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Iron Deficiency and Risk Factors in Pre-Menopausal Females living in Auckland, New Zealand

A thesis presented in partial fulfilment of the requirements for the degree of
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Abstract

Background: Iron Deficiency (ID) is the most common nutrient deficiency worldwide, affecting one third of the world's population. In New Zealand (NZ), the highest rates are found within pre-menopausal females, with previously identified risk factors for ID including low meat intake, heavy menstruation and blood donation. Emerging risk factors such as inflammation and obesity are yet to be explored in NZ, along with the master hormone of iron regulation, hepcidin.

Objectives: To describe iron and hepcidin status within premenopausal females, and identify risk factors for ID.

Methods: Females (n=170) aged 18–45 were recruited. Biomarkers of iron status were measured: Serum ferritin (Sf), haemoglobin, soluble transferrin receptor, hepcidin as well as inflammatory markers C-reactive protein and interleukin-6. Body composition was measured using bioelectrical impedance analysis, and lifestyle factors were assessed using questionnaires, including a previously validated food frequency questionnaire. Variables known to potentially influence iron status were entered into multiple linear regression analysis to identify predictors of Sf.

Results: Iron deficiency was confirmed in 55.8% of participants ($Sf < 30\mu\text{g}\cdot\text{L}^{-1}$). Prevalence of ID did not differ significantly ($p=0.141$) between South Asians (64.3%), NZ Europeans (51.6%), and those of other ethnicities (45.5%). Hepcidin concentrations were higher in those who were iron sufficient ($Sf \geq 30\mu\text{g}\cdot\text{L}^{-1}$) (6.62nM vs 1.17nM, $p<0.001$). South Asian females had higher hepcidin (8.78nM) concentrations, compared to NZ Europeans (6.28nM) and those of other ethnicities (4.89nM) ($p=0.026$). The higher hepcidin concentrations in South Asian participants are possibly associated with these participants having a higher BMI ($p<0.001$), body

fat percentage ($p<0.001$) and interleukin-6 ($p<0.001$) than NZ Europeans and other ethnicities. Hepcidin ($\beta=0.082$, $p<0.001$) and frequency of meat intake ($\beta=0.058$, $p=0.001$) were identified as significant predictors of Sf in NZ Europeans. Hepcidin was the only identified predictor of Sf in South Asians ($\beta=0.138$, $p<0.001$) and those of other ethnicities ($\beta=0.117$, $p<0.002$).

Conclusion: The study confirms a positive relationship between hepcidin and Sf in NZ females, highlighting hepcidin's potential as an emerging biomarker to identify ID. Furthermore, there were differences in hepcidin levels between ethnicities, which may be linked to higher levels of body fat and inflammation.

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List of Abbreviations

Abbreviation	Term
ACD	Anaemia of Chronic Disease
ATP	Adenosine Triphosphate
BIA	Bioelectrical Impedance Analysis
BMI	Body Mass Index
CRP	C-Reactive Protein
DMT-1	Divalent Metal Transporter 1
DNA	Deoxyribonucleic Acid
Fe ²⁺	Ferrous
Fe ³⁺	Ferric
Fe-S	Iron Sulphur
HAMP	Hepcidin Anti-microbial Peptide
Hb	Haemoglobin
ID	Iron Deficiency
IDA	Iron Deficiency Anaemia
IDMH	Iron Deficiency Microcytosis/Hypochromia
IL-6	Interleukin-6
MCH	Mean Cell Haemoglobin
MCV	Mean Cell Volume
NZ	New Zealand
Sf	Serum Ferritin
sTfR	Soluble Transferrin Receptor
TIBC	Total Iron Binding Capacity

Chapter 1: Purpose

1.1 Introduction

Iron is a vital element required to carry out normal physiological functioning, yet it is the most common nutrient deficiency in the world. The deficiency is estimated to affect one third of the world's population (World Health Organisation, 2011). In New Zealand (NZ), the highest prevalence of ID is seen in 31 to 50-year-old females (12.1%) (as defined by serum ferritin (Sf) $< 12\mu\text{g}\cdot\text{L}^{-1}$ and zinc protophoryin $> 60\mu\text{mol}\cdot\text{mol}^{-1}$), with some research suggesting that up to 23% of premenopausal females have ID (Sf $< 20\mu\text{g}\cdot\text{L}^{-1}$) (Heath, Skeaff, Williams, & Gibson, 2001; University of Otago & Ministry of Health, 2011). In the past year alone, NZ's Ministry of Health has spent almost 20 million dollars treating ID, which is a substantial financial burden on NZ's healthcare system (Nyika, 2018).

If ID remains untreated it can progress into anaemia, where iron stores are insufficient to support normal erythropoiesis and subsequently compromises oxygen transport to tissues, which has implications for impaired aerobic performance (Hinton, Giordano, Brownlie, & Haas, 2000). This is likely due to iron's role in oxygen transport through haemoglobin within red blood cells (Radjen et al., 2011).

Iron deficiency anaemia (IDA) presents with a number of unwanted outcomes including: impaired cognitive function and mood regulation; increased risk of infection and poor pregnancy outcomes (McClung & Murray-Kolb, 2013). Changes in cognition during IDA, including reductions in attention, concentration and intelligence quotient in both children and adult females (Falkingham et al., 2010). These changes also extend to increases in irritability, fatigue, and depressive symptoms (Murray-Kolb & Beard, 2007). Iron deficiency has been attributed to reductions in work efficiency and

productivity even in the absence of anaemia (Haas & Brownlie, 2001; Schaaf, Scragg, Metcalf, Grant, & Buchanan, 2000). Females with ID were more likely to report 'constant fatigue' than those with no history of ID (Patterson, Brown, Powers, & Roberts, 2000). Long term, IDA is also associated with development or exacerbation of chronic diseases, in particular cardiovascular disease, with IDA linked to increased risk of damage to myocardial tissue (Klip et al., 2013; Sullivan, 2007). The effects of ID and IDA are of particular concern for pregnant women. Women diagnosed with ID during pregnancy are at increased risk of complications including preterm birth, small for gestational age babies and increased mortality risk for both mother and child (Alwan et al., 2015; Haider et al., 2013).

Conversely, excess iron is toxic to the body (Emerit, Beaumont, & Trivin, 2001). Unbound iron readily donates and receives electrons to generate reactive oxygen species, resulting in damage to proteins, deoxyribonucleic acid (DNA) and cell membranes (Emerit et al., 2001). Therefore, iron status must be tightly regulated within the body. Hepcidin, a hormone synthesised in hepatocytes, is the primary regulator of the body's iron stores and enforces homeostatic control by inhibiting iron movement and utilisation (Nemeth & Ganz, 2009). The magnitude of the hepcidin response within the body is largely influenced by the body's current iron status (Nemeth & Ganz, 2009). When an individual is iron replete hepcidin levels are higher, but during ID hepcidin decreases (Nemeth & Ganz, 2009). However, additional regulatory factors of this hormone include the inflammatory marker interleukin-6 and hypoxia (Nemeth, Rivera, et al., 2004). These factors may up-regulate or down-regulate hepcidin expression within the body and therefore increase or blunt iron utilisation within the body (Nemeth, Tuttle, et al., 2004; Nicolas et al., 2002).

There are certain factors that may increase risk of ID in some individuals. These include non-modifiable risk factors, such as ethnicity and a genetic predisposition to ID and inflammation (Delaney, Esquenazi, Lucas, Zachary, & Leffell, 2004). Additional risk factors include but are not limited to: body composition, dietary patterns (e.g. vegetarianism) and blood loss through menstruation or blood donation (Aigner, Feldman, & Datz, 2014; Beck et al., 2014a; Craig, 2010; Heath, Skeaff, Williams, & Gibson, 2001).

Within NZ, Beck et al. (2014a) demonstrated that blood donation within the past year increased odds of suboptimal iron status ($Sf < 20\mu\text{g}\cdot\text{L}^{-1}$) by up to six times. These findings were in agreement with a study in NZ premenopausal females, where blood donation in the past four months was the strongest risk factor for ID ($Sf < 20\mu\text{g}\cdot\text{L}^{-1}$) (Heath et al., 2001). Dietary factors are also known to affect iron status of NZ females. Heath et al. (2001), showed that a low meat/fish/poultry intake was a significant predictor of ID, while a similar study found that those consuming a dietary pattern high in milk and yogurt had a 1.44 increased odds of ID (Beck et al., 2014a). There is also a higher incidence of ID in certain ethnicities in NZ. Those of Asian ethnicity had five times higher odds of having suboptimal iron status ($Sf < 20\mu\text{g}\cdot\text{L}^{-1}$) (Beck et al., 2014a). In particular, South Asians have been shown to have higher rates of ID ($Sf < 12\mu\text{g}\cdot\text{L}^{-1}$) than both South East Asians and NZ Europeans (Parackal, Smith, & Parnell, 2015).

There are other emerging risk factors that are thought to contribute to the development of ID. For example, it is proposed that those who are obese are at an increased risk of ID due to the inflammatory state associated with increased hepcidin expression (Pustacioglu, Nemeth, & Braunschweig, 2012). However, hepcidin, inflammatory markers and body composition have not been measured in relation to iron status in NZ females.

Research into unexplored risk factors for ID may aid health professionals in identifying individuals who are at an increased risk of ID. Earlier diagnosis and treatment of ID, whether through supplementation or dietary advice, may decrease the incidence of ID and its associated symptoms. This could also aid in preventing progression from ID to IDA and its associated negative health outcomes in NZ. This proactive approach is vital, as the cost of hospitalisation associated with IDA in NZ has more than doubled from 3.2 million to 6.7 million in the past 10 years (Nyika, 2018). The investigation of basal hepcidin concentrations in females within NZ will help provide a better understanding of the extent to which this population can absorb and utilise iron. Additionally, this will contribute to the emerging area of research on hepcidin, helping to identify basal levels for this population.

1.2 Aims

The aim of this research is to determine current iron and hepcidin status as well as risk factors for ID in premenopausal females living in Auckland, New Zealand.

1.2.1 Objectives

1. Measure iron status and hepcidin levels in pre-menopausal females in the Auckland region.
2. Investigate the relationship between iron status, hepcidin expression, inflammatory markers, body composition, blood loss, ethnicity and dietary intake in pre-menopausal females in the Auckland region.

1.2.2 Hypotheses

1. It is hypothesised that the prevalence of ID (serum ferritin $< 30\mu\text{g}\cdot\text{L}^{-1}$) will be significantly higher in South Asian females compared with European females.
2. South Asian females will have higher hepcidin concentrations compared with European females.
3. Determinants of ID will include having a high BMI, low dietary meat intake, high levels of blood loss and lower basal hepcidin concentrations.

1.3 Structure of Thesis

This thesis begins with an introduction to the study and highlights the areas in which further research on ID in NZ females is needed. This is followed by chapter two, which is an extended review of the current literature, outlining the role of iron and hepcidin in the body, how ID is measured and identified, and known risk factors for ID. Chapter three is a manuscript of the study for publication which includes an introduction, methodology of the study, a summary of results and discussion of the findings. Finally, chapter four presents a summary of the research, where the study fits within the existing literature and future directions for research in iron deficiency and hepcidin.

1.4 Researcher's Contributions

Table 1.1: Summary of Researcher's Contributions to Study

Author	Contribution to Thesis
Kimberley Lim MSc Student nutrition and dietetics	Primary author of the thesis. Recruited participants, collected data, processed blood samples, conducted statistical analysis, interpreted results.
Dr Claire Badenhorst Primary Supervisor Lecturer School of Sport, Exercise and Nutrition	Designed research, applied for ethics, recruited participants, collected data, phlebotomist, processed blood samples, assisted with interpretation of results, revised and approved the thesis.
Dr Kathryn Beck Co-Supervisor Associate Professor School of Sport, Exercise and Nutrition	Assisted with research design and data collection, assisted with statistical analysis, revised and approved the thesis.
Dr Pamela Von Hurst Associate Professor School of Sport, Exercise and Nutrition	Assisted with research design and data collection.
Dr Kay Rutherford-Markwick Associate Professor in Pathophysiology School of Health Sciences	Assisted with hepcidin and Interleukin-6 analysis.

Chapter 2: Literature Review

2.0 Introduction

The World Health Organisation has identified children of preschool age (43%), pregnant women (38%) and pre-menopausal females (29%) as being at an increased risk of ID (iron deficiency) (World Health Organisation, 2011). Within New Zealand (NZ), the highest prevalence of ID has been reported in premenopausal females (12.1%) (University of Otago & Ministry of Health, 2011) and certain ethnic groups including Māori, Asian and Pacifica (Grant, Wall, Brunt, Crengle, & Scragg, 2007; Schaaf et al., 2000). Research suggests that genetic factors, donating blood, and a low dietary haem intake are risk factors for the development of ID (Beck et al., 2014a; Parackal, Smith, & Parnell, 2015; Rangan et al., 1997).

Another factor that has a large influence on an individual's *in vivo* iron stores, is the hormone hepcidin. This polypeptide hormone is synthesised in the liver with its primary function to regulate the body's iron stores, by governing absorption and movement of iron (Nemeth & Ganz, 2009). Chronic diseases (such as inflammatory bowel disease and chronic kidney disease) are commonly associated with increased inflammation and corresponding elevated hepcidin levels (Cheng et al., 2013; Gasche, Lomer, Cavill, & Weiss, 2004). These sustained elevations of hepcidin are linked to reduced iron absorption and utilisation (Nemeth & Ganz, 2009). Iron has numerous essential roles within the body, however both iron overload and deficiency are associated with poor health outcomes, and therefore hepcidin's role in regulating iron status is crucial for optimal physiological function.

2.1 Role of Iron in the Body

A female who is iron replete will typically have 2.5g of iron in their body, the majority of which (80%) is found within haemoglobin (Hb) for effective oxygen transport (Beard, 2000). Approximately 20% of this is stored within ferritin, and the remainder is found within other proteins such as myoglobin, and non-haem iron containing proteins (Beard, 2000). An average of 1-2mg of iron is lost daily due to normal red cell mass turnover; gastrointestinal cell turnover and urinary, bile and sweat losses (Bothwell, 2009). Additionally, females lose approximately 1mg of iron per day during menses in their menstrual cycle, putting them at increased risk of ID, with this loss up to five to six times higher in females with menorrhagia (Napolitano et al., 2014). Therefore, it is recommended that regularly menstruating females consume 18mg of iron per day in order to absorb sufficient iron to replace these losses (Australian Government Department of Health and Aging & Ministry of Health, 2006).

Iron is absorbed at the apical membrane of duodenal enterocytes (figure 2.1). After entering the enterocyte, iron is either stored; bound to the body's storage protein (ferritin) or is exported out of the cell through ferroportin export channels which are found on the cell's basolateral membrane (Ponka, Tenenbein, & Eaton, 2015). If exported, iron travels through the blood bound to transferrin, where it gets delivered to various tissues. For example, it may be transported to bone marrow to assist with erythropoiesis of new red blood cells, or to hepatocytes of the liver for storage (Ponka et al., 2015).

Iron is utilised in many physiological processes within the cell (Levi & Roviola, 2009). In the mitochondria, iron is primarily used to synthesise two important structures: iron sulphur (Fe-S) clusters and haem (Levi & Roviola, 2009). Fe-S clusters are used to

assist the production of adenosine triphosphate (ATP) to provide energy for various biochemical reactions (Beinert, 2000). Haem biosynthesis initially occurs in the mitochondria, particularly in erythroid cells and hepatocytes (Nishida & Labbe, 1959). Following this, haem is incorporated into Hb as part of erythropoiesis in the bone marrow (Nishida & Labbe, 1959). Oxygen allosterically binds to each ferrous (Fe^{2+}) ion within haem, of which there are four binding sites in each Hb molecule. Here it plays an important role as oxygen bound to haem iron is transported to tissues for aerobic respiration (Perutz, 1979; Wilson & Reeder, 2008). Approximately 70-80% of the body's iron is found within Hb in circulating erythrocytes (Perutz, 1979). Haem is also incorporated in myoglobin located within muscle tissue where it assists oxygen storage for aerobic respiration (Wilson & Reeder, 2008).

Iron also has an important role in the development of both the innate and acquired immune system, with many iron containing enzymes required for these processes (Ned, Swat, & Andrews, 2003). The ability to acquire iron is important for the proliferation and development of lymphocytes, a type of white blood cell that recognises and produces antibodies to destroy pathogens (Ned et al., 2003). Additionally iron is crucial in neural development, with enzymes responsible for the myelination and proliferation of neural pathways being dependent on iron status (Scott & Murray-Kolb, 2016).

Insufficient iron stores to support these functions within the body will have detrimental effects on numerous physiological functions. Conversely, iron overload is detrimental to the body as there is no dedicated iron excretion pathway (Emerit et al., 2001). This narrow range for optimal homeostasis means that iron absorption must be tightly regulated, and this homeostatic control of iron levels in the body is achieved through the activity of the hormone hepcidin (Nemeth, Tuttle, et al., 2004).

2.2 Hepcidin and Iron Regulation

Hepcidin is a 25 amino acid polypeptide hormone that is primarily synthesised by hepatocytes, however it is also expressed by macrophages, adipocytes and within brain tissue (Nemeth & Ganz, 2009). After discovering that hepatic iron upregulated hepcidin expression, and observing that *HAMP* (hepcidin) gene knock out in mice resulted in iron overload further research into its role in regulating iron was investigated in humans (Nicolas et al., 2001; Pigeon et al., 2001).

Hepcidin elicits its effects by binding to ferroportin, the body's only known iron exporter (Nemeth, Tuttle, et al., 2004). Once bound to ferroportin, hepcidin induces its degradation and lysis resulting in the sequestration of iron within the cell. As a result, iron is unable to be effectively utilised within the body for normal physiological function (figure 2.1) (Nemeth, Tuttle, et al., 2004). Regulation of hepcidin levels are currently known to be controlled by three factors: current iron status, hypoxia, and inflammation (Liu, Davidoff, Niss, & Haase, 2012; Nemeth, Rivera, et al., 2004).

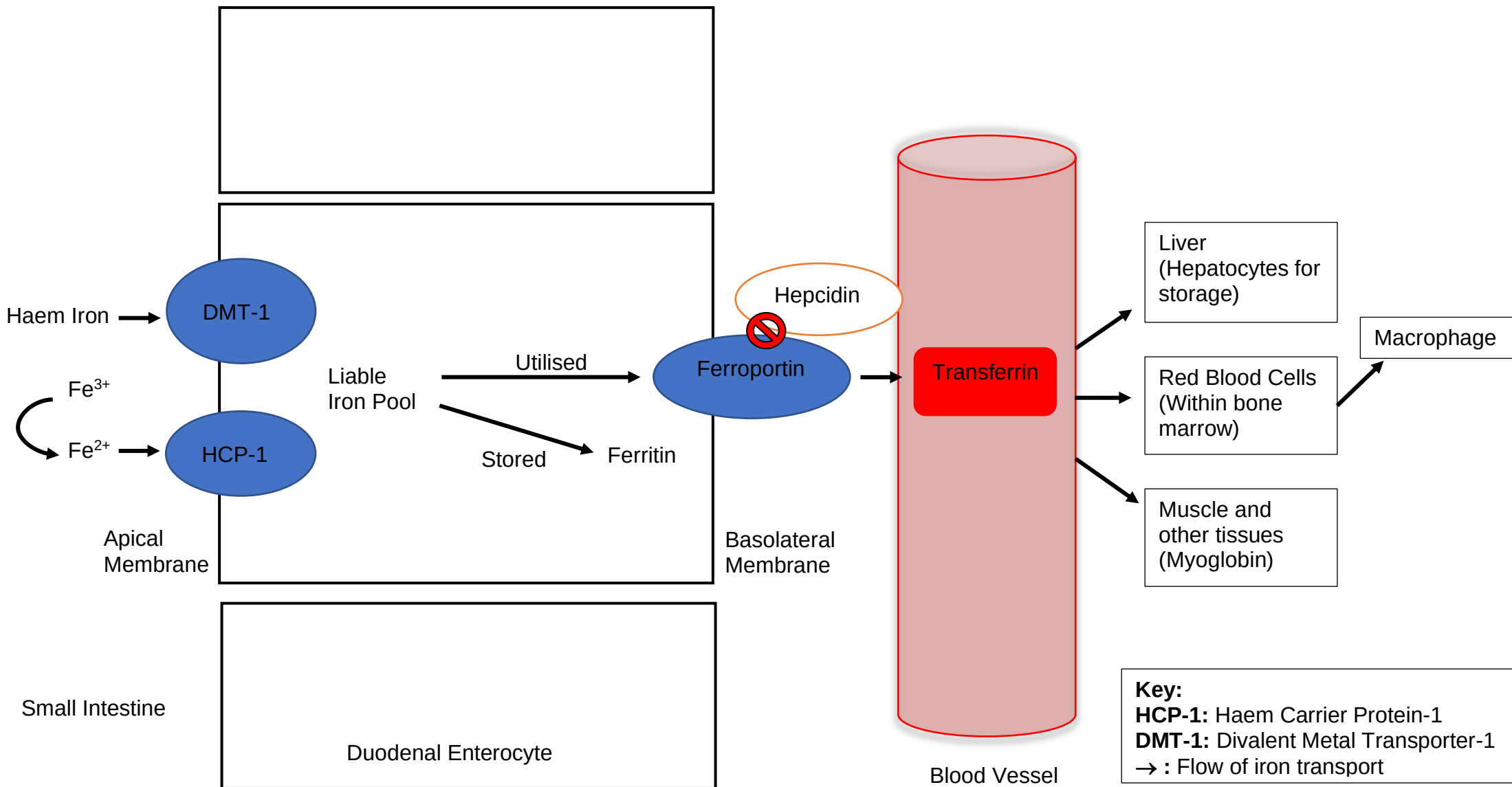


Figure 2.1: Overview of Iron Absorption at the enterocyte, adapted from Ponka, Tenenbein, and Eaton (2015).

An individual's current iron status is a known evolutionary feedback mechanism for hepcidin production. Hepcidin is upregulated when iron status is high to prevent iron overload, and downregulated when iron status is low to aid absorption and utilisation, while limiting the progression of ID to IDA (Nemeth, Rivera, et al., 2004; Nicolas et al., 2001). In addition to this innate evolutionary response, erythroferrone (in hypoxia) and interleukin-6 (IL-6) (during inflammation) allows for further control of hepcidin to avoid large perturbations in the iron stores in the body (Nemeth, Rivera, et al., 2004).

Hepcidin expression appears to be downregulated in periods of low blood oxygen concentrations (hypoxia) (Liu et al., 2012). In a hypoxic state increases in erythropoietic activity suppress hepcidin production via increases in erythroferrone, which is expressed in the early stages of red blood cell production (Liu et al., 2012). The upregulation of erythropoiesis for red blood cell production requires an adequate supply of iron, therefore, the suppression of hepcidin during periods of hypoxia is an appropriate homeostatic response (Liu et al., 2012).

Inflammation appears to upregulate hepcidin production, in particular, the secretion of the inflammatory cytokine IL-6, which stimulates the transcription of the hepcidin gene; *HAMP* (Verga Falzacappa et al., 2007). In humans infused with IL-6 at $30\mu\text{g}\cdot\text{hour}^{-1}$ a rapid increase in hepcidin production was observed, with the highest levels obtained approximately 3 hours post infusion (Nemeth, Rivera, et al., 2004). When compared to pre-infusion values, there was a 34% decrease in serum iron and a 33% decrease in transferrin saturation, suggesting that IL-6 inhibits movement of iron, as a result of the upregulation of hepcidin expression in healthy individuals (Nemeth, Rivera, et al., 2004). This upregulation of hepcidin with inflammation is thought to be an evolutionary mechanism to prevent bacterial growth, by removing iron from the systemic circulation and helping to protect against infection (Drakesmith & Prentice, 2012).

Understanding iron's regulation supports the concept of an optimal range for iron status within the body. Deviations away from this range may have negative health consequences. This remainder of this review will focus on the consequences of iron depletion and risk factors for ID.

2.3 Identification of ID in Research

There is a lack of heterogeneity across biomarkers to identify ID in research, this can make comparison across studies difficult. Table 2.1 summarises the currently used markers of iron status and presents advantages and disadvantages of each.

Within research, Sf values range from $12\text{-}30\mu\text{g}\cdot\text{L}^{-1}$ to identify ID. It has been suggested that using a Sf cut off of $30\mu\text{g}\cdot\text{L}^{-1}$ increased sensitivity for detecting ID from 25% to 92% as compared to $12\mu\text{g}\cdot\text{L}^{-1}$ (Mast, Blinder, Gronowski, Chumley, & Scott, 1994). Therefore, a higher cut-off level for Sf ($30\mu\text{g}\cdot\text{L}^{-1}$) could help in identifying stage 1 ID, allowing clinicians to have a preventative rather than treatment-based approach. Serum ferritin levels are commonly used in conjunction with Hb to identify ID or IDA, as Hb alone is not an accurate measure of iron status (Pfeiffer & Looker, 2017). This is due to Hb being easily affected by other nutrient deficiencies, such as cobalamin and folate (Pfeiffer & Looker, 2017). Haemoglobin levels may also be lowered due to other causes such as: anemia of chronic disease (ACD); haemodilution related to pregnancy and exercise; heavy smoking and hypoxia in individuals residing at high altitudes (Pfeiffer & Looker, 2017).

The second most common form of anaemia appears to be unrelated to iron status and is known as ACD (Weiss & Goodnough, 2005). As the name suggests, this type of anaemia results from long term conditions such as: infections, cancer, auto-immune

diseases (e.g. lupus and rheumatoid arthritis), inflammatory bowel disease and chronic kidney disease (Weiss & Goodnough, 2005). During ACD, it is common to see an increase in inflammatory markers such as C-reactive protein (CRP) and IL-6 (Weiss & Goodnough, 2005). This has consequential effects on iron status, including the upregulation of hepcidin and the increased retention of iron within cells, which limits iron's availability for use in various body tissues and processes (Weiss & Goodnough, 2005). A higher Sf cut-off (of $30\mu\text{g.L}^{-1}$) could also be used in conjunction with inflammatory markers such as IL-6 or CRP to identify the presence of inflammation, which may falsely elevate iron stores (ferritin levels) (Northrop-Clewes, 2008). This reflects a state of functional iron deficiency where iron is ineffectively moved throughout the body, yet Sf increases due to ferritin being a positive acute phase reactant (Cook, 2005). Therefore, the determination of ID via Sf can be confounded by inflammation (Cook, 2005). Soluble transferrin receptor (sTfR) is proposed to be a more reliable indicator of iron status (Mast, Blinder, Gronowski, Chumley, & Scott, 1998), due to levels not being affected by inflammation, theoretically making it a good method of differentiating between ID and ACD (Berlin, Meyer, Rotman-Pikielny, Natur, & Levy, 2011).

Another emerging indicator of iron status is the ratio of sTfR to the log of Sf, known as the sTfR index (Berlin et al., 2011; Infusino, Braga, Dolci, & Panteghini, 2012; Skikne et al., 2011). A meta-analysis that evaluated the efficacy of the sTfR index suggested that both sTfR and the sTfR index are useful to distinguish between ID and ACD (Infusino et al., 2012). However, sTfR produced better odds (odds ratio = 22.9) of predicting ID than the sTfR index (odds ratio = 9.5) (Infusino et al., 2012). One limitation of both sTfR and the sTfR index is the difficulty in standardising a cut-off value for ID across research groups. Studies included in the meta-analysis had cut-

offs ranging from 1.55 to 3.33mg·L⁻¹, with lower values indicating ACD and higher values indicating IDA (Infusino et al., 2012). However, a prospective study in patients with inflammation showed a sTfR index of <1mg·L⁻¹ accurately identified ACD (CRP ≥ 10mg·L⁻¹ or white blood cell count ≥ 10.5 × 10³·μL⁻¹) (Skikne et al., 2011). Inference from this area of research suggests that more studies are required to define the overall accuracy of both sTfR and the sTfR index for ID and ACD identification within various populations.

Hepcidin is another potential biomarker that could be used to differentiate between IDA and ACD (Pfeiffer & Looker, 2017). Like ferritin, hepcidin is an acute phase reactant and elevated levels would indicate inflammation, likely related to ACD (Nemeth et al., 2003). However, there is considerable intra-individual variability, and assay protocols are not standardised (Kroot et al., 2012). Due to this high intra-individual variability, hepcidin may be a more useful marker to track iron status changes over time for an individual, rather than being used to compare between individuals. However, more research needs to be done within this area before hepcidin can be used routinely as a marker of iron status (Kroot et al., 2012; Murphy, Witcher, Luan, & Wroblewski, 2007; van der Vorm et al., 2016).

Table 2.1: Summary of Haematological Markers for Iron Status in Research				
Biomarker	Description	Cut-off	Advantages	Limitations
Serum ferritin (Sf)	An acute phase protein that is reflective of the amount of iron stored within the cell	12-20 $\mu\text{g}\cdot\text{L}^{-1}$ 30 $\mu\text{g}\cdot\text{L}^{-1}$ may be a more sensitive marker during inflammation	Easy to analyse, generally correlates well with iron status	Increased during inflammation
Haemoglobin (Hb)	Iron containing protein found within erythrocytes	120 $\text{g}\cdot\text{L}^{-1}$ (Non-pregnant females) 110 $\text{g}\cdot\text{L}^{-1}$ (Pregnant females) 140 $\text{g}\cdot\text{L}^{-1}$ (Males)	Can be measured instantaneously with a fingerpick, indicates anaemia	Doesn't indicate the cause of anaemia, decreased during haemodilution. A late marker of low iron stores.
Soluble transferrin receptor (sTfR)	The concentration of transferrin receptor that circulates in the serum, it is proportional to transferrin receptors on cells within the body.	Women: 0.75–1.5 $\text{mg}\cdot\text{L}^{-1}$ Men: 0.75–1.75 $\text{mg}\cdot\text{L}^{-1}$	Not affected by inflammation. Useful to differentiate between IDA and ACD	Expensive, therefore typically only done in research, cut off values vary, lack of international standard
% Transferrin Saturation	The percentage of transferrin that is bound to serum iron. Calculated by serum iron/total iron binding capacity $\times 100\%$	20%	Not affected by inflammation. Useful to differentiate between IDA and ACD	Variation of serum iron
Soluble Transferrin Receptor Index	The ratio of soluble transferrin receptor to log serum ferritin	$< 1\text{mg}\cdot\text{L}^{-1}$ indicates ACD, varies	Useful to differentiate between IDA and ACD	Cut off values vary, still an emerging marker
Hepcidin	A hepatic hormone responsible for regulating the absorption of iron	Typically low during ID and high during periods of inflammation.	Useful to differentiate between IDA and ACD	High intra-individual variability, standardisation of assay protocol still developing
Zinc Protoporphyrin	A protein that gets incorporated into haem when iron status is low	$> 100\text{mcmol}\cdot\text{mol}^{-1}$ Hb indicates iron deficient erythropoiesis	Useful to differentiate between IDA and ACD. Early indicator of ID	Not an accurate indicator of iron status by itself
IDA – Iron deficiency anaemia; ACD – Anaemia of chronic disease				
Adapted from Clenin et al. (2015)				

2.3.1 Classification of Iron Deficiency

Iron status is broadly classified into three categories, and cut-off values for biomarkers vary for each stage, as detailed in table 2.2. When iron absorption is lower than is actively utilised, there is a negative iron balance. This negative balance results in the depletion of ferritin, the body's stored iron (Clénin et al., 2015). The decline in serum ferritin (Sf) levels to $< 30\mu\text{g}\cdot\text{L}^{-1}$ is an indicator of stage one ID without anaemia. At this stage iron is unable to support healthy tissue function (Clénin et al., 2015). Concurrently, zinc is incorporated into protoporphyrin instead of ferrous iron, resulting in an increase in zinc protoporphyrin, serving as an additional marker for stage 1 ID (Clénin et al., 2015; Labbé, Vreman, & Stevenson, 1999). During stage 1 ID, soluble transferrin receptor (sTfR) increases, reflecting the increase in iron transport and requirements of the cell as iron stores decline progressively in the body (Berlin et al., 2011). Although there is a lack of an international standard cut off, normal sTfR levels have been shown to average around $0.75\text{--}1.5\text{ mg}\cdot\text{L}^{-1}$ in females and $0.75\text{--}1.75\text{ mg}\cdot\text{L}^{-1}$ in males (Clénin et al., 2015). In stage 1 ID, mean cell volume and Hb do not change, suggesting that this initial stage of ID does not have an effect on blood cell formation (Clénin et al., 2015).

If iron balance remains negative, then iron stores may become insufficient to support effective erythropoiesis. This is known as stage 2 ID, where mean cell volume (MCV), a measure of the size of the red blood cells, falls to $< 80\text{fl}$, and cells are classified as microcytic (Clénin et al., 2015). An additional measure of stage 2 ID, is mean cell haemoglobin (MCH), which is the average quantity of haemoglobin present in a red blood cell (Jolobe, 2000). When MCH drops cells appear pale and lack colour, known as hypochromia ($< 28\text{pg}$) (Jolobe, 2000). Therefore stage two ID is referred to as ID

microcytosis/hypochromia (IDMH) (Clénin et al., 2015). Associated serum markers of this stage include; $Sf < 30\mu g \cdot L^{-1}$, decreased MCV and MCH, however Hb levels remain normal (Clénin et al., 2015).

Left untreated or undiagnosed, a negative iron balance will ultimately result in stage 3 ID known as iron deficiency anaemia (IDA). At this stage ferritin and Hb levels are reduced, indicating compromised erythropoiesis (Clénin et al., 2015; World Health Organisation, 2007). The ineffective erythropoiesis is detrimental for normal physiological functioning (Radjen et al., 2011), due to the impairment of oxygen carrying capacity to tissues, such as muscles and organs (Radjen et al., 2011). This ultimately results in symptoms such as: fatigue, shortness of breath, pallor, dizziness and heart palpitations (Cook, 2005).

Table 2.2: Comparison of Biomarkers at Each Stage of ID, Including the WHO's Cut-offs for ID.					
	Stage 1 ID	Stage 2 IDMH	Stage 3 IDA	ACD	WHO cut off
Serum Ferritin	< 30µg·L ⁻¹	< 20µg·L ⁻¹	< 12µg·L ⁻¹	>30µg·L ⁻¹ typically much increased	12-15µg·L ⁻¹
Zinc Protoporphyrin	>100 mcmol·mol Hb ⁻¹	>100 mcmol·mol Hb ⁻¹	>100 mcmol·mol Hb ⁻¹	Not defined	> 70-80 µg·dL ⁻¹ red blood cells
Soluble transferrin receptor	Women: >0.75 –1.5mg·L ⁻¹ Men: >0.75-1.75mg·L ⁻¹	Women: >0.75 –1.mg·L ⁻¹ Men: >0.75-1.75mg·L ⁻¹	Women: >0.75 –1.5mg·L ⁻¹ Men: >0.75-1.75mg·L ⁻¹	Women: 0.75 –1.5mg·L ⁻¹ Men: 0.75-1.75mg·L ⁻¹	Not defined
Mean Cell Volume	Normal	< 80 fL	< 80 fL	Typically normal (> 70fL)	< 67-81 fL
Mean Cell Haemoglobin	Normal	< 28 pg	< 28 pg	Normal-low	Not defined
Haemoglobin	Women: >120g·L ⁻¹ Men: >140g·L ⁻¹	Women: >120g·L ⁻¹ Men: >140g·L ⁻¹	Women: <120g·L ⁻¹ Men: <140g·L ⁻¹	Typically 100-110g·L ⁻¹	Women: <120g·L ⁻¹ Men: <130g·L ⁻¹
Transferrin Saturation	> 20%	< 20%	< 20%	Reduced	< 20%
Total Iron Binding Capacity	> 3.6µg·L ⁻¹	> 3.9µg·L ⁻¹	> 4.1µg·L ⁻¹	< 3µg·L ⁻¹	4-6µg·L ⁻¹ (calculated from transferrin)
C-Reactive Protein	< 3mg·L ⁻¹	< 3mg·L ⁻¹	< 3mg·L ⁻¹	> 5mg·L ⁻¹	Not defined
ID – Iron deficiency; IDMH – Iron deficiency with microcytosis/hypochromia; IDA – Iron deficiency anaemia; ACD – Anaemia of chronic disease; WHO – The World Health Organisation Adapted from Clenin et al. (2015), Weiss & Goodnough, (2005), Gibson, (2005) and World Health Organisation, (2007).					

2.3.2 Populations at Risk of Iron Deficiency

Globally, ID disproportionality affects certain regions, with the WHO identifying the highest incident rates occurring in South-East Asia (41.9%), the Eastern Mediterranean (37.6%) and African Regions (42.6%) (World Health Organisation, 2011). Although these can be somewhat attributed to poor nutrition in these developing regions, ID is still prevalent in immigrants that have moved to developed countries, such as NZ, as demonstrated in table 2.3 (Stoltzfus, 2003). In addition to ethnic discrepancies in ID presentation, children of preschool age (43%), pregnant women (38%) and pre-menopausal females who are regularly menstruating (29%) are at increased risk of ID (World Health Organisation, 2011). This is due to increased iron requirements with growth and during pregnancy, as well as increased iron losses through menstruation. Table 2.3 summarises the prevalence of ID in NZ females which ranges from 5.2% to 23%, which is considerably high compared to other western countries (Heath et al., 2001). For example, in The Netherlands, Denmark and Australia ID rates in females have been reported as 16%, 12.1% and 7.2% respectively, all defined by the same Sf level of $20\mu\text{g}\cdot\text{L}^{-1}$ (Brussaard & Brants, 1997; Milman, Rosdahl, Lyhne, Jørgensen, & Graudal, 1993; Rangan et al., 1997). Discussion for the increased risk of ID in this population group will be presented in the following sections.

Table 2.3 Summary of studies investigating the prevalence of ID in New Zealand Females

Study	Population Group	Prevalence	Iron status definition
Beck et al. (2014a)	Auckland pre-menopausal females (n=375)	18.7% ID 5.3% IDA	ID (Sf < 20µg·L ⁻¹ ; Hb ≥ 120g·L ⁻¹) IDA (Sf < 20µg·L ⁻¹ ; Hb < 120g·L ⁻¹)
Beck et al. (2012)	Female university students (n=233)	10.4% ID 2.9% IDA	ID (Sf < 20µg·L ⁻¹ ; Hb ≥ 120g·L ⁻¹) IDA (Sf < 20µg·L ⁻¹ ; Hb < 120g·L ⁻¹)
Parackal et al. (2014) (data from the 2008/09 NZ National Nutrition Survey)	Female South Asians over 15 years (n=40)	13.8% ID 4.6% IDA	ID (Sf < 12µg·L ⁻¹ ; zinc protoporphyrin > 60 µmol·mol ⁻¹) IDA (Sf < 12µg·L ⁻¹ ; zinc protoporphyrin > 60 µmol·mol ⁻¹ and Hb < 120g·L ⁻¹)
Parackal et al. (2014) (data from the 2008/09 NZ National Nutrition Survey)	Female East and South East Asians over 15 years (n=55)	9.1% ID 7.1% IDA	ID (Sf < 12µg·L ⁻¹ ; zinc protoporphyrin > 60µmol·mol ⁻¹) IDA (Sf < 12µg·L ⁻¹ ; zinc protoporphyrin > 60µmol·mol ⁻¹ and Hb < 120g·L ⁻¹)
Parackal et al. (2014) (data from the 2008/09 NZ National Nutrition Survey)	Female NZ Europeans over 15 years (n=1169)	6.7% ID 3.4% IDA	ID (Sf < 12µg·L ⁻¹ ; zinc protoporphyrin > 60µmol·mol ⁻¹) IDA (Sf < 12µg·L ⁻¹ ; zinc protoporphyrin > 60µmol·mol ⁻¹ and Hb < 120g·L ⁻¹)
University of Otago and Ministry of Health 2008/09 NZ National Adult Nutrition Survey	Females aged 19-30 years (n=240)	5.2% ID 1.2% IDA	ID (Sf < 12µg·L ⁻¹ ; zinc protoporphyrin > 60µmol·mol ⁻¹) IDA (Sf < 12µg·L ⁻¹ ; zinc protoporphyrin > 60µmol·mol ⁻¹ and Hb < 120g·L ⁻¹)
University of Otago and Ministry of Health 2008/09 NZ National Adult Nutrition Survey	Females aged 31-50 years (n=508)	12.1% ID 6.3% IDA	ID (Sf < 12µg·L ⁻¹ ; zinc protoporphyrin > 60µmol·mol ⁻¹) IDA (Sf < 12µg·L ⁻¹ ; zinc protoporphyrin > 60µmol·mol ⁻¹ and Hb < 120g·L ⁻¹)
Heath et al. (2001)	Dunedin pre-menopausal females (n=384)	23% ID 2% IDA	ID (Sf < 20µg·L ⁻¹ ; Hb ≥ 120g·L ⁻¹) IDA (Sf < 20µg·L ⁻¹ ; Hb < 120g·L ⁻¹)
ID – Iron deficiency; IDA – Iron deficiency anaemia; Sf – Serum ferritin; Hb – haemoglobin			

2.4 Dietary intake

Certain dietary patterns are known to put individuals at increased risk of poor iron status. The most recent NZ National Nutrition Survey conducted in 2008/2009 indicated that one third of teenage females did not meet the recommended dietary intake for iron of $18\text{mg}\cdot\text{day}^{-1}$ (University of Otago & Ministry of Health, 2011). Using a validated food frequency questionnaire in pre-menopausal NZ women, a low total meat intake ($< 79\text{g}$ per day) was the sole dietary predictor of mild iron deficiency ($\text{Sf} < 20\mu\text{g}\cdot\text{L}^{-1}$), but not anaemia ($\text{Hb} \geq 120\text{g}\cdot\text{L}^{-1}$) (Parackal et al., 2015). These results are supported by Beck et al. (2014a), who reported that a low adherence to a 'meat and vegetable' dietary pattern was associated with mild ID ($\text{Sf} < 20\mu\text{g}\cdot\text{L}^{-1}$) in premenopausal females. Epidemiological studies consistently show that vegetarians have lower Sf, indicating poorer iron status, however, in an national survey conducted in the United States, levels of IDA are the same between vegetarian and non-vegetarian women (Hunt, 2003).

A low dietary haem intake is thought to be increasing in NZ due to a rise in vegetarianism as well as immigration from South Asian countries (Roy Morgan Research, 2016; Statistics New Zealand, 2013). Certain religious and ethnic groups within NZ are more likely to follow a diet that is lower in animal foods, for example South Asian females were more likely to have never eaten red meat (25%), chicken (26%) and processed meat (55%) as compared to Europeans (Parackal et al., 2015).

2.4.1 Haem vs Non-Haem Iron Absorption

Non-haem iron, found predominately in plant based foods is acknowledged to not be as well absorbed as haem iron from meat sources (Hunt & Roughead, 1999; Monsen et al., 1978). In a randomised control trial, pre-menopausal women were shown to have a 3.5 fold higher absorption of iron from a non-vegetarian diet as compared to a lacto-ovo-vegetarian diet, even though both diets contained similar amounts (13mg) of iron (Hunt & Roughead, 1999). This is due to non-haem iron being easily oxidised from iron's soluble form, ferrous (Fe^{2+}) to its insoluble form, ferric (Fe^{3+}) in the alkaline environment of the gut (Spiro, Pape, & Saltman, 1967). Non-haem iron must be in its soluble form for absorption across the enterocyte via the divalent metal transporter-1 (DMT-1) (figure 2.1). The DMT-1 is also non-specific for ferrous, which means that other minerals such as zinc also compete with non-haem iron for absorption (Zoller et al., 2001). Conversely, haem iron is more bioavailable as it enters the enterocyte via haem carrier protein 1, which is specific to haem (Le Blanc, Garrick, & Arredondo, 2012). For this reason, vegetarian diets require approximately 80% more iron to compensate for the reduced bioavailability of non-haem iron (Australian Government Department of Health and Aging & Ministry of Health, 2006; Hunt, 2003). In the absence of this increased iron intake, vegetarians will likely increase their risk of acquiring insufficient iron through dietary sources, creating a negative iron balance that may contribute to their increased risk of ID (Craig, 2010; Haider, Schwingshackl, Hoffmann, & Ekmekcioglu, 2018).

2.4.2 Enhancers of Iron Absorption

Due to the non-specificity of the DMT-1 for non-haem iron, there are certain nutrients that interact with non-haem iron that act to either enhance or inhibit non-haem iron's absorption. Vitamin C (ascorbic acid) is known to increase iron's absorption, as it allows iron to remain in its soluble ferrous form for absorption via the DMT-1 (Conrad, Umbreit, & Moore, 1999). Foods high in vitamin C have been shown to increase the absorption of non-haem iron when consumed in the same meal (Hans Brise & Leif Hallberg, 1962; Thankachan, Walczyk, Muthayya, Kurpad, & Hurrell, 2008). Other compounds such as phytates and polyphenols are known to inhibit iron's absorption by binding to iron in the gut's lumen and preventing its absorption via the DMT-1 (Conrad et al., 1999). Ascorbic acid has been shown to protect against these inhibitory effects, with a 30mg dose of ascorbic acid increasing iron absorption in the presence of phytates from maize bran (Siegenberg et al., 1991).

Other nutrients are also known to enhance iron's absorption (Beck et al., 2014b). A review of dietary determinants of iron status, showed the majority of studies report a positive correlation between iron status and meat (or dietary haem) intake, thought to be linked to both haem iron and a meat/poultry/fish factor that enhances non-haem iron absorption (Beck et al., 2014b). Finally, organic acids such as those found in fruits and vegetables have been shown to enhance iron's absorption, however increased consumption of fruit and vegetables has not been associated with improved iron status (Beck et al., 2014b).

2.4.3 Inhibitors of Iron Absorption

Calcium fortified meals have been shown to reduce non-haem iron absorption by up to 39% in a dose dependent manner (Hallberg, Brune, Erlandsson, Sandberg, & Rossander-Hultén, 1991). Within NZ, Beck et al. (2014a), showed that those with a dietary pattern high in milk and yoghurt had a 1.44 times increased odds of suboptimal iron status ($Sf < 20\mu\text{g}\cdot\text{L}^{-1}$). Interestingly, unlike other nutrients, calcium also inhibits haem iron absorption (Hallberg, Rossander-Hulthén, Brune, & Gleerup, 1993). It has been hypothesised that calcium's inhibitory effect is due to the cellular localisation of the DMT-1 at the apical cell membrane, decreasing iron's absorption (Thompson, Sharp, Elliott, & Fairweather-Tait, 2010). However, it's mechanism for inhibiting haem iron is not confirmed and likely due to a combination of various pathways (Lynch, 2000).

Phytates, commonly found in wholegrain foods that are high in fibre are also known to have an inhibitory effect on iron absorption (Hallberg, Brune, & Rossander, 1989). However, a review by Beck et al. (2014b), only found one study that showed a positive association with fibre intake and Sf . Similarly, polyphenols and tannins found in tea and coffee may inhibit iron absorption (Disler et al., 1975). However, consumption of these drinks has only been shown to affect Sf in populations with marginal iron status (Beck et al., 2014b).

Although these nutrient interactions have been shown to affect iron's absorption, this does not necessarily translate to an effect on an individual's iron status. Long term studies looking at iron status have shown no correlation between specific nutrient intake and iron status (Asakura et al., 2009; Mølgaard, Kæstel, & Michaelsen, 2005; Pynaert et al., 2009).

Neither vitamin C or calcium intake was found to be significantly associated with iron status (as measured by Sf and sTfR) in Belgian premenopausal females (Pynaert et al., 2009). These findings aligned with a study in Japanese females, which found no association with vitamin C or calcium intake and iron status, as measured by Sf and Hb (Asakura et al., 2009). In addition, a review of studies that supplemented healthy menstruating females with calcium ($\geq 1000\text{mg}\cdot\text{day}^{-1}$) for 3-48 months found no effect on iron status (Bendich, 2001). Although the mechanisms of iron absorption have been investigated, further research in this area is still required to better understand these nutrient interactions on an individual's iron status in the long term. These studies suggest that dietary patterns, may have a stronger effect on iron status than assessing individual nutrients.

2.4.4 Interaction with Other Nutrients

There is emerging research indicating that other nutrient deficiencies may affect iron's absorption and subsequently iron status. One of these is vitamin D, with vitamin D deficiency frequently linked to both ID and IDA in cross-sectional studies (Lee et al., 2015; Sim et al., 2010).

One proposed explanation for vitamin D's effect on iron status is its ability to reduce inflammation. In vitro, vitamin D has been shown to promote the differentiation of monocytes into macrophages, which prevents the release of inflammatory cytokines (Mousa, Misso, Teede, Scragg, & de Courten, 2016). This in turn would result in lower levels of inflammation and circulating hepcidin. Therefore, increasing absorption and utilisation of iron within the body. A secondary explanation is the direct regulation of hepcidin gene expression through vitamin D (Zughaier, Alvarez, Sloan, Konrad, &

Tangpricha, 2014). Calcitriol (1, 25-dihydroxyvitamin D3) has been shown to suppress *HAMP* gene expression in macrophages (Zughaier et al., 2014), which would also result in decreased hepcidin and increase iron utilisation. However, further research in this area is required to investigate vitamin D's effect on iron status in humans.

Vitamin A is another nutrient potentially linked to iron status. The active form of vitamin A (β -carotene) has been shown to enhance the absorption of non-haem iron from various iron fortified grains (García-Casal et al., 1998). A review that assessed the efficacy of vitamin A supplementation on iron status showed that the simultaneous use of both supplements were more effective at preventing IDA (as measured by Hb) than either micronutrient alone (Michelazzo, Oliveira, Stefanello, Luzia, & Rondó, 2013). However, limited research has been done on the relationship between vitamin A intake and iron status (Beck et al., 2014b).

2.5 Non-Dietary Risk Factors for Iron Deficiency

There are known factors that either have been shown or are hypothesised to put an individual at a higher risk of having poor iron status. Some have been shown to contribute to the high prevalence of ID in NZ premenopausal females. This section discusses each of these risk factors and the mechanisms behind their contribution to sub-optimal iron status.

2.5.1 Obesity and Inflammation

Obesity is an emerging risk factor for ID, and is thought to be linked to the inflammatory state that is caused by excess adiposity (Aigner et al., 2014). The inflammatory state is associated with increased secretion of pro-inflammatory cytokines (Ferrante, 2007). In particular, the secretion of IL-6 which is known to affect iron status by stimulating transcription of the hepcidin gene; *HAMP* (Verga Falzacappa et al., 2007). This increased expression of hepcidin then causes iron to be trapped within the cell and leads to decreased iron absorption and utilisation (Nemeth, Tuttle, et al., 2004). A high BMI ($\geq 30\text{kg}\cdot\text{m}^{-2}$) has been associated with increased expression of IL-6 thereby increasing the risk of ID (Aigner et al., 2014). In an Australian study of females aged 18-25 years, hepcidin levels were higher in those with a BMI $> 35\text{kg}\cdot\text{m}^{-2}$ (3.30nM) than those who had a BMI between $27.5\text{kg}\cdot\text{m}^{-2}$ and $29.9\text{kg}\cdot\text{m}^{-2}$ (1.88nM) (Cheng et al., 2013). Further, a higher Sf ($46\mu\text{g}\cdot\text{L}^{-1}$ vs $31\mu\text{g}\cdot\text{L}^{-1}$), but lower serum iron ($13.0\mu\text{mol}\cdot\text{L}^{-1}$ vs $17.2\mu\text{mol}\cdot\text{L}^{-1}$) was observed in those individuals with a BMI $> 35\text{kg}\cdot\text{m}^{-2}$, indicating a mechanism similar to that of ACD (Cheng et al., 2013). Inference from the research would suggest increased inflammation as a result of body composition could be a possible explanation for the high prevalence of ID observed in individuals who are overweight or obese.

2.5.2 Ethnicity

Within NZ, Asian females were reported to be five times more likely to be iron deficient than European females (Beck et al., 2014a). In another study, Māori, Pacifica and Asian females aged 15-18 years were found to have a higher risk of ID than Europeans

(Schaaf et al., 2000). These differences between ethnicities may be due to aforementioned differences in dietary practices between ethnicities, however, there are also other factors that may predispose certain ethnicities to ID, including increased inflammation and genetics.

In a systematic review of studies from the USA, UK, Europe, Canada and NZ the ethnic minority groups of African Americans, Hispanics and South Asians were consistently reported to have higher levels of inflammation; with a CRP $> 3\text{mg}\cdot\text{mL}^{-1}$ commonly reported in these ethnicities (Nazmi & Victora, 2007). Possible explanation for these high levels of inflammation include low socioeconomic status (linked to increased stress levels); higher levels of adiposity and higher rates of smoking (Nazmi & Victora, 2007; Ranjit et al., 2007). This observation of elevated inflammation would predispose certain ethnicities to having higher basal hepcidin levels and possibly reduced iron absorption and utilisation, thus increasing their risk of ID (Nemeth, Rivera, et al., 2004). However, hepcidin levels within these at-risk ethnicities have not been measured so the extent to which they can absorb, recycle and regulate iron is currently unknown.

2.5.3 Heparidin

Another possible explanation for the differences in iron status is ethnic variations in hepcidin levels. Dutch pre-menopausal women have a median basal hepcidin concentration of 4.10nM (Galesloot et al., 2011), while other Eastern European females have a reported average hepcidin concentration of 1.69nM (Ilkovska, Kotevska, Trifunov, & Kanazirev, 2016). In healthy US and Italian women, the normal range for hepcidin was reported as 6.10-102.5nM with a median of 23.30nM (Ganz, Olbina, Girelli, Nemeth, & Westerman, 2008). Potential explanation for the higher rates

found in this study were that the analysis only included healthy participants, as those with ID had undetectable hepcidin levels (Ganz et al., 2008). In an Australian study on female adolescents, hepcidin concentrations were less than 1.79nM in 80% of ID participants (Cheng et al., 2013). As current iron status is a known feedback mechanism for hepcidin production, the findings of lower hepcidin concentrations in the Australian adolescent females could be explained by low iron status ($Sf < 15\mu\text{g}\cdot\text{L}^{-1}$) in these participants.

2.5.4 Genetics

Another possible contributing factor is a genetic predisposition to inflammation and ID itself (Delaney et al., 2004). There is research that has demonstrated a positive correlation between iron absorption (from maize flour fortified with ferrous sulphate) in mother and child pairs, suggesting inheritable factors may explain some of the intra-individual variance in iron absorption (Zimmermann, Harrington, Villalpando, & Hurrell, 2010). Inherited factors include single nucleotide polymorphisms (a type of genetic mutation) that can be passed from mother to child (Lee, Halloran, Trevino, Felitti, & Beutler, 2001). It is thought that this family correlation with iron absorption is due to one, or a combination of these polymorphisms that is inherited (Zimmermann et al., 2010).

A commonly known polymorphism is in the gene that codes for the transferrin protein (Lee et al., 2001). A mutation in this allele (Tf G277S) is thought to result in a less stable transferrin protein, or one that has a lower affinity for iron meaning that either plasma transferrin decreases or less iron is transported in the blood (Lee et al., 2001). Interestingly, the mutant variant was more common in those of European ancestry

than other ethnicities (such as African American, Hispanic and Asian) (Lee et al., 2001). European premenopausal females that are homozygous for the mutation had a five times higher risk of IDA ($\text{Hb} < 120\text{g}\cdot\text{L}^{-1}$, $\text{Sf} < 20\mu\text{g}\cdot\text{L}^{-1}$) than those who did not have the allele (Lee et al., 2001). However, another study undertaken in premenopausal women with $\text{Sf} < 30\mu\text{g}\cdot\text{L}^{-1}$ showed no significant differences in iron status or non-haem iron absorption in those with or without the mutation, suggesting there are other factors that influence iron status and absorption (Sarria et al., 2007). This research indicates that these genetic differences in iron absorption could be an underlying mechanism as to why certain ethnicities are at higher risk of ID.

2.5.5 Blood Loss

Blood loss is a common cause of ID within NZ (Beck et al., 2014a) and internationally (Zimmermann & Hurrell, 2007). Heath et al., (2001) identified three primary ways in which blood loss contributes to ID in premenopausal females in NZ: menstrual blood loss, blood donation and nosebleeds.

It is estimated that around 1mg of iron per day during menses of the menstrual cycle is lost, however this can increase up to five to six times during menstruation with menorrhagia (Napolitano et al., 2014). Napolitano et al. (2014), identified that females with menorrhagia (as defined as duration of menstruation > 5 days, and mean blood loss of $> 80\text{mL}$) were more likely to have depleted iron stores (median $\text{Sf } 5.7\mu\text{g}\cdot\text{L}^{-1}$) than those with normal menses (median $\text{Sf } 29\mu\text{g}\cdot\text{L}^{-1}$). Females are most likely to experience menorrhagia in their early (11 – 15 years) and late reproductive years (> 40 years) (Janssen, Scholten, & Heintz, 1997; World Health Organisation, 1986). These changes in menstrual cycle length, duration and heaviness can be explained

by the changes in the oestrogen and progesterone observed with age (Janssen et al., 1997). During the first few years of menstruation, the hypothalamic-pituitary ovarian axis is still immature, which often results in long, heavy anovulatory cycles (World Health Organisation, 1986). Individuals with a BMI > 23kg·m⁻² are also at increased risk of these heavy anovulatory cycles, due to excess adiposity being linked to reproductive hormonal changes (Janssen et al., 1997; Rowland et al., 2002; Santoro et al., 2004). Similarly, those with polycystic ovarian syndrome (PCOS) often experience reproductive hormonal changes which also result in long, heavy anovulatory cycles (Cheng et al., 2013). These variances in cycle length and tendency towards heavier menstruation mean that females at the beginning and end of their menstrual years, who have a BMI > 23kg·m⁻² or those with PCOS lose more iron compared to normally menstruating females.

A longer duration of menstruation has been identified as risk factor for ID in investigations undertaken in NZ females (Beck et al., 2014a; Heath et al., 2001). Menstrual blood loss was estimated using a validated recall questionnaire including questions quantifying number sanitary products used and heavy days per menstrual cycle (Heath et al., 2001). For every additional day of menstruation, participants had a 1.4 times increased odds of having suboptimal iron status (Sf < 20µg·L⁻¹; Hb ≥ 120g·L⁻¹) (Beck et al., 2014a). Conversely, women whose duration of menstruation was less than four days had a decreased risk of ID (Sf < 20µg·L⁻¹) (Heath et al., 2001). This research indicates that those females who experience heavy menstruation should be screened more regularly for ID.

Contraceptive choice can also influence menstrual loss and therefore iron status. Studies have shown that those on oral contraception are more likely to have optimal iron stores, as oral contraception can decrease blood menstrual loss by 50% (Beck et

al., 2014a; Bellizzi & Ali, 2018; Galan et al., 1998; Heath et al., 2001). Conversely, intra-uterine devices (particularly copper), were shown to increase menstrual losses by up to 100% (Callark, Litofsky, & DeMerre, 1966; Guttorm, 1971; Liedholm, Rybo, Sjöberg, & Sölvell, 1975). These increased menstrual losses corresponded with an average decrease in Hb concentration from $135\text{g}\cdot\text{L}^{-1}$ to $128\text{g}\cdot\text{L}^{-1}$ 16 months after insertion (Guttorm, 1971).

Another major contributor to blood loss is blood donation, with approximately 1-1.35mg of iron lost each donation (Coad & Conlon, 2011). Blood donation has been shown to be the most significant modifiable risk factor for suboptimal iron status ($\text{Sf} < 20\mu\text{g}\cdot\text{L}^{-1}$) in NZ premenopausal females, with those who had donated blood in the past year having a six times higher odds of low iron stores ($\text{Sf} < 20\mu\text{g}\cdot\text{L}^{-1}$) than those who had not (Beck et al., 2014a). Another study in a similar population group showed that those who donated blood in the past four months had a seven times higher odds of ID (Heath et al., 2001). Blood donation has also been correlated with a lower iron status in a number of western countries including the Netherlands, Belgium and Australia (Brussaard & Brants, 1997; Pynaert et al., 2009; Rangan et al., 1997).

In NZ studies, 11.5% of premenopausal participants in Auckland had donated blood in the past year (Beck et al., 2014a). In a similar study, 27% of premenopausal females in Dunedin donated blood in the past year (Heath et al., 2001). In NZ, 2.19% of the total population were active blood donors as of June 2019 (New Zealand Blood Service, 2019). In a recent study, it showed that 19.9% of females that had donated blood in the past year had ID ($< 12\mu\text{g}\cdot\text{L}^{-1}$) (Badami & Taylor, 2008). This research suggests that blood donation is common amongst pre-menopausal females and is a likely contributor to ID in this population. Currently, donors are screened for anaemia with a Hb fingerpick, with a cut-off of $120\text{g}\cdot\text{L}^{-1}$, however Sf is not measured prior to

donation (New Zealand Blood Service, 2018). Screening using Sf may be a better way to prevent the development of further negative iron balance in those who already have stage 1 ID and prevent this from further progressing to stage 2 or 3 of ID as a result of blood donation.

2.5.6 Other Determinants of Iron Status

Beck et al. (2014a), demonstrated in NZ premenopausal females, that those with a previous history of ID had a three-fold increased odds of suboptimal iron status ($Sf < 20\mu g \cdot L^{-1}$). This means that once initially diagnosed, these females are likely to have this issue in the future. Therefore, ongoing monitoring of iron status is recommended for these females.

Pregnancy places a significant stress on the mother's iron stores, which is likely due to haemodilution and deposition of iron to the growing foetus (Allen, 2000). An average of 1035mg iron is utilised over a normal full-term pregnancy in addition to normal requirements (Beaton, 2000). For this reason, women with higher parity tend to have an increased risk of ID (Rezk, Marawan, Dawood, Masood, & Abo-Elnasr, 2015; Sekhar, Murray-Kolb, Kunselman, Weisman, & Paul, 2016). A cross sectional study undertaken in pregnant Egyptian women showed that having more than two children and/or having children less than two years apart were significant risk factors for IDA during their second trimester ($Hb < 115g \cdot L^{-1}$ and $Sf < 25\mu g \cdot L^{-1}$) (Rezk et al., 2015). Women in the United States who had more than two children had a 2.27 times higher odds of having IDA ($Hb < 120g \cdot L^{-1}$) compared to those who had two children or less (Sekhar et al., 2016).

Within NZ, Beck et al. (2014a), found that women with suboptimal iron stores were more likely to have had children. However, including a meat and vegetable dietary pattern reduced the odds of suboptimal iron status in women who had children, but not in those without children (Beck et al., 2014a). Heath et al., (2001) did not find any correlation between having children and iron status in NZ premenopausal females. Similarly, there was no significant effect regarding the number of children on iron status (as measured by Sf, transferrin and transferrin saturation) in Australian mono and dizygotic twins (Whitfield, Treloar, Zhu, Powell, & Martin, 2003). A possible explanation for the lack of association between number of children and iron status in these studies is better uptake of prenatal vitamins (which include iron) prior to and during conception in developed countries. A study in Australian mothers showed that 52% took an iron containing supplement during pregnancy (Forster, Wills, Denning, & Bolger, 2009). This is in comparison to other developing countries such as India, where only 30.3% of mothers consumed iron supplements during their pregnancy (Rai et al., 2018). This may suggest that pregnant females should be screened for ID and supplemented as appropriate, to ensure iron stores are optimised during this particularly vulnerable period for ID.

2.6 Conclusion

Iron deficiency is a prevalent nutritional issue in NZ, particularly in pre-menopausal females. The identification of specific risk factors for ID may assist health care professionals with updated methods for ID screening in women that have one or more risk factors for ID. Health care professionals may then be more likely to diagnose ID

early and can aim to have a preventative focus in the treatment of ID before it progresses to severe IDA that exerts numerous adverse health outcomes.

The focus on hepcidin as a marker for ID in females, particularly in at risk ethnicities and those with a high BMI, would contribute to the emerging field of research in the hormone and may indicate an underlying mechanism to explain the link between ID in females and associated risk factors.

Chapter 3: Manuscript

3.0 Abstract

Background: Iron deficiency (ID) is highly prevalent in New Zealand (NZ), with low dietary haem intake and blood loss previously identified as risk factors. However, the influence of the hormone hepcidin in combination with dietary factors on iron status has not been investigated.

Objective: To measure iron status, hepcidin levels, lifestyle and dietary determinants of iron status in premenopausal females.

Methods: Females (n = 170) aged 18 – 45 years residing in Auckland were recruited. Iron status and inflammation were confirmed through serum biomarkers including; serum ferritin (Sf), haemoglobin, soluble transferrin receptor, hepcidin, C-reactive protein and interleukin-6. Lifestyle factors were assessed using a series of questionnaires, including a previously validated food frequency questionnaire. Potential determinants of Sf and hepcidin concentrations were entered multiple linear regression analysis to identify risk factors for ID.

Results: ID was confirmed in 55.8% of participants ($Sf < 30\mu g \cdot L^{-1}$). Although not significantly different ($p = 0.141$), 64.3% and 51.6% of South Asian and NZ Europeans presented with ID respectively. Hepcidin levels were higher in those who were iron sufficient ($Sf \geq 30\mu g \cdot L^{-1}$) (6.62nM vs 1.17nM, $p < 0.001$). South Asian females had higher hepcidin (8.78nM) concentrations, compared to NZ Europeans (6.28nM) ($p=0.018$), a result likely due to South Asians presenting with higher interleukin-6 (inflammation) (1.66 vs 0.63pg·ml⁻¹, $p < 0.001$). Hepcidin ($\beta=0.082$, $p<0.001$) and frequency of meat intake ($\beta=0.058$, $p=0.001$) were identified as significant predictors

of Sf in NZ Europeans, while hepcidin was the only identified predictor in South Asians ($\beta=0.138$, $p<0.001$) and those of other ethnicities ($\beta=0.117$, $p<0.002$).

Conclusions: This is the first study in NZ to show that hepcidin levels strongly predict Sf in premenopausal females. Higher meat intake and hepcidin appear to regulate and improve iron uptake amongst NZ Europeans, while hepcidin was a significant predictor of iron stores amongst all ethnicities.

3.1 Introduction

Iron deficiency (ID) is common in children, pregnant women and premenopausal females worldwide (World Health Organisation, 2011). Previous research has suggested high rates of ID (serum ferritin (Sf) $< 12\mu\text{g}\cdot\text{L}^{-1}$ and zinc protoporphyrin $> 60\mu\text{mol}\cdot\text{mol}^{-1}$) within New Zealand (NZ) as compared to other western countries, with a prevalence of 12.1% seen in 31-50 year old females. Ethnic cohorts within NZ, such as Māori, Pacifica and Asians have been identified at a higher risk of developing ID compared to NZ Europeans (Beck et al., 2014a; University of Otago & Ministry of Health, 2011; Wall, Brunt, & Grant, 2009).

There are numerous acknowledged factors known to contribute to ID. One of which is a low dietary haem intake, as haem iron is more bioavailable than non-haem (Hunt & Roughead, 1999). The most recent (2008/09) NZ national nutrition survey indicated that 15.4% of pre-menopausal females had iron intake below the estimated average requirement of $8\text{mg}\cdot\text{day}^{-1}$ (University of Otago & Ministry of Health, 2011). Additionally, vegetarianism has risen 27% since 2008/09, with one in ten New Zealanders following a vegetarian style diet (Roy Morgan Research, 2016; Woolf, 2019). This dietary pattern is likely to contribute increased risk of ID due to non-haem iron (e.g. from dark leafy greens) being poorly absorbed as compared to haem iron, such as from red meat (Hunt & Roughead, 1999; Monsen et al., 1978).

Blood loss has also been identified as a risk factor for poor iron status (Beck et al., 2014a; Heath et al., 2001; Napolitano et al., 2014; Zimmermann & Hurrell, 2007). Within NZ premenopausal females, primary contributors to blood loss include blood donation, menstrual losses, and nose bleeds, with recent blood donation being the

strongest risk factor for poor iron status (Beck et al., 2014a; Heath et al., 2001). Those that had donated blood in the past year had a six times higher odds of suboptimal iron status than those who had not (Beck et al., 2014a). A long duration of menstruation has also been identified a risk factor for ID in NZ females, with every additional day of menstruation increasing the odds of suboptimal iron status ($Sf < 20\mu\text{g}\cdot\text{L}^{-1}$; $\text{Hb} \geq 120\text{g}\cdot\text{L}^{-1}$) by 1.4 times (Beck et al., 2014a).

An area of emerging research in iron regulation and metabolism that is yet to be explored in non-athletic NZ females, is the role of the hormone hepcidin. Hepcidin is a peptide hormone that is known to inhibit the movement and utilisation of iron within the body by inhibiting its export from enterocytes (Nemeth, Tuttle, et al., 2004). Hepcidin levels in females have been shown to vary between countries and ethnicities (Ganz et al., 2008). However, the expression of hepcidin within NZ females has not been investigated, so the extent to which this population group can absorb or utilise iron is still unknown.

One of the factors that is known to upregulate hepcidin gene (*HAMP*) transcription is inflammation, specifically the inflammatory marker interleukin-6 (IL-6) (Verga Falzacappa et al., 2007). Individuals with high levels of inflammation may be at increased risk of having a poor iron status (Andrews, 2004), due to elevations in hepcidin inhibiting effective iron movement in the body (Ganz & Enns, 2003). Inflammatory derived anaemia is a co-morbidity of numerous inflammatory diseases such as irritable bowel disease and chronic kidney disease (Andrews, 2004). A prominent health condition in NZ associated with an inflammatory state is obesity (Aigner et al., 2014).

New Zealand has one of the highest obesity rates in the world, with one in three adults classified as obese (Ministry of Health, 2017). A high BMI ($\geq 30\text{kg}\cdot\text{m}^{-2}$) has been associated with elevated hepcidin levels, thought to be linked to increased expression of IL-6, proposing obesity is a risk factor for ID (Aigner et al., 2014; Cheng et al., 2013). However, the relationship between body composition and iron status has not been investigated in NZ females, so the extent to which obesity contributes to NZ's ID rates is unknown.

The high incident rates of ID within NZ pre-menopausal females of various ethnicities highlights the need for further investigation on additional risk factors that contribute to ID and the influence of hepcidin expression on iron metabolism. Therefore, this study aims to identify current iron and hepcidin status of pre-menopausal females in the Auckland region, as well as determinants of sub-optimal iron status in this population.

3.2 Methodology

This study was a single cross-sectional study conducted in pre-menopausal females residing in Auckland, NZ. Data collection commenced in July 2018 and concluded in July 2019.

3.2.1 Participants and Recruitment

Participants were invited to complete the study via several different recruitment methods. Initial recruitment targeted South Asian community groups as they have previously been shown to have high rates of ID, and was then widened to all pre-menopausal females. This was done via social media, as well as in person at Massey

University's Albany campus. The poster (appendix A) and information sheet (appendix B) used for recruitment are attached. Prior to each testing session participants were screened to ensure they met the inclusion criteria. Premenopausal females aged 18-45 years of age were eligible to take part. All participants resided in the Auckland region and were fluent in English. Participants were excluded from the study if they had consumed iron supplements ($> 20\text{mg}$ elemental iron) three or more times per week in the last three months, or if they had given blood or received a blood transfusion in the past six months, (Krayenbuehl, Battegay, Breymann, Furrer, & Schulthess, 2011; Pynaert et al., 2009). Females who were currently breastfeeding or pregnant (including pregnancy in the last year) and those who had chronic diseases that are known to affect iron status such as inflammatory bowel disease, chronic kidney disease or coeliac disease (Gasche et al., 2004; Gotloib, Silverberg, Fudin, & Shostak, 2006) were also excluded.

Ethics approval was obtained from Massey University Human Ethics Committee: Southern A (18/12) and all participants provided written informed consent prior to participating.

Participants were either invited to Massey University's Albany campus for data collection; alternatively, testing was undertaken off site in researcher approved community centres, such as the Indian Auckland Association and the NZ Sikh Women's Association. During a single data collection session, participants underwent a body composition analysis, serum blood collection, and completed a series of questionnaires (appendix I). An overview of the study procedure is summarised in appendix C.

3.2.2 Body Composition Measurements

Height was measured to the nearest centimetre using a stadiometer. Body composition was determined using bioelectrical impedance analysis (InBody 230, appendix D). Specifically, the data of interest included total body weight, skeletal muscle mass, fat mass and body fat percentage. Body mass index was calculated using the participant's weight (in kilograms) divided by height (in meters) squared.

3.2.3 Blood Sample Analysis

A single finger prick blood sample was collected to measure haemoglobin using the HemoCue® Hb 201+ System. In addition, a venous blood sample was collected by a trained phlebotomist from the antecubital vein with the participants in a seated and rested position (appendix E). The blood samples were collected using a sterile 21-gauge flashback needle into two 5-ml SST gel separator tubes (DB) and one 3-ml EDTA tube. The SST tubes were allowed to clot for 30-60 minutes at room temperature before being centrifuged at 10°C, 3000rpm for 10 minutes. The serum supernatant was extracted and divided into 1-ml aliquots and were stored at -80°C until analysis. Once all blood samples were collected, serum was sent to Auckland LabPlus (soluble transferrin receptor) and Canterbury Health laboratory (complete iron status; serum iron, Sf, transferrin saturation and CRP). Details on the analysis process have been provided as supplementary material to this thesis (appendices F-H). Serum interleukin-6 (RD Systems Human IL-6 Immunoassay high sensitivity ELISA, D6050) and hepcidin (RD System Human Hepcidin Immunoassay ELISA, HDP250) was analysed via commercially available ELISA kits at Massey University (R&D Systems, 2019a, 2019b).

3.2.4 Health, Demographic and Food Frequency Questionnaires

Questionnaires were conducted online using an iPad or smartphone through the online software Qualtrics. The provided questionnaire was an amalgamation of previously validated questionnaires that collected information on the participant's demographics (e.g. age, ethnicity) and medical history (e.g. medication, contraceptive choice, previous chronic disease and/or ID). Iron containing food groups and menstrual blood loss were determined using previously validated iron food frequency (Beck et al., 2012) and menstrual blood loss questionnaires (Heath et al., 1999).

The sample size was calculated from the following formula: $n = [Z^2 p(1-p)]/d^2$. Based on an estimated prevalence of ID of 12.1% in the population of interest (University of Otago & Ministry of Health, 2011), a 5% precision and 95% level of confidence giving a corresponding z-score of 1.69, a sample of 162 participants was determined to be adequate.

3.2.5 Data Handling and Statistical Analysis

Statistical analysis was carried out using IBM SPSS Statistics 25 for Windows (IBM Corporation, Armonk, NY, USA). Data was tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests, with $p > 0.05$ for either test treated as normally distributed. Data that did not meet the criteria for normality was log transformed and retested. Log transformed data that met the criteria for normal distribution is presented as its geometric mean and 95% confidence interval. Normally distributed data is presented as mean $\pm$ standard deviation, while non-normal data is presented as median (25% and 75% percentiles). Categorical data is reported as number of participants and percentages for each group.

Comparison between participants for parametric continuous data was conducted through independent t-tests and one-way ANOVA. Non-parametric data was compared using Mann-Whitney U and Kruskal Wallis tests. Pearson's Chi-squared test was used to compare categorical variables. For chi-squared test, the expected count for each cell was ≥ 5 , and all groups were independent. For all tests, a p-value of ≤ 0.05 was considered as statistically significant. For significant differences between groups determined using one-way ANOVA; Tukey's HSD post hoc test was used to identify where the difference occurred. For significant differences between groups identified by Kruskal Wallis' test; the Mann-Whitney U test was used to identify where the difference occurred.

Multiple linear regression analysis was used to identify the relationship between Sf and potential risk factors for ID. Frequency of food group consumption (alcohol, tea & coffee, dairy, meat, iron fortified foods, cereals, fruit & vegetable) intake per day/week were entered as continuous variables. Grouping of food groups is detailed in appendix J. Age, body fat percentage and length of period were also entered as continuous variables. Ethnicity, having children, contraceptive method, previous ID and previous blood donation were entered as categorical variables.

As Sf was the dependent variable, and was not initially normally distributed, a natural log transformation was used to meet the assumption for multiple linear regression analysis. There was no multicollinearity between independent variables as tested by variance inflation factor, with < 10 , and a tolerance > 0.2 indicating no multicollinearity. Additionally, Pearson's correlation co-efficient was used to check for correlations between independent variables, with values of ≤ 0.80 treated as lack of correlation. All residuals were independent as tested by the Durbin-Watson test, with a test statistic

of 1-3 indicating no correlation between residuals. The residual scatterplot was reviewed for homoscedasticity, and confirmed by a lack of pattern in the distribution in the residual scatterplot.

3.3 Results

3.3.1 Participant Characteristics

Of the 170 females recruited, five were excluded due to being unable to obtain blood samples, therefore, 165 females were included in the final analysis. Of those included in the analysis, 73 (44.2%) had sufficient iron stores ($Sf \geq 30\mu g \cdot L^{-1}$ and $Hb \geq 120g \cdot L^{-1}$) and 92 (55.8%) had insufficient iron stores ($Sf < 30\mu g \cdot L^{-1}$; $Hb < 120g \cdot L^{-1}$ or $\geq 120g \cdot L^{-1}$), of which 14 (8.5%) had iron deficiency anaemia ($Sf \leq 12\mu g \cdot L^{-1}$; $Hb < 120g \cdot L^{-1}$). Previous research within NZ has used a cut-off of Sf of $< 20\mu g \cdot L^{-1}$ to define iron insufficiency. For comparison, at a cut off for Sf of $< 20\mu g \cdot L^{-1}$, 93 (56.4%) participants were iron sufficient and 71 (43.0%) were iron insufficient. Forty-nine (29.7%) participants had a $CRP \geq 5mg \cdot L^{-1}$, and a correction factor was used to adjust their corrected Sf values for further analysis (Bui et al., 2012).

Table 3.1 summarises the characteristics of the participants. The median age of participants was 27 (22, 36) and 26 (21, 32) years for iron sufficient and insufficient respectively. The largest ethnic group was South Asians ($n = 70$; 42.4%), followed by NZ Europeans ($n = 62$; 37.6%). In terms of frequency per week, consumption of different food groups did not differ significantly between those that were iron sufficient and those that were insufficient, with the exception of meat intake. Frequency of meat intake was significantly higher ($p = 0.006$) in those with sufficient iron stores (9.21

times per week) than those with insufficient iron stores (6.29 times per week). Other demographic and health characteristics did not differ significantly between participants with sufficient and insufficient iron scores.

Table 3.1: Demographics and Dietary Patterns of Iron Sufficient and Insufficient Participants			
Characteristic	Iron Sufficient ^a n = 73 (44.2%)	Iron Insufficient ^b n = 92 (55.8%)	P-value ^c
Age (years) [†]	27.0 (22.0, 36.0)	26.0 (21.0, 32.0)	0.273
BMI (kg·m ²) [‡]	24.4 (23.3, 25.6)	24.2 (23.3, 25.2)	0.753
Body Fat Percentage*	32.3 (9.79)	32.9 (9.78)	0.720
Period Length (days) [†]	5.00 (4.00, 6.00)	5.00 (4.00, 6.00)	0.511
Ethnicity [‡]	South Asian	25 (34.2)	0.141
	NZ European	30 (41.1)	
	Other	18 (24.7)	
Having Children [‡]	23 (31.9)	25 (27.5)	0.534
Contraceptive Choice [‡]	Oral	14 (19.4)	0.477
	Other	13 (18.1)	
	None	45 (62.5)	
Previous Iron Deficiency [‡]	35 (49.3)	39 (43.8)	0.490
Previous Blood Donation [‡]	5 (7.0)	9 (10.0)	0.508
Self-reported Dietary Patterns [‡]	Normal	56 (77.8)	0.113
	Vegetarian/Vegan	8 (11.1)	
	Other	8 (11.1)	
Alcohol (p/w) [†]	0.71 (0.00, 2.17)	0.42 (0.0, 1.67)	0.458
Dairy (p/w) [†]	18.37 (10.12, 28.37)	19.42 (9.25, 28.0)	0.860
Tea and Coffee (p/w) [†]	9.25 (1.75, 18.29)	6.00 (1.08, 15.0)	0.152
Meat (p/w) [†]	9.21 (5.50, 13.96)	6.29 (2.25, 9.83)	0.006
Iron Fortified Cereals (p/w) [†]	1.67 (0.33, 4.33)	1.33 (0.50, 5.67)	0.739
Fruits and Vegetables (p/w) [†]	52.42 (33.7, 64.9)	53.16 (37.3, 73.8)	0.504
Cereals and Grains (p/w) [†]	8.17 (3.58, 13.0)	8.54 (3.75, 13.8)	0.882
^a Serum ferritin ≥ 30µg·L ⁻¹ and Hb ≥ 120g·L ⁻¹ ^b Serum ferritin < 30µg·L ⁻¹ (Hb < 120g·L ⁻¹ or ≥ 120g·L ⁻¹) ^c P value is for a test of differences between groups as assessed by the independent <i>t</i> -test (parametric continuous data), Mann-Whitney U test (non-parametric continuous data) or chi-square test (categorical data) (adequate/expected values). * Mean ± standard deviation [‡] Geometric mean and 95% confidence interval [†] Median and 25 th , 75 th centiles [‡] Number and % of participants p/w: Number of times consumed per week			

Table 3.2 shows that participants with insufficient iron stores had significantly lower Hb ($128\text{g}\cdot\text{L}^{-1}$ vs $133\text{g}\cdot\text{L}^{-1}$) ($p = 0.011$), Sf ($15.8\mu\text{g}\cdot\text{L}^{-1}$ vs $60.8\mu\text{g}\cdot\text{L}^{-1}$) ($p < 0.001$), and hepcidin (1.71nM vs 6.62nM) ($p < 0.001$) than those with sufficient iron stores. Participants with insufficient iron stores had a higher soluble transferrin receptor ($3.34\text{mg}\cdot\text{L}^{-1}$ vs $2.55\text{mg}\cdot\text{L}^{-1}$) ($p < 0.001$) and sTfR/log ferritin ratio (2.72 vs 1.50) ($p < 0.001$). There was no difference in inflammatory markers, interleukin-6 and C-reactive protein, between participants with insufficient and sufficient iron stores.

Table 3.2: Biochemical Markers of Iron Status and Metabolism in Iron Sufficient and Insufficient Participants			
Characteristic	Iron Sufficient ^a n = 73 (44.2%)	Iron Insufficient ^b n = 92 (55.8%)	P-value ^c
Haemoglobin ($\text{g}\cdot\text{L}^{-1}$)*	133 (12.0)	128 (13.0)	0.011
Serum Ferritin ($\mu\text{g}\cdot\text{L}^{-1}$) [†]	60.8 (38.00, 71.00)	15.8 (9.00, 23.0)	<0.001
Soluble Transferrin Receptor ($\text{mg}\cdot\text{L}^{-1}$) [‡]	2.55 (2.14, 2.69)	3.34 (3.14, 3.55)	<0.001
sTfR/Log Ferritin Ratio [†]	1.50 (1.30, 1.80)	2.72 (2.10, 3.80)	<0.001
C-Reactive Protein [†]	0.00 (0.00, 0.00)	0.00 (0.00, 3.00)	0.075
Interleukin-6 ($\text{pg}\cdot\text{mL}^{-1}$) [†]	0.95 (0.54, 1.49)	1.15 (0.62, 1.87)	0.125
Hepcidin (nM) [†]	6.62 (4.21, 11.02)	1.71 (0.59, 3.47)	<0.001
^a Serum ferritin $\geq 30\mu\text{g}\cdot\text{L}^{-1}$ and Hb $\geq 120\text{g}\cdot\text{L}^{-1}$ ^b Serum ferritin $< 30\mu\text{g}\cdot\text{L}^{-1}$ (Hb $< 120\text{g}\cdot\text{L}^{-1}$ or $\geq 120\text{g}\cdot\text{L}^{-1}$) ^c P value is for a test of differences between groups as assessed by the independent <i>t</i> -test (parametric continuous data), Mann-Whitney U test (non-parametric continuous data) * Mean $\pm$ standard deviation [‡] Geometric mean and 95% confidence interval [†] Median and 25 th , 75 th centiles			

3.3.2 Biomarkers: Comparison Between Ethnicities

Table 3.3 summarises the biomarkers, body composition and meat intake between ethnic cohorts. It presents results for those who were iron sufficient and insufficient within each ethnic group for those biomarkers that were significantly different in table 3.2. Splitting the ethnic groups further into iron sufficient/insufficient was deemed appropriate as hepcidin is non-responsive in iron sufficient individuals, and previous

research has indicated hepcidin's influence over iron biomarkers (Nemeth & Ganz, 2009).

Haemoglobin differed significantly between ethnicities for those participants who had sufficient ($p = 0.025$) and insufficient iron stores ($p < 0.001$). South Asians with sufficient iron levels had significantly lower Hb levels ($129\text{g}\cdot\text{L}^{-1}$) than NZ Europeans with sufficient iron levels ($137\text{g}\cdot\text{L}^{-1}$) ($p = 0.019$). Similarly, South Asians with insufficient iron levels had significantly lower Hb ($122\text{g}\cdot\text{L}^{-1}$) than NZ Europeans ($136\text{g}\cdot\text{L}^{-1}$) ($p < 0.001$) and those of other ethnicities ($132\text{g}\cdot\text{L}^{-1}$) ($p = 0.014$) with insufficient iron levels.

Soluble transferrin receptor differed significantly between ethnicities, however, only in individuals classified as iron insufficient ($F(2, 91) = 4.26, p = 0.017$). South Asians with insufficient iron levels had a significantly higher soluble transferrin receptor ($3.63\text{ mg}\cdot\text{L}^{-1}$) than those of other ethnicities with insufficient iron levels ($2.77\text{mg}\cdot\text{L}^{-1}$) ($p = 0.045$).

Interestingly, hepcidin only differed significantly between ethnicities for those participants who were iron sufficient ($H(2) = 7.26, p = 0.026$). South Asians with sufficient iron concentrations had a higher serum hepcidin (8.78nM) than both NZ Europeans (6.28nM) ($U = 232, p = 0.025$) and other ethnicities who were iron sufficient (4.89nM) ($U = 123, p = 0.018$).

Serum ferritin, sTfR/log ferritin ratio and C-reactive protein did not differ significantly between ethnicities, regardless of iron status.

Interleukin-6 differed significantly ($H = 59.7, p < 0.001$) with South Asians presenting with significantly higher serum interleukin-6 ($1.66\text{ pg}\cdot\text{ml}^{-1}$) than NZ Europeans ($0.63\text{ pg}\cdot\text{ml}^{-1}$) ($U = 555, p < 0.001$) as well other ethnicities ($0.80\text{pg}\cdot\text{ml}^{-1}$) ($U = 391, p < 0.001$).

There were significant differences in both BMI ($p = 0.001$) and body fat percentage ($p < 0.001$) between ethnic groups. South Asians ($26.3\text{kg}\cdot\text{m}^{-2}$) had a significantly

higher BMI than NZ Europeans ($23.2\text{kg}\cdot\text{m}^{-2}$) ($p = 0.001$). South Asians (39.1%) also had a significantly higher body fat percentage than NZ Europeans (27.4%) ($p = <0.001$) and those of other ethnicities (30.7%) ($p = <0.001$).

Meat intake differed between ethnicities ($p < 0.001$). Meat frequency was significantly lower ($p < 0.001$) in South Asians (3.5 times per week) when compared to other ethnicities (11.25 times per week) and NZ Europeans (8.33 times per week) ($p = 0.001$). This difference in meat intake could be explained by significant differences in eating pattern between ethnicities ($\chi^2(4) = 26.1, p < 0.001$). South Asians had 5.28 higher odds of being vegetarian than NZ Europeans, and 4.86 times higher odds than other ethnicities. Other ethnicities had 1.08 times higher odds of being vegetarian than NZ Europeans. Frequency of intake of other food groups did not differ significantly between ethnicities.

Table 3.3: Comparison of Iron Status and Factors that Increase the Risk of Iron Deficiency Between Different Ethnicities									
Characteristic		New Zealand European n = 62 (37.6%)		South Asian n = 70 (42.4%)		Other n =33 (20.0%)		P-value ^a	
		Sufficient	Insufficient	Sufficient	Insufficient	Sufficient	Insufficient	P-value sufficient	P-value insufficient
Iron Sufficient/Insufficient [‡]		30 (48.4)	32 (51.6)	25 (35.7)	45 (64.3)	18 (54.5)	15 (45.5)	0.141	
Haemoglobin (g·L ⁻¹)*		137 (11.0)	136 (10.0)	129 (12.0)	122 (12.0)	134 (9.00)	132 (10.0)	0.025	< 0.001
Serum Ferritin (µg·L ⁻¹) [†]		44.8 (39.0, 58.0)	16.1 (10.0, 22.1)	48.0 (32.6, 79.0)	14.0 (7.21, 22.5)	62.0 (43.7, 103)	15.0 (9.00, 24.0)	0.259	0.749
Soluble Transferrin Receptor (mg·L ⁻¹) [♢]		2.64 (2.41, 2.89)	3.13 (2.89, 3.39)	2.48 (2.27, 2.69)	3.63 (3.29, 4.01)	2.51 (2.18, 2.89)	2.97 (2.55, 3.42)	0.573	0.017
sTfR/log ferritin ratio [†]		1.60 (1.30, 1.90)	2.55 (2.05, 3.40)	1.43 (1.30, 1.70)	3.04 (2.20, 4.77)	1.40 (1.20, 1.70)	2.60 (1.80, 3.70)	0.278	0.103
Hepcidin (nM) [†]		6.28 (3.78, 9.88)	1.08 (0.54, 3.56)	8.87 (6.22, 15.1)	1.73 (0.48, 3.50)	4.89 (3.74, 8.47)	1.77 (1.16, 2.23)	0.026	0.944
C-Reactive Protein (mg·L ⁻¹) [†]		0.00 (0.00, 3.00)		0.00 (0.00, 4.00)		0.00 (0.00, 0.00)		0.099	
Interlukein-6 (pg·mL ⁻¹) [†]		0.63 (0.45, 1.01)		1.66 (1.26, 2.44)		0.80 (0.57, 1.12)		<0.001	
BMI (kg·m ²) [♢]		22.9 (22.1, 23.7)		25.7 (24.7, 27.0)		23.8 (22.0, 25.8)		0.001	
Body Fat Percentage*		27.4 (7.58)		39.1 (11.1)		30.7 (9.30)		<0.001	
Self-reported dietary patterns [‡]	Normal	54 (83.1)		36 (49.3)		31 (25.6)		<0.001	
	Vegetarian/Vegan	5 (7.7)		17 (23.3)		3 (8.6)			
	Other	6 (9.2)		20 (27.4)		1 (2.9)			
Meat (p/w) [†]		8.33 (5.75, 11.3)		3.5 (0.0, 8.67)		11.25 (7.08, 16.4)		< 0.001	

^a P value is for a test of differences between groups as assessed by one-way ANOVA (parametric continuous data), Kruskal-Wallis test (non-parametric continuous data) or chi-square test (categorical data) (adequate/expected values).

* Mean ± standard deviation

♢ Geometric mean and 95% confidence interval

† Median and 25th,75th centiles

‡ Number and % of participants

BMI: Body Mass Index

p/w: Number of times consumed per week

3.3.5 Overall Predictors of Serum Ferritin

As there was multicollinearity between ethnicity and meat intake, multiple linear regression analysis was used to identify significant predictors of Sf within each ethnicity. Predictor variables were selected by first testing each individual variable, and those with a $p < 0.200$ were then entered into the final model using the enter method. The following variables did not meet the screening criterion, so were not entered into the linear regression analysis: body fat percentage, period length, having children, contraceptive method, previous ID, blood donation, alcohol intake, dairy intake, tea & coffee intake, iron-fortified food intake, fruits & vegetable intake, grains & cereal intake and serum IL-6.

The model showed that hepcidin and frequency of meat intake accounted for 42.0% of the variation in Sf in NZ Europeans. The model also showed that hepcidin status was a stronger predictor of Sf levels than frequency of meat intake. The model showed when serum hepcidin increases by 1nM, the log of Sf will increase by 0.082 ($1.08\mu\text{g}\cdot\text{L}^{-1}$). When frequency of meat intake increased by one time per week, the log of Sf increased by 0.058 ($1.06\mu\text{g}\cdot\text{L}^{-1}$).

Meat intake was not a significant predictor in either South Asians, or those of other ethnicities. The model showed that hepcidin accounted for 56.2% of the variation in Sf for South Asian participants, and that when serum hepcidin increases by 1nM, the log of Sf will increase by 0.138 ($1.15\mu\text{g}\cdot\text{L}^{-1}$). For those of other ethnicities, hepcidin accounted for 27.0% of the variation in Sf, and when serum hepcidin increases by 1nM, the log of Sf will increase by 0.117 ($1.12\mu\text{g}\cdot\text{L}^{-1}$).

Table 3.4: Multiple Linear Regression Analysis to Identify Predictors of Iron Deficiency Stratified by Ethnicity					
NZ European	Predictor	B	95% CI for B	Standardised B	P-value
	Hepcidin (nM)	0.082	0.044, 0.121	0.454	< 0.001
	Meat Intake	0.058	0.026, 0.091	0.382	0.001
	F(2, 56) = 19.5 p = <0.001 R ² = 0.420 R ² (adj) = 0.398				
South Asian	Predictor	B	95% CI for B	Standardised B	P-value
	Hepcidin (nM)	0.138	0.107, 0.168	0.750	< 0.001
	F(1, 66) = 83.5 p = <0.001 R ² = 0.562 R ² (adj) = 0.555				
Other Ethnicities	Predictor	B	95% CI for B	Standardised B	P-value
	Hepcidin (nM)	0.117	0.045, 0.189	0.519	0.002
	F(1, 31) = 11.1 p = 0.002 R ² = 0.270 R ² (adj) = 0.245				

3.4 Discussion

To the best of our knowledge, we are the first to analyse iron and hepcidin status in non-athletic NZ females, in a cohort with a high representation of South Asian participants, an ethnic group that have been shown to have a high incidence of ID (Parackal et al., 2015).

3.4.1 Prevalence of Iron Deficiency in Auckland Premenopausal Females

The overall prevalence of ID in our cohort was high (55.8%) as compared to previous research in NZ pre-menopausal females. A study undertaken in 18-44 year old females in Auckland found 18.7% of participants had suboptimal iron stores ($Sf < 20\mu\text{g}\cdot\text{L}^{-1}$) (Beck et al., 2014a). Similarly, a study in 18-40 year old premenopausal females in Dunedin, indicated that 23% had mild ID ($Sf < 20\mu\text{g}\cdot\text{L}^{-1}$) (Heath et al., 2001). The NZ National Nutrition Survey indicated the highest prevalence of ID ($Sf < 12\mu\text{g}\cdot\text{L}^{-1}$) was found in women aged 31-50 years at 12.1%. Although we used a higher cut off for ID ($Sf < 30\mu\text{g}\cdot\text{L}^{-1}$) than previous studies, when we used a similar cut off to previous research in NZ, of $Sf < 20\mu\text{g}\cdot\text{L}^{-1}$, the rates of ID (43.0%) of the current study were still comparatively high.

One possible explanation for the high rates of ID in our study was the high rates of vegetarianism amongst participants. Our study had 30.3% of all participants excluding certain types of meat (e.g. being pescatarian), and 14.5% of all participants were either vegetarian or vegan. This is slightly higher than rates found in Dunedin premenopausal females, which found that 22% of participants avoided red meat (Heath et al., 2001). Our results are also higher than rates from the National Nutrition Survey, which showed 7.7% of 19-30 year old and 6.4% of 31-50 year old females had not consumed

red meat within the past four months (University of Otago & Ministry of Health, 2011). A nationwide survey found 11.3% of females followed an 'always or mostly vegetarian' diet (Roy Morgan Research, 2016). More recent findings estimate that one in ten (10%) New Zealanders follow a vegetarian or mostly meat free diet (Woolf, 2019). The increase in vegetarianism appears to be a generational trend, with the most likely age groups to be vegetarian in NZ being those aged 25-34 (13.8%) closely followed by 14-24 year olds (13.3%) (Roy Morgan Research, 2016)

Another potential explanation for the high rates of ID was our high representation of South Asian participants (42.4%). Our study had 37.5% NZ Europeans as compared to 95% in Heath et al. (2001), and 70% in Beck et al. (2014a). In Auckland, Asian premenopausal females, the majority of which were either Chinese (41.8%) or Indian (29.1%), had a five times higher odds of having ID than NZ Europeans (Beck et al., 2014a). Furthermore, in the most recent NZ national nutrition survey, South Asians were more likely than any other ethnic group to have low iron stores ($Sf \geq 12\mu g \cdot L^{-1}$) (Parackal et al., 2015).

3.4.2 Ethnic Differences

Although not statistically significant, our study showed that South Asians had 1.68 higher odds of having ID than NZ Europeans and 2.16 higher odds of having ID than those of other ethnicities. This could potentially be explained by ethnic differences in body composition and inflammation leading to variations in hepcidin levels.

South Asians had a significantly higher body mass index (BMI) ($26.3 kg \cdot m^{-2}$) than NZ Europeans ($23.2 kg \cdot m^{-2}$). Although the difference in BMI was relatively small ($3.1 kg \cdot m^{-2}$), there was a large difference in body fat percentage between the two ethnicities

(39.1% vs 27.4% for South Asians and NZ Europeans respectively). Similarly, South Asians also had a significantly higher body fat percentage than those of other ethnicities (30.7%).

Differences in body composition between ethnicities has also been demonstrated in previous research (Krishnaveni et al., 2005; Rush, Freitas, & Plank, 2009; Wulan, Westerterp, & Plasqui, 2010). For the same BMI, age and gender, Asians have been shown to have a higher body fat percentage than Caucasians, with South Asians having the highest body fat percentage among Asian cohorts (Wulan et al., 2010). It is estimated that Indian women in NZ have around a 8% higher body fat percentage than Caucasians (Rush et al., 2007). Some suggested explanations for these discrepancies in body composition include a combination of environmental factors (such as diet and exercise), genetics and even intrauterine development; as a 'thin-fat' phenotype has been found to be present from childhood in Asian Indian children (Krishnaveni et al., 2005; Wulan et al., 2010).

The higher body fat percentage found in South Asians could be an underlying explanation for why South Asians had significantly higher serum Interleukin-6 (IL-6) levels ($1.66\text{pg}\cdot\text{mL}^{-1}$) than both NZ Europeans ($0.63\text{pg}\cdot\text{mL}^{-1}$) and other ethnicities ($0.80\text{pg}\cdot\text{mL}^{-1}$).

Previous studies in South Asian females have also shown significantly higher IL-6 ($1.94\text{mg}\cdot\text{L}^{-1}$) than Europeans ($1.51\text{mg}\cdot\text{L}^{-1}$) (Peters, Ghouri, McKeigue, Forouhi, & Sattar, 2013). It is estimated that 15-35% of IL-6 found in the blood originates from adipose tissue, and higher levels of IL-6 have previously been observed in individuals with obesity (Eder, Baffy, Falus, & Fulop, 2009). This research indicates that body composition likely plays a role in IL-6 expression.

These higher IL-6 levels could explain why South Asians had a higher serum hepcidin (8.78nM) than both NZ Europeans (6.28nM) and those of other ethnicities (4.89nM). Interestingly, this was only significant for participants who were iron sufficient. Iron status has been identified as the strongest predictor of hepcidin expression, therefore, in those with low Sf, there is a negative feedback mechanism on hepcidin expression to enable effective iron absorption and utilisation within the body. Thus providing a probable explanation for lack of significant difference in hepcidin for those that are iron insufficient (Ganz, 2011).

A positive correlation between BMI and serum hepcidin has been demonstrated in Dutch females (Galesloot et al., 2011). Conversely, a decrease in serum hepcidin concentration has been observed in bariatric surgery patients in response to weight loss (Tussing-Humphreys, Nemeth, Fantuzzi, Freels, Holterman, et al., 2010). The high hepcidin levels in South Asian participants, could be due to high body fat percentage, leading to increased inflammation. However, research in 18 to 25-year-old overweight Australian females ($BMI \geq 27.5 \text{ kg} \cdot \text{m}^{-2}$) has suggested that although increasing obesity was associated with minor disturbances in iron metabolism (lower serum iron, lower transferrin saturation, higher Sf and higher CRP), the effect of inflammation and hepcidin alone was not enough to induce significant changes to iron status (Cheng et al., 2013).

Therefore, we need to consider other factors that are likely to influence iron status. In our study, South Asians had 5.28 higher odds of being vegetarian than NZ Europeans, and 4.86 higher odds than those of other ethnicities. As a result, South Asian participants consumed meat less frequently (3.5 times per week) than NZ Europeans (8.33 times per week) and those of other ethnicities (11.25 times per week). This aligns with findings from the most recent national nutrition survey, with South Asian females

being the most likely ethnic group to have never eaten chicken, red meat and processed meat (Parackal et al., 2015).

A low dietary haem intake has consistently been identified as a risk factor for ID, likely due to haem iron being more easily absorbed than non-haem iron (Craig, 2010; Heath et al., 2001; Hunt, 2003; Parackal et al., 2015). In Dunedin pre-menopausal females, consuming meat/fish/poultry intake > 79g per day was found to be protective against mild ID ($Sf < 20\mu g \cdot L^{-1}$) (Heath et al., 2001). While a study in Auckland premenopausal females with children found that those following a meat and vegetable dietary pattern had a 0.21 reduced odds of having ID ($Sf < 20\mu g \cdot L^{-1}$) (Beck et al., 2014a).

3.4.3 Overall Predictors of Iron Deficiency

Using multiple linear regression analysis, our final model showed that hepcidin and frequency of meat intake were the only significant predictors of Sf in our study population. Meat intake was only correlated with Sf in NZ European participants, whereas hepcidin was a predictor of Sf in all ethnicities.

A positive correlation between serum ferritin and serum hepcidin was also found in Dutch males and females, with a 1% increase in serum ferritin associated with a 0.81%-0.85% increase in serum hepcidin (Galesloot et al., 2011). A study in Australian premenopausal females demonstrated that a 10% increase in red and white meat intake corresponded with a 0.9% increase in Sf, however, they too found hepcidin levels were a stronger predictor of iron stores (Lim et al., 2016), thus reinforcing the findings from our study.

The high levels of hepcidin in iron sufficient South Asians could also explain why this ethnic group had a higher rate of ID (although not significant). Higher hepcidin concentrations causes the lysis of the iron export channels on cell surfaces (ferroportin), disrupting the flow of iron into the plasma. In instances of elevated hepcidin activity iron is sequestered in enterocytes, thereby lowering the amount of dietary iron absorbed by the gut (Ganz, 2011; Leong & Lönnerdal, 2004; Nemeth, Rivera, et al., 2004). Hepcidin's effect on regulating dietary haem absorption is still an emerging area of research, however, in rats injected with hepcidin, significantly reduced mucosal iron uptake was observed (Laftah et al., 2004). Therefore South Asian participants may have altered iron absorption and utilisation due to elevated hepcidin levels and in combination with a low meat intake may have an increased risk of developing ID.

3.5 Conclusion

Our cohort had a high rate of ID compared to previous research undertaken in NZ premenopausal females. This is likely explained by a high representation of South Asian participants as well as the high rates of vegetarianism in our cohort, a rising trend amongst young females.

South Asians were more likely to have a higher BMI, body fat percentage and IL-6 as compared to NZ Europeans and those of other ethnicities. These factors could be possible explanations for iron sufficient South Asians having a higher serum hepcidin concentration than NZ Europeans and those of other ethnicities. This proposes South Asians as an at-risk group for ID development.

Hepcidin and frequency of meat intake were significant predictors of ID in NZ Europeans, with hepcidin a stronger predictor than the latter. Health professionals should continue to be vigilant around the rise in meat restriction as a risk factor for ID, particularly in premenopausal females as this group is among the most likely to restrict their meat intake. Those of South Asian ethnicities should also be highlighted as an at-risk group, and iron status of these individuals should be monitored regularly. The strong correlation of hepcidin with Sf aligns with previous research and continues to highlight hepcidin as an emerging biomarker for identifying ID.

Chapter 4: Conclusion and Recommendations

4.1 Achievement of Aims and Hypotheses

The overall aim of the research was to determine current iron and hepcidin status as well as risk factors for iron deficiency (ID) in premenopausal females living in Auckland, New Zealand (NZ).

The comparatively high rate (55.8%) of ID found in our study suggests that ID continues to be a prevalent health issue amongst premenopausal females in NZ. A low meat intake amongst participants is a potential contributing factor to this high rate of ID, this aligns with the increasing rates of vegetarianism amongst NZ females. Frequency of meat intake was positively correlated with serum ferritin (Sf) in our study. This aligns with previous research which has indicated that a meat/poultry/fish intake great than 79g per day was protective against ID (Heath et al., 2001; Roy Morgan Research, 2016).

Hepcidin levels were consistent with findings from research undertaken in other western countries. The normal range for serum hepcidin has been previously been reported as 6.09nM – 102nM in healthy Italian and American females (Ganz et al., 2008). In Australia, female adolescents with a BMI $\geq 27.5\text{kg}\cdot\text{m}^{-2}$ had a median hepcidin concentration of 2.29nM (Cheng et al., 2013). The overall median serum hepcidin level of our cohort was slightly lower (3.54nM) when compared to Dutch premenopausal females (4.1nM) (Galesloot et al., 2011). This is likely due to many participants in our study presenting with insufficient iron stores (median Sf of $26.23\mu\text{g}\cdot\text{L}^{-1}$), as compared to the Dutch study which had a median Sf of $81.6\mu\text{g}\cdot\text{L}^{-1}$. The low hepcidin levels found in participants with ID reinforces the evolutionary feedback mechanism of iron status

on hepcidin. Low iron status results in lower levels of hepcidin, to allow for increased iron absorption and utilisation (Nemeth, Rivera, et al., 2004).

The percentage of participants with ID was high amongst all ethnicities: South Asians (64.3%), NZ Europeans (50.0%) and those of other ethnicities (45.5%), however, the prevalence of ID did not differ significantly between ethnic groups. This is in contrast to other findings in NZ which has suggested that Asians, Māori and Pacifica have higher rates of ID as compared to Europeans (Beck et al., 2014a; Wall et al., 2009). South Asian males were more likely to have poor iron status than South East Asians and Europeans in the 2008/09 NZ National Nutrition Survey, however it was found that this difference was not significant in females (Parackal et al., 2015). Potential explanation for the significant difference in iron status found in males but not females is that ID rates in females are high among all ethnicities including NZ Europeans (Parackal et al., 2015). Females lose iron through menstruation, whereas the cause of ID in males is more likely due to low dietary iron intake.

Secondly, we hypothesised that South Asian females would have higher hepcidin levels as compared to European females. This was true for participants that were iron sufficient, as South Asian participants had significantly higher median hepcidin levels (8.87nM) as compared to NZ Europeans (6.28nM) and those of other ethnicities (4.89nM). Interestingly, the findings of higher hepcidin levels did not translate into significantly different Sf between ethnicities. However, further research into the iron status of South Asians in NZ is warranted, as South Asian participants who were iron sufficient had lower Hb and higher hepcidin levels.

These differences in Hb and hepcidin could be linked to the higher body fat percentage and IL-6 found in South Asian participants in this study. Obesity is associated with a

low-grade inflammatory state as adipose tissue stimulates production of pro-inflammatory cytokines such as IL-6 (Aigner et al., 2014). Previous research has demonstrated that in obese individuals, this inflammatory state is linked to higher hepcidin levels (Tussing-Humphreys, Nemeth, Fantuzzi, Freels, Guzman, et al., 2010). Higher levels of inflammation (as measured by C-reactive protein and IL-6) have previously been demonstrated in South Asians, as well as in other ethnic groups, such as African Americans and Hispanics (Nazmi & Victora, 2007; Paalani, Lee, Haddad, & Tonstad, 2011).

Obesity induced inflammation is an emerging risk factor for ID (Aigner et al., 2014). Interleukin-6 has been shown to directly stimulate production of hepcidin in hepatocytes (Nemeth et al., 2003). This increase in hepcidin, then leads to the lysis of ferroportin (the sole iron exporter), which in turn causes the sequestration of iron within intracellular compartments, leading to the decline of iron in the extracellular fluid (Drakesmith & Prentice, 2012). Consequently, functional iron utilisation such as Hb within red blood cells begin to drop (Ganz, 2007). This altered iron metabolism is also seen in anaemia of chronic disease, where Sf increases and Hb decreases (Drakesmith & Prentice, 2012). In our study, Hb levels in South Asians were significantly lower compared with NZ Europeans and those of other ethnicities, which potentially also highlights this altered metabolism in our participants.

This also raises the question if the relationship between body composition, inflammation and increased hepcidin levels are also found in other populations known to have high levels of obesity, such as Māori and Pacifica (Ministry of Health, 2017). A higher IL-6 has previously been reported in Māori as compared to those of other ethnicities (Cervantes, Singh, Pendharkar, Bharmal, & Petrov, 2018). Higher rates of ID in Māori and Pacifica as compared to Europeans have also been identified in NZ

children (Wall et al., 2009). However, this hypothesis remains to be investigated within NZ.

The combination of body composition and inflammation may also be linked to other serious health outcomes such as coronary heart disease and insulin resistance. Interleukin-6 is known for its role in the hepatic acute phase response, regulating the hepatic synthesis of CRP, thus elevated IL-6 may increase CRP levels (Yudkin, Kumari, Humphries, & Mohamed-Ali, 2000). This inflammatory response has previously been associated with atherosclerotic plaque formation, increasing risk of coronary disease (Yudkin et al., 2000). This is particularly relevant for South Asian females, as they have the second highest rates of coronary heart disease of any ethnicity in NZ (Thornley et al., 2011). A high IL-6 is also thought to stimulate the hypothalamic–pituitary–adrenal axis, which has been associated with insulin resistance syndrome, characterised by impaired glucose tolerance, high plasma triglycerides, low high density lipoprotein cholesterol, and high blood pressure (Yudkin et al., 2000). Therefore, a high IL-6 could be a contributing factor as to why both coronary heart disease and insulin resistance are commonly associated with those of South Asian ethnicity (McKeigue, Shah, & Marmot, 1991).

Iron deficiency is often linked as a co-morbidity of cardiovascular disease, with up to one third of patients with heart failure also presenting with anaemia (Von Haehling, Jankowska, Van Veldhuisen, Ponikowski, & Anker, 2015). In ID, hepcidin levels are decreased, resulting in the higher uptake of iron of macrophages (Sullivan, 2007). This excess of iron trapped within macrophages in atherosclerotic plaques can result in the generation of harmful reactive oxidation species, leading to foam cell formation and increasing risk of heart attack and stroke (Emerit et al., 2001; Sullivan, 2007). Furthermore, anaemia has been associated with increased mortality in those with

heart failure (Lapice, Masulli, & Vaccaro, 2013). It has been proposed that anaemia results in reduced oxygen delivery to cardiac tissue, which in turn causes increased heart rate and stroke volume, which may lead to ischemia and myocardial cell death (Lapice et al., 2013). This research suggests a possible interplay between hepcidin levels, ID and increased risk of cardiovascular disease that requires future research. As our study demonstrated high rates of ID, this could potentially be a co-morbidity of NZ's high rates of CVD, affecting one in 20 adults, and being the highest cause of mortality in females (Heart Foundation, 2019).

We hypothesised that a high BMI ($\geq 30\text{kg}\cdot\text{m}^{-2}$), low dietary haem intake, high levels of blood loss and lower basal hepcidin concentrations would be determinants of ID in premenopausal females. However, only hepcidin and frequency of meat intake were positively correlated with Sf in our cohort, with hepcidin being a stronger predictor of Sf than meat. This research reinforces the role of hepcidin in iron absorption and movement within the body. A positive correlation between hepcidin and Sf has also been previously identified in Dutch and Australian females, and our study confirms this relationship in NZ females (Galesloot et al., 2011; Lim et al., 2016). Meat intake has also previously been identified as a predictor of iron status in NZ females, with a meat/fish/poultry intake $> 79\text{g}\cdot\text{day}^{-1}$ associated with lower risk of mild ID (Sf $< 20\mu\text{g}\cdot\text{L}^{-1}$).

This positive correlation highlights hepcidin as an emerging biomarker to identify those with ID, as has been previously investigated in Australian blood donors (Pasricha et al., 2011). Hepcidin was positively correlated with Sf (which aligns with the findings from the current study) and negatively correlated with both STfR and the STfR index (Pasricha et al., 2011). The study suggested that a serum hepcidin cut-off of $< 2.87\text{nM}$ identified the highest proportion of participants with ID (86.2%), while a cut-off of $<$

6.45nM had the highest sensitivity and specificity for detecting ID for that population (Pasricha et al., 2011). This highlights the potential for health professionals to identify those who would benefit the most from iron supplementation, as those with low hepcidin levels will likely have the best iron uptake from enterocytes (Young et al., 2009). Using hepcidin as a predictor for those who may potentially respond to oral iron supplementation has also been investigated in patients with IDA (Bregman, Morris, Koch, He, & Goodnough, 2013). Those who responded to oral iron therapy (Hb increased by $\geq 10\text{g}\cdot\text{L}^{-1}$ within 14 days) had a significantly lower hepcidin level (4.05nM) than those who did not respond (13.8nM) (Bregman et al., 2013). The study found that a hepcidin cut-off of $< 7.17\text{nM}$ correctly predicted 81.6% of non-respondents to oral iron therapy (Bregman et al., 2013). However, as it is known that there is high intra-individual variability in hepcidin levels, more research is required to establish a hepcidin cut off to identify ID (Kroot et al., 2012).

4.2 Strengths

To our knowledge, this study was the first in NZ to examine hepcidin levels in non-athletic premenopausal females, and its relationship with iron status, inflammation and body composition. It was also unique in that it had strong representation of South Asian participants, as previous research has had high representation of NZ Europeans, but not other ethnicities (Beck et al., 2014a; Heath et al., 2001).

One of the study's strengths was its focus on hepcidin, and additional emerging factors that are potentially associated with high hepcidin concentrations. In particular, body composition and interleukin-6 (a marker of inflammation) has not been previously explored in relation to iron status in NZ females.

A strength of our analysis was using a higher cut off to identify ID ($Sf < 30\mu\text{g}\cdot\text{L}^{-1}$), than what was previously been used in research, which typically has been $12\text{-}20\mu\text{g}\cdot\text{L}^{-1}$. Research has suggested that using this higher cut-off is a more sensitive marker for identifying ID than either $12\mu\text{g}\cdot\text{L}^{-1}$ or $20\mu\text{g}\cdot\text{L}^{-1}$ (Clénin et al., 2015; Mast et al., 1994). Therefore, our research potentially identified individuals with stage 1 ID, which would not have been identified in previous studies.

Finally, when we interpreted Sf levels, we adjusted for inflammation based on CRP for those participants with a $\text{CRP} > 5\text{mg}\cdot\text{L}^{-1}$. This gave Sf readings that were more reflective of true iron status, as Sf is an acute phase reactant known to be falsely elevated during periods of inflammation (Bui et al., 2012).

4.3 Limitations

To assess dietary intake of foods, we used a food frequency questionnaire (FFQ), which has been previously validated in NZ females to assess iron-related dietary patterns (Beck et al., 2014a). A limitation of this FFQs is that it assessed the frequency of food intake, but not timing of consumption. For example, calcium found in dairy foods is known to inhibit non-haem iron absorption, but only when the two nutrients are co-ingested (Hallberg et al., 1991). We did not collect data on timing of food consumption, and the FFQ may not have been sensitive enough to identify if specific food groups may have influenced iron status. Further, this FFQ did not quantify the amount of foods eaten, so a higher frequency of consumption of a food may not necessarily translate into higher amounts of a food ingested. Also, individuals are more likely to over-report with FFQs, especially when there are many food options presented in a questionnaire, such as with the fruit and vegetables section. A final limitation of

FFQs is that they are dependent on accurate recall of the participant, and therefore can be less reliable than other dietary assessment methods (Gibson, 2005).

Another limitation is that timing of hepcidin was not accounted for, as previous research has suggested there is diurnal variation in hepcidin, with measures taken at 12pm and 8pm significantly higher than samples taken from fasting subjects at 8am (Ganz et al., 2008). The researchers attempted to take all samples before 12pm and no later than 4pm. This may have resulted in increased variation in hepcidin samples within the study, however, this would only be between those participants with sufficient iron levels, as hepcidin levels are suppressed during ID (Ganz et al., 2008), as reported in our study.

Finally, body composition was measured using bioelectrical impedance analysis (BIA). BIA is known to be affected by hydration status, consumption of food and recent exercise which was not controlled for (Kushner, Gudivaka, & Schoeller, 1996). Therefore, this method is considered not as robust for measuring body composition as others, for example dual-energy X-ray absorptiometry or air displacement methods (such as BodPod) (Lee & Gallagher, 2008). Additionally, BIA equations are largely based on Caucasian populations, and research has suggested a need for ethnic specific equations, particularly in the South Asian ethnicity (Rush, Chandu, & Plank, 2006).

4.4 Recommendations and Future Directions for Research

Given the high rates of iron insufficient participants found in our study, it is evident that ID is still a prevalent issue among NZ females. Future research should be directed at

particular groups that are at risk, as well as focusing on using hepcidin to better identify and treat ID.

- Health professionals should focus on South Asians as an at-risk group for ID, as although not significant, this ethnic group had a higher percentage of ID, which is potentially linked to higher levels of body fat, inflammation and hepcidin. They are of additional interest because they are a group that are likely to have a low dietary haem intake.
- With the trend of vegetarianism amongst young females in NZ, an already at-risk group for ID; those females that restrict their meat intake should be regularly monitored for iron status as it is likely that they are not consuming sufficient bioavailable iron to maintain healthy iron stores.
- Continue to investigate the relationship between body composition, inflammation and iron status. There needs to be a specific focus in certain ethnicities that are known to have high body fat percentages, such as South Asians, Māori and Pacifica.
- The positive correlation observed between hepcidin and Sf proposes hepcidin as an emerging biomarker for ID. Future research should investigate how hepcidin could be used to identify ID early, to allow for optimal intervention and treatment.
- Research exploring how hepcidin responds to dietary iron intake is required, to see if there is a way in which health professionals such as dietitians can advise consumption of iron rich foods when hepcidin is low, to support maximal iron uptake from enterocytes.

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Appendix A: Poster for Recruitment

vIVID study

The School of Sport, Exercise and Nutrition is doing a study investigating the relationship between iron and vitamin D deficiency. We will be investigating the hormones, genetics, iron and vitamin D status in premenopausal females in Auckland.



Why are we doing the research?

12 %

Of women are diagnosed with iron deficiency in New Zealand

NZ females were

6 times

more likely to suffer from iron deficiency compared to males

1 in 4

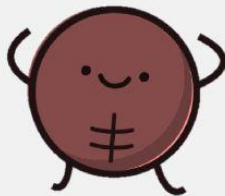
New Zealand Adults had below recommend levels of vitamin D

Who are we looking for?

- Females
- Aged 18-40 years
- Able to communicate in English
- Have not consumed supplements with calcium, vitamin D, vitamin C or iron for 3 months
- Have not donated blood in the past 6 months
- Are not pregnant or currently breastfeeding
- Have no known health conditions that may affect iron status (e.g. inflammatory bowel disease, celiac disease, history of gastric ulcers.)

What will happen?

- We will ask you to complete a screening questionnaire to check your eligibility
- Sign a consent form
- Complete a body composition measurement using a bioelectrical impedance analysis machine (BIA)
- Have your blood pressure measured
- Provide a blood sample. This will be with a trained phlebotomist, and around 2 tsp will be taken.
- Fill in a questionnaire about food, beliefs, physical activity and general health



If you are interested in the study, or would like further details please contact Kimberley Lim MSc student in Nutrition and Dietetics

You can also contact the lead researcher for this study, Dr Claire Badenhurst Lecturer School of Sport, Exercise and Nutrition.

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☎ (09) 414 0800 ext 43410

Appendix B: Information Sheet for Recruitment

Hepcidin, Iron and Vitamin D status in New Zealand Females

Information Sheet

Introduction

We would like to invite you to participate in this study investigating iron and vitamin D status. The purpose of this research is to learn more about iron and vitamin D deficiency in women living in Auckland, New Zealand, and identify risk factors, metabolic biomarkers and genetic factors associated with the development of these nutrient deficiencies. This study is being conducted by a group of researchers at Massey University, Auckland New Zealand.

Please read the Information Sheet carefully before deciding whether to participate.

The details of the researchers for this study are:

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Why are we doing this research?

Within New Zealand approximately 12% of premenopausal females are diagnosed with iron deficiency. Changes in season and lifestyle factors (e.g. clothing, food choice) may be contributing to the development of these nutrient deficiencies. If left untreated these deficiencies can develop into chronic health conditions including poor muscle strength and bone health, poor immune function, type II diabetes and cardiovascular disease. This study will aim to assess the current relationship between hormones, genetics, iron and vitamin D status in women residing in Auckland, New Zealand.

Who are we looking for?

We are looking for 235 females to participate in this study. To take part in the study you should:

- Be between 18-40 years of age
- Be proficient in English (able to speak and read English)
- Not consumed iron, vitamin C or calcium supplements (or supplements that contain these nutrients) in the previous 3 months.
- Not have donated blood for 6 months prior to the study.
- Not be pregnant or currently breastfeeding.
- Have no known health problems likely to influence iron status including inflammatory bowel disease, celiac disease, history of gastric ulcers, disorders of red blood cells, menorrhagia, piles, blood in the urine or malaria.

What will happen?

If you decide to take part in this study after you have read and had time to consider the information contained in this information sheet, you will be asked to complete a short screening questionnaire to ensure that you meet the study criteria. If you meet the inclusion criteria you will be invited to take part in the study. If you are willing to participate, you will be invited to attend a ***single*** session at a pre-allocated time, lasting ***45-60 minutes*** at the School of Sport, Exercise and Nutrition laboratory. The laboratory is located in Building 60, Massey University Oteha Rohe Campus, Albany Highway, Albany, Auckland, New Zealand. Data collection may also occur at a Massey researcher approved community centre. Centres will be required to have private rooms available for blood and body composition assessments to respect the privacy of all participants.

- At this session you will be asked to sign a consent form for participating in the study and you will have the opportunity to ask any questions about the study.
- You will be reimbursed for your travel costs to Albany.
- You will be seated in a quiet room and asked to complete a series of online questionnaires in private.
- The information that will be collected from these questionnaires will include; age, ethnicity, socioeconomic status, medical history (including medication and/or supplement use), skin colour, sun exposure, physical activity level, dietary iron intake, alcohol and smoking status and blood loss. In some cases, we may ask you to complete the questionnaire prior to coming in for data collection.
- In a separate private room your height will be measured using stadiometer. In the same private room your body composition and body weight (lean body mass and percentage body fat) will be measured using the bioelectrical impedance analysis machine (BIA). The BIA measures body composition and percentage of body fat by running a very low level of electrical current through electrodes attached to hands, feet. You will not feel this current. Testing is highly accurate, safe, and quick, completed in less than 5 minutes. Only yourself and a female researcher will be allowed in this room. If you have a pacemaker and/or Implantable Cardioverter Defibrillators then your weight will be collected by a standard scale only.

- In another private room blood pressure will be collected using a sphygmomanometer; an inflatable cuff will be placed around the top of your arm and will be inflated. This may be slightly uncomfortable for you but will not last for a long period of time (~5 seconds). The cuff will then be deflated and your blood pressure will be recorded.
- A blood sample will be taken by a trained female phlebotomist (Dr Claire Badenhorst). The total amount taken will be ~20 ml, equivalent to 4 x teaspoons. We will be measuring iron and vitamin D levels, hepcidin and inflammatory markers (such as C-RP, IL-6) in this blood sample.
- Participants will be provided with water, tea and or coffee and a small snack **following** blood sample collection.



Measurement of blood pressure (right) and body composition via BIA (left)

Data Management

The data will be used only for the purposes of this project and no individual will be identified. Only the investigators and administrators of the study will have access to personal information and this will be kept secure and strictly confidential. Participants will be identified only by a study identification number. Results of this project may be published or presented at conferences or seminars and no individual will be able to be identified.

At the end of this study the list of participants and their study identification number will be disposed of. Any raw data on which the results of the project depend will be retained in secure storage for 10 years, after which it will be destroyed.

A summary of the project findings will be available to all study participants. All participants will be sent this information via email or a personal letter.

Who is funding the research?

The research is funded by Massey University Research Fund, College of Health

Participants Rights:

You are under no obligation to accept this invitation. Should you choose to participate, you have the right to:

- Decline to answer any particular question
- Withdraw from the study at any time, even after signing a consent form (if you choose to withdraw you cannot withdraw your data from the analysis after the data collection has been completed)
- Ask any questions about the study at any time during participation
- Provide information on the understanding that your name will not be used unless you give permission to the researcher
- Be given access to a summary of the project findings when it is concluded

What are the benefits and risks of taking part in this study?

There will be no cost to you for any of the procedures or assessments taken as part of this study. You will receive information on your body measurements, blood pressure at the end of the testing session. Your iron and vitamin D status will be sent out to you (email or mail) at the completion of the study. You will also receive a brief report summarising the main findings of the project via mail or email. You will be provided with a petrol voucher for completing the study on the Albany Campus in Auckland.

The principle benefit of taking part in this study is that you will contribute to a novel area of research and assist us in increasing our understanding of iron and vitamin D deficiency in premenopausal females.

Some people may have a fear of having a blood sample taken or experience discomfort when the blood samples are taken. Occasionally a slight bruising will result. The bruising usually disappears within a day or two. Blood samples will be taken by a trained phlebotomist. There may be social or cultural discomfort from having body composition measurements done or a blood sample taken, however, privacy will be ensured, and you will be treated with respect. You may also be accompanied by a support person if required.

There are no personal risks to your health, but the blood tests could potentially identify the need for follow-up with your health care provider.

Support Processes

If we find that your blood results are outside normal parameters we will discuss with you what action should be taken. You will be advised to talk to your medical practitioner or we can send your results directly to them. If as a result of this you require additional blood tests you may be liable for any costs. You will be able to book into the nutrition clinic following the study to obtain dietary advice from a registered dietitian if desired, however, usual clinic fees will apply.

Project Contacts:

If you have any questions regarding this study, please do not hesitate to contact either of the following people for assistance:

Principal Researcher:

Dr Claire Badenhorst (School of Sport, Exercise and Nutrition)

C.Badenhorst@massey.ac.nz

(09)414 0800

Kimberley Lim

K.Lim@massey.ac.nz



Associated Researchers:

A/Prof Pamela von Hurst

P.R.vonHurst@massey.ac.nz

(09)213 6657

Dr Kathryn Beck

K.L.Beck@massey.ac.nz

(09)213 6662

A/Prof Peter Peeling

P.Peeling@uwa.edu.au

Ethics Committee Approval Statement

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 18/12. If you have any concerns about the conduct of this research, please contact Dr Lesley Batten, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 356 9099 x 85094, email humanethicsoutha@massey.ac.nz.

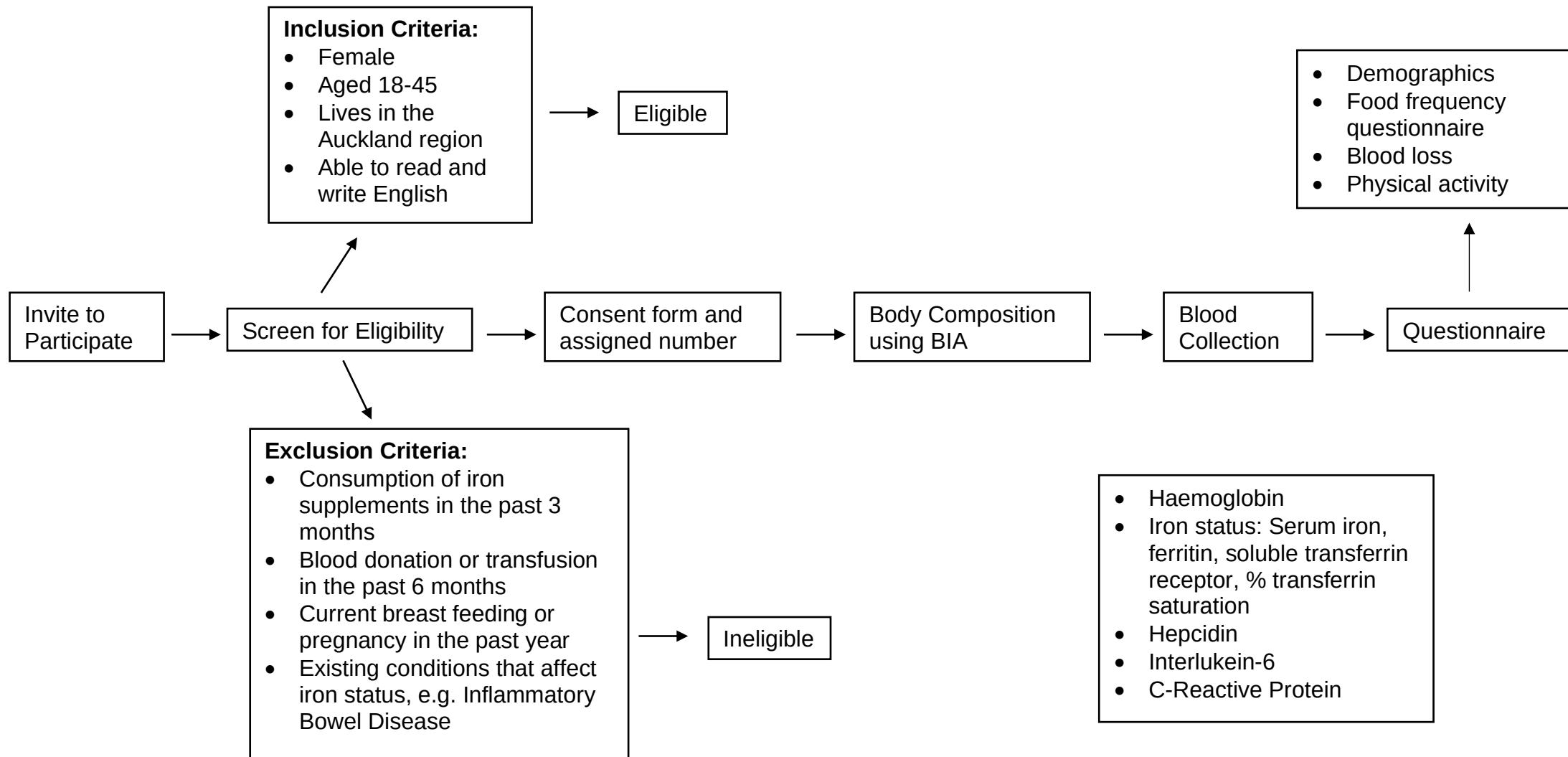
Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this incurred as a result of physical injury.

If your ACC claim is not accepted, you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

Thank you for considering participation in this study

Appendix C: Overview of Study Procedure



Appendix D: InBody230 Standard Operating Procedures



Purpose:

The purpose of the Bioelectrical Impedance Analyser (BIA) is to measure a participant's body composition, specifically their body fat.

Advantages: The advantages of using this particular BIA, is that it is easy to use, compactible, and can fold away. It also has an advantage as it is simple and quick to perform.

Disadvantages: Other equipment such as DEXA will give more accurate results as these results are not as accurate. It also cannot be taken outside in the field, as it needs to be plugged in to a power source and is not waterproof.

Method:

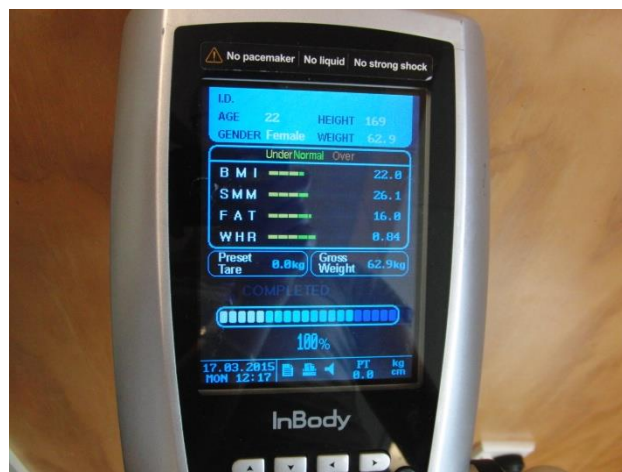
1. Switch on the machine from the back (on/off switch).
2. Wait until the screen starts up.



3. Stand on the electrodes, bare feet only (see step 9 picture).
4. Wait until the weight (kg) calculates.
5. Enter ID number.
6. Then enter your age and height (cm) into the machine.
7. Enter your gender by pressing the male/female button.
8. Once this information has been entered you then press enter.
9. Next step you must hold rods with thumb on the electrodes.



10. Stand with arms away from body and wait until the machine is 100% calibrated.



11. Now you can take your hands off the rods but feet must stay on the machine.
12. Take down your recordings of your proposed BMI (Body Mass Index), SMM (Skeletal Muscle Mass), FAT, WHR (Weight to height ratio) and Body mass (Weight in kg).
13. Now you can come off the machine and it will be ready for the next person.

Appendix E: Obtaining a Venous Blood Sample

Background

The purpose of obtaining venous blood samples in a safe and effective manner to assist in the diagnosis and ongoing management of an adult patient.

Scope

Applies to all medical practitioners, staff, who perform peripheral intravenous cannulation on trial participants of adult age.

Authorities and Responsibilities

It is expected that all staff performing venipuncture should have undertaken suitable training and have been evaluated as competent to perform this procedure by a registered health professional. MidCentral Health have trained HN&H staff.

At IFNHH, the laboratory clinician, laboratory manager and associated research scientists who hold certification in peripheral intravenous cannulation may perform this procedure. From time to time, qualified nursing staff and phlebotomists will be contracted to perform this procedure in trial participants.

Each trial participant must be assessed prior to venipuncture. If the venipuncture is judged as being too complex for the level of skill of the person performing the procedure, then the patient must be referred to a more experienced health professional,.

The laboratory's scientific staff are responsible for ordering the blood tests required and which are according to the parameters of the particular study being conducted, and in accordance with MUHEC.

Prerequisites

The following key points must be considered prior to venipuncture:

Vigorous hand exercise or "pumping" by the trial participant in order to distend the vein can cause the test results to be inaccurate and therefore must be avoided. Having the trial participant make a fist is adequate to achieve this result.

If the tourniquet is left on for more than one minute, the results of some tests may be abnormally high. If there is difficulty or delay in drawing blood, the tourniquet should be loosened for approximately a minute, and then tightened again before entering the vein.

Careful assessment of the trial participant should be made prior to venipuncture to identify whether the proposed site is suitable. Sites that should not be used include:

- Veins which have extensive scarring or a haematoma present.
- If a patient has lymphedema, the affected limb should not be used for venipuncture as the lymphedema could be exacerbated.

Procedure

Equipment

- Vacutainer
- Size 20 or 21g vacutainer needle OR Syringe with 20g or 21g needle OR size 20/21g

- Winged infusion device.
- Sharps container
- Blood tube(s)
- Tourniquet
- Alcohol swabs
- Non-sterile gloves
- Gauze swabs
- Adhesive plaster dressing i.e. Airstrip.
- 18 gauge needle (if syringe and needle method is used).

Health and Safety:

Staff:

- 1) Apply standard precautions when dealing with human blood samples. See IFNHH Safety Manual.
- 2) This is an aseptic procedure, and aseptic technique should be followed.
- 3) Disposable latex gloves should be worn. Before using these gloves, check whether the trial participant is allergic to latex and where necessary use non-latex gloves (e.g. nitrile)
- 4) Vacutainer evacuated blood collection system is the preferred method to reduce the incidence of needlestick injuries and blood spillage.
- 5) Needles should be placed in a sharps container once venipuncture is performed.
- 6) If a needlestick injury occurs refer to the IFNHH safety manual and follow procedure
- 7) Identify and plan safe actions/stance e.g. Semi-squat.

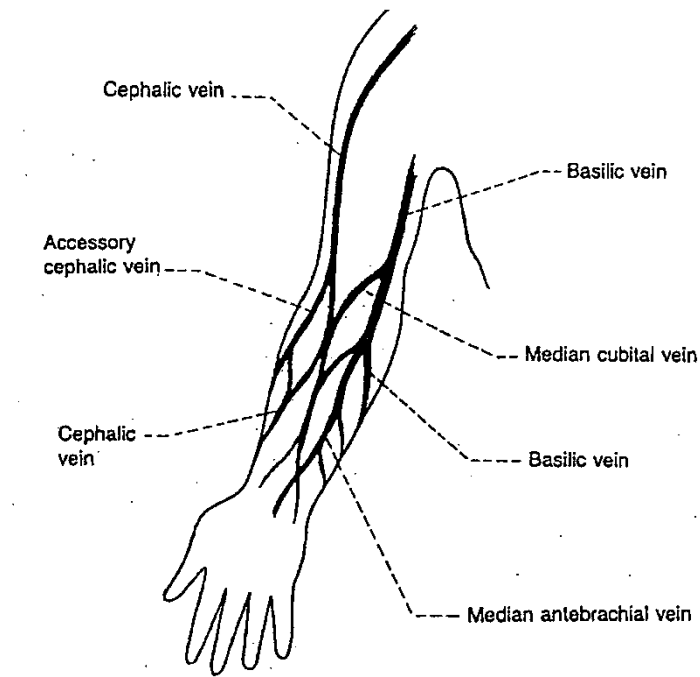
Trial Participant

- 1) If venipuncture is unsuccessful on the third consecutive attempt, the procedure should be stopped and the trial participant referred to a more experienced health professional.
- 2) To ensure that disinfection occurs, the insertion site must be cleaned well using an alcohol swab and the area left to dry prior to venipuncture. This will also reduce the risk of haemolysis of any specimen collected and prevent a burning sensation to the trial participant when the venipuncture is performed.
- 3) To prevent preservative contamination, the recommended sequence for blood tubes is:

☞	Blood cultures (sterile samples)	
☞	Plain	- red top (glass)
☞	Citrate	- blue
☞	SST-II Gel	- red top (with clot activator)
☞	PST-II Gel	- green top
☞	EDTA	- pink top
☞	EDTA	- lavender top
☞	Fluoride	- grey top
☞	ESR	- black
- 4) For restless or very apprehensive trial participants arrange to have an assistant to help you. The assistant will focus on the person's needs and safety during the procedure.

Venipuncture Sites

For adults, blood is usually drawn from a vein in or around the antecubital fossa (refer to the diagram below).



Veins in antecubital region

Performing the Procedure

- 1) Accurately identify the trial participant. Immediately after taking a sample the identification details of the trial participant and the date and time of the collection must be written onto the sample. The minimum information required on all specimens is the trial participants surname, first name(s), and date of birth. Explain the procedure and reassure the trial participant.
- 2) Position the trial participant with his/her arm extended and supported on a pillow and extends the arm to form a straight line from the shoulder to the wrist.
- 3) Wash hands.
- 4) Put on gloves.
- 5) Apply tourniquet around the arm 8-10cm above the venipuncture site. Allow vein to fill and distend ensuring that the flow of blood is not stopped for more than a minute before the blood is drawn.
- 6) Select the venipuncture site. Ask the trial participant to clench their hand into a fist. If a vein is still not apparent, ask the trial participant to hang their arm downward. Firm tapping of the vein site with the index and second finger a few times will cause the vein to dilate. Applying a warm damp cloth to the site for five minutes may have the same result.
- 7) Clean the venipuncture site with an alcohol swab. Allow the skin to dry. Do not touch the site after cleaning it. If you have to relocate the vein with a finger before puncturing the skin, ensure that asepsis is maintained.
- 8) Grasp the trial participant's arm near the venipuncture site using the thumb to draw their skin tight.
- 9) With the needle bevel facing up, line the needle with the vein, penetrating the skin and entering the vein at an angle of approximately 10-20 degrees. Hold the barrel firmly, to prevent movement of the needle in the trial participant's arm, and push the tube stopper over the needle. Keep the tube below the puncture site while the needle is in the vein. Ask the trial participant to open his/her fist when the blood starts to flow into the tube.
- 10) Release the tourniquet slowly.
- 11) When the last tube has been filled, remove it from the holder and gently remove the needle from the venipuncture site. Tubes containing an anticoagulant, e.g. blue, lavender – need to be inverted several times in order to mix the anticoagulant. If using a syringe instead of a vacutainer, ensure sufficient blood is withdrawn for the required tests. Replace existing needle with 18 gauge needle. Gently pierce the rubber top of the blood tube with the needle to minimise the risk of haemolysis.
- 12) Apply a gauze swab or cotton wool to the venipuncture site for 2-3 minutes. Do not bend the elbow as sufficient pressure cannot be applied in this position and bruising may occur.
- 13) Dispose of the needle directly into sharps container. If using a steel butterfly needle cut needle off into the sharps container, taking care to prevent injury. Do not recap needles. Dispose of equipment into designated containers in approved manner.

- 14)** Record collection details on requisition form and label blood tubes immediately after collection.
- 15)** Carefully check the site to ensure the bleeding has stopped and apply an adhesive over the site. Instruct the trial participant to leave the adhesive in place for at least 15 minutes. If the trial participant is allergic to an adhesive, apply gauze and tape.
- 16)** Forward specimen to laboratory in a biohazard bag after placing form into side pocket.
- 17)** Wash hands.

Appendix F: Soluble Transferrin Receptor – Assay Protocol

0020763454122-501V9.0

STFR

Tina-quant Soluble Transferrin Receptor

Order information

REF	CONTENT	Analyzer(s) on which cobas c pack(s) can be used
20763454 122	Tina-quant Soluble Transferrin Receptor (80 tests)	System-ID 07 6345 4
12148331 122	Preciset sTfR (5 x 1 mL)	Codes 750-754
12148340 122	sTfR Control Set	
	Level I (2 x 3 mL)	Level I Code 211
	Level II (2 x 3 mL)	Level II Code 212
04489357 190	Diluent NaCl 9 % (50 mL)	System-ID 07 6869 3

cobas®

English

System information

For cobas c 311/501 analyzers:

STFR: ACN 665

For cobas c 502 analyzer:

STFR: ACN 8665

Intended use

In vitro test for the quantitative determination of soluble transferrin receptor (sTfR) in human serum and plasma on Roche/Hitachi cobas c systems.

Summary^{1,2,3,4,5,6,7,8}

The transferrin receptor is an integral membrane glycoprotein having a molecular weight of 190 kilodaltons. It consists of two identical subunits linked by disulfide bridges. Each of the monomers has an 85 kD C-terminal component which can bind an iron-laden transferrin molecule. Proteolysis leads to the soluble form of the transferrin receptor (sTfR). In plasma, the soluble transferrin receptor is present in the form of a complex with transferrin having a molecular weight of approximately 320 kD. The serum concentration of sTfR is directly proportional to the concentration of the receptor on the membrane.

The uptake of iron by the body's cells is controlled by expression of the transferrin receptor (TfR). If the intracellular iron stores are exhausted - corresponding to a ferritin concentration of less than 12 µg/L - then more TfR is expressed. The affinity of the transferrin receptor to transferrin depends on the latter's loading state. As 80-95 % of the transferrin receptor molecules are localized on erythropoietic cells, the TfR concentration (and hence also the sTfR concentration) reflects the iron requirement of these cells. When iron deficiency exists, the sTfR concentration in serum rises even before the hemoglobin concentration is significantly depressed. The sTfR concentration can therefore describe the functional iron status while ferritin reflects the iron storage status. A precise assessment of the iron status can be obtained by determining the sTfR index (= sTfR concentration/log ferritin concentration).

As - in contrast to ferritin - the concentration of sTfR is not affected by acute-phase reactions, acute liver function disorders or malignant tumors, it is possible to differentiate between anemia of chronic disease (ACD) and iron deficiency anemia (IDA). Elevated sTfR values are also found in polycythemia, hemolytic anemia, thalassemia, hereditary spherocytosis, sickle cell anemia, megaloblastic anemia, myelodysplastic syndrome and vitamin B₁₂ deficiency. Elevated sTfR concentrations occur during pregnancy when there is a deficiency of functional iron. Therapy with rhEPO can be monitored via the sTfR concentration.

Parameter	Change	IDA	ACD	IDA + ACD
Ferritin	iron stores	↓	↑	— or ↑
TIBC/TRSF	iron status	↑	↓	or —
Serum iron	iron status	↓	↓	↓
sTfR	functional iron deficiency	↑	—	↑

↓ decreased, ↑ increased, — unchanged

Test principle⁹

Particle enhanced immunoturbidimetric assay.

Human soluble transferrin receptor agglutinates with latex particles coated with anti-soluble transferrin receptor antibodies. The precipitate is determined photometrically.

Reagents - working solutions

R1 TES/HCl buffer: 20 mmol/L, pH 7.7; NaCl: 500 mmol/L; preservative

R2 Latex particles coated with monoclonal anti-human sTfR antibodies (mouse); TRIS/HCl buffer: 20 mmol/L, pH 8.0; preservative

R1 is in position A and R2 is in position B.

Precautions and warnings

For in vitro diagnostic use.

Exercise the normal precautions required for handling all laboratory reagents.

Disposal of all waste material should be in accordance with local guidelines. Safety data sheet available for professional user on request.

For USA: Caution: Federal law restricts this device to sale by or on the order of a physician.

Reagent handling

Ready for use

Carefully invert reagent container several times prior to use to ensure that the reagent components are mixed.

Mix cobas c pack well before placing on the analyzer.

Storage and stability

STFR

Shelf life at 2-8 °C: See expiration date on cobas c pack label.

On-board in use and refrigerated on the analyzer: 12 weeks

Diluent NaCl 9 %

Shelf life at 2-8 °C: See expiration date on cobas c pack label.

On-board in use and refrigerated on the analyzer: 12 weeks

Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable.

Serum.

Plasma: Li-heparin plasma

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

See the limitations and interferences section for details about possible sample interferences.

Sample stability claims were established by experimental data by the manufacturer or based on reference literature and only for the temperatures/time frames as stated in the method sheet. It is the responsibility of the individual laboratory to use all available references and/or its own studies to determine specific stability criteria for its laboratory.

Stability:	3 days at 15-25 °C
	7 days at 2-8 °C
	4 weeks at (-15)-(-25) °C (freeze only once)

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- See "Order information" section
- General laboratory equipment

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for serum and plasma**cobas c 311 test definition**

Assay type	2-Point End		
Reaction time/Assay points	10 / 8-17		
Wavelength (sub/main)	800/570 nm		
Reaction direction	Increase		
Unit	mg/L (mg/dL, nmol/L)		
Reagent pipetting	Diluent (H ₂ O)		
R1	110 µL	-	
R2	110 µL	-	
Sample volumes	Sample	Sample dilution	
		Sample	Diluent (NaCl)
Normal	2 µL	-	-
Decreased	10 µL	10 µL	90 µL
Increased	2 µL		

cobas c 501 test definition

Assay type	2-Point End		
Reaction time/Assay points	10 / 13-25		
Wavelength (sub/main)	800/570 nm		
Reaction direction	Increase		
Unit	mg/L (mg/dL, nmol/L)		
Reagent pipetting	Diluent (H ₂ O)		
R1	110 µL	-	
R2	110 µL	-	
Sample volumes	Sample	Sample dilution	
		Sample	Diluent (NaCl)
Normal	2 µL	-	-
Decreased	10 µL	10 µL	90 µL
Increased	2 µL		

cobas c 502 test definition

Assay type	2-Point End		
Reaction time/Assay points	10 / 13-25		
Wavelength (sub/main)	800/570 nm		
Reaction direction	Increase		
Unit	mg/L (mg/dL, nmol/L)		

Reagent pipetting	Diluent (H ₂ O)		
R1	110 µL	-	
R2	110 µL	-	
Sample volumes	Sample	Sample dilution	
		Sample	Diluent (NaCl)
Normal	2 µL	-	-
Decreased	10 µL	10 µL	90 µL
Increased	4 µL		

Calibration

Calibrators	S1: H ₂ O S2-S6: Preciset sTfR
Calibration mode	RCM2
Calibration frequency	Full calibration • daily and after reagent lot change • as required following quality control procedures

Calibration interval may be extended based on acceptable verification of calibration by the laboratory.

Traceability: This method has been standardized against an in-house reference preparation.

Quality control

For quality control, use control materials as listed in the "Order information" section.

In addition, other suitable control material can be used.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

Calculation

Roche/Hitachi **cobas c** systems automatically calculate the analyte concentration of each sample.

Conversion factors:	mg/L x 11.8 = nmol/L ^{a)}
	nmol/L x 0.085 = mg/L
	mg/L x 0.1 = mg/dL
	mg/dL x 10 = mg/L

a) Based on a molecular mass of 85 kDa for circulating transferrin receptor.

Limitations – interference

Criterion: Recovery within ± 10 % of initial value at a sTfR concentration of 2.00 mg/L (0.20 mg/dL).

Icterus:¹¹ No significant interference up to an I index of 60 for conjugated and unconjugated bilirubin (approximate conjugated and unconjugated bilirubin concentration: 1026 µmol/L or 60 mg/dL).

Hemolysis:¹¹ No significant interference up to an H index of 800 (approximate hemoglobin concentration: 497 µmol/L or 800 mg/dL).

Lipemia (Intralipid):¹¹ No significant interference up to an L index of 1000. There is poor correlation between the L index (corresponds to turbidity) and triglycerides concentration.

Rheumatoid factors: No significant interference from rheumatoid factors up to a concentration of 750 IU/mL.

High dose hook-effect: No false result occurs up to an sTfR concentration of 80 mg/L.

The antibodies are specific for sTfR. There is no cross-reactivity with diferritotransferrin, apotransferrin or ferritin under the assay conditions.

Drugs: No interference was found at therapeutic concentrations using common drug panels.^{12,13}

In very rare cases, gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.¹⁴

As for any assay employing mouse antibodies, erroneous findings may be obtained from samples taken from patients who have been treated with monoclonal mouse antibodies or have received them for diagnostic purposes.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

ACTION REQUIRED

Special Wash Programming: The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi cobas c systems. The latest version of the carry-over evasion list can be found with the NaOH-SMS-SmpCln1+2-SCCS Method Sheets. For further instructions refer to the operator's manual. cobas c 502 analyzer: All special wash programming necessary for avoiding carry-over is available via the cobas link, manual input is required in certain cases.

Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.

Limits and ranges

Measuring range

0.50-40.0 mg/L (5.9-472 nmol/L, 0.05-4.00 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:2 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 2.

Lower limits of measurement

Lower detection limit of the test

0.50 mg/L (5.9 nmol/L, 0.05 mg/dL)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying 3 standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

Expected values¹⁵

Men (n = 208)	2.2-5.0 mg/L	26-59 nmol/L	0.22-0.50 mg/dL
(aged 18-60 years)			
Women (n = 211)	1.9-4.4 mg/L	22-52 nmol/L	0.19-0.44 mg/dL
(aged 18-45 years)			

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using human samples and controls in an internal protocol with repeatability (n = 21) and intermediate precision (3 aliquots per run, 1 run per day, 21 days). The following results were obtained:

Repeatability	Mean	SD	CV
	mg/L	mg/L	%
	(nmol/L, mg/dL)	(nmol/L, mg/dL)	
sTfR Control Set Level 1	2.16 (25.5, 0.216)	0.03 (0.35, 0.002)	1.5
sTfR Control Set Level 2	6.82 (80.5, 0.682)	0.06 (0.71, 0.006)	0.9
Human serum 1	1.93 (22.8, 0.193)	0.04 (0.47, 0.004)	2.1
Human serum 2	3.38 (39.9, 0.338)	0.04 (0.47, 0.004)	1.3
Intermediate precision	Mean	SD	CV
	mg/L	mg/L	%
	(nmol/L, mg/dL)	(nmol/L, mg/dL)	
sTfR Control Set Level 1	2.05 (24.2, 0.205)	0.08 (0.94, 0.008)	4.0

sTfR Control Set Level 2	6.67 (78.7, 0.667)	0.11 (1.30, 0.011)	1.6
Human serum 3	1.37 (16.2, 0.137)	0.05 (0.59, 0.005)	3.8
Human serum 4	12.1 (143, 1.21)	0.2 (2.36, 0.02)	1.4

Method comparison

sTfR values for human serum and plasma samples obtained on a Roche/Hitachi cobas c 501 analyzer (y) were compared with those determined using the corresponding reagent on a Roche/Hitachi 917 analyzer (x).

Sample size (n) = 119

Passing/Bablok ¹⁶	Linear regression
y = 0.976x + 0.260 mg/L	y = 0.979x + 0.244 mg/L
r = 0.957	r = 1.000

Values ranged from 1.41 to 39.9 mg/L (16.6 to 471 nmol/L, 0.141 to 3.99 mg/dL).

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A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Appendix G: Standard Operating Procedure – Ferritin

Test Identifier Information				
Registration Code	FER			
Method	Chemiluminescent Microparticle Immunoassay (CMIA) Abbott i2000 Architect, Abbott Reagent			
External Price	\$7.68(Exclusive of GST)			
Specimen Collection				
Patient Specimen	4.5 ml heparinised PST, SST or plain tube			
Paediatric Specimen	600 uL heparinised or plain			
Instructions for Referral to CHLabs				
Aliquot Instructions	500 uL plasma or serum Refrigerated for up to 7 days or frozen			
Aliquot Transport to CHL	Refrigerated or frozen			
CHLabs Laboratory				
Department	Biochemistry - Core			
Contact Details	Elly Barnes			
Contact Phone Number	03 3640397 ext: 80397			
Test Availability	Mon - Sun 24 hrs			
Turnaround Time	2-3 hours			
Reference Interval	Reference Intervals		Reference Interval	
	Age Range			
	0 – 15 years		15 – 150	
	Female		Male	
	Age Range	Reference Interval	Age Range	Reference Interval
	>15 – 50 years	20 – 200	>15 – 30 years	20 – 350
	> 50 years	20 – 350	> 30 years	20 – 500
Delphic Number Test Number	3302			

Appendix H: Standard Operating Proceedure – CRP

Test Identifier Information	
Registration Code	UCRP
Method	An immunoturbidometric method analysed on Abbott c series analyser, Abbott reagents
Refer to Group	Iron Studies, Plasma ;
External Price	\$9.98(Exclusive of GST)
Specimen Collection	
Patient Specimen	4.5 ml heparinised PST, SST or plain tube
Paediatric Specimen	600 uL lithium heparin blood
Instructions for Referral to CHLabs	
Aliquot Instructions	100 uL plasma
Aliquot Transport to CHL	Ambient
CHLabs Laboratory	
Department	Biochemistry - Core
Contact Details	Elly Barnes
Contact Phone Number	03 3640397 ext: 80397
Test Availability	Mon - Sun, 24 hrs
Turnaround Time	2-3 hrs
Reference Interval	Reference Range: < 5 mg/L
Delphic Number Test Number	3206

Appendix I: Health and Lifestyle Questionnaire

vIVID Questionnaire

Start of Block: Default Question Block

Demographics and medical history questionnaire

Please fill in Section 1 of this questionnaire. If you require any help please ask one of the researchers

Q1 Please fill in you vIVID study ID number

End of Block: Default Question Block

Start of Block: Demographics and Medical History

Q2 How old are you? (e.g. if 20 years, then please just write 20)

Q3 Please state the country you were born in?

Q4 If you live in New Zealand but were NOT born here, when did you first arrive to live in New Zealand? Please indicate the month and year e.g. February 2000

Q5 Which ethnic group do you identify with?

Q6 What is your first language?

Q113 Do you have children?

☐ Yes

☐ No

Display This Question:

If Do you have children? = Yes

Q7

How many children do you have

Display This Question:

If Do you have children? = Yes

Q8

When was your youngest child born __/__/____(DD/MM/YYYY)

Q9 Are you currently taking any contraception such as

	Yes	No
Oral Contraception	☐	☐
Patch Contraception	☐	☐
Contraception by injection	☐	☐
Intra-uterine device	☐	☐

Display This Question:

If Are you currently taking any contraception such as = Yes

Q11 If yes, how long have you been using this contraceptive method (e.g. 3 months or 1 year)

Q13 Have you been pregnant within the last year

☐ Yes

☐ No

End of Block: Demographics and Medical History

Start of Block: Section 2: Lifestyle

Q14 Do you smoke?

☐ Yes

☐ No

Display This Question:

If Do you smoke? = Yes

Q15 If yes how many cigarettes do you smoke a day?

Q16 How would you describe you current eating pattern?

☐ Eat a variety of all foods, including animal products

☐ Eat eggs, dairy, fish and chicken but avoid other meats

☐ Eat eggs and dairy products but avoid all meat and fish

☐ Eat eggs but avoid dairy products, all meat and fish

☐ Eat no animal products

☐ Other

Q17 Do you avoid any particular foods for cultural or religious reasons

☐ Yes

☐ No

Display This Question:

If Do you avoid any particular foods for cultural or religious reasons = Yes

Q19 If yes, what type of diet do you follow?

Q20 Have you dieted strictly in the last year?

☐ Yes

☐ No

Display This Question:

If Have you dieted strictly in the last year? = Yes

Q131 If yes then please describe

End of Block: Section 2: Lifestyle

Start of Block: Section 3: Health

Q21 Have you suffered from any acute or chronic illness in the last year?

☐ Yes

☐ No

Display This Question:

If Have you suffered from any acute or chronic illness in the last year? = Yes

Q22 Please provide the diagnosis, who diagnosed you, date and any further details

Q25 Have you ever suffered from low iron stores, iron deficiency or iron deficiency anaemia?

☐ Yes

☐ No

Display This Question:

If Have you ever suffered from low iron stores, iron deficiency or iron deficiency anaemia? = Yes

Q26 Please provide the diagnosis, who diagnosed you, date and any further details

Q27 Have you ever been treated for iron deficiency or iron deficiency anaemia?

☐ Yes

☐ No

Display This Question:

If Have you ever been treated for iron deficiency or iron deficiency anaemia? = Yes

Q28 Type of treatment, duration and any further details

Q29 Do you have or have you had any medical condition which resulted in blood loss?

☐ Yes

☐ No

Display This Question:

If Do you have or have you had any medical condition which resulted in blood loss? = Yes

Q30 If yes, please describe and give approximate dates

Q31 Have you had a blood transfusion in the last year?

☐ Yes

☐ No

Display This Question:

If Have you had a blood transfusion in the last year? = Yes

Q32 If yes, do you know why you received the transfusion?

Q33 Have you had any blood loss (other than your periods or nose bleeds) such as wounds, regular scratches from contact sport, blood in stools or urine in the past year?

☐ Yes

☐ No

Display This Question:

If Have you had any blood loss (other than your periods or nose bleeds) such as wounds, regular scra... = Yes

Q34 If yes, then please describe

Q35 Are you currently taking any medication (excluding nutritional supplements)?

☐ Yes

☐ No

Display This Question:

If Are you currently taking any medication (excluding nutritional supplements)? = Yes

Q36 If yes, please state what medication you are taking and why

Display This Question:

If Have you been pregnant within the last year = Yes

Q37 Have you breastfed a baby in the current year?

☐ Yes

☐ No

End of Block: Section 3: Health

Start of Block: Section 4: Supplements

Q39 Did you take any vitamin and/or mineral capsule/tablets at any time during the past year?

☐ Yes

☐ No

Display This Question:

If Did you take any vitamin and/or mineral capsule/tablets at any time during the past year? = Yes

Q40 If yes, please list the brand name of the supplement, the type of supplement, the number taken and the frequency of intake and the dose (including units)?

Note, it is important to obtain the amount and types of iron, vitamin C and calcium in any supplement if that information is available

e.g. Healtheries, Iron & vitamin C. 1 tablet taken every 2nd day, ferrous gluconate (170mg) providing elemental iron (20mg) and vitamin C (40 mg)

If you are unable to remember the details please send us an email with your supplement details

Q41 Did you take any other dietary supplements such as plain wheat bran (unprocessed bran, not 'All Bran', or breakfast cereal), fibre tablets, Noni juice, lecithin, evening primrose oil, performance enhancers, protein supplements, etc at any time during the past year?

☐ Yes

☐ No

Display This Question:

If Did you take any other dietary supplements such as plain wheat bran (unprocessed bran, not 'All B... = Yes

Q42 If yes, please list the brand name of the supplement, the type of supplement, the number taken and the frequency of intake and the dose (including units)?

eg. Nutralife Evening Primrose Oil, 3 capsules taken per day, contains 1000mg evening primrose oil,

End of Block: Section 4: Supplements

Start of Block: Section 5: Blood Donation/ Nose Bleeds / Menstrual Cycle

The questions regarding blood loss were adapted from (Heath, Skeaff, & Gibson, 1999).

End of Block: Section 5: Blood Donation/ Nose Bleeds / Menstrual Cycle

Start of Block: Iron Food Frequency Questionnaire

The full food frequency questionnaire is adapted from (Beck, 2013).

End of Block: Iron Food Frequency Questionnaire

Start of Block: Survey completed

Q133 Thank you for taking the time to complete this questionnaire. We greatly appreciate all the information and opinions you have provided. Survey answers will remain anonymous, but group data may be extracted and presented.

Thank you for your assistance with the research study on Iron and Vitamin D status.

End of Block: Survey completed

Appendix J: Grouping of Food Categories from Food Frequency Questionnaire

Food Group	Foods Included
Alcohol	Beer, cider, red wine, white wine, spirits and ready to drink alcoholic beverages
Tea and Coffee	Black tea, coffee, herbal and fruit teas
Dairy	Cheese (all types), cream, sour cream, cream cheese, fromage frais, milk, soy milk, coconut milk, condensed milk, ice cream, buttermilk, yoghurt, seekhand, milk pudding, burfi, desserts made from milk and foods soaked in yoghurt (e.g. dahiwada, dhokra, raita, kudhi)
Meat	Beef, chicken, turkey, duck, lamb, mutton, pork, veal, liver, kidney, offal, ham, bacon, venison, mutton, rabbit, corned beef, Haleem, beef jerky, sausages, luncheon, black pudding, meat pies, fish, mussels, pippi, paua, cockles, oysters, scallops, crabs, crayfish, kina, squid, prawn, shrimp
Iron Fortified Foods	Weetbix, cornflakes, rice bubbles, all bran, sultana bran, light and fruity, special k, cocopops, milo cereal, nutrigrain, iron fortified bread, iron fortified crackers, marmite, vegemite
Fruit and vegetables	Apples, bananas, green banana, citrus fruits (e.g. orange, tangelo, tangerine, mandarin, grapefruit, lemon), kiwifruit, pears, stone fruit (e.g. apricots, nectarines, peaches, plums, lychees), avocadoes, olives, feijoas, persimmon, tamarillos, grapes, mango, watermelon, pawpaw, other melons (e.g. honey dew, rock melon), pineapple, rhubarb, canned fruit salad, strawberries, blackberries, cherries, blueberries, boysenberries, loganberries, cranberries, gooseberries, raspberries, sultanas, raisins, currants, figs, dried apricots, prunes, dates, figs, mulberry, mixed dried fruit, potato, kumara, green beans, broad beans, runner beans, asparagus, broccoli, cabbage, brussel sprouts, capsicum, carrots, cauliflower, corn, courgette, zucchini, cucumber, gerkins, beetroot, radishes, lettuce, mushrooms, onions, leeks, celery, tomatoes, peas, green beans, spinach, silver beet, swiss chard, watercress, puha, whitloof, chicory, kale, chard, collards, chinese kale, bok choy, pumpkin, squash, yams, parsnip, taro leaves (palusami)
Breads and Cereals	Porridge, rolled oats, oat bran, oat meal, muesli (All varieties), white rice, biriyani, pilau, khitchri, brown rice, noodles, pasta, couscous, polenta, bulgur wheat (e.g. tabbouleh), wheat germ, wheat bran