The impact of vitamin D status on hepcidin and iron status in premenopausal females living in Auckland, New Zealand.

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Abstract

Background: Iron deficiency impacts female health, potentially leading to reduced immunity, cognitive function and physical performance. Hepcidin is a hormone that regulates iron homeostasis via the iron export channel ferroportin, subsequently controlling iron absorption, export and recycling. Supplementation of vitamin D has been demonstrated to reduce hepcidin concentration via direct transcription of the hepcidin gene (*HAMP* gene). However, the role of vitamin D and the impact on hepcidin and iron status has yet to be fully investigated.

Aim: To investigate the associations between serum 25 hydroxyvitamin D (s-25(OH)D), hepcidin and iron status in premenopausal females living in Auckland New Zealand (NZ). The secondary aim is to investigate potential determinants of vitamin D status in premenopausal females.

Methods: Pre-menopausal females aged 18-45 years, living in Auckland, New Zealand (NZ) participated in this cross-sectional study. Body composition was measured using bioelectrical impedance analysis and included: height, weight and body fat %. S-25(OH)D, inflammatory (CRP and IL-6), and iron biomarkers (serum ferritin, haemoglobin, soluble transferrin receptor and hepcidin) were measured from a venous blood sample. A series of questionnaires were completed to assess demographic and lifestyle factors, including: medical history, skin colour, sun exposure and dietary iron intake. Statistical analysis was undertaken using SPSS statistics 27 for windows (IBM).

Results: Of the 160 participants included in the final analysis, 60 were NZ European, 67 South Asian and 33 from 'other' ethnicities. South Asians had significantly higher body fat % and IL-6 concentration (38.34% and 1.66 $pg \cdot mL^{-1}$ respectively) compared to NZ Europeans, (27.49% and 0.63 $pg \cdot mL^{-1}$ respectively, $p<0.001$). South Asians had significantly lower s-25(OH)D concentrations compared to NZ Europeans (33.59 $nmol \cdot L^{-1}$ vs 74.84 $nmol \cdot L^{-1}$, $p<0.001$). In NZ Europeans, higher s-25(OH)D concentrations were seen in those with lower (≤3.5nM) hepcidin concentration $p=0.0046$. Conversely, in South Asians, higher s-25(OH)D concentration was seen in those with higher (>3.5nM) hepcidin concentrations, $p=0.038$. There was no significant association in the 'other' ethnicities and no associations between s-

25(OH)D and iron status/serum ferritin. Key determinants of s-25(OH)D were ethnicity, age and body fat %.

Conclusion: The positive relationship between s-25(OH)D and hepcidin concentration in the South Asian women was unexpected, although possibly explained by higher IL-6 concentration, body fat % and lower s-25(OH)D concentration identified in the South Asian ethnic group, requiring further research.

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Table of contents

List of tables

List of figures

List of appendices

List of abbreviations

Abbreviation	**Term**
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D3
s-25(OH)D	Serum 25-hydroxyvitamin D
BMI	Body Mass Index
CHD	Coronary Heart Disease
CRP	C-Reactive Protein
CYP27A1	Cytochrome P450 27
CYP27B1	Enzyme 1-α-hydroxylase
CYP2R1	Cytochrome P450 2R1
D_2	Ergocalciferol
D_3	Cholecalciferol
DCytb	Duodenal Cytochrome B
DMT1	Divalent Metal Transporter 1
DNA	Deoxyribonucleic Acid.
DS-25(OH)D	Deseasonalised Serum 25(OH)D
EGF	Epidermal Growth Factor
Fe	Iron
Fe^{2+}	Ferrous Iron
Fe^{3+}	Ferric Iron
H^+	Hydrogen
HAMP	Hepcidin Anti-Microbial Peptide
Hb	Haemoglobin
HCP1	Haem Iron Transporter
HGF	Hepatocyte Growth Factor
HPA	Hypothalamic-Pituitary-Adrenal
ID	Iron Deficiency
IDA	Iron Deficiency Anaemia
IDE	Iron Deficient Erythropoiesis

IDMH	Iron Deficiency with Microcytosis and/or Hypochromia
IL-6	Interleukin-6
LPS	Lipopolysaccharide
JAK1-STAT3	Janus Kinase2-signal Transducer and Activator of Transcription-3
MCH	Mean Cellular Haemoglobin
MCP-1	Monocyte Chemoattractant Protein-1
MCV	Mean Cellular Volume
MID	Mild Iron Deficiency
mRNA	Messenger Ribonucleic acid
NAID	Nonanaemic Iron Deficiency
NZ	New Zealand
PAI-1	Plasminogen activator inhibitor 1
RBC	Red Blood Cells
RDI	Recommended Dietary Intake
Sf	Serum Ferritin
sTfR	Soluble Transferrin Receptor
Th1	Type 1 T Helper Cells
Th2	Type 2 T Helper Cells
Th17	Type 17 T Helper Cells
UVβ	Ultraviolet B
VDR	Vitamin-D Receptor
ZnPP	Zinc-Protoporphyrin

Chapter 1: Purpose

1.1 Introduction

Iron (Fe) is a trace mineral that is required to support a number of our body's functions, including: oxygen transport and storage, production of red blood cells, oxidative metabolism and deoxyribonucleic acid (DNA) synthesis (Beard et al., 1996; Mühlenhoff et al., 2003; Zimmermann & Hurrell, 2007). Negative iron balance, as a result of inadequate intake or increased losses of iron, can lead to severe iron deficiency (ID) (defined as serum ferritin (Sf) <12ug·L^{-1} and zinc protoporphyrin >60µmol·mol^{-1}), that if left uncorrected can lead to decreased haemoglobin concentrations below the lower limit of 120g·L^{-1}, known as iron deficiency anaemia (IDA) (Clénin et al., 2015; University of Otago and Ministry of Health, 2011). Iron deficiency anaemia may be associated with chronic health conditions, including but not limited to kidney and cardiovascular disease, contributing to decreased quality of life in these patients (Beard et al., 1996; Cappellini et al., 2020; Clénin et al., 2015). Despite the importance of this trace mineral for healthy physiological functioning, ID is one of the most common nutritional deficiencies impacting 29% of non-pregnant females globally in 2011 (World Health Organization, 2015). From 1997 to 2008/09 in New Zealand (NZ), the prevalence of ID in females increased from 2.9% to 7.2%, with higher prevalence in Māori and Pacific ethnicities (University of Otago and Ministry of Health, 2011). Due to the increasing rates of ID in NZ and the association between ID and lower health status, research into the causes and effective treatment of iron status is required to improve quality of life for females.

Maintaining iron homeostasis is essential in the prevention of ID and/or iron excess and is primarily managed by the hormone hepcidin, encoded by the *HAMP* gene (Nemeth, Tuttle, et al., 2004). High concentrations of hepcidin in the blood, lead to sequestration of iron in storage cells as ferritin, reduced recycling of iron by macrophages and reduced absorption of iron from foods via the enterocytes lining the duodenum (Nemeth, Tuttle, et al., 2004). As a consequence, persistently elevated hepcidin concentrations are thought to increase the risk of ID (Nemeth, Rivera, et al., 2004; Nicolas et al., 2001). As a hormone, hepcidin demonstrates diurnal variation, with lowest levels occurring in the morning, increasing throughout the day

and declining overnight (Troutt et al., 2012). In addition, hepcidin activity is known to be regulated by iron status, inflammation (in particular the inflammatory cytokine interleukin-6 (IL-6)) and erythropoietic activity (Clénin et al., 2015; Nemeth, Rivera, et al., 2004). It appears that the strongest determinant of hepcidin activity is the individual's iron status, for example, if an individual is iron sufficient and iron concentration increases post food ingestion, hepcidin production is stimulated (Sangkhae & Nemeth, 2017). However, if an individual is iron deficient, hepcidin activity appears to be blunted in order to maximise iron uptake and recycling (Sangkhae & Nemeth, 2017). Hepcidin is upregulated by IL-6 during acute and chronic inflammation, therefore in chronic health conditions (e.g. obesity, chronic kidney disease, inflammatory bowel disease) defined by persistent and elevated inflammation, individuals are considered to be at risk of developing ID (Sangkhae & Nemeth, 2017). Research regarding the role of hepcidin in iron homoeostasis is still in its infancy, and while there is some evidence on the impact of hepcidin in chronic health conditions and exercise (Gafter-Gvili et al., 2019; Peeling et al., 2014), the variations in the general population and the influence of this hormone on iron treatment strategies is still largely unknown. Currently, researchers are still looking at effective strategies to maximise iron absorption. To date investigation include: morning vs evening or alternative day iron supplementation (Stoffel, Zeder, et al., 2020), food/nutrients that may maximise iron absorption for example via the co-ingestion of iron with vitamin C (ascorbic acid) (Beck, Conlon, Kruger, & Coad, 2014; Hallberg, Brune, et al., 1987), or reducing hepcidin activity via vitamin D supplementation (Arabi et al., 2020; Bacchetta et al., 2014; Fuzi & Mushtaq, 2019).

Vitamin D is able to regulate the hormone hepcidin, directly and indirectly (Bacchetta et al., 2014; Zughaier et al., 2014). The binding of 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) directly to the vitamin D receptor present on the *HAMP* gene leads to the downregulation of *HAMP* gene expression subsequently reducing hepcidin concentrations (Bacchetta et al., 2014). $1,25(OH)_2D_3$ has also been shown to reduce hepcidin concentration indirectly via the reduction of the inflammatory cytokine, IL-6 (Zughaier et al., 2014). The reduction in hepcidin concentration as a result of adequate/optimal vitamin D levels may lead to increased levels of iron in the blood. This may improve systemic iron levels and iron regulation and reduce risk of developing ID (Bacchetta et al., 2014; Malczewska-Lenczowska et al., 2018). Serum 25-

hydroxyvitamin D (s-25(OH)D) concentration, the main biomarker of vitamin D status, is impacted by ethnicity, skin pigmentation, season, sun exposure, latitude and body mass index (BMI) (Cairncross et al., 2017; Cheng et al., 2010; Rockell et al., 2006; Snijder et al., 2005; Van Dam et al., 2007).

South Asian females in NZ are at risk of suboptimal s-25(OH)D concentrations, partly due to avoidance of sun exposure and indoor lifestyles (von Hurst et al., 2010). Other ethnic groups which have been identified as at risk include African and Middle Eastern ethnicities due to their naturally dark skin and/or their cultural or religious dress requirements that provide full-body coverage. This behaviour decreases the amount of skin exposed to the sun (Ministry of Health and Cancer Society of New Zealand, 2012). Vitamin D deficiency has been linked to skin colour (Van Dam et al., 2007), as individuals with darker skin pigmentation (increased levels of melanin), are likely to have reduced efficiency of vitamin D synthesis due to decreased absorption of ultraviolet B (UVβ) rays (Clemens et al., 1982; Webb et al., 1988). Sun exposure, the primary stimulus for vitamin D synthesis, is associated with increased s-25(OH)D concentrations (Van Dam et al., 2007). In NZ, seasonal variation is observed in s-25(OH)D concentrations, with increased concentrations during the summer/autumn months (November-February) compared to the winter/spring months (August-October) (Bolland et al., 2008; Rockell et al., 2006). This is partly due to lower UVβ angle of the sun and decreased sun exposure during winter months (Bolland et al., 2008). Body mass index and body fat percentage has also been found to have an inverse relationship with s-25(OH)D concentration, such that individuals with a higher BMI (BMI at or above 30) and greater adiposity tend to have a lower s-25(OH)D concentrations, due to increased storage of vitamin D in adipose tissue and decreased release of endogenously produced vitamin D into circulation (Cheng et al., 2010; Mazahery et al., 2015; Snijder et al., 2005).

Low s-25(OH)D concentration has been identified to increase the risk of ID in elite female athletes (Malczewska-Lenczowska et al., 2018). In further support of this relationship, a double-blind randomised placebo-controlled trial explored the effect of vitamin D supplementation on hepcidin concentration and iron status in a healthy adult population

(Smith et al., 2017). Here the authors reported that 1-week post 250,000 IU oral bolus of vitamin D3, hepcidin concentrations were significantly reduced but no significant changes in ferritin concentration were identified (Smith et al., 2017).

In NZ, ID prevalence is higher in South Asian females when compared to Caucasian females (Lim et al., 2020). Between 2006-2018, Asian and Middle Eastern populations have nearly doubled in size, with 63% of the Asian and 51% of the Middle Eastern population located in Auckland (Statistics New Zealand, 2018a, 2018b). With increasing immigration rates and increased risk of both vitamin D and ID, research into the prevention of both micronutrient deficiencies is essential. Currently, there is limited evidence for the association between vitamin D status, hepcidin and iron status, particularly in different ethnicities (South Asian, Caucasian and Middle Eastern). While some published research has shown an association between vitamin D supplementation and iron status, there is limited research on the association between endogenous variations in s-25(OH)D concentration and the impact this may have on systemic hepcidin concentration and iron status. Additionally, minimal research has investigated determinants (lifestyle, physiological or anatomical) which may influence the s-25(OH)D, hepcidin and iron status relationship. The aim of the study is to develop our understanding of the interactions between s-25(OH)D concentration, hepcidin and iron status in New Zealand females and potential determinants (ethnicity, body composition and inflammation) which may impact both their iron and vitamin D status.

1.2 Aims

Primary aim:

1. To investigate the association between s-25(OH)D, hepcidin and iron status in premenopausal females living in Auckland New Zealand.

Secondary aim:

2. To investigate potential determinants of vitamin D status, including: body composition, ethnicity, inflammatory markers, sun exposure, skin colour and seasonality in premenopausal females living in Auckland New Zealand.

1.2.1 Objectives

1. To describe vitamin D, iron and hepcidin status in healthy premenopausal females aged 18-45 years living in Auckland New Zealand.
2. To investigate associations between s-25(OH)D, hepcidin and iron status in premenopausal females living in Auckland New Zealand.
3. To investigate associations between s-25(OH)D and ethnic groups (NZ European and South Asian), inflammatory markers, body composition, sun exposure, skin colour, seasonality in premenopausal females living in Auckland New Zealand.

1.2.2 Hypotheses

1. Low s-25(OH)D concentration is associated with increased hepcidin concentration and subsequently lower iron status in South Asian and NZ European premenopausal females living in Auckland New Zealand.
2. South Asian premenopausal females will have lower s-25(OH)D concentrations compared to NZ European females.
3. Increased sun exposure, lighter skin colour and Summer/Autumn will be associated with increased s-25(OH)D concentration.
4. Higher body fat % will be associated with increased inflammation (IL-6), reduced s-25(OH)D concentration, iron and increased hepcidin concentration.

1.3 Structure of thesis

This thesis begins with an introduction on iron and maintenance of iron homeostasis via the regulation of the hormone hepcidin. It discusses the link between hepcidin and vitamin D, and the importance of conducting further research into the three components while outlining the aim of this research. Chapter two is a detailed literature review, expanding further on each micronutrient and associations between iron, hepcidin and vitamin D as well as factors which may impact them. Chapter three is the original research manuscript, this includes the abstract, introduction, methodology, results, discussion and conclusion. Finally, chapter four summarises the overall conclusions which were reached from the study, the impact that this research will have in NZ, the strengths and limitations of this study as well as future recommendations/directions which may aid in increasing knowledge on the impact of vitamin D on iron status.

1.4 Researcher's contributions

Table 1: Summary of Researcher's Contributions to Study

Author	**Contribution to Thesis**
Anna (Anya) Greenwood MSc Student nutrition and dietetics	Primary author of thesis. Analysed data and 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, supported with results analyses and interpretation, reviewed and approved thesis.
Prof Pamela von Hurst Secondary supervisor Associate professor School of Sport, Exercise and Nutrition	Assisted with research design and data collection, supported with results analyses and interpretation, reviewed and approved thesis.
Dr Hajar Mazahery Post Doc	Assisted with statistical analysis and interpretation of results.
Kimberly Lim Dietitian	Collected data, recruited participants and processed blood samples.
Dr Kathryn Beck Associate Professor in Pathophysiology School of Health Sciences.	Assisted with research design and data collection.

Chapter 2: Literature review

2.0 Introduction

Iron and vitamin D are essential micronutrients for us to consume to avoid adverse physiological outcomes (Clénin et al., 2015; Holick, 2011). Iron depletion and/or deficiency occurs when iron losses exceed iron intake and subsequently impact: oxygen transport and storage, DNA synthesis, energy production and immune function (Musallam & Taher, 2018). Therefore, maintaining iron within a normal range (table 2), is considered essential for optimal physiological functioning and health. Iron is acquired mainly from food and is kept in homeostatic balance through micronutrient recycling by macrophages and controlled iron absorption/release from cells, with both actions regulated via the hormone hepcidin (Lopez et al., 2016; Nemeth, Tuttle, et al., 2004). The *HAMP* gene encodes the hormone hepcidin, which is involved in the regulation of ferroportin export channels in response to iron status, inflammation, hypoxia and erythropoiesis (Nemeth, Tuttle, et al., 2004; Rauf et al., 2020).

Vitamin D, unlike iron, is mainly acquired via skin exposure to the sun, which initiates the process of forming the active molecule, 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), from 7-dehydrocholesterol (Holick, 2011). Consequently, vitamin D status is influenced by sun exposure, seasonality, latitude and skin pigmentation (Delshad et al., 2019; Nessvi et al., 2011). Factors that affect both iron and vitamin D status include ethnicity, obesity, dietary intake and physical activity (Beck, Conlon, Kruger, Heath, et al., 2014; Delshad et al., 2019; Nessvi et al., 2011). In particular, it has been demonstrated that both iron and serum 25-hydroxyvitamin D (s-25(OH)D) concentrations have an inverse relationship with BMI/body fat %, with prevalence of both micronutrient deficiencies tending to be higher in South Asians compared to Caucasians (Lim et al., 2020; Touvier et al., 2015).

A potential association between vitamin D and iron could be mediated by the hormone hepcidin (Bacchetta et al., 2014; Smith et al., 2017). In vitro studies have shown vitamin D to reduce the *HAMP* mRNA, leading to a reduction of circulating hepcidin which, subsequently increases systemic iron levels (Bacchetta et al., 2014). Supplementation studies have also investigated this micronutrient interaction, showing a decrease in hepcidin concentration

with vitamin D supplementation (Smith et al., 2017). Furthermore, cross-sectional studies have shown a higher prevalence of anaemia in those who have lower s-25(OH)D concentrations (Smith et al., 2015). Knowledge of the interactions between iron, hepcidin and vitamin D is required to understand how these two micronutrients may impact overall status.

2.1 Role of iron in the body

Iron (Fe) is a trace mineral acquired from food in two forms: haem iron and non-haem iron, both absorbed via different pathways into the duodenal enterocyte cells (figure 1) (Zimmermann & Hurrell, 2007). Haem iron is absorbed directly into the enterocyte via haem iron transporter (HCP1) and is then further reduced via enzymatic processes to ferrous iron (Fe^{2+}) (Zimmermann & Hurrell, 2007). Non-haem iron is present in two forms in the gut lumen: ferric iron (Fe^{3+}) or Fe^{2+}, if in Fe^{3+} form, it is reduced to Fe^{2+} via the duodenal cytochrome B (DCytB) and is then transported into the enterocyte via the divalent metal transporter (DMT1) with the help of the hydrogen (H^{+}) electrochemical gradient (Zimmermann & Hurrell, 2007). Fe^{2+} is either stored as ferritin in the enterocyte or transported out into the circulation via ferroportin where it is oxidised to Fe^{3+} by iron oxidase hephaestin and is bound to transferrin (iron transport protein) (Zimmermann & Hurrell, 2007).

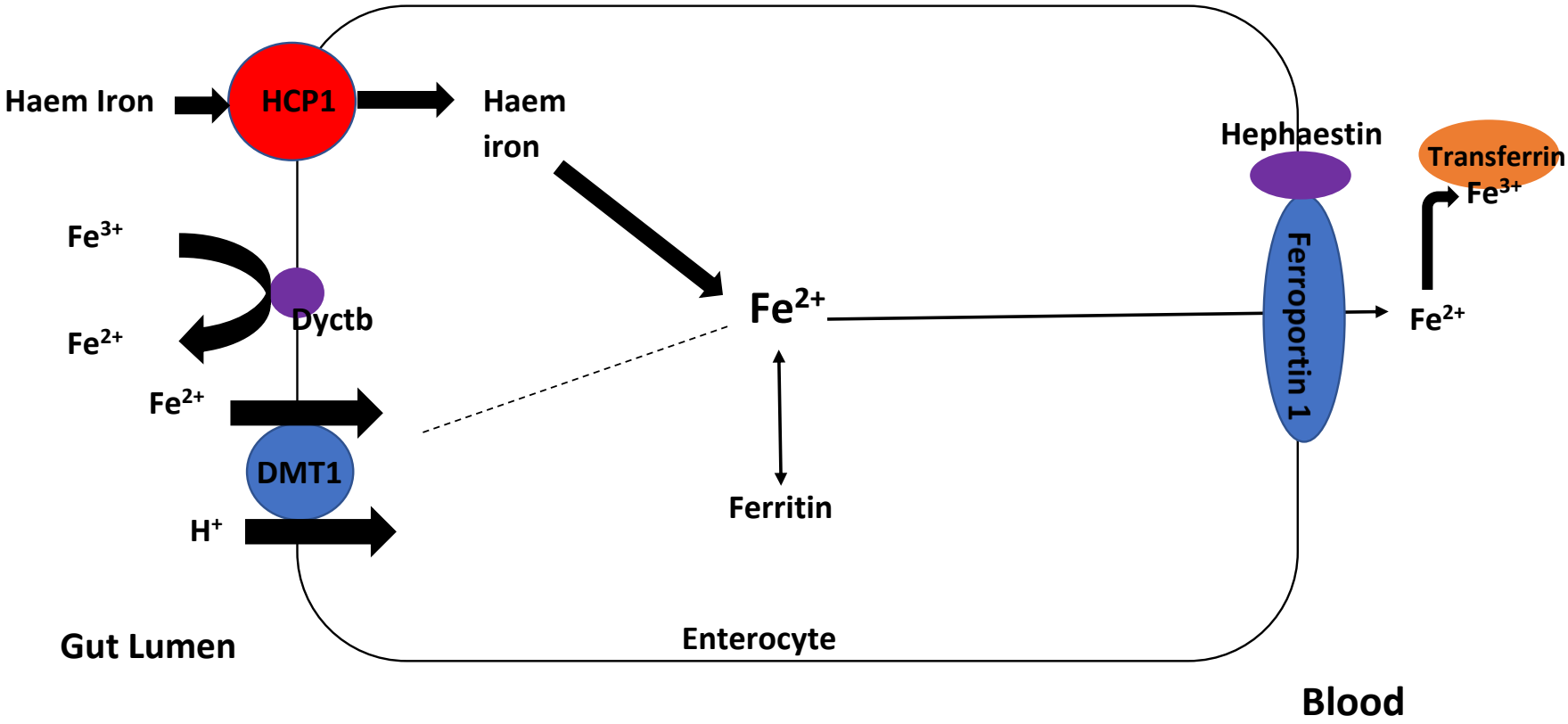


Figure 1: Overview of iron absorption at the enterocyte, adapted from Zimmermann and Hurrell (2007).

The human body contains 3-5g of iron, this is distributed between three main compartments: haemoglobin protein (oxygen-carrying protein in erythrocytes), myoglobin protein (muscle cells) and enzymes (Clénin et al., 2015). In addition to oxygen transport in systemic circulation, iron has multiple roles in cells. This includes: energy production in mitochondria through the redox reactions in the electron transport chain, DNA replication and repair through enzymes which require iron-sulphur clusters to form active proteins, regulation of cell cycle and support of immune system response as a co-factor of immune cells and support of T-cells maturation (Clénin et al., 2015; Musallam & Taher, 2018).

The human body losses an average of 1-2mg of iron daily, as a result of urinary losses, sweating, skin desquamation, gastrointestinal losses and menstrual bleeding (Lopez et al., 2016). These losses under normal conditions are counter balanced mainly by dietary intake, intestinal absorption and iron recycling by macrophages (Lopez et al., 2016). However, increased blood losses through blood donation, heavy or prolonged menstrual bleeding and nose bleeds can lead to increased iron losses, subsequently reducing iron stores and increasing the risk of iron deficiency (ID) (Heath et al., 2001).

Exercise can increase the risk of ID through the cumulative effects of sweating, haematuria, gastrointestinal bleeding and haemolysis (occurring due to foot strike) (Sim et al., 2019). An up-regulation of inflammatory cytokines has been observed immediately post exercise, leading to an increase in hepcidin (~3-6 hours post exercise) and subsequent decrease in iron absorption and recycling (Sim et al., 2019). The post exercise hepcidin response in regularly active individuals can result in accelerated iron losses if not accounted for by increased dietary intake, increasing risk of ID. An inadequate dietary iron intake can result in a negative iron balance which can lead to iron depletion and ID (Zimmermann & Hurrell, 2007).

Iron deficiency can often progress to iron deficiency anaemia (IDA), where a negative iron balance has been uncorrected and the depletion of iron stores has resulted in adverse physiological outcomes (Lopez et al., 2016). Symptoms associated with IDA,

may include fatigue, paleness, dyspnoea, headaches, dry skin and hair and neurocognitive dysfunction (Lopez et al., 2016). Due the prominent role of iron in oxygen transport and delivery, IDA causes a decline in physical performance and cognitive function in adulthood (Lopez et al., 2016). Impaired immune function in iron deficient individuals has also been observed due to iron's role as a cofactor in immune cells (Musallam & Taher, 2018). Furthermore, during infancy, depleted iron stores impact neurocognitive and motor development, with abnormalities persisting for up 10 years following iron repletion (Cappellini et al., 2020; Congdon et al., 2012).

2.1.1 Iron deficiency

The association between ID and adverse physiological outcomes requires an understanding of the different stages of ID and changes to blood markers that will aid in identifying and subsequently monitoring interventions, as described in table 2. Stage 1, is typically when there are insufficient iron stores (decreased serum ferritin (Sf)) leading to an increase in zinc-protoporphyrin (ZnPP) (Clénin et al., 2015). This may occur as a replacement when there are low iron levels and is inserted into the protoporphyrin molecule in haemoglobin (Clénin et al., 2015; World Health Organization, 2007). There is also a subtle increase in soluble transferrin receptor (sTfR) concentration, suggesting an increase in tissue iron needs (Clénin et al., 2015). During stage 1, haematopoiesis is unchanged: haemoglobin, mean cellular volume (MCV) and mean cellular haemoglobin (MCH) will still be present in the normal range (Clénin et al., 2015). Stage 1 is known as nonanaemic iron deficiency (NAID) (Clénin et al., 2015).

Stage 2 occurs when iron stores continue to decrease and as a result, young red blood cells (RBC) may become reduced in size (microcytic), pale (hypochromic) and haemoglobin concentration start to decrease, lowering MCV and MCH (Clénin et al., 2015). As the reduction in iron stores continues to progress, MCV and MCH is likely to drop below the lower limit of the normal range, this is classed as iron deficiency with microcytosis and/or hypochromia (IDMH) (Clénin et al., 2015). As the red blood cells indices are now impacted (both MCV and MCH) IDMH is seen as impacting

haematopoiesis, however changes to haemoglobin concentrations are not yet observed (Clénin et al., 2015). Stage 3 occurs when there are insufficient iron stores to support adequate and healthy haematopoiesis and as a result haemoglobin concentration drops below the lower limit of the normal range (>120g·L^{-1} for females), this is defined as IDA (Clénin et al., 2015).

The presence of inflammation can impact iron biomarkers. Inflammation stimulates the acute phase response leading to changes in concentration of acute phase proteins (Namaste et al., 2017). Ferritin, an acute phase protein, production is stimulated by cytokines during the acute phase response resulting in elevated ferritin levels independent of iron homeostasis (Namaste et al., 2017). A possible way of dealing with this in an inflammatory state (e.g. chronic inflammation conditions) is increasing the cut-off threshold from <12-15µg·L^{-1} to <30µg·L^{-1} (World Health Organization, 2007), as well as using a combination of biomarkers that are not influenced by inflammation: sTfR and ZnPP (Clénin et al., 2015). C-reactive protein (CRP, normal range <3mg·L^{-1}), is a good indicator to assess for infection and inflammation and can help control for high Sf concentration in screening (World Health Organization, 2007).

Table 2: Biomarkers of ID at each stage, including WHO cut-offs for ID.

	Stage 1: NAID	**Stage 2: IDMH**	**Stage 3: IDA**	**WHO cut-offs for IDA**
Serum ferritin ($\mu g \cdot L^{-1}$)	<30	<30	<12-15	<15
Zinc protoporphyrin ($\mu mol \cdot mol^{-1}$)	<50	>100	>100	40-70
Soluble transferrin receptor ($mg \cdot L^{-1}$)	0.75-1.5	0.75-1.5	0.75-1.5	Undefined.
Mean cellular volume (fl (10^{-12}))	>80	<80	<80	<81
Mean cellular haemoglobin (pg (10^{-9}))	>28	<28	<28	Undefined.
Haemoglobin ($g \cdot L^{-1}$)	>120	>120	<120	<120

ID = Iron deficiency, IDMH= iron deficiency with microcytosis and/or hypochromia, IDA= iron deficiency with anaemia, NAID= non anaemic iron deficiency.
Adapted from Clénin et al. (2015) and World Health Organisation, (2007).

2.1.2 Prevalence of iron deficiency in New Zealand females

Prevalence of ID in females has increased from 2.9% in 1977 to 7.2% in 2008/09 (University of Otago and Ministry of Health, 2011). The last New Zealand adult nutrition survey (NZANS) occurred in 2008/09 which reported ID in 7.2% of females and further 3.5% of females classed as IDA (University of Otago and Ministry of Health, 2011). Since the NZANS survey, two more studies have been undertaken in the Auckland region investigating ID prevalence (table 3). The most recent study found that 55.8% and 8.5% of pre-menopausal females were classed as ID and IDA respectively (Lim et al., 2020). The higher prevalence of ID in this study compared to NZANS is due to different cut-off values for ID (NZANS stage 3: Sf<12 $\mu g \cdot L^{-1}$, Lim et al., 2020; stage 1: Sf <30 $\mu g \cdot L^{-1}$), a smaller population group studied and the high proportion of high-risk population (South Asian females) represented in the study.

Biomarkers and classification of ID/IDA in the New Zealand (NZ) population differ between numerous studies and are summarised in table 3. As stated above, the NZANS used Sf<12 $\mu g \cdot L^{-1}$ as a cut off marker for ID and incorporated ZnPP as a biomarker. Whereas Heath et al. (2001) and Beck et al. (2014) classified ID as Sf < 20 $\mu g \cdot L^{-1}$. Using a higher cut-off value for Sf (~25-30 $ug \cdot L^{-1}$) can lead to a higher portion of the study population being classified as ID due to this being a more sensitive diagnosis threshold (Clénin et al., 2015; Yu et al., 2013). This does however create some barriers when comparing outcomes of different studies and needs to be considered during ID prevalence interpretation. Regardless, with seemingly increasing rates of ID (particularly stage 1) in NZ, it's important to identify strategies to prevent the degradation to stage 2 and 3 ID. To achieve this, an understanding of iron regulation through the activity of the hormone hepcidin is required.

Table 3: Summary of studies investigating the prevalence of ID and IDA in NZ females.

Study	Population group	Prevalence	Iron status definition	C-reactive protein cut-off
(University of Otago and Ministry of Health, 2011), NZ adult nutrition survey.	NZ females aged 31-50.	ID: 12.1% IDA: 6.3%	ID: Sf <12 $\mu g \cdot L^{-1}$; zinc protoporphyrin >60$\mu mol \cdot mol^{-1}$ IDA: Sf <12 $\mu g \cdot L^{-1}$; zinc protoporphyrin >60$\mu mol \cdot mol^{-1}$ and Hb <120 $g \cdot L^{-1}$	>8$mg \cdot L^{-1}$
(University of Otago and Ministry of Health, 2011), NZ adult nutrition survey.	NZ females aged 19-30.	ID: 5.2% IDA: 1.2%	ID: Sf <12 $\mu g \cdot L^{-1}$; zinc protoporphyrin >60$\mu mol \cdot mol^{-1}$ IDA: Sf <12 $\mu g \cdot L^{-1}$; zinc protoporphyrin >60$\mu mol \cdot mol^{-1}$ and Hb <120 $g \cdot L^{-1}$	>8$mg \cdot L^{-1}$
(Lim et al., 2020)	Pre-menopausal females, 18-45, years, living in Auckland, NZ (n=165)	ID: 55.8% (92) IDA: 8.5% (14)	ID: Sf <30 $\mu g \cdot L^{-1}$; Hb <120 $g \cdot L^{-1}$ or ≥120 $g \cdot L^{-1}$ IDA: Sf ≤12 $\mu g \cdot L^{-1}$; Hb <120 $g \cdot L^{-1}$	>5$mg \cdot L^{-1}$
(Beck, Conlon, Kruger, Heath, et al., 2014)	Pre-menopausal females aged 18-44 years living in Auckland, New Zealand (n=375)	ID: 18.7% (70) IDA: 5.3% (20)	ID: Sf < 20 $\mu g \cdot L^{-1}$ IDA: Sf <20 $\mu g \cdot L^{-1}$; Hb <120 $g \cdot L^{-1}$	>10$mg \cdot L^{-1}$
(Heath et al., 2001)	Pre-menopausal females aged 18-40, living in Dunedin, NZ (n=335)	MID: 23% (87) IDE: 4% (15) IDA: 2% (8)	MID: Sf <20$\mu g \cdot L^{-1}$; Hb ≥120 $g \cdot L^{-1}$ IDE: Sf <12$\mu g \cdot L^{-1}$ IDA: Sf <12$\mu g \cdot L^{-1}$; Hb <120 $g \cdot L^{-1}$	>10$mg \cdot L^{-1}$
ID= iron deficiency, MID= mild iron deficiency, IDE= iron deficient erythropoiesis, IDA= iron deficiency anaemia, Sf= serum ferritin, Hb= haemoglobin, NZ=New Zealand.				

2.2 The role of hepcidin in iron regulation

Hepcidin is a 25-amino acid peptide hormone whose primary role is maintaining iron homeostasis (Rauf et al., 2020; Rishi & Subramaniam, 2017). Hepcidin is encoded by the *HAMP* gene found primarily in hepatocyte cells (Rauf et al., 2020). Hepcidin exerts its action on ferroportin (iron exporter channel) which is involved in: the release of recycled iron, dietary iron absorption from the intestine and movement of iron from hepatocytes into systemic circulation (Nemeth, Tuttle, et al., 2004).

The binding of hepcidin to ferroportin causes the internalisation and degradation of the iron exporter channel, reducing the export of iron from the cells into the circulatory system (Nemeth, Tuttle, et al., 2004). As such, hepcidin is considered a negative iron regulator (Nemeth, Tuttle, et al., 2004). The impact of hepcidin concentration on systemic iron levels may have a detrimental impact on an individual's health, especially if hepcidin concentrations are either too low or too high (Nemeth, Tuttle, et al., 2004). Excessively low or blunted hepcidin levels may result in iron tissue overload known as hemochromatosis (Nemeth, Tuttle, et al., 2004). Whereas constantly high levels of hepcidin are likely to result in hypoferremia (Nemeth, Tuttle, et al., 2004). For example, chronic inflammatory disease/s may increase hepcidin due to the presence of persistent inflammation (and the presence of increased interleukin-6 (IL-6)), resulting in a state of functional iron deficiency (a state of hypoferremia), where iron stores cannot be accessed or utilised (Nemeth, Rivera, et al., 2004).

Inflammation is just one of the regulatory factors affecting hepcidin concentration, others include iron stores, hypoxia, erythropoiesis, growth factors (hepatocyte growth factor (HGF) and epidermal growth factor (EGF)), summarised in table 4. Hormones, testosterone, oestrogen and progesterone have also been noted to affect hepcidin concentration and are summarised in table 4 (Rishi & Subramaniam, 2017). More recently, vitamin D supplementation and variations in endogenous vitamin D status have also been investigated as a possible method in increasing iron stores through the inhibitory effect on hepcidin transcription (Bacchetta et al., 2014).

Table 4: Summary of factors impacting hepcidin transcription and the subsequent impact on iron levels.

Impact factors	Hepcidin transcription	Iron levels
Inflammation (Interleukin-6)	Increased	↓iron absorption/recycling ↓ release from iron stores =↓iron bioavailability
Iron loading, iron excess	Increased	↓iron absorption/recycling ↓ release from iron stores =↓iron bioavailability
Low iron stores or anaemia (e.g. iron deficiency)	Decreased	↑iron absorption/recycling ↑release from iron stores = ↑iron bioavailability
Hypoxia	Decreased	↑iron absorption/recycling ↑release from iron stores = ↑iron bioavailability
Erythropoiesis	Decreased	↑iron absorption/recycling ↑release from iron stores = ↑iron bioavailability
Growth factors (EGF and HGF)	Decreased	↑iron absorption/recycling ↑release from iron stores = ↑iron bioavailability
Testosterone	Decreased	↑iron absorption/recycling ↑release from iron stores = ↑iron bioavailability
Oestrogen	Decreased	↑iron absorption/recycling ↑release from iron stores = ↑iron bioavailability
Progesterone	Increased	↓iron absorption/recycling ↓ release from iron stores =↓iron bioavailability

HGF= hepatocyte growth factor, EGF= epidermal growth factor
Table adapted from (Sangkhae & Nemeth, 2017) and (Rauf et al., 2020)

2.3 Vitamin D roles in the body

Vitamin D is a prohormone and fat-soluble vitamin that is mainly acquired from skin exposure to the ultraviolet β (UVβ) rays (Holick et al., 1980). The 7-dehydrocholesterol molecule present in our skin is converted to previtamin D3 and further converted to vitamin D3 in a temperature-dependent thermal isomerization, as seen in figure 2 (Holick et al., 1980). Vitamin D binding protein binds to the isomerized product allowing it to enter the circulation and be transported to the liver where it is hydroxylated to 25-hydroxyvitamin D ($25(OH)D_3$) by the enzyme cytochrome P450 2R1 (CYP2R1) and cytochrome P450 27 (CYP27A1), (Holick, 2011; Sassi et al., 2018). Historically, it was known that $25(OH)D_3$ conversion to the active form 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), by the enzyme 1-α-hydroxylase (CYP27B1), occurred exclusively in the kidneys (Holick, 2011). However, the presence of CYP27B1 and the vitamin D receptor (VDR) has been identified in cells and tissues outside the kidneys including: epithelial cells, placental cells, bone cells (osteoblasts, osteoclasts, osteocytes, chondrocytes), immune cells, endocrine glands (e.g. parathyroid gland, thyroid gland, pancreatic islets), brain, liver and endothelia suggesting that production and effect of vitamin D may occur in various tissues and organs throughout body (Bikle et al., 2018). Vitamin D is also found in the form ergocalciferol (D_2), produced from the irradiation of ergosterol present in plants and fungi (Holick, 2011). Once absorbed in the intestines, D_2 binds to the vitamin D binding protein and enters the circulation and continues through the same pathway as described for D_3 above, with the final product being the active form $1,25(OH)_2D_2$ (Holick, 2011).

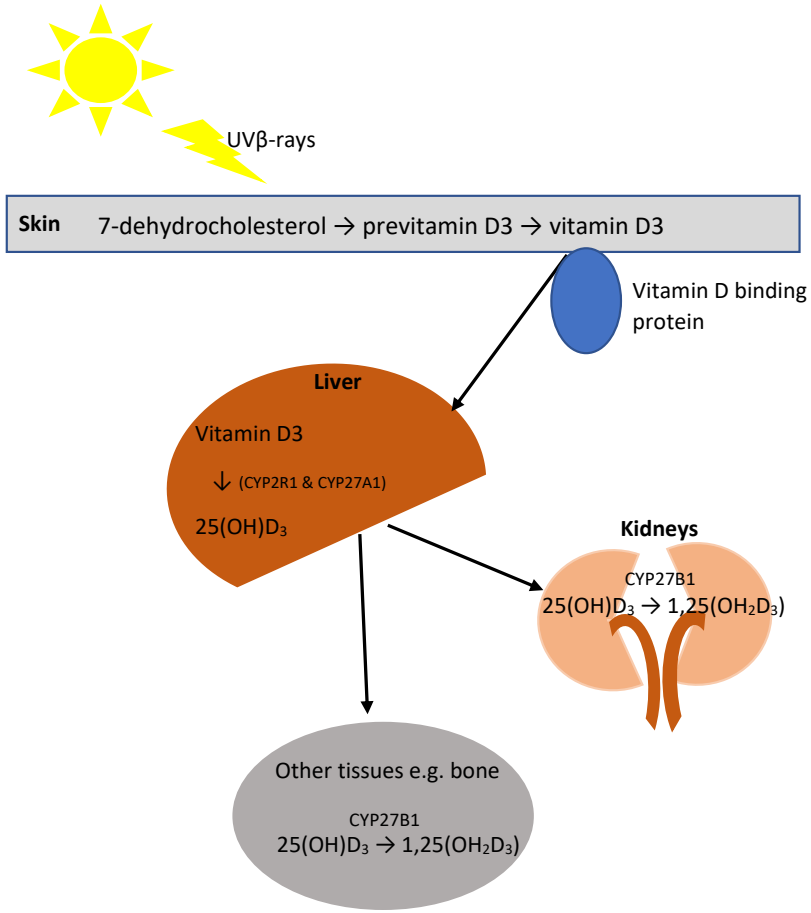


Figure 2: Overview of synthesis and metabolism of vitamin D, adapted from Holick (2011)

One of the earliest recognised roles of vitamin D was the maintenance of calcium and phosphorus homeostasis, supporting bone mineralisation (DeLuca, 2004). Vitamin D, as a hormone, can increase calcium concentration through the mobilization of calcium from bone (DeLuca, 2004). In addition, it supports calcium and phosphate absorption in the intestines and reabsorption of calcium from the distal renal tubules (DeLuca, 2004). The presence of CYP27B1 enzyme in immune cells suggests that vitamin D also has a role in the modulation of the immune system. $1,25(OH)_2D_3$ is known to regulate the innate immune system by stimulating chemokine production, autophagy, and phagolysosomes (Sassi et al., 2018). In addition, it is also known to induce antimicrobial peptides and play a role in microbiota: increasing physical barrier composition, influencing gut microbiota composition, reducing gut permeability, and subsequently reducing inflammation (Sassi et al., 2018). In regard to the adaptive immune response, $1,25(OH)_2D_3$ provides an anti-inflammatory effect, reducing the inflammatory response of the system via the inhibition of type 1 T helper (Th1) and Th17 cells, thus subsequently reducing the production of pro-inflammatory cytokines (e.g., IL-6, IL-2, and TNF-α), while upregulating the activity of the T regulatory Treg and Th2 cells (Sassi et al., 2018).

With the involvement of vitamin D in immune, epithelial, bone, brain and liver function, vitamin D deficiency has been found to be associated with increased risk of infection (Sassi et al., 2018). Furthermore, Vitamin D deficiency has been associated with increased risk of diabetes, sarcopenia and multiple sclerosis (Munger et al., 2004; Palomer et al., 2008; Remelli et al., 2019; Scragg et al., 2004).

2.3.1 Vitamin D deficiency

With vitamin D having a crucial role in supporting bone and immune function, it is important to be able to monitor, and measure an individual's vitamin D status. 25(OH)D is the main circulating vitamin D metabolite, comprising of both D_2 and D_3, and is used as the serum biomarker to measure an individual's vitamin D status (Dirks et al., 2018). $1,25(OH)_2D_3$ is a tightly regulated metabolite and may not be the most

accurate method for determining vitamin D status as it is regulated in a specific reference range (Dirks et al., 2018). Vitamin D concentrations that define deficiency, insufficiency and sufficient levels are still controversial, with countries and experts all defining the adequate and inadequate cut-offs differently (Dirks et al., 2018). The different stages of vitamin D status and the defining concentrations currently used in NZ are summarised below in table 5.

Table 5: Vitamin status and the corresponding s-25(OH)D concentration, (Ministry of Health, 2012).

	Serum 25(OH)D concentration ($nmol \cdot L^{-1}$)
Vitamin D toxicity	>125
Sufficient vitamin D	>50
Insufficient vitamin D	25.0-49.9
Vitamin D mild-moderate deficiency	12.5-24.9
Severe vitamin D deficiency	<12.5

2.3.2 Prevalence of vitamin D deficiency in New Zealand.

The last NZ Adult Nutrition Survey in 2008/09 (N=4721) investigated the vitamin D status, with results identifying a mean s-25(OH)D concentration for NZ adults of 63.0 $nmol \cdot L^{-1}$ (Ministry of Health, 2012). As shown in table 5, in NZ, this is classed as sufficient vitamin D status (Ministry of Health, 2012). In NZ, 5.4% of females were found to be vitamin D deficient (Ministry of Health, 2012).

Investigations of vitamin D status in NZ adults are summarised below in table 6. Definitions for vitamin D status that were used in the studies are the same as those listed in table 5. The exception being, Narang et al. (2020), where the authors defined deficiency as deaseasonalised s-25(OH)D <40 $nmol \cdot L^{-1}$ and Ingra et al. (2018) who defined insufficiency 50-74 $nmol \cdot L^{-1}$. Furthermore, it should be noted that some studies categorise participants as either insufficient or sufficient, while others further break this down to insufficient, deficient and/or very deficient. This creates inconsistencies when comparing prevalence outcomes of the studies.

In NZ, 68.1% of adults had sufficient vitamin D concentration in 2008/09 (Ministry of Health, 2012). In Auckland specifically, 49% of postmenopausal females had insufficient vitamin D levels (Bolland et al., 2007). When considering various ethnic groups only, 40.3% of Middle Eastern females and 16% of South Asian females appear to have sufficient vitamin D levels (Mazahery et al., 2015; von Hurst et al., 2010).

Table 6: Summary of studies investigating the prevalence of vitamin D deficiency in the NZ adult population.

Study	Population	Vitamin D status	Deficiency definition.
(Narang et al., 2020)	Adults living in Auckland, NZ, aged 50-84 (using ViDA study dataset). N=5106	Deficient: 11.9% (607)	Deficient: DS-25(OH)D <40 $nmol \cdot L^{-1}$
(Ingram et al., 2018)	Males and Females, ≥18 years with chronic plaque psoriasis in Auckland NZ. N=101	Sufficient: 23.8% (24) Insufficient: 40.6% (41) Deficient: 29.7% (30) Very deficient: 4.9% (5)	Sufficient: 75-250 $nmol \cdot L^{-1}$ Insufficient: 50-74 $nmol \cdot L^{-1}$ Deficient: 25-49 $nmol \cdot L^{-1}$ Very deficient: <25 $nmol \cdot L^{-1}$
(Mazahery et al., 2015) Part 1	Middle Eastern females aged >20. N= 43	Sufficient: 0 Insufficient: 25.6% (11) Deficient: 46.5% (20) Very deficient:28.0% (12)	Sufficient: >50 $nmol \cdot L^{-1}$ Insufficient: 25-50 $nmol \cdot L^{-1}$ Deficient: 12-25 $nmol \cdot L^{-1}$ Very deficient: <12.5 $nmol \cdot L^{-1}$
(Mazahery et al., 2015) Part 2	Middle Eastern females aged 20-50. N=62.	Sufficient: (>75 $nmol \cdot L^{-1}$): 3.2% (2) Sufficient (50-75 $nmol \cdot L^{-1}$): 37.1% (23) Insufficient: 50.0% (31) Deficient: 9.7% (6).	Sufficient: >50 $nmol \cdot L^{-1}$ and >75 $nmol \cdot L^{-1}$ Insufficient: 25-50 $nmol \cdot L^{-1}$ Deficient: 12-25 $nmol \cdot L^{-1}$ Very deficient: <12.5 $nmol \cdot L^{-1}$
(Bolland et al., 2007)	Healthy postmenopausal females and older men, Auckland, New Zealand. N=1606	Insufficient (females): 49% Insufficient (men): 9%	Insufficient: <50 $nmol \cdot L^{-1}$
(Rockell et al., 2006)	New Zealanders aged 15 years or over. N=2,946	Insufficiency: 48% Deficiency: 3%	Insufficiency: ≤50 $nmol \cdot L^{-1}$ Deficiency: ≤17.5 $nmol \cdot L^{-1}$

(Lucas et al., 2005)	Healthy postmenopausal females living in Auckland, NZ N= 1,606	Deficient (summer): 0-3% Deficient (winter): 6-16% Insufficient (summer): 28-58% Insufficient (winter): 56-74%	Deficient= <25 nmol·L^{-1} Insufficient: <50 nmol·L^{-1}
(von Hurst et al., 2010)	South Asian females living in Auckland, NZ N=228	Sufficient: 16% <25 nmol·L^{-1}: 43%	Sufficient: >50 nmol·L^{-1}
(Ministry of Health, 2012) Findings from the 2008/09 New Zealand Adult nutrition survey.	NZ adults aged >15 years N=4721	Sufficient: 68.1% Insufficient: 27.1% Mild-moderate deficiency: 4.6% Severe deficiency: 0.2%	Defined in table 5.
DS-25(OH)D= deseasonalised serum 25(OH)D.			

Factors that appear to prominently affect both micronutrients are BMI and ethnicity. These factors will be discussed below and should be taken into consideration when analysing vitamin D or iron status of various populations.

2.4 Risk factors impacting iron and vitamin D status

Body composition impacts both s-25(OH)D and iron concentration, due to increased body fat (Nemeth, Rivera, et al., 2004; Wortsman et al., 2000). Furthermore, vitamin D and iron status vary between ethnic groups, with South Asian seen as an at risk population of developing both micronutrient deficiencies (Lim et al., 2020; von Hurst et al., 2010). Diet, particularly low meat intake is associated with decreased iron status but has minimal impact on vitamin D status (Lim et al., 2020; Touvier et al., 2015). Instead, vitamin D is mainly impacted by sun exposure, seasonality, latitude and skin pigmentation (Touvier et al., 2015; Webb et al., 1988). Exercise is positively associated with s-25(OH)D concentration, partially due to increased sun exposure, but is negatively associated with iron concentration due to the increase of exercise induced hepcidin and the cumulative effect of iron loss mechanism during exercise (Brock et al., 2010; Sim et al., 2019).

2.4.1 Body composition

Body composition impacts both s-25(OH)D and iron concentration. Obesity is a low-grade inflammatory condition associated with increased adipose tissue and defined as BMI of ≥30kg·m^2 (Calabro & Yeh, 2007; World Health Organization, 2021). Adipose tissue consists of adipocyte cells which secrete inflammatory factors including TNF-alpha, leptin, adiponectin, resistin, plasminogen activator inhibitor 1 (PAI-1), IL-6 and angiotensinogen (Calabro & Yeh, 2007). Obese individuals tend to have elevated IL-6 concentration when compared to lean individuals (Eder et al., 2009). Furthermore, increased adipose tissue has been linked to an increased storage of vitamin D, resulting in decreased circulating 25(OH)D (Wortsman et al., 2000). As previously mentioned, one of the inflammatory factors released by adipocytes is IL-6, and is known to impact iron concentration through the upregulation of hepcidin concentration (Nemeth, Rivera, et al., 2004).

IL-6 has been shown to upregulate *HAMP* transcription, increasing hepcidin concentrations via the Janus kinase2-signal transducer and activator of transcription-3 (JAK1-STAT3) pathway, leading to sequestration of iron in cells and subsequently reducing blood iron levels (Rauf et al., 2020). This is considered part of the innate immune response where by the sequestering of iron and prevention of iron release into systemic circulation prevented invading bacteria from accessing it for bacterial growth (Rauf et al., 2020). Prolonged elevation in IL-6 will result in sustained increases in hepcidin concentrations that can result in functional ID, where iron turnover (recycling and utilisation) is impaired even if the individual presents with adequate iron stores (Clénin et al., 2015). Other inflammatory cytokines, such as IL-1α and TNF-α, currently appear to have no impact on hepcidin mRNA, however, this is still an area of investigation (Nemeth et al., 2003). IL-6 concentration has been shown to be increased in overweight and obese individuals compared to lean individuals (Gregor & Hotamisligil, 2011). The increase in hepcidin as a result of increased IL-6 and body fat may prevent individuals from accessing or effectively recycling/absorbing iron and may result in the presentation of ID symptoms due to the state of functional ID (Stoffel, El-Mallah, et al., 2020). A similar presentation of ID is observed in individuals presenting with chronic inflammatory diseases. Here the risk of ID may not be a result of insufficient iron intake but is rather from functional ID where the body is unable to access stored iron for normal physiological processes due to the inflammatory driven increases in hepcidin (Clénin et al., 2015).

A large cross-sectional study with NHANES III participants found that increasing BMI was associated with increased inflammation, decreased serum iron and increased Sf, indicating a state of functional ID (Ausk & Ioannou, 2008). Hepcidin concentrations appear to be positively associated with BMI, with two studies reporting a higher BMI was related to increased hepcidin concentration and decreased iron absorption (Stoffel, El-Mallah, et al., 2020; Tussing-Humphreys et al., 2010). With regards to vitamin D, an inverse relationship appears to be evident for BMI. A longitudinal study with participants older than 65 years, found a significant association with higher BMI

and lower s-25(OH)D concentration (Snijder et al., 2005). The Framingham Heart Study produced similar results with decreased s-25(OH)D concentrations associated with higher adiposity (Cheng et al., 2010). Smaller comparative studies identified similar results; with decreased s-25(OH)D and $1,25(OH)_2D_3$ concentrations in obese individuals when compared to non-obese individuals (Parikh et al., 2004; Wortsman et al., 2000). Lower body fat % has been associated with increased changes in s-25(OH)D post consumption of 50,000 IU or 100,000 IU of vitamin D_3 in 62 Middle Eastern females (aged 20-50) (Mazahery et al., 2015).

Prevalence of obesity in NZ is high; in 20019/20, 30.9% of adults above 15 years of age were classed as obese, this is currently estimated to be 1.24 million adults residing in NZ (Ministry of Health, 2020). The combination of a high prevalence of obesity and the inference from research highlighting the association between increased BMI and reduced iron and s-25(OH)D concentration, highlights the risk of developing deficiencies in both micronutrients in this cohort of the population.

2.4.2 Ethnicity

Prevalence of vitamin D deficiency and ID varies between ethnic groups, with South Asian considered as an at risk group in NZ (Beck, Conlon, Kruger, Heath, et al., 2014; Nessvi et al., 2011; von Hurst et al., 2010). The odds of suboptimal iron status in Asians (observed in 41.8% Chinese and 29.1% Indian ethnicity) was 5 times greater than Europeans (Beck, Conlon, Kruger, Heath, et al., 2014). Another study looking at vitamin D status in South Asians (91% from India, 6% Sri Lanka and 3% from Pakistan), found only 16% of the study group had adequate s-25(OH)D concentration (≥50 $nmol \cdot L^{-1}$), suggesting that this group is at a high risk of both micronutrient deficiencies (von Hurst et al., 2010).

Supporting these results, South Asians residing in Auckland NZ, had 1.68 higher odds of developing ID compared to NZ Europeans and other residing ethnicities (Lim et al., 2020). Some potential explanations for this pattern could be explained by the increased body fat percentage, BMI and IL-6 concentrations (inflammation) that was

observed in the South Asians compared to Europeans (Lear et al., 2009). These factors are all known to contribute to increased hepcidin concentration and decreased iron levels (Lim et al., 2020). Alongside this, South Asians consumed meat less frequently when compared to other ethnicities, a factor that may further exacerbate lower iron levels (Lim et al., 2020).

Prevalence of vitamin D deficiency in South Asian, African Americans and Middle Eastern individuals is also thought to be related to sun avoidance, indoor lifestyle, cultural or religious dress requirements and skin pigmentation which is discussed in more detail later in this literature review (Ministry of Health and Cancer Society of New Zealand, 2012; von Hurst et al., 2010).

Between 2006-2018, the total Asian population had nearly doubled in size, with 707,598, ~15.1% of the total population permanently living in NZ in 2018 (Statistics New Zealand, 2018a). The increasing growth of the total Asian population and the high incidence of both iron and vitamin D deficiency, will not only decrease the overall health status of these individuals but also impact the NZ healthcare system. Knowledge of the association between the ethnic group and risk of micronutrient deficiencies is required to help improve and sustain their health status. Furthermore, to improve micronutrient status in both Asian ethnicity and others residing in NZ, it is important to consider the dietary intake of these individuals.

2.4.3 Dietary patterns

Both iron and vitamin D can be obtained from dietary sources, however it is noted that vitamin D is acquired from food sources in minimal quantities when compared to endogenous synthesis following sun exposure (Holick, 2011; Touvier et al., 2015). Therefore, it may be considered that the impact of dietary vitamin D intake on status is minimal. Whereas dietary iron intake has been seen to impact iron status, and may be a primary factor contributing to the increased risk of developing ID (Beck, Conlon, Kruger, & Coad, 2014).

Iron is found in two forms: haem and non-haem (Monsen et al., 1978). Haem iron, mainly found in meat/fish/poultry, is more readily absorbed (15-35%) compared to non-haem iron (1-10%), found in: cereals, vegetables, legumes, fruits and animal products (Monsen et al., 1978; Sharp & Srai, 2007). Haem iron is more bioavailable as its absorbed as an intact molecule through HCP1, compared to non-haem iron, which is predominantly present in ferric form and must be reduced to ferrous form prior to absorption (figure 1) (Sharp & Srai, 2007). Supporting this mechanism, Sf was found to be positively associated with both haem and non-haem iron intake, with haem-iron a stronger predictor of Sf stores than non-haem iron (Fleming et al., 1998; Young et al., 2018). Non-haem iron absorption can be increased through enhancers or decreased through inhibitors that are present in the diet.

A known enhancer of non-haem iron absorption is ascorbic acid (vitamin C) (Diaz et al., 2003; Hurrell et al., 2006). Main inhibitors of non-haem iron absorption are calcium (dairy based products, e.g. milks, yoghurt) and phytates (cereals and legumes) (Beck et al., 2013; Hallberg, Rossander, et al., 1987). Tannins (present in tea and coffee) are also viewed as an iron inhibitor (Fleming et al., 1998; Pynaert et al., 2009), however literature provides mixed results with other studies finding no effect of these inhibitors on non-haem iron absorption (Heath et al., 2001).

Increased consumption of meat/poultry/fish, and therefore haem-iron, is positively associated with increased Sf concentration (Beck et al., 2013; Heath et al., 2001; Lim et al., 2020; Lim et al., 2016). Furthermore, individuals who follow a predominately vegetarian diet and/or low meat intake, low haem-iron, are more likely to be at increased risk of developing ID (Heath et al., 2001; Lim et al., 2020). A predominantly non-haem based diet, classed as vegetarian, is a dietary pattern seen to be increasing in NZ, with one in three individuals identifying as vegetarian (Food Fronteir & Life Health Foods, 2019; Morgan, 2016). Furthermore, 24% of meat eaters between 2019/20 have reduced their meat consumption, potentially placing the NZ population at increased risk of ID (Food Fronteir & Life Health Foods, 2019)

Research supporting a positive association between dietary intake and s-25(OH)D concentration is minimal (Brock et al., 2010). Positive associations are mainly related to either the use of supplemental vitamin D or vitamin D fortified foods (Brock et al., 2010; Van Dam et al., 2007; Zgaga et al., 2011). Most evidence supports sun exposure and seasonality as the main determinant of vitamin D status with no significant relationship between dietary intake and s-25(OH)D concentration (Kühn et al., 2014; Lucas et al., 2005; Touvier et al., 2015).

2.4.4 Other impacts (physical activity, sun exposure, seasonality, latitude, skin pigmentation).

The key determinant in prevention of vitamin D deficiency is skin exposure to UVβ rays and therefore sun exposure (Bolland et al., 2007; Touvier et al., 2015). There is an apparent seasonal variation in s-25(OH)D, with higher concentration occurring during the summer months (November-February), when compared to winter months (August-October) (Bolland et al., 2008; Lucas et al., 2005; Rockell et al., 2006). This is highly related to increased skin coverage (increased clothes worn) in winter and is also largely influenced by latitude of the country and the angle of UVβ rays (Lucas et al., 2005; Rockell et al., 2006). During winter and higher latitudes (further from equator), the angle of the sun is increased (Webb et al., 1988). This will increase the travel distance and filtration of the UVβ rays, decreasing the UVβ rays reaching the earth's surface, which will subsequently reduce the stimulus for vitamin D synthesis (Lucas et al., 2005; Webb et al., 1988). Latitude variation in s-25(OH)D concentrations can be seen between different countries, as well as in New Zealand (Nessvi et al., 2011; Webb et al., 1988). Auckland (North Island) residents tend to have a higher s-25(OH)D concentration, mean of 51.3nmol·L^{-1}, with the city located at a latitude of 37^{o}S (Nessvi et al., 2011). Whereas, residents living in Dunedin (South Island) at 45^{o}S, have lower s-25(OH)D concentration, mean of 45.2 nmol·L^{-1}, demonstrating an inverse relationship between s-25(OH)D concentration and latitude (Nessvi et al., 2011).

Increase in physical activity tends to be positively associated with s-25(OH)D concentration, however this may be a reflection of time spent outdoors and increased sun exposure (Brock et al., 2010; Lucas et al., 2005). Researchers investigating this relationship, tend to include time spent outdoors e.g., gardening, as a determinant of physical activity (Freedman et al., 2013; Lucas et al., 2005). Therefore, physical activity may not be a true determinant of s-25(OH)D concentration but rather a representation of potential sun exposure. Touvier et al. (2015), isolated physical activity by adjusting for sun exposure and discovered a positive association, however more research is required in this area.

Increased physical activity impacts iron status, through the increase in hepcidin concentration as a response to increased IL-6 levels following exercise (Sim et al., 2019). Exercise also increases iron losses through cumulative effects of haemolysis, sweating, haematuria and gastrointestinal bleeding (Sim et al., 2019). Iron deficiency risk is increased in those individuals who are active when compared to individuals with a more sedentary lifestyle (Crouter et al., 2012), however the risk appears particularly heightened in the female athlete population (Auersperger et al., 2013; Di Santolo et al., 2008).

Increased skin pigmentation is also associated with decreased 25(OH)D concentration. Melanin, the skin pigment, has been seen to reduce the absorption of UVβ rays, as such individuals with dark skin tend to have reduced synthesis of vitamin D, and therefore reduced s-25(OH)D concentrations (Clemens et al., 1982; Webb et al., 1988). Observational studies have demonstrated lower s-25(OH)D concentrations in individuals with darker skin pigment compared to individuals with lighter skin pigment (Cairncross et al., 2017; Delshad et al., 2019; Mitchell et al., 2012). Substantial research has been performed on determinants of iron and vitamin D independently, however a small and developing area of research is investigating the interaction between vitamin D, hepcidin and iron, and the potential impact of vitamin D status on iron status. The next section will discuss literature which has investigated this interaction.

2.5 Interaction between vitamin D, hepcidin and iron

Recent research has shown an association between low s-25(OH)D concentration and low iron levels, via the regulation of the hormone hepcidin in both *in vitro* and *in vivo* studies (Bacchetta et al., 2014; Malczewska-Lenczowska et al., 2018; Smith et al., 2017). Vitamin D appears to decrease hepcidin mRNA expression directly and indirectly, which may subsequently result in increased iron levels (Bacchetta et al., 2014; Zughaier et al., 2014). This interaction has been highlighted as a potential method for reducing the risk of developing IDA and associated physiological health outcomes (Bacchetta et al., 2014; Malczewska-Lenczowska et al., 2018). The initial studies on this interaction were first performed *in vitro*, where more detail about interactions and pathways could be explored.

Bacchetta et al. (2014), undertook *in vitro* studies which formed the basis for the interaction between vitamin D metabolites and hepcidin. Treatment with vitamin D metabolites on cultured human and mouse monocytes and hepatocytes decreased *HAMP* mRNA expression by 0.5-fold (Bacchetta et al., 2014). This appeared as the result of a direct regulation and binding of $1\alpha,25(OH)_2D_3$ to VDR present on the *HAMP* promoter gene (Bacchetta et al., 2014). Supporting these findings, treatment of human monocytic cells, THP1, with lipopolysaccharide (LPS) and doses ranging from 5nM-40nM of $1\alpha,25(OH)_2D_3$, decreased hepcidin concentration and increased ferroportin expression (Zughaier et al., 2014). An indirect pathway was also explored, where dose-dependent provision of $1\alpha,25(OH)_2D_3$ reduced both IL-6 and IL-1β inflammatory cytokine production. As such there is a possible association between suppression of hepcidin by vitamin D metabolites due to the suppression of inflammatory cytokines (Zughaier et al., 2014).

2.5.1 Cross sectional studies

A significant increase in the risk of developing anaemia was observed in individuals with s-25(OH)D concentrations <50 $nmol \cdot L^{-1}$ compared to individuals with s-25(OH)D concentration >50 $nmol \cdot L^{-1}$ (Smith et al., 2015). This American US cohort (n=638),

consisted of Caucasian (72%), African American (23%), Asian (5%) and other ethnicities (Smith et al., 2015). Further, it was observed that the risk was greater in African Americans (darker skin pigmentation) compared to Caucasians (lighter skin pigmentation), demonstrating the impact of skin pigmentation on 25(OH)D concentration (Clemens et al., 1982; Smith et al., 2015). Investigations in a different cohort, consisting of 3258 pre- and post-menopausal Korean females, observed that females with lower s-25(OH)D (<29.8nmol·L^{-1}) concentration were at increased risk of IDA (Shin & Shim, 2013).

It was observed in female athletes that those with s-25(OH)D concentration <75 nmol·L^{-1}, had higher (32%) prevalence of ID compared to vitamin D sufficient group (11%) (Malczewska-Lenczowska et al., 2018). Alternatively, they reported that 75% of athletes with ID also had low s-25(OH)D concentrations compared with 48% of athletes with normal iron status and high s-25(OH)D concentrations (Malczewska-Lenczowska et al., 2018). The cross-sectional studies appear to highlight a potential association between a low s-25(OH)D concentration and an increased risk of developing ID/IDA.

2.5.2 Randomised control trials and open label interventions: Supplemental studies

A single arm supplementation study in seven healthy males and females was undertaken requiring consumption of a single oral dose of vitamin D2 supplementation (100,000 IU) (Bacchetta et al., 2014). An increase in s-25(OH)D serum concentration and 34% decrease in serum hepcidin concentration in 24-hours of supplement ingestion was observed (Bacchetta et al., 2014). However, no significant changes were observed in iron biomarkers (Sf concentration, iron binding capacity or sTfR) (Bacchetta et al., 2014). Even though changes have been observed in hepcidin concentration, more prolonged studies may be required to investigate whether vitamin D impacts long term iron status and regulation.

A one-time oral dose of vitamin D3 (250,000 IU) was provided to twenty-eight healthy adults, predominately females and of Caucasian ethnicity, aged 18-65 years, to investigate the impact on hepcidin, inflammatory markers and Sf concentration (Smith et al., 2017). Following one week after supplement consumption, hepcidin concentrations had decreased by 73% in the supplementation group compared to placebo group, with no significant changes in pro-inflammatory cytokines (IL-6, IL-8, IL-1β and monocyte chemoattractant protein-1 (MCP-1)) or Sf concentrations (Smith et al., 2017). These results tend to favour the direct regulation of hepcidin via vitamin D supplementation, due to the reductions occurring independent of inflammatory cytokines.

The impact of vitamin D3 supplementation was then explored in 50 premenopausal females aged 19-49 years, with low iron stores (Sf levels <20µg·L-1) over an 8-week period (Fuzi & Mushtaq, 2019). Participants consumed iron fortified cereals (containing 9mg of Fe) either with vitamin D3 supplementation (1,500 IU) or placebo daily (Fuzi & Mushtaq, 2019). A significant increase was observed in haemoglobin concentration and haematocrit level in the group receiving vitamin D3 supplementation, however no significant changes were observed in Sf concentration (Fuzi & Mushtaq, 2019). Contrary to previous studies, no significant changes were observed in the hepcidin concentration (Fuzi & Mushtaq, 2019). One possible explanation for the different results may be due to involving participants with low iron stores, compared to healthy adults, as hepcidin levels are suppressed during periods of low iron stores to help increase iron absorption and release, increasing overall iron status (Rauf et al., 2020). This study does however highlight a possible intervention for iron deficient females through the combination of vitamin D supplementation and iron fortified foods to help improve iron status. Compliance with iron supplementation is low, due to adverse gastrointestinal events (Cancelo-Hidalgo et al., 2013). Therefore, this approach may be a more desirable for treatment for iron deficiency.

In NZ, it has been frequently demonstrated that there is an increased risk of ID and vitamin D deficiency, particularly in the South Asian population. However, no research

has been undertaken investigating the interaction between the micronutrients and the potential mediator, hepcidin, in the different ethnic groups currently residing in NZ.

2.5.3 Where to now?

Previous research has investigated the impact of vitamin D supplementation on iron status and hepcidin concentration in the short-term (days to few weeks) (Bacchetta et al., 2014; Zughaier et al., 2014), and observed increasing prevalence of ID/IDA in individuals who have low s-25(OH)D concentrations in various populations (Malczewska-Lenczowska et al., 2018; Shin & Shim, 2013; Sim et al., 2010). However, there are still limited published studies investigating this association, with no published studies investigating BMI, sun exposure and ethnicity as associated co-factors.

Further research is required to gain a better understanding between the interaction of vitamin D, hepcidin, iron status, potential lifestyle and genetic determinants. Undertaking studies in a healthy adult population may aid an understanding the determinants of these micronutrient deficiencies, which can be translated to proactive interventions that may be utilised by health care professionals and the public to help with the management and treatment of these micronutrient deficiencies.

2.6 Conclusion

Iron and vitamin D deficiencies are prevalent in the NZ population, with an increased risk for those of South Asian and Middle Eastern ethnicities and in individuals with an increased BMI. Hepcidin is a known regulator of iron levels and emerging research is also providing evidence of the role of vitamin D in hepcidin regulation, therefore, further exploration of the interaction between vitamin D, hepcidin and iron in the NZ population, particularly the South Asian and Middle Eastern ethnic groups is essential. In-depth knowledge of this association can help provide a basis for management and prevention

of the two micronutrient deficiencies, thus improving overall health status of the NZ population.

Chapter 3: Research study manuscript

3.0 Abstract

Background: An association has been identified between vitamin D and iron levels. Increased concentration of serum 25(OH)D has been shown to decrease hepcidin concentration, through the downregulation of *HAMP* transcription, which may lead to increased concentration of serum ferritin. Female South Asians are at risk of both iron and vitamin D deficiency; therefore it is essential to investigate the associations between these two micronutrients in an at risk population.

Aim: To investigate the associations between serum 25(OH)D, hepcidin and iron status in premenopausal females living in Auckland New Zealand (NZ). Secondary aim is to investigate potential determinants of vitamin D status in premenopausal females.

Methods: Pre-menopausal females aged 18-45 years, living in Auckland, NZ, participated in a cross-sectional study. Body composition was measured using bioelectrical impedance and included height, weight and body fat %. Serum 25(OH)D, inflammatory (CRP and IL-6), and iron biomarkers (serum ferritin, haemoglobin, soluble transferrin receptor and hepcidin) were measured. A series of validated questionnaires were completed to assess demographic and lifestyle factors, including medical history, skin colour, sun exposure and dietary iron intake. Statistics analysis was undertaken using IBM SPSS.

Results: Of the 160 participants, 60 were NZ European, 67 were South Asian and 33 were from the 'other' ethnic groups. South Asians had significantly higher body fat % and IL-6 concentration (38.34% and 1.66 $pg \cdot mL^{-1}$, respectively), compared to NZ Europeans, (27.49% and 0.63 $pg \cdot mL^{-1}$ respectively, $p<0.001$). South Asians had significantly lower serum 25(OH)D concentrations compared to NZ Europeans (33.59 $nmol \cdot L^{-1}$ vs 74.84 $nmol \cdot L^{-1}$, $p<0.001$). In NZ Europeans, higher serum 25(OH)D concentration were seen in those with lower (≤3.5nM) hepcidin concentration $p=0.0046$. Conversely, in South Asians, higher serum 25(OH)D concentration was seen in those with higher (>3.5nM) hepcidin concentrations, $p=0.038$. There was no significant association in the 'other' ethnic group and no associations between

serum 25(OH)D and iron status/serum ferritin. Key determinants of serum 25(OH)D were ethnicity, age and body fat %.

Conclusion: The positive relationship between s-25(OH)D and hepcidin concentration in the South Asian women was unexpected, although possibly explained by significantly higher IL-6 concentration, body fat % and lower s-25(OH)D concentration. Ethnicity, age and body fat % were identified as key determinants of s-25(OH)D concentration.

3.1 Introduction

Iron deficiency anaemia (IDA) impacted ~29% of non-pregnant females (aged 12-50 years) globally in 2011 (World Health Organization, 2015). Recent research in New Zealand (NZ), Auckland, has observed high rates of iron deficiency (ID), particularly in the NZ South Asian population, with 55.8% of premenopausal females classed as ID (serum ferritin (Sf) <30 $ug \cdot L^{-1}$; haemoglobin (Hb) <120 $g \cdot L^{-1}$ or ≥120 $g \cdot L^{-1}$) (Lim et al., 2020). Iron supports oxygen transport and energy production, therefore a deficiency can result in reduced physical performance, cognitive function, health and wellbeing of the individual presenting with ID (Lopez et al., 2016).

To ensure iron levels are maintained in a normal range, hepcidin a peptide hormone, has been shown to regulate endogenous iron stores (Nemeth, Tuttle, et al., 2004). Encoded by the *HAMP* gene, hepcidin regulates iron homeostasis by controlling iron absorption, movement from hepatocytes and recycling from macrophages via the body's only known iron exporter channel, ferroportin (Nemeth, Tuttle, et al., 2004). When hepcidin concentration is increased, degradation and internalisation of ferroportin channels is increased resulting in reduced movement of iron into circulation (Nemeth, Tuttle, et al., 2004). Hepcidin appears to be suppressed when iron stores are low and in hypoxia when there is an increased erythropoietic demand (Nemeth, Tuttle, et al., 2004). Whereas it appears to be stimulated when an individual has high iron stores or presents with increased inflammation (Nemeth, Rivera, et al., 2004). Research has identified that the inflammatory cytokine, interleukin-6 (IL-6), is key in inducing the elevated hepcidin levels during inflammation (Rauf et al., 2020).

More recently research has demonstrated that increased serum 25-hydroxyvitamin D (s-25(OH)D) concentrations may also decrease hepcidin concentration via the suppression of the *HAMP* mRNA expression (Bacchetta et al., 2014).

Vitamin D is mainly acquired from skin exposure to ultraviolet β (UVβ) rays from the sun, therefore vitamin D status of the individual is strongly impacted by sun exposure, seasonality, latitude and skin pigmentation (Clemens et al., 1982; Touvier et al., 2015). In 2008/09, 27.1% of adults over the age of 15 years living in NZ had insufficient vitamin D levels (s-25(OH)D, 25-49nmol·L-^{1}) and 4.9% were vitamin D deficient (s-25(OH)D <25 nmol·L^{-1}) (Ministry of Health, 2012). Even though vitamin D is found in some foods, the impact of dietary intake on vitamin D status is minimal (Touvier et al., 2015). Conversely, iron dietary intake has been shown to strongly influence iron status. A vegetarian diet consisting mainly of non-haem iron dietary sources is associated with lower iron status due to the decreased iron absorption rates when compared to haem iron (animal-based sources) (Lim et al., 2020). Therefore, it is essential to consider individuals dietary intakes when assessing iron status.

Obesity, defined as ≥30kg·m^{2}, and increased body fat % is associated with an increased presence of the inflammatory cytokine, IL-6, a known up-regulator of hepcidin concentration (Eder et al., 2009; Nemeth et al., 2003; World Health Organization, 2021). Increased inflammatory levels (typically assessed by the presence of CRP and IL-6) have been observed in individuals with an increased ratio of android fat/total body fat and was subsequently associated with increased serum hepcidin concentration and impaired iron homeostasis (Stoffel, El-Mallah, et al., 2020). Vitamin D is a fat-soluble vitamin, therefore increased adipose tissue results in increased sequestration of 25(OH)D, subsequently resulting in decreased s-25(OH)D concentrations (Wortsman et al., 2000). Increased body fat % has been identified to decrease response to oral vitamin D supplementation, resulting in decreased s-25(OH)D concentrations (Mazahery et al., 2015). Prevalence of obesity in NZ is high, with 30.9% of adults of NZ adults considered to be obese in 2019/2020 (Ministry of

Health, 2020). Therefore, when considering vitamin D and iron status in population studies, body composition must be considered as a co-factor.

Female South Asians appear to have a higher prevalence of both micronutrient deficiencies, research identified that South Asians are 1.68 times more likely to develop ID compared to NZ Europeans (Lim et al., 2020). Research has also identified that that South Asians have lower s-25(OH)D (37.0nmol·L^{-1}) compared to NZ Europeans (57.9 nmol·L^{-1}) (Nessvi et al., 2011). With the total Asian population nearly doubling in size between 2006-2018, with 707,598 permanently living in NZ in 2018 (Statistics New Zealand, 2018a), the importance of investigating the association between vitamin D and iron status in different ethnic cohorts to help manage micronutrient deficiencies and health is of high priority.

The association between vitamin D, hepcidin and iron status in premenopausal females has not been investigated in NZ. It's important to investigate the possible association between the two micronutrients taking into consideration the impact of the regulating hormone, hepcidin and lifestyle factors that could affect both iron and vitamin D status. Therefore, this study aims to investigate the association between vitamin D status, hepcidin and iron status and potential determinants in premenopausal females living in Auckland, NZ.

3.2 Methodology

This was a cross-sectional study in pre-menopausal females living in Auckland, New Zealand. Data collection commenced in July 2018 and concluded in July 2019.

3.2.1 Participants and recruitment

Participants were recruited through posters, flyers, email contacts, newspaper articles, advertisements, social media and community groups. Screening was preformed via a questionnaire prior to participants taking part in the study.

Inclusion criteria was: healthy premenopausal females, aged between 18-45 years (participants over the age of 45 were included if an active menstrual cycle was confirmed) and of South Asian, Middle Eastern or Caucasian ethnicity living in Auckland, NZ. Participants were not recruited if they were: pregnant or breastfeeding, had been pregnant in the last year, had a chronic health condition which may impact iron status (e.g. menorrhagia, coeliac disease or kidney disease), donated or transfused blood in the last 6 months or had been consuming iron supplements (>20mg elemental iron), 3-4 times a week in the 3 months prior to the trial.

Ethics approval was obtained from Massey University Human Ethics Committee: Southern A (18/12).

Prior to data collection, the participants were provided with an information sheet that informed them of the study procedures. They were allowed time to ask any questions related to the study before signing the consent form. Participants were randomly allocated a study identification number to ensure anonymity.

The following formula was used to calculate the sample size: N = [Z2 p(1-p)]/d2. A 95% level of confidence, 5% precision and based on an estimation of 12.1% prevalence of ID in the population of interest (University of Otago and Ministry of Health, 2011) provided a corresponding Z-score of 1.69. A sample size of 162 participants was determined to be adequate.

A single data collection session was completed at Massey University's Auckland campus or a researcher approved community centre. Data collected from the participants included: body composition analysis, venous blood collection and a series of online questionnaires.

3.2.2 Body composition measurements

Height (to the nearest cm) was measured using a stadiometer by a trained researcher following a standardised procedure. Height was recorded three times and then averaged. Body composition was measured using a bioelectrical impedance analysis (InBody 230). The data recorded included total body weight and body fat percentage. BMI was calculated using participants weight (kg) divided by their height (M) squared.

3.2.3 Blood sample analysis

Haemoglobin was measured using a HemoCue Hb 201+ system via a single finger prick blood sample. This was done because this method is based on whole blood and did not require freezing. Remaining blood samples were collected by a trained phlebotomist who collected a venous blood sample from the antecubital vein while the participant was in a seated and rested position. The blood samples were collected using a sterile 21-gauge flashback needle into two 5-mL SST gel separator tubes 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^{o}C, 1000rcf for 10 minutes. The serum supernatant was removed and divided into 1-ml aliquots and stored at -80^{o}C until analysis. Once all blood sample were collected, serum was sent to Auckland LabPlus and Canterbury Health laboratory for markers of iron status, inflammation (C-Reactive Protein (CRP)), Sf, soluble transferrin receptor (STfR) and s-25(OH)D. Serum interleukin-6 (RD Systems Human IL-6 Immunoassay high sensitivity ELISA, D6050) and hepcidin (RD System Human Hepcidin Immunoassay ELISA, HDP250) were analysed via commercially available ELISA kits at the Human Nutrition Research Unit at Massey University, Auckland.

3.2.4 Questionnaires

Questionnaires was conducted online using the software Qualtrics on iPads or on personal mobile devices of the participants. Information obtained included demographic (e.g. age, ethnicity) and medical history (e.g. medications, previous chronic disease, previous iron diagnosis and use of iron supplements).

Participants were asked about their menstrual blood loss and blood donation as both are known to affect iron status (Heath et al., 1999). This was then followed by the Fitzpatrick survey for skin colour (Fitzpatrick, 1988) and the sun exposure survey adapted from von Hurst, Stonehouse & Coad, 2010 (von Hurst et al., 2010). Physical activity levels were assessed via the New Zealand Physical Activity Questionnaire Short Form (NZPAQ-SF) (SPARC et al., 2004). The iron food frequency questionnaire was completed to estimate iron related dietary intake of participants (Beck, Conlon, Kruger, Heath, et al., 2014).

3.2.5 Data handling and statistical analysis

Statistical analysis was carried out using SPSS statistics 27 (IBM). All data was tested for normality using the Kolmogorov-Smirnov and Shapiro Wilk test. Non-normally distributed data was log transformed to obtain normality. Normally distributed data is reported as mean ± standard deviation (SD) or geometric mean (95% confidence interval). Data that was not normality distributed is reported as median (25th, 75th percentiles). Categorical data is reported as count and percentage.

Comparison between participants and ethnic groups for parametric data was conducted through One-Way ANOVA, followed by Tukeys post hoc tests to identify where the differences occurred. Comparisons between non-parametric data was conducted through Kruskal Wallis test. For identified significant differences, Mann-Whitney U was conducted using NZ European as reference category and a Bonferroni correction (0.05/2=0.025). Pearson's Chi-squared test was used to compare categorical variables, with the expected cell count for each cell ≥0.05 and all variables considered to be independent. To meet the expected cell count for Chi square test, categorical variables, seasonality, skin colour, sun exposure and BMI categories were collapsed into two groups. Sf cut-off value for ID is <30 $\mu g \cdot L^{-1}$ as this is an indicator of stage one ID and is a more sensitive marker for identifying ID (Clénin et al., 2015).

Spearman's correlation co-efficient tests were conducted to check for correlations between independent variables. A p-value of ≤0.05 was considered significant. Effect size (r) value of 0.1, 0.3 and 0.5 represents small, medium and large size effect, respectively.

Analysis of covariance (ANCOVA) was used to compare serum 25(OH)D (s-25(OH)D) across different iron parameter categories (hepcidin, ferritin, Hb, and iron status) while adjusting for possible covariates where appropriate (IL-6, body fat %, log-ferritin and season of enrolment). The interaction effect of covariates on s-25(OH)D and iron parameters were examined. Because we found a trend for an interaction effect of ethnicity on the relationship between s-25(OH)D and hepcidin (p=0.06), all analyses were stratified by ethnicity and the results are reported for each ethnic group separately. A hepcidin stratification cut-off point of ≤3.5nM and >3.5nM was used. This is based on the median hepcidin concentration of our cohort and hepcidin concentration ≤3.09nM was identified to be the cut-off point for increased iron absorption rates (Galetti et al., 2021).

Multiple linear regression was conducted to investigate predictors of s-25(OH)D. As s-25(OH)D was the dependant variable and non-normally distributed, it was square root transformed to meet assumptions. All residuals were independent as tested by Durbin Watson test, with values between 1-3. The residuals were normally distributed as confirmed by histogram and Kolmogorov-Smirnov normality test. Collinearity tolerance demonstrated no multicollinearity which was confirmed by variance inflation ≤10 and tolerance statistics ≥0.2.

3.3 Results

Participant characteristics

Of the 170 females recruited, 10 participants were excluded: 5 had no blood samples, one had s-25(OH)D concentration of 264nmol·L-1 and 4 bloods were unmatched to questionnaire, therefore a total of 160 were included in the final analysis for this study (presented in figure 3). Out of the 160 participants, 15 donate blood, with 6 of those participants donating blood in the last year. Of those included in the final analysis, 51 participants (32%) reported irregular menstrual bleeds (regular menstrual bleed are every 24-34 days).

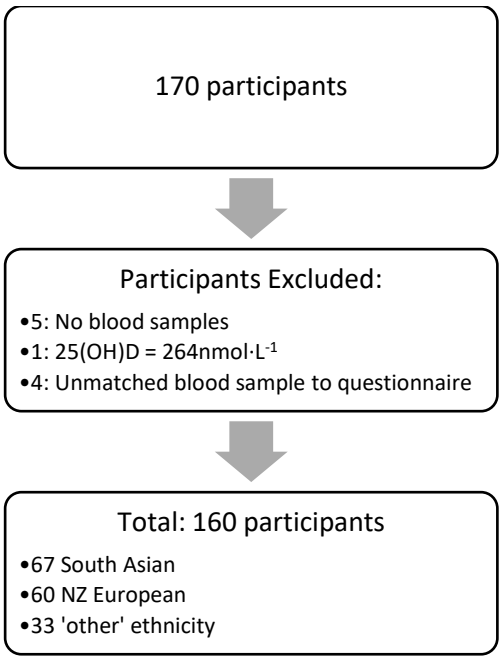


Figure 3: Flow diagram of participant numbers included and excluded from study

Characteristics of participants are stratified according to their ethnicity, summarised demographics are presented in table 7. South Asians were the largest ethnic group compromising of 67 (42%) participants, followed by NZ European (n=60; 38%) and 'other' ethnic group (n=33; 21%). The 'other' ethnic group included Middle Eastern, other Asian, Māori and Pacifica. South Asians were significantly older compared to NZ Europeans (p<0.001). Of all participants, 67% mostly/often enjoy spending time in the sun, with only 33% reporting sometimes/never. Blood samples were collected across the year, with the majority of South Asians (56%) enrolled during summer/autumn,

and most of the NZ Europeans (52%) enrolled during winter/spring. Meat intake included all poultry, red meat (e.g., venison, lamb, beef), pork, game meats, processed meats and seafood, and the number/frequency of servings per week is reported. NZ Europeans had significantly higher meat intake per week compared to South Asians and 'other' ethnic groups (p=0.002 and p=0.011, respectively). South Asians had significantly higher BMI and body fat % compared to NZ Europeans.

Table 7: Participant demographics and body composition for the total population and stratified by ethnic group.

Variables	**Total** n=160	**NZ European** n=60	**South Asian** n=67	**Other**[f] n=33	***p*-value**[K]
Age (years)[a]	26 (22, 36)	23 (20,30)	35 (27, 40)[e]	23 (21,28)	<0.001
Skin colour[b]					
Reddish/very pale/pale	82 (52%)	54 (66%)	9 (11%)	19 (23%)	<0.001
Light/ dark brown	76 (48%)	5 (7%)	57 (75%)	14 (18%)	
Sun exposure[b]					
Mostly/Often	106 (67%)	54 (51%)	31 (29%)	21 (20%)	<0.001
Sometimes/Never	53 (33%)	6 (11%)	35 (66%)	12 (23%)	
Season of enrolment[b]					
Summer/Autumn	71 (44%)	14 (20%)	40 (56%)	17 (24%)	<0.001
Winter/Spring	89 (56%)	46 (52%)	27 (30%)	16 (18%)	
Meat intake (p/w)[a]	7 (2, 11)	8 (5, 11)	3 (0, 9)[e]	11 (7, 16)[e]	<0.001
Anthropometrics and body composition					
BMI[c] (kg/m^2)	24.3 (23.6, 25.0)	22.9 (22.1, 23.7)	25.8 (24.7, 27.2)[e]	23.8 (21.9, 25.9)	0.001
BMI categories[b,g]**:**					
Underweight/ Healthy-weight	95 (59%)	44 (46%)	30 (32%)	21 (22%)	0.004
Overweight/Obese	65 (41%)	16 (25%)	37 (57%)	12 (19%)	
Body fat %[d]	33 (9.8)[h]	27 (7.7)	38 (8.8)[e]	31 (9.4)	<0.001

<32%[e]	77 (48%)	44 (57%)	14 (18%)	19 (25%)	<0.001
≥32%[e]	83 (52%)	16 (19%)	53 (64%)	14 (17%)	

[a] Median (25th, 75th centile).
[b] Count (percentage)
[c] Geometric mean (95th confidence interval).
[d] Mean ± SD [e] Based on median.
P/w: Number/frequency of servings per week.
[e] Significantly different to NZ Europeans.
[f] 'other' ethnic groups includes: Middle Eastern, other Asian, Māori and Pacifica.
[g] Underweight: <18.50, healthy weight: 18.50-24.99, overweight: 25.00-29.99, obese: ≥30.00.
[h] total body fat % was non-normally distributed data, however was reported as Mean ± SD for consistency. The median value was 31.13 (29.64, 32.71)
[K] Kruskal Wallis test was conducted with p=0.05 as significant, followed by Mann-Whitney U using NZ/European as reference category and a Bonferroni correction (0.05/2=0.025) to identify where the difference occurred. Pearson's Chi-squared test was used to compare categorical variables.

Table 8 summarises the biomarkers for the total sample (n=160) and is stratified according to ethnic groups. The median s-25(OH)D for the total population was 54 (33, 73) nmol·L^{-1}. Ninety-four (59%) of the participants had concentrations ≥50 nmol·L^{-1}, 39 (24%) of participants had s-25(OH)D concentration between 26-49nmol·L^{-1} and 27 (17%) of participants had s-25(OH)D concentration ≤25nmol·L^{-1}. Of the total sample, 71 (44%) of participants had sufficient iron stores (Sf ≥30µg·L^{-1} and Hb ≥120 g·L^{-1}) and 89 (56%) of participants had insufficient iron stores (Sf <30µg·L^{-1} and Hb <120 g·L^{-1} or ≥120 g·L^{-1}). Participants who had CRP concentration ≥5 mg·L^{-1}, had a correction factor used to adjust Sf values (Bui et al., 2012).

South Asians had significantly lower s-25(OH)D (p<0.001) and Hb (p<0.001) concentrations and significantly higher IL-6 (p<0.001) concentrations when compared to the NZ European cohort. Furthermore, NZ Europeans had significantly higher s-25(OH)D (p<0.001) concentrations compared to 'other' ethnic groups. There was no significant difference in ferritin, hepcidin, sTfr and CRP concentrations between the different ethnic groups.

Table 8: Iron, vitamin D, hepcidin and inflammatory biomarkers for the total population and stratified according to ethnic groups.

Biomarker	Total	NZ European N=60	South Asian N=67	Others[j] N=33	*p*-value[l]
Haemoglobin ($g \cdot L^{-1}$)[a]	131 (13)	137 (11)	124 (13)[f]	133 (10)	<0.001
Serum Ferritin ($ug \cdot L^{-1}$)[b]	24 (21, 28)[h]	25 (21, 30)	20 (16, 25)	31 (22, 45)	0.149
Hepcidin (nM)[b]	4.3 (3.8, 4.9)[h]	4.2 (3.4, 5.1)	4.4 (3.6, 5.5)	4.6 (3.5, 6.0)	0.947
>3.5[d]	78 (50%)	30 (39%)	32 (41%)	16 (21%)	0.996
≤3.5[d]	79 (50%)	30 (38%)	33 (42%)	16 (20%)	
Soluble Transferrin receptor ($mg \cdot L^{-1}$)[c]	2.9 (2.5, 3.5)	2.8 (2.4, 3.4)	3.0 (2.6, 3.7)	2.6 (2.3, 3.3)	0.077
Iron status[g]					0.239
Sufficient[d]	71 (44%)	28 (39%)	25 (35%)	18 (25%)	
Insufficient[d]	89 (56%)	32 (36%)	42 (47%)	15 (17%)	
Interleukin-6 ($pg \cdot mL^{-1}$)[c]	1.1 (0.6, 1.7)	0.6 (0.5, 1.0)	1.7 (1.3, 2.5)[f]	0.8 (0.6, 1.1)	<0.001
>1.1 [e,d]	71 (45%)	11 (16%)	53 (75%)	7 (10%)	<0.001
≤1.1[e,d]	86 (55%)	49 (57%)	12 (14%)	25 (29%)	
C-Reactive protein[c]	0 (0,3)	0 (0, 3)	0 (0,4)	0 (0,0)	0.102
s-25(OH)D ($nmol \cdot L^{-1}$)[b]	48 (44, 53)[h]	75 (69, 81)	34 (29, 38)[f]	44 (37, 52)[f]	<0.001
≥50$nmol \cdot L^{-1}$[d]	94 (59%)	57 (61%)	22 (23%)	15 (16%)	<0.001
26-49$nmol \cdot L^{-1}$[d]	39 (24%)	3 (8%)	24 (62%)	12 (31%)	
≤25$nmol \cdot L^{-1}$[d]	27 (17%)	0	21 (78%)	6 (22%)	

[a] Mean ± SD
[b] Geometric mean (95% Cl)
[c] Median (25^{th}, 75^{th} centile).
[d] Count (percentage).
[e] Based on median.
[f] Significantly different to NZ Europeans.
[g] Sufficient: Serum ferritin ≥30$ug \cdot L^{-1}$ and Hb ≥120 $g \cdot L^{-1}$. Insufficient; Serum ferritin <30ug·L-1 (Hb <120 $g \cdot L^{-1}$ or ≥120 $g \cdot L^{-1}$)
[h] The data was non-normally distributed, however to keep table consistent was reported as geometric mean. The median values are: serum ferritin of 26 (14, 44), hepcidin of 3.5 (1.6, 6.4), and s-25(OH)D of 54 (33, 73).
[l] Kruskal Wallis test and one-way ANOVA was conducted with *p*=0.05 as significant, followed by Mann-Whitney U or Tukeys post hoc test using NZ/European as reference category and a Bonferroni correction (0.05/2=0.025) to identify where the difference occurred. Pearson's Chi-squared test was used to compare categorical variables.
[j] other ethnic groups includes: Middle Eastern, other Asian, Māori and Pacifica.

Relationship between serum 25(OH)D, iron parameters, inflammatory markers and body fat %:

Table 9 reports correlations between biomarkers, s-25(OH)D, hepcidin, ferritin, and known related biomarkers stratified according to ethnic groups. Hepcidin concentration was negatively correlated with s-25(OH)D concentration in NZ Europeans (p=0.02, medium effect size). In the South Asian participants, hepcidin was positively correlated to s-25(OH)D (p=0.049, small to medium effect size), for the r values (effect size) please refer to table 3. Hepcidin positively correlated with ferritin in all ethnic groups ($p \leq 0.05$, large effect size) and negatively with sTfr in all ethnic groups ($p<0.005$, medium to large effect size). Ferritin was negatively correlated with sTfr in NZ Europeans and South Asians ($p \leq 0.001$, medium to large effect size) and positively associated with haemoglobin in South Asians ($p<0.05$, small to medium effect size).

Table 10 reports correlations between body fat %, IL-6, s-25(OH)D, hepcidin and ferritin stratified according to ethnic groups. Body fat percentage was positively correlated with IL-6 concentration in both South Asians and NZ Europeans (r=0.30, p=0.017 and r=0.254, p=0.050 respectively).

Table 9: Correlations between s-25(OH)D, hepcidin, ferritin and known determinants stratified according to ethnic groups.

Variables	NZ European						South Asian						Others					
	s-25 (OH)D		Hepcidin[a]		Ferritin		s-25(OH)D		Hepcidin[a]		Ferritin		s-25(OH)D		Hepcidin[a]		Ferritin	
	CC	*P*-value	CC	*P*-value	CC	*P*-value	CC	*P*-value	CC	*P*-value	CC	*P*-value	CC	*P*-value	CC	*P*-value	CC	*p*-value
Hepcidin	-0.29	0.02	-	-	-	-	0.25	0.049	-	-	-	-	0.01	0.96	-	-	-	-
Ferritin	-0.05	0.69	0.43	0.001	-	-	0.08	0.55	0.78	<0.001	-	-	0.06	0.72	0.49	0.005	-	-
Soluble transferrin receptor	-0.16	0.22	-0.33	0.010	-0.40	0.001	-0.21	0.09	-0.54	<0.001	-0.64	<0.001	0.01	0.94	-0.40	0.023	-0.14	0.46
Haemoglobin	-0.15	0.25	0.15	0.26	0.17	0.19	0.22	0.08	0.30	0.016	0.25	0.045	-0.20	0.26	-0.003	0.99	0.08	0.64
C-reactive protein	0.20	0.47	-0.03	0.85	-0.09	0.49	0.14	0.26	0.04	0.74	-0.02	0.88	0.22	0.23	0.096	0.60	-0.09	0.62
Interleukin-6	0.18	0.16	-0.01	0.95	-0.04	0.77	-0.08	0.52	-0.13	0.29	-0.79	0.71	0.02	0.92	0.08	0.66	0.18	0.33

CC: correlation coefficient.
[a] categorical (>3.5 or ≤3.5).

Table 10: Correlations between body fat %, IL-6, s-25(OH)D, hepcidin and ferritin stratified according to ethnic groups.

Variables	NZ European		South Asian		Others	
	Body fat %		Body fat %		Body fat %	
	CC	*P*-Value	CC	*P*-Value	CC	*P*-Value
Inetrleukin-6	0.25	0.050	0.30	0.017	0.22	0.92
s-25(OH)D	-0.15	0.27	-0.08	0.52	0.02	0.92
Hepcidin[a]	0.10	0.46	0.07	0.60	0.14	0.44
Ferritin	0.17	0.18	-0.02	0.89	0.02	0.91

CC: correlation coefficient.
[a] categorical (>3.5 or ≤3.5).

Table 11 summarises the association of s-25(OH)D with hepcidin, ferritin, haemoglobin and iron status stratified by ethnicity. There was a trend for an interaction effect of ethnicity on the association of s-25(OH)D with hepcidin (p=0.06). In NZ Europeans higher s-25(OH)D concentrations, 79.68 (71.49, 88.81) nmol·L^{-1}, were seen in those with lower (≤3.5nM) hepcidin concentrations. While NZ Europeans with lower s-25(OH)D concentrations, 70.29 (63.11, 78.28) nmol·L^{-1}, tended to have higher hepcidin concentrations (>3.5nM) p=0.046 (figure 4a). Conversely in South Asians, higher s-25(OH)D concentrations, 38.17 (30.70, 47.46) nmol·L^{-1}, was seen in those with higher (>3.5nM) hepcidin concentrations, compared to South Asians presenting with lower (≤3.5) hepcidin concentrations, 29.43 (24.82, 34.88) nmol·L^{-1}, p=0.038 (figure 4b). No association was observed between s-25(OH)D and hepcidin in the 'other' ethnic group (figure 4c) and between s-25(OH)D and other iron parameters in all ethnic groups (p>0.05).

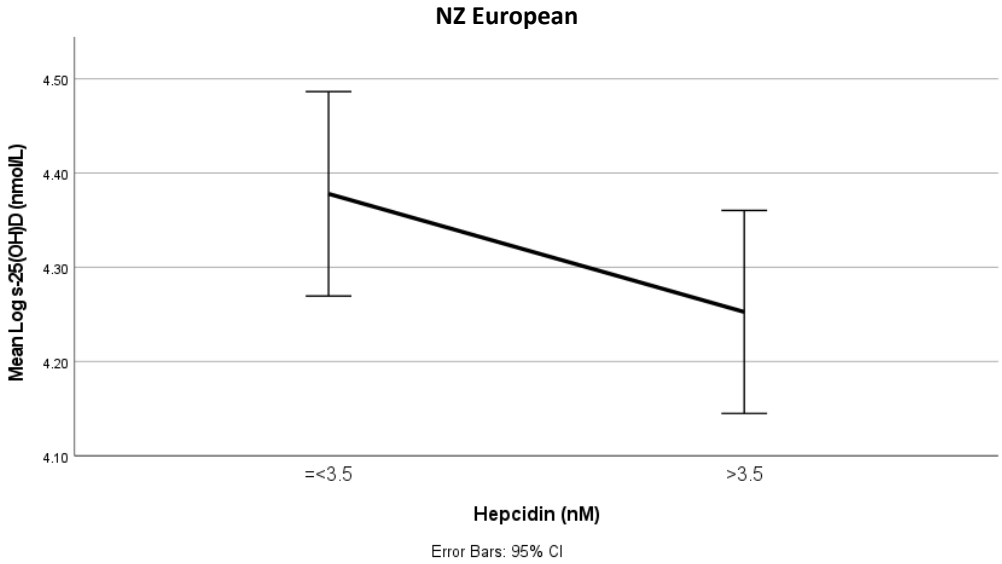
Table 11: Associations between s-25(OH)D, hepcidin, serum ferritin, haemoglobin and iron status, stratified according to ethnic groups.

	s-25(OH)D, nmol·L^{-1}			
	Total	**NZ EU**	**South Asian**	**Others[g]**
Hepcidin (nM) [c]				
≤3.5	47 (40, 53)[f]	80 (71, 89)	29 (25, 35)	44 (33, 58)
>3.5	50 (44, 57)[f]	70 (63, 78)	38 (31, 47)	45 (35, 58)
p-value[e]	0.380	0.046	0.038	0.849
Serum Ferritin (µg·L^{-1}) [c]				
<30	46 (41, 52)[f]	74 (66, 84)	34 (29, 40)	40 (31, 53)
≥30	50 (44, 58)[f]	75 (69, 83)	33 (25, 43)	49 (38, 61)
p-value[e]	0.498	0.927	0.799	0.173
Haemoglobin (g·L^{-1}) [b]				
<120	34 (24, 61)	93 (69, 97)	28 (21, 45)	54 (34, 69)
≥120	59 (38, 76)	73 (64, 87)	39 (24, 53)	48 (31, 65)
p-value[e]	0.066	0.649	0.437	0.535
Iron status[c,d]				
Insufficient	54 (30)[a]	75 (66, 85)	33 (29, 39)	40 (30, 53)
Sufficient	58 (27)[a]	75 (68, 82)	34 (26, 44)	48 (38, 60)
p-value[e]	0.533	0.918	0.864	0.297

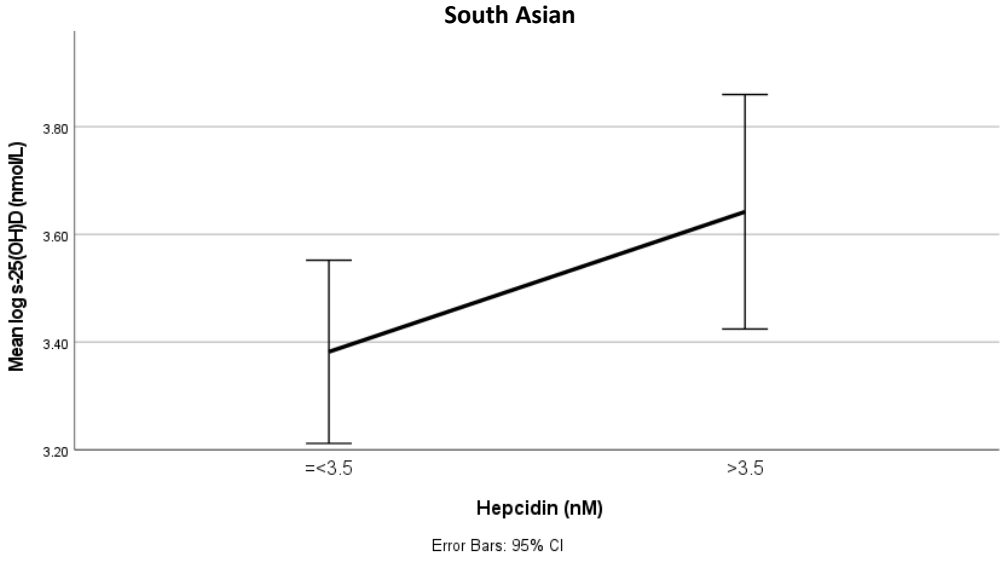
[A] Mean (±SD).
[B] Median (25th, 75th centile).
[C] Geometric mean (95th centile).
[d] Sufficient; Serum ferritin ≥30ug·L^{-1} and Hb ≥120 g·L^{-1}. Insufficient: Serum ferritin <30ug·L-1 (Hb <120 g·L^{-1} or ≥120 g·L^{-1})

[e] ANCOVA was conducted to identify significant differences using p value of ≤0.05 as significant.
[f] Data was non-normally distributed but was reported as geometric mean for consistency. The median values are: hepcidin ≤3.5 was 53 (27, 76), hepcidin >3.5 was 58 (37, 70), serum ferritin <30 was 51 (31, 69) and serum ferritin ≥30 59 (37, 75).
[g] other ethnic groups includes: Middle Eastern, other Asian, Māori and Pacifica.

***(a) Simple line mean of log s-25(OH)D (nmol/L) by hepcidin (nM)**



***(b) Simple line mean of log s-25(OH)D (nmol/L) by hepcidin (nM)**



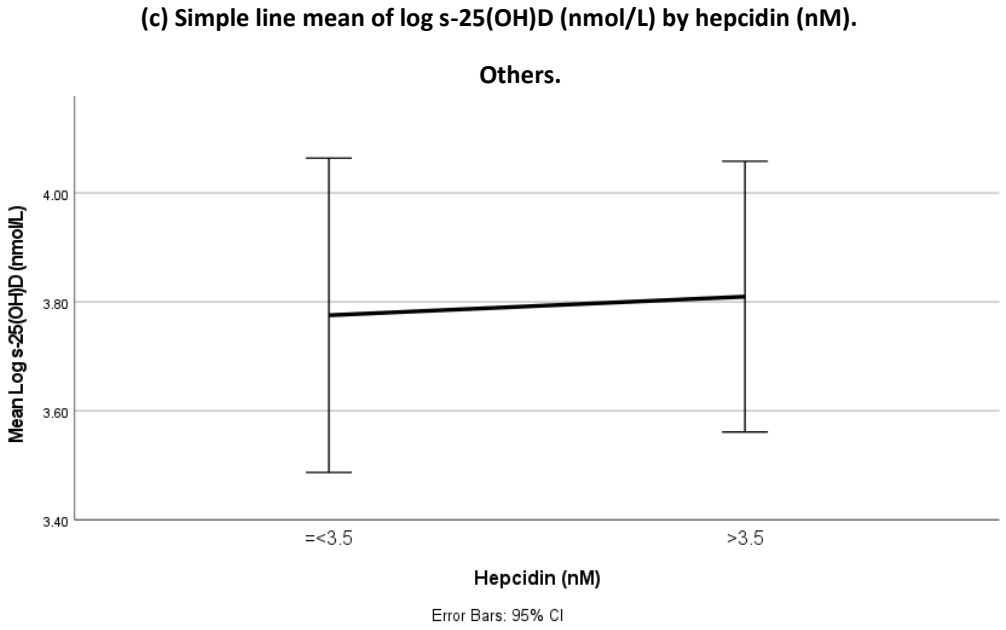


Figure 4: The association of s-25(OH)D (nmol·L^{-1}) with hepcidin (nM) in NZ Europeans (4a), South Asians (4b) and 'other' ethnic groups (4c). * s-25(OH)D is significantly different across hepcidin categories (≤3.5 vs <3.5 nM)

Determinants of s-25(OH)D:

Table 12 reports results from multiple linear regression model in relation to determinants of s-25(OH)D. The multiple linear regression model identified that 45.4% of variation in sqrt s-25(OH)D is explained by ethnicity, age, body fat %, sun exposure and season of enrolment, with this model being significant (p<0.001). The model identified that being of South Asian ethnicity strongly influenced s-25(OH)D concentration, followed by 'other' ethnic group, the participants age and finally body fat %. Being of South Asian and 'other' ethnicities, having a lower age (<30 years) and higher body fat % (≥32%) were associated with a lower sqrt s-25(OH)D concentration. Figure 5 illustrates that South Asians and 'other' ethnic group had significantly lower s-25(OH)D concentration compared to NZ Europeans.

Table 12: Multiple linear regression analysis to identify predictors of s-25(OH)D concentration.

SQRT s-25(OH)D	Coefficient (B)	s.e. (B)	95% CI B	Standardised B	R^2	P-value
Model					0.454	<0.001
Intercept	9.48	0.81	7.88, 11.09	-		<0.001
South Asian [a]	-2.83	0.37	-3.56, -2.10	-0.717		<0.001
'Other' ethnicity [b]	-1.78	0.35	-2.46, -1.09	-0.369		<0.001
Age [c]	0.67	0.28	0.12, 1.23	0.170		0.018
Body fat % [d]	-0.49	0.28	-1.04, 0.06	-0.125		0.080

Abbreviations: Cl; confidence interval, SQRT s-25(OH)D; square root transformed s-25(OH)D.
Regression equation: Square root s-25(OH)D (nmol/L)= 9.48-(2.83x South Asian) –(1.78x'others') +(0.67xage) – (0.49xbodyfat%). F (7, 148) =17.56, p<0.001.
Enter method technique. Following variables were included in the model: ethnicity, BF%, IL-6, sun exposure, season of enrolment and age.
[a] South Asian was coded as; South Asian=1 and no South Asian=0. Being of South Asian ethnicity was associated with a lower concentration of 2.83nmol·L^{-1} in sqrt s-25(OH)D.
[b] 'other' ethnicity was coded as; 'others'=1 and not 'others=0. Being of other ethnicities was associated with a lower concentration of 1.78nmol·L^{-1} in sqrt s-25(OH)D.
[c] Age was coded as; <30 years=1 and ≥30 years=2. Being ≥30 years old was associated with a higher concentration of 0.67nmol·L^{-1} in sqrt s-25(OH)D.
[d] Body fat % was coded as <32%=1 and ≥32%=2. Having a body fat % of 32% or more was associated with a lower concentration of 0.49nmol·L^{-1} in sqrt s-25(OH)D.

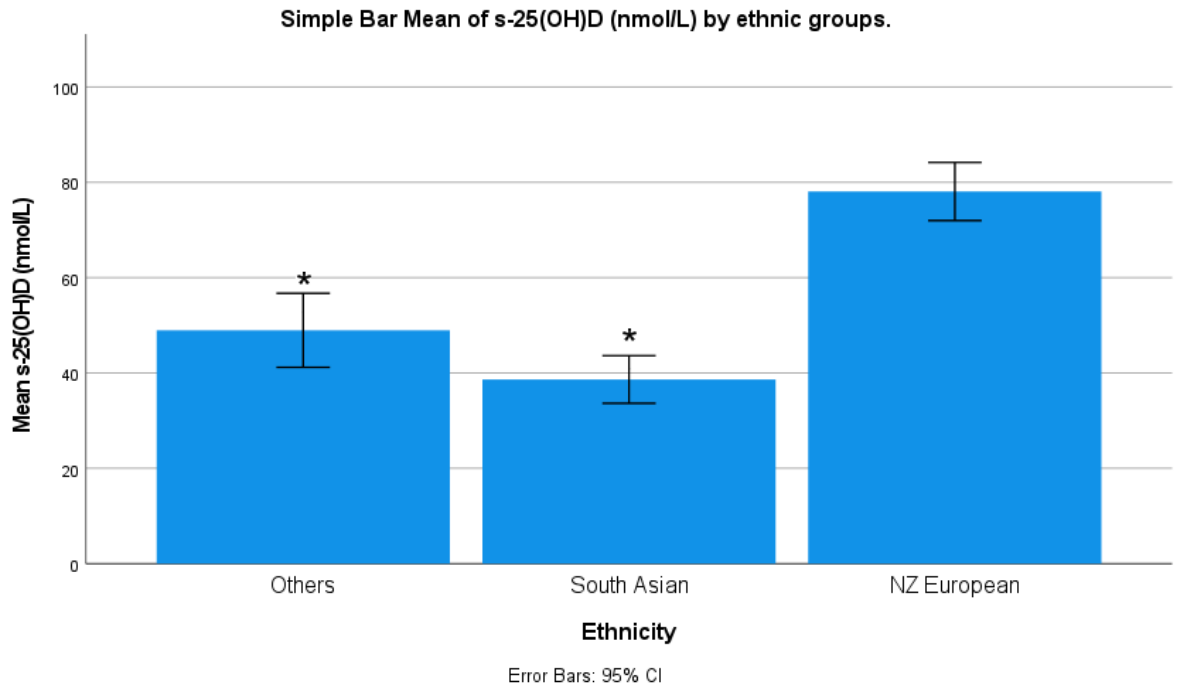


Figure 5: s-25(OH)D (nmol·L^{-1}) concentration across ethnic groups.
* South Asians and 'other' ethnic group had significantly lower mean s-25(OH)D concentration compared to NZ Europeans.

3.4 Discussion

Associations between s-25(OH)D, hepcidin and serum ferritin:

This research has identified an association between s-25(OH)D and hepcidin concentration, specifically in South Asian and NZ European premenopausal females. No association was identified between s-25(OH)D and Sf concentration.

NZ Europeans who tended to have higher s-25(OH)D concentration, ~80 nmol·L^{-1}, had lower (≤3.5nM) hepcidin concentration. Vitamin D supplementation studies have demonstrated similar associations. One week post supplemental dosing of vitamin D_3 in healthy adults (n=28), a 73% decrease in hepcidin was observed (Smith et al., 2017). Bacchetta et al. (2014), also observed a decrease in hepcidin concentration by 34%, 24 hours post a single oral dose of vitamin D_2. The direct mechanism behind the reduction of hepcidin concentration appears to involve the binding of $1\alpha,25(OH)_2D_3$ to the vitamin D receptor present on the *HAMP* promoter gene, decreasing mRNA expression, and subsequently resulting in reduced hepcidin levels (Bacchetta et al., 2014).

South Asians who tended to have higher s-25(OH)D concentration, ~38 nmol·L^{-1}, were found to have higher (>3.5) hepcidin concentration. This is the opposite to the association seen in the NZ Europeans and results reported in previous other ethnic cohorts (Caucasian, African American and Korean). To our knowledge, this association has not been reported in any of the previously published literature.

IL-6 is known to be released by adipose tissue and is directly correlated with adiposity (Eder et al., 2009). Elevated IL-6 concentrations have been observed in obese individuals compared to lean individuals (Eder et al., 2009). In our cohort, South Asians had significantly higher body fat % (38.34%) and BMI (25.88, kg/m^2) compared to NZ Europeans (27.49% and 22.91 kg/m^2, respectively). Therefore, it is possible that the higher body fat % could be a contributing factor to the increased IL-6 concentration observed in South Asians. Body composition has been demonstrated to be ethnically

dependent (Lear et al., 2009; Wulan et al., 2010). For the same BMI, Asians are frequently reported to have higher body fat % compared to Caucasians, this is particularly prominent in South Asians, Malay and Chinese (Wulan et al., 2010). Furthermore, it has been observed that South Asian females have higher fat to lean mass ratio compared to Aboriginal, Chinese and European females (Lear et al., 2009). Specifically in NZ, Asian Indians were identified to have 8% higher body fat % compared to Europeans (Rush et al., 2007). Potential factors that could be contributing to the varying body composition in ethnic groups include: genetics, environmental and intra-uterine development (Wulan et al., 2010). Extensive research demonstrates that IL-6 is an up-regulator of *HAMP* gene transcription and hepcidin concentration (Nemeth, Rivera, et al., 2004). Therefore, it is possible that the higher body fat % and IL-6 levels may be contributing factors to the increased hepcidin levels and the inverse association with s-25(OH)D levels observed in the South Asian participants. Vitamin D demonstrates anti-inflammatory actions through the down regulation of T helper 1 cells, subsequently reducing the production of pro-inflammatory cytokines, including IL-6 (Sassi et al., 2018). In addition, an in vitro study demonstrated a potential indirect mechanism in reducing hepcidin expression through dose-dependent $1,25(OH)_2D_3$, reducing pre-hepcidin cytokines, IL-6 and IL-1β (Zughaier et al., 2014). With vitamin D expressing anti-inflammatory effects and with low s-25(OH)D concentration observed in the South Asian participants, there may be a possible dose-dependent effect of vitamin D in the reduction of IL-6 concentration in the body. However, this remains to be investigated in future research.

Another potential confounding factor to consider with regards to the hepcidin and s-25(OH)D association in South Asians, is the significantly lower geometric mean of s-25(OH)D concentration (36 $nmol \cdot L^{-1}$) in this group of participants. Only 33% of South Asians were identified as having adequate vitamin D levels (≥50$nmol \cdot L^{-1}$). In comparison, 95% of NZ Europeans had adequate s-25(OH)D concentration with a geometric mean of 75 $nmol \cdot L^{-1}$. In NZ, South Asians have been identified as high risk for vitamin D deficiency (von Hurst et al., 2010). In a cohort of 228 South Asian

females, the median s-25(OH)D concentration was 27.5 nmol·L^{-1}, with only 16% of the cohort presenting with sufficient (≥50nmol·L^{-1}) s-25(OH)D concentration (von Hurst et al., 2010). Another ethnically diverse cohort in NZ have reported means of s-25(OH)D concentration as 51 nmol·L^{-1} in NZ Europeans, 42 nmol·L^{-1} in Māori and 37 nmol·L^{-1} in Pacific (Rockell et al., 2006). The higher reported means of s-25(OH)D concentration in other ethnicities further highlights the increased risk of vitamin D deficiency that is present in the South Asian population. There is the possibility that the low s-25(OH)D concentration observed in South Asians may not be sufficient to exert a suppressive effect on *HAMP* transcription that was observed in NZ Europeans and previous research. Thus, further research may seek to determine if there is a dose dependent effect of s-25(OH)D on hepcidin concentration.

There was no association between s-25(OH)D and Sf or iron status in the current study. Previously published studies have identified associations between s-25(OH)D and Sf, while some studies found no association. In female athletes, the percentage of iron deficient subjects was higher in the vitamin D deficient group (32%) compared to vitamin D sufficient athletes (11%) (Malczewska-Lenczowska et al., 2018). In the same cohort, the vitamin D deficient group had lower Sf concentrations and higher sTfR levels (Malczewska-Lenczowska et al., 2018). In support of these results, individuals with s-25(OH)D <50nmol·L^{-1} had increased odds of anaemia compared to individuals with s-25(OH)D ≥50nmol·L^{-1}, this association was observed in African Americans but not in Caucasians (Smith et al., 2015). Conversely, in a vitamin D_3 supplementation trial, there was no significant changes in Sf concentration one week post supplement (Smith et al., 2017). If the trial was undertaken over a longer period (month/s vs 1 week), an increase in Sf concentration could have been observed supporting the s-25(OH)D, hepcidin and Sf relationship.

The hepcidin cut-off for stratification used in the study (>3.5 or ≤3.5) was based upon the median hepcidin concentration of our cohort. However, recent research has demonstrated increased iron absorption rates when hepcidin concentrations fall below ≤3.09nM (Galetti et al., 2021). In this research, NZ Europeans who had higher

s-25(OH)D concentrations, also tended to have hepcidin concentrations ≤3.5nM. Lower hepcidin concentrations increase iron absorption rates, physiologically supporting the attainment of adequate iron levels (geometric mean Sf 25 $\mu g \cdot L^{-1}$). South Asians who tended to have higher s-25(OH)D concentration, tended to have hepcidin levels >3.5nM, previously indicative of lower iron absorption rates (Galetti et al., 2021). Therefore, the higher hepcidin levels in this ethnic group may contribute to their increased risk of ID (geometric mean of Sf 20 $\mu g \cdot L^{-1}$). Despite the low levels of Sf (<30 $\mu g \cdot L^{-1}$) observed in both ethnic groups, the reduced hepcidin concentration in NZ Europeans, observed in those with higher s-25(OH)D concentrations, would suggest improved regulation of the hepcidin levels in response to current iron status. However, in South Asians, despite the reduced Sf concentration, hepcidin remained elevated. This may indicate in this cohort that additional confounding factors may strongly influence the natural decline in hepcidin concentration when iron stores are reduced, thus impacting iron homeostasis. The observed difference between NZ Europeans and South Asians would suggest potential ethnic consideration for both iron and vitamin D deficiency diagnosis. Additional ethnic specific health considerations, including the increased IL-6 concentration and body fat % identified in South Asians but not in NZ Europeans and the impact this may have on nutrient metabolites.

Serum 25(OH)D determinants:

The determinants of s-25(OH)D identified by our logistic model included: ethnicity, age and body fat %. Being of South Asian ethnicity was strongly associated with a lower concentration of s-25(OH)D. As discussed previously, South Asians had significantly lower s-25(OH)D concentrations compared to NZ Europeans. Previously in NZ, significant differences in s-25(OH)D between ethnic groups had been identified, with s-25(OH)D concentrations highest in NZ Europeans compared to Māori, Polynesian or South/East Asian decent (Bolland et al., 2008). Supporting these results, 52.2% of South Asians had s-25(OH)D <40nmol$\cdot L^{-1}$ compared to only 8.2% of NZ Europeans (Narang et al., 2020). A possible explanation for this is the difference in skin colour between ethnic groups. Ethnicity can be related to skin colour which is

observed in this study as 75% of South Asians reported to have light/dark brown skin colour compared to 5% of NZ Europeans. Similar results have been observed in children, with 57% of South Asian children reporting dark/brown skin colour and 56% of NZ European children reporting medium skin colour (Delshad et al., 2019). In this cohort, South Asian females tended to have darker skin colour and lower s-25(OH)D concentrations compared to NZ Europeans. The skin pigment, melanin, is known to reduce absorption of UVβ rays, reducing synthesis of vitamin D, subsequently reducing circulating 25(OH)D (Clemens et al., 1982). Previous studies have also observed lower s-25(OH)D concentrations in those with darker skin colour (Cairncross et al., 2017; Mitchell et al., 2012). Behaviours and attitudes towards the sun identified in South Asians could also be a contributing factor to the low s-25(OH)D concentrations (von Hurst et al., 2010). South Asians were found to have increased concerns for developing skin cancer due to the strength of the NZ sun, which led to the avoidance of sun exposure (von Hurst et al., 2010). In support of these findings, our research has identified that 51% of South Asians sometimes/never enjoy spending time outside compared to only 10% of NZ Europeans. As sun exposure is the main source of vitamin D, decreased exposure can result in decreased s-25(OH)D concentration.

Another determinant of s-25(OH)D concentration and a contributing factor of low s-25(OH)D concentrations in South Asians is body fat %. As discussed previously, South Asians had a significantly higher body fat % compared to NZ Europeans. Vitamin D is a fat-soluble vitamin and higher adipose tissue is associated with increased sequestration and decreased circulating 25(OH)D (Wortsman et al., 2000). Obese individuals have been observed to have significantly lower s-25(OH)D compared to lean controls (Wortsman et al., 2000). In support of this, increased response to oral vitamin D supplementation has been observed in those with lower body fat % compared to individuals with higher body fat % (Mazahery et al., 2015). The low s-25(OH)D concentrations in South Asians compared to NZ European further supports the ethnic and body composition considerations for changes in vitamin D thresholds to help prevent vitamin D deficiency in the South Asian population.

Our logistic model had identified that increased age (≥30years) is associated with increased concentration of s-25(OH)D. This differs from published research, where increased age was associated with decreased s-25(OH)D concentration (Bolland et al., 2008; Lucas et al., 2005; Rockell et al., 2006). In older adults, capacity of production of previtamin D3 is decreased, reducing the production of the active vitamin D molecule (MacLaughlin & Holick, 1985). During the recruitment process of our study, screening of vitamin D supplementation was not an exclusion factor. This could have led to the potential relationship observed between age and vitamin D concentration as older individuals tend to have increased intake of supplementation (O'Brien et al., 2017).

3.5 Conclusion

In conclusion, in NZ Europeans, those with a higher s-25(OH)D concentration have a decreased (≤3.5nM) hepcidin concentration. This is the opposite in South Asians, where those with a higher s-25(OH)D concentration, tended to have higher (>3.5nM) hepcidin concentrations. Potential confounding factors which led to this observation include the significantly higher IL-6 concentration and body fat % observed in South Asians compared to NZ Europeans. Body fat % is known to impact IL-6 concentration, which is further known to increase hepcidin concentration. South Asians also had significantly lower s-25(OH)D concentrations compared to NZ Europeans. These observations could be a result of a potential dose-dependent effect of vitamin D on IL-6 and hepcidin concentration. Ethnicity is a key determinant of s-25(OH)D concentration, with those of South Asian ethnicity tending to have lower s-25(OH)D concentrations compared to NZ Europeans. Increased age and decreased body fat % were also associated with increased s-25(OH)D concentrations.

South Asians are at risk for vitamin D and iron deficiency and should be monitored regularly. Furthermore, when undertaking biochemical and health analysis, their IL-6 concentration and body fat % should be taken into consideration as both may be influential factors in metabolism of nutrients. Due to the inverse relationship of s-25(OH)D and hepcidin concentration identified in South Asians, compared to NZ

Europeans, further research is needed to increase knowledge on the dose-dependent effect of vitamin D on hepcidin concentrations and iron regulation.

Chapter 4: Conclusion and recommendations

4.0 Achievements of aims and hypothesis:

The main aim of the study was to investigate the associations between s-25(OH)D, hepcidin and iron concentration in premenopausal females. An interaction effect of ethnicity on the relationship between s-25(OH)D and hepcidin concentration (p=0.06) was observed, therefore analysis was stratified according to three ethnic groups: NZ European, South Asian and 'other'. This created clear associations between s-25(OH)D and hepcidin concentrations that otherwise would not have been identified. NZ Europeans who had higher s-25(OH)D concentration tended to have lower (≤3.5nM) hepcidin concentration. These findings align with previous research. Vitamin D supplementation has been observed to increase s-25(OH)D concentration and decrease hepcidin concentration in 24 hours and after one week post the supplemental dose (Bacchetta et al., 2014; Smith et al., 2017). Mechanisms identified to be behind the decrease of hepcidin concentration involves the binding of 25(OH)D to the *HAMP* promoter gene, subsequently decreasing hepcidin transcription (Bacchetta et al., 2014). In this study, no significant association was identified between s-25(OH)D and serum ferritin (Sf)/iron status. However, these findings suggest that long term, NZ Europeans who tended to have higher s-25(OH)D concentrations may be at decreased risk of iron deficiency (ID), due to decreased concentration of hepcidin. This would need to be investigated further in a separate study.

In South Asians, it was identified that those with higher s-25(OH)D tended to have higher (>3.5nM) hepcidin concentration. This is the opposite that was observed in NZ Europeans and does not align with previous research. Potential confounding factors that may have contributed to this result include higher body fat % and IL-6 concentration identified in South Asians compared to NZ Europeans. IL-6, an inflammatory cytokine, is released from and is positively correlated with adipose tissue (Eder et al., 2009). Increased IL-6 concentrations have been observed in obese individuals when compared to lean individuals (Eder et al., 2009). Furthermore, Asian females have higher body fat % and higher fat to lean mass ratio when compared to Caucasian and European females (Lear et al., 2009; Wulan et al., 2010). A

positive correlation between IL-6 concentration and body fat % in NZ Europeans and South Asians was identified in our study. The positive association is demonstrated to affect South Asians more than NZ Europeans in our cohort due to significantly higher body fat % and IL-6 concentration observed in the South Asian participants.

In support of our results, increased IL-6 concentration in South Asians when compared to NZ Europeans, has been identified previously, where 30% of the increased IL-6 concentration was explained by body fat % in South Asian participants (Peters et al., 2013). IL-6 is known to increase hepcidin concentration through the upregulation of *HAMP* transcription (Nemeth, Rivera, et al., 2004). Therefore, the higher IL-6 concentration identified in South Asians in our study, could be the contributing factor to the increased hepcidin concentration identified in those with lower s-25(OH)D concentration.

Another potential confounding factor that may have contributed to the inverse relationship between s-25(OH)D and hepcidin concentration observed in South Asian participants, is the significantly lower s-25(OH)D concentration, compared to NZ Europeans. Of the NZ Europeans, 95% were identified to have adequate s-25(OH)D concentrations (≥50nmol·L^{-1}) compared to 33% of South Asians. Increased concentration of s-25(OH)D through the supplementation of vitamin D, has been shown to decrease hepcidin concentration by 73% (Smith et al., 2017). Vitamin D metabolite, 1α,25$(OH)_2D_3$, may also indirectly reduce hepcidin expression via the reduction of pro-inflammatory cytokines, IL-6 and IL-1β (Zughaier et al., 2014). This combination of low s-25(OH)D concentration observed in South Asian participants and potentially reduced anti-inflammatory actions of vitamin D, suggests a potential dose-dependent effect of vitamin D in reducing IL-6 and hepcidin concentration.

The hepcidin cut-off used for stratification in our analysis (>3.5 or ≤3.5), was based of the median hepcidin concentration in our cohort. However, recent research has identified that hepcidin concentrations ≤3.09nM, typically results in increased iron absorption rates, facilitating the correction of reduced iron levels when in a negative iron balance (Galetti et al., 2021). This research clearly highlighted this iron homeostatic mechanism, showing that

when iron concentration decreases, there is a resulting decrease in hepcidin concentrations to support increased iron absorption and use (Nemeth, Tuttle, et al., 2004). In our study, both NZ Europeans (25 $\mu g \cdot L^{-1}$) and South Asian (20 $\mu g \cdot L^{-1}$) females had geometric mean of Sf <30 $\mu g \cdot L^{-1}$ (insufficient Sf concentration). The decreased Sf concentrations should result in decreased hepcidin concentration as part of typical iron regulation. However, this was only observed in NZ Europeans who tended to have higher s-25(OH)D concentrations. This suggests that vitamin D may have a dose dependent effect on reducing IL-6 and hepcidin concentrations. Key factors influencing s-25(OH)D concentration are body fat % and sun exposure (Mazahery et al., 2015; Touvier et al., 2015). The combination of higher body fat % and decreased sun exposure (discussed in the next section) observed in the South Asian cohort, may have resulted in the reduced s-25(OH)D concentration. The low concentration of s-25(OH)D may have a decreased influence on both direct and indirect regulation of hepcidin concentration, suggesting further investigations into the dose-dependent effect of vitamin D on iron regulation is warranted.

The secondary aim was to identify the determinants of s-25(OH)D concentration. It was hypothesised that increased sun exposure, summer/autumn, lighter skin colour, lower body fat % and NZ European females will be associated with increased s-25(OH)D concentration. This was partly true, as our models indicates that ethnicity and body fat % are key determinants of s-25(OH)D concentration. In our cohort, South Asians had significantly lower s-25(OH)D concentration compared to NZ Europeans. Ethnicity has been identified to impact s-25(OH)D previously, with higher s-25(OH)D concentrations in NZ European compared to Māori, Polynesian and South/East Asian (Bolland et al., 2008). This observation may be related to skin colour differences between ethnic groups. In our study, 75% of South Asians reported having light/dark brown skin colour compared to only 5% of NZ Europeans. This has previously been reported in children, with 57% of South Asian having dark/brown skin colour (Delshad et al., 2019). In our study, darker skin colour was more prominent in South Asians, who also tended to have lower s-25(OH)D concentrations. These findings align with previous research, where it has been observed that those with darker skin colour tended to have lower s-25(OH)D concentration (Cairncross et al., 2017; Delshad et al., 2019; Mitchell et al., 2012). Darker skin has reduced ability to absorb UVβ rays due to the increased presence of melanin

skin pigment, resulting in reduced synthesis of vitamin D and reduced circulating 25(OH)D (Clemens et al., 1982). Attitudes and behaviours towards the sun may also be a possible explanation for why South Asians tend to have lower s-25(OH)D concentration. South Asians tend to have increased concern for developing skin cancer due to the strength of the NZ sun, subsequently resulting in decreased sun exposure (von Hurst et al., 2010). In support of these results, our research has identified that 51% of South Asians sometimes/never enjoy spending time outside compared to only 10% of NZ Europeans.

Another confounding factor contributing to decreased s-25(OH)D concentration in South Asians, is significantly higher body fat %, which has also been identified by our model as a key determinant. As discussed previously, South Asians have significantly higher body fat % compared to NZ Europeans. Increased body fat %, increases storage of vitamin D, subsequently decreasing circulating 25(OH)D (Wortsman et al., 2000). Randomised controlled trials providing vitamin D supplements have previously noted that increased body fat %, decreases the response to vitamin D oral supplements, resulting in decreased s-25(OH)D concentration (Mazahery et al., 2015). Body composition should be taken into consideration when measuring and analysing s-25(OH)D concentration, across all ethnic groups, as this impacts 25(OH)D metabolism.

Our model also identified increasing age is associated with increased s-25(OH)D concentration. This is the opposite of previously published research, which demonstrated that increasing age is associated with decreased s-25(OH)D concentrations (Bolland et al., 2008; Rockell et al., 2006). Capacity to produce previtamin D3 decreases with age, resulting in reduced production of the active vitamin D molecule (MacLaughlin & Holick, 1985). Our observation could be a result of not excluding the use of vitamin D supplementation prior to participation in the study. As individuals age, supplementation use tends to increase, which may result in slightly elevated s-25(OH)D concentration compared to those younger (O'Brien et al., 2017) and could have impacted the results that were reported in this research. Therefore, future research may need to consider this factor when doing longitudinal research on vitamin D status in female participants.

4.1 Strengths

Our study was the first cross sectional study to have investigated the association between s-25(OH)D, hepcidin and Sf in healthy premenopausal females living in Auckland, NZ. Previous cross-sectional studies have focused on the potential association between s-25(OH)D and Sf, not investigating impact on hepcidin concentrations (Malczewska-Lenczowska et al., 2018; Smith et al., 2015). Previous large cross-sectional studies included participants with chronic kidney disease in their analyses while controlling for other confounding factors (Monlezun et al., 2015; Shin & Shim, 2013; Sim et al., 2010; Smith et al., 2015). Therefore, our study was the first to include participants who were healthy, without additional co-morbidities and analysed all three biomarkers (s-25(OH)D, hepcidin and Sf concentration).

A strength of our recruitment and analysis was the high portion of South Asians in our cohort, subsequently leading to an analysis stratified by ethnicity. Ethnicity was found to impact the relationship of s-25(OH)D and hepcidin concentration (p=0.06) in our study. Consistent with our results, ethnicity had been found to impact both s-25(OH)D and iron (Bolland et al., 2008; Lim et al., 2020). In stratifying our data by ethnicity, we were able to identify an association between s-25(OH)D and hepcidin concentration. Furthermore, we were able to see the difference between NZ Europeans and South Asians, which has demonstrated a key outcome of a potential dose-dependent effect of vitamin D on hepcidin concentration, which will require further research.

Another strength of our study was using a Sf correction factor for those who had CRP values ≥5mg·L^{-1} (Bui et al., 2012). This increased the number of participants that we could analyse in our study by including those who had CRP values above ≥5mg·L^{-1}.

4.2 Limitations

A limitation of our study during data collection was the timing of s-25(OH)D samples. The majority of South Asians had samples collected during the summer months, when s-25(OH)D concentrations tends to be higher. Compared to NZ Europeans, where the majority of blood samples were collected during the winter months, when s-25(OH)D

tends to be lower, due to seasonal variation (Bolland et al., 2008). In addition, due to naturally lower s-25(OH)D concentrations in South Asians, the difference of s-25(OH)D concentration may have been greater if participants had been measured in one season. Furthermore, seasonal variation also impacts vitamin D diagnosis (Bolland et al., 2008). Individuals who were reported to have adequate levels during the summer period, may have suboptimal levels during the winter/spring months (Bolland et al., 2008). Therefore, if our blood samples were collected during the winter months or a higher cut-off point was used for the summer months (60-75 $nmol \cdot L^{-1}$), we may see lower vitamin D sufficiency prevalence in this cohort.

Another limitation was participants who used vitamin D supplementation or multivitamins containing vitamin D were not excluded from the study. This could have led to older participants consuming vitamin D supplements, increasing their s-25(OH)D concentrations and potentially affecting the results of key determinants of vitamin D status.

Body composition was measured using bioelectrical impedance analysis (BIA). This is a limitation in itself, as it is influenced by ethnic variation (Dehghan & Merchant, 2008). The equation used in this study during BIA is based mainly on a Caucasian population, and was not ethnic specific (Dehghan & Merchant, 2008). As populations differ in fat distribution, body density and proportion of limb length, particularly South Asians, this can result in increased error in body composition measurements (Dehghan & Merchant, 2008). To increase accuracy, ethnic specific equations should be used during BIA assessment in health screening and research trials (Dehghan & Merchant, 2008).

4.3 Recommendations and future directions for research

The association between s-25(OH)D and hepcidin concentration differed significantly between South Asians and NZ Europeans. It is evident that a potential metabolic difference exists between the two ethnic groups. Future research should focus on a

potential dose-dependent effect of vitamin D, directly and indirectly, on hepcidin concentrations.

- South Asians who had higher s-25(OH)D concentration, tended to have increased hepcidin concentration. This was the opposite for NZ Europeans: who had higher s-25(OH)D and decreased hepcidin concentration. Researching the potential dose-dependent effect of vitamin D on hepcidin concentration, either directly or indirectly, can help increase knowledge and aid health professionals in providing recommendations to manage iron and vitamin D status.
- Continue researching the associations between s-25(OH)D, hepcidin, and Sf, particularly in different ethnic groups. There was an ethnic difference between NZ Europeans and South Asians in our cohort, therefore focusing on dose-dependent effect of vitamin D can help to further explain these associations. This can help further develop knowledge in this area and aid in potential changes in iron and vitamin D thresholds to suit ethnic differences.
- Research focusing on South Asian population and how their body composition may impact their health status. South Asians were identified to had significantly higher body fat % and IL-6 concentration, which aligns with previous research and has been observed to increase the risk of CHD. Vitamin D and iron status can both impact and further exacerbate the symptoms of CHD. Therefore, risk factors in South Asians could help increase awareness of factors contributing to CHD and lead to future interventions to be implemented and researched.
- Health professionals should focus on South Asians as at-risk group for both vitamin D and iron deficiency. In our study it was identified that South Asians had significantly lower s-25(OH)D concentration and the combination of both low ferritin and high hepcidin concentration impacts iron status. Awareness of these trends in South Asians, can help aid health professionals in their recommendations towards clients, e.g. Dietitians can be more aware of the deficiencies, and recommend regular testing to help prevent decline in these nutrients.

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Appendices

Appendix A: Ethics Approval Letter

Date: 16 April 2018

Dear Dr Claire Badenhorst

Re: Ethics Notification - **SOA 18/12** - **Hepcidin, Iron and Vitamin D status in South Asian and Middle Eastern females.**

Thank you for the above application that was considered by the Massey University Human Ethics Committee: **Human Ethics Southern A Committee** at their meeting held on **Monday, 16 April, 2018**.

Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

Dr Brian Finch
Chair, Human Ethics Chairs' Committee and Director (Research Ethics)

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Appendix B: Questionnaire

vIVID Questionnaire

Start of Block: Default Question Block

Please fill in you vIVID study ID number

_____

End of Block: Default Question Block

Start of Block: Demographics and Medical History

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

_____

Please state the country you were born in?

_____

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

_____

_____

_____

_____

_____

Which ethnic group do you identify with?

_____

What is your first language?

_____

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

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

_____

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

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

_____

Have you been pregnant within the last year

○ Yes

○ No

End of Block: Demographics and Medical History

Start of Block: Section 2: Lifestyle

Do you smoke?

○ Yes

○ No

Display This Question:

If Do you smoke? = Yes

If yes how many cigarettes do you smoke a day?

_____

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

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

If yes, what type of diet do you follow?

_____

Have you dieted strictly in the last year?

- ○ Yes
- ○ No

Display This Question:

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

If yes then please describe

_____

End of Block: Section 2: Lifestyle

Start of Block: Section 3: Health

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

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

_____

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

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

_____

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

Type of treatment, duration and any further details

_____

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

If yes, please describe and give approximate dates

_____

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

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

_____

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

If yes, then please describe

_____

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

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

_____

Display This Question:

If Have you been pregnant within the last year = Yes

Have you breastfed a baby in the current year?

- ○ Yes
- ○ No

End of Block: Section 3: Health

Start of Block: Section 4: Supplements

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

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

_____

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

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)?

_____

End of Block: Section 4: Supplements

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

Do you donate blood

- ○ Yes
- ○ No

Display This Question:

If Do you donate blood = Yes

If yes, when did you last donate

_____

Display This Question:

If Do you donate blood = Yes

How many times have you donated blood in the past year?

_____

Do you get nose bleeds

- Yes
- No

Display This Question:

If Do you get nose bleeds = Yes

If yes, how often do you get a nose bleed? Please indicate how many times a month (e.g. 3 x a month) or how many times a year (e.g. 2 x a year)

_____

Display This Question:

If Do you get nose bleeds = Yes

How heavy are your nose bleeds

- Heavy
- Medium
- Light

How old were you when you had your first menstrual period?

_____

Have you had a menstrual period in the last 6 months

- ○ Yes
- ○ No

How regular (around every 28 days) are your menstrual periods?

- ○ Regular
- ○ Irregular

Can you recall the date that your last menstrual period started? (DD/MM/YYYY)

_____

How many days do you usually have between periods? (For instance, counting from the first day of your last period to the day you expect your next period to start)

_____

How many days does your period usually last?

_____

We need to ask the following questions to help us estimate how heavy your periods are

How many 'heavy' (going through more than 3 tampons or pads) days do you have during your period?

_____

How many 'light' (going through less than 2 tampons or pads) days do you have during your period?

_____

On a 'heavy' day how many pads and/or tampons do you use?

_____

What absorbency level are you pads or tampons?

	Super plus	Super	Regular	Light	Mini
Pads absorbency	○	○	○	○	○
Tampons absorbency	○	○	○	○	○

If you use tampons, which brand of tampon do you usually use?

	Care Free	Tampax applicator	Tampax tampets	Libra fleur	Other
Tampon brand	○	○	○	○	○

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

Start of Block: Physical Activity

We would like to ask you about the time you spent being physically active in the last 7 days up to yesterday. Please do not include activity you have done today

By 'active' we mean doing anything using your muscles

Think about activities at work, school or home, getting from place to pace, and any activities you did for exercise, sport, recreation or leisure.

Q67 Walking: During the last 7 days, on how many days did you walk at a brisk pace - a brisk pace is a pace at which you are breathing harder than normal? This includes walking at work or school, while getting from place to place, at home and at any activities that you did solely for recreation, sport, exercise or leisure.

Think ONLY about brisk walking done for at least 10 minutes at a time

Please give you answer is days per week. (e.g. 2 days per week)

_____

How much time did you typically spend <b>walking at a brisk pace on each of those days? Please give your answer in hours and minutes (e.g. 1 h and 5 minutes)

_____ Hours
_____ Minutes

Moderate Physical Activity: During the last 7 days, on how many days did you do moderate physical activities? 'Moderate' activities make you breathe harder than normal, but only a little - like carrying light loads, bicycling at a regular pace, or other activities like this. Do NOT include walking of any kind. Think only about those physical activities done for at least 10 minutes at a time. Please give you answer in days per week (e.g. 1 day per week)

_____

How much time did you typically spend on each of those days doing moderate physical activities?

_____ Hours
_____ Minutes

Vigorous Physical Activity: During the last 7 days, on how many days did you do vigorous physical activities? 'Vigorous' activities make you breathe a lot harder than normal ('huff and puff') - like heavy lifting, digging, aerobics, fast bicycling, or other activities like this. Think only about those physical activities done for at least 10 minutes at a time. Please give your answer in days per week (e.g. 1 day per week)

_____

Q72 How much time did you typically spend on each of those days doing vigorous physical activities?

_____ Hours
_____ Minutes

Frequency of Activity: Thinking about all your activities over the last 7 days (including brisk walking), on how many days did you engage in: At least 30 minutes of moderate activity (including brisk walking) that made you breathe a little harder than normal, or At least 15 minutes of vigorous activity that made you breathe a lot harder than normal ('huff and puff')? Answer in days per week (e.g. 5 days per week)

_____

End of Block: Physical Activity

Start of Block: Sun Exposure Questionnaire

We would like to know your thoughts and attitudes to sun exposure and sunbathing. Please select the statement that best expresses your thoughts. Please read each statement and select ONE ANSWER for each of the following questions

---

Click to write the question text

	Mostly	Often	Sometimes	Never
I enjoy spending time outside in the sun	○	○	○	○
During summer I sunbathe	○	○	○	○

Click to write the question text

	Strongly Disagree	Disagree	Neutral	Agree	Strongly Agree	No Opinion
I believe that the sunlight can be good for your health	○	○	○	○	○	○
I believe that the sunlight is bad for your health	○	○	○	○	○	○
If I know I am going to be outside, I will use sunscreen	○	○	○	○	○	○

What is your main source for protection from the sun?

- ○ Clothing
- ○ Sunscreen
- ○ Hat
- ○ Combination of clothing, hat and sunscreen
- ○ I don't use any protection
- ○ I don't go out in the sun

My main reason for avoiding the sun is:

- ○ Cultural or religious reasons
- ○ Public health messages say to avoid the sun
- ○ Specific health reasons
- ○ I don't want darker skin
- ○ I don't avoid the sun

I would spend more time in the sun if:

- ○ I had somewhere private to sunbathe
- ○ I had more time
- ○ I wasn't worried about skin cancer
- ○ I wouldn't spend more time in the sun

Do you want to make any comments about sun exposure?

_____

End of Block: Sun Exposure Questionnaire

Start of Block: Skin Type Chart

Genetic disposition

	0) Light blue, Grey, Green	1) Blue, Grey or Green	2) Blue	3) Dark Brown	4) Brownish Black
What is the colour of your eyes	○	○	○	○	○

Click to write the question text

	0) Sandy Red	1) Blond	2) Chestnut/Dark Blond	3) Dark Brown	4) Black
What is the natural colour of your hair	○	○	○	○	○

Click to write the question text

	0) Reddish	1) Very pale	2) Pale with Beige tint	3) Light brown	4) Dark brown
What is the colour of your skin (non-exposed areas)	○	○	○	○	○

Click to write the question text

	0) Many	1) Several	2) Few	3) Incidental	4) None
Do you have freckles on un-exposed areas of your skin?	○	○	○	○	○

Reaction to Sun Exposure

	0) Painful redness, blistering, peeling	1) Blistering followed by peeling	2) Burns sometimes followed by peeling	3) Rarely burns	4) Never had any burns
What happens when you stay in the sun too long	○	○	○	○	○

Click to write the question text

	0) Hardly or not at all	1) Light colour tan	2) Reasonable tan	3) Tan very easy	4) Turn dark brown quickly
To what degree do you turn brown	○	○	○	○	○

Click to write the question text

	0) Never	1) Seldom	2) Sometimes	3) Often	4) Always
Do you turn brown within several hours after sun exposure?	○	○	○	○	○

Click to write the question text

	0) Very sensitive	1) Sensitive	2) Normal	3) Very resistant	4) Never had a problem
How does your face react to the sun?	○	○	○	○	○

Tanning Habits

	0) More than 3 months ago	1) 2 to 3 months ago	2) 1 to 2 months ago	3) Less than a month ago	4) Less than 2 weeks ago
When did you last expose your body to the sun (or artificial sunlamp/ tanning cream)?	○	○	○	○	○

End of Block: Skin Type Chart

Start of Block: Iron Food Frequency Questionnaire

When answering this questionnaire consider your intake of food over the PAST MONTH. To help you do this please think of an event in your life that happened ONE month ago and think about your eating patterns since that date. Example: Consider if you have sugar in all your drinks during the day

as well as added to other food items and indicate how many times in the day you are consuming sugar. E.g. drinking 2 cups of coffee with sugar and 4 cups of tea with sugar, one bowl of cereal with sugar and sugar on pancakes at dinner resulting in choosing the category (4 Plus times per day)

Meat and chicken

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Beef (e.g. roast, steak, chops, schnitzel, silverside, casseroles, stew, stir fry, curry/shak, hamburger meat, mince dishes, kebab)	○	○	○	○	○	○	○	○	○
Chicken, turkey or duck (e.g. roast, fried, steamed, BBQ, casseroles, stew (e.g. fesenjan), stir fry, curry/shak, fried takeaway chicken, kebab)	○	○	○	○	○	○	○	○	○
Lamb, hogget or mutton (e.g. roast, steak, chops, BBQ, casseroles, stew (e.g. gheymeh), stir fry,	○	○	○	○	○	○	○	○	○

curry/shak, kebab)									
Pork (e.g. roast, chops, steak, casserole, casseroles, stir fry, curry/shak)	○	○	○	○	○	○	○	○	○
Veal	○	○	○	○	○	○	○	○	○
Liver, kidney, other offal (Including pate, tongue)	○	○	○	○	○	○	○	○	○
Ham, bacon	○	○	○	○	○	○	○	○	○
Game meats (e.g. venison, mutton bird, rabbit)	○	○	○	○	○	○	○	○	○
Corn beef, canned	○	○	○	○	○	○	○	○	○
Haleem	○	○	○	○	○	○	○	○	○

Prepared Meat

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Beef Jerky, biltong	○	○	○	○	○	○	○	○	○
Sausages, frankfurters, saveloys	○	○	○	○	○	○	○	○	○
Luncheon sausage, salami, brawn, pastrami	○	○	○	○	○	○	○	○	○
Black pudding	○	○	○	○	○	○	○	○	○
Meat pies	○	○	○	○	○	○	○	○	○

Fish and seafood

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Fresh and frozen fish (e.g. snapper, tarakihi, gurnard, flounder, hoki, salmon, white bait, shark, eel)	○	○	○	○	○	○	○	○	○	○
Battered and crumbed fish (e.g. fish fingers, fish cakes)	○	○	○	○	○	○	○	○	○	○
Canned and bottled fish (e.g. tuna, salmon, herrings, sardines)	○	○	○	○	○	○	○	○	○	○
Mussels, pipi, paua, cockles, oysters	○	○	○	○	○	○	○	○	○	○
Scallops, crab sticks, crab, squid, crayfish, kina	○	○	○	○	○	○	○	○	○	○
Prawns, shrimps	○	○	○	○	○	○	○	○	○	○

Eggs

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Eggs – boiled, fried, poached, scrambled, raw and egg based dishes including quiche, soufflés, frittatas, omelettes, kuku	○	○	○	○	○	○	○	○	○

Nuts

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Peanuts, mixed nuts, macadamias, pecan, hazelnuts, brazil nuts, walnuts, cashews, pistachios	○	○	○	○	○	○	○	○	○
Almonds	○	○	○	○	○	○	○	○	○
Pumpkin seeds, sunflower seeds, pinenuts	○	○	○	○	○	○	○	○	○
Sesame seeds, tahini	○	○	○	○	○	○	○	○	○

Legumes

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Tofu, soybeans, tempeh	○	○	○	○	○	○	○	○	○
Beans in sauce (e.g. baked beans, chilli beans)	○	○	○	○	○	○	○	○	○
Beans (canned or dried) (e.g. black beans, butter beans, haricot beans, red kidney beans, white kidney beans, green mung beans, refried beans)	○	○	○	○	○	○	○	○	○
Lentils (e.g. adasi)	○	○	○	○	○	○	○	○	○
Peas (e.g. chick peas, hummus, falafels, split peas, cow peas)	○	○	○	○	○	○	○	○	○
Dahl (All varieties)	○	○	○	○	○	○	○	○	○
Poppadoms, papad (Lentil flour)	○	○	○	○	○	○	○	○	○

Lentil based dish

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Khitchri	○	○	○	○	○	○	○	○	○
Chevda (Bombay mix, savoury snack made with split gram dhal)	○	○	○	○	○	○	○	○	○
Ganthia/Sev (Chickpea flour made into a paste then made into spaghetti and fried)	○	○	○	○	○	○	○	○	○
Kachori (Ball shaped pastry filled with chopped peas or dry mung dhal)	○	○	○	○	○	○	○	○	○

Dairy products

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Cheese (e.g. Cheddar, Colby, Edam, Tasty, blue vein, camembert, parmesan, gouda, feta, paneer, processed)	○	○	○	○	○	○	○	○	○
Cottage cheese, ricotta cheese	○	○	○	○	○	○	○	○	○
Cream, sour cream, cream cheese, cheese spreads, fromage frais (All varieties)	○	○	○	○	○	○	○	○	○
Milk (cow's milk) as a drink (e.g. flavored milk, milk shakes)	○	○	○	○	○	○	○	○	○
Milk (cow's milk) (All varieties) added to drinks (e.g. in tea, coffee), chai	○	○	○	○	○	○	○	○	○
Soy Milk	○	○	○	○	○	○	○	○	○
Coconut milk	○	○	○	○	○	○	○	○	○

Condensed milk	○	○	○	○	○	○	○	○	○
Ice cream, kulfi	○	○	○	○	○	○	○	○	○
Buttermilk (Lassi)	○	○	○	○	○	○	○	○	○
Yoghurt, lebneh, kashk,dhai, yoghurt added to water/soda (e.g. Doogh drink, Aashe doogh, Shish barak, Shakriya)	○	○	○	○	○	○	○	○	○
Seekhand, think sweet yogurt with liquid drained out	○	○	○	○	○	○	○	○	○

Dairy based foods/ Meals that have a diary base

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Milk puddings such as rice pudding (Kheer), custard,	○	○	○	○	○	○	○	○	○
Burfi	○	○	○	○	○	○	○	○	○
Desserts made from milk powder and milk (e.g. Jelabi, Gulab Jaman, Rasmalai)	○	○	○	○	○	○	○	○	○
Foods made and soaked in yogurt (e.g. Dahiwada, Dhokra, Raita, Kudhi)	○	○	○	○	○	○	○	○	○

Fruit

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Apples	○	○	○	○	○	○	○	○	○
Bananas, green bananas	○	○	○	○	○	○	○	○	○
Citrus fruits (e.g. orange, tangelo, tangerine, mandarin, grapefruit, lemon)	○	○	○	○	○	○	○	○	○
Green kiwifruit	○	○	○	○	○	○	○	○	○
Zespri, gold kiwifruit	○	○	○	○	○	○	○	○	○
Pears, nashi pears	○	○	○	○	○	○	○	○	○
Stone fruit (e.g. apricots, nectarines, peaches, plums, lychees)	○	○	○	○	○	○	○	○	○
Avocadoes, olives	○	○	○	○	○	○	○	○	○
Feijoas, persimmon, tamarillos	○	○	○	○	○	○	○	○	○
Grapes	○	○	○	○	○	○	○	○	○
Mango	○	○	○	○	○	○	○	○	○
Watermelon	○	○	○	○	○	○	○	○	○
Pawpaw (Papaya), other melons	○	○	○	○	○	○	○	○	○

(e.g. honey dew, rock melon)									
Pineapple	○	○	○	○	○	○	○	○	○
Rhubarb	○	○	○	○	○	○	○	○	○
Fruit salad, canned	○	○	○	○	○	○	○	○	○
Strawberries, blackberries, cherries, blueberries, boysenberries, loganberries, cranberries, gooseberries, raspberries	○	○	○	○	○	○	○	○	○
Sultanas, raisins, currants, figs	○	○	○	○	○	○	○	○	○
Dried apricots, prunes, dates, figs, mulberry, mixed dried fruit	○	○	○	○	○	○	○	○	○

Vegetables

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Potato (e.g. boiled, mashed, baked, roasted, fried, chips)	○	○	○	○	○	○	○	○	○
Kumara (e.g. boiled, mashed, baked,	○	○	○	○	○	○	○	○	○

roasted, fried, chips)									
Green beans, broad beans, runner beans, asparagus	○	○	○	○	○	○	○	○	○
Broccoli (All varieties)	○	○	○	○	○	○	○	○	○
Red cabbage	○	○	○	○	○	○	○	○	○
Cabbage (All varieties), brussel sprouts	○	○	○	○	○	○	○	○	○
Capsicum, peppers (All varieties)	○	○	○	○	○	○	○	○	○
Carrots	○	○	○	○	○	○	○	○	○
Cauliflower	○	○	○	○	○	○	○	○	○
Corn (All varieties)	○	○	○	○	○	○	○	○	○
Courgette, zucchini, cucumber, gerkins or marrow (all varieties)	○	○	○	○	○	○	○	○	○
Beetroot	○	○	○	○	○	○	○	○	○
Radishes (All varieties)	○	○	○	○	○	○	○	○	○
Lettuce	○	○	○	○	○	○	○	○	○
Mushrooms	○	○	○	○	○	○	○	○	○

Onions (All varieties), leeks, celery	○	○	○	○	○	○	○	○	○
Tomatoes (All varieties)	○	○	○	○	○	○	○	○	○
Peas, green	○	○	○	○	○	○	○	○	○
Spinach, silver beet, swiss chard (All varieties)	○	○	○	○	○	○	○	○	○
Other green leafy vegetables (e.g. watercress, puha, whitloof, chicory, kale, chard, collards, chinese kale, bok choy)	○	○	○	○	○	○	○	○	○
Pumpkin, squash, yams	○	○	○	○	○	○	○	○	○
Parsnip	○	○	○	○	○	○	○	○	○
Taro leaves (Palusami)	○	○	○	○	○	○	○	○	○

Breakfast cereals or porridge

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Porridge, rolled oats, oat bran, oat meal	○	○	○	○	○	○	○	○	○

Muesli (All varieties)	○	○	○	○	○	○	○	○	○
Weetbix (All varieties)	○	○	○	○	○	○	○	○	○
Cornflakes or rice bubbles	○	○	○	○	○	○	○	○	○
Bran based cereals (All varieties e.g. All Bran, Sultana Bran)	○	○	○	○	○	○	○	○	○
Light and fruity cereals (e.g. Special K, Light and tasty)	○	○	○	○	○	○	○	○	○
Chocolate based cereals (e.g. Milo cereal, CocoPops)	○	○	○	○	○	○	○	○	○
Sweetened cereals (e.g. Nutrigrain, Fruit Loops, Honey Puffs, Frosties)	○	○	○	○	○	○	○	○	○
Breakfast drinks (e.g. Up and Go)	○	○	○	○	○	○	○	○	○

Grains

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
White rice, biriyani, pilau, khitchri	○	○	○	○	○	○	○	○	○
Brown rice	○	○	○	○	○	○	○	○	○
Instant noodles	○	○	○	○	○	○	○	○	○
Pasta, noodles (White)	○	○	○	○	○	○	○	○	○
Pasta, noodles (Whole wheat)	○	○	○	○	○	○	○	○	○
Couscous, polenta	○	○	○	○	○	○	○	○	○
Bulgur wheat (e.g. Tabbouleh)	○	○	○	○	○	○	○	○	○
Wheat germ, wheat bran (Flakes)	○	○	○	○	○	○	○	○	○

Breads, cakes, biscuits and crackers

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
White bread and rolls (Including specialty breads such as Foccacia, Panini, Pita, Naan, Barbari, Crumpets, Pizza bases, Tortilla's, Burrito, Roti, Rotla, Bhatura, Paratha, Farsi poori, Kaak)	○	○	○	○	○	○	○	○	○
Brown bread and rolls (Including multigrain, wholegrain, whole meal breads), Chapatti	○	○	○	○	○	○	○	○	○
Breads fortified with iron (e.g. Mighty White Tip Top bread)	○	○	○	○	○	○	○	○	○
Fruit and currant bread / buns	○	○	○	○	○	○	○	○	○
White flour muffins (All varieties)	○	○	○	○	○	○	○	○	○
Whole meal	○	○	○	○	○	○	○	○	○

muffins (All varieties)									
Cakes (All varieties except chocolate and fruit cake)	○	○	○	○	○	○	○	○	○
Chocolate cake	○	○	○	○	○	○	○	○	○
Fruit cake	○	○	○	○	○	○	○	○	○
Biscuits, plain sweet	○	○	○	○	○	○	○	○	○
Biscuits, chocolate or chocolate covered	○	○	○	○	○	○	○	○	○
Crackers (e.g. crisp bread, water crackers, rice cakes, cream crackers, Cruskits, Mealmates	○	○	○	○	○	○	○	○	○
Iron fortified crackers (e.g. Vita wheat)	○	○	○	○	○	○	○	○	○
Pancakes, poori	○	○	○	○	○	○	○	○	○

Miscellaneous foods and snacks

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day

Marmite/Vegemite	○	○	○	○	○	○	○	○	○
Chocolate spread (e.g. Nutella)	○	○	○	○	○	○	○	○	○
Peanut butter	○	○	○	○	○	○	○	○	○
Almond/Cashew nut butter	○	○	○	○	○	○	○	○	○
Butter or margarine	○	○	○	○	○	○	○	○	○
Cooking oil (All varieties- sunflower, cornflower)	○	○	○	○	○	○	○	○	○
Ghee (clarified butter)	○	○	○	○	○	○	○	○	○
Soup, vegetable based, homemade or canned	○	○	○	○	○	○	○	○	○
Soup meat based, homemade or canned	○	○	○	○	○	○	○	○	○
Sugar, Gur (All varieties) added to food or drinks	○	○	○	○	○	○	○	○	○
Jam, marmalade, honey or syrups (e.g. dates, syrup, jaggery)	○	○	○	○	○	○	○	○	○
Muesli or cereal bar (All varieties)	○	○	○	○	○	○	○	○	○
Chocolate covered Muesli or cereal bar (all varieties)	○	○	○	○	○	○	○	○	○
Potato crisps	○	○	○	○	○	○	○	○	○
Milk chocolate	○	○	○	○	○	○	○	○	○
Dark chocolate	○	○	○	○	○	○	○	○	○
White chocolate	○	○	○	○	○	○	○	○	○

Halwa (Dessert made from syrup, vegetables or fruit)	○	○	○	○	○	○	○	○	○
Mesub (Flour, ghee, sugar dessert)	○	○	○	○	○	○	○	○	○
Pak/Sero (Semolina flour, ghee and sugar dessert)	○	○	○	○	○	○	○	○	○
Ladoos (Orange sweet round balls made from ghee and flour)	○	○	○	○	○	○	○	○	○

Alcohol

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Beer, Cider (All varieties)	○	○	○	○	○	○	○	○	○
Red Wine	○	○	○	○	○	○	○	○	○
White wine	○	○	○	○	○	○	○	○	○
Spirits (All varieties)	○	○	○	○	○	○	○	○	○
Ready to drink alcoholic beverages (e.g. Smirnoff Ice, Vodka Cruiser)	○	○	○	○	○	○	○	○	○

Non-alcoholic beverage

	I never eat this food	Less than once a month	1 to 3 times a month	Once per week	2 to 3 times per week	4 to 6 times per week	Once per day	2 to 3 times per day	4 plus times per day
Complan, Sustagen (All varieties)	○	○	○	○	○	○	○	○	○
Milo	○	○	○	○	○	○	○	○	○
Hot chocolate, Cocoa, Ovaltine, Nesquik	○	○	○	○	○	○	○	○	○
Coffee (All varieties)	○	○	○	○	○	○	○	○	○
Black tea	○	○	○	○	○	○	○	○	○
Herbal tea, Fruit tea	○	○	○	○	○	○	○	○	○
Cordial (Including syrups, powders e.g. blackcurrent, orange)	○	○	○	○	○	○	○	○	○
Fruit and vegetable juices (All varieties)	○	○	○	○	○	○	○	○	○
Sports drinks (e.g. Powerade, Gatorade)	○	○	○	○	○	○	○	○	○
Energy drinks (e.g. Red Bull, or V energy drink)	○	○	○	○	○	○	○	○	○
Water (Including tap, bottled or sparkling)	○	○	○	○	○	○	○	○	○

Rus (Sweet mango canned drink)	○	○	○	○	○	○	○	○	○

End of Block: Iron Food Frequency Questionnaire

End of Block: Iron Food Frequency Questionnaire

Start of Block: Survey completed

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

End of Block: Survey completed