0.34	1.67 ± 0.61	0.445	1.55 ± 0.35	1.73 ± 0.66	0.356
sTfR-F index[a]	1.01 (1.52)	1.02 (1.60)	0.652	0.95 (1.50)	1.01 (1.42)	0.839
Hepcidin (ng/ml)[a]	5.70 (24.6)	8.25 (24.3)	0.123	5.80 (24.6)	7.40 (24.0)	0.298
CRP (mg/l)[a]	3.55 (13.2)	4.63 (27.1)	0.690	3.51 (13.2)	4.75 (13.0)	0.626
>10.0 mg/l (%)	4.17	20.0		4.76	13.3	

Mean ± SD or median (range)

CRP, C-reactive protein; HPHI, higher-protein and higher-haem iron; LPLI, lower-protein and lower-haem iron; sTfR, soluble transferrin receptor; sTfR-F, soluble transferrin receptor-ferritin index; Tsat, transferrin saturation

Reference ranges – Hb: 115-165 g/l; serum iron: 10.0-30.0 µmol/l; Tsat: 12.0-45.0%; serum ferritin: 15.0-165 µg/l; sTfR: 0.74-2.39 mg/l; CRP: 0.11-4.52 mg/l; hepcidin: 1.92-32.40 ng/ml

[a]Natural log transformation performed on the serum ferritin, sTfR-F, hepcidin and CRP variables

Weight loss

Both diets independently led to significant weight loss at six (HPHI: 9.31 ± 8.87%, $p<0.001$; LPLI: 5.08 ± 5.99%; $p=0.001$) and 12 months (HPHI: 9.79 ± 13.0%, $p=0.003$; LPLI: 4.56 ± 7.15%, $p=0.027$). Between the diets, weight loss in HPHI was approximately double that of LPLI, although this was not statistically significant (Figure 5.1). Similar between-diet outcomes were observed for waist circumference at six (HPHI: -7.8 ± 1.3; LPLI: -3.7 ± 1.0 cm; $p=0.30$) and 12 months (HPHI: -7.9 ± 1.8; LPLI: -2.4 ± 0.8 cm; $p=0.36$). Other anthropometric outcomes have also been published (Appendix D5).[219] At six months, there were 18 non-responders (HPHI: $n=8$; LPLI: $n=10$) who had lost <5% of initial weight while 13 responders (HPHI: $n=10$; LPLI: $n=3$) achieved losses of ≥10%. By 12 months, 27 of the 36 participants who completed the trial had lost either <5% (HPHI: $n=6$; LPLI: $n=8$) or ≥10% (HPHI: $n=9$; LPLI: $n=4$) of weight. The distribution of responders and non-responders was not significantly different between the diets (six months: $p=0.127$; 12 months: $p=0.153$).

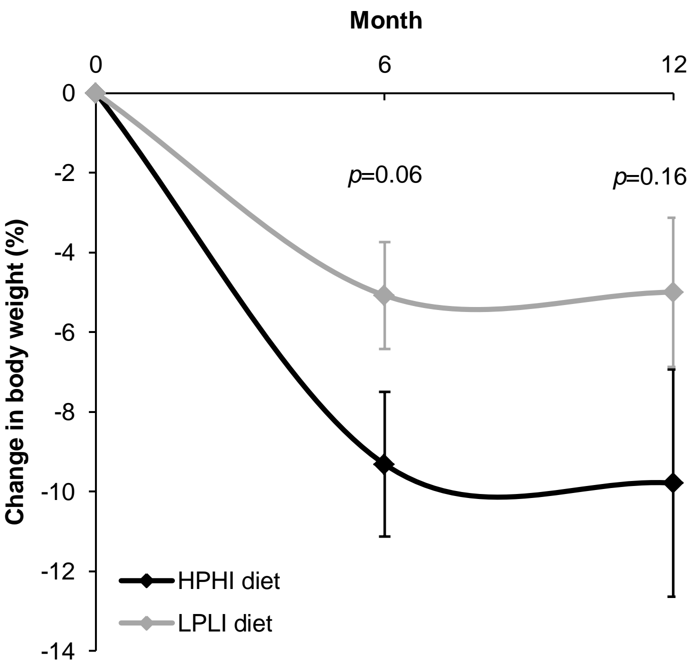


Figure 5.1. Percent loss of initial weight between the diets (Mean ± SE; HPHI, higher-protein and higher-haem iron; LPLI, lower-protein and lower-haem iron)

Dietary compliance and estimated iron intake

No significant UCR difference was observed between the diets at baseline (HPHI: 33.5 ± 7.43; LPLI: 30.6 ± 6.94; $p=0.194$). At six months, UCR was significantly higher on the HPHI diet (HPHI: 38.4 ± 9.67; LPLI: 30.5 ± 7.31; $p=0.023$) which was consistent with the protein prescription. However, this difference was no longer significant at 12 months (HPHI: 35.0 ± 7.40; LPLI: 33.7 ± 5.49; $p=0.547$), indicating reduced compliance.

As expected, mean estimated iron intake in HPHI was significantly higher than the LPLI diet (all $p<0.001$) and was close to the dietary prescription for both groups (Table 5.2). No significant intake differences were observed between responder groups in either diet (Table 5.3). All HPHI participants met the EAR for dietary iron while a number of participants on the LPLI diet did not (Table 5.2). Pearson's tests revealed no significant correlations between dietary iron intake and serum ferritin ($r=0.109$; $p=0.547$ at 12 months).

Table 5.2. Comparison of biochemistry and micronutrient intake between the diets at six and 12 months

Biochemical or intake variable	**6 months**		***p* value**	**12 months**		***p* value**
	HPHI diet (*n*=24)	**LPLI diet (*n*=20)**		**HPHI diet (*n*=21)**	**LPLI diet (*n*=15)**	
Hb (g/l)	130 ± 11	128 ± 7	0.318	132 ± 12	126 ± 11	0.075
Serum iron (µmol/l)	14.3 ± 6.4	15.0 ± 4.5	0.564	15.6 ± 5.3	15.5 ± 8.8	0.242
Tsat (%)	21.9 ± 10.3	22.1 ± 7.4	0.410	23.7 ± 8.6	23.5 ± 14.4	0.244
Serum ferritin (µg/l)[a]	46.0 (107)	37.0 (141)	0.052	52.0 (132)	39.0 (65.0)	0.021
<15.0 µg/l (%)	12.5	5.00		9.52	20.0	
sTfR (mg/l)	1.50 ± 0.39	1.72 ± 0.69	0.335	1.46 ± 0.30	1.68 ± 0.71	0.145
sTfR-F index[a]	0.80 (1.58)	0.98 (2.55)	0.057	0.89 (1.04)	1.05 (2.96)	0.024
CRP (mg/l)[a]	2.36 (14.9)	2.65 (21.8)	0.597	2.22 (25.8)	3.95 (12.0)	0.784
>10.0 mg/l (%)	12.5	20.0		14.3	6.67	
Iron intake (mg/day)	11.9 ± 2.7	8.3 ± 1.6	<0.001	11.4 ± 1.9	8.5 ± 1.5	<0.001
<EAR (%)	0.00	30.0		0.00	26.7	
<RDI (%)	95.8	95.0		90.5	93.3	

Mean ± SD or median (range)

CRP, C-reactive protein; EAR, estimated average requirement; HPHI, higher-protein and higher-haem iron; LPLI, lower-protein and lower-haem iron; sTfR, soluble transferrin receptor; sTfR-F, soluble transferrin receptor-ferritin index; RDI, recommended dietary intake; Tsat, transferrin saturation

Reference ranges – Hb: 115-165 g/l; serum iron: 10.0-30.0 µmol/l; Tsat: 12.0-45.0%; serum ferritin: 15.0-165 µg/l; sTfR: 0.74-2.39 mg/l; CRP: 0.11-4.52 mg/l

Nutrient reference values for iron – EAR: 8.00 mg/day, RDI: 18.0 mg/day

[a]Natural log transformation performed on the serum ferritin, sTfR-F and CRP variables

Iron and inflammatory status between diets

Comparison of iron and inflammatory markers between the diets is presented in Table 5.2. At 12 months, HPHI participants showed changes reflective of increased iron stores with significantly higher ferritin [HPHI: 52.0 (132) vs. LPLI: 39.0 (65.0) µg/l; p=0.021] and lower sTfR-F [HPHI: 0.89 (1.04) vs. LPLI: 1.05 (2.96); p=0.024]. In contrast, these markers remained stable in LPLI participants throughout the trial. CRP decreased from baseline in both diet groups, although no significant between-diet differences were observed. Use of contraceptive medication was a significant confounder of several iron markers including serum ferritin (p=0.032), sTfR (p=0.019) and sTfR-F (p=0.001) (confounding effect on 12-month data reported for brevity).

Of the six (HPHI: n=4; LPLI: n=2) participants identified as iron deficient at recruitment, three (HPHI: n=2; LPLI: n=1) were able to maintain normal ferritin following 12 months of intervention. Conversely, in those with normal baseline iron status, four participants (HPHI: n=2; LPLI: n=2) developed iron deficiency upon trial completion and had to be referred to their family physician for follow-up treatment. The number of HPHI participants presenting with low ferritin decreased from baseline to 12 months whereas the opposite was observed in the LPLI diet group (Table 5.1 and Table 5.2).

Iron and inflammatory status between responder groups

Table 5.3 shows the biochemical comparisons between diet non-responders (<5% weight loss) and responders (≥10% weight loss). Within each diet at six months, HPHI responders showed significantly lower sTfR-F [non-responders: 1.03 (0.92) vs. responders: 0.76 (0.40); p=0.019] while LPLI responders exhibited a trend for higher ferritin [non-responders: 27.0 (133) vs. responders: 69.0 (28.0) µg/l; p=0.053] and significantly lower sTfR [non-responders: 1.73 ± 0.72 vs. responders: 1.59 ± 0.93 mg/l; p=0.011]. By 12 months, the tendency for those with ≥10% weight loss to show a more favourable iron profile was only apparent in LPLI responders who had higher serum iron (non-responders: 13.5 ± 6.3 vs. responders: 20.0 ± 12.7 µmol/l; p=0.002), transferrin saturation (non-responders: 19.4 ± 9.1%; responders: 29.8 ± 20.3%; p=0.001) and lower sTfR (non-

responders: 1.92 ± 0.68 vs. responders: 1.24 ± 0.52 mg/l; p=0.034). The HPHI diet appeared to have a greater impact on CRP reduction with successful weight loss than LPLI (p=0.085 at 12 months), although not significantly so. Similar to the between-diet analysis, contraceptive medication was a significant confounder for transferrin saturation (p=0.031) in the HPHI diet and for serum iron (p=0.001), transferrin saturation (p=0.001) in the LPLI diet group.

Table 5.3. Comparison of biochemistry and micronutrient intake between non-responders (<5% loss of initial weight) and responders (≥10% loss of initial weight) within the diets at six and 12 months

Biochemical or intake variable	HPHI diet						LPLI diet					
	Weight loss at 6 months		*p* value	Weight loss at 12 months		*p* value	Weight loss at 6 months		*p* value	Weight loss at 12 months		*p* value
	<5% (*n*=8)	≥10% (*n*=10)		<5% (*n*=6)	≥10% (*n*=9)		<5% (*n*=10)	≥10% (*n*=3)		<5% (*n*=8)	≥10% (*n*=4)	
Hb (g/l)	128 ± 11	129 ± 9	0.590	131 ± 14	133 ± 10	0.840	129 ± 7	134 ± 7	0.613	127 ± 11	129 ± 6	0.060
Serum iron (μmol/l)	13.6 ± 8.5	15.8 ± 5.0	0.400	16.7 ± 6.3	14.1 ± 3.3	0.769	14.5 ± 3.7	17.0 ± 4.6	0.453	13.5 ± 6.3	20.0 ± 12.7	0.002
Tsat (%)	19.6 ± 11.8	25.5 ± 9.1	0.878	24.7 ± 10.5	23.2 ± 5.7	0.608	21.9 ± 7.0	25.3 ± 9.0	0.161	19.4 ± 9.1	29.8 ± 20.3	0.001
Serum ferritin (μg/l)[a]	42.0 (77.0)	59.5 (91.0)	0.137	51.0 (75.0)	70.0 (104.0)	0.172	27.0 (133.0)	69.0 (28.0)	0.053	27.0 (52.0)	51.5 (16.0)	0.263
<15.0 μg/l (%)	0.00	0.00		33.3	0.00		0.00	0.00		50.0	0.00	
sTfR (mg/l)	1.47 ± 0.37	1.32 ± 0.25	0.241	1.48 ± 0.31	1.32 ± 0.26	0.658	1.73 ± 0.72	1.59 ± 0.93	0.011	1.92 ± 0.68	1.24 ± 0.52	0.034
sTfR-F index[a]	1.03 (0.92)	0.76 (0.40)	0.019	1.04 (0.99)	0.63 (0.46)	0.145	1.06 (2.14)	0.62 (0.89)	0.050	1.12 (2.52)	0.67 (0.69)	0.223
CRP (mg/l)[a]	5.43 (14.69)	0.92 (12.67)	0.145	3.78 (17.33)	1.06 (25.57)	0.085	2.17 (13.82)	5.16 (21.16)	0.310	3.45 (11.97)	5.14 (7.74)	0.830
>10.0 mg/l (%)	0.25	10.0		16.7	11.1		20.0	33.3		12.5	0.00	
Iron intake (mg/day)	11.8 ± 2.5	12.3 ± 3.4	0.953	12.3 ± 2.9	11.0 ± 1.1	0.244	8.9 ± 1.8	7.7 ± 1.9	0.299	8.7 ± 0.9	7.9 ± 1.8	0.911
<EAR (%)	0.00	0.00		0.00	0.00		10.0	33.3		25.0	25.0	
<RDI (%)	100	90.0		83.3	88.9		90.0	100		87.5	100	

Mean ± SD or median (range)

CRP, C-reactive protein; EAR estimated average requirement; HPHI, higher-protein and higher-haem iron; LPLI, lower-protein and lower-haem iron; sTfR, soluble transferrin receptor; sTfR-F, soluble transferrin receptor-ferritin index; RDI, recommended dietary intake; Tsat, transferrin saturation

Reference ranges – Hb: 115-165 g/l; serum iron: 10.0-30.0 μmol/l; Tsat: 12.0-45.0%; serum ferritin: 15.0-165 μg/l; sTfR: 0.74-2.39 mg/l; CRP: 0.11-4.52 mg/l

Nutrient reference values for iron – EAR: 8.00 mg/day, iron RDI: 18.0 mg/day

[a]Natural log transformation performed on the serum ferritin, sTfR-F and CRP variables

DISCUSSION

This study evaluated the impact of two energy-restricted diets with contrasting protein and haem iron content on iron and inflammatory markers in young women. Results showed that during medium to longer term energy restriction, the HPHI diet had a propensity for increasing iron stores more readily although serum ferritin was maintained within normal limits for most individuals on both diets. Responders on the LPLI diet who lost ≥10% of initial weight also showed a more favourable iron profile compared to non-responders who lost <5% of initial weight.

Iron homeostasis in adults is essential for tissue oxygen delivery, cell growth and immune function,[76, 245] with inadequate body iron impacting adversely on physical performance, work productivity and cognitive function.[76, 245] The tendency for greater iron stores (ferritin) in the HPHI diet group was comparable to a similar study conducted in middle-aged women.[238] The substantial proportion of individuals identified with iron deficiency (17%) at recruitment also highlights the clinical relevance of HPHI diets for maintaining iron status, particular in young women with higher requirements for this nutrient.

Meeting age and gender-appropriate nutrient recommendations can be difficult for young women undergoing energy restriction.[32] This was reflected in our study whereby the HPHI and LPLI diets did not reach the RDI for iron, even after detailed modelling and manipulation of the meal plans. In women with greater nutritional needs such as those who experience high menstrual iron losses,[7] adopting energy-restricted diets may become problematic. The persistence and new development of iron deficiency in a number of our participants was similar to a previous study describing an increase in nutrient deficiency with weight reduction via a nutritionally complete formula diet.[246] This highlights the importance of careful nutritional planning and monitoring during dietary weight management in young women, particularly if energy restriction is sustained over a prolonged period of time.

Contrary to our expectations, mean CRP and hepcidin concentration were not greatly elevated at baseline, which was most likely due to the modest obesity and absence of comorbidities in this cohort.[247] Hence, diet therapy and weight loss would only have brought about a minor (if any) decrease in these markers. This finding indicates that unlike previous studies,[31, 117] significant weight loss associated reductions in hepcidin may only occur in individuals who are severely obese and/or burdened with comorbid conditions and greater levels of inflammation.[31, 214, 248] Despite low baseline hepcidin, higher circulating iron levels were still observed in LPLI responders who lost ≥10% of weight. While a superior iron profile in LPLI responders vs. non-responders is likely to be the result of better diet quality and compliance, these findings do not rule out a possible standalone benefit of weight loss on iron status that is independent of significant hepcidin reduction. Higher haem iron intake may have masked the iron-related benefits of weight loss, which explains the absence of significant differences between responder groups in the HPHI diet.

This study enrolled young women, a nutritionally vulnerable population for which limited clinical research is available. Limitations of this study include the high rate of attrition (leading to small sample size) and absence of longitudinal hepcidin assessment. Interestingly, similar difficulties with attrition was recently reported in a systematic review of weight management interventions in young adults,[37] highlighting the inherent challenges of conducting research in this population.

In conclusion, this study supports the use of well-designed energy-restricted diets for weight loss and reasonable maintenance of iron status in young, healthy, overweight and obese women. Despite insignificant changes to inflammatory status, loss of ≥10% initial weight was associated with a superior iron profile which was evident under conditions of limited haem iron intake. This may reflect greater diet quality and compliance, or the influence of iron regulatory pathway(s) independent of significant inflammatory hepcidin reduction.

ACKNOWLEDGEMENTS

We would like to acknowledge Ms Zahra Munas for the clinical work with participants in this study.

CONFLICT OF INTEREST AND FUNDING DISCLOSURE

This study was supported by Meat and Livestock Australia. Authors declare that Meat and Livestock Australia did not have any influence on the results or conclusions drawn.

CHAPTER 6.

A candidate gene approach to identifying differential iron responses of overweight and obese young women to an energy-restricted haem iron-rich diet

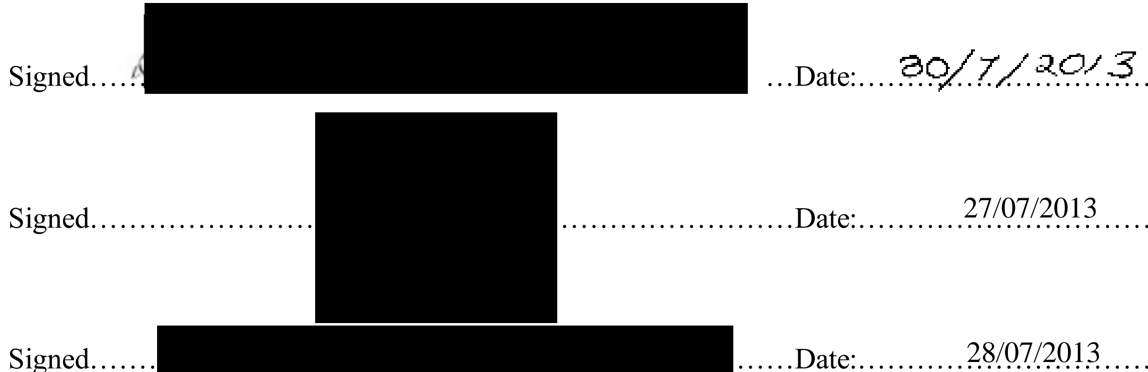
PUBLICATION STATEMENT

The work presented in this chapter is largely based on the following brief report that has been submitted for publication at the time of examination:

Cheng HL, Hancock DP, Rooney KB, Steinbeck KS, Griffin HJ, O'Connor HT. A candidate gene approach to identifying differential iron responses of young overweight and obese women to an energy-restricted haem iron-rich diet. *Eur J Clin Nutr* (in preparation for resubmission)

As co-authors of the paper 'A candidate gene approach to identifying differential iron responses of young overweight and obese women to an energy-restricted haem iron-rich diet' we confirm that Hoi Lun Cheng has made the following contributions:

- Conception and design of the research – selected appropriate biochemical markers for analysis (with guidance from all co-authors), researched and selected target SNP, designed oligonucleotide primers and conducted initial sequencing work required for genotype determination (with guidance from DPH)
- Data collection, biochemical and genetic analysis – recruited and collected data for the final 27% of participants in the randomised controlled trial, conducted all biochemical analyses involving ELISA, conducted all genotype determination assays
- Statistical analysis and interpretation of the findings – completed all statistical analyses and interpreted most of the findings presented (with guidance from all co-authors)
- Writing the paper and critical appraisal of content – primarily responsible for drafting and editing the manuscript following peer review (with guidance from all co-authors)



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ABSTRACT

Although young women following energy-restricted diets are at increased risk of iron deficiency, weight management trials rarely examine the longitudinal impact of genetic polymorphisms on iron metabolism. This exploratory study examined how the *TMPRSS6* rs855791 polymorphism influenced iron and hepcidin status in 76 young (18-25 years) women with BMI ≥27.5kg/m^2, and in a subset of this cohort, iron responses to two 12-month iso-energetically restricted diets [higher-protein, higher-haem iron (HPHI) vs. lower-protein, lower-haem iron (LPLI) diet] were explored. While all mean/median values were within normal range, individuals hetero- or homozygous for the T allele showed significantly lower serum iron (coefficient=-3.116; p=0.047) and higher hepcidin (coefficient=0.607; p=0.023) at baseline than those homozygous for the alternative C allele. Following 12 months of dieting, T hetero- and homozygotes were less able to maintain serum iron (p=0.028) and transferrin saturation (p=0.043) while on the HPHI, but not LPLI diet. Whilst rs855791 did not influence ferritin concentration (HPHI: p=0.89; LPLI: p=0.91), a significant diet × time effect was observed (p=0.026). In conclusion, this study confirms the iron-lowering effect of the T allele on serum iron and hepcidin at baseline. Lower serum iron and transferrin saturation observed in T hetero- and homozygotes following the HPHI diet suggest that rs855791-associated iron variability may be dependent on haem iron and/or protein intake. These small but significant variations highlight the need for studies incorporating more polymorphisms and dietary patterns to evaluate gene-nutrient interactions that affect iron status during weight management.

Keywords: TMPRSS6, iron deficiency, weight loss, young women

INTRODUCTION

Young women are at increased risk of iron deficiency.[6] Recent evidence indicates that obesity may exacerbate this risk secondary to inflammation and elevated hepcidin, resulting in unfavourable changes to iron metabolism.[8, 18, 249] However, inflammation and hepcidin were not found to be significant contributors to iron abnormality in a recent study of young healthy overweight and obese women (Chapter 4).[228] The primary issue observed was simple iron deficiency, suggesting that iron disruptions related to excess adiposity may be dependent on the degree of obesity and presence of obesity-related comorbidities.[214, 228] Although iron deficiency is globally the most common nutrient deficiency with a high prevalence amongst young women,[76] the influence of genetics on iron is rarely explored in longitudinal clinical trials, particularly in weight management interventions involving energy restriction where iron intake may be limited.[32]

The rs855791 variant in the transmembrane protease, serine 6 (*TMPRSS6*) gene is a single nucleotide polymorphism (SNP) that has been identified to be strongly associated with iron status in healthy populations.[21, 26] The *TMPRSS6* gene encodes matriptase-2, a transmembrane serine protease that is involved in negative regulation of hepcidin production.[250] The SNP rs855791 involves a non-synonymous cytosine (C) to thymine (T) nucleotide substitution that corresponds to an alanine to valine change in the amino acid sequence. Mutations or variations in the *TMPRSS6* gene (such as SNP rs855791) resulting in blunted matriptase-2 activity, are believed to cause increased hepcidin expression and greater attenuation of iron absorption.[250] Recent evidence indicates that rs855791 may also exert an unidentified hepcidin-independent effect on iron metabolism.[175]

Genome-wide association studies have shown significant associations between rs855791 and various haematological and iron traits.[20, 26, 172] However, no research to date has reported the effect of rs855791 on iron responses to dietary protein and haem iron manipulation during restricted energy intake. In order to gain a further insight into the impact of rs855791 on iron metabolism in

young overweight and obese women, we performed a number of exploratory analyses by: (1) comparing baseline iron and hepcidin status between young women of differing rs855791 genotype, and (2) assessing how rs855791 genotype may influence iron-related responses to two iso-energetically restricted diets with contrasting protein and haem iron content.

METHODS

Study design and participants

This study reports results from young overweight and obese women recruited to two previous studies (a larger cross-sectional study and a subsequent randomised controlled trial) from which sufficient DNA was extracted from frozen blood samples. In the cross-sectional study (Chapter 4), iron, inflammatory and hepcidin status were analysed in a community sample of healthy women aged 18-25 years with a measured BMI ≥27.5 kg/m^2.[228] A subset of these participants then went on to complete a randomised controlled trial comparing the efficacy of two diets with contrasting macronutrient and haem iron content (Chapter 5). One of these diets was higher in protein and haem iron (HPHI diet), whereas the other was an iso-energetically restricted diet containing lower protein and haem iron content (LPLI diet).[251] Exclusions were applied during participant recruitment to minimise confounding on weight, iron and inflammatory marker outcomes (Appendix B1).[228, 251]

Briefly, the HPHI diet provided 32% protein; 41% carbohydrate; 25% fat and 12.2 mg iron daily, whereas the LPLI diet provided 20% protein; 58% carbohydrate; 21% fat and 9.9 mg iron (Appendix B8). Haem iron, estimated as 40% of total iron from meat, poultry, and fish,[237] was calculated at 1.90 and 0.40 mg/day for the HPHI and LPLI diets respectively. Both diets met the Australian estimated average requirement but not the recommended dietary intake for iron (HPHI: 68%; LPLI: 55%).[79] Methods used for estimating dietary iron intake are described in Chapter 5.

Biochemical analysis

Haemoglobin, serum iron, transferrin saturation and serum ferritin were analysed at a nationally accredited commercial diagnostic laboratory. Plasma was frozen for retrospective analysis of soluble transferrin receptor (sTfR), hepcidin and C-reactive protein (CRP). Haemoglobin was measured using the Sysmex Instrument (Roche Diagnostics Australia, Sydney, Australia), with anaemia defined as haemoglobin <120 g/l. Serum iron, transferrin saturation and serum ferritin were measured using the Roche Modular E170 Immunoassay Analyser (Roche Diagnostics

Australia, Sydney, Australia). Reference ranges used were 120-165 g/l for haemoglobin; 10.0-30.0 µmol/l for serum iron; 12.0-45.0% for transferrin saturation; and 15.0-165.0 µg/l for serum ferritin. Plasma sTfR and CRP were analysed using commercial ELISA kits (R&D Systems, Minneapolis, USA). Reference ranges used were 0.74-2.39 mg/l for sTfR; and 0.11-4.52 mg/l for CRP. sTfR values were converted from nmol/l to mg/l.[181] Plasma hepcidin was measured only at baseline by on-line extraction coupled to liquid chromatography-tandem mass spectrometry using the Xevo TQ MS (Waters Corporation, Milford, USA).[221] Inter-assay accuracy and coefficient of variation were 95% and 8.2% respectively. Assay sensitivity was 2.00 ng/ml, with values below the detectable range defined as 1.00 ng/ml. The reference range used for hepcidin was 1.92-32.40 ng/ml.[222, 223]

DNA analysis

DNA was extracted from frozen packed cell samples using a Wizard® Genomic DNA Extraction Kit (Promega Corporation, Madison, USA) in accordance with the manufacturer's instructions.

A pair of forward and reverse oligonucleotide primers intended to amplify 167 base pairs surrounding the SNP rs855791 were generated using the primer design program Primer3,[252] and verified using an in-silico polymerase chain reaction (PCR) tool.[253] The custom primers were obtained from Sigma-Aldrich Australia.

The 167 base pair sequence surrounding the target SNP was amplified and purified in five of the 76 DNA samples by PCR and gel electrophoresis respectively. Gel-purified PCR products (or amplicons) were sent to an accredited national research facility (Australian Genome Research Facility Ltd, Sydney, Australia) for sequence determination using Sanger sequencing techniques (Applied Biosystems, Foster City, USA). Sequencing results were verified against published data with a sequence alignment tool.[254] The remaining 71 genomic DNA samples were genotyped using TaqMan® SNP Allelic Discrimination on the 7500 Fast Real-time PCR System platform (Applied Biosystems, Foster City, USA). Three Sanger-sequenced DNA samples (one of each zygosity) were selected as positive controls in the TaqMan® genotyping analyses. Please refer to Appendix

C1 for further details on the: DNA extraction method; custom primer and DNA target sequences; PCR reaction set-up; amplicon purification method; and sequence determination and verification procedures. A broad explanation of the TaqMan® SNP Allelic Discrimination technique and output is presented in Appendices C2 and C3.

Ethics

Both the cross-sectional study and randomised controlled trial were registered with the Australian New Zealand Clinical Trials Registry (ACTRN12613000072718 and ACTRN12609000307202) and approved by the Ethics Review Committee of the Sydney South West Area Health Service and the University of Sydney Ethics Committee. Signed informed consent was obtained from all participants (the participant consent forms used for both studies are presented in Appendices B3 and B5).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20 (IBM Corporation, Armonk, USA). Variables were assessed for normality with natural log transformations performed where appropriate. Descriptive data are presented as percentages, mean ± SD or median (interquartile range). To explore the differences in iron marker concentrations between differing genotype groups, univariate ANOVA was used to analyse the cross-sectional data based on the co-dominant (CC vs. CT vs. TT) and dominant [CC vs. combined CT and TT (defined as the 'mixed group')] genetic models. Confounders including BMI, waist circumference, lnCRP, ethnicity, self-reported use of hormonal contraceptives and polycystic ovary syndrome were incorporated as covariates. Using the longitudinal data, the impact of SNP rs855791 on iron alterations across three key time points of the clinical trial (baseline, six and 12 months) was assessed using the dominant genetic model only. This was performed using repeated measures ANOVA with effect size reported as partial eta-squared (η_p^2). Significant confounders including percent loss of initial weight at 12 months, mean dietary iron intake across 12 months and contraceptive medication use were included as covariates in all repeated measures analyses.

RESULTS

Participants

From the 114 participants recruited to the larger cross-sectional study, sufficient DNA for genotyping was extracted from 76 participants. T was the less frequent allele (0.44) with genotypic distribution in Hardy-Weinberg equilibrium (*p*=0.68). A comparison of the observed and theoretical genotype distributions (based on the global minor allele frequency) is presented in Table 6.1.[255] Of the 76 participants who were genotyped, 27 completed the 12-month weight management intervention with 15 randomised to the HPHI diet (CC: *n*=4; CT: *n*=9; TT: *n*=2), and 12 to the LPLI diet (CC: *n*=5; CT: *n*=6; TT: *n*=1).

Table 6.1. Comparison of the observed and theoretical (based on the global minor allele frequency) genotype distributions

Genotype (*n*=76)	Genotype distribution			Hardy-Weinberg *p* value
	Observed		Theoretical[a]	
	n	%	%	
C homozygote	22	29	36	
CT heterozygote	41	54	48	0.68
T homozygote	13	17	16	

C, cytosine allele; T, thymine allele

[a]Theoretical distribution obtained from the Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. dbSNP accession:[rs855791], (dbSNP Build ID: [86/137]). Available from: http://www.ncbi.nlm.nih.gov/SNP/

A summary of baseline age, BMI and biochemical marker concentrations is presented in Table 6.2. The baseline characteristics of participants from whom DNA was extracted were similar to those with no available DNA. Furthermore, no significant differences were observed between participants who were only included in the cross-sectional analysis and those in the longitudinal trial (Table 6.2).

Table 6.2. Comparison of baseline biochemical markers between participants with/without available DNA and between participants recruited to the cross-sectional study only and those who completed the longitudinal trial

Biochemical marker	Participants without DNA (*n*=38)	Participants with DNA (*n*=76)	*p* value	Participants with DNA CSS (*n*=49)	Clinical trial (*n*=27)	*p* value
Age (years)	22.5 ± 1.9	22.1 ± 2.5	0.35	22.0 ± 2.5	22.5 ± 2.4	0.82
BMI (kg/m^2)	33.8 ± 4.8	33.6 ± 4.2	0.81	33.5 ± 4.4	33.8 ± 3.8	0.77
Hb (g/l)	131 ± 9	130 ± 9	0.80	130 ± 9	131 ± 8	0.50
Serum iron (µmol/l)	14.9 ± 7.6	15.5 ± 6.5	0.67	15.3 ± 6.5	15.7 ± 6.0	0.40
Tsat (%)	22.4 ± 11.7	22.5 ± 10.1	0.96	22.0 ± 10.3	23.2 ± 9.5	0.71
Serum ferritin[b] (µg/l)	41.5 (44.0)	33.0 (31.0)	0.47	31.0 (34.0)	34.0 (26.0)	0.73
sTfR (mg/l)	1.52 ± 1.47	1.65 ± 0.45	0.14	1.67 ± 0.42	1.60 ± 0.51	0.67
sTfR-F[b]	0.89 (0.74)	1.05 (0.59)	0.20	1.08 (0.59)	1.01 (0.40)	0.86
Hepcidin[b] (ng/ml)	6.50 (11.0)	6.95 (8.00)	0.36	8.40 (9.25)	5.60 (4.60)	0.99
Hepcidin/ferritin ratio[b]	0.20 (0.21)	0.20 (0.17)	0.63	0.21 (0.19)	0.15 (0.14)	0.64
Hepcidin/Tsat ratio[b]	0.34 (0.56)	0.31 (0.48)	0.19	0.36 (0.55)	0.29 (0.21)	0.86
Age (years)	3.59 (5.79)	3.85 (5.91)	0.83	3.52 (6.08)	3.85 (5.56)	0.77

Mean ± SD or median (interquartile range)

CSS, cross-sectional study; DNA, deoxyribonucleic acid; Hb, haemoglobin; sTfR, soluble transferrin receptor; sTfR-F, soluble transferrin receptor-ferritin index; Tsat, transferrin saturation

[a]Regression models adjusted for BMI, waist circumference, C-reactive protein, ethnicity, contraception and polycystic ovary syndrome

[b]Natural log transformation performed for the serum ferritin, sTfR-F, hepcidin, hepcidin/ferritin and hepcidin/Tsat variables

Baseline iron biomarkers of participants of differing rs855791 genotype

Initial observation of baseline biochemical concentrations across the three genotypes indicated a tendency for lower serum iron and transferrin saturation with each additional T allele. The inverse was observed for serum ferritin, sTfR and hepcidin (both adjusted and unadjusted for ferritin and transferrin saturation) (Table 6.3). Univariate analysis using the co-dominant model (Table 6.3) showed that hepcidin/transferrin saturation ratio was significantly different between the three genotype groups (CC: coefficient=-0.741, SE=0.319; CT: coefficient=0.012, SE=0.305; *p*=0.007). Of all potential confounders, use of contraceptive medication had a significant impact on serum iron (coefficient=6.245; SE=1.756; *p*=0.001), transferrin saturation (coefficient=7.321; SE=3.103;

p=0.022), sTfR (coefficient=-0.339; SE=0.133; *p*=0.013) and sTfR-F (coefficient=-0.285; SE=0.133; *p*=0.036).

A dominant genetic model was also adopted to explore the iron-lowering effect of the T allele. This was done by combining CT heterozygotes and T homozygotes into a single group (defined as the 'mixed group') and analysing iron traits against C homozygotes. Using this alternative model, the mixed group had significantly lower serum iron (coefficient=-3.116; SE=1.532; *p*=0.047), higher hepcidin (coefficient=0.607; SE=0.260; *p*=0.023) and higher hepcidin/transferrin saturation ratio (coefficient=0.750; SE=0.226; *p*=0.002) than C homozygotes (Table 6.3). Higher sTfR (coefficient=0.225; SE=0.115; *p*=0.056) and hepcidin/ferritin ratio (coefficient=0.406; SE=0.203; *p*=0.050) exhibited by the mixed group were close to significance. The influence of contraceptive medication on serum iron (coefficient=6.004; SE=1.731; *p*=0.001), transferrin saturation (coefficient=7.150; SE=3.042; *p*=0.022), sTfR (coefficient=-0.353; SE=0.130; *p*=0.009) and sTfR-F (coefficient=-0.296; SE=0.131; *p*=0.027) were similar to the co-dominant model.

Table 6.3. Co-dominant and dominant genetic analysis of biochemical differences between genotypes

Biochemical marker[a]	Co-dominant genetic model CC (n=22)	CT (n=41)	TT (n=13)	p value	Dominant genetic model CC (n=22)	CT and TT / mixed group (n=54)	p value
Hb (g/l)	131 ± 7	131 ± 9	128 ± 10	0.573	131 ± 7	130 ± 9	0.437
Serum iron (µmol/l)	17.5 ± 7.2	15.1 ± 6.3	13.4 ± 4.8	0.096	17.5 ± 7.2	14.7 ± 6.0	0.047
Tsat (%)	24.0 ± 10.7	22.6 ± 10.2	19.7 ± 8.9	0.515	24.0 ± 10.7	21.9 ± 9.9	0.270
Serum ferritin[b] (µg/l)	25.0 (34.0)	33.0 (42.0)	35.0 (19.0)	0.697	25.0 (34.0)	34.0 (36.0)	0.395
<15.0 µg/l (%)	18.0	19.5	7.7		18.0	16.7	
sTfR (mg/l)	1.59 ± 0.42	1.67 ± 0.49	1.72 ± 0.39	0.132	1.59 ± 0.42	1.68 ± 0.46	0.056
sTfR-F[b]	1.07 (0.65)	1.01 (0.59)	1.19 (0.55)	0.689	1.07 (0.65)	1.05 (0.59)	0.483
Hepcidin[b] (ng/ml)	4.00 (5.00)	7.50 (8.95)	10.00 (7.45)	0.075	4.00 (5.00)	8.55 (8.30)	0.023
Hepcidin/ferritin ratio[b]	0.18 (0.16)	0.20 (0.23)	0.27 (0.21)	0.141	0.18 (0.16)	0.21 (0.20)	0.050
Hepcidin/Tsat ratio[b]	0.22 (0.26)	0.36 (0.47)	0.50 (0.36)	0.007	0.22 (0.26)	0.43 (0.45)	0.002

Mean ± SD or median (interquartile range)

CC, cytosine allele homozygote; CT, cytosine and thymine allele heterozygote; Hb, haemoglobin; sTfR, soluble transferrin receptor; sTfR-F, soluble transferrin receptor-ferritin index; TT, thymine allele homozygote; Tsat, transferrin saturation

[a]Regression models adjusted for BMI, waist circumference, C-reactive protein, ethnicity, contraceptive medication use and polycystic ovary syndrome

[b]Natural log transformation performed for the serum ferritin, sTfR-F, hepcidin, hepcidin/ferritin and hepcidin/Tsat variables

Effect of rs855791 on iron alterations induced by two energy-restricted diets differing in protein and haem iron content

Due to small sample size and limited T homozygote representation, only the dominant genetic model was employed for analysis of longitudinal data (Figure 6.1). HPHI participants in the mixed group showed significantly lower absolute serum iron (η_p^2=0.475, *p*=0.028) and transferrin saturation (η_p^2=0.418, *p*=0.043) than C homozygotes on the same diet. A significant genotypic effect on serum iron (η_p^2=0.110, *p*=0.38) and transferrin saturation (η_p^2=0.118, *p*=0.36) was not observed in participants following the LPLI diet.

Analysis of absolute serum ferritin concentration across 12 months showed no significant effect of SNP rss855791 within either diet (HPHI: *p*=0.89; LPLI: *p*=0.91). Both HPHI genotype groups exhibited increased ferritin concentration at 12 months, while a slight decrease in serum ferritin was observed in both LPLI genotype groups. Consequently, the effect of diet on absolute ferritin concentration was assessed using repeated measures ANOVA (covariates included: rs855791, contraceptive medication and percent loss of initial weight at 12 months), and a significant diet × time interaction (η_p^2=0.160; *p*=0.026) was found. No significant SNP or diet effects were observed for absolute haemoglobin, sTfR or sTfR-F levels. Mean/median values for all biomarkers remained within normal range throughout the trial and across all diet and genotype groups.

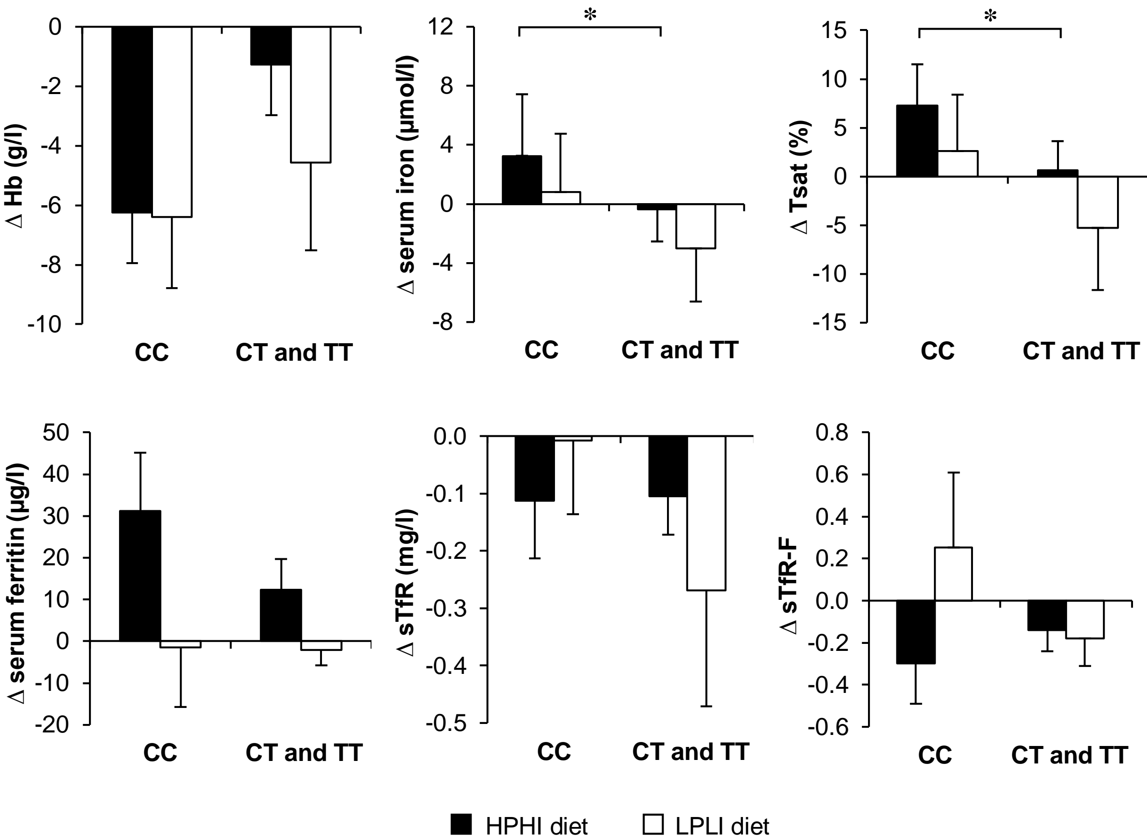

Figure 6.1. Comparison of 12-month iron alterations induced by the higher-protein, higher-haem iron (HPHI) and lower-protein, lower-haem iron (LPLI) diets between C homozygotes and the mixed group (CT heterozygotes and T homozygotes) [Asterisks denote significant SNP effects on absolute serum iron and Tsat levels (p<0.05); mean ± SE; Hb, haemoglobin; sTfR, soluble transferrin receptor; sTfR-F, soluble transferrin receptor-ferritin index; Tsat, transferrin saturation; Δ, change from baseline]

DISCUSSION

The purpose of this study was to explore the influence of SNP rs855791 on iron metabolism in young overweight and obese women at baseline, and across a 12-month dietary intervention using either a HPHI or LPLI diet. Outcomes from the baseline analysis support current literature suggesting a significant relationship between rs855791, serum iron and hepcidin concentration. Furthermore, our longitudinal results indicate iron-related responses to altered protein and haem iron intake during energy restriction can vary across participants with differing rs855791 genotype. With young women at higher risk of iron inadequacy compared to other age and gender groups,[32] a greater understanding of how SNP rs855791 may affect iron metabolism during dietary energy restriction can aid selection of suitable diets that minimise the risk of suboptimal iron status.

A more novel angle of this research involves the adoption of a candidate gene approach to assessing longitudinal effects of rs855791 on iron status during energy restriction. Analysis of the longitudinal data showed that participants with one or both copies of the T allele (i.e. the mixed group), and who were following the HPHI diet exhibited lower serum iron and transferrin saturation after 12 months of intervention than C homozygotes. Interestingly, an equivalent trend was not observed in LPLI participants, which may reflect insufficient power or suppressed hepcidin-mediated regulation of gut iron absorption in the context of limited protein and haem iron intake.

SNP rs855791 did not significantly affect serum ferritin in participants following either dietary intervention. While obesity and inflammation have been reported to elevate serum ferritin,[8] the use of ferritin as a marker of iron stores is justified in this cohort (Chapter 4).[228] As supported by the significant diet × time interaction observed across 12 months, dietary intake appeared to exert a greater relative impact on serum ferritin concentration than the SNP itself. This suggests that a higher-protein and haem iron diet can be beneficial for increasing iron stores during energy restriction regardless of rs855791 genotype.

In addition to assessing genotypic effects on longitudinal iron alterations, this study also confirms previous research reporting a significant effect of SNP rs855791 on iron status. Baseline outcomes from our cross-sectional cohort identified the T allele (coding for valine) as the minor, iron-lowering allele. The minor allele frequency observed was reasonably consistent with the global minor allele frequency (0.40) reported on the National Center for Biotechnology Information SNP database,[255] and with the frequency reported in Australian adolescents of European ancestry (0.42).[20]

In line with previous research reporting an association between rs855791 and serum iron levels,[26] a significant negative trend for serum iron was observed with each additional T allele in our cross-sectional cohort. Although significant associations were not observed for other iron markers in this study, significant genome-wide relationships between rs855791, haemoglobin, transferrin saturation and sTfR have been reported.[20, 21, 172] Contrary to our expectations, serum ferritin tended to be higher in T homozygotes despite adjustment for potential inflammatory effects. The reason for this is unclear although the majority of genetic studies do not support a significant link between this SNP and serum ferritin.[20, 22, 27, 171, 172] The significant effect of diet on ferritin concentration in the longitudinal analysis suggests that external factors such as iron intake may have contributed to the contradictory baseline result. Future studies that incorporate additional dietary patterns such as vegetarianism (which can limit iron intake and bioavailability) can help to determine the relative impact of SNP rs855791 and dietary iron intake on ferritin variation between individuals.

The trend for C homozygotes in the cross-sectional cohort to have lower hepcidin and hepcidin/transferrin saturation ratio (which was clearest in the dominant genetic model) corroborates recent findings reported in an Italian genetic isolate population.[29, 175] These studies (which to date are the only studies to measure the association between rs855791 and serum hepcidin directly) found significantly higher hepcidin in T vs. C homozygotes,[29] as well as associations between rs855791, serum iron and transferrin saturation.[29, 175] Interestingly, serum hepcidin was only able to partially explain these significant genotype-iron associations, which

indicates the possibility of unidentified hepcidin-independent iron regulatory mechanisms at play.[175]

This study is not without its limitations. As genetic analysis was not the primary study objective at the time of participant recruitment, the selected sample size was not powered to detect small biochemical differences between genotypes. The significance of our results may have also been compromised due to measurement of only one SNP which is often inadequate for detection of substantial phenotypic variation. Related polymorphisms including SNP rs4820268 are likely to have also contributed to iron variation.[21] We were also unable to assess the effects of diet and rs855791 on hepcidin alteration as it was not measured longitudinally.

In conclusion, the iron-lowering T allele of SNP rs855791 was associated with lower serum iron and higher hepcidin concentration at baseline. Lower serum iron and transferrin saturation observed in T hetero- and homozygotes following the HPHI diet suggest that rs855791-associated iron variability may be dependent on haem iron and/or protein intake. As overall iron marker concentrations were within normal range throughout the 12 months of the intervention, the clinical significance of these findings remain to be established. As young women are known to be at higher risk of iron inadequacy compared to other population groups,[32] a greater understanding of how genetic variation affects iron metabolism can facilitate selection of suitable diets for minimising iron deficiency risk during weight loss. This exploratory study demonstrates the need for larger longitudinal studies that examine a wider range of iron-related SNPs and dietary patterns (e.g. vegetarianism) to more clearly evaluate gene-nutrient interactions that contribute to iron variation during weight management in young women.

CONFLICT OF INTEREST

This work was supported by a grant from Meat and Livestock Australia. The authors declare that Meat and Livestock Australia had no influence on any part of the data analysis or interpretation processes, including the conclusions drawn. The authors declare no other conflicts of interest.

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CHAPTER 7.

Conclusions

SUMMARY OF FINDINGS

A growing number of studies suggest that obesity is associated with disturbances to iron metabolism secondary to elevated inflammation and hepcidin. However, there is limited research incorporating the measurement of hepcidin and iron markers, such as sTfR and the sTfR-ferritin index (sTfR-F), which are appropriate for use under inflammatory conditions. This is presumably due to higher costs associated with analysis and the absence of standardised reference ranges. Existing studies have focussed on paediatric, mixed adult or morbidly obese populations with little research conducted in young women who are at increased risk of both weight gain and iron deficiency. To address the paucity of literature in this demographic, this thesis contains a series of studies investigating the impact of excess weight, weight loss and genetics on iron status in a cohort of healthy, free-living overweight and obese young women.

The primary aims of the studies in this thesis were to:

1. systematically review the evidence on the relationship between obesity, inflammation and iron status in adults;
2. examine the prevalence of iron deficiency, severity of obesity-related iron disturbances, and hepcidin concentration in a community sample of young, healthy overweight and obese Australian women;
3. assess the impact of two energy-restricted diets with contrasting protein and haem iron content, and weight loss on iron status during a clinical weight management trial; and
4. investigate the influence of the common *TMPRSS6* rs855791 polymorphism on iron and hepcidin status, as well as physiological iron responses to altered protein and haem iron intake during energy restriction.

Findings from the systematic review in Chapter 3 support an association between obesity and iron disturbances. Existing literature of measurements in obese individuals, particularly those with BMI >35 kg/m^2, showed higher ferritin concentration and lower transferrin saturation than non-obese comparison groups. This result was consistent with typical iron alterations observed in

inflammatory conditions. Higher haemoglobin in the obese also indicated that chronic tissue hypoxia syndromes, such as obstructive sleep apnoea, may influence haematological marker levels. No definitive conclusions could be drawn for sTfR and hepcidin due to the limited amount of data available. In the few studies that did measure these markers, higher levels of sTfR and hepcidin in the obese suggest that some degree of functional iron disruption may be present.

To examine whether similar disruptions to iron metabolism also occur in young, healthy overweight and obese women, the cross-sectional study described in Chapter 4 was conducted to address the second aim of this thesis. Results from this study showed mild iron and inflammatory alterations with increasing obesity. This was reflected by the significant associations observed between increasing BMI and lower serum iron, lower transferrin saturation, and higher CRP. Despite this, the main finding of this study was the unexpectedly low ferritin and hepcidin concentrations observed in our cohort. Hypoferritinaemia was present in 17% of participants, with many also showing hepcidin concentrations below 5 ng/ml. Hence, simple iron deficiency and not obesity-related hypoferraemia, was found to be the main iron-related abnormality in this sample.

Simple iron deficiency is often a result of inadequate iron intake, and obtaining sufficient iron may be even more challenging for young women undergoing energy restriction. As few studies have monitored iron status during diet-induced weight reduction, the study in Chapter 5 was conducted to explore the effects of manipulating protein and haem iron intake on iron status in a subset of the cross-sectional cohort undergoing a 12-month weight management intervention. Median serum ferritin levels were maintained within normal limits in both diet groups throughout the 12 months, although only participants on the higher-protein and haem iron diet were able to increase iron stores during the trial. Participants who lost ≥10% of initial weight tended to show a more favourable iron profile, and this was particularly evident in the lower-protein and haem iron diet group who showed higher serum iron and transferrin saturation, and lower sTfR concentration. While this observation may simply reflect greater diet quality and compliance in the more successful participants, it is possible that weight reduction itself (provided that the diet is

reasonably nutrient-rich) may be beneficial for improving iron status despite the absence of significant hepcidin elevation at baseline.

As significant obesity-related iron disturbances were not observed in this sample of young women, a final exploratory study was conducted to examine the potential influence of genetics on individual iron variation. The rs855791 polymorphism in the *TMPRSS6* gene was selected due to its significant association with various iron markers, its high frequency in the general population and evidence of its hepcidin-independent impact on iron status. Outcomes from this analysis revealed that participants who were homozygous for the C allele showed the most favourable iron profile. Furthermore, C homozygotes on the higher-protein and haem iron diet tended to have greater serum iron and transferrin saturation levels than those who were hetero- and homozygous for the alternative T allele. These findings suggest that the rs855791 polymorphism can significantly influence iron and hepcidin status at baseline, although its significance appears to be reduced when dietary protein and haem iron intake during energy restriction are limited.

NUTRITIONAL AND CLINICAL IMPLICATIONS

The findings from this thesis highlight the importance of monitoring and appropriately managing simple iron deficiency in young overweight and obese women. The high prevalence of hypoferritinaemia in combination with low hepcidin observed in this sample suggest an aetiology of inadequate intake and/or excessive loss of iron.

In spite of recent evidence showing iron disruptions with excess weight and inflammation, outcomes from the cross-sectional analysis suggest that the routine iron marker serum ferritin, with its cut-off value at <15 µg/l, appears to be a sound measure of iron deficiency in young women not burdened with significant obesity-related comorbidities. However, caution must be exercised when utilising ferritin in those with a BMI >35 kg/m^2 as inflammatory-related iron disruptions may be more prominent. In order to more accurately assess true iron status in these individuals, analysis of additional biochemical markers including sTfR, sTfR-F and hepcidin should be considered in those with class II obesity and above. Moreover, potential confounding from elevated inflammation should also be accounted for when attempting to gauge iron status in any individual. This can include the measurement of acute phase proteins and proinflammatory cytokines such as CRP, α - 1-acid glycoprotein, IL-6 and TNF-α, or at minimum, avoiding iron assessment during periods of overt infection.

Simple iron deficiency clearly remains an issue for a substantial proportion of young Australian women including those who are overweight and obese. Designing energy-restricted diets that provide sufficient iron to meet Australian recommended intakes for women at this age is also challenging. Therefore, it would be prudent in the clinical setting, to conduct initial screening for suboptimal iron status in young women who seek dietetic and medical advice for weight management. This is especially relevant for women with greater iron needs such as those with a history of iron deficiency, PCOS, heavy or prolonged menses and/or those who report marginal iron intake.

As demonstrated by the dietary intervention study described in Chapter 5, practical implementation of well-designed and nutrient-rich diets allow for reasonable preservation of iron status during 12 months of energy restriction in most young women. Successful losses of ≥10% of initial weight may also have a positive impact on iron levels independent of significant hepcidin reduction. As weight loss is known to bring about other important health benefits such as reduced cardiovascular and metabolic disease risk, weight management using nutritionally balanced diet plans should be encouraged in this group. Young overweight and obese women who possess greater iron needs (such as those mentioned in the paragraph above) may particularly benefit from adopting energy-restricted diets higher in protein and haem iron for superior maintenance of iron homeostasis. Practitioners should however, thoroughly assess iron status and needs before recommending these diets, given the possible link between haem iron intake and risk of colorectal cancer.[256]

Exploration of the rs855791 polymorphism and its associations with various biochemical iron traits in Chapter 6 demonstrates the significant role of genetics in determining individual iron variability. The question of whether higher-protein and haem iron diets can be beneficial to those with the iron-lowering T allele remains unclear as the significant effect of rs855791 on iron status appeared to diminish under conditions of limited protein and haem iron intake. Replication of this study using a larger sample and a greater range of SNPs is warranted to clarify the benefits of increasing iron intake for those genetically predisposed to having lower iron status, and to establish the clinical significance of findings reported in this exploratory analysis.

STUDY LIMITATIONS

Low statistical power was a primary limiting factor in the studies described in Chapters 5 and 6. Various issues contributed to the small sample size of the 12-month dietary intervention study. A rigorous selection criterion was enforced at the recruitment stage to minimise confounding on the weight and iron outcomes. Hence, exclusion of some lifestyle choices common in young women such as vegetarianism and smoking made a number of volunteers ineligible to participate in the trial. High attrition was also experienced in this study. Illness and an inability to attend face-to-face sessions, particularly due to lack of time and frequent changes in living situation, were reported as common reasons for attrition. Few studies have attempted to examine weight management in this age group relative to paediatric and older cohorts. Difficulty with recruitment and retention of participants may explain the lack of studies, yet more research in young women is needed given the greater risk of weight gain and obesity compared to other populations. Similar issues to those reported in this research have been summarised in a recent systematic review of weight management studies in young adults.[37] However, reasons for the recruitment and retention difficulties experienced in these studies were often not provided, making improvement to future weight management study designs for similar age groups more difficult.

The cross-sectional design of Chapter 4, while providing some estimate of prevalence in this convenience sample, is not able to provide evidence of causality between obesity, iron, hepcidin and inflammatory status. Although an absence of significant hypoferraemia and inflammation in majority of the sample indicate that excess weight may precede the development of significant comorbidities and functional iron disturbances, larger longitudinal studies are required to examine the evolution of iron and hepcidin-related disruptions that accompany the development of obesity. Additionally, collection of baseline dietary iron intake in this cohort would have also allowed for a more thorough evaluation of possible contributors to simple iron deficiency.

Due to the high costs associated with measuring plasma hepcidin at present, the dietary intervention study in Chapter 5 is limited by the lack of longitudinal hepcidin measurement. As results from the cross-sectional study did not indicate the presence of substantial baseline hepcidin elevation, it was decided that hepcidin would not be analysed at the six- and 12-month time points as significant decreases accompanying weight loss (as hypothesised in Chapter 1) was deemed to be unlikely. Future measurement of hepcidin at these time points is possible, pending budgetary allowances, which will clarify the effect of diet-induced weight loss on hepcidin alterations in this group of young overweight and obese women.

Low DNA yield from a number of frozen biological specimens also contributed to low statistical power observed in the genetic study (Chapter 6). As genetic investigation was not a primary objective of the cross-sectional and weight loss intervention studies, DNA was not extracted from fresh blood. Instead, a small aliquot of packed cells from each participant was frozen for potential genetic analysis at a later stage. Consequently, a number of these frozen samples may have contained insufficient white cells for extraction of adequate DNA. Strategies such as repetition of the extraction process on remaining blood and reducing the final rehydration volume to 20 μl were employed to maximise the yield and concentration of the final DNA sample.

FUTURE RESEARCH

The outcomes of this thesis have implications for future directions in obesity and iron-related research. The systematic review in Chapter 3 argues for more widespread use of sTfR and hepcidin, as well as the appropriate controlling of confounders such as obstructive sleep apnoea which is common in obesity. Consideration of these factors is necessary in future studies as a more accurate assessment of true iron and haematological status allows for the development of suitable clinical care plans for overweight and obese individuals who exhibit abnormal and clinically significant biochemical profiles.

Results from Chapter 4 suggest that obesity-related comorbidities may be more important than excess adiposity *per se* for the development of substantial inflammatory iron disruptions in this age group. Studies are required to identify these specific comorbidities and to clarify the causal relationships between obesity, inflammation and hepcidin elevation. Some studies have reported altered hepcidin and ferritin concentration with type 2 diabetes.[257] However, the question of whether diabetes precedes the manifestation of iron and hepcidin abnormalities, or vice versa, remains to be established. More research is also required to examine potential relationships between iron, hepcidin and other comorbid conditions such as cardiovascular disease, the metabolic syndrome and non-alcoholic hepatosteatosis. Although total and abdominal body fat was not significantly associated with the iron biomarkers measured in the cross-sectional study, the role of body fat distribution and the relative contribution of abdominal/android vs. gynoid fat, particularly in respect to gender differences also merits further investigation.

In healthy overweight and obese populations undergoing dietary weight management, larger studies including the measurement of hepcidin at longitudinal time points will provide further insight into how body iron regulation alters with energy restriction, weight loss and dietary macronutrient and iron manipulation. Research is also needed to evaluate the influence of other common iron-related polymorphisms such as the *TMPRSS6* SNP rs4820268 under various dietary contexts including vegetarian- and veganism. A greater understanding of these gene-nutrient interactions may

potentially help to direct future nutrigenetic research into personalised nutrition, or the tailoring of dietary recommendations in accordance to individual iron needs.

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APPENDICIES

APPENDIX A: SUPPLEMENTARY MATERIAL FOR CHAPTER 3

A1. MEDLINE electronic search strategy

A2. Quality item checklist

A3. Summary of outcomes for non-bariatric surgery studies (age, BMI, haemoglobin and ferritin)

A4. Summary of outcomes for bariatric surgery studies (age, BMI, haemoglobin and ferritin)

A5. Summary of outcomes for non-bariatric surgery studies (transferrin saturation, soluble transferrin receptor, hepcidin and C-reactive protein)

A6. Summary of outcomes for bariatric surgery studies (transferrin saturation, soluble transferrin receptor, hepcidin and C-reactive protein)

1. MeSH – Anemia, Iron-deficiency
2. Keyword – Anemi*
3. Keyword – Anaemi*
4. Keyword – Iron Deficien*
5. Keyword – Iron Deple*
6. MeSH – Obesity; abdominal obesity; morbid obesity
7. Keyword – Obes*
8. Keyword – Overweight
9. 1 OR 2 OR 3 OR 4 OR 5
10. 6 OR 7 OR 8
11. 9 AND 10

A1. Electronic search strategy used to search the MEDLINE database with no limits.

Similar strategies were used for other electronic information sources, modified to comply with search rules of each database. (Asterisk denotes the use of truncation)

A2. Quality item checklist

Author (Country)	**Study population sufficiently described? (age, gender, menopausal status)**	**Sampling procedures sufficiently described? (sampling frame, sample size)**	**Appropriate participant exclusion criteria?**	**Outcomes collected using appropriate methods? (measured anthropometry, fasting status, blood collection by venipuncture)**	**All important confounders accounted for (gender, age/menopausal status, ethnicity, dietary iron intake, oral contraceptive use, blood donation, iron treatment, alcohol consumption, menorrhagia or amenorrhoea, haemorrhage or gastrointestinal bleeding, acute infection, liver disease or haemochromatosis, inflammatory disorders, chronic disease)**
Aasheim *et al*, 2009 (Sweden, Norway)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	No (not reported: height and weight)	No (confounders accounted for: age)
Ahmed *et al*, 2008 (Australia)	No (menopausal status assumed by age)	Yes	No (excluded subjects with elevated ferritin)	Yes	No (confounders accounted for: gender, age/menopausal status, haemochromatosis)
Amaral *et al*, 1984 (USA)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	No (not reported: height, weight, venipuncture)	No (confounders accounted for: liver disease)
Anna *et al*, 2009 (USA) [Abstract]	Yes	Yes	No exclusion criteria reported	Yes	No (confounders accounted for: gender)
Anty *et al*, 2008 (France)	No (menopausal status assumed by age)	No (sample size determination not reported)	Yes	No (not reported: height, weight, venipuncture)	No (confounders accounted for: gender, menorrhagia, gastrointestinal bleeding, acute infection, liver disease or haemochromatosis)
Ausk & Ioannou, 2008 (USA)	Yes	Yes	Yes	Yes	No (confounders accounted for: gender, age, menopausal status, ethnicity, dietary iron intake, blood donation, alcohol consumption)
Beard *et al*, 1997 (USA)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	Yes	No [confounders accounted for: gender, liver disease, chronic disease (insulin-dependent diabetes mellitus)]

Author (Country)	**Study population sufficiently described? (age, gender, menopausal status)**	**Sampling procedures sufficiently described? (sampling frame, sample size)**	**Appropriate participant exclusion criteria?**	**Outcomes collected using appropriate methods? (measured anthropometry, fasting status, blood collection by venipuncture)**	**All important confounders accounted for (gender, age/menopausal status, ethnicity, dietary iron intake, oral contraceptive use, blood donation, iron treatment, alcohol consumption, menorrhagia or amenorrhoea, haemorrhage or gastrointestinal bleeding, acute infection, liver disease or haemochromatosis, inflammatory disorders, chronic disease)**
Bekri *et al*, 2006 (France)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	No (not reported: height, weight, venipuncture)	No [confounders accounted for: non-alcoholic steatohepatitis, haemochromatosis, chronic disease (diabetes)]
Belgaumkar *et al*, 2011 (UK) [Abstract]	No (gender and menopausal status not reported)	No (sampling frame and sample size determination not reported)	No exclusion criteria reported	No data collection procedures reported	No consideration or adjustment of confounders reported
Brolin *et al*, 1998 (USA)	Yes	No (sample size determination not reported)	Yes	No (none reported)	No (confounders accounted for: gender, menopausal status)
Cooper *et al*, 1999 (Australia)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	No (not reported: fasting status, venipuncture)	No consideration or adjustment of confounders reported
Coupaye *et al*, 2009 (France)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	No (not reported: venipuncture)	No consideration or adjustment of confounders reported
de Luis *et al*, 2013 (Spain)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	No (not reported: venipuncture)	No (confounders accounted for: gender, iron treatment/drugs, renal disease, thyroid disease)
Ernst *et al*, 2009 (Switzerland)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	No (not reported: venipuncture)	No (confounders accounted for: gender)
Fricker *et al*, 1990 (France)	Yes	No (sample size determination not reported)	Yes	Yes	No (confounders accounted for: gender, menopausal status, oral contraceptive use, blood donation, iron treatment/drugs, amenorrhoea, chronic disease)

Author (Country)	**Study population sufficiently described? (age, gender, menopausal status)**	**Sampling procedures sufficiently described? (sampling frame, sample size)**	**Appropriate participant exclusion criteria?**	**Outcomes collected using appropriate methods? (measured anthropometry, fasting status, blood collection by venipuncture)**	**All important confounders accounted for (gender, age/menopausal status, ethnicity, dietary iron intake, oral contraceptive use, blood donation, iron treatment, alcohol consumption, menorrhagia or amenorrhoea, haemorrhage or gastrointestinal bleeding, acute infection, liver disease or haemochromatosis, inflammatory disorders, chronic disease)**
Gasteyger *et al*, 2006 (Switzerland)	Yes	No (sample size determination not reported)	Yes	No (not reported: venipuncture)	No (confounders accounted for: gender, menopausal status, ethnicity, oral contraceptive use, liver disease)
Hamalainen *et al*, 2012 (Finland)	No (menopausal status not available)	No (sample size determination not reported)	Yes	Yes	No (confounders accounted for: gender, age, smoking, physical activity, inflammation)
Kretsch *et al*, 1998 (USA)	Yes	No (sample size determination not reported)	No (may have excluded subjects with anaemia)	Yes	No (confounders accounted for: gender, age/menopausal status, oral contraceptive use, menorrhagia or amenorrhoea, acute infection, liver disease, inflammatory disorders, chronic disease)
Lecube *et al*, 2006 (Spain)	Yes	No (sample size determination not reported)	No (excluded subjects with ferritin <10 ng/ml)	No (not reported: height, weight, venipuncture)	No [confounders accounted for: gender, menopausal status, ethnicity, blood donation, iron treatment, alcohol consumption, haemorrhage, acute infection, haemochromatosis, chronic disease (type 2 diabetes)]
Micozzi *et al*, 1989 (USA)	No (menopausal status not reported)	Yes	No (may have excluded subjects with anaemia)	No (haemoglobin analysis by haemoglobinometer)	No (confounders accounted for: gender, age, chronic disease)
Murer *et al*, 2012 (Switzerland) [Abstract]	No (gender and menopausal status not reported)	No (sampling frame and sample size determination not reported)	No exclusion criteria reported	No (not reported: fasting status, venipuncture)	No (confounders accounted for: inflammation)
Ortiz Espejo *et al*, 2011 (Spain)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	None reported	No consideration or adjustment of confounders reported

Author (Country)	Study population sufficiently described? (age, gender, menopausal status)	Sampling procedures sufficiently described? (sampling frame, sample size)	Appropriate participant exclusion criteria?	Outcomes collected using appropriate methods? (measured anthropometry, fasting status, blood collection by venipuncture)	All important confounders accounted for (gender, age/menopausal status, ethnicity, dietary iron intake, oral contraceptive use, blood donation, iron treatment, alcohol consumption, menorrhagia or amenorrhoea, haemorrhage or gastrointestinal bleeding, acute infection, liver disease or haemochromatosis, inflammatory disorders, chronic disease)
Palomar *et al*, 2005 (Spain)	Yes	No (sample size determination not reported)	Yes	No (not reported: fasting status, venipuncture)	No consideration or adjustment of confounders reported
Payne & DeWind, 1969 (USA)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	No (not reported: fasting status, venipuncture)	No consideration or adjustment of confounders reported
Skroubis *et al*, 2006 (Greece)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	None reported	No consideration or adjustment of confounders reported
Smith *et al*, 2013 (UK) [Abstract]	No (gender and menopausal status not reported)	No (sample size determination not reported)	No exclusion criteria reported	None reported	No (confounders accounted for: gender, age, iron treatment/drugs, chronic disease)
Świtoniak & Król, 1992 (Poland)	No (menopausal status not reported)	No (sample size determination not reported)	No exclusion criteria reported	None reported	No (confounders accounted for: age, gender)
Thurnheer *et al*, 2012 (Switzerland)	No (menopausal status not reported)	No (sample size determination not reported)	No exclusion criteria reported	No (not reported: venepuncture)	No (confounders accounted for: gender)
Toh *et al*, 2009 (Australia)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	None reported	No [confounders accounted for: gender (only for haemoglobin)]

Author (Country)	**Study population sufficiently described? (age, gender, menopausal status)**	**Sampling procedures sufficiently described? (sampling frame, sample size)**	**Appropriate participant exclusion criteria?**	**Outcomes collected using appropriate methods? (measured anthropometry, fasting status, blood collection by venipuncture)**	**All important confounders accounted for (gender, age/menopausal status, ethnicity, dietary iron intake, oral contraceptive use, blood donation, iron treatment, alcohol consumption, menorrhagia or amenorrhoea, haemorrhage or gastrointestinal bleeding, acute infection, liver disease or haemochromatosis, inflammatory disorders, chronic disease)**
Tussing-Humphreys *et al*, 2009 (USA)	Yes	Yes	Yes	No (haemoglobin analysis by haemoglobinometer)	No (confounders accounted for: gender, menopausal status, ethnicity, blood donation, alcohol consumption, amenorrhoea, gastrointestinal bleeding, acute infection, haemochromatosis, inflammatory disorders)
Yanoff *et al*, 2007 (USA)	No (menopausal status not reported)	No (sample size determination not reported)	Yes	No (not reported: venipuncture)	No (confounders accounted for: gender, age, ethnicity, gastrointestinal bleeding, liver disease, inflammatory disorders, chronic disease)

A3. Summary of outcomes for non-bariatric surgery studies (age, BMI, haemoglobin and ferritin)

Study (Country)	Strata/Group	Age (years)		BMI (kg/m^2)		Haemoglobin (g/l)		Ferritin (µg/l)	
		Obese	Non-obese	Obese	Non-obese	Obese	Non-obese	Obese	Non-obese
Ahmed *et al*, 2008	Male (Normal)	≥25	≥25		<25				144 (118-144)[a]
(Australia)	Female <50 y (Normal)	25 – 49	25 – 49		<25				38.1 (33.8-43.1)[a]
	Female ≥50 y (Normal)	≥50	≥50		<25				64.7 (57.8-73.3)[a]
	Male (Overweight)	≥25	≥25		25 – 30				147 (134-160)[a]
	Female <50 y (Overweight)	25 – 49	25 – 49		25 – 30	–	–		42.1 (35.8-49.7)[a]
	Female ≥50 y (Overweight)	≥50	≥50		25 – 30				75.9 (68.0-85.0)[a]
	Male (Obese)	≥25	≥25	>30				150 (130-172)[a]	
	Female <50 y (Obese)	25 – 49	25 – 49	>30				50.4 (42.1-60.3)[a]	
	Female ≥50 y (Obese)	≥50	≥50	>30				69.4 (59.7-78.8)[a]	
Anna *et al*, 2009	BMI <25		42.4 ± 22.4		22.3 ± 2.0		140 ± 16		
(USA)	BMI 25.1 – 30		48.5 ± 19.1		27.4 ± 1.5		139 ± 15	–	–
[Abstract]	BMI 30.1 – 35	48.1 ± 18.9		32.1 ± 1.3		140 ± 15			
	BMI >35	45.5 ± 17.7		40 ± 4.9		138 ± 15			
Ausk & Ioannou, 2008	BMI <25	18 – >70	18 – >70		<25		140 ± 20		106 ± 179
(USA)	BMI 25 – 30				25 – 30		145 ± 18		144 ± 219
	BMI 30 – 35			30 – 35		143 ± 25		145 ± 236	
	BMI 35 – 40			35 – 40		140 ± 30		147 ± 253	
	BMI ≥40			≥ 40		139 ± 22		146 ± 259	
Beard *et al*, 1997	VLED 1758 kJ/day	41 ± 37.4						40.1 ± 25.0[b]	
(USA)	VLED 2763 kJ/day	39 ± 37.1	–	38.1 ± 7.8	–	–	–	26.7 ± 16.5[b]	–
	VLED 3349 kJ/day	46 ± 22.6						32.0 ± 23.1[b]	
Fricker *et al*, 1990 (France)	N/A	30.8 ± 9.3	30.5 ± 7.0	31.9 ± 4.0	20.7 ± 1.4	137 ± 9	129 ± 10	48.0 ± 44.3	25.8 ± 19.5

Study (Country)	Strata/Group	Age (years)		BMI (kg/m^2)		Haemoglobin (g/l)		Ferritin (µg/l)	
		Obese	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**
Hamalainen *et al*, 2012 (Finland)	Female MetS	54.4 ± 5.7	–	30.8 ± 5.3	–	141 ± 10	–	94 ± 75	–
Kretsch *et al*, 1998 (USA)	N/A	25 – 42	–	31.5 ± 4.1	–	131 ± 4	–	33.1 ± 24.8	–
Lecube *et al*, 2006 (Spain)	N/A	56.9 ± 9.4	57.4 ± 11.1	39.7 ± 7.9	25.0 ± 2.7	135 ± 9	133 ± 8	70.5 ± 50.3	69.5 ± 61.5
Micozzi *et al*, 1989 (USA)	BMI <27.4				< 27.4		136 ± 0.3[c]		
	BMI 31.1 – 36.1	25 – 74	25 – 74	31.1-36.1		137 ± 0.3[c]		–	–
	BMI >36.1			>36.1		138 ± 0.3[c]			
Murer *et al*, 2012 (Switzerland) [Abstract]	N/A	40.7 ± 12.8	–	39.2 ± 7.5	–	–	–	35.8 ± 38.7	–
Świtoniak & Król, 1992 (Poland)	Underweight		27.4 ± 2.0		17.7 ± 1.0		132 ± 6		
	Normal weight		38.0 ± 4.0		21.8 ± 1.2		130 ± 13	–	–
	Overweight		39.6 ± 3.3		26.4 ± 2.4		137 ± 12		
	Obese	40.2 ± 3.6		32.1 ± 3.2		137 ± 10			
Yanoff *et al*, 2007 (USA)	N/A	38.6 ± 9.7	37.2 ± 11.2	38.4 ± 6.3	25.6 ± 3.1	135 ± 11	135 ± 11	81.1 ± 88.8	57.6 ± 88.7

Data presented as mean ± SD unless otherwise specified

BMI, body mass index; MetS, metabolic syndrome; N/A, not applicable; VLED, very-low-energy diet

[a]Geometric mean (95% confidence interval)

[b]Data obtained from measurement of plasma ferritin

[c]Mean ± standard error (conversion to mean ± SD was not possible due to lack of information regarding sample size)

A4. Summary of outcomes for bariatric surgery studies (age, BMI, haemoglobin and ferritin)

Study (Country)	**Strata/Group**	**Age (years)**		**BMI (kg/m^2)**		**Haemoglobin (g/l)**		**Ferritin (µg/l)**	
		Obese	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**
Aasheim *et al*, 2009 (Sweden, Norway)	GBP group	35 ± 7	–	54.8 ± 17.8	–	143 ± 61	–	–	–
	DS group	36 ± 5		55.2 ± 18.8		143 ± 65			
Amaral *et al*, 1984 (USA)	Male	35.0 ± 6.8	–	103 ± 25.7% above ideal body weight	–	156 ± 15	–	–	–
	Female	35.8 ± 6.2				140 ± 10			
Anty *et al*, 2008 (France)	N/A	39 (30.8-47.0)[a]	–	43.8 (40.9-47.9)[a]	–	137 (128-142)[a]	–	45.0 (22.0-78.5)[a]	–
Bekri *et al*, 2006 (France)	Lean controls		29.0 (25-38)[b]		21 (20.5-21.5)[b]				
	Obese	37.5 (30.7-43.2)[b]		46.2 (40.0-49.4)[b]					
	Obese with diabetes	47.0 (39.3-48.8)[b]		42.3(37.8-49.6)[b]		139 ± 15	–	112 ± 125	–
	Obese with NASH	34.5 (29.9-41.3)[b]		44.8 (40.8-48.9)[b]					
Belgaumkar *et al*, 2011 (UK) [Abstract]	N/A	45 ± 9	–	60.0 ± 11.3	–	134 ± 13	–	81.7 ± 66.7[c]	–
Brolin *et al*, 1998 (USA)				Mean weight:					
	Placebo group	37.0 ± 8.0	–	129 ± 27 kg	–	130 ± 10	–	56 ± 58	–
	Intervention group	36.6 ± 8.0		126 ± 21.6 kg		130 ± 10		87 ± 81	
Cooper *et al*, 1999 (Australia)	N/A	23 – 59	–	31.6 – 52.7	–	135 ± 9	–	94 ± 90	–
Coupaye *et al*, 2009 (France)	AGB group	35 ± 10	–	43 ± 3	–	129 ± 10	–	86.3 ± 75.4	–
	GBP group	43 ± 10		49 ± 8		131 ± 12		96.4 ± 89.3	

Study (Country)	Strata/Group	Age (years)		BMI (kg/m^2)		Haemoglobin (g/l)		Ferritin (µg/l)	
		Obese	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**
de Luis *et al*, 2013 (Spain)	BMI 35-40	43.4 ± 12.8		36.4 ± 4.1		142 ± 14		89.6 ± 15.1	
	BMI 40-45	43.1 ± 15.1		42.6 ± 1.5		138 ± 12		59.1 ± 84.3	
	BMI 45-50	44.7 ± 12.5	–	47.5 ± 1.3	–	139 ± 11	–	101.4 ± 97.0	–
	BMI >50	45.1 ± 11.8		55.4 ± 5.3		140 ± 14		65.1 ± 51	
Ernst *et al*, 2009 (Switzerland)	N/A	40.5 ± 11.6	–	44.9 ± 6.2	–	143 ± 15	–	73.5 ± 72.9	–
Gasteyger *et al*, 2006 (Switzerland)	N/A	35.4 ± 6.5	–	43.8 ± 4.1	–	140 (no SD)	–	61.3 (no SD)[d]	–
Ortiz Espejo *et al*, 2011 (Spain)	Obese controls	48	–	–	–	–	–	64.8 ± 78.5	–
Palomar *et al*, 2005 (Spain)	N/A	40.1 ± 11.6	–	46.9 ± 6.3	–	129 ± 22	–	49.0 ± 52.3	–
Payne & DeWind, 1969 (USA)	N/A	34.6	–	Mean weight: 140.9 kg	–	14.2 ± 1.9[e]	–	–	–
Skroubis *et al*, 2006 (Greece)	RYGBP group	32.6 ± 9.4	–	45.6 ± 4.9	–	140 ± 10	–	41.4 ± 45.0	–
	BPD group	36.1 ± 9.8		57.2 ± 6.1		140 ± 24		71.6 ± 82	
Smith *et al*, 2013 (UK) [Abstract]	Normoglycaemic	36.5 ± 12.2		46.5 ± 6.8				105 ± 93	
	Pre-diabetic	44.4 ± 11.5	–	47.9 ± 7.4	–	–	–	118 ± 125	–
	Diabetic	50.9 ± 10.3		46.6 ± 7.7				129 ± 151	

Study (Country)	Strata/Group	Age (years)		BMI (kg/m^2)		Haemoglobin (g/l)		Ferritin (μg/l)	
		Obese	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**
Thurnheer *et al*, 2012 (Switzerland)	Male					151 ± 19	–	174 ± 128	–
	Female	41.4 ± 10.8	–	48.5 ± 11.5	±	140 ± 20		62 ± 52	
Toh *et al*, 2009 (Australia)	RYGBP group (male)	48 ± 10		43.1 ± 5.2		146 ± 13		170 ± 190	
	RYGBP group (female)		–		–	132 ± 7	–		–
	SG group (male)	46 ± 12		51.0 ± 11.2		140 ± 14		152 ± 140	
	SG group (female)					136 ± 9			
Tussing-Humphreys *et al*, 2009 (USA)	N/A	35.6 ± 7.2	37.0 ± 8.7	50.1 ± 11.0	21.9 ± 4.9	122 ± 12[f]	120 ± 11[f]	26.3 ± 19.7[f]	18.4 ± 17.8[f]

Data presented as mean ± SD unless otherwise specified

AGB, adjustable gastric banding; BPD, biliopancreatic diversion; BMI, body mass index; DS, duodenal switch; GBP, gastric bypass; N/A, not applicable; NASH, non-alcoholic steatohepatitis; RYGBP, Roux-en-Y gastric bypass; SG, sleeve gastrectomy

[a]Median (interquartile range)

[b]Median (95% confidence interval)

[c]Ferritin data likely reported in incorrect units (data originally reported in mg/l)

[d]Ferritin data likely reported in incorrect units (data originally reported in μmol/l)

[e]Haemoglobin data reported in mg. per cent

[f]Geometric mean (95% confidence interval)

A5. Summary of outcomes for non-bariatric surgery studies (transferrin saturation, soluble transferrin receptor, hepcidin and C-reactive protein)

Study (Country)	Strata/Group	Transferrin saturation (%)		sTfR (mg/l)		Hepcidin (ng/ml)		CRP (mg/l)	
		Obese	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**
Ahmed *et al*, 2008	Male (Normal)								
(Australia)	Female <50 y (Normal)								
	Female ≥50 y (Normal)								
	Male (Overweight)								
	Female <50 y (Overweight)	–	–	–	–	–	–	–	–
	Female ≥50 y (Overweight)								
	Male (Obese)								
	Female <50 y (Obese)								
	Female ≥50 y (Obese)								
Anna *et al*, 2009	BMI <25								
(USA)	BMI 25.1 – 30	–	–	–	–	–	–	–	–
[Abstract]	BMI 30.1 – 35								
	BMI >35								
Ausk & Ioannou, 2008	BMI <25		28 ± 20						
(USA)	BMI 25 – 30		27 ± 18						
	BMI 30 – 35	24 ± 12		–	–	–	–	–	–
	BMI 35 – 40	22 ± 15							
	BMI ≥40	20 ± 17							
Beard *et al*, 1997	VLED 1758 kJ/day	18.0 ± 7.5							
(USA)	VLED 2763 kJ/day	22.3 ± 9.1	–	–	–	–	–	–	–
	VLED 3349 kJ/day	20.0 ± 4.5							
Fricker *et al*, 1990 (France)	N/A	18 ± 8	20 ± 7	–	–	–	–	–	–
Hamalainen *et al*, 2012 (Finland)	Female MetS	–	–	2.8 ± 0.9	–	–	–	3.1 ± 3.4[a]	–

Study (Country)	Strata/Group	Transferrin saturation (%)		sTfR (mg/l)		Hepcidin (ng/ml)		CRP (mg/l)	
		Obese	Non-obese	Obese	Non-obese	Obese	Non-obese	Obese	Non-obese
Kretsch *et al*, 1998 (USA)	N/A	23.4 ± 6.7	–	–	–	–	–	–	–
Lecube *et al*, 2006 (Spain)	N/A	29.8 ± 10.8	30.8 ± 10.7	1.38 ± 0.38[b]	1.16 ± 0.34[b]	–	–	–	–
Micozzi *et al*, 1989 (USA)	BMI <27.4		29.0 ± 0.3[c]						
	BMI 31.1 – 36.1	26.9 ± 0.3[c]		–	–	–	–	–	–
	BMI >36.1	24.1 ± 0.3[c]							
Murer *et al*, 2012 (Switzerland) [Abstract]	N/A	–	–	3.9 ± 1.9	–	2.79 ± 2.93	–	3.81 ± 5.24	–
Świtoniak & Król, 1992 (Poland)	Underweight		26.7 ± 5.0						
	Normal weight		24.5 ± 9.8	–	–	–	–	–	–
	Overweight		27.2 ± 10.2						
	Obese	28.4 ± 11.4							
Yanoff *et al*, 2007 (USA)	N/A	20.3 ± 9.9	23.0 ± 9.9	1.92 ± 0.60	1.79 ± 0.61	–	–	7.5 ± 6.7	3.4 ± 6.7

Data presented as mean ± SD unless otherwise specified

BMI, body mass index; CRP, C-reactive protein; MetS, metabolic syndrome; N/A, not applicable; sTfR, soluble transferrin receptor; VLED, very-low-energy diet

[a]High sensitivity CRP results reported

[b]sTfR data likely reported in incorrect units (data originally reported in mg/dl)

[c]Mean ± standard error (conversion to mean ± SD was not possible due to lack of information regarding sample size)

A6. Summary of outcomes for bariatric surgery studies (transferrin saturation, soluble transferrin receptor, hepcidin and C-reactive protein)

Study (Country)	Strata/Group	Transferrin saturation (%)		sTfR (mg/l)		Hepcidin (ng/ml)		CRP (mg/l)	
		Obese	Non-obese	Obese	Non-obese	Obese	Non-obese	Obese	Non-obese
Aasheim *et al*, 2009 (Sweden, Norway)	GBP group DS group	–	–	–	–	–	–	14 ± 42.6[a] 14 ± 31.0[a]	–
Amaral *et al*, 1984 (USA)	Male Female	–	–	–	–	–	–	–	–
Anty *et al*, 2008 (France)	N/A	19 (46-26)[b]	–	–	–	–	–	10.0 (7.0-18.0)[b,c]	–
Bekri *et al*, 2006 (France)	Lean controls	19.6 ± 8.3	–	–	–	–	–		1 (1-3.3)[d]
	Obese							8.7 (13.9-16.1)[d]	
	Obese with diabetes							10.0 (7.2-13.6)[d]	
	Obese with NASH							9.0 (4.3-14.3)[d]	
Belgaumkar *et al*, 2011 (UK) [Abstract]	N/A	–	–	–	–	–	–	–	–
Brolin *et al*, 1998 (USA)	Placebo group Intervention group	23 ± 9 27 ± 15	–	–	–	–	–	–	–
Cooper *et al*, 1999 (Australia)	N/A	–	–	–	–	–	–	–	–
Coupaye *et al*, 2009 (France)	AGB group GBP group	21.6 ± 11.9 19.6 ± 8.1	–	–	–	–	–	12.2 ± 8.8 11.4 ± 7.9	–

Study (Country)	**Strata/Group**	**Transferrin saturation (%)**		**sTfR (mg/l)**		**Hepcidin (ng/ml)**		**CRP (mg/l)**	
		Obese	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**
de Luis *et al*, 2013 (Spain)	BMI 35-40 BMI 40-45 BMI 45-50 BMI >50	–	–	–	–	–	–	–	–
Ernst *et al*, 2009 (Switzerland)	N/A	–	–	–	–	–	–	–	–
Gasteyger *et al*, 2006 (Switzerland)	N/A	–	–	–	–	–	–	–	–
Ortiz Espejo *et al*, 2011 (Spain)	Obese controls	–	–	–	–	–	–	–	–
Palomar *et al*, 2005 (Spain)	N/A	31.5 ± 19.2	–	–	–	–	–	–	–
Payne & DeWind, 1969 (USA)	N/A	–	–	–	–	–	–	–	–
Skroubis *et al*, 2006 (Greece)	RYGBP group BPD group	–	–	–	–	–	–	–	–
Smith *et al*, 2013 (UK) [Abstract]	Normoglycaemic Pre-diabetic Diabetic	25.9 ± 10.2 22.6 ± 9.6 21.6 ± 9.3	–	–	–	–	–	–	–
Thurnheer *et al*, 2012 (Switzerland)	Male Female	–	–	–	–	–	–	–	–

Study (Country)	**Strata/Group**	**Transferrin saturation (%)**		**sTfR (mg/l)**		**Hepcidin (ng/ml)**		**CRP (mg/l)**	
		Obese	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**	**Obese**	**Non-obese**
Toh *et al*, 2009 (Australia)	RYGBP group (male) RYGBP group (female) SG group (male) SG group (female)	–	–	–	–	–	–	7.4 ± 5.8[e]	–
Tussing-Humphreys *et al*, 2009 (USA)	N/A	14.2 ± 8.3[f]	18.2 ± 10.7[f]	2.76 ± 0.82[f]	2.00 ± 0.57[f]	88.02 ± 100.77[f]	9.70 ± 13.33[f]	7.5 ± 5.4[f]	0.8 ± 1.5[f]

Data presented as mean ± SD unless otherwise specified

AGB, adjustable gastric banding; BPD, biliopancreatic diversion; CRP, C-reactive protein; DS, duodenal switch; GBP, gastric bypass; N/A, not applicable; NASH, non-alcoholic steatohepatitis; RYGBP, Roux-en-Y gastric bypass; SG, sleeve gastrectomy; sTfR, soluble transferrin receptor

[a]Data obtained from a subgroup (*n*=15)

[b]Median (interquartile range)

[c]Data obtained from a subgroup (*n*=55)

[d]Median (95% confidence interval)

[e]Data obtained from RYGBP group (*n*=103)

[f]Geometric mean (95% confidence interval)

APPENDIX B: SUPPLEMENTARY MATERIAL FOR CHAPTERS 4 AND 5[a]

B1. Study protocol for the cross-sectional and longitudinal dietary intervention studies

B2. Participant information sheet for the cross-sectional study

B3. Participant consent form for the cross-sectional study

B4. Participant information sheet for the longitudinal dietary intervention study

B5. Participant consent form for the longitudinal dietary intervention study

B6. Dietary prescription provided to participants randomised to the higher-protein, higher-haem iron diet

B7. Dietary prescription provided to participants randomised to the lower-protein, lower-haem iron diet

B8. Comparison of nutritional profiles of the higher-protein, higher-haem iron and lower-protein, lower-haem iron diets based on seven day models designed for a 23 year old female with light activity

B9. Seven day model of the higher-protein, higher-haem iron diet

B10. Seven day model of the lower-protein, lower-haem iron diet

[a]Acknowledgement: the materials presented in Appendix B were prepared by Dr Hayley Griffin

B1. Study protocol for the cross-sectional and longitudinal dietary intervention studies

Weight Loss for Overweight Women (WOW) Study Protocol

A. Study Design

The study is a randomised controlled trial comparing a higher-protein, red meat based "Total Wellbeing" style diet to an iso-energetic conventional higher carbohydrate, white meat based diet.

Numbered envelopes containing the diet to be followed will be used to randomly allocate the diet status of each subject. These envelopes will be prepared by a researcher not involved in the recruitment or seeing of subjects according to a random block design per every four subjects.

The subjects will not be told the explicit nature of the opposing diet (i.e. difference in protein and carbohydrate) in order to keep them blinded to the intervention. They will be told only that there are two different dietary plans, which are both nutritionally adequate, restricted in energy, low in fat and include low GI foods, however one is a red meat based diet and the other is a white meat based diet.

They will be required to follow the diet and a standardised exercise plan for a period of 12 months (30 mins of brisk walking on most, preferably all, days), which is in line with the recommendations in the Australian National Physical Activity guidelines for Adults.

The study is divided into three phases. The first phase is to recruit subjects and obtain baseline measures. In this phase subjects will attend an initial screening visit where their suitability and willingness to participate will be assessed, informed consent obtained, baseline tests organised and questionnaires completed.

This is followed by a treatment phase of six months where subjects will be advised to follow the prescribed diets and standardised exercise programme and undergo further follow-up tests. During this six month phase, subjects will attend the clinic weekly for three months and then fortnightly until six months.

The subjects will be requested to maintain their diet and exercise programmes for a further six months with monthly clinic follow-up so that post intensive treatment success can be evaluated.

B. Subjects

Subjects will be recruited from a number of methods, including:

- The Metabolism and Obesity Service (MOS) at Royal Prince Alfred Hospital (RPAH) Sydney. This service provides specialised assessment and treatment to approximately 500 new obese (Body Mass Index [BMI] >35 kgm^{-2}) subjects annually. Subjects are referred by local general practitioners, specialist endocrinologists, other specialist clinicians and allied health practitioners.
- Advertisements of the study via flyers in tertiary education campuses
- Advertisement of the study via the Central Sydney Area Health Service Intranet
- Advertisement of the study via the University of Sydney Student Intranet
- Advertisement of the study in local newspapers

Inclusion Criteria:

- Healthy females aged 18-25 years
- BMI ≥ 27.5 kgm^{-2}
- Able to attend scheduled visits at MOS (RPAH)
- Willing to include red meat in the diet four or more times a week
- Fluent in English
- Adequate contraception maintained throughout the study

Exclusion Criteria:

- Significant medical conditions e.g. malignancy; cardiovascular, respiratory, gastrointestinal, renal or liver disease
- Significant immune function conditions including auto-immune diseases e.g. lupus or subjects undergoing chemotherapy treatment
- Diabetes mellitus
- Secondary cause of obesity e.g. Cushing's syndrome, hypothyroidism
- Psychiatric illness
- Severe untreated obstructive sleep apnoea (Epworth Sleepiness Scale ≥10)
- Previous bariatric surgery
- Medications known to affect appetite, metabolic rate, weight and immune function
- Severe binge eating (Binge Eating Questionnaire ≥27)
- Smoking
- Vegetarianism
- Pregnancy or breastfeeding
- Severe mobility problems
- Severe eating disorder such as bulimia nervosa or binge eating disorder
- Use of certain dietary supplements
- Iron deficiency anaemia i.e. Haemoglobin (Hb) must be normal

PROTOCOL FOR IRON LEVELS IN RANDOMISED CONTROL TRIAL:

For newly recruited participants whose baseline iron levels are found to be suboptimal, they will require varying degrees of iron supplementation prior to starting the trial to ensure optimal health upon commencement of the diet.

Protocol 1: Hb normal, ferritin normal, but serum iron low
Recruit subject as per normal method.

Protocol 2: Hb normal, but ferritin low (± low serum iron)
Subject to take Herron Iron Complex (2 tablets, 5 days/week to reduce risk of gastrointestinal upset) for 3 months. If ferritin is just low (i.e. 13-14 μgL^{-1}) then subject to take tablets for only 1 month and followed by review.

Subject can be recruited for trial once ferritin levels are normalised. Ferritin levels will be reviewed again at 6 and 12 months.

Protocol 3: Hb low
Exclude and refer to local GP.

Protocol 4: Ferritin high (>200)
Consider screening for haemochromatosis.

PROTOCOL FOR DIETARY SUPPLEMENTS IN RANDOMISED CONTROLLED TRIAL:

Protocol 1: Iron supplements

A) Subjects that have been diagnosed with iron deficiency and are currently taking (or recently ceased) iron supplementation:

These subjects can only be recruited once the treating doctor has ceased iron supplementation. Subjects will need to organise for their doctor to perform a blood test to ensure iron levels are normal. If levels are abnormal, they will need to continue the regimen and be tested again in the future.

Once they have ceased supplementation and their iron levels are normal, they can be recruited.

B) For subjects that HAVE been taking iron tablets without evidence of established iron deficiency:

Recruit once they have ceased iron supplementation.

Protocol 2: Zinc supplements

Exclude

Protocol 3: Multivitamin supplements

Document exactly what supplement(s) they were taking (e.g. brand, dose, frequency) and record the contents. Exclude if tablets contain a high dose of zinc (>20mg). If supplements contain a high dose of iron, follow the iron tablet protocol. If contain low doses of zinc and iron (<10mg and <5mg respectively), then recruit but allow a minimum of 2 weeks between last tablet taken and venepuncture.

Protocol 4: Vitamin e.g. B, C supplements

Recruit, however allow a minimum of 2 weeks between when last taken and venepuncture.

Protocol 5: Fish oil supplements

Exclude if taking 5 or more capsules per day (~1.5g n-3 fatty acids). If taking less than this then recruit, however allow a minimum of 2 weeks between when last taken and venepuncture.

During the trial, all subjects are asked to not take any dietary supplements and avoid donating blood. Subjects are able to maintain their usual blood pressure or lipid management medication. However ANY changes to medication need to be reported to researchers.

C. Study Parameters

1. *Weight and Body Composition*

1.1 Weight and Body Mass Index (BMI)

Body weight (light clothing, no shoes) will be recorded on an electronic digital platform scale (Teraoka DS-260; Teraoka Seiko, Tokyo, Japan) with a capacity of 300 kg and accuracy to 0.1 kg. Height will be measured to nearest 0.5 cm with a wall-mounted stadiometer (Hyssna Limfog AB, Hyssna, Sweden).

1.2 Waist Circumference and Waist-to-Hip Ratio (WHR)

Waist and hip circumference (light clothing) will be measured to nearest 0.5 cm with a metal retractable tape (Lufkin W606PM; Cooper Industries, Sparks, USA). Waist circumference will be measured at the narrowest circumference above the level of the umbilicus (the midpoint between the last rib and the iliac crest). Hip circumference will be measured at the maximal gluteal protrusion (Lohman et al 1998).

1.3 Blood Pressure

Blood pressure will be measured using an automatic machine (OMRON Healthcare Co. Ltd., Kyoto, Japan) and manually (ACCOSON Dekamet, England) before the walking test at baseline, week 2, 3 months, 6 months and 12 months with a large cuff if necessary after the subject has been seated for 15-20 minutes.

1.4 Bio-electrical Impedance Analysis (BIA)

Body composition will be measured by bio-electrical impedance using the tetra-polar surface electrode technique. BIA involves the use of a small electrical current that is passed through the body for quick measurement of body composition. It is approved for use during pregnancy and in neonates. BIA measurements will be taken after a four hour fast according to a strict standardised protocol used by the staff of MOS.

Note: The initial BIA machine used broke mid-trial, and was replaced with a new one.

1.5 Dual-energy X-ray Absorptiometry (DXA)

Body composition will also be measured by DXA on a Lunar Prodigy (GE Healthcare, Giles, UK) using 8.6 software unless body shape or mass precludes the use of the DXA apparatus. The radiation dose subjects will be exposed to does not exceed 0.03mSv and side effects are negligible. DXA measurements will be performed by a trained technician in the metabolic unit at RPAH.

2. DIET AND EATING BEHAVIOURS

2.1 24 Hour Recall

This will be done "randomly" (subjects are unaware of scheduled 24 hour recall assessments) on the weeks that the food diary is to be analysed. The food diary will be analysed for the period that corresponds to weeks 4, 8, 12, 25 and 51 therefore this will be done at weeks 5, 9, 13, 26 and 52. This is so that the recall will be completed after the diary to be analysed has been completed to minimise the risk of participants changing what they ate during the week that is to be analysed.

It will be done using the multiple-pass method by a researcher trained in this methodology. Amounts will be quantified in household measures with the assistance of a photographic atlas of food portion sizes if required (Nelson, Atkinson & Meyer 1997).

This information will then be analysed using FoodWorks Version 6.0.25175 (Xyris Software, Brisbane, Australia) incorporating the AusNut (all foods) revision 18, AusFoods (brands) revision 11 and Nuttab 2006 revision 1 databases. Analyses will be performed for a 23 year old female doing light activity by a trained researcher using a standard protocol for the entry of food items. Food selections will be made using the description closest to the item. Due to limitations in the database, modelling is not going to be performed for all nutrients (e.g. vitamin B_{12}, fatty acids).

2.2 3 Day Food Diary

Although a weighed food diary is considered to be the 'gold standard' dietary method for recording food intake, these diaries are labour intensive for the respondent and compliance is a problem. The use of semi-quantative household measures is much easier for the subject and similar results have been obtained using this method. Due to the length of the study and the respondent burden from other aspects of measurement, semi-quantative household measures have been selected for the recording of dietary information.

The diary will be kept weekly (3 days/week) for the first three months, fortnightly (3 days/fortnight) for the following three months and once a month (3 days/month) for the last six months. Food diary analysis (two weekdays and one weekend day) will be undertaken at weeks 4, 8 12, 25 and 51. Analysis will be as reported for the 24 hour recall above.

Subjects will be completing more diaries than will be analysed, so that they gain benefits from completing a diary (i.e. being aware of what they are eating etc.) and are also unaware of the weeks selected for dietary analysis. To improve the completeness of food record keeping, subjects will be urged to report exactly what they eat and every effort will be made for them to feel comfortable reporting digressions from the diet. In addition, one set of electronic household scales and a brief training session on how to use household measures will be provided to subjects when the diary is introduced.

2.3 Daily Hunger and Desire to Eat Scores

Subjects will rate hunger for the entire day on a 10 cm visual analogue scale (VAS) ranging from "not at all hungry" at one end to '"extremely hungry" at the other. Desire to eat will also be rated on a 10cm VAS from "not at all strong" to "extremely strong". Subjects will be instructed to summarise their hunger/desire to eat into these rating scales to reflect how they felt over the day. The VAS will be placed at the bottom of each page in the food diary as a reminder for subjects to complete this on the days food intake is recorded.

2.4 Health Log

Subjects will be asked to record their health daily during the trial using the following criteria that have been used in studies measuring immune function (Nieman et al, 1990; Nieman et al, 1998). This provides some guide to the general health of the subjects during the study.

1. No health problems today
2. Cold symptoms (runny, stuffy nose, sore throat, coughing, sneezing, coloured discharge)
3. Flu symptoms (fever, headache, general aches and pains, fatigue, weakness, chest discomfort, cough)
4. Nausea, vomiting, diarrhoea
5. Muscle, joint or bone problems, injury
6. Other health problem (describe).

2.5 Infection Frequency

Subjects will complete a subjective rating on how often they become sick with an infection (upper respiratory tract) such as a cold or flu using a 10 cm visual analogue scale (VAS) ranging from at one end "always sick with infections" to "never sick with infections" (i.e. the ends of the scales represent the extremes that they only rarely experience). Subjects will be asked complete this scale at baseline, 6 months and 12 months.

2.6 Three Factor Eating Questionnaire (TFEQ)

The TFEQ measures restrained eating or the cognitive control of eating behaviour, the disinhibition of control (tendency to break restraint) and perceived hunger. It is a validated questionnaire that is used at MOS for obese patients.

The TFEQ is estimated to take no more than twenty minutes to complete and will be completed at baseline, the end of 3, 6 and 12 months of treatment and finally at post-trial follow-up.

2.7 Binge Eating Questionnaire

The Binge Eating Questionnaire is a self-report measure designed to assess the extent and severity of binge eating problems in the obese. During the trial, use of this questionnaire will help to identify differences in the binge eating tendency between the two diets. It is a questionnaire that has been validated in obese patients (Gormally et al, 1982), and is estimated to take no more than twenty minutes to complete and will be

completed at baseline, the end of 3, 6 and 12 months of treatment and finally at post-trial follow-up.

We will be excluding those subjects with a severe binge eating questionnaire score (≥27), but will discuss management options with them.

2.8 Harter Self Perception Questionnaire

Concepts such as self-esteem, self-image and perceived competence are central to an individual's overall perception of their self-worth. Low levels of these constructs have been reported in obese individuals and are observed to improve with weight loss.

The Harter Self Perception Assessment has been developed to assess global self-worth and the importance of success in each of eleven specific domains for the individual (sociability, job competence, nurturance, athletic abilities, physical appearance, adequate provider, morality, household management, intimate relationships, intelligence, sense of humour).

The Harter Self Perception Questionnaire is a validated questionnaire that is used at MOS for obese patients. It is estimated to take no more than twenty minutes to complete and will be completed at baseline, the end of 3, 6 and 12 months.

2.9 Food Habits Questionnaire

This questionnaire measures fat intake and food habits. It has been validated as suitable for assessing food habits relevant to controlling weight and is a questionnaire used at MOS as a part of clinical care. The food habits questionnaire is estimated to take no more than twenty minutes to complete and will be completed at baseline, the end of 3, 6 and 12 months.

2.10 Weight Cycling Questions

Weight cycling will be assessed by modifying the question used recently by Shade et al (2004) to ask "within the last two, five and ten years when you were not pregnant or sick, did you ever lose 5 kg or more on purpose?" In addition we will ask subjects to report their minimum and maximum (non-pregnant) weight (if >18 years), the maximum amount of weight they had lost at one time and the methods used for weight loss.

2.11 24 Hour Urea Nitrogen

24 hour urea nitrogen will be measured as 24 hour urine urea excretion using the Roche Modular instrument with Roche kits at Laverty Pathology Laboratory, Sydney. This test will be done at baseline, 3, 6 and 12 months to assess kidney function and determine dietary compliance to the protein prescription.

Participants will be provided with instructions for urine collection to ensure collection accuracy.

3. METABOLIC PARAMETERS

i) Collection and Processing

3.1 Venepuncture

Blood will be drawn at recruitment, 6 and 12 months by trained staff at Medical Centre Pathology RPAH. Recruitment blood samples will be used to confirm subject eligibility e.g. iron levels (iron deficiency anaemia), glucose (diabetes) and TSH (Thyroid Stimulating Hormone) (thyroid disease).

Blood will be collected from the antecubital vein with the subject fasting and seated. The following criteria will also be used (specifically designed for the immune markers): no strenuous exercise or alcohol for 24 hrs, adequate sleep, no topical corticosteroids for 48 hrs, no symptoms of infection or systemic antihistamines or corticosteroids for a week, no aspirin for two days and no immunizations for 3 weeks prior to the draw.

3.2 Plasma and Packed Red Cell Storage: The University of Sydney

The study researcher will collect all blood samples and transport on ice to the Endocrine and Metabolic Unit at RPA for processing and distribution. Heparin and EDTA plasma samples will be stored in 1 mL aliquots within polypropylene tubes temporarily at -20°C then transported in dry ice to The University of Sydney (Cumberland Campus) for long term storage at -80°C in the School of Exercise and Sport Science.

Blood samples for analysis at Laverty Pathology will be transported to the commercial laboratory via courier.

ii) Parameters to be Measured – Laverty Pathology

3.4 Liver Function Tests (LFT), Urea, Creatinine, Glucose & Lipids

All chemistries (LFT, urea, creatinine, glucose) and lipids (total cholesterol, TG, LDL and HDL) will be measured from serum using the Roche Modular instrument with Roche kits.

3.5 Routine Iron Studies

Iron studies (including serum iron, transferrin and ferritin) will be done from serum using the Roche Modular E170 immunoassay analyser with Roche kits.

3.6 Thyroid Stimulating Hormone (TSH)

Serum TSH will be measured by Immunochemiluminometric assay (ICMA) using Siemens Advia Centaur immunoassay analyser with Siemens kits.

3.7 Full Blood Count (FBC)

FBC will be measured from serum using the Sysmex instrument being a Roche platform.

3.8 Zinc

Zinc (a potential confounder for immune function) will be measured from plasma using the Perkin-Elmer instrument with Inductivity Coupled Plasma Mass Spectroscopy (instrument model Perkin-Elmer Elan DRC Plus).

3.9 Immune Markers

Lymphocyte subsets will be measured from whole blood by flow cytometry using the Multi IMK test (lyse no wash) method.

iii) Parameters to be Measured – The University of Sydney

3.10 Inflammatory Markers

Inflammatory markers such as C-Reactive Protein (CRP), Tumour Necrosis Factor-Alpha (TNF-α) and Interleukin-6 (IL-6) will be measured from fasting plasma samples using a commercial enzyme-linked immunosorbent analysis (ELISA) kit.

Note: ELISA results from the TNF-α and IL-6 kits were invalid (absorbances for the standard curve and most samples were below the lower limit of detection) and hence were not used in the final analysis.

3.11 Insulin

Fasting plasma insulin will be measured using a commercial ELISA kit.

3.12 Appetite Markers

Adipocyte hormones Leptin and Adiponectin, as well as Ghrelin and Gastric Inhibitory Peptide (GIP) will be measured from fasting plasma samples using commercial ELISA kits.

3.13 Soluble Transferrin Receptor (sTfR)

sTfR will be measured from fasting plasma samples using commercial ELISA kits.

3.14 Genetic Analysis

DNA will be extracted from packed red blood cells. Single nucleotide polymorphism and polymerase chain reaction technologies will be applied by one of the researchers to identify potential candidate genes that may affect response to a particular diet (e.g. hepatic lipase gene [LIPC], *PPAR gamma 2* and the leptin receptor gene) or iron metabolism (e.g. hepcidin and ferritin).

iv) Parameters to be Measured – Other

3.15 Resting Metabolic Rate (RMR)

RMR will be measured with the subject in a semi-reclined position using a portable bedside metabolic monitor (Deltatrac II Metabolic Monitor, Datex-Ohmeda, Helsinki, Finland) by a trained technician in the metabolic unit at RPAH.

Subjects will be fasted for 12 hours (including no medications) and will not have performed any strenuous exercise 24 hours prior to the measurement. They will be rested for a minimum of 30 minutes prior to measurement. They will be made to feel comfortable and relaxed and will be familiarised with the procedure.

Nitrogen excretion will be estimated to be 12g/24 hr for all subjects. Predicted energy expenditure is calculated using the Harris Benedict equation. Actual energy expenditure is calculated on the Deltatrac according to the equation derived by Weir (de V Weir 1949).

4. PHYSICAL FITNESS/ACTIVITY

4.1 Sub-Maximal Oxygen Uptake (VO_2)

The cardiovascular fitness of subjects will be assessed using a sub-maximal walk test on a treadmill (Technogym, Gambettola, Italy). Subjects are required to achieve three or four steady state heart rates when walking at **4 km/hr** while the treadmill grade increases by **2%** every 3 minutes. Ratings of perceived exertion will be collected at each steady state using the Borg Scale (Borg, 1982).

The week 1 treadmill test will be used to familiarise subjects with the test, and determine the appropriate grades at which to test them. The test will be repeated at week 2 (to obtain a baseline reading following familiarisation), 6 and 12 months to track fitness during and after the trial.

4.2 Pedometer

Subjects will be asked to wear a pedometer (Yamax SW-200, Yamax Corporation, Tokyo, Japan) to monitor daily activity for the first three months, then one week a month between 3 and 6 months.

4.3 3 Day Activity Diary

The energy expenditure of subjects will be assessed using a modified physical activity diary (Bouchard et al 1983). This is completed for 3 days (two weekdays and one weekend day). Subjects record their activity for every 15 minute period, which is converted into total metabolic activity.

4.4 Exercise Record

Subjects will record any physical activity (type and duration) daily during the trial. This is another method to assess the level of exercise subjects do while participating in the trial, and will also act as a behaviour modification tool as subjects will be more aware of their physical activity.

5. SLEEP

Epworth Sleepiness Questionnaire

While a full sleep study is the best way to diagnose obstructive sleep apnoea, this is not practical for this trial in terms of time and cost. Therefore in order to identify subjects at higher risk of sleep apnoea (one of the exclusion criteria), the Epworth Sleepiness Scale (ESS) will be used to screen patients. The ESS is a measure of general daytime sleepiness, an important symptom of sleep apnoea. Subjects scoring 10 or more on the ESS will be excluded from the trial.

6. CROSS-SECTIONAL STUDY (CSS)

In addition to the longitudinal trial outlined above, we will determine the prevalence of iron deficiency, inflammation and measure immunocompetence in a wider sample of overweight and obese women aged 18-25 years.

We plan to measure an additional 50 subjects over the duration of the study. Combining data with the longitudinal trial cohort provides a total of 120 subjects. We will evaluate the effect of weight cycling/dieting behaviour on the abovementioned parameters as well as the relationships between excess weight, inflammation and differential iron markers.

These women will fit all inclusion/exclusion criteria for the longitudinal trial with the exception of:

- Being unable to participate in the trial due to their inability to attend scheduled visits
- Being unwilling to include red meat in their diet
- Being unable to maintain adequate contraception throughout the study
- Having severe untreated obstructive sleep apnoea, severe eating disorder, iron deficiency anaemia or severe mobility problems
- Have not donated blood in the past 3 months
- Are not undergoing metformin treatment

PROTOCOL FOR IRON LEVELS IN CSS

Protocol 1: **Hb normal, ferritin normal, but serum iron low**
Recruit subject as per normal method.

Protocol 2: **Hb normal, but ferritin low (± low serum iron)**
Refer to local GP and request treatment.

Protocol 3: **Hb low**
Refer to local GP and request treatment.

Protocol 4: **Ferritin high (>200)**
Subject may need to be screened for haemochromatosis.

In addition, there is a separate protocol for dietary supplements for those included in the cross-sectional study:

PROTOCOL FOR DIETARY SUPPLEMENTS IN CSS

Protocol 1: **Iron supplements**
Exclude. Note: If subject has been taking (or given after blood donation) normal iron supplements i.e. not Ferro-Gradumet, and willing to wait, can be recruited after 3 month "wash out" from last tablet.

Protocol 2: **Zinc supplements**
Exclude

Protocol 3: **Multivitamin supplements**
Document the exact supplement(s) taken (e.g. brand, dose, frequency) and record the contents. Exclude if tablets contain a high dose of zinc or iron. Recruit if contain low doses of zinc and iron, however allow a minimum of 2 weeks between when last taken and the venepuncture.

Protocol 4: **Vitamin e.g. B, C supplements**
Recruit, however allow a minimum of 2 weeks between when last taken and the venepuncture.

Protocol 5: **Fish oil supplements**
Exclude if taking 5 or more capsules per day (~1.5g n-3). If taking less than this then recruit, however allow a minimum of 2 weeks between when last taken and the venepuncture.

Subjects in the cross-sectional study will be asked if they have a secondary cause of obesity, however they will NOT be screened for thyroid function as per the longitudinal trial.

6.1 Parameters to be Measured (Also Included in the Longitudinal Trial)

As with the longitudinal trial participants, these subjects will provide a weight/dieting history in addition to a baseline blood test. Parameters to be collected include:

- Weight, BMI, BIA, Waist circumference & WHR
- TFEQ
- Binge eating questionnaire
- Food habits questionnaire
- Weight cycling questions
- FBC, routine iron studies & sTfR
- Immune/Inflammatory markers
- Epworth Sleepiness Scale

6.2 Parameters to be Measured (Not Included in the Longitudinal Trial)

Frozen plasma samples will be transported to the Princess Alexandria Hospital (Brisbane, Australia) for measurement of plasma hepcidin by an on-line extraction coupled to liquid chromatography-tandem MS method (Franklin et al, 2010) using the Xevo TQ MS (Waters Corporation, Milford, USA).

B2. Participant information sheet for the cross-sectional study

Level 6 West Main Building
Missenden Rd
Camperdown NSW 2050 AUSTRALIA
Phone: 61 2 95154220 Fax: 61 2 95155820
Email: mos@email.cs.nsw.gov.au

The effect of weight cycling on iron status, inflammation and immune function in young, overweight and obese women

INFORMATION FOR PARTICIPANTS

Introduction

You are invited to take part in a research study that examines the effect of weight cycling on iron status, inflammation and immune function. The objective is to measure iron levels, inflammation and the immune systems of young women and then investigate how weight cycling can affect these factors. This research study is part of larger trial that is comparing the effectiveness of a red meat based, low fat diet and a white meat based, low fat diet on weight loss, immune function, blood fats, glucose, insulin and iron levels.

Weight gain in young women is often associated with frequent and unsuccessful dieting, yet not enough research has occurred on weight management strategies for this group. This research study will provide valuable information on how changes in weight can affect young women.

The study is being conducted within this institution by the following researchers:

- Dr Helen O'Connor, Lecturer, Department of Exercise and Sport Science, The University of Sydney
- Prof Kate Steinbeck, Conjoint Professor University of Sydney, Faculty of Medicine & Senior Staff Specialist in Endocrinology & Adolescent Medicine, Royal Prince Alfred Hospital
- Dr Kieron Rooney, Lecturer, Department of Exercise and Sport Science, The University of Sydney
- Hayley Griffin, PhD student, The University of Sydney
- Helen Cheng, Research Assistant, The University of Sydney

This study is being sponsored by Meat and Livestock Australia, under a Red Meat and Human Nutrition Research and Development Grant.

Study Procedures

If you agree to participate in this study, you will be asked to sign the Participant Consent Form. You will then be asked to complete a number of procedures in order to determine your degree of obesity, your weight history and health status. Each procedure will need to be done only once for this study, and they should all be completed during one or two clinic visits. Each questionnaire should not take more than 20 minutes to complete (i.e. less than 1 1/2 hours to complete all of them). The procedures involved include:

- Weight, height, waist and hip measurements
- Three diet/eating behaviour questionnaires and weight cycling history
- Sleep questionnaire
- Bio-electrical impedance (BIA): This involves small electrodes being attached to your ankle and wrist that pass a current through your body and is pain and risk free. It is used in order to assess your body composition assess so we can determine how much of the body is comprised of fat and lean muscle.
- Blood test: You will be asked to provide a 25 ml blood sample in order to measure your full blood count, iron status, immune and inflammatory markers. DNA will be isolated from your blood sample for gene analysis. This genetic analysis allows the researchers to study your genetic code in order to identify if you express specific genes that may relate to your weight. The blood would be taken from a vein in your arm by an experienced pathologist.

Most of these tests are performed routinely at the Metabolism and Obesity Services regardless of your participation in this study. If you have a referral for the Metabolism and Obesity Services, you will be referred back for routine treatment after completing this study.

Risks

All medical procedures - whether for diagnosis or treatment, routine or experimental – involve some risk of injury. In addition, there may be risks associated with this study that are presently unknown and unforeseeable. In spite of all precautions, you might develop medical complications from participating in this study.

Blood collection involves some discomfort at the site from which the blood is taken. There is also a risk of some minor bruising at the site, which may last one to two days.

It is also important that women participating in this study are not pregnant and do not become pregnant during the course of the study. If you are a woman of child-bearing potential and there is any possibility that you are pregnant, the researchers will perform a pregnancy (urine) test before you start in the study. If necessary, you should use reliable contraception (such as oral or implanted contraception, an IUD or have had a tubal ligation) during the course of the study. If at any time you think you may have become pregnant, it is important to let the researchers know immediately.

Participants will be able to maintain their usual blood pressure or lipid management medication during this study. However, any changes to medications should be reported to the researchers.

Benefits

While we intend that this research study furthers medical knowledge and may improve treatment options for weight gain in young women in the future, it may not be of direct benefit to you.

Compensation for injuries or complications

If you suffer any injuries or complications as a result of this study, you should contact the study doctor as soon as possible, who will assist you in arranging appropriate medical treatment. If you are eligible for Medicare, you can receive any medical treatment required to treat the injury or complication, free of charge, as a public patient in any Australian public hospital. In the unlikely event that you experience psychological distress during this study, an appropriate referral will be made.

In addition, you may have a right to take legal action to obtain compensation for any injuries or complications resulting from the study. Compensation may be available if your injury or complication is sufficiently serious and is caused by unsafe drugs or equipment, or by the negligence of one of the parties involved in the study (for example, the researcher, the hospital, or the treating doctor). You do not give up any legal rights to compensation by participating in this study.

Costs

Participation in this study will not cost you anything, nor will you be paid.

Voluntary Participation

Participation in this study is entirely voluntary. You do not have to take part in it. If you do take part, you can withdraw at any time without having to give a reason. Whatever your decision, please be assured that it will not affect your medical treatment or your relationship with the staff who are caring for you.

Confidentiality

All the information collected from you for the study will be treated confidentially, and only the researchers named above will have access to it. The study results may be presented at a conference or in a scientific publication, but individual participants will not be identifiable in such a presentation.

Further Information

When you have read this information, Helen Cheng will discuss it with you further and answer any questions you may have. If you would like to know more at any stage, please feel free to contact her on (02) 9515 4235 or 0434 362 515.

This information sheet is for you to keep.

Ethics Approval

This study has been approved by the Ethics Review Committee (RPAH Zone) of the Sydney South West Area Health Service, and by the Human Research Ethics Committee of The University of Sydney. Any person with concerns or complaints about the conduct of this study should contact either:

- The Secretary of the Ethics Review Committee (RPAH Zone) of the Sydney South West Area Health Service on (02) 9515 6766 and quote protocol number X09-0107
- The Manager of Ethics Administration at The University of Sydney on (02) 9351 4811 and quote reference number 8286

Version No. : #11
Date: 03/06/09

B3. Participant consent form for the cross-sectional study

Level 6 West Main Building
Missenden Rd
Camperdown NSW 2050 AUSTRALIA
Phone: 61 2 95154220 Fax: 61 2 95155820
Email: mos@email.cs.nsw.gov.au

The effect of weight cycling on iron status, inflammation and immune function in young, overweight and obese women

PARTICIPANT CONSENT FORM

I, ... *[name]*

of
....*[address]*

have read and understood the Information for Participants on the abovenamed research study

and have discussed the study with ....

I have been made aware of the procedures involved in the study, including any known or expected inconvenience, risk, discomfort or potential side effect and of their implications as far as they are currently known by the researchers.

I freely choose to participate in this study and understand that I can withdraw at any time.

I also understand that the research study is strictly confidential.

I hereby agree to participate in this research study.

NAME: ....

SIGNATURE: ....

DATE: ....

NAME OF WITNESS: ....

SIGNATURE OF WITNESS: ....

Version No. : #9
Date: 19/02/09

B4. Participant information sheet for the longitudinal dietary intervention study

Level 6 West Main Building
Missenden Rd
Camperdown NSW 2050 AUSTRALIA
Phone: 61 2 95154220 Fax: 61 2 95155820
Email: mos@email.cs.nsw.gov.au

Comparison of red meat based, low fat diet versus white meat based, low fat diet on weight loss, immune function, hunger, metabolic parameters and iron status in young, overweight and obese women

INFORMATION FOR PARTICIPANTS

Introduction
You are invited to take part in a research study comparing the effectiveness of a low fat red meat (RM) and white meat (WM) diet on weight loss, immune function, hunger, metabolic parameters and iron status. The objective is to investigate whether a RM or WM diet is more effective for weight loss and controlling hunger, as well as improving immune function, blood fats, glucose, insulin and iron levels in young women.

Weight gain in young women is often associated with frequent and unsuccessful dieting, yet not enough research has occurred on weight management strategies for this group. This research study will provide valuable information on the wellbeing of young women who are consuming either a RM or WM based weight loss diet. In order to participate in this study, you will need to be prepared to eat either red or white meat at least four times each week.

The study is being conducted within this institution by the following researchers:

- Dr Helen O'Connor, Lecturer, Department of Exercise and Sport Science, The University of Sydney
- Prof Kate Steinbeck, Conjoint Professor University of Sydney, Faculty of Medicine & Senior Staff Specialist in Endocrinology & Adolescent Medicine, Royal Prince Alfred Hospital
- Dr Kieron Rooney, Lecturer, Department of Exercise and Sport Science, The University of Sydney
- Hayley Griffin, PhD student, The University of Sydney
- Helen Cheng, Research Assistant, The University of Sydney

This study is being sponsored by Meat and Livestock Australia, under a Red Meat and Human Nutrition Research and Development Grant.

Study Procedures
If you agree to participate in this study, you will be asked to sign the Participant Consent Form. You will then be asked to complete a number of procedures to determine your weight, body composition (i.e. fat and lean muscle mass), health status, fitness, diet/eating/sleeping behaviours and resting metabolic rate. A description of these procedures is provided below.

These procedures will tell us if you are eligible to participate in this study. If you are not eligible, and have a referral for the Metabolism and Obesity Services, you will be referred back for routine treatment. If you are eligible and interested in participating, you will be allocated to one of the two dietary plans by a process called randomisation. The term randomisation means you have an equal chance of being allocated to either group, similar to tossing a coin.

You will be asked to follow the prescribed dietary plan for 12 months, and to attend the clinic (for approximately 1 hour) once per week for the first 3 months, then once per fortnight from 3 to 6 months, and finally once per month from 6 to 12 months. During these visits you will receive information on your diet and some of the study procedures will be performed. Each questionnaire on sleep and eating behaviours should not take more than 20 minutes to complete (i.e. less than 2 hours to complete all of them) and you will be able to fill them out at home if this option is more convenient. However, you may be required to attend the clinic at other times for certain tests. You will also be advised to undertake some form of physical activity/exercise e.g. walking for 30 minutes each day, and record in a diary your food intake and how you are feeling. You will be given appropriate log sheets to record these items.

The following procedures will occur at the clinic:

- Weight, height, waist and hip measurements
- Diet history (including past weight cycling) and four diet/eating behaviour questionnaires
- Physical activity and sleep questionnaires
- Body composition via dual-energy x-ray absorptiometry (DEXA) scan and bio-electrical impedance (BIA): DEXA will be done three times and involves a whole body scan with x-rays. BIA will be done four times and involves small electrodes being attached to your ankle and wrist that pass a current through your body. Both methods are pain free.
- Blood test: A 35 ml blood sample will be taken at three different times in order to measure lipids (including cholesterol), glucose, insulin, liver function tests, creatinine, urea, full blood count, iron status, appetite markers, immune and inflammatory markers. DNA will be isolated from your blood sample for gene analysis. This genetic analysis allows the researchers to study your genetic code in order to identify if you express specific genes that may relate to your weight. The blood would be taken from a vein in your arm by an experienced pathologist.
- Resting metabolic rate: This will be done three times and involves you lying still for around 40 minutes under a clear plastic hood while your breathing is monitored.
- Sub-maximal VO_2 treadmill test: This will be done three times. This test involves supervised walking, not running, on a treadmill and having your heart rate monitored.

You will be asked to complete the following procedures, outside the clinic:

- Food and health diary: You will be asked to keep a 3-day record of your food and drink intake, a record of how hungry you feel and how well you are feeling regularly throughout the study.
- Pedometer: You will need to wear a pedometer regularly during the first half of the study and to record the readings. A pedometer is a non-intrusive devise that is attached to your clothing.
- Urine collection for 24hr Urea Nitrogen test: You will be asked to collect all of your urine for 24 hours and then bring it into the clinic for processing four times during the study.

Following completion of the study, you will be asked to attend a clinic visit (for approximately 2 hours) 1 and 2 years post-trial where long-term sustainability of your diet will be assessed.
The following procedures will occur during this clinic visit:

- Weight, height, waist and hip measurements
- Four diet/eating behaviour questionnaires
- One physical activity questionnaire
- Dieting and weight cycling history since completion of trial
- Physical activity history since completion of trial
- Sub-maximal VO_2 treadmill test

Risks
All medical procedures - whether for diagnosis or treatment, routine or experimental – involve some risk of injury. In addition, there may be risks associated with this study that are presently unknown and unforeseeable. In spite of all precautions, you might develop medical complications from participating in this study.

The risks of participating in this study are related to the following procedures:

- Blood test: Blood collection involves some discomfort at the site from which the blood is taken. There is also a risk of some minor bruising at the site, which may last one to two days. If this occurs, you will be given suitable advice.
- DEXA Scan: This involves exposure to a very small amount of radiation. As part of everyday living, everyone is exposed to naturally occurring background radiation and receives a dose of about 2 millisieverts (2 mSv) each year. The effective dose from this study is 0.03 mSv (i.e. 0.01 mSv from each DEXA scan). At this dose level, no harmful effects of radiation have been demonstrated and the risk is negligible. Any person who would like further information on radiation should contact Jocelyn Towson, Radiation Safety Officer at RPAH, on (02) 9515 6023.
- Resting metabolic rate (RMR): Some patients may feel slightly claustrophobic when placed under the clear plastic hood. If this is the case, the procedure will be stopped immediately.

Please inform the researchers if you have participated in any other research studies using radiation in the last five years. Please keep this information sheet in a safe place for the next five years and show it to the researchers if you volunteer for any more studies using radiation.

It is also important that women participating in this study are not pregnant and do not become pregnant during the course of the study. If you are a woman of child-bearing potential and there is any possibility that you are pregnant, the researchers will perform a pregnancy (urine) test before you start in the study. If necessary, you should use reliable contraception (such as oral or implanted contraception, an IUD or have had a tubal ligation) during the course of the study. If at any time you think you may have become pregnant, it is important to let the researchers know immediately.

Participants will be able to maintain their usual blood pressure or lipid management medication during this study. However, any changes to medications should be reported to the researchers.

Benefits
As a participant of this study, you will receive regular dietary counselling and information sessions in a standard clinical setting. You will be supported to reduce your weight, improve any metabolic complications associated with excess weight and increase your physical activity. It is anticipated that both the RM and WM diet groups will lose weight during the study. You will also receive additional testing to the normal patients attending the Metabolism and Obesity Services, which includes body composition measurement by DEXA and blood tests which measure immune and inflammatory markers. This means that your treatment plan will be more holistic.

Compensation for injuries or complications
If you suffer any injuries or complications as a result of this study, you should contact the study doctor as soon as possible, who will assist you in arranging appropriate medical treatment. If you are eligible for Medicare, you can receive any medical treatment required to treat the injury or complication, free of charge, as a public patient in any Australian public hospital. In the unlikely event that you experience psychological distress during this study, an appropriate referral will be made.

In addition, you may have a right to take legal action to obtain compensation for any injuries or complications resulting from the study. Compensation may be available if your injury or complication is sufficiently serious and is caused by unsafe drugs or equipment, or by the negligence of one of the parties involved in the study (for example, the researcher, the hospital, or the treating doctor). You do not give up any legal rights to compensation by participating in this study.

Costs

Participation in this study will not cost you anything, nor will you be paid. However, you will be given vouchers for Coles or Woolworths supermarkets in order to help cover the cost of meat or chicken purchases. Vouchers will be given out at regular intervals during the trial. If you complete the full 12 months of the trial, five vouchers each to the value of $20 will be given. Therefore, the maximum total value of the vouchers given to you during the trial will be $100. If you withdraw early from the trial, you will only be given vouchers for the period prior to withdrawal (i.e. you will not receive all five vouchers).

In addition, you will be given a set of kitchen scales to use during the trial to help you measure food items. If you complete the full 12 months of the trial, you will be able to keep the scales. However if you withdraw early from the trial, you will be asked to return the scales so that other participants can use them.

Voluntary Participation

Participation in this study is entirely voluntary. You do not have to take part in it. If you do take part, you can withdraw at any time without having to give a reason. Whatever your decision, please be assured that it will not affect your medical treatment or your relationship with the staff who are caring for you.

Confidentiality

All the information collected from you for the study will be treated confidentially, and only the researchers named above will have access to it. The study results may be presented at a conference or in a scientific publication, but individual participants will not be identifiable in such a presentation.

Further Information

This information sheet is for you to keep. When you have read this information, Helen Cheng will discuss it with you further and answer any questions you may have. If you would like to know more at any stage, please feel free to contact her on (02) 9515 4235 or 0434 362 515.

Ethics Approval

This study has been approved by the Ethics Review Committee (RPAH Zone) of the Sydney South West Area Health Service, and by the Human Research Ethics Committee of The University of Sydney. Any person with concerns or complaints about the conduct of this study should contact either:

- The Secretary of the Ethics Review Committee (RPAH Zone) of the Sydney South West Area Health Service on (02) 9515 6766 and quote protocol number X05-0085
- The Manager of Ethics Administration at The University of Sydney on (02) 9351 4811 and quote reference number 8286.

Version No. : #12
Date: 16/04/09

B5. Participant consent form for the longitudinal dietary intervention study

Level 6 West Main Building
Missenden Rd
Camperdown NSW 2050 AUSTRALIA
Phone: 61 2 95154220 Fax: 61 2 95155820
Email: mos@email.cs.nsw.gov.au

Comparison of red meat based, low fat diet versus white meat based, low fat diet on weight loss, immune function, hunger, metabolic parameters and iron status in young, overweight and obese women

PARTICIPANT CONSENT FORM

I, ...*[name]*

of ....*[address]*

have read and understood the Information for Participants on the abovenamed research study

and have discussed the study with ....

I have been made aware of the procedures involved in the study, including any known or expected inconvenience, risk, discomfort or potential side effect and of their implications as far as they are currently known by the researchers.

I freely choose to participate in this study and understand that I can withdraw at any time.

I also understand that the research study is strictly confidential.

I hereby agree to participate in this research study.

NAME: ....

SIGNATURE: ....

DATE: ....

NAME OF WITNESS: ....

SIGNATURE OF WITNESS: ....

Version No. : #11
Date: 24/02/09

B6. Dietary prescription provided to participants randomised to the higher-protein, higher-haem iron diet

Foods that you will need to eat each day

Food type	*Units allowed*	*Unit conversion*
HIGH FIBRE CEREAL	**1 unit per day**	1 unit = 1 weetbix plus ½ cup all bran = 40 g high fibre breakfast cereal (*eg* Sultana Bran, Fibre Plus) = 1 slice wholegrain bread
DAIRY	**2 units per day**	1 unit = 250 ml skim milk = 200 g diet yoghurt = 200 g low-fat custard or dairy dessert = 25 g cheddar cheese or other full-fat cheese = 50 g reduced fat cheese (less than 10 percent fat)
** **LEAN PROTEIN**	**2 units per day (for dinner)**	1 unit = 100 g raw weight of lean meat *You should eat 2 units of lean red meat (beef, lamb, veal) for dinner 4 times each week. Eat 2 units of fish for dinner twice a week and 2 units of lean other white meats (chicken, turkey, pork) for dinner once per week.*
	1 unit per day (for lunch)	1 unit = 100 g raw weight of lean meat (tinned or fresh fish, chicken, turkey, pork, ham, beef, lamb) = 2 eggs
WHOLEGRAIN BREAD	**3 units per day**	1 unit = one 35 g slice of wholegrain bread *You can replace one unit of bread each day with any of the following:* 1 unit = 2 crispbread, such as Ryvita = 1 medium potato (150 g) = 1/3 cup cooked rice or noodles = 1/2 cup cooked pasta = 1/3 cup baked beans, cooked lentils, kidney beans or other legumes = 3/4 cup cooked couscous
FRUIT	**2 units per day**	1 unit = 150 g fresh or tinned, unsweetened fruit = 150 ml unsweetened fruit juice = 20 g dried fruit

VEGETABLES	**2 ½ units per day**	1 unit = 1 cup cooked vegetables (see list on following pages) *It is recommended that you eat ½ cup of salad vegetables and 2 cups of cooked vegetables per day. Always include some green leafy vegetables such as broccoli, spinach, brussel sprouts etc.*
FATS AND OILS	**3 units per day**	1 unit = 1 teaspoon canola or olive oil = 1 teaspoon canola or olive oil based normal margarine = 2 teaspoons canola or olive oil based light margarine = 1 teaspoon curry paste or pesto in oil = 20 g avocado = 7 g nuts or seeds
SOUP LOW KILOJOULE	**1 unit per day (optional extra)**	1 unit = 1 cup (250 ml) any packet soup that has less than approximately 40 kilocalories (or 160 kilojoules) per serve (*eg* Home Brand Chicken Noodle soup mix, Continental French Onion Classic Recipe soup, Continental Spring Vegetable Classic Recipe soup)
ALCOHOL	**2 units <u>per week</u> (optional extra)**	1 unit = 150 ml wine = 300 ml bitter or draught beer (not stout) = 400 ml reduced alcohol beer = 50 ml spirits *If you prefer not to drink, you can replace your units of alcohol for a treat or snack:* 1 unit = 20 g chocolate = 1 plain muesli bar = 2 plain sweet biscuits = other treat up to the value of 430 kJ

** It is essential you eat the lean protein units daily – these are <u>compulsory</u> foods

Free Foods

These foods contain minimal energy and will not affect your weight loss

Vegetables/salad (up to 2 ½ cups per day)

asparagus * artichoke * green beans * bean sprouts * broccoli
brussel sprouts * beetroot * carrot * cabbage * capsicum * cauliflower
celery * champignon * chilli * chives * choko * corn * cucumber * fennel
lettuce * marrow * mushroom * onion * rhubarb * radish * peas * pumpkin
silver beet * spinach * swede * tomato * turnip * zucchini

Condiments

artificial sweeteners * stock cubes * clear soups * diet topping
diet jelly * no oil or oil free salad dressings * tomato sauce * BBQ sauce
soy sauce * chilli sauce * pickles * mustard * vegemite * mint sauce * horseradish
curry powder/paste * parsley * garlic * ginger * lemon * vinegar * herbs * spices

Drinks

water * diet cordial * diet soft drink
mineral water (unflavoured) * tea/coffee * Bonox/Bovril

A lot of energy can be consumed when drinking fluids
Tea/coffee is allowed freely, but make sure you use artificial sweetener if you like to add sugar. A cappuccino is allowed but must be exchanged for 125 ml (½ unit) of your milk ration. You may only have fruit juice as part of your fruit ration. Wine, beer and other alcoholic beverages are allowed as per your alcohol quota.

You may find that recipes normally prepared at home need to be modified to fit with the eating plan
It could mean that you need to check the quantity of each ingredient. It is best to weigh any meat before eating it, unless it is already in a pre-weighed pack. Thickeners and gravies are allowed but are to be used in small amounts. Dressings and mayonnaise may be used on salad dishes as long as they are the low joule or no oil varieties.
You can always add your own measured canola oil to these dressings.

B7. Dietary prescription provided to participants randomised to the lower-protein, lower-haem iron diet

Foods that you will need to eat each day

	Food type	*Units allowed*	*Unit conversion*
	CEREAL	**1 unit per day**	1 unit = 2 weetbix = 40 g breakfast cereal (*eg* Just Right, Sustain) = 1 slice wholegrain bread
	DAIRY	**2 units per day**	1 unit = 250 ml high calcium skim milk (*eg* Shape No Fat, Pura Tone) = 200 g diet yoghurt = 200 g low-fat custard or dairy dessert = 25 g cheddar cheese or other full-fat cheese = 50 g reduced fat cheese (less than 10 percent fat)
**	**LEAN PROTEIN**	**1 unit per day**	1 unit = 80 g raw weight of lean meat *You should eat 1 unit of lean white meat (chicken, turkey, pork) for dinner 4 times each week. Eat 1 unit of fish for dinner twice a week and 1 unit of lean red meat (beef, lamb, veal) for dinner once per week.*
**	**WHOLEGRAIN BREAD**	**3 units per day**	1 unit = one 35 g slice of wholegrain bread *You can replace one unit of bread each day with any of the following:* 1 unit = 2 crispbread, such as Ryvita = 1 medium potato (150 g) = 1/3 cup cooked rice or noodles = 1/2 cup cooked pasta = 1/3 cup baked beans, cooked lentils, kidney beans or other legumes = 3/4 cup cooked couscous
	RICE or PASTA	**1 unit 6 times per week**	1 unit = 120 g cooked rice or pasta
	FRUIT	**2 units per day**	1 unit = 150 g fresh or tinned, unsweetened fruit = 150 ml unsweetened fruit juice = 20 g dried fruit

VEGETABLES	**2 ½ units per day**	1 unit = 1 cup cooked vegetables (see list on following pages) *It is recommended that you eat ½ cup of salad vegetables and 2 cups of cooked vegetables per day. Always include some green leafy vegetables such as broccoli, spinach, brussel sprouts etc.*
FATS AND OILS	**3 units per day**	1 unit = 1 teaspoon canola or olive oil = 1 teaspoon canola or olive oil based normal margarine = 2 teaspoons canola or olive oil based light margarine = 1 teaspoon curry paste or pesto in oil = 20 g avocado = 7 g nuts or seeds
SHORTBREAD BISCUITS	**2 units per day**	1 unit = 1 biscuit (*eg* Arnotts Glengarry, Arnotts Arno Shortbread)
SOUP LOW KILOJOULE	**1 unit per day (optional extra)**	1 unit = 1 cup (250 ml) any packet soup that has less than approximately 40 kilocalories (or 160 kilojoules) per serve (*eg* Home Brand Chicken Noodle soup mix, Continental French Onion Classic Recipe soup, Continental Spring Vegetable Classic Recipe soup)
ALCOHOL	**2 units <u>per week</u> (optional extra)**	1 unit = 150 ml wine = 300 ml bitter or draught beer (not stout) = 400 ml reduced alcohol beer = 50 ml spirits *If you prefer not to drink, you can replace your units of alcohol for a treat or snack:* 1 unit = 20 g chocolate = 1 plain muesli bar = 2 plain sweet biscuits = other treat up to the value of 430 kJ

** It is essential you eat the lean protein and wholegrain bread units daily – these are <u>compulsory</u> foods

Free Foods

These foods contain minimal energy and will not affect your weight loss

Vegetables/salad (up to 2 ½ cups per day)

asparagus * artichoke * green beans * bean sprouts * broccoli
brussel sprouts * beetroot * carrot * cabbage * capsicum * cauliflower
celery * champignon * chilli * chives * choko * corn * cucumber * fennel
lettuce * marrow * mushroom * onion * rhubarb * radish * peas * pumpkin
silver beet * spinach * swede * tomato * turnip * zucchini

Condiments

artificial sweeteners * stock cubes * clear soups * diet topping
diet jelly * no oil or oil free salad dressings * tomato sauce * BBQ sauce
soy sauce * chilli sauce * pickles * mustard * vegemite * mint sauce * horseradish
curry powder/paste * parsley * garlic * ginger * lemon * vinegar * herbs * spices

Drinks

water * diet cordial * diet soft drink
mineral water (unflavoured) * tea/coffee * Bonox/Bovril

A lot of energy can be consumed when drinking fluids
Tea/coffee is allowed freely, but make sure you use artificial sweetener if you like to add sugar. A cappuccino is allowed but must be exchanged for 125 ml (½ unit) of your milk ration. You may only have fruit juice as part of your fruit ration. Wine, beer and other alcoholic beverages are allowed as per your alcohol quota.

You may find that recipes normally prepared at home need to be modified to fit with the eating plan
It could mean that you need to check the quantity of each ingredient. It is best to weigh any meat before eating it, unless it is already in a pre-weighed pack. Thickeners and gravies are allowed but are to be used in small amounts. Dressings and mayonnaise may be used on salad dishes as long as they are the low joule or no oil varieties.
You can always add your own measured canola oil to these dressings.

B8. Comparison of nutritional profiles of the higher-protein, higher-haem iron and lower-protein, lower-haem iron diets based on seven day models designed for a 23 year old female with light activity

Nutrient intake per day	HPHI diet	LPLI diet	EAR	RDI	AI	UL
Energy (kJ)	5614.64	5602.41	-	-	-	-
kJ from protein (%)	32.46	20.35	-	-	-	-
kJ from fat (%)	25.00	21.04	-	-	-	-
kJ from saturated fat (%)	7.53	6.86	-	-	-	-
kJ from carbohydrate (%)	41.49	57.56	-	-	-	-
kJ from alcohol (%)	1.05	1.05	-	-	-	-
kJ from others (%)	0.09	?	-	-	-	-
Protein (g)	107.1	67.09	37	46	-	-
Total fat (g)	37.91	31.86	-	-	-	-
Saturated fat (g)	11.41	10.39	-	-	-	-
Polyunsaturated fat (g)	5.69	4.75	-	-	-	-
Monounsaturated fat (g)	16.23	12.83	-	-	-	-
Fat as polyunsaturated (%)	17.07	16.99	-	-	-	-
Fat as monounsaturated (%)	48.69	45.87	-	-	-	-
Fat as saturated (%)	34.24	37.13	-	-	-	-
Cholesterol (mg)	297.67	87.06	-	-	-	-
Carbohydrate (g)	137.85	190.58	-	-	-	-
Sugars (g)	73.09	83.24	-	-	-	-
Starch (g)	63.58	106.11	-	-	-	-
Alcohol (g)	2.04	2.04	-	-	-	-
Dietary fibre (g)	22.68	23.83	-	-	25	-
Thiamin (mg)	1.63	1.76	0.9	1.1	-	-
Riboflavin (mg)	2.54	2.5	0.9	1.1	-	-
Niacin equivalents (mg)	47.44	31.68	11	14	-	35
Vitamin B_6 (mg)	?	?	1.1	1.3	-	50
Vitamin B_{12} (µg)	?	?	2	2.4	-	-
Vitamin C (mg)	156.43	159.67	30	45	-	-
Total folate (µg)	332.45*	355.2*	320	400	-	1000
Total vitamin A equivalents (µg)	1057.79	1307.11	500	700	-	3000
Sodium (mg)	2186.23	1939.55	-	-	460-920	2300
Potassium (mg)	3554.05	3095.82	-	-	2800	-
Magnesium (mg)	318.36	268.11*	255	310	-	350
Calcium (mg)	907.51*	876.95*	840	1000	-	2500
Phosphorus (mg)	1725.45	1282.3	580	1000	-	4000
Iron (mg)	12.19*	9.85*	8	18	-	45
Zinc (mg)	11.74	7.63*	6.5	8	-	40

AI, adequate intake; EAR, estimated average requirement; HPHI, higher-protein and higher-haem iron; LPLI, lower-protein and lower-haem iron; RDI, recommended dietary intake; UL, upper limit of intake
?Nutrients that were unable to be computed using FoodWorks Version 6.0.25175
*Nutrients that were below the recommended dietary intake (RDI)

B9. Seven day model of the higher-protein, higher-haem iron diet

Day	Meal	Food	Quantity
Monday	Breakfast	Yoghurt,Skim/Low Fat,Fruit,Artificially Sweetened	200g
		Bread,Mixed Grain	35g
		Banana,common,raw,peeled	150g
		Tea,black,brewed from leaf/teabags,regular	600 ml
	Lunch	Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	2 tsp
		Salmon,Pink,Canned In Water,No Added Salt,Drained	100g
		Cheese,Cheddar(Mild,Tasty,Vintage)	25g
		Tomato,Raw	30g
		Cucumber,Lebanese,Raw	20g
		Spinach,English,Raw	6g
		Apple,Green,Raw,Unpeeled	150g
		Water,plain,drinking	600 ml
	Dinner	Beef,Steak,Fillet,Raw,Fat Trimmed	200g
		Oil,Canola(Include Rape Seed Oil)	1 tsp
		Potato,Boiled,Without Added Salt,With Skin(Unpeeled),Fat Not Added	150g
		Broccoli,Cooked,Fat Not Added	65g
		Capsicum,Red,Cooked/Canned,Fat Not Added In Cooking	50g
		Squash,Button,Cooked,Fat Not Added In Cooking	75g
		Salad,Side,Without Dressing,NFS	1 cup
		.Diet coke [Soft drink]	500 ml
Tuesday	Breakfast	.Sanitarium Weet-Bix(regular) [Breakfast cereal]	1 biscuit
		.Kellogg's All bran [Breakfast cereal]	0.5 cup
		Milk,Fluid,Skim/Nonfat(Fat<0.16%)	250 ml
		Pineapple,raw,peeled	150g
		Water,plain,drinking	500 ml
	Lunch	Soup,Tomato,Smooth,Red Energy,Fr Dry Mix,Reconst W/Water	1 cup
		Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	2 tsp
		Ham,Leg(Separable Fat),Fat Trimmed	100g
		Tomato,Raw	90g
		Juice,orange,commercial,unsweetened	150 ml
		Water,plain,drinking	500 ml
		.Cadbury dairy milk [Chocolate]	20g
	Dinner	Lamb,Trim Lamb(No Separable Fat),Raw	200g
		Garlic,raw,peeled	0.5 Clove
		Oil,Sesame	0.5 tsp
		Shallot,Raw	1 serve (2 shallot bulbs)
		Chili,red,raw	5g
		Cabbage,Bok Choy,Cooked,Fat Not Added In Cooking	1 cup (cooked)
		Snowpea(Pea Pod),Cooked,Fat Not Added In Cooking	75g
		Sauce,Oyster,Asian,Commercial	0.08 cup
		Sauce,Chili,Asian,Commercial	0.06 cup
		Bread,Mixed Grain	35g
		Margarine Spread,Monounsaturated,Canola	0.5 tsp
		Yoghurt,Skim/Low Fat,Vanilla,Artificially Sweetened	200g
		Cordial,other fruit juice,artif sweetened,rec dilution	600 ml
Wednesday	Breakfast	Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	2 tsp
		Vegemite	2 tsp
		Yoghurt,Skim/Low Fat,Fruit,Artificially Sweetened	200g
		Coffee,black,from instant,regular,NS strength	250 ml
		Milk,Fluid,Skim/Nonfat(Fat<0.16%)	20 mL
		Water,plain,drinking	400 ml

Day	Meal	Food	Quantity
Wednesday	Lunch	Beef,Steak,Fillet,Raw,Fat Trimmed	100g
		Oil,Olive	1 tsp
		Lettuce,Mignonette,Raw	2 leaf
		Capsicum,Red,Raw	20g
		Bean Sprout,Raw	15g
		Onion,Spring,Raw	15g
		Chili,red,raw	10g
		Juice,lime	10 ml
		Sauce,Fish,Asian,Commercial	0.25 tsp
		Sauce,soy,commercial	0.25 tsp
		Garlic,raw,peeled	0.4g
		Bread,Mixed Grain	70g
		Custard,Low Fat,Vanilla,Commercial	200g
		Water,plain,drinking	500 ml
	Dinner	Snapper,Raw	200g
		Broccoli,Cooked,Fat Not Added	0.5 cup (nfs)
		Carrot,Cooked,Fat Not Added In Cooking	0.5 cup (nfs)
		Bean,Green,Cooked,From Raw,Fat Not Added In Cooking	0.25 cup (chopped)
		Capsicum,Red,Cooked/Canned,Fat Not Added In Cooking	0.25 cup (chopped)
		Fruit salad,fresh	300g
		Water,plain,drinking	500 ml
Thursday	Breakfast	.Kellogg's Sultana bran [Breakfast cereal]	40g
		Milk,Fluid,Skim/Nonfat(Fat<0.16%)	250 ml
		Peach,Canned In Natural Juice,Drained	150g
		Tea,black,brewed from leaf/teabags,regular	500 ml
	Lunch	.Westons ryvita(original whole rye) [Biscuit]	2 biscuit (crispbread)
		Tuna,Canned In Water,Drained	100g
		Onion,Mature,Raw	1 thin slice
		Tomato,Raw	0.5 cup (chopped/sliced)
		Cucumber,Lebanese,Raw	0.5 cup (chopped/diced)
		Cheese,Feta,Reduced Fat	50g
		Oil,Olive	1 tsp
		Mineral Water,Natural	500 ml
	Dinner	Beef,Steak,Fillet,Raw,Fat Trimmed	200g
		Oil,Olive	2 tsp
		Pepper,Black/White	0.5 tsp
		Fennel,Cooked,Fat Not Added In Cooking	0.25 cup
		Zucchini,Cooked,Fat Not Added In Cooking	0.5 cup (nfs)
		Capsicum,Red,Cooked/Canned,Fat Not Added In Cooking	0.5 cup (chopped)
		Mushroom,Cooked/Canned,Fat Not Added In Cooking	0.25 cup (sliced)
		Juice,lemon,fresh	0.06 cup
		Bread,Mixed Grain	70g
		Apple,Red,Raw,Unpeeled	150g
		Water,plain,drinking	600 ml
Friday	Breakfast	Bread,Mixed Grain	35g
		Margarine Spread,Monounsaturated,Canola	1 tsp
		Egg,poached	1 Average
		Yoghurt,Skim/Low Fat,Fruit,Artificially Sweetened	200g
		Juice,apple	150 ml
		Water,plain,drinking	600 ml
	Lunch	Bread,Mixed Grain	70g
		Ham,Leg(Separable Fat),Fat Trimmed	50g
		Pickle,Mixed,In Brine,NFS	1 tsp
		Cheese,Cheddar,Reduced Fat(50%Reduction)	50g
		Lettuce,Cos,Raw	0.5 cup (nfs)
		Banana,common,raw,peeled	150g
		.Diet coke [Soft drink]	1 bottle(500ml)

Day	Meal	Food	Quantity
Friday	Dinner	Barramundi,Raw	200g
		Oil,Olive	1 tsp
		Garlic,raw,peeled	0.5 Clove
		Juice,lemon,commercial	1 tsp
		Capers	1 tb
		Lettuce,Common,Raw	0.5 cup (nfs)
		Carrot,Raw	0.5 cup (chopped)
		Tomato,Cherry,Raw	0.5 cup (cherry tomato)
		Snowpea Sprout,Raw	0.5 cup
		Vinegar	1 tb
		Bread,Mixed Grain	35g
		Margarine Spread,Monounsaturated,Canola	1 tsp
		Water,plain,drinking	600 ml
Saturday	Breakfast	.Uncle Tobys Fibreplus [Breakfast cereal]	40g
		Milk,Fluid,Skim/Nonfat(Fat<0.16%)	250 ml
		Apricot,Dried,Raw	20g
		Coffee,black,from instant,regular,NS strength	250 ml
		Milk,Fluid,Skim/Nonfat(Fat<0.16%)	20 mL
		Water,plain,drinking	300 ml
	Lunch	Bread,Mixed Grain	105g
		Turkey,Breast,Deli-Sliced	100g
		Rocket,Raw	0.25 cup (shredded/chopped)
		Tomato,Raw	0.25 cup (chopped/sliced)
		Custard,Low Fat,Vanilla,Commercial	200g
		Water,plain,drinking	500 ml
	Dinner	Chicken,Breast,Raw,Without Skin	200g
		Oil,Olive	3 tsp
		Tomato Puree	1 tb
		Garlic,raw,peeled	0.5 Clove
		Asparagus,Cooked,Fat Not Added In Cooking	0.5 cup
		Carrot,Cooked,Fat Not Added In Cooking	0.5 cup (nfs)
		Pumpkin,Boiled,Without Added Salt,Fat Not Added In Cooking	0.5 cup (nfs)
		Cauliflower,Cooked,Fat Not Added In Cooking	0.5 cup
		Grape,Green,No Seeds,Raw	150g
		Wine,Red	150 mL
		Water,plain,drinking	300 ml
Sunday	Breakfast	Egg,Scrambled,Fat Not Added In Cooking	2 medium egg (55-64g)
		Tomato,Grilled	0.25 cup (chopped)
		Mushroom,Cooked/Canned,Fat Not Added In Cooking	0.25 cup
		Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	1 tsp
		Coffee,black,from instant,regular,NS strength	250 ml
		Milk,Fluid,Skim/Nonfat(Fat<0.16%)	20 mL
		Water,plain,drinking	500 ml
	Lunch	Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	1.5 tsp
		Chicken,Breast,Raw,Without Skin	100g
		Lettuce,Common,Raw	1 medium leaf
		Tomato,Raw	3 thin slice
		Beetroot,canned,drained	2 Slices
		Cucumber,Lebanese,Raw	3 slice (2.5cm dia,0.5cm thick)
		Apple,Green,Raw,Unpeeled	150g
		Mineral Water,Natural	500 ml

Day	Meal	Food	Quantity
Sunday	Dinner	Lamb,Trim Lamb(No Separable Fat),Raw	100g
		Snowpea(Pea Pod),Cooked,Fat Not Added In Cooking	50g
		Pea,Green,Cooked,From Raw,Fat Not Added In Cooking	50g
		Rocket,Raw	25g
		Mint,Raw	0.25 cup
		Cheese,Feta,Reduced Fat	50g
		Vinegar	0.25 tb
		Oil,Canola(Include Rape Seed Oil)	0.5 tsp
		Mustard,Cream Type,Commercial	0.25 tsp
		Yoghurt,Skim/Low Fat,Vanilla,Artificially Sweetened	200g
		Date,Dried	20g
		Cordial,citrus fruit juice,artif sweetened,rec dilution	600 ml

B10. Seven day model of the lower-protein, lower-haem iron diet

Day	Meal	Food	Quantity
Monday	Breakfast	Yoghurt,Skim/Low Fat,Fruit,Artificially Sweetened	200g
		Apple,Green,Raw,Unpeeled	150g
		Bread,Mixed Grain	35g
		Margarine Spread,Monounsaturated,Canola	1 tsp
		.Vegemite [Yeast extract]	1 tsp
		Tea,black,brewed from leaf/teabags,regular	500 mL
	Lunch	Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	2 tsp
		Baked Beans,Canned In Tomato Sauce	0.33 cup
		Cheese,Cheddar(Mild,Tasty,Vintage)	25g
		Carrot,Raw	0.25 cup (chopped)
		Celery,Raw	0.25 cup (strips)
		.Cadbury dairy milk [Chocolate]	20g
		.Diet coke [Soft drink]	500 mL
	Dinner	Chicken,Breast,Raw,Without Skin	80g
		Broccoli,Cooked,Fat Not Added	65g
		Capsicum,Red,Cooked/Canned,Fat Not Added In Cooking	50g
		Squash,Button,Cooked,Fat Not Added In Cooking	75g
		Salad,Side,Without Dressing,NFS	1 cup
		Rice,white,boiled	120g
		Juice,orange,commercial,unsweetened	150 ml
		Water,plain,drinking	500 mL
		.Arnotts glengarry [Biscuit]	2 biscuit
Tuesday	Breakfast	.Sanitarium Weet-Bix(regular) [Breakfast cereal]	2 biscuit
		.Shape [Milk]	250 ml
		Strawberry,Raw	150g
		Water,plain,drinking	500 ml
	Lunch	Soup,Tomato,Smooth,Red Energy,Fr Dry Mix,Reconst W/Water	1 cup
		Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	2 tsp
		Lettuce,common,raw	1 Leaf
		Tomato,Raw	2 thin slice
		Carrot,Raw	1 large strip (7.5cm long)
		Beetroot,canned,drained	1 Slices
		.Arnotts glengarry [Biscuit]	2 biscuit
		Banana,common,raw,peeled	150g
		Water,plain,drinking	500 ml
	Dinner	Pork,butterfly steak,raw,lean	80g
		Garlic,raw,peeled	0.5 Clove
		Oil,Sesame	1 tsp
		Shallot,Raw	1 serve (2 shallot bulbs)
		Chili,red,raw	5g
		Cabbage,Bok Choy,Cooked,Fat Not Added In Cooking	1 cup (cooked)
		Snowpea(Pea Pod),Cooked,Fat Not Added In Cooking	75g
		Sauce,Oyster,Asian,Commercial	0.08 cup
		Sauce,Chili,Asian,Commercial	0.06 cup
		Noodle,Asian,NS Type,Cooked,Fat Not Added In Ckg	0.33 cup
		Yoghurt,Skim/Low Fat,Vanilla,Artificially Sweetened	200g
		Cordial,other fruit juice,artif sweetened,rec dilution	600 ml
Wednesday	Breakfast	Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	2 tsp
		Honey,All Types	2 tsp
		Yoghurt,Skim/Low Fat,Fruit,Artificially Sweetened	200g
		Coffee,black,from instant,regular,NS strength	250 ml
		.Shape [Milk]	20 mL
		Water,plain,drinking	400 ml

Day	Meal	Food	Quantity
Wednesday	Lunch	Bean,Red Kidney,Canned,Drained	0.33 cup (drained)
		Pasta,Regular,Cooked,Fat Not Added In Cooking,With Added Salt	120 g (dry)
		Carrot,Raw	0.3 cup (chopped)
		Capsicum,Red,Raw	0.3 cup (nfs)
		Corn,Canned,Fat Not Added	0.3 cup (baby corn,chopped)
		Onion,Spring,Raw	0.1 cup (chopped)
		Dressing,Salad,Fat Free,Vinegar-Based,NFS	0.25 cup
		Apple,Green,Raw,Unpeeled	150g
		Custard,Low Fat,Vanilla,Commercial	200g
		Water,plain,drinking	500 ml
	Dinner	Snapper,Raw	80g
		Broccoli,Cooked,Fat Not Added	0.5 cup (nfs)
		Carrot,Cooked,Fat Not Added In Cooking	0.5 cup (nfs)
		Bean,Green,Cooked,From Raw,Fat Not Added In Cooking	0.25 cup (chopped)
		Capsicum,Red,Cooked/Canned,Fat Not Added In Cooking	0.25 cup (chopped)
		Bread,Mixed Grain	35g
		Margarine Spread,Monounsaturated,Canola	1 tsp
		.Arnotts shortbread [Biscuit]	2 biscuit
		Apricot,dried	20g
		Water,plain,drinking	500 ml
Thursday	Breakfast	.Kellogg's Just right [Breakfast cereal]	40g
		.Shape [Milk]	250 ml
		Peach,Canned In Natural Juice,Drained	150g
		Tea,black,brewed from leaf/teabags,regular	500 ml
	Lunch	.Westons ryvita(original whole rye) [Biscuit]	2 biscuit (crispbread)
		Cheese,Cheddar(Mild,Tasty,Vintage)	25g
		Onion,Mature,Raw	1 thin slice
		Tomato,Raw	0.4 cup (chopped/sliced)
		Cucumber,Lebanese,Raw	0.4 cup (chopped/diced)
		Lettuce,Cos,Raw	0.2 cup (nfs)
		Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	2 tsp
		.Vegemite [Yeast extract]	2 tsp
		Juice,apple	150 ml
		Water,plain,drinking	500 ml
	Dinner	Beef,Steak,Fillet,Raw,Fat Trimmed	80g
		Oil,Olive	1 tsp
		Pepper,Black/White	0.5 tsp
		Fennel,Cooked,Fat Not Added In Cooking	0.25 cup
		Zucchini,Cooked,Fat Not Added In Cooking	0.5 cup (nfs)
		Capsicum,Red,Cooked/Canned,Fat Not Added In Cooking	0.5 cup (chopped)
		Mushroom,Cooked/Canned,Fat Not Added In Cooking	0.25 cup (sliced)
		Juice,lemon,fresh	0.06 cup
		Rice,white,boiled	120g
		.Arnotts glengarry [Biscuit]	2 biscuit
		Mineral Water,Natural	500 ml
Friday	Breakfast	Bread,Mixed Grain	35g
		Margarine Spread,Monounsaturated,Canola	1 tsp
		Baked Beans,Canned In Tomato Sauce	0.33 cup
		Banana,common,raw,peeled	150g
		Water,plain,drinking	600 ml
	Lunch	Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	2 tsp
		Cheese,Cheddar,Reduced Fat(50%Reduction)	50g
		Tomato,Raw	0.25 cup (chopped/sliced)
		Salad,Side,Without Dressing,NFS	0.75 cup
		.Arnotts shortbread [Biscuit]	2 biscuit
		.Diet coke [Soft drink]	1 bottle(500ml)

Day	Meal	Food	Quantity
Friday	Dinner	Chicken,Breast,Raw,Without Skin	80g
		Tomato Puree	3 tb
		Garlic,raw,peeled	0.5 Clove
		Asparagus,Cooked,Fat Not Added In Cooking	0.5 cup
		Carrot,Cooked,Fat Not Added In Cooking	0.25 cup (nfs)
		Pumpkin,Boiled,Without Added Salt,Fat Not Added In Cooking	0.5 cup (nfs)
		Cauliflower,Cooked,Fat Not Added In Cooking	0.25 cup
		Pasta,Regular,Cooked,Fat Not Added In Cooking,With Added Salt	120g
		Yoghurt,Skim/Low Fat,Vanilla,Artificially Sweetened	200g
		Grape,Green,No Seeds,Raw	150g
		Water,plain,drinking	600 ml
Saturday	Breakfast	.Kellogg's Sustain [Breakfast cereal]	40g
		.Shape [Milk]	250 ml
		Watermelon,raw,peeled	300g
		Water,plain,drinking	500 mL
	Lunch	Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	2 tsp
		Rocket,Raw	0.25 cup (shredded/chopped)
		Tomato,Raw	0.25 cup (chopped/sliced)
		Carrot,Raw	0.25 cup (grated)
		Beetroot,Canned,Drained	0.25 cup (slices)
		Potato,Dry-Baked,With Skin(Unpeeled)	150g
		Oil,Olive	1 tsp
		Custard,Low Fat,Vanilla,Commercial	200g
		Water,plain,drinking	500 ml
	Dinner	Barramundi,Raw	80g
		Garlic,raw,peeled	0.5 Clove
		Juice,lemon,commercial	1 tsp
		Capers	1 tb
		Lettuce,Common,Raw	0.5 cup (nfs)
		Carrot,Raw	0.25 cup (chopped)
		Tomato,Cherry,Raw	0.5 cup (cherry tomato)
		Snowpea Sprout,Raw	0.25 cup
		Vinegar	1 tb
		Pasta,Regular,Cooked,Fat Not Added In Cooking,With Added Salt	120g
		.Arnotts glengarry [Biscuit]	2 biscuit
		Wine,Red	150 mL
		Water,plain,drinking	300 ml
Sunday	Breakfast	Bread,Mixed Grain	70g
		Margarine Spread,Monounsaturated,Canola	2 tsp
		Tomato,Grilled	0.5 cup (chopped)
		Mushroom,Cooked/Canned,Fat Not Added In Cooking	0.5 cup
		Coffee,black,from instant,regular,NS strength	250 ml
		.Shape [Milk]	20 mL
		Water,plain,drinking	500 ml
	Lunch	Rice,white,boiled	120g
		Pea,Green,Cooked,From Frozen,Fat Not Added In Cooking	0.2 cup
		Carrot,Cooked,Fat Not Added In Cooking	0.2 cup (diced fr fresh)
		Corn,Canned,Fat Not Added	0.1 cup
		Bread,Mixed Grain	35g
		Margarine Spread,Monounsaturated,Canola	1 tsp
		Apple,Green,Raw,Unpeeled	150g
		Yoghurt,Skim/Low Fat,Fruit,Artificially Sweetened	300g
		Mineral Water,Natural	500 ml

Day	Meal	Food	Quantity
Sunday	Dinner	Pork,butterfly steak,raw,lean	80g
		Juice,apple	0.06 cup
		Sauce,Cranberry,Commercial	0.125 cup (sauce)
		Honey	1 tsp
		Juice,orange,commercial,unsweetened	1 tsp
		Couscous,Cooked,Fat Not Added In Cooking	0.75 cup (cooked)
		Broccoli,Cooked,Fat Not Added	0.33 cup (nfs)
		Snowpea(Pea Pod),Cooked,Fat Not Added In Cooking	0.33 cup
		Pumpkin,Dry-Baked	0.33 cup
		.Arnotts shortbread [Biscuit]	2 biscuit
		Date,Dried	20g
		Cordial,citrus fruit juice,artif sweetened,rec dilution	600 ml

APPENDIX C: SUPPLEMENTARY MATERIAL FOR CHAPTER 6

C1. Detailed methods used for genetic analysis

C2. Background information on the TaqMan® SNP Allelic Discrimination technique

C3. Background information on interpreting the TaqMan® SNP Allelic Discrimination output

C1. Detailed methods used for genetic analysis

DNA extraction

The procedures outlined below were largely in accordance with the specified protocol for extracting DNA from frozen blood samples (Promega Corporation, Madison, USA).

Cells were lysed in sterile 1.5ml microcentrifuge tubes containing 300 µl of thawed (from frozen storage) heparin packed red blood cells and 900 µl of cell lysis solution. Tubes were incubated for 10 minutes at room temperature. After incubation, cell nuclei were isolated by centrifugation at 13,000 × *g* for 20 seconds (room temperature) with removal of as much supernatant as possible. The cell lysis and nuclei isolation procedures were then repeated to maximise removal of cell debris. Tubes were then vortexed vigorously for 15 seconds to resuspend the isolated nuclei.

Following resuspension, cell nuclei were lysed by adding 300 µl of nuclei lysis solution, mixed (by repeated pipetting) and incubated for approximately 60 minutes at 37 ºC. During this step, 1.5 µl of RNAse solution was also added to each tube to eliminate RNA contaminants. Subsequently, 100 µl of protein precipitation solution was added to each tube of nuclear lysate and centrifuged at 13,000 × *g* for 3 minutes (room temperature) to induce precipitation and separation of unwanted protein contaminants. The supernatant (containing solubilised genomic DNA) from each tube were transferred into new sterile 1.5 ml microcentrifuge tubes (correspondingly labelled) containing 300 µl of room temperature isopropanol and inverted several times to induce precipitation of DNA strands. Tubes were then centrifuged at 13,000 × *g* for 60 seconds (room temperature) and the isopropanol supernatant discarded carefully (to ensure that the precipitated DNA pellet does not dislodge from the wall of the tubes).

A final wash of the DNA pellets was performed by adding 300 µl of 70% ethanol to each tube and gently inverting several times. Tubes were centrifuged at 13,000 × *g* for 60 seconds (room temperature) again with the ethanol supernatant discarded carefully. Each tube was then inverted and the DNA pellet (lodged on the wall of each tube) was air-dried for 15 minutes. Finally, the air-

dried DNA samples were rehydrated in 10mM Tris-HCl (pH 7.4) and 1mM EDTA (pH 8.0) (1× TE buffer) and incubated overnight at 4 ºC (see note below). Rehydrated DNA samples were transferred to longer term storage at -20 ºC the following day. Concentration of the rehydrated DNA was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA).

N.B. DNA pellets were initially rehydrated in 100 µl of 1× TE buffer as per the kit protocol. However, due to low DNA yield from some of the blood samples that were initially isolated (likely due to low white cell concentration), the rehydration volume was adjusted to 20 µl so as to maximise the concentration of the rehydrated DNA.

DNA target sequence amplification

PCR was employed to amplify the 167 base pair target sequence containing the SNP rs855791. The custom primer and target sequences are shown in Appendix Table C1.1, with details of the PCR reaction set-up outlined in Appendix Table C1.2.

Appendix Table C1.1. Custom oligonucleotide primer and target sequences

	Tm (ºC)	Any SC	3' SC	Sequence (5' to 3')
Primers				
TMPRSS6fwd	64.1	8.00	2.00	TCTGCAGAAAGTGGATGTGC
TMPRSS6rev	63.5	2.00	0.00	GCATCCTTTCTCCCTCCTCT
Target sequence		6.00	2.00	TCTGCAGAAAGTGGATGTGCAGTTGATC CCACAGGACCTGTGCAGCGAGG[C/T]C TATCGCTACCAGGTGACGCCACGCATGC TGTGTGCCGGCTACCGCAAGGGCAAGAA GGATGCCTGTCAGGTGAGTCCCCCGGGC ATGGGAGGGAGAGAGGAGGGAGAAAGGA TGC

Tm, melting temperature; SC, self-complementarity

Appendix Table C1.2. PCR reaction set-up and thermal cycler program

PCR reaction set-up (total volume 50 µl)	Thermal cycler program	
- 5 µl genomic DNA	95 ºC × 5 min	
- 1.5 µl TMPRSS6fwd primer (5 µM)[a]	95 ºC × 1 min	repeated for 35 cycles
- 1.5 µl TMPRSS6rev primer (5 µM)[a]	60 ºC × 1 min	
- 5 µl 10× PCR buffer[b]	72 ºC × 1 min	
- 5 µl dNTPs (2 mM)	72 ºC × 7 min	
- 1 µl Taq polymerase (5 units/µl)	4 ºC × ∞	
- 31 µl sterile nuclease free water		

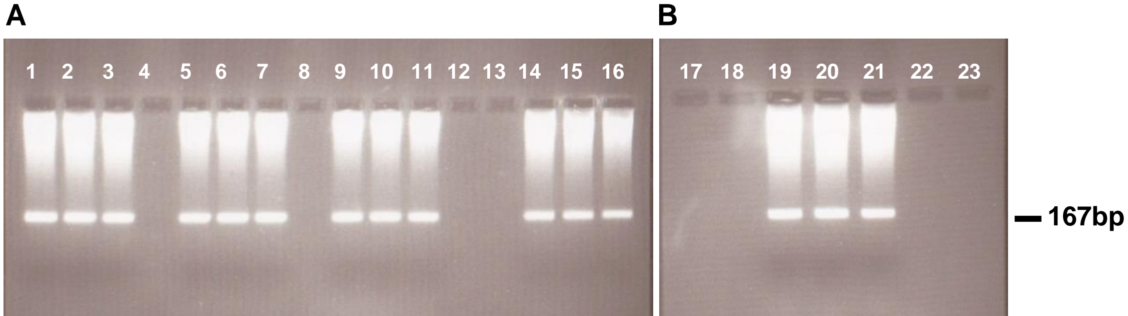
DNA, deoxyribonucleic acid; dNTPs, deoxyribonucleotide triphosphates; PCR, polymerase chain reaction; ∞, indefinite period of time

[a]rehydrated in 10mM Tris-HCl (pH 7.4) and 1mM EDTA (1× TE buffer)

[b]10× PCR buffer contains 200 mM Tris-HCl (pH 8.4) and 500 mM KCl

PCR product (amplicon) purification

Following amplification, 10 µl of 6× Orange G loading dye was added to each PCR product and then loaded onto 3.5% agarose gels made in 1× TAE buffer (40 mM Tris-acetate, pH 8.0; 20 mM sodium acetate; 1 mM EDTA) and containing 50 µl of 500 µg/ml ethidium bromide solution. DNA purification was performed by running gel electrophoresis in 1× TAE buffer at 80 volts for 30 minutes (or when loading dye bands had travelled approximately halfway across the gel). DNA bands of the purified amplicons were visualised using an ultraviolet (UV) transilluminator as shown in Appendix Figure C1.1.

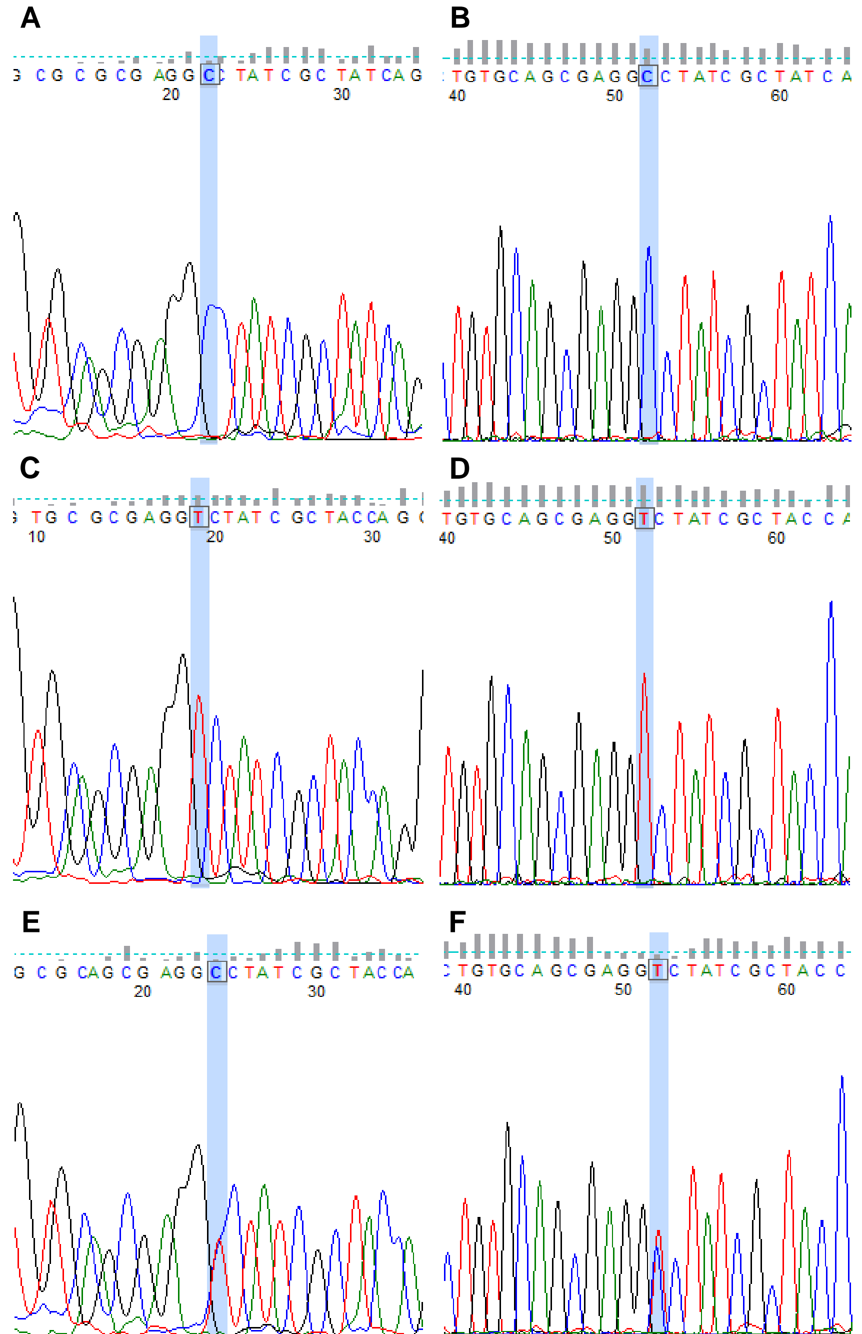


Appendix Figure C1.1. Agarose gel-purified amplicons visualised under ultraviolet (UV) light (Four samples, each taking three wells, were loaded onto gel A with each sample divided by a minimum of one empty lane to ensure clear separation of DNA; one sample was loaded onto gel B for logistical reasons)

Purified DNA bands were cut out and recovered by Spin-X centrifuge filtration (Corning Incorporated, Corning, USA) at 13,000 × *g* for 15 minutes. To precipitate the amplicons from solution, 3 M sodium acetate (1/10th of the volume of each amplicon solution), 100% ethanol (2½ volumes of each amplicon solution) and 2 µl of 1 mg/ml glycogen (carrier) were added to each tube and stored at -20 ºC for overnight precipitation. Samples were centrifuged the following day at 13,000 × *g* for 15 minutes (at 4 ºC) and the supernatant was discarded (ensuring the amplicon pellet does not dislodge from the wall of the tube). A final wash with 500 µl of 70% ethanol and centrifugation (13,000 × *g* for 15 minutes at 4 ºC) was performed before the amplicon pellets were air-dried and rehydrated in 10 µl of sterile nuclease free water.

Amplicon sequencing

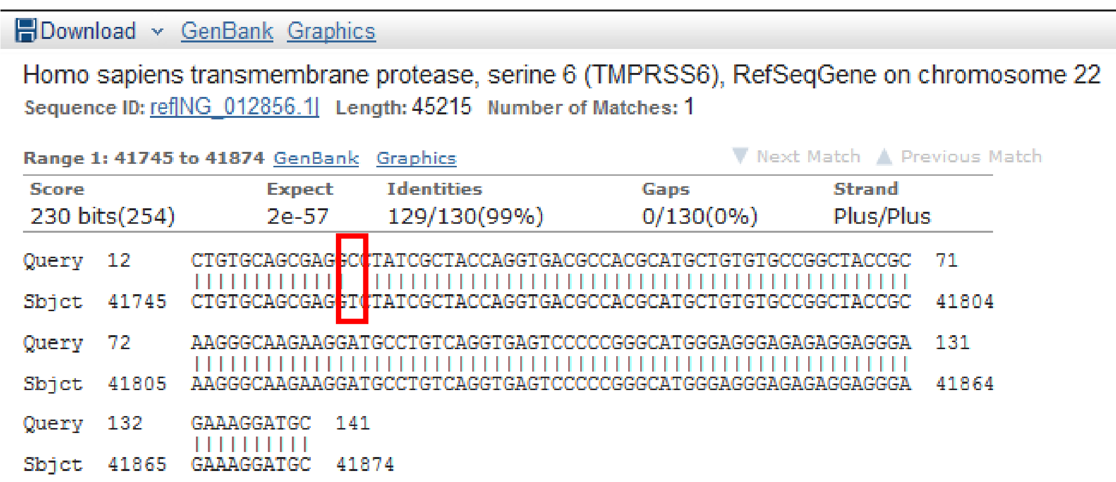
Amplicons were prepared for sequencing in accordance with the sample submission requirements outlined by the Australian Genome Research Facility Ltd, Sydney, Australia. Results showed one participant as homozygous for the C allele, another as homozygous for the T allele, and the remaining three participants as heterozygotes. The sequencing results of three participants (one of each zygosity) are presented in Appendix Figure C1.2.



Appendix Figure C1.2. Amplicon sequencing results from three participants [C allele homozygosity as reflected by the blue cytosine signal spike on the (A) forward and (B) reverse complement sequences; T allele homozygosity by the red thymine signal spike on the (C) forward and (D) reverse complement sequences; and CT heterozygosity by the mixed blue cytosine and red thymine signal spikes on the (E) forward and (F) reverse complement sequences]

Verification of sequencing results

To ensure that the SNP rs855791 was captured within the amplicons generated from the two custom oligonucleotide primers, sequencing results were verified against online published data. The sequence alignment results from one amplicon sample are shown in Appendix Figure C1.3.



Appendix Figure C1.3. Sequence alignment and verification results (Amplicons generated from one DNA sample showing a 99% match with published data on the *TMPRSS6* gene; the 1% mismatch reflects the rs855791 polymorphism as highlighted by the red box)

TaqMan® SNP Allelic Discrimination set-up

Three of the five participants with known rs855791 genotype (from sequencing) were selected as positive controls for genotyping analysis on the remaining participants (n=71). Prior to analysis, genomic DNA samples were diluted to a concentration of 30 ng/µl with sterile nuclease free water. Reactions were arranged on 96-well PCR reaction plates (Life Technologies Australia Pty Ltd, Mulgrave, Australia) based on the diagram shown in Appendix Figure C1.4. Details of the genotyping reaction set-up are outlined in Appendix Table C1.3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	U01	U01	U09	U09	U17	U17	U25	U25	U33	U33
B	NTC	NTC	U02	U02	U10	U10	U18	U18	U26	U26	U34	U34
C	+ve (CC)	+ve (CC)	U03	U03	U11	U11	U19	U19	U27	U27	U35	U35
D	+ve (CC)	+ve (CC)	U04	U04	U12	U12	U20	U20	U28	U28	U36	U36
E	+ve (CT)	+ve (CT)	U05	U05	U13	U13	U21	U21	U29	U29	U37	U37
F	+ve (CT)	+ve (CT)	U06	U06	U14	U14	U22	U22	U30	U30	U38	U38
G	+ve (TT)	+ve (TT)	U07	U07	U15	U15	U23	U23	U31	U31	U39	U39
H	+ve (TT)	+ve (TT)	U08	U08	U16	U16	U24	U24	U32	U32	U40	U40

Appendix Figure C1.4. Real-time PCR plate set-up for genotyping analysis (Four wells were assigned to positive controls of each zygosity and the no template control/blank; reactions for all unknown DNA samples were performed in duplicate; NTC, no template control; +ve, positive controls; U, unknown samples)

Appendix Table C1.3. Genotyping reaction set-up and thermal cycler program

Genotyping reaction set-up (total volume 25 µl)	Thermal cycler program
- 2.5 µl genomic DNA (30 ng/µl)	60 ºC × 1 min
- 1.25 µl 20× TaqMan® SNP Genotyping Assay	95 ºC × 10 min
- 12.5 µl 2× TaqMan® Universal PCR Master Mix	95 ºC × 15 sec } repeated for 40 cycles
- 8.75 µl sterile nuclease free water	60 ºC × 1 min }
	60 ºC × 1 min

DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism

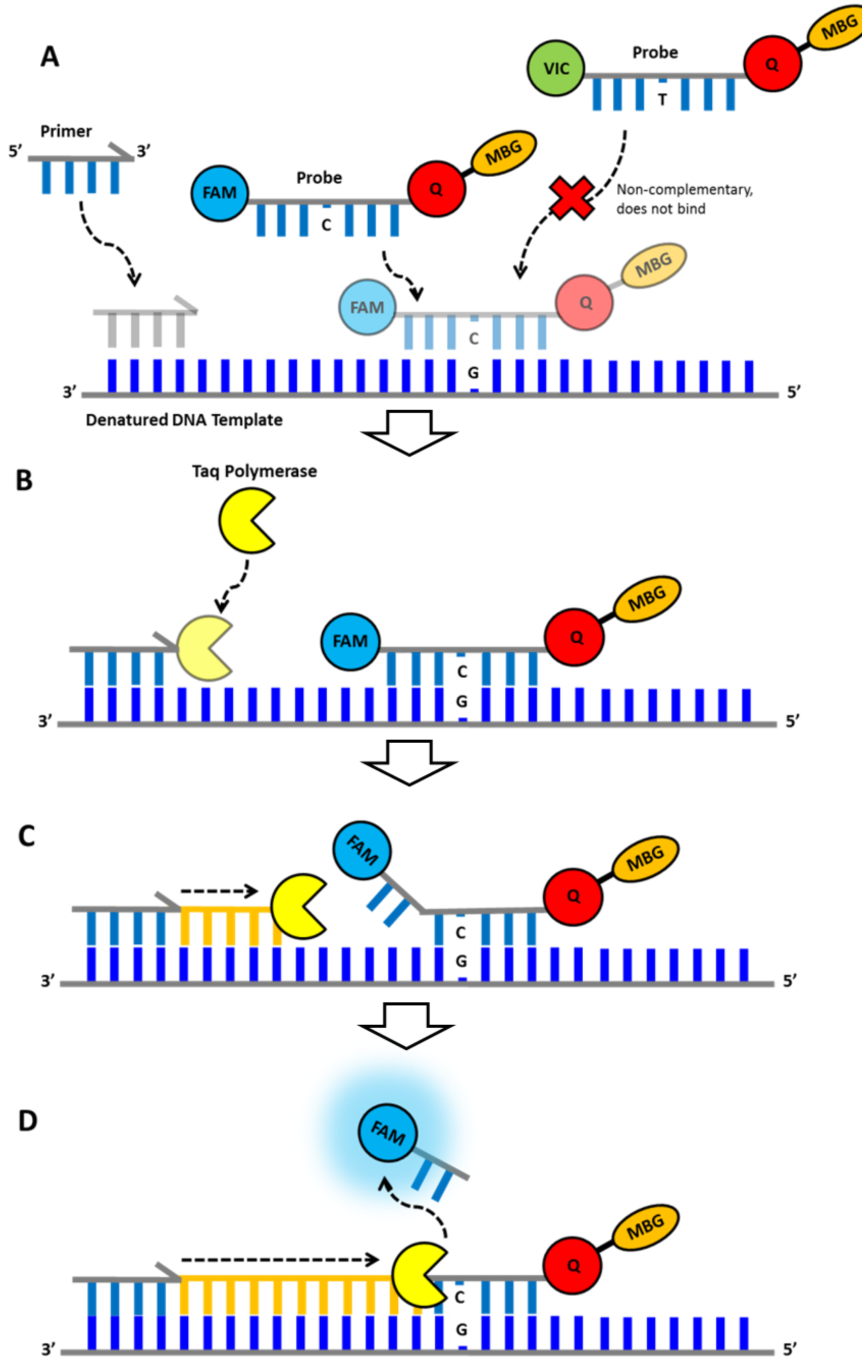
C2. Background information on the TaqMan® SNP Allelic Discrimination technique

TaqMan® SNP Allelic Discrimination technique

TaqMan® SNP genotyping is an allelic determination technique that uses a real-time PCR system platform (Applied Biosystems, Foster City, USA) and relies primarily on the activity of two reagents: (1) 20× SNP Genotyping Assay; and (2) 2× TaqMan® Universal PCR Master Mix (Life Technologies Australia Pty Ltd, Mulgrave, Australia). The 20× SNP Genotyping Assay contains two primers for amplifying the sequence surrounding the SNP rs855791 (similar to the custom oligonucleotide primers used for target sequence amplification) and two TaqMan® Minor Groove Binder (MGB) probes. The TaqMan® MGB probes are small amplicons that bind to their complementary sequence directly on the SNP site of the DNA strand. These specialised probes are attached to one of two reporter dyes (FAM™ or VIC® corresponding to the C or T allele respectively) and a dye-quencher that inhibits fluorescence of the reporter dye whilst it is still attached to the probe. The 2× TaqMan® Universal PCR Master Mix contains a specialised Taq DNA polymerase, deoxyribonucleotide triphosphates (dNTPs), as well as dye-signal reference and buffer components for optimal allelic determination performance. The theoretical concept of this genotyping technique is outlined below and illustrated visually in Appendix Figure C2.1.

During real-time PCR, DNA denaturation occurs resulting in formation of single-stranded DNA templates that are free to bind (anneal) with the primers and probes from the SNP Genotyping Assay reagent. The probes anneal directly to the SNP site (which is between the two primers) on the DNA strand. Depending on genotype, an individual may only have C or T allele DNA templates (indicating that only the FAM™ or VIC® probes can anneal to the SNP sites) or they may have a combination of both DNA template types (indicating that both probes can anneal to their complementary SNP sites). Taq polymerase, which has both DNA synthesising (3’→5’ polymerase activity) and degrading (5’→3’ exonuclease activity) properties, subsequently attaches to the 3’ end of the primers on the DNA template and generates a short amplicon sequence up to the location where the probe is annealed to the SNP site. At this stage, Taq polymerase 5’ exonuclease activity cleaves the probe resulting in separation of the FAM™ or VIC® reporter dye

from its quencher. Once the FAM™ or VIC® dye is released from its quencher, it fluoresces at a specific frequency which is detected by the real-time PCR system. Hence, if fluorescence is only detected from the FAM™ reporter dye, this indicates homozygosity for the C allele. Conversely, T allele homozygosity is reflected by fluorescence from the VIC® reporter dye only. If fluorescence is detected from both reporter dyes, this indicates heterozygosity.



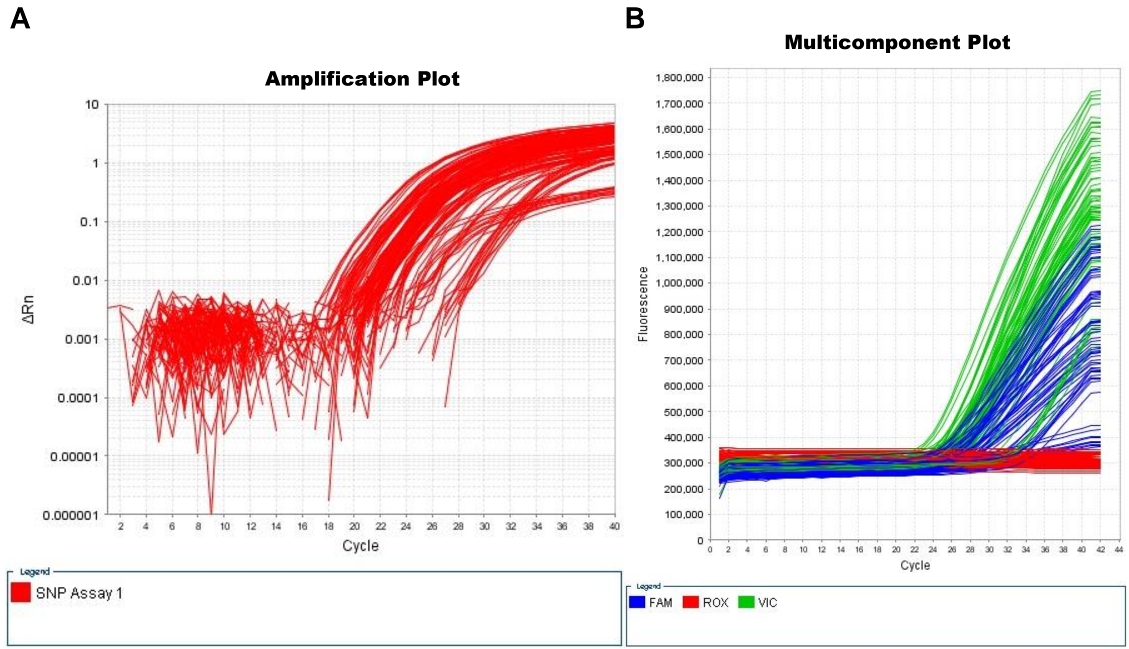
Appendix Figure C2.1. An illustrated summary of the TaqMan® SNP genotyping technique [(A) the Minor Groove Binder (MGB) probe anneals directly to the SNP site of the DNA template while the primer binds nearby; (B) once the primer and probe are attached, Taq polymerase (in yellow) binds to the 3' end of the primer; (C) Taq polymerase generates an amplicon sequence (in orange) up to the SNP site; (D) Taq polymerase cleaves the MGB probe leading to the detachment of the FAM™ reporter dye (in blue) from its quencher (in red) resulting in fluorescence of FAM™]

C3. Background information on interpreting the TaqMan® SNP Allelic Discrimination output

TaqMan® SNP Allelic Discrimination outputs

A number of results are provided by the real-time PCR system following genotype analysis, with important outputs being the change in fluorescent signals, the 'call' and the allelic discrimination plot.

As the reporter dyes fluoresce at two distinct frequencies, an increase in fluorescence at the FAM™ or VIC® specific frequency indicates the presence of the C or T allele respectively (Appendix Figure C3.1).



Appendix Figure C3.1. Amplification and multicomponent plots [(A) amplification and (B) multicomponent plots showing detectable increases in fluorescent signals from the 20th cycle of the real-time PCR system; increases in FAM™ (in blue) and VIC® (in green) signals are shown in the multicomponent plot on the right]

Theoretically, the detection of both signals in one DNA sample indicates heterozygosity for the SNP. However, results from analysing the sequenced DNA samples show the presence of some background VIC® fluorescence despite C homozygosity. This suggests some inappropriate binding of the VIC® probe to non-complementary SNP sites. Although the cause of this is not entirely clear, we believe that it may be due to a phenomenon known as the 'wobble' where pairing of non-complementary bases occasionally occurs during translation.[a] This phenomenon is observed in natural DNA replication and partly explains redundancy in the genetic code.[b] It is known to be a cause of DNA mutation.[a]

Despite the presence of the 'wobble' phenomenon, the inclusion of sequenced positive controls on each PCR plate provides a benchmark of expected FAM™ and VIC® signal sizes for each zygosity. The genotype of the unknown DNA samples is determined (or 'called') based on how closely their signals match those of the positive controls (Appendix Figure C3.2). Information on the size of each fluorescent signal and the call made is then visually summarised in the allelic discrimination plot (Appendix Figure C3.3).

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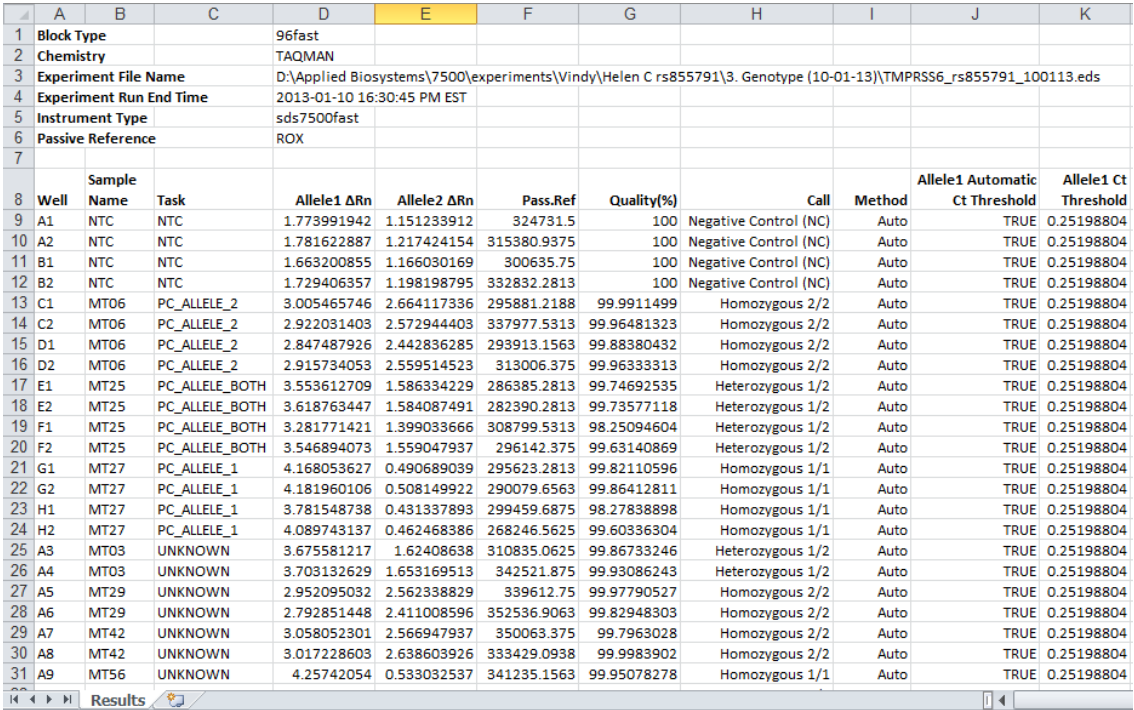
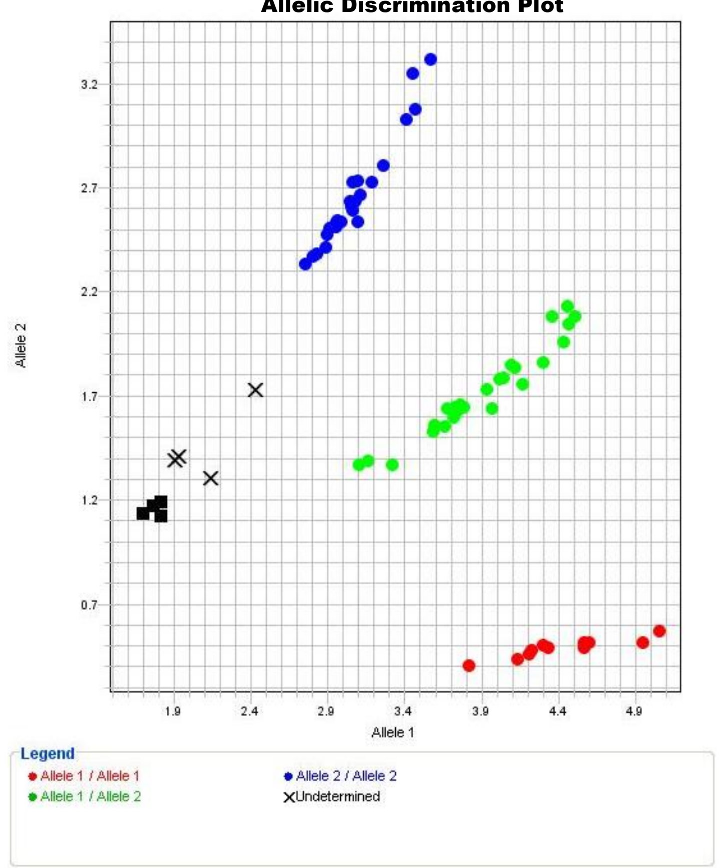
[a]Pray L. DNA Replication and Causes of Mutation. *Nature Education* 2008; 1.
[b]Ardell DH, Sella G. On the evolution of redundancy in genetic codes. *J Mol Evol* 2001; 53: 269-81.



	A	B	C	D	E	F	G	H	I	J	K
1	**Block Type**			96fast							
2	**Chemistry**			TAQMAN							
3	**Experiment File Name**			D:\Applied Biosystems\7500\experiments\Vindy\Helen C rs855791\3. Genotype (10-01-13)\TMPRSS6_rs855791_100113.eds							
4	**Experiment Run End Time**			2013-01-10 16:30:45 PM EST							
5	**Instrument Type**			sds7500fast							
6	**Passive Reference**			ROX							
7											
8	**Well**	**Sample Name**	**Task**	**Allele1 ΔRn**	**Allele2 ΔRn**	**Pass.Ref**	**Quality(%)**	**Call**	**Method**	**Allele1 Automatic Ct Threshold**	**Allele1 Ct Threshold**
9	A1	NTC	NTC	1.773991942	1.151233912	324731.5	100	Negative Control (NC)	Auto	TRUE	0.25198804
10	A2	NTC	NTC	1.781622887	1.217424154	315380.9375	100	Negative Control (NC)	Auto	TRUE	0.25198804
11	B1	NTC	NTC	1.663200855	1.166030169	300635.75	100	Negative Control (NC)	Auto	TRUE	0.25198804
12	B2	NTC	NTC	1.729406357	1.198198795	332832.2813	100	Negative Control (NC)	Auto	TRUE	0.25198804
13	C1	MT06	PC_ALLELE_2	3.005465746	2.664117336	295881.2188	99.9911499	Homozygous 2/2	Auto	TRUE	0.25198804
14	C2	MT06	PC_ALLELE_2	2.922031403	2.572944403	337977.5313	99.96481323	Homozygous 2/2	Auto	TRUE	0.25198804
15	D1	MT06	PC_ALLELE_2	2.847487926	2.442836285	293913.1563	99.88380432	Homozygous 2/2	Auto	TRUE	0.25198804
16	D2	MT06	PC_ALLELE_2	2.915734053	2.559514523	313006.375	99.96333313	Homozygous 2/2	Auto	TRUE	0.25198804
17	E1	MT25	PC_ALLELE_BOTH	3.553612709	1.586334229	286385.2813	99.74692535	Heterozygous 1/2	Auto	TRUE	0.25198804
18	E2	MT25	PC_ALLELE_BOTH	3.618763447	1.584087491	282390.2813	99.73577118	Heterozygous 1/2	Auto	TRUE	0.25198804
19	F1	MT25	PC_ALLELE_BOTH	3.281771421	1.399033666	308799.5313	98.25094604	Heterozygous 1/2	Auto	TRUE	0.25198804
20	F2	MT25	PC_ALLELE_BOTH	3.546894073	1.559047937	296142.375	99.63140869	Heterozygous 1/2	Auto	TRUE	0.25198804
21	G1	MT27	PC_ALLELE_1	4.168053627	0.490689039	295623.2813	99.82110596	Homozygous 1/1	Auto	TRUE	0.25198804
22	G2	MT27	PC_ALLELE_1	4.181960106	0.508149922	290079.6563	99.86412811	Homozygous 1/1	Auto	TRUE	0.25198804
23	H1	MT27	PC_ALLELE_1	3.781548738	0.431337893	299459.6875	98.27838898	Homozygous 1/1	Auto	TRUE	0.25198804
24	H2	MT27	PC_ALLELE_1	4.089743137	0.462468386	268246.5625	99.60336304	Homozygous 1/1	Auto	TRUE	0.25198804
25	A3	MT03	UNKNOWN	3.675581217	1.62408638	310835.0625	99.86733246	Heterozygous 1/2	Auto	TRUE	0.25198804
26	A4	MT03	UNKNOWN	3.703132629	1.653169513	342521.875	99.93086243	Heterozygous 1/2	Auto	TRUE	0.25198804
27	A5	MT29	UNKNOWN	2.952095032	2.562338829	339612.75	99.97790527	Homozygous 2/2	Auto	TRUE	0.25198804
28	A6	MT29	UNKNOWN	2.792851448	2.411008596	352536.9063	99.82948303	Homozygous 2/2	Auto	TRUE	0.25198804
29	A7	MT42	UNKNOWN	3.058052301	2.566947937	350063.375	99.7963028	Homozygous 2/2	Auto	TRUE	0.25198804
30	A8	MT42	UNKNOWN	3.017228603	2.638603926	333429.0938	99.9983902	Homozygous 2/2	Auto	TRUE	0.25198804
31	A9	MT56	UNKNOWN	4.25742054	0.533032537	341235.1563	99.95078278	Homozygous 1/1	Auto	TRUE	0.25198804

Results

Appendix Figure C3.2. A snapshot of the real-time PCR genotyping output (Columns 'Allele1 ΔRn' and 'Allele2 ΔRn' denote the magnitude of the change in VIC® and FAM™ fluorescent signals respectively; the 'Call' column shows the genotype of the unknown DNA samples determined according to the fluorescence patterns of the positive controls)



Appendix Figure C3.3. Allelic discrimination plot [The X and Y axes indicate the size of the VIC® and FAM™ fluorescent signals respectively; the coloured markers signify successful genotype determination (blue: C homozygote; green: heterozygote; red: T homozygote) whereas the black crosses indicate undetermined calls; the black markers represent the no template controls or blanks]

APPENDIX D: PUBLISHED MANUSCRIPTS RELATED TO THIS THESIS

(See PDF documents in CD-ROM titled 'Appendix D')

D1. O'Connor H, Munas Z, Griffin H, Rooney K, Cheng HL, Steinbeck K. Nutritional adequacy of energy restricted diets for young obese women. *Asia Pac J Clin Nutr* 2011; 20: 206-11

D2. Cheng HL, Bryant, C, Cook R, O'Connor H, Rooney K, Steinbeck K. The relationship between obesity and hypoferraemia in adults: a systematic review. *Obes Rev* 2012; 13: 150-61

D3. Cheng HL, Bryant C, Rooney K, Steinbeck K, Griffin H, Petocz P, O'Connor H. Iron, hepcidin and inflammatory status of young healthy overweight and obese women in Australia. *PLoS ONE* 2013; 8: e68675. doi:10.1371/journal.pone

D4. Cheng HL, Griffin HJ, Bryant CE, Rooney KB, Steinbeck KS, O'Connor HT. Impact of diet and weight loss on iron and zinc status in overweight and obese young women. *Asia Pac J Clin Nutr* 2013; 22: 574-82

D5. Griffin HJ, Cheng HL, O'Connor HT, Rooney KB, Petocz P, Steinbeck KS. Higher protein diet for weight management in young overweight women: a 12-month randomized controlled trial. *Diabetes Obes Metab* 2013; 15: 572-5

D6. Supplementary material for Griffin HJ, Cheng HL, O'Connor HT, Rooney KB, Petocz P, Steinbeck KS. Higher protein diet for weight management in young overweight women: a 12-month randomized controlled trial. *Diabetes Obes Metab* 2013; 15: 572-5