The effect of dietary components on non-haem iron absorption in healthy and iron-deficient women

Thesis submitted in accordance with the requirements of the University of Chester for the degree of Doctor of Philosophy

SALMA FAEZA AHMAD FUZI

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Declaration

"The material being presented for examination is my own work and has not been submitted for an award of this or another HEI except in minor particulars which are explicitly noted in the body of the thesis. Where research pertaining to the thesis was undertaken collaboratively, the nature and extent of my individual contribution has been made explicit."

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Date Salma Faeza Ahmad Fuzi

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1,25(OH)D	1,25-dihydroxyvitamin D/ 1, 25-dihydroxycholecalciferol/ calcitriol
25(OH)D	25-hydroxyvitamin D/ 25-hydroxycholecalciferol/ calcidiol
7-DHC	7-dehydrocholesterol
ACKD	Anaemia of chronic kidney disease
ACP	Acute phase protein
AP	Ascorbyl palmitate
BMI	Body mass index
$Ca^{2+}HPO_4^{2-}$	Calcium hydrogen phosphate
CaBP	Calbindin
CDC	Centre for Disease Control And Prevention
COMA	Committee on the Medical Aspects of Food Policy
CRP	C-reactive protein
CV	Coefficient of variation
CYPs	High-capacity cytochrome P450 oxidases
DcytB	Duodenal cytochrome B
DMT-1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DRV	Dietary Reference Value
ECaC	Epithelial calcium channel
EDTA	Ethylenediaminetetraacetec acid
ELFA	Enzyme linked fluorescent assay
ELISA	Enzyme-linked immunosorbent assay
ESA	Erythropoietin stimulating agents
FAO	Food and Agriculture Organisation of the United Nations
FBC	Full blood counts
Fe2+	Ferrous
Fe3+	Ferric
$^{57}FeSO_4$	Ferrous sulphate solution
FFQ	Food frequency questionnaires
FGF23	Fibroblast growth factor 23
FP	Ferric pyrophosphate
FPN	Ferroportin
GDF15	Growth differentiation factor 15
HAMP	Hepcidin antimicrobial peptide
Hb	Haemoglobin

HCP-1	Haem carrier protein 1
Hct	Haematocrit
HFE	Haemochromatosis gene
HH	Hereditary haemochromatosis
HIF	Hypoxia inducing factor
HJV	Hemojuvelin
HO	Haem oxygenase
HPLC	High performance liquid chromatography
ICP-MS	Inductively-coupled plasma mass spectrometry
ICS	Iron-containing sulphur
ID	Iron deficiency
IDA	Iron deficiency anaemia
IL	Interleukin
IOM	Institute of Medicine
IRIDA	Iron-refractory iron deficiency anaemia
IU	International Unit
JAKs	Janus kinases
LIP	Labile iron pool
LPS	Lipopolysaccharide
LRNI	Lower reference nutrient intake
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
NaFeEDTA	Sodium iron ethylenediaminetetraacetec acid
NDNS	National Diet and Nutrition Survey
PBMC	Peripheral blood mononuclear cells
PCBP1	Poly(rC) binding protein 1
PCR	Polymerase chain reaction
PHA-L	Proteins of lectin
PIS	Participant information sheet
Pre-D3	Pre vitamin D3
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor-kappaB ligand
RBC	Red blood cells
RCT	Randomised controlled trial
RDW	Red cell width

RNI	Reference nutrient intake
ROS	Reactive oxygen species
SACN	Scientific Advisory Committee on Nutrition
SF	Serum ferritin
SPR	Solid phase receptacle
STAT3	Signal transducer and activator of transcription 3
STEAP3	Six-transmembrane epithelial antigen of prostate 3
S-Tfn	Serum transferrin
sTfR	Soluble transferrin receptor
TF	Transferrin
TF-I	Transferrin-iron complexes
TfR	Transferrin receptor
TfR2	Transferrin receptor 2
TIBC	Total iron binding capacity
TM	Test meal
TMAH	Tetramethylphenylammonium hydroxide
TMPRSS6	Transmembrane protease, serine 6
TWSG1	Twisted gastrulation protein homologue 1
UNICEF	United Nations Children’s Fund
UNU	United Nations University
UVB	Ultraviolet-B
VDBP	Vitamin D binding protein
VDD	Vitamin D deficiency
VDR	Vitamin D receptor
VDRA	Vitamin D receptor agonist
WHO	World Health Organisation

ABSTRACT

Two clinical trials investigating the effect of modulating two dietary components, tea containing polyphenols and vitamin D aimed at improving non-haem iron absorption and iron status recovery, were carried out in a cohort of healthy and iron deficient UK women, respectively.

Tea has been shown to be a potent inhibitor of non-haem iron absorption but it remains unclear whether the timing of tea consumption relative to a meal influences iron bioavailability, with limited published evidence, especially in human trials. The aim of the first study was to investigate the effect of tea consumption on non-haem iron absorption and to assess the effect of time interval of tea consumption on non-haem iron absorption relative to an iron-containing meal, in a cohort of healthy female participants using a stable iron isotope (^{57}Fe).

Twelve participants (mean age (± S.D): 24.8 ± 6.9 years) were administered a standardised breakfast porridge meal, extrinsically labelled with 4 mg of $^{57}FeSO_4$ on 3 occasions consecutively, with a 14-day time interval between each test meal (TM). The isotope-labelled test meal was administered with (i) water (TM I); (ii) tea administered simultaneously with a meal (TM II); and (iii) tea administered 1 hour post-meal (TM III). A reference iron dose was also administered (3 mg $^{57}FeSO_4$ with 35 mg ascorbic acid) without a test meal 14-days after the final test meal (TM III). Fasted venous blood samples were collected for iron isotopic analysis and measurement of iron status biomarkers, including plasma hepcidin concentration. Iron isotopic analysis was carried out using ICP-MS, and fractional iron absorption was estimated by the erythrocyte iron incorporation method.

Mean (± S.D) fractional iron absorption was 5.69 ± 8.5% (TM I), 3.57 ± 4.2% (TM II) and 5.73 ± 5.4% (TM III). Mean fractional iron absorption was found to be significantly higher (2.2%) with TM III compared to TM II ($p = 0.046$). Mean total iron absorbed was also found to be significantly higher (0.043 mg) when tea was consumed 1-hour post-meal (TM III) compared to when tea was administered simultaneously with test meal (TM II) ($p= 0.049$). A 2-fold reduction in the inhibition effect of tea (relative to water) was observed, from 37.2% in TM II to 18.1% in TM III. Plasma hepcidin concentration was found to be associated with fractional iron absorption ($r=-0.475$, $p=0.007$) and total iron absorbed ($r=-0.560$, $p=0.004$).

This study demonstrates that tea consumed with an iron-containing test meal simultaneously leads to decreased iron absorption, and that a 1-hour time interval between a meal and tea consumption attenuates the inhibitory effect, resulting in increased iron absorption. These findings are not only important in relation to the management of iron deficiency, but should also influence dietary advice, especially to those at risk of iron deficiency.

Vitamin D has recently been suggested to be involved in regulating iron absorption by its action on hepcidin suppression, but there is paucity of supporting literature, especially in human trials. The objective of the second study was to investigate the effect of an 8-week high dose of daily vitamin D3 supplementation (1500 IU) consumed with 60-grams of iron-fortified breakfast cereal containing 9 mg iron on haematological indicators and hepcidin response in a cohort of marginally and iron deficient premenopausal UK female participants. A total of 50 participants (mean age (± S.D): 27.4 ± 9.4 years) were randomised to receive either 1500 IU (38 µg) of vitamin D3 or placebo. Both groups were instructed to consume iron-fortified breakfast cereals with 200 ml of semi-skimmed milk, with either vitamin D3 or a placebo daily according to the group assigned. Participants attended 3 clinics at baseline, 4 weeks and 8 weeks, and fasted venous blood samples were collected for measurement of iron and vitamin D status biomarkers at each time point. Anthropometric measurements were performed at each time point, and a 3-day food diary was provided at baseline and post-intervention. Mixed model repeated measures analysis of variance (ANOVA) was performed to determine the effect of intervention and the interaction with time points for all iron and vitamin D status biomarkers.

There was a significant increase in the two main iron status biomarkers: haemoglobin concentration and haematocrit % from baseline (Week 0) to post-intervention (Week 8) in the vitamin D group, but not in the placebo group. It was demonstrated that mean (±S.D) haemoglobin concentration increase by 0.36 ± 0.71 g/dl from baseline to post-intervention in the vitamin D group was significantly higher than the placebo group ($p=0.037$). Mean (±S.D) haematocrit % increase from baseline to post-intervention was also significantly higher in the vitamin D group (1.82 ± 3.12 %) compared to the placebo group ($p=0.032$). It was also established in the present study that baseline concentrations of haemoglobin and plasma 25(OH)D had a significant effect on participants' response to the intervention. Interestingly, it was also observed that improvement in plasma vitamin D binding protein concentrations were strongly associated with the recovery of the red blood cell ($r=0.653$, $p=0.002$), mean corpuscular volume ($r=0.612$, $p=0.004$), haematocrit levels ($r=0.751$, $p=0.0001$), haemoglobin ($r=0.638$, $p=0.002$) and mean corpuscular haemoglobin concentrations ($r=-0.592$, $p=0.006$).

This study demonstrates that vitamin D3 supplements (1500 IU), consumed daily with iron-fortified breakfast cereal is a novel iron absorption enhancer in premenopausal women with marginal and low iron stores. An upregulation in plasma vitamin D binding protein concentration was demonstrated to be associated with the restoration of participants' iron status, however, the underpinning mechanism was unclear, which needs further investigations. These findings are clinically relevant, especially in the UK where iron deficiency prevalence is currently 16% in women of child-bearing age with vitamin D deficiency prevalence that could reach up to 39% in adults during the winter months.

CHAPTER 1

General Introduction

1.0 INTRODUCTION

1.1 IRON

Most living organisms require iron, which is one of the essential trace elements (Ganz, 2013) that plays a role in numerous important biochemical functions at the cellular level, including biosynthesis, cell proliferation, oxygen transportation and energy metabolism (Wang & Pantopoulos, 2011). Iron exists in two main oxidation states, the ferrous (Fe^{2+}) or ferric (Fe^{3+}) state, and ferric iron is readily converted to ferrous iron with the presence of reducing agents, or ferrous iron is converted to the ferric form facilitated by molecular dioxygen (Aisen, Enns, & Wessling-Resnick, 2001). Due to it's ability to occur in two different states, reduced and oxidised iron is capable of transferring electrons, facilitating reactions, forming complexes with proteins, and binding to oxygen (Aisen et al., 2001), but the ferric form of iron has low solubility that leads to poor bioavailability (Wang & Pantopoulos, 2011). However, the free form of iron can be lethal, resulting in significant cell and tissue damage due to the production of radical scavengers known as reactive oxygen species (ROS) (Silva & Faustino, 2015). Humans developed their own mechanism to take advantage of the presence of iron by utilising it for metabolism purposes whilst diminishing the ROS activities. This manipulation of iron, however, requires specific proteins to facilitate iron homeostasis processes which include intestinal absorption, and transportation to circulatory system, body utilisation, storage and functional sites (SACN, 2010; Silva & Faustino, 2015).

A typical adult human comprises of approximately 3-4 g iron, and the largest iron depot (2-3 g) is in haemoglobin of the erythrocytes (Ganz, 2013). The incorporation of body iron into haemoglobin is required for the development of erythroid precursors or mature red blood cells (RBC) (Andrews, 1999) and transportation of oxygen (Wang & Pantopoulos, 2011). **Figure 1.1** illustrates the iron distribution in different human tissues, which shows that about 1-2 mg of iron is mobilised across the human body daily in the non-pathological state. The distribution begins with the absorption of dietary iron by the intestinal enterocytes which enters the circulation, and forms iron-transferrin complexes to be distributed to various tissues (Andrews, 1999). Iron is distributed for utilisation in muscle fibres that exists in myoglobin form (300 mg), in the bone marrow (300 mg), and the greater proportion in erythrocytes as haemoglobin (1.8 g). The repository form of iron can be found in parenchymal hepatocytes of the liver (1 g) or in the macrophages of reticuloendothelial system (600 mg) (Andrews, 1999). Macrophages are responsible for iron recycling from the

degraded senescent RBCs (Knutson & Wessling-Resnick, 2003). As iron has an immediate turnover and rapid changes in the utilisation, the mobilisation of the recycled iron into plasma is tightly controlled to ensure that plasma iron concentrations are constantly in a balanced state (Ganz, 2013).

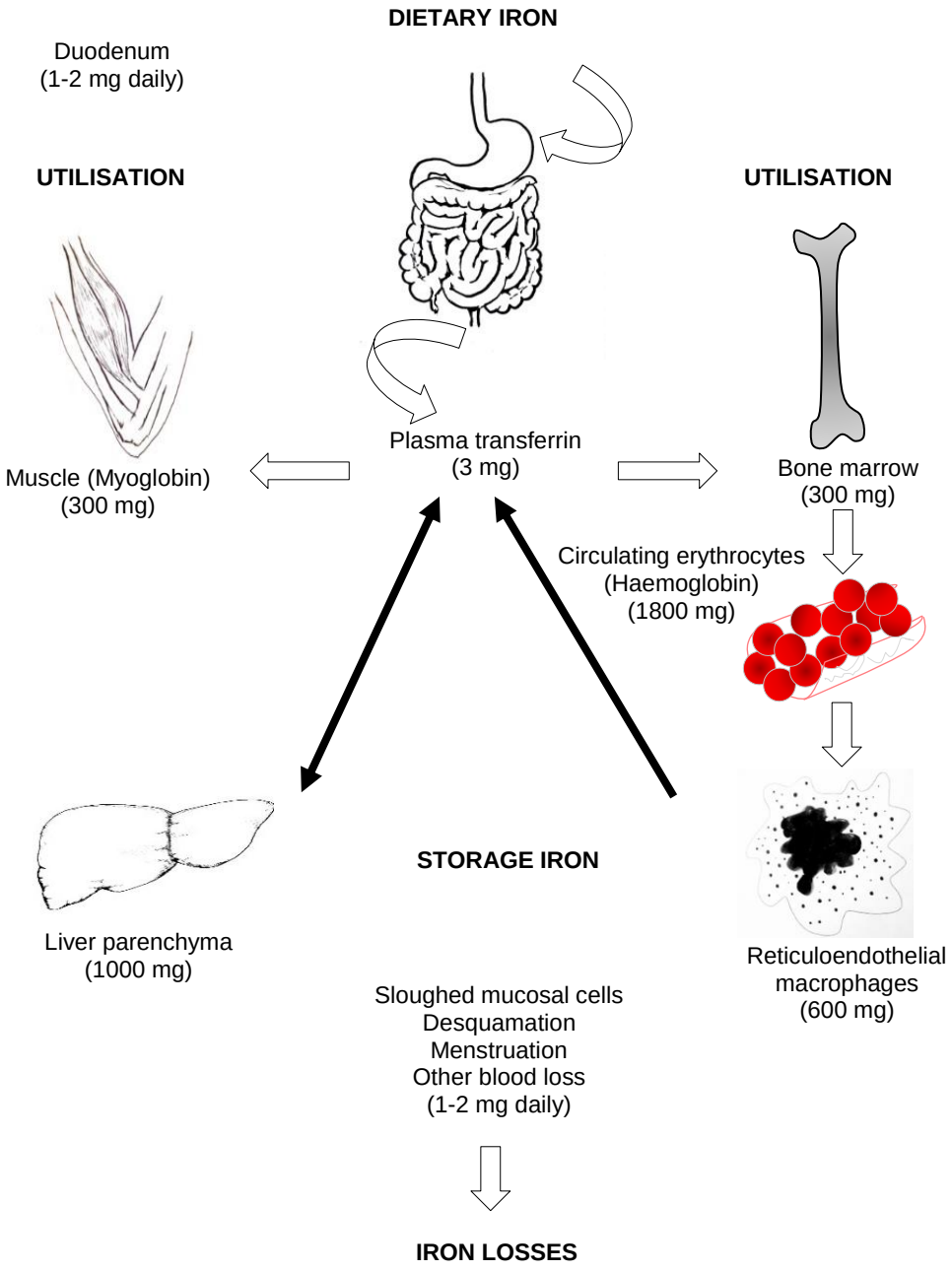


Figure 1.1 Iron distribution in typical adults - adapted from Andrews (1999)

1.2 IRON METABOLISM

As iron is not routinely excreted by specific mechanisms, iron metabolism does not involve typical pathways, as with other metals. Iron metabolism is different in that a greater amount of the daily iron requirement (~90%) is obtained endogenously, from the breakdown of circulating RBCs. Iron losses, especially for premenopausal women occur from the monthly menstrual cycle, in addition to average obligatory losses from various sources such as skin, the urinary tract, airways and the intestine (Hurrell & Egli, 2010). It was reported that a healthy man will lose an average of nearly 14 µg/kg body weight from gastrointestinal and urinary tracts, bile or dermis, which is estimated to be between 0.9 to 1.0 mg/day (Green et al., 1968). Taking into account these losses and the physiological needs for growth, iron must be obtained from the diet in order to maintain optimal iron balance (Hurrell & Egli, 2010).

Tightly-regulated iron homeostasis exists to ensure sufficient body iron content and plasma iron concentrations by controlling intestinal dietary iron uptake and regulating iron release from the macrophages (Ganz, 2013). Different transporter proteins and enzymes are specifically required for the transportation and handling of iron, as well as for the iron homeostatic control (Andrews & Schmidt, 2007), described further in **Table 1.1**. There are 3 prime cells that are individually vital in iron homeostasis including; (i) duodenal enterocytes which are involved in iron uptake, (ii) macrophages which are involved in iron recycling and (iii) hepatocytes which are involved in iron storage (Ganz, 2013). The key step in iron metabolism is the transfer of iron from enterocytes, macrophages and hepatocytes to the plasma, which is regulated by a peptide hormone, known as hepcidin that is produced in liver. Hepatocytes and macrophages are the iron depot for the proportion of iron not transported into plasma, whilst iron that is retained in enterocytes will be sloughed off after 2-5 days (Ganz, 2013). For this reason, iron body content is indicated by the segregation of iron between enterocytes and the plasma (Donovan et al., 2005).

1.2.1 Intestinal dietary iron absorption at duodenal enterocytes

A substantial proportion of physiological iron can be obtained from two sources; via the placenta during the foetus stage of life, or through intestinal uptake after birth. Dietary iron becomes the chief source of iron, as the iron accumulated during the foetus phase is rapidly utilised in the first few months postpartum (Fuqua, Vulpe, & Anderson, 2012). Dietary iron can be classified into haem or non-haem iron, which has different pathways of absorption. However, the absorption of haem iron is poorly understood, whilst non-haem iron absorption has been extensively discussed (Ganz, 2013). Additionally, the bioavailability of non-haem iron is influenced by the presence of iron enhancers such as meat and ascorbic acid, or iron inhibitors such as tannins or phytates that are predominantly found in plants (Abbaspour, Hurrell, & Kelishadi, 2014).

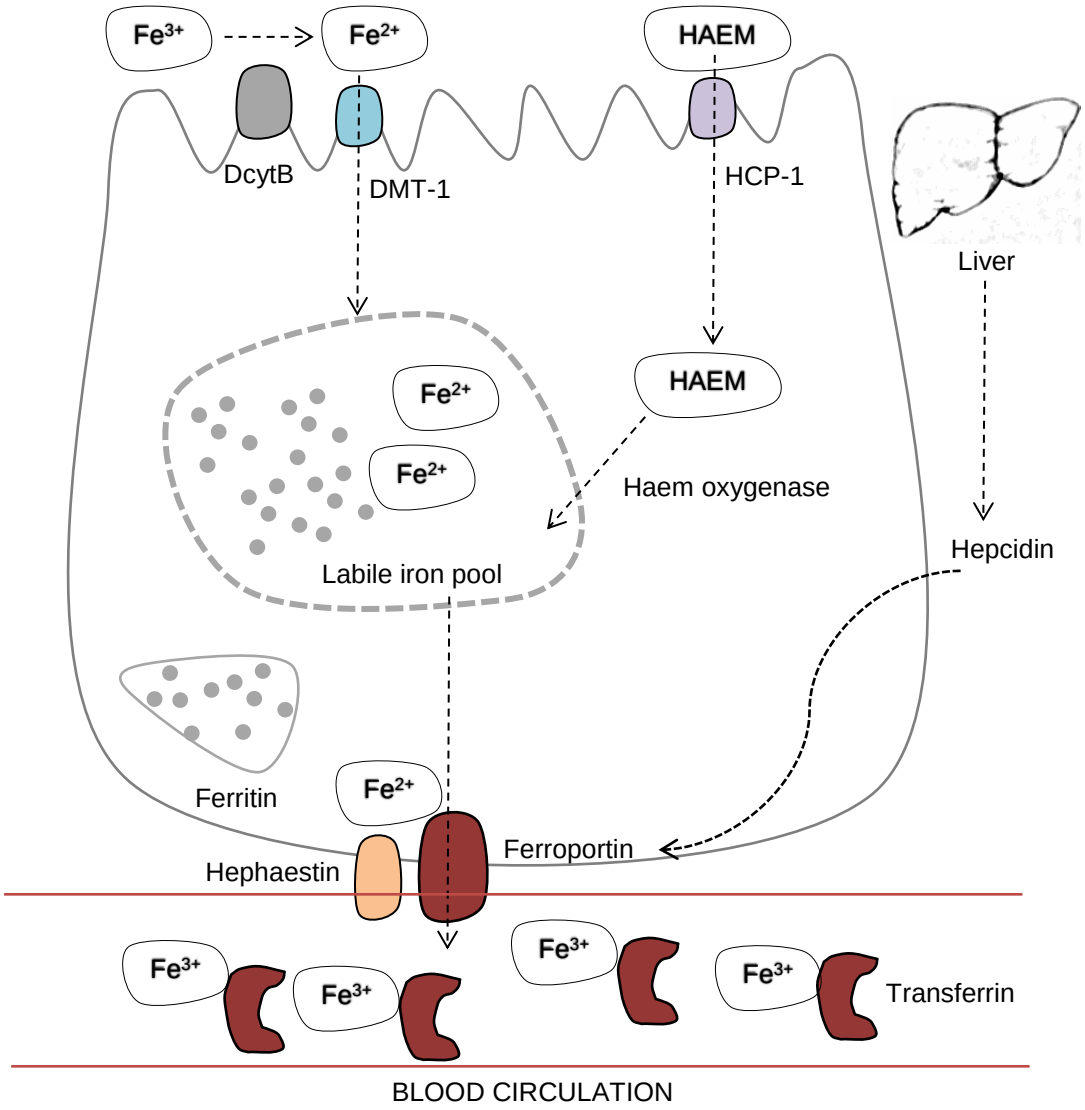
As excess of iron may lead to release of the ROS, intestinal iron uptake is tightly regulated to; (i) supply an adequate amount of iron needed for regular human body functions, (ii) rapidly respond to physiological requirements, such as pregnancy, and (iii) restrain iron uptake when there is a sufficient amount of iron in a human body. The disruption in regulation of intestinal iron absorption may result in clinical outcomes such as iron overload, that are characterised by increased iron uptake in spite of replete iron stores (Fuqua et al., 2012). This iron loading, or hereditary haemochromatosis (HH) is caused by a genetic mutation, affecting hepcidin antimicrobial peptide (HAMP) gene expression that results in low hepcidin concentration (Gulec, Anderson, & Collins, 2014). On the contrary, there are few syndromes or diseases that can cause a lowered intestinal iron absorption. One of the most described types of this disorder is iron-refractory iron deficiency anaemia (IRIDA), which is characterised by increased concentration of hepcidin due to a mutation in gene transmembrane protease, serine 6 (*TMPRSS6)*, and the presence of anaemia, with no response to oral iron therapy (Heeney & Finberg, 2014).

Intestinal iron absorption is comprised of a number of processes, including; (i) the transportation of iron across the apical membrane of the duodenum, (ii) the storage of iron as ferritin, or mobilisation from stores subject to requirements, (iii) transportation of iron across the enterocytes, and (iv) iron transfers across the basolateral membranes. **Table 1.1** summarises the description of the key molecules and enzymes involved in the absorption process, including the functions of each molecule or enzyme in the steps involved in intestinal iron absorption.

Table 1.1 Molecules and enzymes involved in the regulation of intestinal iron absorption

Molecules/Enzymes
1. Duodenal cytochrome B (DcytB) ▪ A ferric reductase brush border enzyme that reduces dietary iron ferric (Fe^{3+}) into ferrous iron (Fe^{2+}). ▪ Assumed to be a haem protein that utilises ascorbic acid in the reduction process, and is highly expressed in the presence of iron deficiency & hypoxia (McKie et al., 2001).
2. Divalent metal transporter 1 (DMT-1) ▪ Transfers Fe^{2+} across cell membranes. ▪ Acts as a proton symporter, which works effectively in a low pH environment (Gunshin et al., 1997).
3. Haem carrier protein 1 (HCP-1) ▪ Transports haem iron as intact into the enterocyte (Shayeghi et al., 2005). ▪ The mechanism is not fully established. Postulated that haem iron is degraded by haem oxygenase and enters the same iron pool with non-haem iron (Andrews & Schmidt, 2007).
4. Ferritin ▪ A protein that stores iron in the enterocytes in order to regulate the export of iron into circulation (Ganz, 2013). ▪ There is no established mechanism on how iron is transported from ferritin to bind to iron exporter (Ganz, 2013).
5. Ferroportin (FPN) ▪ Exports iron from the enterocytes into the blood circulation via hephaestin that mediates the oxidisation of Fe^{2+} to Fe^{3+} (Vulpe et al., 1999). ▪ Exists at the enterocytes membranes (Canonne-Hergaux, Donovan, Delaby, Wang, & Gros, 2006) and hepatocyte surface (Ramey et al., 2010).
6. Transferrin (TF) ▪ The iron carrier protein that primarily offers high-affinity binding sites for plasma iron which the concentrations depends on local conditions (Andrews & Schmidt, 2007). ▪ High in concentration when there is high availability of iron in circulation due to recent duodenal uptake (Andrews & Schmidt, 2007). ▪ Low in concentration when iron is used by erythroid precursor cells to produce RBCs in the bone marrow (Andrews & Schmidt, 2007).
7. Hepcidin ▪ Peptide hormone that is produced in the liver (Ganz, Olbina, Girelli, Nemeth, & Westerman, 2008). ▪ Regulates iron relocation from enterocytes, macrophages and hepatocytes into plasma (Ganz, 2013).

Figure 1.2 shows the iron uptake process from the cell into the blood circulation, which involves several different transporters and enzymes.



Key:

Ferric (Fe^{3+}); Ferrous (Fe^{2+}); Duodenal cytochrome B (DcytB); Divalent metal transporter 1 (DMT1); Haem carrier protein 1 (HCP1)

Figure 1.2 Haem and non-haem intestinal iron absorption pathways in humans

Non-haem iron is composed mainly from an inorganic form of iron (Fe^{3+}) and is reduced to the absorbable form of ferrous (Fe^{2+}) by duodenal cytochrome b (DcytB), a ferric reductase enzyme. Once reduced into ferrous form, divalent metal transporter 1 (DMT1) transports the iron into the intracellular iron pool in the enterocyte, known as labile iron pool (LIP). Unlike non-haem iron, haem iron is absorbed intact into the enterocyte via haem carrier protein 1 (HCP-1), degraded by haem oxygenase (HO) and released into the same pool (LIP) joining iron from the non-haem pathway. The absorbed iron will be at this point either (a) stored in enterocytes as ferritin, (b) utilised, e.g. for instance to produce haem, or (c) exported from the basolateral membrane into the blood circulation via the transporter ferroportin (FPN1). As transferrin will only receive a ferric form of iron, ferrous iron is re-oxidised into the ferric form via hephaestin and subsequently binds transferrin in blood circulation to be distributed to various tissues in the body (Gulec et al., 2014; Sharp & Srai, 2007; Steele, Frazer, & Anderson, 2005).

1.2.2 Utilisation of iron at various tissues for cellular functions

In a non-pathological state, 75% of iron is utilised in the development of erythroid precursors and RBC production (Andrews & Schmidt, 2007). Once iron is absorbed, it is bound to transferrin (TF) and forms transferrin-iron complexes (TF-I) (Andrews & Schmidt, 2007). Iron utilisation begins with the binding of TF-I complexes with transferrin receptor (TfR) that is expressed by the erythroid precursors, preparing for delivery to the required cells for cellular functions (Silva & Faustino, 2015). Subsequent to the binding, the TF-I/TfR complex undergoes a clathrin-mediated endocytosis, which causes changes in the shape of the complex that results in the release of iron (Fe^{3+}) (Andrews & Schmidt, 2007; Hentze, Muckenthaler, & Andrews, 2004).

A ferric reductase known as six-transmembrane epithelial antigen of prostate 3 (*STEAP3)* is found in high concentrations in haematopoietic tissues, which is responsible to reduce ferric iron to ferrous iron before transportation into a cytoplasm (Ohgami et al., 2005). After the iron is released, *STEAP3* reduces the ferric iron (Fe^{3+}) into the ferrous form (Fe^{2+}) (Andrews & Schmidt, 2007; Silva & Faustino, 2015), before the iron transporter known as divalent metal transporter 1 (DMT-1) **(Table 1.1)** transfers the reduced iron (Fe^{2+}) into the cytoplasm across the endosome membrane (Fleming et al., 1998). Both TF and TfR are reused for another cycle of iron binding and absorption, following the recycling process to the cell membrane surface (Hentze et al., 2004).

Poly(rC) binding protein 1 (PCBP1) is an RNA-binding protein that is found in the cytosol and nucleus of mammal cells (Shi, Bencze, Stemmler, & Philpott, 2008). Iron that leaves endosomes will move into mitochondria, directly or mediated by PCBP1 (Shi et al., 2008). A protein known as mitoferrin-1, encoded by the *SLC25A37* gene is the chief iron importer which facilitates iron transport into the mitochondria membrane (Shaw et al., 2006). Once in the mitochondria, iron is then used to initiate haem synthesis and subsequently used to produce haemoglobin and RBC (Andrews & Schmidt, 2007). Iron is also utilised in the biosynthesis of iron-containing sulphur (ICS) protein that is needed for transfer of electrons (Silva & Faustino, 2015) and oxidative phosphorylation (Richardson et al., 2010). The mechanism through which muscle assimilates iron to produce myoglobin is unclear and poorly understood (Andrews & Schmidt, 2007).

1.2.3 Storage and recycling of iron

Since there is no mechanism in place to secrete iron, and intestinal uptake accounts for only 10% of iron requirements per day, iron recycling involves iron already available within the system, in order to ensure a satisfactory amount of circulating iron (Andrews & Schmidt, 2007). The amount of circulating iron is primarily a derivative of aged and deteriorated RBCs that are phagocytosed by splenic macrophages. The engulfed RBCs undergo lysis and iron is released and transported to plasma transferrin once haemoglobin is catabolised by haem oxygenase (Andrews & Schmidt, 2007). Whilst ferroportin (FPN) mediates the iron transport from macrophages to bind to transferrin and distribute to specific tissues (Hentze, Muckenthaler, Galy, & Camaschella, 2010), some iron is retained in macrophages as a depot (Andrews & Schmidt, 2007). Iron is stored predominantly as ferritin in hepatocytes to serve two key functions; to ensure the absence of free iron that may damage cells, and to ensure sufficient iron mobilisation when there is demand (Silva & Faustino, 2015). Ferritin is comprised of 24-subunits of both heavy and light polypeptide chains which forms a spherical shape capable of storing more than 4500 iron atoms inside the core of the sphere (Wang & Pantopoulos, 2011). Ferritin is not only found in serum, but also in the cell cytosol, mitochondria and nucleus. Iron is released to the required cells by degradation of lysosome and proteasome (Silva & Faustino, 2015), depending on the iron levels, whether it is depleted or sufficient (De Domenico, Ward, & Kaplan, 2009).

1.3 REGULATION OF IRON METABOLISM

Iron levels are maintained homeostatically via several mechanisms, and iron uptake, recycling and storage are part of these mechanisms. However, iron uptake may be subject to changes, resulting in iron release from the storage and cells. Under increased demands, iron uptake, as well as released iron from macrophage storage will be rapidly increased. On the contrary, stored iron increases when there is iron overload due to increased iron absorption to avoid higher concentrations of free iron (Finch, 1994; Gavin, McCarthy, & Garry, 1994). Iron metabolism is strictly regulated at cellular and systemic levels (Silva & Faustino, 2015). Ferritin, the iron storage protein which is produced in all mammalian cells regulates iron metabolism at the cellular level by storing iron when there is excess, and releasing iron with demand (Andrews & Schmidt, 2007). Hepcidin has been reported to be a systemic iron regulator (Andrews & Schmidt, 2007).

1.3.1 Role of hepcidin in regulating iron metabolism

Hepcidin is produced in the liver and this peptide hormone is suggested to be partly responsible for regulating iron balance by obstructing intestinal iron absorption (Gulec et al., 2014). The *HAMP* gene which was first discovered at the long arm of chromosome 19, is where hepcidin is produced, consisting of a single peptide and 25 acid amino sequence (Hunter, Fulton, Ganz, & Vogel, 2002). Hepcidin expression is triggered when ferritin concentrations are high, and also when there is inflammation or infection, which leads to a decrease amount of circulating iron. The iron transporter ferroportin is eventually degraded following the internalisation, which is caused by the interaction between hepcidin-ferroportin. Conversely, hepcidin production is downregulated in the presence of iron deficiency, pregnancy and tissue hypoxia (Gulec et al., 2014). In a normal and healthy individual, the downregulation process of iron uptake takes place when there are adequate iron stores, however, in hereditary haemochromatosis patients, iron will accumulate in various organs including, the pancreas, heart, liver or other tissues as a result of increased iron absorption (Gulec et al., 2014). Hepcidin expression is regulated by different stimuli occurs at systemic levels, including; (i) circulating iron concentrations, (ii) hypoxia, (iii) erythropoiesis, (iv) depleted or excess iron and (v) presence of inflammation. The expression is greater with the presence of infections or inflammations, iron overload and increased serum iron, whilst it reduced in response to erythropoiesis, hypoxia and iron deficiency (Lin et al., 2007; Nicolas et al., 2002; Vokurka, Krijt, Sulc, & Necas, 2006), described individually as follows.

1.3.1.1 Circulating iron concentrations: presence of iron overload or iron deficiency

The expression of *HAMP* gene regulated by iron levels is necessary to avert the incidence of iron overload and iron deficiency in humans. There are different proteins involved in regulating hepcidin expression, and mutations in these proteins causes decreased hepcidin which leads to iron overload, or increased hepcidin, which results in iron deficiency (Ganz, 2011). The mutations in haemochromatosis (HFE), transferrin receptor 2 (TfR2), and hemojuvelin (HJV) genes are associated with a human hereditary haemochromatosis disorders, whilst the mutation in *TMPRSS6* gene is related to severe iron deficiency (Ganz, 2011).

1.3.1.2 Infection and inflammation

Iron deficiency may be caused by infection due to utilisation of iron by pathogens to proliferate, and for survival. Further inflammation in the gut can reduce intestinal iron uptake and systematically iron can be retained iron in macrophages, mediated by hepcidin expression. The retained iron will subsequently lead to low circulating iron concentrations (Ganz, 2011). Different pro-inflammatory cytokines (IL-6, IL-22) are released when pathogens are identified as foreign substance by cells, such as macrophages (Silva & Faustino, 2015). The cytokine subsequently forms a ligand with its receptor that activates the Janus kinases (JAKs) which then triggers the pathway of transcription protein known as signal transducer and activator of transcription 3 *(STAT3)* that leads to transcription of *HAMP* gene expression (Wrighting & Andrews, 2006), rendering iron unavailable for pathogens to proliferate (Silva & Faustino, 2015).

1.3.1.3 Hypoxia/anaemia and erythropoiesis

Under hypoxia or oxygen deficits, hypoxia inducing factor (HIF) is postulated to downregulate *HAMP* gene expression (Nicolas et al., 2002), but the HIF mechanism of action in inhibiting hepcidin expression is not well-established (Silva & Faustino, 2015). With hypoxia or anaemia, the production of the hormone erythropoietin, leads to the initiation of erythropoiesis. *HAMP* gene expression is reduced in parallel to this, to increase iron uptake in enterocytes, allowing an adequate amount of iron for erythropoiesis (Nicolas et al., 2002).

Suppression of *HAMP* gene expression in erythropoiesis has been shown to be facilitated by either erythropoietin hormone (Pinto et al., 2008), or molecules originating from the bone marrow known as growth differentiation factor 15 (GDF15) (Tanno et al., 2007) and twisted gastrulation protein homologue 1 (TWSG1) (Tanno et al., 2009).

Figure 1.3 (A) and (B) describes the role of hepcidin in regulating iron absorption (Vyoral & Petrák, 2005). **Figure 1.4** summarises the role of hepcidin in regulating iron metabolism. The liver is responsible for expressing hepcidin, a peptide hormone that functions to downregulate iron absorption in iron metabolism homeostasis (Tandara & Salamunic, 2012). The liver is considered the chief organ in iron metabolism homeostasis as it produces hepcidin and stores the majority of iron (Detivaud et al., 2005). The interaction of hepcidin-ferroportin causes internalisation of the iron exporter, which results in degradation by the lysosome. Since ferroportin is responsible for delivering iron to plasma, the uptake is reduced when there is a lower concentration of ferroportin at the membrane, causing the interference in iron flow from the main sites of circulating iron, such as macrophage, liver and enterocytes into blood circulation (Ganz, 2013). This then leads to decreased transferrin saturation and ultimately low iron availability (Nemeth et al., 2004; Tandara & Salamunic, 2012).

To sum up, iron metabolism involves the process of (i) iron uptake at intestinal level, (ii) iron utilisation for the production of RBCs, and (iii) iron storage and iron recycling as there is no mechanism for iron to be excreted. Different proteins and enzymes are involved at each stage in iron metabolism, and iron homeostasis is regulated by a tightly-controlled mechanism at systemic level by the action of hepcidin, which is expressed depending on iron concentrations, presence of inflammation, hypoxia and rate of erythropoiesis.

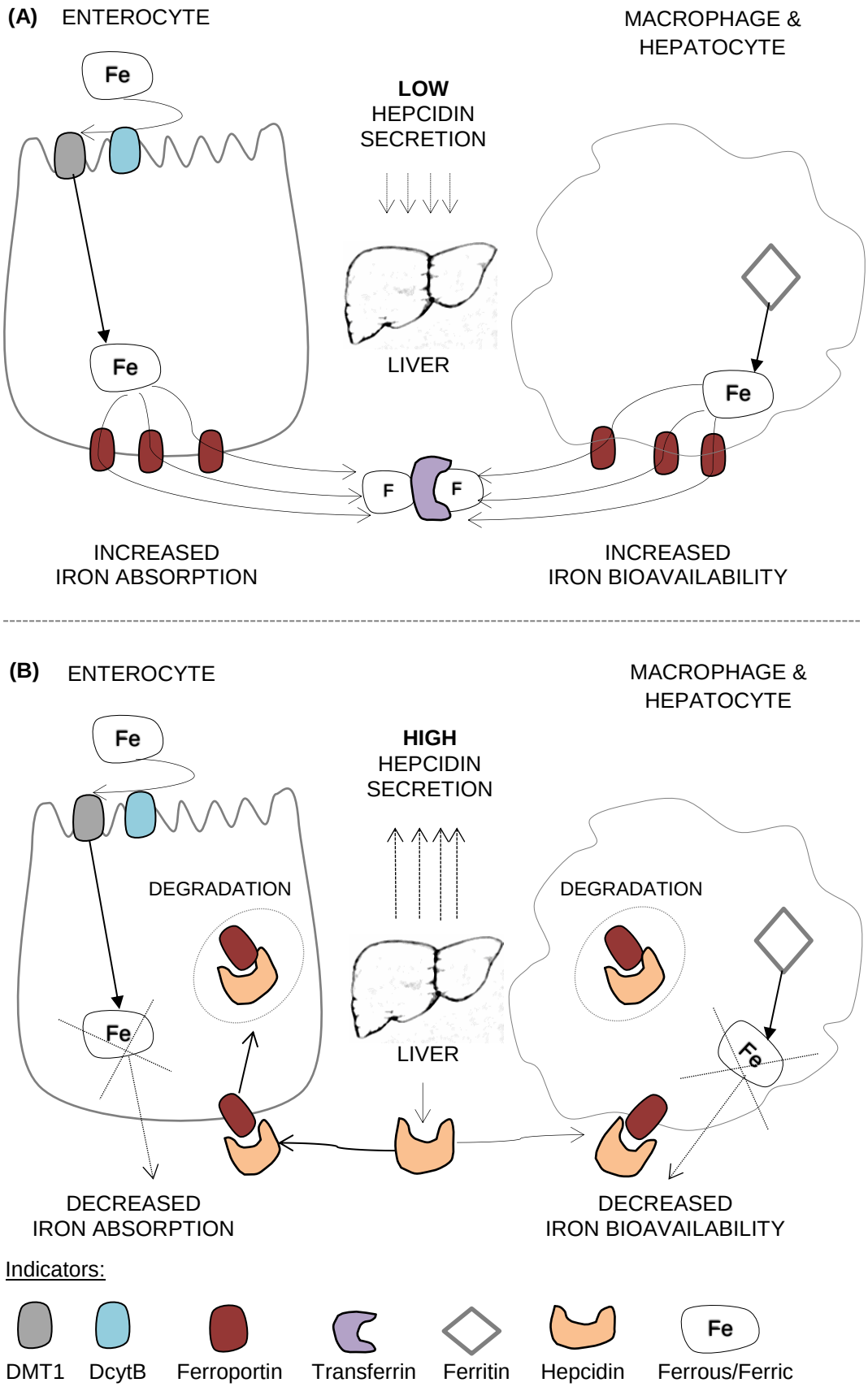


Figure 1.3 **Regulation of non-haem iron absorption with (A) low hepcidin expression (B) high hepcidin expression - adapted from Vyoral and Petrák (2005)**

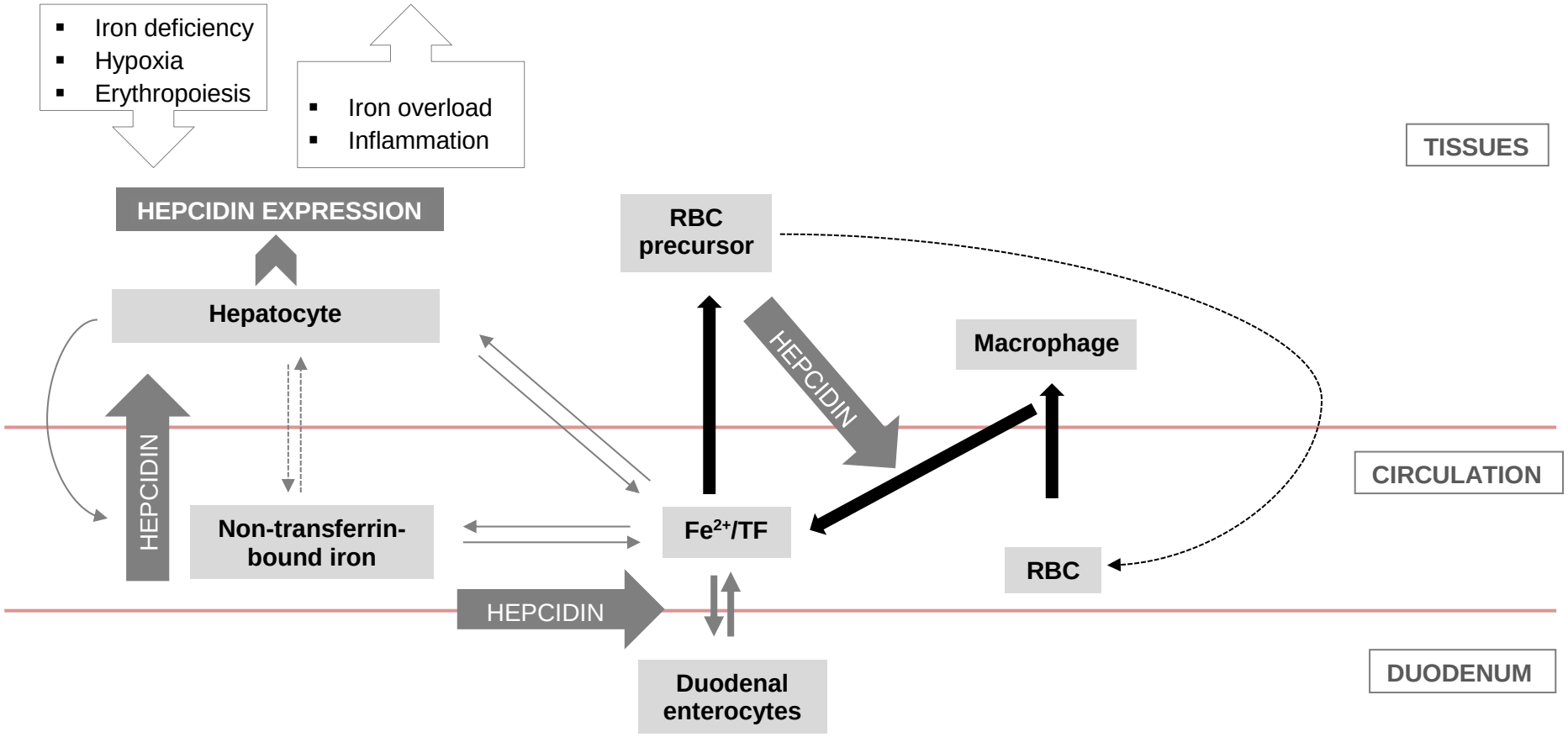


Figure 1.4 Regulation of iron metabolism by hepcidin - adapted from Tandara and Salamunic (2012)

1.4 IRON IN THE DIET

1.4.1 Dietary iron bioavailability

Iron bioavailability is defined as “the extent to which iron is absorbed from the diet and used for normal body function” (Hurrell & Egli, 2010) that is sometimes interchangeably used with the word “absorption”, which is defined by SACN as “the passage of a nutrient, or other dietary constituent, from the intestinal lumen into the body. It usually comprises the uptake of a nutrient into the enterocytes of the gut mucosa and its subsequent transfer across the cell and into the body” (SACN, 2010). Iron will not be principally absorbed and transported unless there are systemic demands, thus, bioavailability is not as critical in dictating iron absorption. Nevertheless, bioavailability becomes fundamental when there is an increased iron requirement, as different type of diets or component within the diet will have an impact on the bioavailability (SACN, 2010). Iron is less bioavailable, particularly when there is unvaried diet pattern, for instance in the developing countries where the dietary intake comprised of legume and cereal-based food sources (Zimmermann, Chaouki, & Hurrell, 2005).

Dietary-based iron sources exist in two major forms; haem or non-haem iron (Hurrell & Egli, 2010), and the differences between these forms are summarised in **Table 1.2**. Iron can also be obtained from other sources, such as iron fortifiers, that are used for food fortification and iron supplements, which are available in tablet or capsule form (SACN, 2010). There is no established method to quantify the amount of haem iron, therefore, the difference between the amount of total iron content and non-haem iron is assumed to be the total amount of haem iron (SACN, 2010).

Table 1.2 Comparison between haem and non-haem iron

	Haem iron	Non-haem iron
[1]Primary food sources	Meat, poultry and fish	Fruit, vegetable, cereal, pulses, legumes, eggs
[2]Bioavailability	High, between 15-35% and not affected by dietary components	Low, between 2-20% and greatly influenced by dietary components
[3]Amount in diet	Low	High

[1]FAO/WHO (2001); [2]Hurrell and Egli (2010); [3]Abbaspour et al. (2014)

1.4.2 Dietary iron intake of the UK adult population

The NDNS provides data on dietary intake of the UK population. It was shown that the mean daily intake, in general, has reduced from 14 mg/day in the 1950s to 10 mg/day in the 1990s, and remained unchanged (Fairweather-Tait, 2004). Mandatory fortification of wheat flour and breast milk alternatives are the currently implemented community health approach improving iron intake in the UK population (SACN, 2010). However, it is evident in the NDNS population data that the dietary iron intake is lower in women as opposed to men, and premenopausal women in particular, are an at-risk groups susceptible to iron deficiency. A review on the quality of UK women's diet by Ruxton and Derbyshire (2010) concluded that women aged 19-50 years consumed a diet characterised by a low intake of vegetable, fruit and oily fish, but high in saturated fat and salt. Micronutrients which include folic acid, vitamin D, calcium, and iron intakes, in particular, are still below the recommended level, and women of child-bearing aged are at risk of developing iron deficiency (Ruxton & Derbyshire, 2010).

Earlier NDNS population data has shown that a fifth of women of reproductive age failed to meet the requirement of lower reference nutrient intake (LRNI) for iron intake (Henderson et al., 2003). The percentage of women with LRNI for iron is increased when data was combined in the 4th year of the survey to 23% (Bates et al., 2014). Recent evidence from the NDNS data shows that the percentage of women with LRNI for iron has increased to 27% (Public Health England, 2016). Insufficient dietary iron intake increases individual risk of iron deficiency, particularly in women of childbearing-age, and iron deficiency may result in various health consequences demonstrated in previous studies in different settings (Ruxton & Derbyshire, 2010).

Table 1.3 summarises the findings on dietary intake of iron for women and men aged 19-64 years from the UK NDNS carried out from 2008-2014. For women, in particular, it can be observed that the mean daily iron intake was less than 80% of the RNI, which is 14.8 mg for women aged 19-64 years. Almost 30% of women consumed iron below the recommended intake (LRNI). Cereal and cereal products represent the highest percentage of food contributes to iron intake.

Table 1.3 NDNS population data for dietary iron intake of UK men and women aged 19-64 years

NDNS	[a]Mean (±S.D) daily iron intake (mg)/ [b](Percentage of RNI)		[c]Percentage of participants with LRNI	[d]Percentage contribution of food groups
[1]2008/09-2010/11 (Year 1-3)	Women	Not available (79%)	20%	Not available
	Men	Not available (137%)	1%	
[2]2008/09-2010/12 (Year 1-4)	Women	9.6 ± 3.0 mg (78%)	23%	Cereal & products: Women (38%), Men (40%)
	Men	11.7 ± 4.0 mg (135%)	1%	Meat & products: Women (19%), Men (20%)
				Vegetable & potato: Women (17%), Men (16%)
				[d]Other groups : Women and men (1-5%)
[3]2012/13-2013/14 (Year 5-6)	Women	9.4 ± 3.1 mg (76%)	27%	Cereal & products: Women (39%), Men (41%)
	Men	11.5 ± 4.0 mg (133%)	1%	Meat & products: Women (17%), Men (22%)
				Vegetable & potato: Women (17%), Men (15%)
				[d]Other groups : Women and men (1-4%)

RNI values: 14.8 mg/day; LRNI values: 8 mg/day for women aged 19-50 years; 8.7 mg/day; LRNI values: 4.7 mg/day for men aged 19-50 years
Mean and/or SD represents the data for women and men aged 19-64 years
[a]The mean daily iron intake represents the mean daily intakes from food sources only (not including dietary supplements)
[b]The mean daily iron intake as a percentage of Reference Nutrient Intake (RNI)
[c]The proportion of participants with mean daily iron intake below the Lower Reference Nutrient Intake (LRNI)
[d]The percentage contribution of food groups to average daily iron intake : Other food groups include milk and its products; eggs and egg dishes; fish and fish dishes savoury snacks; nuts and seeds; fruits; sugars, preserves and confectionery
[1]Henderson et al. (2003); [2]Bates et al. (2014);[3]Public Health England (2016)

1.5 IRON STATUS

Iron status can be described as a spectrum that ranges between the presence of iron overload and iron deficiency anaemia (IDA) at the lower end. The presence of IDA is due to a negative iron balance, as a result of a continuous iron depletion for a long period of time (UNICEF/WHO, 1999).

Iron status is a measure of the individual's body iron level characterised by physiological requirements. Both iron deficiency and iron overload occur due to the failure of iron homeostasis and results in flaws at functional and structural levels (SACN, 2010). **Figure 1.5** represents the spectrum of iron status, indicated by principal iron status biomarkers, including ferritin, transferrin receptor and hepcidin.

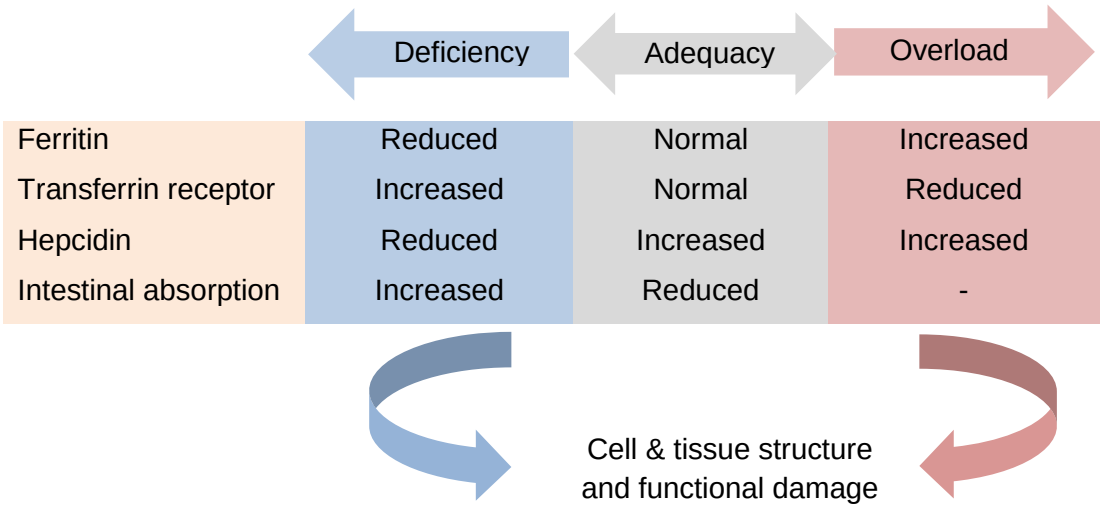


Figure 1.5 Spectrum of iron status - adapted from SACN (2010)

1.5.1 Anaemia

Anaemia is one of the major worldwide public health concerns that generally affects not only population health, but also the social and economic development of both industrial and non-industrialised countries (WHO, 2008). A WHO report on anaemia prevalence across different countries worldwide reported that anaemia is present across all age groups, but is often observed amongst young children and pregnant women. Iron deficiency has been identified to be the main aetiology of anaemia and marked as one of the most prevailing micronutrient deficiencies, affecting approximately 2 billion individuals worldwide (UNICEF/WHO, 1999).

It was estimated that nearly 30-60% of non-pregnant women are anaemic in developing countries, with Asia and Africa being two of the leading countries affected by anaemia (UNICEF/WHO, 1999). Anaemia is the result of a combination of causes which could be considered separately, however, these anaemia aetiologies are more frequently linked to each other (WHO, 2008). Up to half of all anaemia cases are attributable to iron deficiency (WHO/UNICEF/UNU, 2001), and the proportions hinge on numerous factors, for instance, geographical aspects or different age groups (WHO, 2008).

Anaemia can typically be diagnosed at the population level with the measurement of haemoglobin concentration that is straightforward and reasonably inexpensive. However, the sole use of haemoglobin concentration to determine the aetiology of anaemia should be interpreted cautiously (WHO, 2008). WHO normal threshold of haemoglobin concentrations of < 12 g/dl for non-pregnant women aged > 15 years to define anaemia have been internationally established and implemented (WHO/UNICEF/UNU, 2001). Anaemia prevalence of ≥ 40% is considered a public health issue, but not regarded as public health problem with the prevalence of ≤ 4.9% (WHO/UNICEF/UNU, 2001). The prevalence of anaemia in Europe between 1993 and 2005 in non-pregnant women was 19%, which approximates to nearly 41 million women, which indicates a mild public health concern (WHO, 2008). Despite the multifactorial causes of anaemia, measuring haemoglobin concentration to establish anaemia may be used as an indirect means of detecting iron deficiency (WHO, 2008).

1.5.2 Iron deficiency (ID) and iron deficiency anaemia (IDA)

Iron deficiency is a condition that is characterised by depleted iron stores that either develop into iron deficiency anaemia or remain at depleted levels with no progression (Camaschella, 2015). Iron deficiency with the presence of anaemia signifies reduced iron stores, with the presence of smaller and paler RBCs characterised by low MCV and MCH levels (Camaschella, 2015). In order to appraise the global prevalence of iron deficiency, it is a common practice to use levels of anaemia as a surrogate and it is observed that prevalence differs in developed countries compared to developing countries (WHO/UNICEF/UNU, 2001).

ID and IDA occurrences in developing countries have been attributable to inadequate dietary iron intake, infection by a worm that leads to blood loss, or a combination of both. On the contrary, dietary patterns such as a vegetarian diet and pathological disorders such as gastrointestinal-related illness are the generic causes of ID and IDA in developed countries (Camaschella, 2015). The main factors that generally lead to the incidence of IDA are poor dietary iron consumption; low iron bioavailability from meals especially with the presence of polyphenols and phytates; and higher physiological demands during menstruating, pregnancy or puberty (WHO, 2008).

The indications of ID in tissues are often elusive and treatment with iron may not lead to the restoration of iron stores. ID has been demonstrated to be associated with various health problems related to slowing development and cognitive impairment, particularly in children. As it is constantly recurring, IDA may also exist without distinct symptoms and typically may not be diagnosed. However, it is classically linked to disability in maintaining focus, reduced work output, fatigue and lethargy. A severe stage of IDA may increase individuals to risk of infection or heart failure, restless leg syndrome and affected numerous birth outcomes in pregnant mothers (Camaschella, 2015).

The evaluation of iron status, by measuring arrays of iron parameters is fundamental to determine the specific stage, due to a broad spectrum of iron status. It was specified that the use of several parameters is necessary as the use of a single parameter to define deficiency, adequacy or excess is not appropriate (Camaschella, 2015; SACN, 2010).

1.5.3 Measurement of iron status using different iron blood biomarkers

Iron biomarkers can be classified into 3 different categories that indicate either (i) iron function; (ii) iron delivery; and (iii) iron stores. Functional iron can be used as biomarker indicating iron utilised in production of the RBCs; iron delivery and supplies represents the biomarkers that are associated with the supply of iron to tissues; whilst iron stores represents the biomarkers that specify the iron depot in tissues (British Nutrition Foundation, 1995; SACN, 2010). Haemoglobin concentrations, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) levels give measurements of functional iron. Serum iron, transferrin saturation, and soluble transferrin receptor (sTfR) indicate the iron supplies to tissues, whilst ferritin and total iron binding capacity (TIBC) represents iron repository (British Nutrition Foundation, 1995).

The presence of anaemia can be confirmed with a single biomarker of haemoglobin concentration, however, diagnosing iron deficiency is more intricate and requires interpretation of combined iron status biomarkers (Lopez, Cacoub, Macdougall, & Peyrin-Biroulet, 2016). The diagnosis starts with observation of hypochromic and microcytic red cells indicated by reduced in MCH and MCV concentrations due to the effect of long-term iron deficiency (Lopez et al., 2016).

Ferritin concentration has been demonstrated to be well-associated with iron storage, on condition that there is no presence of inflammation (WHO/CDC, 2004), and deemed to be the best measurement to identify iron deficiency (Lopez et al., 2016). Iron stores to the level of below 15 µg/l, indicated by serum ferritin concentration, is reported as a depleted iron storage, however, a higher concentration of ferritin may be observed with inflammation despite low iron stores, causing the interpretation to be more complex (WHO/CDC, 2004). This, however, can be controlled by measuring acute phase protein (ACP) and a typical ACP is C-reactive protein (CRP) which has a rapid response to infection but also rapidly diminishes in concentration (WHO/CDC, 2004).

When iron deficiency is present, there will be a decline in serum iron and transferrin saturation but an increase in TIBC and sTfR signifies the total iron available to initiate erythropoiesis in response to iron deficiency (Lopez et al., 2016). Finally, the use of bone marrow (Perls' stain) aspiration is still regarded the benchmark method to detect iron deficiency. Despite its high specificity and that it is not influenced by infections, the method is costly, invasive, gives an unpleasant experience to the patients and the use of recombinant human erythropoietin affects the results (Lopez et al., 2016). **Table 1.4** summarises the principal iron biomarkers typically used to define iron status in adults.

Table 1.4 Principal biochemical iron parameters with indications of iron status assessment in adults

Iron parameters	Indication	Normal threshold	Presence of ID	Presence of IDA
1. Hb	Anaemia	Males : 13-18 g/dl Females : 12-16 g/dl	Normal	Low
2. MCV	RBC size (micro or macrocytic)	80-96 fL	Normal	<80
3. MCH	Amount of hb in RBC (hypo or normochromic)	27-32 pg	Normal	<27
4. Ferritin	Proportions of iron storage	Males : 15-300 µg/l Females : 15-200 µg/l	<30	<10
5. Hepcidin	Iron intestinal-uptake regulator	No established threshold	Low	Very low
6. Iron	Amount of iron that binds to transferrin in blood	10-30 µmol/l	Low	Low
7. TF saturation	Amount of circulating transferrin/iron complex	16-50%	≥16	<16
8. sTfR	Balance between iron demands and supplies	No established threshold	High	High

Hb: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular volume; TF: transferrin; sTfR: soluble transferrin receptor; ID: iron deficiency; IDA: iron deficiency anaemia
Threshold of Hb, ferritin, iron, TF saturation : SACN (2010), adapted the values from British Nutrition Foundation (1995)
Threshold of MCV,MCH: Kumar and Clark (2009), MCV threshold varies due to no established threshold
Threshold of hepcidin, sTfR – varies depending on methods of measurement and assay manufacturer's recommendation
Presence of ID and IDA were based on review by Camaschella (2015)
Indication of each parameter was based on report by WHO/CDC (2004)

Premenopausal women are an at-risk group with respect to iron deficiency (Gregory, Foster, Tyler, & Wiseman, 1990), and are more prone to mild iron deficiency in comparison to post-menopausal women (Heath, Skeaff, Williams, & Gibson, 2001). In the most recent combined population data of NDNS between 2008 - 2012, it was reported that 9.9% women aged 19-64 years were anaemic (haemoglobin concentration < 12 g/dl), 15.5% were ID (plasma ferritin concentration < 15 µg/l) and 4.7% were IDA based on thresholds of both iron biomarkers (Bates et al., 2014). Apart from menstrual losses in premenopausal women, another factor that leads to iron deficiency is a lack of iron intake from the diet (Bendich, 2001). Whilst the iron RNI for females aged 19-50 years is 14.8 mg/day (Department of Health, 1991), dietary iron intake has constantly remained at borderline levels in the UK population (Gibson & Ashwell, 2003).

1.5.4 Prevalence of anaemia, ID and IDA in the UK adult population

Prevalence of anaemia, ID and IDA reported in the NDNS that represents the UK population used the threshold suggested by the WHO/UNICEF/UNU (2001). For non-pregnant women aged ≥15 years, in particular, anaemia was defined as haemoglobin concentration of < 12 g/dl, ID was defined as serum ferritin concentration of < 15 µg/l and IDA is the combination of both biomarkers. The determination of the iron status of the UK population, reported in the NDNS did not use multiple biomarkers due to different thresholds available for other biomarkers, recommended by manufacturers of assays or laboratories which are not internationally established. Iron deficiency is predominantly prevalent in women, as opposed to men (Fairweather-Tait, 2004), and it was evident in the recent NDNS population data. It was reported that 4% of men were anaemic (mean (±S.D) 14.8 ± 10.3 g/dl), 2% was iron deficient (mean (±S.D) 139 ± 91.7 µg/l), and only 1% classified as IDA (Public Health England, 2016). It can be observed that the prevalence of anaemia and IDA in women did not substantially change, however, there was a decline in the prevalence of women with low serum ferritin concentration despite the overall normal concentration of both iron biomarkers **(Table 1.5)**.

Table 1.5 NDNS population data for prevalence of anaemia, ID and IDA of women aged 19-64 years

NDNS	[1]2008/09-2010/11 (Year 1-3)	[2]2008/09-2010/12 (Year 1-4)	[3]2012/13-2013/14 (Year 5-6)
Haemoglobin concentration			
Mean ± SD (g/dl)	13.3 ± 9.8	13.2 ± 9.8	13.2 ± 9.5
% below threshold	9.8	9.9	9.0
Serum ferritin concentration			
Mean ± SD (µg/l)	56.0 ± 52.0	56.0 ± 53.6	56.0 ± 57.7
% below threshold	16.6	15.5	10
Hb & serum ferritin concentrations			
% below threshold	3.3	4.7	3

[1]Henderson et al. (2003); [2]Bates et al. (2014); [3]Public Health England (2016)

One of the many reasons that anaemia, ID or IDA are still prevalent is due to methodological difficulties in the execution of corrective measures of this widespread nutritional deficiency (Lynch, 2011). Hence, it is important to identify and implement the approach that is effective to tackle this nutritional deficiency.

1.6 STRATEGIES FOR RECOVERY OF IRON STATUS

In a review by Aspuru, Villa, Bermejo, Herrero, and López (2011), it was stated that the management of ID and IDA due to insufficient dietary iron intake involves the normalisation of iron intake in daily food consumption. The aim of preventive measures are to restore and sustain a sufficient level of iron status, predominantly in the highly susceptible group of young children, adolescents, premenopausal women, pregnant and lactating women (Milman, 2011) and ultimately improve the quality of life (Lopez et al., 2016). A report on the management of IDA by WHO/UNICEF/UNU (2001) summarised the corrective and preventive measures into 2 different categories of (i) a food-based approach that comprises dietary modification and iron fortification or (ii) iron supplementation.

1.6.1 Dietary modification

Dietary modification involves strategies that are mainly focused on the education or dissemination of appropriate information, tailored specifically for iron. The emphasis should be on promoting and selecting iron-rich food sources; inclusion of iron absorption enhancers such as meat and ascorbic acid; exclusion of iron absorption inhibitors such as phytates and polyphenol; and appropriate ways of processing and cooking foods to retain the nutrient of interest (WHO/UNICEF/UNU, 2001). This dietary-based approach is not only specific to iron, but to the long-term overall diet as the aim is not only to increase the amount of iron in the diet but to improve overall bioavailability of the diet (WHO/UNICEF/UNU, 2001). **Figure 1.6** summarises the steps to implement this strategy that can be implemented in different populations.

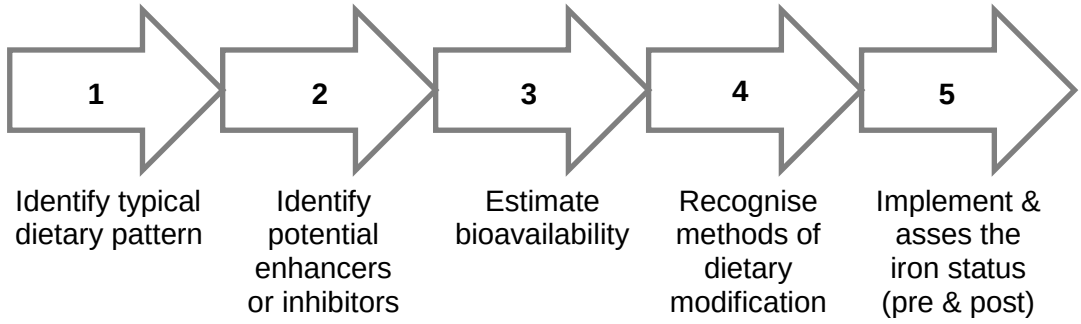


Figure 1.6 Step-by-step dietary modification approach to recovery of iron status - adapted from WHO/UNICEF/UNU (2001)

The first step in determining a specific meal pattern of the population can be achieved by using a food frequency questionnaire (FFQ), as the methods developed at step 4 are tailored specifically for a particular population or at-risk group. In developing countries where the iron intake is mostly derived from plant-based sources, or the majority of the population are vegetarians, ID is more prevalent (Zimmermann et al., 2005), therefore, promotion of the incorporation of iron absorption enhancers into the diet is crucial (Aspuru et al., 2011).

However, one of the greatest limitations of dietary modification is that this approach is demanding, as amending dietary patterns of the population can be challenging (Aspuru et al., 2011). In addition, women, in particular, have been reported to not have a tendency to alter their dietary intake to maintain optimal iron status despite higher physiological requirement, especially during pregnancy (Milman, 2011).

1.6.2 Iron fortification

Iron fortification is one of the dietary-based approaches aimed at the general population and can be a practical, efficient, sustainable and cost-effective approach if appropriately implemented (Aspuru et al., 2011; WHO/UNICEF/UNU, 2001). However, this approach requires joined-up efforts from the legislation sector or government, food manufacturers and also the food users (the population). The success of this approach also depends on (i) the choice of food vehicles, which are selected specifically to suit intended population, easily accessed and vastly consumed; and (ii) iron fortifiers that do not change the sensory appeals of the food vehicle which are normally staples or commonly consumed food items such as flour, salt or sugar (WHO/UNICEF/UNU, 2001).

For instance, mandatory iron addition into white and brown flour has been implemented in the UK since 1953, and the most used iron fortifiers are ferrous sulphate, elemental iron powder, and ferric ammonium citrate (SACN, 2010), which highlights the use of staple food items to cater for specific needs of UK population that use flour to prepare pasta or bread in their daily diet. The challenging aspect of this approach is to select the appropriate iron fortifiers to incorporate into the chosen food vehicles, as different fortifiers have their own pros and cons.

Ferrous sulphate has always been used as a reference if the comparison is made with regards to the different bioavailability of iron fortifiers (Hurrell, 2002a). However, because of its tendency to cause colour changes and become rancid if the foods are stored for long periods, the use of ferrous sulphate as a fortifier is often limited (SACN, 2010). Compared to ferrous sulphate, elemental iron powders will not cause organoleptic changes but their bioavailability differs between individual elemental iron powders, depending on how they are processed. Processing will have an impact on their shape, surface area and porosity which will ultimately affect their solubility in gastric acid (Hurrell, 1997). Electrolytic iron, hydrogen/carbon monoxide reduced iron and carbonyl iron are among elemental iron powders that are commonly used in fortification of cereal products. Novel iron compounds, as opposed to these specified conventional iron compounds that are available and used in fortification studies, are summarised in **Table 1.6**.

Table 1.6 Novel iron compounds used as fortifiers in iron fortification

Iron compound	Description	Cons
NaFeEDTA	▪ Highly absorbed compared to other iron compounds ▪ Prevent complex formation between iron & inhibitors	Costly, may cause colour changes in certain food vehicle
Ferrous bis-glycinate	▪ Manufactured by independent company (Albion Laboratories, Utah, USA) with unknown composition ▪ Claimed to be capable of preventing iron having contact with inhibitors	Expensive, may lead to change in colour and also cause fat oxidation in certain food vehicles
Haemoglobin	▪ Add to foods as a fortifier in a dried bovine RBC form ▪ Highly absorbed and inaccessible to inhibitors	Demanding in terms of production and storage, unattractive red colour to be used in food vehicle
Encapsulated iron compounds	▪ The outer coatings function to avoid organoleptic changes associated with the use of conventional compounds such as ferrous sulphate and fumarate ▪ The frequently utilised coating substances are maltodextrin, ethyl cellulose or hydrogenated oils	Heat labile

NaFeEDTA: sodium iron ethylenediaminetetraacetec acid

Information extracted from review by Hurrell (2002b)

In the UK, there is no population data available on the contribution of iron-fortified foods from the total daily iron intake, as this information was not assessed in the NDNS. It is known that main food items that are mandatorily fortified with iron fortifiers in the UK are white and brown wheat flours and most breakfast cereals which are frequently iron-fortified voluntarily by a number of food manufacturers (SACN, 2010). In general, it was reported in the NDNS that cereal and cereal products contributed to approximately 50% of daily iron intake across all age groups (Bates et al., 2014).

1.6.3 Iron supplementation

Iron supplementation has been used predominantly in developing countries, to manage ID in at-risk groups such as women, adolescents, young children (WHO/UNICEF/UNU, 2001), and has been advocated to be the primary corrective measure to be utilised to stabilise haemoglobin concentration and iron depletion if IDA is present (Aspuru et al., 2011). Iron supplementation for adults including premenopausal women can be classified into 2 categories; (i) correction of ID without anaemia as preventive measures at community level with 12-weeks of 60 mg/day of iron supplementation and (ii) therapeutic supplementation of IDA at health care level with 12-weeks of 120 mg/day of iron supplementation (WHO/UNICEF/UNU, 2001). Administration of iron supplements is frequently linked to a low compliance due to classically-associated adverse effects of gastrointestinal discomfort, nausea and black stool (WHO/UNICEF/UNU, 2001).

Ferrous sulphate has been suggested to be primarily used as an oral iron supplement, with other commercially available ferrous salts (ferrous fumarate, ferrous glutamate, ferrous lactate, ferrous succinate) as options if intolerant to ferrous sulphate, as these specified ferrous salts have comparable bioavailability (Aspuru et al., 2011). To enhance the absorption, it was advocated that the administration of oral iron supplements should be followed by ascorbic acid-rich juice and consumed following the meal (Aspuru et al., 2011). Iron supplements can be consumed with a meal to avoid the undesirable adverse effects, but it has been shown that this will result in decreased absorption by approximately 40%, and efficiency may be enhanced by taking the supplements in separate small doses (Lopez et al., 2016). This approach aims to replenish the depleted iron stores, and results may not be observed in a short amount of time, hence the need for high adherence (Aspuru et al., 2011). Following the administration of iron supplements, an increase in reticulocyte counts should be observed at the earliest at 4 days and is optimised after 7 to 10 days. A recovery of 2 g/dl of haemoglobin concentration is expected following iron supplementation, and the increment in concentration is typically established beginning the second week, hence the suggestion of 12-weeks of supplementation to continually replenish the iron stores (Lopez et al., 2016). However, the response to supplementation differs between individuals, depending on several other factors such as baseline haemoglobin concentration and overall iron status; the proportion of physiological-related losses; iron uptake; or other factors such as the presence of infections (Pavord et al., 2012).

The administration of intravenous iron supplements such as iron sucrose or the recently developed, iron carboxy maltose (Aspuru et al., 2011) is recommended typically in patients who have poor tolerance and low adherence to oral supplements (Clark, 2008). Parenteral iron supplements should only be considered when the oral iron supplements show no progression after thorough investigations have been conducted, considering the possible causes including poor compliance, malabsorption, or presence of infection (Lopez et al., 2016).

In the UK, evidence from NDNS population data shows that the intake of iron supplements in women aged 19-64 years represents only approximately 3%, with the approximately 15% for all single supplements including vitamin C and vitamin D (Bates et al., 2014; Henderson et al., 2003). The guideline to manage ID and IDA during pregnancy has been published after a joint review by several organisations representing UK-based medical experts recommending the consumption of ferrous salts, instead of ferric salts due to higher bioavailability (Pavord et al., 2012).

Pasricha et al. (2010) regarded a dietary-based approach as secondary to supplementation, as dietary iron intake as a single approach is insufficient in the treatment of IDA. However, it was evident that iron supplementation has low adherence as a result of it's adverse effects despite its efficacy in the recovery of haemoglobin and ferritin concentrations (Pavord et al., 2012).

1.7 IMPLEMENTATION OF STRATEGIES FOR MANAGING ANAEMIA AND IRON DEFICIENCY

Combatting anaemia or iron deficiency requires a cohesive approach, as its occurrence is suggested to be multifactorial. Iron supplements have been widely used to correct iron deficiency in at-risk groups, whereas, in the general population, either dietary pattern modification or iron fortification in selected food vehicles may be implemented (WHO, 2008). Due to various factors affecting iron absorption, it is challenging to design an efficient strategy to tackle iron deficiency, especially at population level (Fairweather-Tait & Collings, 2010). Extensive literature is available from intervention studies investigating the effect of utilising dietary-based approaches; including dietary pattern modification, iron fortification and iron supplementation on the improvement of iron status. However, previous studies have reported inconsistent findings on the effectiveness of the utilised approach on iron status recovery. Dietary modification has been regarded as the strategy that requires long-term goal (Pasricha, Drakesmith, Black, Hipgrave, & Biggs, 2013) as altering the individual's preference of food has been one of the barriers to improving

iron status through dietary modification (Lynch, 2011). Evidence from the most recent experimental trials has shown that iron fortification, which is considered as a medium-term strategy (Pasricha et al., 2013), will not completely improve general iron status, especially in countries where iron fortification is mandatory, including UK (SACN, 2010). The use of iron compounds in fortifying foods is one of the practical approaches to tackling iron deficiency (Ferreira da Silva, Dutra-de-Oliveira, & Marchini, 2004). Oral iron supplementation is widely used to improve iron status, especially ferrous sulphate, but efficacy is limited due to its adverse effects and low bioavailability, which have initiated studies using other iron compounds (Ferreira da Silva et al., 2004) such as iron polymaltose complex and iron protein succinylate (Santiago, 2012). The classic related adverse effects associated with iron supplementation include gastrointestinal discomfort (abdominal pains, constipation), black stools, nausea, vomitting and appetite loss, causing poor adherence which leads to inefficiency of intervention (Souza, Batista Filho, Bresani, Ferreira, & Figueiroa, 2009).

Extensive literature is available from intervention studies investigating the effects of dietary-based approaches (dietary pattern modification or iron fortification) and a therapeutic approach (iron supplementation) on the improvement of iron status, reporting inconsistent findings. Scientific evidence from systematic reviews carried out to date that includes experimental, or/and observational studies also reveal discrepancies in the efficacies of these iron interventions in the recovery of iron status. For instance, a systematic review was carried out to assess the effect of added iron, in the form of supplements, fortified foods, and iron-rich dietary sources on iron status biomarkers in healthy adults by Casgrain, Collings, Harvey, Hooper, and Fairweather-Tait (2012). The main finding of the review was, that added iron from supplements, fortifiers or dietary source improved iron status, reflected by increased in principal iron biomarkers; haemoglobin (0.51 g/dl, CI: 0.37, 0.65 g/dl, $p<0.00001$) and serum ferritin concentrations (9.19 µg/l, CI: 6.63, 11.75 µg/l, $p<0.00001$) (Casgrain et al., 2012). Despite the increase in iron status biomarkers following intervention, high levels of heterogeneity were observed between studies, and of the 41 RCTs included in the review, 24 studies were evaluated as very much at risk of bias (Casgrain et al., 2012). Inclusion criteria of selected studies that were not specific to one type of iron intervention may have led to high level of heterogeneity, therefore, results presented should be interpreted prudently.

Gera, Sachdev, and Boy (2012) carried out another meta-analysis but focusing solely on the effect of iron-fortified foods on iron biomarkers replicated the findings by demonstrating the effectiveness of iron fortification intervention in improving iron status. It was reported that haemoglobin (0.42 g/dl, CI: 0.28, 0.56, $p<0.001$) and serum ferritin (1.36 µg/l, CI: 1.23, 1.52 µg/l, $p<0.001$) concentrations at post-intervention increased after iron-fortified food consumption. Lower risk of anaemia and iron deficiency were also observed following the intervention (Gera et al., 2012). However, the observation revealed a high level of heterogeneity for both iron biomarkers and level of bias is not summarised as a whole but available in a separate supplemental data. The inclusion of only fortification studies makes the selection criteria tighter, but no control on age range and sex may have contributed to the high level of heterogeneity, thus, cautious interpretation is also necessary particularly on the impact on iron status following the iron intervention.

A systematic review by Jackson, Williams, McEvoy, MacDonald-Wicks, and Patterson (2016) carried out recently to determine the effectiveness of animal meat consumption, as part of dietary modification strategy, on the improvement of iron status in adults reported different observation from the previous 2 meta-analyses. Contradictory findings were observed in the pool of experimental studies, reporting mixed findings on the effectiveness of consuming the different type of meats on haemoglobin or serum ferritin concentrations, irrespective of the duration of studies and amount of meat consumed (Jackson et al., 2016). Inconsistent findings were also demonstrated from the observational studies included in the review which investigated the impact of vegetarian and omnivore diets on iron status, however, 70% of the studies showed increase in serum ferritin concentrations following omnivore diet as opposed to a vegetarian diet (Jackson et al., 2016). The review included 49 studies, including only 8 experimental studies, of which 3 was regarded as high quality out of only 7 studies. Due to the heterogeneity of the studies included in the review, no meta-analysis was carried out in this review. This shows that limited studies are available to date, especially from the RCTs, to reach a conclusive judgement on the effectiveness of incorporating the use of more meat in the diet (as part of a dietary modification strategy) in improving iron status.

It is well established that a wide range of studies have been carried out addressing different strategies in managing anaemia, ID or IDA, and in support of the mentioned systematic reviews, appraisal of the most recently published individual experimental studies employing individual or/and integrated strategies to improve iron status, particularly in women also revealed disparities in findings **(Table 1.7)**.

Table 1.7 Experimental studies investigating the effect of different types of interventions on the recovery of iron status

Study	Participant	Intervention	Main findings
A) Dietary modification			
Blanton (2013) United States	Women, n= 43 18-30 years	Beef or non-beef lunch 16-week	No significant difference between intervention and control groups in all iron status indices.
Remark: Moderate amount of beef (85 g) supplied in the study did not sufficient to improve iron status indices. Participants were not deficient at baseline, which may have contributed to the non-significant increase as no physiological demands for iron.			
McArthur, Petocz, Caterson, and Samman (2012) Australia	Women, n=76 18-35 years	A: Pork-diet + placebo B: Control diet + placebo C: Control diet +supplement 12-week	SF concentration significantly higher at post-intervention in C compared to A & B groups.
Remark: Iron intake, which is high at baseline, was similar at post-intervention in A, indicates 100 g did not sufficient to increase iron status indices. The supplement used contained ascorbic acid, which may have contributed to enhanced iron absorption.			

SF: Serum ferritin

Table 1.7 Experimental studies investigating the effect of different types of interventions on the recovery of iron status

Study	Participant	Intervention	Main findings
B) Iron fortification			
Karl et al. (2010) United States	Women, n=142 21 ± 4 years	Iron-fortified food bars or placebo food bars 9-week	Hb concentration significantly higher at post-intervention in the intervention group compared to placebo.
Remark: Despite the high iron content of the food bars (28 mg), other iron status did not improve, may be due to iron loss during the process of the fortified bars, and incorporation of iron enhancer may have aided in the iron absorption.			
Andersson et al. (2010) Switzerland	Women, n=142, 18-40 years	A: Fortified margarine (FP) B: Fortified margarine (Na) C: Placebo margarine 32-week	SF concentration significantly higher at post-intervention in B compared to A and C groups.
Remark: The study employed a random serial sampling to assess the iron status indices at 3 times throughout study duration (except baseline and post-intervention), not a fixed time point for all participants, which may have affected the interpretation of results in terms of the difference in participants' response to the intervention.			
Blanco-Rojo et al. (2011) Spain	Women, n=130, 18-35 years	Iron-fortified juice or placebo juice 16-week	SF concentration significantly higher at post-intervention in the intervention group compared to placebo.
Remark: Highly bioavailable iron compound (microencapsulated iron pyrophosphate) used in the fortified juice, as well as the readily available ascorbic acid in juice, may have aided in the absorption, hence the improvement in SF concentration.			
Beck, Conlon, Kruger, Coad, and Stonehouse (2011) Australia	Women, n=89, 18-44 years	A: Iron-fortified cereal + kiwi B: Iron-fortified cereal + banana 16-week	SF concentration significantly higher at post-intervention in A compared to B.
Remark: The study included no control group that consumed only iron-fortified cereals and the ascorbic acid in kiwi fruits administered was higher than in banana, which may have contributed to the enhanced iron absorption.			

Hb: haemoglobin; FP: ferric pyrophosphate; Na: sodium iron edetate or NaFeEDTA

Table 1.7 Experimental studies investigating the effect of different types of interventions on the recovery of iron status

Study	Study population	Intervention	Main findings
C) Iron supplementation			
Hoppe, Brün, Larsson, Moraeus, and Hulthén (2013) Switzerland	Women, n= 77 *24 years	A: haem blood-based bread B: ferrous fumarate 35mg C: ferrous fumarate 60mg D: iron-free supplement 12-week	No significant difference between A and B, C, D groups in all iron status indices.
Remark: Cautious interpretation of results necessary as two types of placebos used due to company shut down; no bread control to compare with the blood-based group. Adverse effects reported were gastrointestinal discomforts in supplement groups.			
Ortiz et al. (2011) Colombia/Argentina	Pregnant women, n=80 *>16 years	A: iron poly maltose (IP) B: ferrous sulphate (FS) 12-week / 100 mg	SF concentration significantly higher at post-intervention in group A compared to group B.
Remark: Group A reported fewer adverse effects indicating better safety and higher adherence, group B reported vomiting, nausea and constipation. IP was given in 2 different forms of single dose container or film tablet, FS in conventional tablet form, no placebo group.			
Vaucher, Druais, Waldvogel, and Favrat (2012) France	Women, n=198 18-50 years	A: ferrous sulphate (FS) B: placebo 12-week / 80 mg	SF and haemoglobin concentrations significantly higher at post-intervention in group A compared to group B.
Remark: The significant increase observed in SF, Hb, and other biomarkers may be due to the prolong-released type of FS used in the study. Classic adverse effects symptoms related to gastrointestinal discomforts reported in the intervention group.			
Berber et al. (2014) Turkey	Women, n=104 19-60 years	A: iron succinylate flacon B: iron glycine sulphate 12-week / 80 mg	Haemoglobin concentration significantly higher at post-intervention in iron ferrous group (B) compared to iron ferric group (A)
Remark: A total of 104 randomised and partake in the study, but the study included only 64 in the analyses mostly due to absence at the control visit (after 12-week) (n=17) and hypermenorrhoea (n=15). FS was not included as a control as a comparison of the efficacy.			

*SD and range for age not available

1.8 THE EFFECT OF DIETARY COMPONENTS ON NON-HAEM IRON ABSORPTION AND RECOVERY OF IRON STATUS

The influence of dietary components on non-haem iron absorption have been established in numerous single meal studies using an iron isotope technique (SACN, 2010). Iron absorption and bioavailability may be reduced in the presence of calcium, polyphenols, phytates, or peptides derived from incomplete digested protein. However, iron uptake has been shown to increase in the presence of muscle tissues which is haem source of iron, or ascorbic acid which is capable of reducing ferric to ferrous iron to be absorbed in the duodenum (Hurrell & Egli, 2010). As previously demonstrated in various studies in different settings, ascorbic acid has been suggested to enhance iron absorption, dose dependently (Conrad & Umbreit, 1993) by reducing iron into the absorbable ferrous (Fe^{2+}) form as well as functioning as a chelating agent (Abbaspour et al., 2014). Earlier studies have shown the capability of ascorbic acid in counteracting the effect of dietary iron inhibitors, such as phytate, polyphenols, calcium and milk components, by increasing both native and supplemental iron, but not significantly affecting the inhibitory effect of polyphenols from plant-based foods (Abbaspour et al., 2014). However, the enhancing effect of ascorbic acid has been shown to be diminished due to its lack of stability with cooking, processing and storage (Teucher, Olivares, & Cori, 2004). A mechanism describing how meat or its elements, contribute to the enhanced effect on non-haem iron absorption has not been established, due to discrepancies in findings (SACN, 2010). Elements in phytic acid, known as *myoinositol hexakisphosphate* (Hurrell & Egli, 2010), and tannin (Disler et al., 1975b) or galloyl esters (Hurrell, Reddy, & Cook, 1999) in polyphenols have been postulated and demonstrated to be responsible for the reduction of non-haem iron absorption. The formation of insoluble complexes with iron, rendering iron to be unavailable for the uptake has been suggested to be the mechanism of action of these components (Khokhar & Apenten, 2003; Nielsen, Tetens, & Meyer, 2013). Calcium affects both haem and non-haem iron absorption (Hallberg, Rossander-Hulthèn, Brune, & Gleerup, 1993), however, there are limited and inconsistent findings on the impact of calcium, milk components or animal proteins on non-haem iron absorption (Abbaspour et al., 2014).

There are a number of methods that can be utilised to quantify iron absorption in human subjects, which include isotopes (radio or stable), whole body counting following administration of radio-labelled isotopes, plasma kinetic studies, or chemical balance studies (International Atomic Energy Agency, 2012). However, it has been demonstrated that the use of stable iron isotopes is the most effective approach to properly determine dietary iron absorption as well as iron utilisation (SACN, 2010).

Iron bioavailability can be directly quantified using stable iron isotopes by measuring the estimation of isotope incorporation into the erythrocyte (Fairweather-Tait, 2001) or whole body counting (Benito & Miller, 1998). Stable or radioactive forms of isotopes are available and test meals used in the research can be either extrinsically (directly added to test meal) or intrinsically (added during the production of food) labelled. Generic radioactive isotopes (^{55}Fe or ^{59}Fe) have been used in the earliest isotope-nutrition research, however, these radioisotopes can only be used in research involving non-vulnerable groups such as non-pregnant women or healthy adults (International Atomic Energy Agency, 2012). For ^{59}Fe, direct measures of iron absorption can be carried out using whole body counting methods, but not for ^{55}Fe as this isotope does not emit gamma rays (ɣ). The ingested tracer can be measured as early as one hour after ingestion, and again after 14 days to allow incorporation. Using whole body counting methods, the amount of iron ingested and retained in the systems will indicate absolute value of iron absorption.

In contrast, stable isotopes cannot be measured with whole body counting (Wienk, Marx, & Beynen, 1999). However, stable isotopes can be applied in groups of children, infants or pregnant women as they carry no adverse effects with no radiation discharge (International Atomic Energy Agency, 2012). The incorporation of stable isotopes in the erythrocyte nevertheless, is the preferred method particularly in humans (Benito & Miller, 1998), in which iron absorption can be estimated based on the mass of the isotopes measured in blood via mass spectrometry (Wienk et al., 1999). Using the data collected from mass spectrometry analysis, the mass ratio (before and after test meal consumption) is used to calculate the amount of iron absorbed (International Atomic Energy Agency, 2012). Stable iron isotope technique measures the fraction of absorbed dietary iron, and its incorporation into the erythrocytes 14-days post-dosing as a measure of iron utilisation or bioavailability (International Atomic Energy Agency, 2012).

The whole blood samples collected before and after the isotope-dosing are typically measured using mass spectrometry to obtain an iron ratio that will be used in the estimation of fractional iron absorption (International Atomic Energy Agency, 2012). Different types of mass spectrometry have their own pros and cons, however, the key principle behind the measurement is the capability of these instruments to separate and quantify the different mass of the iron isotopes of interest (International Atomic Energy Agency, 2012). Using the ratio of iron gathered by the instrument, fractional iron absorption is estimated using a series of published equations (International Atomic Energy Agency, 2012). **Figure 1.7** illustrates the process of determining iron absorption from a test meal.

The principle behind the use of isotopes is that the absorbed iron in a labelled meal, be it with the stable or radioactive type will end up in a common non-haem iron pool. The absorption can then be measured in the assumed common pool by following what happens to the isotopes which function as a tracer (Benito & Miller, 1998). There are 5 important stages involved in human iron bioavailability studies: (i) recruitment of human participants; (ii) administration of labelled test meals with the selected isotopes after overnight fast; (iii) abstaining from food and drink a few hours after test meal administration; (iv) iron isotopic analysis from blood collected 14-days post meal administration and (v) estimation of fractional iron absorption (Benito & Miller, 1998).

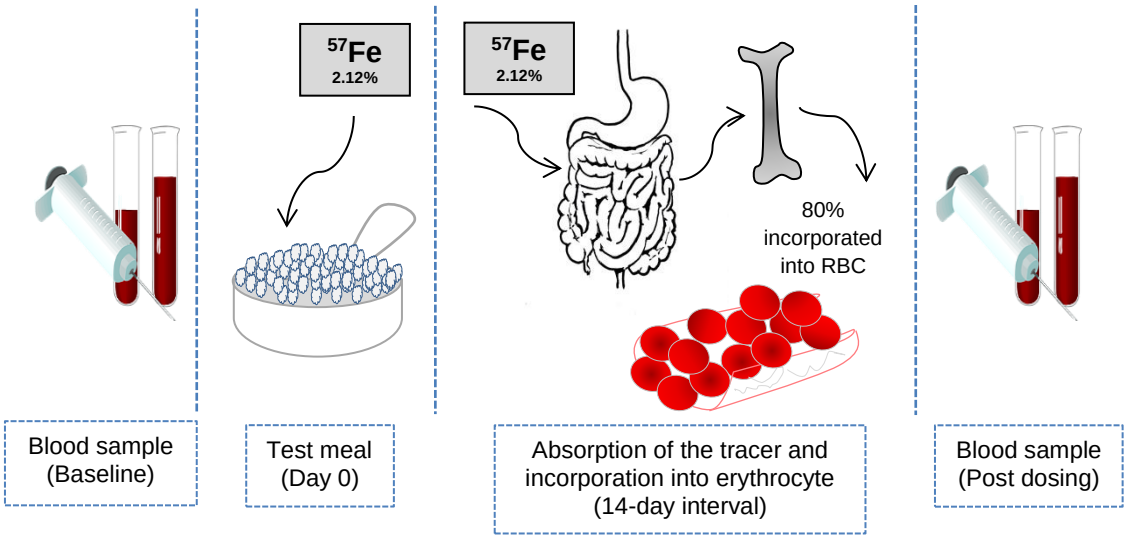


Figure 1.7 **Determination of fractional iron absorption - adapted from International Atomic Energy Agency (2012) and International Atomic Energy Agency (2014)**

Estimation of fractional iron absorption involves the use of published equations beginning with determination of blood volumes of research participants. Blood volume of the participants is needed to estimate the circulating body iron pool using a published equation (McKie et al., 2001), and can be obtained by experiments or using a fixed value (Wienk et al., 1999) of 65 per kg of body weight (International Atomic Energy Agency, 2012). Alternatively, a published equation by Shayeghi et al. (2005) can also been used to accurately calculate blood volume, which takes into account the height and weight of the female participants.

With the initial information on blood volumes gathered, the circulating iron pool can be estimated. The estimated circulating iron can then be used to calculate the amount of tracer incorporated into the erythrocyte (Andrews, 2005; Latunde-Dada, Simpson, & McKie, 2006). At this stage, the outcome from isotopic analysis of whole blood collected at baseline and post-dosing (expressed as ratio), as well as the natural abundance, atomic mass and atomic weight of tracer are used as part of calculation.

The percentages of isotopes absorbed and incorporated into erythrocyte has been suggested to depend on individual iron status, ranging from 80% for a healthy individual to 96% for those with iron deficiency anaemia (Wienk et al., 1999). Generally, the final fractional iron absorption (%) is estimated with the 80% assumption (International Atomic Energy Agency, 2012). However, differences in background iron status will contribute to the inter-variability between the subjects, and the use of a reference iron dose will correct the variation between subjects and enable comparison of the fractional iron absorption to be made between different iron bioavailability studies (Wienk et al., 1999).

It was agreed by experts that the isotope-labelling method is the gold standard in measuring iron bioavailability in human studies, especially for micronutrients such as iron, zinc, folate as well as vitamin A (Casgrain, Collings, Harvey, Boza, & Fairweather-Tait, 2010). However, it was stated in a report on iron and health by SACN in 2010 that findings on non-haem iron absorption from single meal studies which have used isotope methods, are inconsistent. The inconsistencies were due to the presence of enhancers and inhibitors in the meal used, causing an increase or reduction in iron absorption.

1.8.1 Dietary inhibitory factors of iron absorption

According to a review on iron by Abbaspour et al. (2014), phytates, polyphenols, calcium and some proteins are classified as the main inhibitors of iron absorption. Calcium has been demonstrated to affect both, non-haem and haem iron absorption (Hallberg et al., 1993). However, as phytates and polyphenols are mainly derived from plant-based diet and commonly lead to significantly reduced iron absorption (Abbaspour et al., 2014; Davidsson, 2003), the review focused on these two main inhibitors. Approximately 90% of phytate in the western-style diet is derived from cereals, such as bran or oat, and has been shown to inhibit iron absorption in a dose-dependent manner in a number of bioavailability studies since the late 80s (Zijp, Korver, & Tijburg, 2000).

A study to investigate iron absorption using two different strains of isotope-labelled cowpea (red and white) with fortifiers (NaFeEDTA and ferrous sulphate) was carried out in 16 Dutch students aged 18-40 years. Cowpeas are known to have considerable amounts of non-haem iron compared to rice and maize, and a higher content of polyphenols and phytic acid (Abizari et al., 2012). A low range of fractional iron absorption was observed in both white cowpeas (1.2-1.7%) and red cowpeas (0.9-1.4%), irrespective of fortifiers used (NaFeEDTA or ferrous sulphate). Lower iron absorption in red cowpeas was observed in the study (non-significant) due to higher polyphenol content in red strain. Irrespective of strain, NaFeEDTA enhanced the iron absorption more than $FeSO_4$ ($p<0.05$).

It was deduced that the use of a single meal consisting of a combination of both polyphenol and phytic acid molar ratio to iron (PP and PA : iron) may have exaggerated the inhibition effect, as no significant difference was observed between strains even though the red strain had higher polyphenol content (Abizari et al., 2012). The study, however, did not include any control test meal to allow comparison to be made.

In India, several studies carried out among low socio-economic groups showed that dietary iron consumption is below the recommended amount. Iron absorption was expected to be low, as the diets were largely plant-derived (rice, pulses and vegetables) (Thankachan, Muthayya, Walczyk, Kurpad, & Hurrell, 2007) with large amounts of phytates. In a study carried out in 45 iron deficient (ID) and 15 iron replete (IR) women aged 18-35 years, it was reported that iron absorption from the rice-based meal in the ID subjects (8.3%) were significantly higher (>3-fold) compared to IR subjects (2.7%) ($p<0.05$) (Kalasuramath, Kurpad, & Thankachan,

2013). The women were assigned to 4 groups of 15, three groups for ID women (millet, rice or wheat-based meal) and 1 group for IR (rice-based meal) women for comparison. The study found a higher iron absorption from rice and wheat meals (8.3 and 11.2% respectively) compared to millet meal (4.6%) within the ID group ($p<0.05$). It was postulated that the addition of cooked tomato that is high in ascorbic acid in the wheat meal had to some degree aided absorption (Kalasuramath et al., 2013), as wheat flour is normally rich in phytates (Hurrell, 2002a). Additionally, the combination of millet and green leafy vegetables known to be high in phytates and tannins may have possibly contributed to the increase in the inhibitory effect, resulting in the lowest iron absorption of 4.6%.

Bæch et al. (2003) used a known phytate-rich test meal, administered with different amounts of pork in 45 healthy women to determine a dose-dependent effect of using meat as a dietary enhancer to promote higher iron absorption. The women were assigned to consume isotope-labelled meals of rice with tomato sauce with 0, 25, 50 or 75 g of pork. It was shown that iron absorption was not significantly higher with pork when 25 g pork was given (7.1%) compared to the control meal (5.9%) but iron absorption was significantly higher with 50 g and 75 g (8.6% and 9.2%) compared to when no pork was added ($p<0.001$). Additionally, iron absorption was higher in the meal administered with 75 g pork compared to 25 g ($p<0.05$). It was suggested that a higher intake of meat, combined with a diet high in phytate, particularly in developing countries, may be useful in improving iron absorption (Bæch et al., 2003).

Instead of adding an enhancer into a phytate-rich meal, Petry, Ines, Christophe, Thomas, and Richard (2010) applied a dehulling and dephytinisation processes, to reduce the polyphenol and phytate content of test meals, resulting in higher iron absorption. The study was carried out in 49 women aged 18-45 years, grouped into 3 separate studies using isotope-labelled whole bean porridge meal (control) or bean porridge meals which had been either dehulled, dephytinised or a combination of both. It was found that dehulling did not appear to increase iron absorption as predicted, but lowered it from 2.6% (control meal) to 1.6% ($p<0.001$). However, iron absorption increased 2.6-fold from 2.4% to 8.7% ($p<0.001$) following the removal of both phytates and polyphenols by both dephytinisation and dehulling (Petry et al., 2010).

Petry, Egli, Campion, Nielsen, and Hurrell (2013) demonstrated that genetic modification of beans leads to a higher iron absorption in a study using iron isotope-labelled bean meal with a reduced concentration of phytic acid by >90% (bean known as lpa) but not polyphenol content. A cohort of 20 healthy women aged 18-30 years were administered two varieties of bean porridge (W and B) with modified polyphenol (PP) compared to parent-line with native phytic acid (PA): (a) lpa-B: high PP, low PA; (b) lpa-W: low PP, high PA; (c) wt-B: parent-line B and (d) wt-W: parent-line W. Comparing modified PA beans with the parent line, it was demonstrated that there was a higher iron absorption from the lpa-B compared to wt-B (6.14 to 3.84%, $p<0.001$) and lpa-W compared to wt-W (3.99 to 2.68%, $p<0.001$). It was shown that, despite the large difference in polyphenol content, there was no difference in iron absorption ($p=0.09$) and total iron absorbed ($p=0.26$) between the 2 varieties. This finding is practical especially in the countries where beans are staple foods, however, careful interpretation is needed if extrapolating the finding with mixed diets as other nutrient interactions may be present resulting in different magnitude of iron absorption.

Petry et al. (2016) carried out a further study comparing 3 types of beans including lpa beans (lpa), bio-fortified beans which has high iron content (bb) and control beans (cb), administered with either rice or potatoes in 29 women aged 18-30 years, with plasma ferritin concentrations < 25 µg/l. It was found that the same modified bean (lpa bean) caused side gastro-intestinal effects to the participants and that the iron absorption was not significantly different between lpa, bb and cb (8.6%, 7.3% and 8.0% respectively), consistent with the total iron absorbed of 421 µg, 431 µg and 278 µg. Four of the participants dropped out from the study, due to adverse events of vomitting, whilst seven complained about symptoms associated with gastro-intestinal discomfort such as diarrhoea and nausea. The events occurred following consumption of lpa beans, not with bb or cb beans, leading to the discovery of the presence of PHA-L (proteins of lectin) which affected the digestive tract of women. This effect was due to the fact that the lpa bean utilised in the study was not properly cooked, which results in a complex formation of pectinic acid and cations (Petry et al., 2016).

Phytate (inositol hexaphosphate or pentaphosphate), mainly found in cereals and polyphenols, and also derived from plant-based diets caused inhibition of iron absorption by chelating the non-haem iron, rendering it unavailable for the uptake at luminal level (Beck, Conlon, Kruger, & Coad, 2014). However, polyphenol is not only found in vegetables, but also in beverages such as tea that contains phenolic compounds (iron-binding properties) that bind to either catechol or galloyl groups (Zijp et al., 2000).

1.8.1.1 Inhibitory effects of tea-containing polyphenol

With the exception of water, tea, which originates from a plant scientifically known as *Camellia sinensis* and is considered to be one of the most frequently consumed drinks in the world (Vuong, 2014). Tea leaves are manufactured to either produce green, black or oolong teas (Łuczaj & Skrzydlewska, 2005). Unfermented green tea is made by either pan frying or steaming the leaves to ensure the inactivation of the enzyme polyphenol oxidase. This process results in catechin concentrations of 30-42% (dry weight) which is responsible for the sharpness and bitterness of the tea (Dufresne & Farnworth, 2001). Contrary to green tea, black tea is made by crushing the tea leaves to promote activation of polyphenol oxidase which leads to lower concentrations of catechins (10-12% dry weight). The oxidation products which include theaflavins (3-6% dry weight) and thearubigins (10-20% dry weight) give the tea its distinctive black colour (Haslam, 2003). Tea leaves, which are made into dried form, consist of chlorophyll, enzymes, caffeine, carbohydrates, protein, theanine, and the richest component of flavonoids, which consist of mainly catechins (Vuong, 2014).

In a review on tea and health by Vuong (2014), tea was found to be capable of exhibiting health benefits such as reduction in risk of cancer, diabetes, cardiovascular disease or obesity, however, there were discrepancies in the reported literature which was mostly derived from observational epidemiological studies. Despite its health benefits, it was also suggested that phenolic components in black tea may disrupt iron uptake, resulting in poor iron status, especially in at-risk groups such as pregnant women, children, or the elderly (Gardner, Ruxton, & Leeds, 2006). In addition, tea has been identified as the most popular beverage, predominantly in the countries where iron deficiency is prevalent (Disler et al., 1975a).

1.8.1.2 Intervention studies: effect of tea consumption on iron status

Intervention studies demonstrating that consumption of tea can lead to poor iron status are scarce. However, Prystai, Kies, and Driskell (1999) carried out a study in a group of 9 adults (5 females, 4 males) aged 22-43 years who were given a controlled diet with either black tea, decaffeinated black tea, green or no tea (control) for 14 days. Faecal analysis revealed that there was no significant difference between male and female subjects for each type of tea, including control, but urinary analysis showed a lower value of iron in females compared to males for control diet (no tea) ($p<0.05$). Iron balance [intake - (faecal + urinary)] was found to be more negative when green tea was administered compared to other teas ($p<0.001$). Iron balance was lower with tea consumption, regardless of type, compared to control, however, no difference was observed between genders. A metabolic study, especially which involves collection of urine and faeces may yield accurate findings, however, this study was limited by its low number of participants which limit interpretation to a general population.

Breet, Kruger, Jerling, and Oosthuizen (2005) demonstrated that black tea did not have an impact on iron status in 150 children aged 6-15 years, grouped into black or rooibos tea (control) groups, administered with 400 ml tea and brown bread at school, daily for 16 weeks. Unexpectedly, mean (± 95% CI) haemoglobin concentrations were found to be higher at the end of study (13.2 g/dl, CI: 13.0, 13.4) compared to 12.8 g/dl (CI: 12.5, 13.2) at baseline in the black tea group ($p=0.002$). In the black tea group, it was also observed that there were significant changes between baseline and 16-weeks in MCV, MCH, S-Tfn, and TIBC ($p<0.0001$), but not for transferrin saturation, serum iron and serum ferritin. The mean (± 95% CI) MCV, S-Tfn and TIBC were significantly higher at 16-weeks (77.2 fl, CI: 76.2, 78.2; 2.71 g/l, CI: 2.54, 2.99; 60.6 µmol/l, CI: 56.8, 67.0) but lower for MCH (24.7 pg, CI: 24.8, 24.7). However, no significant difference was observed between groups in all iron status biomarkers measured.

No effect on iron status following tea consumption was reported by Schlesier et al. (2012) in shorter study of 4-weeks in 34 healthy adults (25 females, 9 males) aged 19-32 years. It was a cross-over study, and all participants were required to consume 1 litre of green or black tea at home, with meal but without milk for 4 weeks. Iron status biomarkers including haemoglobin, haematocrit, serum ferritin, serum iron, and TIBC were not affected in male subjects with black or green tea consumption.

However, in women, serum ferritin concentration was significantly lower after 4 weeks of black tea consumption (mean ±S.D not provided) ($p<0.05$), but not green tea. Other iron status biomarkers were found to be unaffected by consumption of black or green tea in both gender (Schlesier et al., 2012).

1.8.1.3 Cross-sectional studies: association between tea consumption and iron status

Table 1.8 summarises cross-sectional studies carried out to date, investigating the association between tea consumption and iron status. Tea consumption did not lead to poor iron status, resulting in either anaemia, iron deficiency or iron deficiency anaemia, based on the findings reported. Consumption of tea as part of normal diet will not influence the iron status, especially for a generally healthy population (Mennen et al., 2007). A dietary pattern which may promote iron absorption, such as a diet high in ascorbic acid as observed in the study by Mehta, Pritchard, and Stegman (1992), or high in meat (Milman, Pedersen, Ovesen, & Schroll, 2004) may counteract the inhibition effect of tea, as no association between tea consumption and iron status was established in these studies. It was suggested that the association observed in the experimental studies may not represent actual dietary habits as they are carried out under controlled environments, not in free-living population (Hogenkamp, Jerling, Hoekstra, Melse-Boonstra, & MacIntyre, 2008). These studies, which are mainly part of larger scale studies e.g. national surveys, however, do not use specific measures to quantify the consumption of tea, despite the use of blood biomarkers to determine iron status. Validated food frequency questionnaires (FFQ), or tea questionnaires may not be a true representation of subjects' tea intake, as recording food in an FFQ may result in underestimation due to focusing on current intake compared to previous intake (Palacios, Trak, Betancourt, Joshipura, & Tucker, 2015).

Table 1.8 Cross-sectional studies: association between tea consumption and poor iron status

Study	Study population	Tea consumption	Prevalence of anaemia/ ID/ IDA	Association (tea and poor iron status)
Mehta et al., 1992 USA	Adults, n= 11684 19-74 years	3.1 cups/day	Hb : 5.3%	No association
Galan et al., 1998 France	Adults, n= 9931 35-60 years	Not available	Hb : 10.4% SF : 29.8% Hb & SF : 5.1%	No association with either Hb (p=0.74) or SF (p=0.31)
Milman et al., 2004 Denmark	Men, n= 2612 40-70 years	6 cups/day including coffee	SF : 0.4-0.5% Hb & SF : 0.08-0.16%	Negative association with serum ferritin (p<0.0001)
Keskin et al., 2005 Turkey	Children, n=1014 12-13 years	4.8-8.5 cups/week	SF : 19.1% Hb & SF : 3.9%	No statistical test performed but ID prevalence high in group that consumed more tea
Mennen et al., 2007 France	Adults, n=2593 42-68 years	256-351ml/day (B) 150-185 ml/day (G) 104-140 ml/day (H)	SF : 38-165 µg/l SF : 42-501 µg/l SF : 45 – 168 µg/l (Prevalence N/A)	No association with SF across type of tea (black, green, herbal) (p>0.05)
Hogenkamp et al., 2008 South Africa	Adults, n=1360 15-65 years	56.6-85.1 ml	SF : 6.8-21.6% Hb & SF : 3.2-14.6%	No association with either Hb (men p=0.33, women p=0.49) or SF (men p=0.059, women p=0.49)

Hb – Haemoglobin (g/dl) indicates anaemia; SF-Serum ferritin (µg/l) indicates iron deficiency (ID); Combined Hb and SF indicates - iron deficiency anaemia (IDA); NA: not available

Mehta et al. (1992); Galan et al. (1998); Milman et al. (2004);Keskin et al. (2005); Mennen et al. (2007); Hogenkamp et al. (2008)

1.8.1.4 Iron isotope-labelled studies: effect of tea consumption on iron status

Disler et al. (1975a) showed that 200 ml of tea consumed with a simple iron salt solution ($FeCl_3$), labelled with iron isotope, reduced iron absorption to 6.2% compared to 21.7% when the $FeCl_3$ was administered with water ($p<0.05$). Iron absorption of another simple salt solution ($FeSO_4$), reduced to 11.2% when administered with tea, with added ascorbic acid, as opposed to 30.9% when administered with water ($p<0.01$). Disler et al. (1975a) also used a simple test meal of intrinsically labelled bread and demonstrated a 3-fold reduction from 10.4% to 3.3% ($p<0.001$) when the tea was administered with the labelled bread, compared to when it was administered with water. The galloyl component of polyphenol in tea may have caused the reduced iron uptake (Brune, Rossander, & Hallberg, 1989).

A simple bread test meal was also used in a study carried out by Hurrell et al. (1999) investigating the effect of various polyphenol-containing beverages, including different types of tea on non-haem iron absorption in the US which included 77 adults aged 19-40 years. In addition to observing reductions in iron absorption when tea was administered with bread, compared to water, the study also demonstrated a dose-dependent effect of tea concentration on iron absorption. It was shown that iron absorption decreased to 0.59%, compared to 6.58% with water as control ($p<0.001$). Diluting the same tea to 50% and 25% also significantly increased iron absorption to 1.05% and 1.18% respectively ($p<0.001$) (Hurrell et al., 1999). However, these studies were carried out using a simple test meal that did not contain a mixture of potential enhancers or inhibitors, which may potentially affect iron absorption. A simple iron salt solution without the use of test meal also may have affected the iron absorption in different ways, compared to when an actual labelled test meal was used.

1.8.2 Dietary enhancing factors of iron absorption

One of the current approaches used to improve iron nutrition in the UK includes compulsory fortification of flour and breast milk substitutes which target at-risk groups e.g. infants, young children, and pregnant women, as well as the overall population. Other than consuming iron rich food sources, the advice given includes to consume food sources that are high in ascorbic acid to maximise iron absorption as ascorbic acid, as well as meat, are the most researched potential enhancers of iron absorption (SACN, 2010). Extensive scientific evidence on the role of ascorbic acid in amplifying iron absorption, using different test meals has been reported since the 1950s (Cook & Reddy, 2001), and studies since the 2000s have focused more

on alternatives to ascorbic acid. One recent bioavailability study demonstrated the potential role of ascorbic acid in enhancing iron absorption in 47 healthy children aged 6-7 years (Davidsson, Walczyk, Zavaleta, & Hurrell, 2001). The test meal used was an isotope-labelled powdered-based breakfast drink with white bread, administered with either ascorbic acid solution or Na_2EDTA to investigate the effect of these two compounds on iron absorption from the same standardised test meal. It was demonstrated that iron absorption was significantly increased (8.2%) when a higher ascorbic acid:iron ratio used (1.6:1) compared to 5.1% when a 0.6:1 ratio was used ($p<0.01$). When Na_2EDTA was used, there was a significantly higher iron absorption of 3.8%, 3.5% and 3.7% ($p<0.01$) compared to 2.9%, 2.2% and 2.4%, respectively, when no enhancer was added to the test meal. On the contrary, Cook and Reddy (2001) demonstrated that with a complete diet, the effect of ascorbic acid on iron absorption was less evident compared to when a single test meal is used. Twelve generally healthy adult subjects aged between 20-38 years were recruited and participated in 4 different absorption studies. A hamburger meal was used as a control, where a further 3 types of diet; self-selected (SS), high in vitamin D diet (HC), and low vitamin C diets (LC) were consumed at home as and self-recorded for the duration of 5 days each. Instructions were given on how to choose a high or low vitamin C diet. It was reported that no significant difference was observed in fractional iron absorption between the three diets despite the difference in total ascorbic acid intake between the 3 diets. Iron absorption was generally higher in the HC diet (7.69%) as opposed to SS diet (4.57%) and LC diet (5.69%), as expected. This may be due to the fact that several meals eaten as part of the diet in a day may have an impact on the how ascorbic acid or any other dietary factors in the meal (polyphenol, animal tissue etc) interact, causing a lesser effect on iron absorption, compared to when overnight-fasted subjects were given a single meal in a controlled environment. A randomised crossover study was carried out in Switzerland by Fidler, Davidsson, Zeder, and Hurrell (2004) in 10 women aged 20-26 years to assess the dose effect of erythorbic acid, an alternative to ascorbic acid on iron absorption. Erythorbic acid is a stereoisomer of ascorbic acid but dissimilar to ascorbic acid merely in the aspect of relative position of hydrogen and hydroxyl groups on the fifth carbon atom in the molecule. The subjects were administered 4 isotope-labelled wheat-based cereals: (a) without any enhancer as a control; (b) with erythrobic acid (31.5 & 63 mg) and (c) ascorbic acid (63 mg). It was observed that iron absorption was higher ($p<0.0001$) in meals with erythrobic acid, irrespective of concentration (10.8% and 18.8%), compared to the control meal, without any enhancer (4.1%). A similar observation on iron absorption was reported with

ascorbic acid (11.7%) when compared to the control meal (p=0.0004). However, when ascorbic acid is compared against erythrobic acid, there was a higher absorption only when higher concentration of erythrobic is used (63 mg) (p=0.0002). Ascorbyl palmitate (AP), another substitute for ascorbic acid has shown to be effective in enhancing iron absorption. Pizarro et al. (2006) carried out a study in 14 women aged 35-45 years, and used intrinsically labelled bread with different AP content (0, 222, 445, 890 mg) administered at different occasions. The iron absorption did not increase from 10.5% (control bread) with 222 mg AP (9.3%), but significantly increased to 14.6 % with 445 mg AP and 20.2% with 890 mg AP ($p<0.001$). The thermostable characteristics of AP have been demonstrated to be useful in promoting iron absorption in bread with high content of phytate (Pizarro et al., 2006) and also, the kinetics of erythrobic acid that prolongs its presence in duodenum have also proved to be beneficial in promoting higher iron absorption (Fidler et al., 2004).

A study carried out in Chile in 13 women (32-50 years) which also aimed to focus on the effect of ascorbic acid on iron absorption used a sweetened lemonade drink, administered with different isotope-labelled noodle-based test meals (Olivares, Pizarro, Hertrampf, Fuenmayor, & Estévez, 2007). Iron absorption was reported to be not significantly different between bread (9.9%), noodles (8.5%) and noodle soup (8.1%). However, there was a significant increase from 8.1 to 14.7% (p=0.02) when lemonade was consumed with the noodle soup, as compared to when no lemonade was consumed, as anticipated. Ascorbic acid in the lemonade, consumed as part of the meal may have promoted the iron absorption (Olivares et al., 2007).

A study by Cercamondi, Egli, Zeder, and Hurrell (2014) recently added to the available evidence demonstrating the efficiency of ascorbic acid in promoting higher iron absorption, and counteracting the inhibition effect of polyphenols in cereal porridge. The study was carried out in 18 healthy women aged 18-40 years and the test meal used was isotope-labelled sorghum-based porridge, administered with either: (a) no enhancer (control); (b) 40 mg ascorbic acid or (c) reduced-polyphenol porridge (treated with laccase). It was demonstrated that iron absorption from the porridge with ascorbic acid was 3-fold higher (13.6%), compared to the control meal (4.6%) ($p<0.001$), but not with the laccase-treated porridge (4.6%, p=0.4). This further demonstrates that ascorbic acid can be useful as an enhancer, especially in a meal high in polyphenols and phytic acid (Cercamondi et al., 2014).

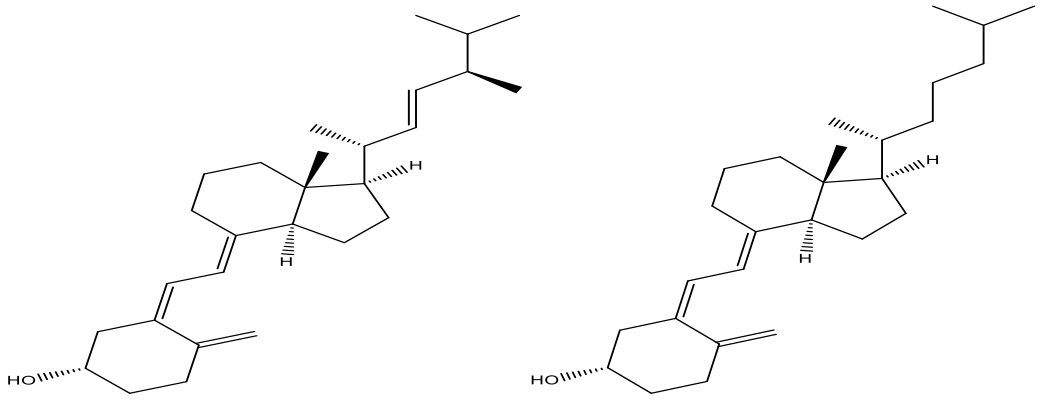
It has been long established that ascorbic acid has the capability of reducing ferric iron to the ferrous form, enabling iron to form soluble complexes to be absorbed (Hurrell & Egli, 2010). Additionally, ascorbic acid is capable of counteracting the effect of iron absorption inhibitors such as polyphenols or calcium in milk products (Abbaspour et al., 2014), however, as it is thermolabile (Pizarro et al., 2006), and can be degraded with processing, storage or cooking may limit its capability to enhance iron absorption (Teucher et al., 2004).

Vitamin D has recently been suggested to be a potential iron absorption enhancer by its role in the erythropoiesis process, but there is limited evidence available, especially linking haemoglobin and 25-hydroxyvitamin D (25(OH)D) concentrations in human studies (Sim et al., 2010). In addition, an *in vitro* study carried out in human cell lines demonstrated that administration of 25(OH)D and 1,25-dihydroxyvitamin D (1,25(OH)D) for 6 hours reduced hepcidin expression (Bacchetta et al., 2013), which may have facilitated iron status regulation. Observational studies, however, report inconsistent findings in relation to the connection between vitamin D deficiency and iron deficiency. For instance, vitamin D deficiency was shown to result in higher risk of anaemia in participants aged >17 years (Sim et al., 2010) but iron deficiency and anaemia not found to be associated with vitamin D deficiency in a study carried out in children (McGillivray et al., 2007).

1.8.2.1 Enhancement effects of vitamin D

Vitamin D has been shown to play role in calcium (Christakos et al., 2013), and phosphate homeostasis (Bikle, 2014) and bone health (Brouwer-Brolsma et al., 2013). Recent observational and experimental studies have suggested that vitamin D has a role in other human physiological systems including cardiovascular, brain, pancreas, muscle and immune system, but the findings were inconsistent (Brouwer-Brolsma et al., 2013). There are two main sources of vitamin D; consumption from the daily diet, or skin synthesis (Christakos et al., 2013). The dietary sources of vitamin D are relatively limited, mostly found in low amounts in an animal-based sources such as meat but abundant in fatty fish (Bikle, 2014), fish liver oils (DeLuca, 2004) or fortified foods (Christakos et al., 2013). Vitamin D, which is a secosteroid hormone, exists in two major forms; D3 or cholecalciferol and D2 or ergocalciferol (SACN, 2016), which are both utilised in vitamin D-fortified foods and supplements (Holick et al., 2011). The additional methyl group and a double bond in the secosteroid skeleton cause the D2 to have higher molecular mass than the D3

(SACN, 2016). **Figure 1.8** shows the structure of vitamin D in D2 and D3 forms (Norman, 2008).



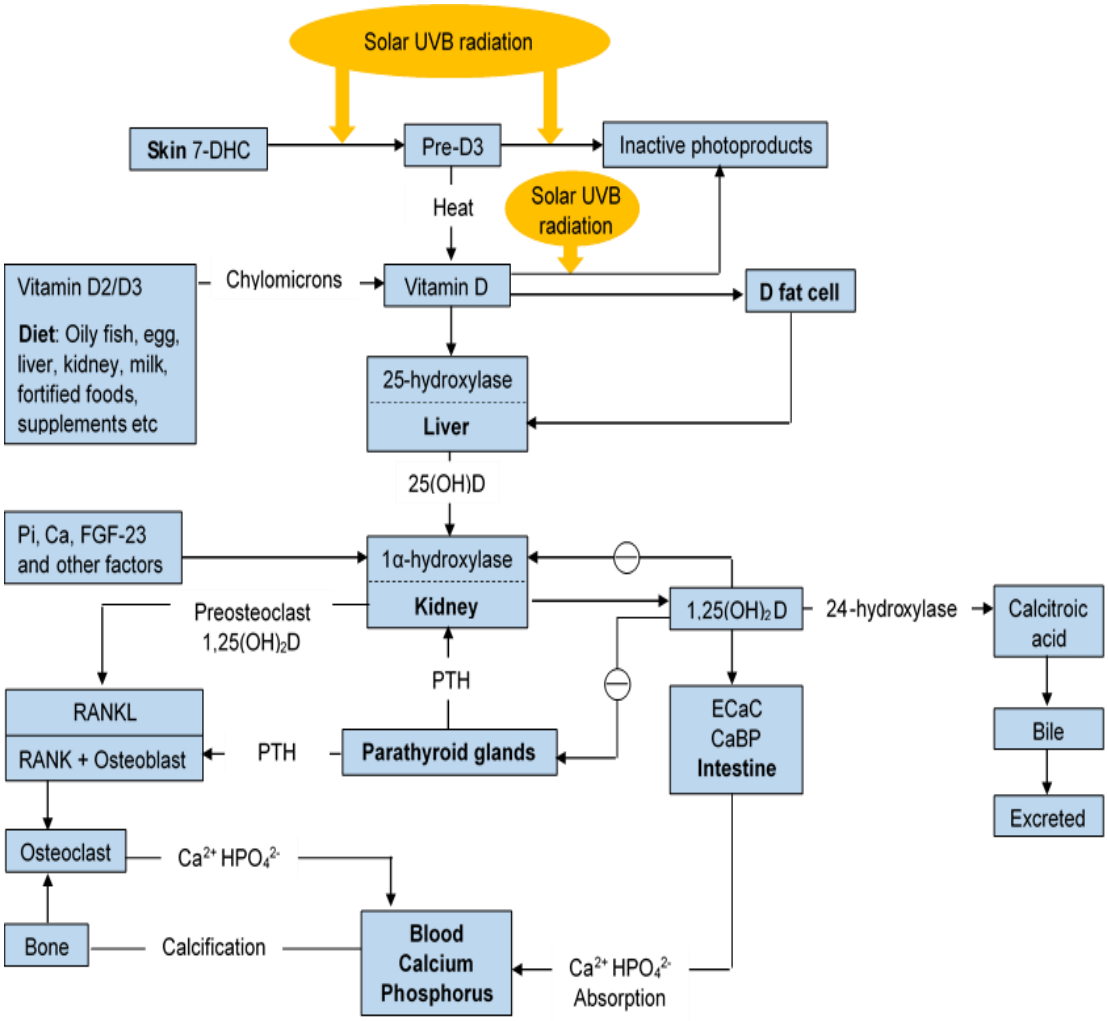
VITAMIN D2 VITAMIN D3

Figure 1.8 Structure of vitamin D2 and D3

Vitamin D3 is endogenously produced in humans or animal skin via non-enzymatic reactions, facilitated by ultraviolet-B (UVB) radiation from the sun ranging between 280-315 nm (SACN, 2016) that converts 7-dehydrocholesterol (7-DHC) found in the epidermis to form pre-vitamin D3 (pre-D3). As this pre-D3 is thermodynamically volatile, it undergoes a further thermal isomerisation reaction that produces vitamin D3 (Adams & Hewison, 2010). The degree of formation of pre-D3 in the skin is subject to the intensity of UVB and individual's skin pigmentation (Holick, 2008). Dietary-based sources of vitamin D become pivotal when the synthesis via skin is insufficient, which may due to limited exposure to sunlight (SACN, 2016). Vitamin D2 is regarded as the primary analogue of vitamin D (Bikle, 2014), and is produced predominantly in plants and fungi from irradiation process of yeast sterol ergosterol (DeLuca, 2004; Holick et al., 2011).

Skin-synthesised vitamin D3 diffuses into circulation after it enters the extracellular fluid (Holick et al., 2011). Conversion of vitamin D3 to the active and stable metabolite of vitamin D known as 25-hydroxyvitamin D3 (25OH-D) or calcidiol, takes place in the liver (Adams & Hewison, 2010) by 25-hydroxylases or high-capacity cytochrome P450 oxidases (CYPs) that originate from either mitochondria or endoplasmic reticulum (Bikle, 2014). 25(OH)D subsequently binds to vitamin D binding protein (VDBP) (SACN, 2016), and is transported to the kidney or other tissues, hydroxylated into the active metabolite, 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$ or calcitriol) by 1α-hydroxylase (Bikle, 2014). 1,25(OH)D binds to vitamin D receptor (VDR) and functions as a steroid, after being mobilised to the

targeted tissues (Christakos et al., 2013). Dietary-based sources of vitamin D are transferred by the lymphatic system into the circulation, after being integrated into chylomicrons, and undergo the same processes of metabolism as from cutaneous synthesis (Holick, 2008). Homeostasis regulation functions to control the conversion rate to the active metabolite in kidney and depends on the parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) concentrations (Henry, 2011). **Figure 1.9** summarises the overall overview of vitamin D synthesis and metabolic pathway.



Key:
7-DHC: 7-dehydrocholesterol; pre-D3: pre vitamin D3; 25(OH)D: 25-hydroxyvitamin D; Pi: phosphate ions; Ca: calcium; FGF23: fibroblast growth factor 23; 1,25(OH)2D: 1,25-dihydroxyvitamin D; RANKL: receptor activator of nuclear factor-kappaB ligand; ECaC: epithelial calcium channel; CABP: calbindin; $Ca^{2+}HPO_4^{2-}$: calcium hydrogen phosphate

Figure 1.9 Vitamin D: acquisition, metabolism and biological functions - adapted from Holick et al. (2011)

25(OH)D which has a longer half-life (Norman, 2008) is the main circulatory form of vitamin D (Christakos et al., 2013), hence is extensively used as a vitamin D status biomarker, expressed in nmol/l or ng/ml (SACN, 2016). Assays that quantify the serum/plasma concentration of 25(OH)D have been extensively utilised in research to assess vitamin D status (Hilger et al., 2014). There is no internationally established threshold to define vitamin D adequacy (Thacher & Clarke, 2011), however, serum/plasma concentration 25(OH)D of < 25 nmol/l is regarded as a clinical vitamin D deficiency, associated with an indication of bone-related disorders such as bone pain and muscle weakness (van Schoor & Lips, 2011). There are several thresholds used worldwide to define vitamin D status for different age groups **(Table 1.9)**, which supports by the significant variations in vitamin D deficiency prevalence observed across different countries, ranging between 2-90% (Hilger et al., 2014).

Table 1.9 Vitamin D status based on different thresholds of serum/plasma 25(OH)D concentration (nmol/l)

Organisation/Reference	Deficiency	Insufficiency	Sufficiency
UK SACN (SACN, 2016)	< 25	-	> 25
IOM (Ross, Taylor, Yaktine, & Del Valle, 2011)	<30	30-50	>50
The Endocrine Society (Holick et al., 2011)	<50	52.5-72.5	75-250
Vitamin D Council	0-75	77.5-97.5	100-200
Vieth (2005)	0-25	25-40	40-100

To convert to ng/ml: divide nmol/l by 2.5

Vieth (2005): Desirable (75-160 nmol/l), Toxicity (>220 nmol/l); Vitamin D Council: Toxicity (375 nmol/l)

A recently published SACN report for UK population has recommended <25 nmol/l of serum 25(OH)D as the threshold to define vitamin D deficiency for individual aged >4 years old (SACN, 2016). **Table 1.10** summarises the NDNS population data for vitamin D status of women and men aged 19-64 years between 2008 and 2014 based on serum 25(OH)D concentration (Bates et al., 2014; Henderson et al., 2003; Public Health England, 2016).

The table shows mean (±S.D) serum 25(OH)D concentrations, with the percentage of women and men who had mean (±S.D) serum 25(OH)D concentrations below < 25 nmol/l, which is classified as vitamin D deficiency by the UK SACN. Based on the newly proposed threshold by the SACN, UK adults were not deficient across all 3 NDNS population data, however, if the IOM threshold is applied, the reported mean (±S.D) serum 25(OH)D concentrations were classified as insufficient. Approximately 1/5 of adults (between 15-24%) in the UK were vitamin D deficient based on the proposed SACN 25(OH)D threshold of <25 nmol/l.

Not taking into account sex and seasonal variations, mean (±S.D) serum 25(OH)D concentration of adults was 45.4 ± 24.8 nmol/l (Bates et al., 2014). The NDNS population data for mean (±S.D) serum 25(OH)D concentrations of adults aged 19-64 years were at the lowest in January-March (34.8 ± 22.9 nmol/l), followed by April-June (44.2 ± 24.4 nmol/l), October-December (45.6 ± 22.7 nmol/l) and at the highest were in July-September (57.5 ± 23.4 nmol/l) (Bates et al., 2014).

Table 1.10 NDNS population data for vitamin D status of adults aged 19-64 years

NDNS		Mean (±S.D) serum 25(OH)D concentrations (nmol/l)	Proportion of adults with mean (±S.D) serum 25(OH)D concentrations < 25 nmol/l (%)
2008/09-2010/11	Men	45.6 ± 22.7	17.1
(Year 1-3)	Women	49.6 ± 25.6	18.6
2008/09-2010/12	Men	43.5 ± 23.9	24.0
(Year 1-4)	Women	47.3 ± 25.6	21.7
2012/13-2013/14	Men	42.4 ± 21.0	22.0
(Year 5-6)	Women	45.3 ± 21.1	15.0

2008/09-2010/11 (Year 1-3) (Henderson et al., 2003); 2008/09-2010/12 (Year 1-4) (Bates et al., 2014); 2012/13-2013/14 (Year 5-6) (Public Health England, 2016)

Even though food-based sources of vitamin D are available, they are predominantly limited to animal origin foods such as oily fish (Brouwer-Brolsma et al., 2013), causing difficulty in sustaining sufficient vitamin D status (Adams & Hewison, 2010). The vitamin D recommended intake in different populations worldwide is currently set to between 100-800 IU (Adams & Hewison, 2010), and 400 IU specific to UK population (SACN, 2016). The range between 100-800 IU is regarded as underestimated, contributes to the difficulty in maintaining sufficient vitamin D status, hence, the need for additional vitamin D from supplement form (Adams & Hewison, 2010).

There is no international consensus on the optimal dietary intake of vitamin D (Brouwer-Brolsma et al., 2013), however, a recent RNI of 400 IU/day (10 µg) was proposed for general UK population aged >4 years, including pregnant and lactating women (SACN, 2016). The RNI proposed includes the daily intake from either naturally-contained vitamin D food sources, vitamin D-fortified foods or supplements. **Table 1.11** summarises the recommended dietary and supplement intakes from various sources worldwide.

Table 1.11 Overview of vitamin D dietary and supplement recommendations for adults

Reference	Dietary	Supplements (D2 or D3)
UK SACN (SACN, 2016)	400 IU/day (10 µg)	400 IU/day (10 µg)
IOM (Ross et al., 2011)	600 IU/day (15 µg)	600 IU/day (15 µg)
The Endocrine Society (Holick et al., 2011)	600 IU/day (15 µg)	6000 IU/day (150 µg) or 50000 IU/week (1250 µg) (8 weeks)
Vitamin D Council	5000 IU/day (125 µg)	5000 IU/day (125 µg)
Vieth (2005)	200 IU/day (5 µg)	4000 IU/day (100 µg)

The mean (±S.D) daily dietary intake of vitamin D for adults aged 19-64 years was 2.8 ± 2.1 µg and was higher in men (3.1 ± 2.3 µg) compared to women (2.6 ± 1.9 µg) (Bates et al., 2014). Recently compiled and published NDNS population data showed that the daily dietary intake of vitamin D in adults has not changed as compared to the earlier NDNS data by Bates et al. (2014), with overall mean (±S.D) daily dietary intake of vitamin D for adults of 2.8 ± 2.1 µg, 3.1 ± 2.3 µg (men), and slightly lower in women (2.5 ± 1.9 µg) (Public Health England, 2016). The food group contributing to vitamin D dietary intake the most in adults was meat and meat products (29%), followed by egg and egg dishes (19%), fish and fish dishes (15%) and fat spread (13%) (Public Health England, 2016).

1.8.2.2 Association between iron and vitamin D deficiencies: human observational studies

An association between vitamin D deficiency and a higher risk of anaemia was reported in a cross-sectional study carried out in adolescents and adults of both sexes aged 17+ years who attended 11 medical centres in the California, USA as part of routine health care examinations (Sim et al., 2010). Participants were classified as anaemic based on Hb concentration of <11 g/dl and vitamin D deficient based on serum 25(OH)D concentration of <75 nmol/l. Of the 554 participants who were included in the study, more than half (57%) were found to be not deficient in vitamin D. Anaemia prevalence in the vitamin D deficient group (48.8%) was found to be significantly higher in the non-deficient group (35.7%, $p<0.01$). However, the mean (SD not provided) haemoglobin concentration was not significantly lower in vitamin D deficient group (11.0 g/dl) compared to non-deficient group (11.7 g/dl, $p=0.12$). As opposed to only 1/5 of the participants in the non-deficient group, half of the participants in the vitamin D deficient group were administered with erythropoietin stimulating agents (ESA), suggesting anaemia is commonly associated with the incidence of ACKD, and possibly vitamin D deficiency. The study, however, used a threshold of 75 nmol/l to define vitamin D deficiency, which may have also included participants that are classified as sufficient based on the IOM recommendation (Ross et al., 2011). It is demonstrated by the fact that only 2% of the total of 43% vitamin D deficient participants were severely deficient, based on the threshold of <25 nmol/l by IOM. In addition, the iron stores, indicated by the mean (SD not provided) plasma ferritin concentration of > 15 µg/l in both groups were in the normal range, and were in fact, significantly higher in the vitamin D deficient group (364 µg/l) compared to non-deficient group (189 µg/l, $p=0.01$). The study included a wide range of ages and both sexes, which may have confounded

the findings related to incidence of anaemia and vitamin D deficiency, reported in the study. Despite the nature of observational studies that do not provide a causal effect, the study demonstrated appropriate evidence to instigate further investigation on the link between these two deficiencies.

A weak but significant linear association between haemoglobin and serum 25(OH)D concentrations (r=0.221, p=0.026) was observed in another cross-sectional study carried out in 102 Korean children aged 3-24 months who attended a medical centre in Korea (Jin, Lee, & Kim, 2013). Participants were classified into 3 groups: ID (Hb >11 g/dl, plasma ferritin ≤ 12 µg/l), IDA (Hb ≤ 11 g/dl, plasma ferritin ≤ 12 µg/l) and normal. Vitamin D status was defined by using serum 25(OH)D threshold of ≤ 50 nmol/l (deficiency), 50-75 nmol/l (insufficiency) and >75 nmol/l (normal). It was observed that vitamin D deficiency was more predominant in IDA (67%) compared to ID (53%) and normal (29%) groups. It was shown in the mean (±S.D) serum 25(OH)D concentration, as it was the lowest in the IDA (45.3 ± 28.5 nmol/l), as opposed to the ID (57.5 ± 55.8 nmol/l) and normal groups (81.8 ± 60.8 nmol/l) (Jin et al., 2013). The study is limited by the unequal number of participants in each group and may be subject to selection bias due to the nature of the study. The study included only children who attended the medical centre, who have a complete specific set of biochemical data (Hb, MCV, red cell width, platelets, serum ferritin, serum iron, TIBC, serum transferrin saturation, calcium, alkaline phosphatase and serum 25(OH)D concentrations), which may prone to selection bias. However, serum iron concentration was found to be one of the factors that affected serum 25(OH)D concentration by regression analyses, which may require further investigation on the long-term consequences linking iron and vitamin D deficiencies.

In a larger scale of cross-sectional study, Lee et al. (2015) reported that the incidence of vitamin D deficiency was higher in anaemic female participants (65%), compared to only 40% anaemic male participants with mean (SE) of 34.3(1.3) and 41(2.8) nmol/l, respectively. The study included a Korean nationally representative data derived from 2526 participants aged 10-20 years, and used different thresholds of Hb (female: <12 g/dl, male:<13 g/dl); serum ferritin (<12 µg/l) and serum transferrin saturation (<16%) to define iron status. A serum 25(OH)D threshold of <37.5 nmol/l was used to classify participants as deficient, or insufficient (37.5-72.5 nmol/l) and sufficient (≥75 nmol/l) (Lee et al., 2015). It can be concluded that vitamin D deficiency observed in the participants lead to increased risk of anaemia, and was more prevalent in female participants (Lee et al., 2015). The study was limited to the nature of cross-sectional study that is unable to determine a causal effect, and also

the inclusion of both sexes may have also have impacted up on the findings as menstrual losses in females may cause iron deficiency that leads to anaemia. However, the study included a large sample size and the findings in the study are consistent with the observation in previous studies that reported concurrent incidence of iron and vitamin D deficiencies in similar individuals.

A cross-sectional study which included data obtained from 263 children aged 3 months-12 years who attended an outpatient department of a hospital in New Delhi, India reported a higher anaemia incidence in vitamin D deficient participants (66%) compared to vitamin D sufficient participants (35%) (Sharma, Jain, & Dabla, 2015). Participants were stratified into 3 groups based on serum 25(OH)D threshold of ≤ 30 nmol/l (deficiency), 30-75 nmol/l (insufficiency) and >75 nmol/l (sufficiency). Anaemia was considered present with Hb <11 g/dl, MCV < 70 fl and red cell width (RDW) >15%, and IDA was present with serum ferritin < 12 µg/l, TIBC > 450 µg/dl, or transferrin saturation < 15%. Mean (±S.D) haemoglobin concentration was reported to be significantly lower in the vitamin D deficient group (9.86 ± 2.6 g/dl) compared to sufficient group (11.25 ± 1.9 g/dl, $p<0.01$) but no significant difference was observed between groups in serum ferritin concentration. A moderate, but significant linear association ($r=0.317$, $p=0.013$) between serum 25(OH)D and haemoglobin concentrations was observed. A multiple regression test carried out discovered that there was an increased risk of vitamin D deficiency in participants who were anaemic ($p<0.001$) (Sharma et al., 2015), also consistent with findings from the previous studies. The study showed that concurrent incidence of anaemia and vitamin D deficiency are prevalent in infants and young children, which should be addressed, as physiological requirements of iron and vitamin D are crucial at this age group for normal body functions.

Based on evidence gathered from these recent cross-sectional studies, it can be established that there is a link between anaemia and vitamin D deficiency, demonstrated across different populations. However, it should be noted that different observational studies have employed various thresholds, especially in defining vitamin D status as there is no established international threshold. The different thresholds used in each study may have an effect on the reported figures of prevalence, causing difficulty in the comparison and interpretation.

1.8.2.3 Effect of vitamin D on iron status: human intervention studies

A single-blinded randomised controlled trial was carried out in 30 IDA patients recruited from an outpatient department in a medical institute in India, aged 15-60 years who had IDA (Hb < 12 g/dl, serum ferritin < 15 µg/l) and serum 25(OH)D of < 50 nmol/l, to investigate the effect of intramuscular vitamin D3 therapy on iron status (Sooragonda et al., 2014). All participants were administered with iron intravenously in separate doses for 12 weeks but were randomised into a vitamin D therapy (600, 000 IU) and placebo group. No significant effect of intervention on haemoglobin or serum ferritin concentrations was observed between the vitamin D and placebo groups. In addition, no association between serum 25(OH)D and haemoglobin concentration was observed, contrasting from the findings of observational studies. It was established that a 12-week intramuscular bolus dosage of vitamin D3 did not improve the iron status of patients diagnosed with IDA (Sooragonda et al., 2014). It should be noted that the study was a single-blinded RCT, did not report baseline concentrations of serum ferritin despite the change reported within both groups, and the small sample size may have limited the study to observe a significant change in iron status indices.

An 8-week randomised controlled trial in 200 hypertensive patients who attended an outpatient department of a hospital in Austria, aged >18 years with a low serum 25(OH)D concentration of < 75 nmol/l was carried out to determine the effect of vitamin D supplementation on haemoglobin concentrations (Ernst et al., 2016). Participants were randomised into 2 groups to consume either 7 drops/day of vitamin D3 oily drops (2800 IU) or matching placebo. The following thresholds were used as participants' inclusion criteria: anaemia (Hb < 12.5 g/dl); vitamin D deficient (< 30 nmol/l); vitamin D insufficient (30-49.9 nmol/l) and borderline vitamin D (50-74.9 nmol/l). It was observed that vitamin D supplementation did not have a significant effect on the haemoglobin concentration, or any other full blood count indices. Participants were generally non-anaemic at baseline in both groups and remained non-anaemic at post-intervention (Ernst et al., 2016). It was shown in the study that supplementation of vitamin D3 (2800 IU) did not improve hypertensive patients' iron status as hypothesised. Despite the strong study design and large sample size, the baseline concentration of haemoglobin (>14 g/dl) and serum 25(OH)D (>50 nmol/l) were clearly in the normal ranges, which may have influenced the participants' response to the intervention. It should also be noted that hypertensive patients may have other underlying problems that may have contributed to the non-significant improvement in iron status.

No significant improvement in iron status biomarkers including haemoglobin concentration were observed in a randomised controlled trial carried out in Norway following a longer duration of 16-weeks vitamin D3 supplementation (Madar et al., 2016). The study was carried out in 251 healthy adults of both sexes aged 18-50 years, randomised into 3 groups, consuming 1 tablet/day of vitamin D3 (1000 IU), vitamin D3 (400 IU) and placebo. It was observed that supplementation of vitamin D3 of both doses, analysed pooled or separately, had no significant effect on all iron status biomarkers of participants, including serum ferritin and haemoglobin concentrations between the vitamin D and placebo groups (Madar et al., 2016). The study included no clear definition of serum 25(OH)D concentration as participants' inclusion criteria, but overall mean (±S.D) serum 25(OH)D concentration at baseline for all participants was reported to be 29 ± 17.6 nmol/l, with over half proportion of participants (53%) with serum 25(OH)D concentration <25 nmol/l. In addition, it was shown that baseline serum 25(OH)D concentration did not associate with any of the iron status biomarkers (Madar et al., 2016). The non-significant improvement in the iron status following the vitamin D supplementation may be due to the fact that the participants included in the study were not iron depleted or anaemic at baseline, as it has been shown that physiological demand of iron dictates the rate of iron absorbed. A different approach was employed by Hennigar et al. (2016) using a calcium/vitamin D-fortified snack bar with the aim to improve iron status in 152 healthy adult soldiers of both sexes aged 18-42 years who had undergone military training for 9 weeks. The participants were randomised to receive the fortified (1032 mg/13.7µg Ca/vitamin D3 per snack bar) or placebo snack bars and instructed to consume 2 bars daily. The main finding yielded a similar observation from the previous studies that used vitamin D supplements, with no significant effect reported on any of the iron biomarkers, including plasma ferritin and haemoglobin concentrations following fortified food consumption between the fortified and non-fortified placebo groups. However, high compliance of the fortified foods was supported with the significantly higher mean (±S.D) vitamin D dietary intake in the intervention group at post-intervention (27.8 ± 5.13 µg) as opposed to the placebo group (5.3 ± 3.45 µg, $p<0.05$) (Hennigar et al., 2016). The study was limited to the fact that there was no pre-defined iron or vitamin D status in recruiting the participants and the short duration of the study, but the double-blinded study design was one of the strengths. The non-significant improvement observed in the iron status biomarkers may be due to the normal baseline concentration of both haemoglobin (> 12 g/dl) and serum ferritin concentrations (> 50 µg/l), which is associated with low homeostatic iron demands.

A randomised controlled trial investigating the effect of iron-fortified fruit juice consumption on both iron and vitamin D status was carried out in 41 women aged 18-35 years, with low iron stores (serum ferritin < 40 µg/l) (Blanco-Rojo et al., 2013). The participants were randomised to receive 500 ml/day of iron-fortified fruit juice containing 18 mg of iron, or placebo fruit juice for a period of 16 weeks. There were significantly higher mean (±S.D) haemoglobin and serum ferritin concentrations at post-intervention, relative to baseline, in the iron-fortified group (13.7 ± 0.7 g/dl, 33.5 ± 16.9 µg/l) , as compared to placebo group (13.1 ± 0.7 g/dl, 20.2 ± 14.6 µg/l) at $p<0.01$ and $p<0.05$, respectively. No significant difference was observed between the groups on the serum 25(OH)D concentration. The study showed that more than half of the participants (53.6%) were vitamin D deficient based on serum 25(OH)D threshold of < 50 nmol/l at baseline, with an overall mean (±S.D) of 50.2 ± 14.0 nmol/l. Despite a non-significant improvement in serum 25(OH)D concentration, the study demonstrated a positive and significant association between serum 25(OH)D concentration and transferrin saturation ($p=0.007$, r value not provided), but not with serum ferritin or haemoglobin concentrations (Blanco-Rojo et al., 2013). The high baseline concentration of serum 25(OH)D concentration with no additional vitamin D in the iron-fortified juice may have contributed to the non-significant change of serum 25(OH)D concentration at post-intervention. The inclusion of iron deficient participants lead to a significant improvement in most iron status biomarkers, however, it should also be noted that the fortified juice used in the study contained added ascorbic acid in the form of liquid that is highly bioavailable, hence, this may have contributed to the improvement in principal iron status biomarkers.

Toxqui et al. (2013) carried out a randomised controlled trial by incorporating both iron and vitamin D in skimmed milk, to investigate the effect of fortified skimmed milk consumption on iron status in 129 iron deficient Spanish women aged 18-35 years with serum ferritin < 30 µg/l. The participants were randomised to receive iron/vitamin D fortified skimmed milk (Fe/D group) or iron-fortified skimmed milk (Fe group) and were required to consume the 500 ml skimmed milk containing 15 mg iron and/or 5 µg vitamin D according to the assigned group for 16 weeks. No significant difference was observed in serum ferritin and haemoglobin concentrations between the groups, but significantly higher erythrocyte counts, haematocrit % and haemoglobin concentration were observed at 8 weeks (interim) in the Fe/D group. At 8 weeks, mean (±S.D) haemoglobin concentration was significantly higher in the Fe/D group (13.3 ± 0.8 g/dl) compared to Fe group (13.0 ± 0.7 g/dl) ($p<0.05$), but not significantly higher at post-intervention (Toxqui et al.,

2013). Negligible effect of the added vitamin D on the improvement of iron stores may be due to milk components (whey, casein, calcium) in the skimmed milk used as the fortification vehicle in the study, which may have reduced the iron absorption. The serum ferritin concentration at baseline was > 20 µg/l in both groups and remained below 30 µg/l at all time points and at post-intervention, despite the high content of iron added to the fortified skimmed milk. It should also be noted that the serum ferritin threshold used in the study was higher (<30 µg/l) as opposed to a lower threshold proposed by WHO of < 12 µg/l to define ID (WHO/UNICEF/UNU, 2001).

Based on the evidence gathered from the recent intervention studies, it can be established that not all iron status biomarkers were improved in vitamin D interventions. Most improvements were observed when iron deficient participants were included in the studies, compared to generally healthy participants who had low systemic iron requirements, indicating that initial participants' iron status background at the start of interventions may dictate the response to vitamin D interventions. Additionally, factors such as (i) amount of iron and vitamin D (low, high or none); (ii) forms of iron and vitamin D (fortified foods or supplements); and (iii) duration of interventions, contributes to inconsistencies in the reported findings.

1.8.2.4 Effect of vitamin D on iron status: *in vivo and in vitro* studies

Based on the evidence that anaemia is linked to both incidence of vitamin D deficiency and increased hepcidin concentration in kidney patients (ACKD), Bacchetta et al. (2013) carried out an *in vitro* study investigating the role of vitamin D in regulation of hepcidin expression, as hepcidin is known for its role as iron regulator. It was shown that treating different cell lines (human PBMC and THP1 monocytes, and HepG2 hepatocytes) with 100 nM of 25(OH)D and 5 nM of 1,25(OH)D for a duration of 6 hours reduced the expression of *HAMP*, the gene responsible for hepcidin production. It was observed that hepcidin expression was significantly reduced by at least 0.4-fold when treated with 25(OH)D and 1,25(OH)D ($p<0.001$) compared to control vehicle treatment. It was suggested that treating the cell lines with 25(OH)D and 1,25(OH)D caused direct inhibition on transcription of the *HAMP* gene (Bacchetta et al., 2013), which may have ultimately lead to increased iron absorption.

Based on findings in the *in vitro* study that demonstrated the effect of vitamin D on the regulation of hepcidin, Bacchetta et al. (2013) carried out a pilot human study using 7 healthy participants, orally supplemented with vitamin D2 bolus dose of 100, 000 IU once. Evidence from this human study supported the *in vitro* findings, that serum hepcidin concentration was significantly reduced to 34% (mean ±S.D not provided) following 24 hours of supplementation ($p<0.05$). Analysis of serum for the next 72 hours of after vitamin D supplementation showed that the serum hepcidin concentration was further decreased to 33% ($p<0.01$) (mean ±S.D not provided). However, instead of increasing, mean (±S.D) serum ferritin concentration was found to have significantly decreased to 138.8 ± 25.0 µg/l (24 hours) to 124.5 ± 21.7 µg/l (72 hours) ($p=0.04$) (Bacchetta et al., 2013). It should be noted that participants included in the study were not iron depleted, but generally healthy, which may have explained the observation. It was reported in the study that the baseline serum ferritin concentration of participants was described as between 18-160 µg/l for female, and 18-2710 µg/l for male (Bacchetta et al., 2013), which is considered iron replete based on the WHO serum ferritin threshold of >12 µg/l (WHO/UNICEF/UNU, 2001). In addition to iron status biomarkers, the study also included the measurement of serum PTH concentration (n=6) and it was reported that mean (±S.D) serum PTH concentration was significantly lower at 72 hours (51.3 ± 7.5 pg/ml) compared to 58.9 ± 6.4 pg/ml at 24 hours ($p=0.06$) (Bacchetta et al., 2013). A small sample size was a limitation of this pilot study, but the significant decline observed in serum hepcidin concentration despite the small sample size, in addition to the evidence from the *in vitro* study is sufficient to initiate larger scale investigations especially RCTs.

A recent experimental study that included both *in vitro* study and *in vivo* pilot human trial was carried out by Zughaier, Alvarez, Sloan, Konrad, and Tangpricha (2014), based on the evidence available on the connection between anaemia and vitamin D deficiency by measuring hepcidin expression following vitamin D treatment/intervention. The *in vitro* study used a monocyte cell line (THP-1), treated with 1,25(OH)D with doses between 5-40 nM. The cell line was induced with lipopolysaccharide (LPS) to cause inflammation, and the *HAMP* gene expression was quantified with polymerase chain reaction (PCR) (Zughaier et al., 2014). After 6 hours, hepcidin expression was found to be significantly suppressed to 15-fold (LPS/20 nM 1,25-OHD) and 20-fold (LPS/40 nM 1,25-OHD) compared to control ($p<0.05$). Ferroportin, which is an iron exporter, was found to be increased, as a result of the reduced hepcidin, but only significantly increased with a 40 nM dose of

1,25(OH)D ($p<0.05$) (Zughaier et al., 2014). This indicates that with the presence of inflammation, vitamin D suppresses the transcription of the *HAMP* gene, and increases ferroportin concentration, to ultimately transport iron for utilisation. In addition, the treatment of 1,25(OH)D at both doses resulted in a significantly higher concentration of NRAMP1, the endosomal iron transporter ($p<0.05$). Pro-hepcidin cytokines (IL6 and IL1β) were found to be significantly reduced by at least 2-fold with a 20 nM dose of 1,25(OH)D ($p<0.05$) and further reduced with 40 nM dose ($p<0.05$), supporting the proposed theory that vitamin D regulates hepcidin by reducing the release of these cytokines (Zughaier et al., 2014). Zughaier et al. (2014) included 38 kidney patients, who were at stage 2 and 3 of ACKD in the *in vivo* part of the study. The patients were randomised to receive oral administration of vitamin D3 (50 000 IU/week), and every other week for 40 weeks or placebo for 12 weeks. There was no data provided on the change of biomarkers measured following the supplementation, but a moderate, significant, negative association was found between the serum hepcidin and serum 25(OH)D concentrations ($r=-0.38$, $p=0.02$) ($n=38$). However, it should be taken into consideration that mean (±S.D) baseline concentration of serum 25(OH)D concentration was significantly higher in the placebo group (82.3 ± 21.3 nmol/l) compared to the intervention group (68.8 ± 15.8 nmol/l) ($p=0.03$). The study demonstrated that bolus administration of vitamin D3 affected the serum hepcidin concentration which may lead to increase in iron status biomarkers. However, the study was carried out in kidney patients, who are commonly associated with anaemia due to chronic disease, and results may not be able to be generalised to the general population, healthy or iron deficient.

To the best of our knowledge, these were the only experimental trials that combined both *in vitro* and *in vivo* studies investigating the potential mechanism of the role of vitamin D in iron homeostasis, by regulation of transcription of the *HAMP* gene that is responsible for hepcidin production, which is known to function as an iron regulator. However, the in vivo part of the studies used a bolus concentration of vitamin D2 or D3 and were carried out in a small sample of healthy participants (Bacchetta et al., 2013) and ACKD patients (Zughaier et al., 2014). There is a scarcity of randomised controlled trials, investigating the effect of the vitamin D supplementation administered routinely, as an iron absorption enhancer on iron status especially in a population who are at risk of iron deficiency.

1.8.2.5 Proposed mechanism of the effect of vitamin D on iron homeostasis

The underlying mechanism behind the effect of vitamin D on iron regulation is poorly understood (Madar et al., 2016), and the proposed theory was based on observations reported in studies carried out in patients with disease complication-derived anaemia, such as haemodialysis patients (Miskulin et al., 2016; Naini, Hedaiati, Gholami, Pezeshki, & Moinzadeh, 2015) or critically ill patients (Smith et al., 2016b). Vitamin D has recently been suggested to play role in erythropoiesis, but there is limited evidence available, especially linking haemoglobin and serum 25(OH) concentrations in humans (Sim et al., 2010).

A double-blind randomised controlled trial was carried out in 64 renal patients undergoing haemodialysis treatment aged 18-80 years who had a concurrent incidence of vitamin D deficiency, anaemia, and were administered with erythropoietin stimulating agent (ESA) to manage anaemia at the time of recruitment (Naini et al., 2015). Patients were included using the following thresholds; Hb concentration <11 g/dl, serum 25(OH)D concentration < 75 nmol/l, and randomised to receive placebo or vitamin D 50 000 IU/week) for 12 weeks and subsequently every 3 weeks, for a total of 16 weeks. No significant difference was observed in haemoglobin concentration between the two groups, however, the dose of ESA was found to be significantly lower at post-intervention in the intervention group ($p<0.001$) (data not provided) (Naini et al., 2015). Mean (±S.D) haemoglobin concentrations were similar at baseline (9.8 ± 1.6, 10.1 ± 1.7 g/dl) and post-intervention (10.6 ± 1.1, 11.2 ± 1.2 g/dl) for male and female participants, respectively. The reduced ESA dose in the intervention group as opposed to the placebo group indicates that vitamin D may play role in increasing erythropoiesis and that anaemia is effectively managed. However, the study was carried out in set of renal patients and cannot be generalised to the general population, despite the strong double-blind RCT study design.

Another randomised controlled trial carried out in haemodialysis patients diagnosed with vitamin D deficiency, however, observed no significant effect of vitamin D2 supplementation on ESA utilisation (Miskulin et al., 2016). The 6-month study was carried out in 276 patients with serum 25(OH)D concentration of < 75 nmol/l at baseline, who were prescribed with vitamin D receptor agonist (VDRA) treatment and ESA. The participants were randomised to receive either vitamin D2 (50 000 IU/week) or placebo. As expected, mean (±S.D) serum 25(OH)D concentration was significantly higher at post-intervention in the intervention group (98.0 ± 37.3 nmol/l)

compared to placebo group (43.8 ± 18.5 nmol/l). No significant difference in the ESA weekly dose between groups was observed which may indicate the ineffectiveness of vitamin D supplementation in reducing anaemia, as demonstrated in the study by Naini et al. (2015). However, the participants included in the trial were generally elderly with a mean (±S.D) age of 61.1 ± 13.6, with a high incidence of diabetes (46%) and hypertension (30%), with comorbidities such as ischaemic heart disease and heart failure (both 29%) (Miskulin et al., 2016), which may have indirectly affected the findings in the study.

A pilot randomised controlled study carried out in 28 healthy adults aged 18-65 years in the USA by Smith et al. (2016a) also hypothesised that vitamin D therapy would lead to a reduction in the release of pro-hepcidin cytokines which cause suppression of hepcidin expression and ultimately increase in iron uptake. Participants were randomised to receive either an oral dose of vitamin D3 (250 000 IU) or matching placebo and measurement of blood biomarkers was carried out at baseline and 1-week post-intervention (Smith et al., 2016a). It was observed that plasma hepcidin concentration (geometric mean, 95% CI) significantly decreased by 73% at post-intervention in the vitamin D group (2.4, 0.8-7.4 ng/ml) compared to the placebo group (9.0, 4.8-16.7 ng/ml) (p=0.04). However, no significant increase in plasma ferritin concentration was observed between the groups (Smith et al., 2016a). Vitamin D supplementation may have affected the hepcidin expression directly, without affecting the release of cytokines as previously demonstrated in the *in vivo* studies and patients, who were likely to have existing inflammation (Smith et al., 2016a). The non-significant increase observed in the plasma ferritin concentrations, despite the significant effect of vitamin D on hepcidin expression, may be due to the short study duration and small sample size, which are the limitation of the study.

Based on existing evidence from observational, human interventions and *in vitro* studies, anaemia was clearly associated with the incidence of vitamin D deficiency, though there is no clear mechanism (Sim et al., 2010). The proposed theory, however, revolves around the mechanism of action of vitamin D that affects hepcidin expression, pro-inflammatory cytokine production, and rate of erythropoiesis. Erythroid precursor cells are responsible to express 1,25(OH)D receptors, which then stimulates the production and development of progenitor cell, that are required in erythropoiesis. The compromised concentration of the vitamin D active metabolite, 1,25(OH)D will then change the rate of erythropoiesis (Santoro et al., 2015; Sim et al., 2010). This active metabolite has also been suggested to improve

the responsiveness of patients that were administered with ESA in managing anaemia, by improving erythropoiesis, thus decreasing the use of ESA when supplementing with vitamin D (Ernst et al., 2016). The involvement of inflammatory cytokines in the proposed mechanism is particularly related to anaemia due to inflammation mostly in chronic disease patients. The release of inflammatory cytokines leads to increased hepcidin concentration, known as its role as an iron regulator (Smith et al., 2016a). The use of vitamin D has been shown to decrease hepcidin expression and increase ferroportin to export more iron and ultimately decrease the incidence of anaemia (Madar et al., 2016).

On the basis of the aspects covered in this general introduction, it can be summarised that iron, as an element, is vital as it is required in most human metabolic processes. It also shows that individual's iron status, measured by various iron biomarkers, is affected by the mechanism of action of various molecules or enzymes that play roles in iron homeostasis. In the context of UK population, in particular, dietary intake of iron does not achieve the recommended RNI and there is the mild prevalence of anaemia and iron deficiency, especially in premenopausal women, which is the focus of this present research. Despite the established strategies proposed in the management of poor iron status, the sole approach to either dietary modification, iron fortification or iron supplementation will not lead to the recovery of iron status. Therefore, it is important to carry out well-designed intervention studies that are focus on the utilisation of more than sole strategy in the management of anaemia and ID.

Despite extensive literature available emphasising the influence of different dietary factors (ascorbic acid or polyphenols) on iron absorption, it is apparent that few iron absorption intervention studies have been carried out in the UK population investigating the effect of tea consumption on iron uptake. According to the NDNS, UK adults consume approximately 1114 grams/day of tea, coffee, and tea (Bates et al., 2014), therefore understanding the link between tea consumption and iron uptake will be potentially useful.

Previous studies addressing this area have been carried out outside of the UK using different types of test meals, however, there is limited evidence available to date, particularly using a typical meal relative to UK dietary patterns. In addition, to the best of our knowledge, no human studies addressing the impact of the time interval of tea consumption, relative to an iron-containing test meal, on iron absorption have been carried out in the UK. Apart from an animal study carried out by Disler et al. (1975b) using 10 male Sprague rats and a human study investigating the time interval effect of tea and coffee consumption, respectively on iron absorption (Morck, Lynch, & Cook, 1983), there is no available evidence to date, that a 1-hour time interval will attenuate the inhibitory effect of tea on iron absorption.

As discussed earlier, a peptide hormone hepcidin plays a key role in the regulation of iron homeostasis, however, there is little mechanistic research in this area especially in humans (Roe, Collings, Dainty, Swinkels, & Fairweather-Tait, 2009). In addition, there is recently emerging evidence of the utilisation of vitamin D, as a novel iron absorption enhancer that acts through the suppression of hepcidin, the main iron regulator. However, there is a paucity of intervention studies, especially randomised controlled trials that investigate the effect of vitamin D supplement consumption as a routine therapy, on the recovery of iron status in individuals with low iron stores. Previous studies were mostly cross-sectional studies which cannot establish a causal-effect relationship, utilising bolus concentrations of vitamin D or fortified in a food vehicle, carried out in patients, and cannot be generalised to the general population, or *in vitro* studies which cannot be directly extrapolated to humans.

1.9 AIMS AND OBJECTIVES

The aims and objectives of the present research was to manipulate the effect of potential inhibitors and enhancers of iron absorption to improve the overall iron status in premenopausal women. The research was divided into two clinical trials that were aimed to increase the iron bioavailability and iron status by (a) modulating the time interval of tea consumption, that is known to be an iron inhibitor, to increase iron absorption from a porridge meal in a cohort of healthy UK women, and (b) incorporating vitamin D supplementation, as a potential novel iron enhancer, in combination with the consumption of an iron-fortified breakfast cereal to improve iron status of the UK women with marginal and low iron stores.

The first clinical trial was aimed to assess the effect of time interval of tea consumption relative to an iron-containing test meal on non-haem iron absorption from a typical western breakfast specific to UK population, using a stable iron isotope (^{57}Fe) in non-pregnant women as well as investigating the potential role of hepcidin in iron metabolism.

The second clinical trial was designed to investigate the effect of 8-weeks vitamin D3 supplementation consumed with an iron-fortified breakfast cereal on haematological indicators and hepcidin response in marginally and iron deficient UK women. The study integrated 2 strategies proposed to tackle incidence of iron deficiency, by using vitamin D3 supplements that may potentially act as a novel iron absorption enhancer, in combination with the consumption of iron-fortified foods, in order to improve iron status in the UK premenopausal women with low iron stores. In addition to investigating the efficacy, this study is also designed to assess the effect of a high dose of vitamin D3 (1500 IU) on iron metabolism, by measuring plasma hepcidin concentrations, in addition to plasma PTH and VDBP concentrations to investigate a possible mechanism that links vitamin D and iron deficiency, as postulated from the existing literature.

CHAPTER 2

Materials and Methods

2.1 MATERIALS

2.1.1 INSTRUMENTS

Instruments used for each measurement in the study are summarised in **Table 2.1**.

Table 2.1 Instruments used for anthropometric, blood biomarkers and iron isotopic measurements

Measurement	Instruments	Supplier
Anthropometric	Digital stadiometer	SECA, Hamburg, Germany
	Electronic weighing scale	
Blood biomarkers	Coulter counter	Beckman Coulter Inc, California, USA
	Automated immuno-analyser	Biomerieux, Marcy-l'Etoile, France
	Microplate washer	Thermo Fisher Scientific, Massachusetts, USA
	Microplate reader	Biochrom Ltd, Massachusetts, USA
Iron isotope ratio	Mass spectrometry	Agilent Technologies Ltd, Tokyo, Japan

2.1.2 CHEMICALS/REAGENTS

Table 2.2 outlines the chemicals and reagents used in the study.

Table 2.2 Chemicals and reagents used for acid-washing procedure, iron isotopic analysis and full blood counts measurements

Procedure	Chemicals/reagents	Supplier
Acid washing	Deionised water (through deioniser)	Purite Ltd, Thame, UK
	Nitric acid	Fisher Chemical, Loughborough, UK
Iron isotopic analysis	Ultrapure water	Sigma Aldrich Inc, Missouri, USA
	Tetramethylphenylammonium hydroxide (TMAH)	Sigma Aldrich Inc, Missouri, USA
Full blood counts	Coulter reagents and coulter rinse shutdown diluent	Beckman Coulter Inc, California, USA

2.1.3 ELISA ASSAY KITS

The ready-to-use reagents, built in strip form used for plasma ferritin determination and plasma 25(OH)D total were obtained from Biomerieux (Marcy-l'Etoile, France). Commercial Human ELISA kits for C-reactive protein (CRP), soluble transferrin receptor (sTfR), with quality control sets (low, medium, high) were purchased from R&D Systems Inc (Minneapolis, USA). The commercial ELISA kits for human hepcidin were purchased from Sincere Biotech Co. Ltd (Beijing, China) and R&D Systems Inc (Minneapolis, USA) for experimental study 1 and 2, respectively. Human Intact parathyroid hormone (PTH) ELISA kit and 25(OH)D were purchased from Calbiotech Inc (California, USA) complete with 2 sets of quality controls for each kit. The human Fe (ferritin) ELISA kit was purchased from Elabscience Biotechnology Co Ltd (Bethesda, USA). ELISA kit for plasma 25(OH)D and ferritin concentrations were used to re-analyse the blood samples which were below the detection limit of the mini VIDAS automated immuno-analyser.

Quality controls (low, medium and high) including expected range were obtained for each kit from the similar company supplying the specified ELISA kits. For iron isotopic analysis, an iron (Fe) pure single element standard was purchased from Perkin Elmer, Massachusetts, USA.

2.1.4 FOODSTUFFS

Instant porridge (Sainsbury's Express Porridge Original), tea bag (Yorkshire Everyday Black Tea), and white granulated sugar (Fairtrade International, Bonn, Germany) were purchased from Sainsbury's UK Ltd, and semi skimmed milk portions were obtained from Lakeland Dairies Co-op Society UK Ltd. Ultrapure water to prepare the test meal and tea were obtained from Sigma Aldrich, Inc, UK. Ascorbic acid powder used in the preparation of the reference iron dose was purchased from Sports Supplements Ltd (Essex, UK).

2.2 METHODS

2.2.1 PARTICIPANTS

2.2.1.1 Experimental Study 1 (Chapter 3)

Twelve premenopausal women aged between 19 to 40 years old were included in the study based on criteria described in **Table 2.3**. Participants were recruited using convenience sampling within the University of Chester, UK only. Posters (Appendix A) and emails (Appendix B) were used as a medium to recruit the potential participants amongst staff and students. All participants were given a participant information sheet (PIS, Appendix C) and briefed on the study protocol before the beginning of the study. The eligible participants also provided written informed consent (Appendix D) prior to the start of the study.

Table 2.3 Inclusion and exclusion criteria of participants

Inclusion criteria	Exclusion criteria
Women aged 19-40 years	History of gastrointestinal and metabolic disorders
Healthy	Have donated blood in the past 6 months
Non-pregnant nor lactating	Regular consumption of nutritional supplements

2.2.1.2 Experimental Study 2 (Chapter 4)

The inclusion criteria were premenopausal women, healthy, aged 19-49 years, and non-pregnant nor lactating. The women were included after screening session based on plasma ferritin concentration of < 20 µg/l and plasma 25(OH)D concentration of < 250 nmol/l. The women were excluded if they had a history of gastrointestinal and metabolic disorders, had donated blood since the past 6 months, and regularly consumed nutritional supplements.

2.2.2 SAMPLE SIZE

2.2.2.1 Experimental Study 1 (Chapter 3)

Sample size was estimated using iron absorption data from a study by Derman et al. (1977) carried out in adult women aged between 21-71 years in India. The study aimed to investigate the inhibition effect of tea or coffee on iron absorption from maize meal, labelled with radioisotopes ^{55}Fe or ^{59}Fe.

It was reported in the study that mean (±S.D) iron absorption was 6.7 ± 6.2% when the test meals were administered with tea and 34.0 ± 23.0% when the test meals were administered without tea. With an effect size of 1.3, the total sample size required in the proposed study was 10. Allowing for a 20% drop out rate, the total sample size required to demonstrate a significant difference in iron absorption between consumption of meal with and without tea was estimated to be 12 (power=0.95, α err prob=0.05). Sample size was estimated using of G Power Software Version 3.1.7 (Faul, Erdfelder, Lang, & Buchner, 2007).

2.2.2.2 Experimental Study 2 (Chapter 4)

Sample size was estimated using serum ferritin concentrations (µg/l) from a double-blind placebo-controlled study carried out by Scholz-Ahrens et al. (2004) in women aged 20-40 years with serum ferritin concentrations of < 22 µg/l. The study was designed to determine the effect of 8 weeks iron-fortified milk supplementation on iron stores. At the beginning of study, mean (±S.D) serum ferritin concentrations were not significantly different between the iron-fortified group (13.3 ± 6.90 µg/l) and control group (12.6 ± 6.78 µg/l) (p=0.69). At the end of study, it was observed that mean (±S.D) ferritin concentrations were 17.65 ± 11.85 µg/l in the iron-fortified group, compared to 10.60 ± 8.13 µg/l in the control group (p=0.01). With an effect size of 0.7, the total sample size required in the present study was 26/group (Power=0.80, α err prob=0.05). Allowing for a 20% drop-out rate, the total sample size required to demonstrate a significant difference in iron stores between vitamin D group and placebo was estimated to be 62 (31 participants/group). The sample size was estimated using of G-Power Software (Version 3.1.7).

2.2.3 STUDY DESIGN

2.2.3.1 Experimental Study 1 (Chapter 3)

A non-randomised controlled trial to investigate the effect of tea on non-haem iron absorption using a porridge meal labelled with a stable iron isotope (^{57}Fe) was carried out in 12 participants over a period of 56 days at University of Chester, UK. The data collection started in November, 2014 and the final clinic was completed in March, 2015. All participants were administered three standardised test meals and a reference iron dose at 4 separate clinics **(Table 2.4)**.

Table 2.4 Administration of test meals according to clinic

Clinic	Day	Test meal (Instant porridge)
1	0	Test meal I administered with water (control)
2	14	Test meal II with tea administered simultaneously with a meal
3	28	Test meal III with tea administered 1 hour post-meal
4	42	Reference iron dose administered without a test meal

2.2.3.1.1 Trial registration/Ethical approval

The study was registered at clinicaltrials.gov (Protocol ID: 14/NW/0310 S-LS17042014, ClinicalTrials.gov ID: NCT02365103). The study protocol was approved by the National Research Ethics Service Committee North West-Greater Manchester East, Manchester, UK (REC reference: 14/NW/0310, IRAS Project ID 154775) (Appendix E).

2.2.3.1.2 Study protocol

All the eligible participants were invited to partake in the study and provided informed consent at the beginning of the study. The participants were required to fast for approximately 10 hours prior to their scheduled clinic and expected to attend 5 clinics in total at the clinical laboratory within the Department of Clinical Sciences & Nutrition at the University of Chester over a period of 56 days between 8-10 am. The schematic study protocol is illustrated in the flow chart **(Figure 2.1)**.

All participants were asked to attend a total of 5 clinic sessions (day 0, 14, 28, 42 and 56) with a 14-day interval between clinics to allow the incorporation of the isotope into the erythrocyte. A standardised test meal of porridge extrinsically labelled with $^{57}FeSO_4$ solution was given to the participants with water or tea depending on the clinics, under supervision of researcher. The participants were not permitted to consume food or drink for 3-4 hours after the administration of the labelled test meal. All the containers used to administer the test meals were washed with ultrapure water to ensure complete consumption of both test meals and isotope.

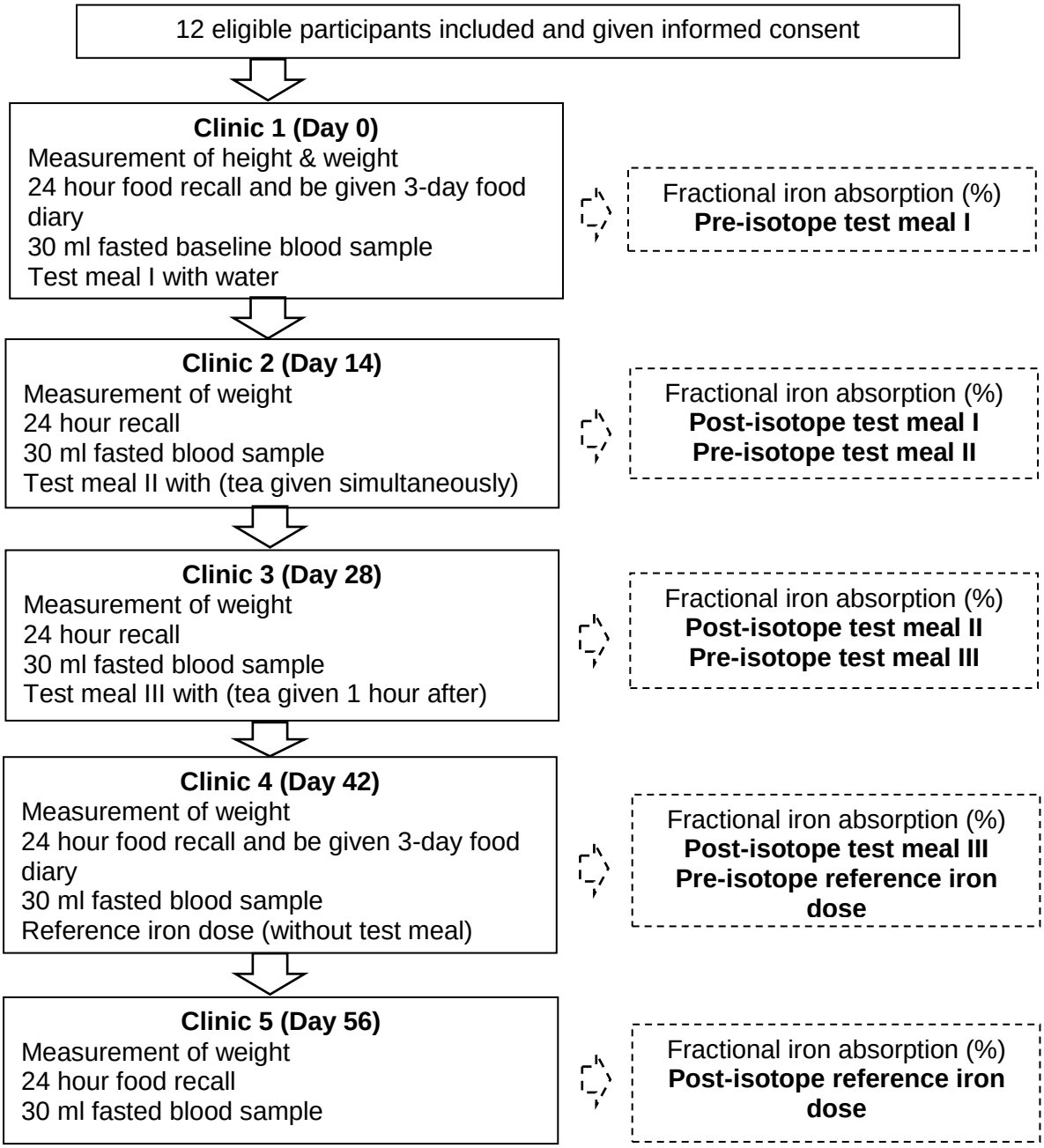


Figure 2.1 Study protocol (administration of test meals)

2.2.3.1.2.1 Clinic 1 (Day 0)

The participants' height and weight were measured and a 30 ml fasted blood sample was collected in trace element-free EDTA blood collection tubes for isotopic analysis and iron status measurements. The whole blood sample collected at every clinic was used for iron isotopic analysis and FBC measurement and plasma sample was used for iron status measurements.

The researcher conducted a 24-hour food recall interview and participants were given a 3-day food diary to record their dietary intakes at home. Participants were then administered test meal I, with water to serve as a control.

2.2.3.1.2.2 Clinic 2 (Day 14)

The participant's weight was measured and a 30 ml fasted blood sample was collected in trace element-free EDTA blood collection tubes. The researcher conducted a 24-hour food recall interview and participants were then administered test meal II, simultaneously with tea.

2.2.3.1.2.3 Clinic 3 (Day 28)

The same processes in Clinic 2 were repeated, with participants were then given test meal III, and tea was administered 1 hour post-meal.

2.2.3.1.2.4 Clinic 4 (Day 42)

The same processes in Clinic 2 were repeated, with participants were then administered a reference iron dose and were given another 3-day food diary to record their dietary intakes.

2.2.3.1.2.5 Clinic 5 (Day 56)

The same processes in Clinic 2 were repeated, with the collection of final whole blood sample without administration of any test meal.

2.2.3.1.3 Stable iron isotopes

A single isotope technique was employed in the study using a stable iron isotope ^{57}Fe. The stable iron isotope ^{57}Fe was purchased from Trace Sciences (Ontario, Canada) in metal form (300 mg), converted to sterile form of ferrous sulphate solution ($^{57}FeSO_4$) and tested for endotoxin by Anazao Health Corporation (Florida, USA) (Appendix F). The enriched stable iron isotopes were transferred and kept in individual vials, flushed with nitrogen before being sealed and stored at -20^{o}C until test meal administration by the researcher. The specified dose for test meals and reference iron dose used in the study were 4 mg and 3 mg respectively.

2.2.3.1.3.1 Preparation of labelled isotope ^{57}Fe

The dissolution process to produce a clear $^{57}FeSO_4$ solution safe for human consumption was carried out by the Anazao Health Corporation. Following complete dissolution of the substance, which resulted in a clear and colourless solution, the solution was filtered over 0.5 µm membrane. The prepared $^{57}FeSO_4$ solution was shipped to the Department in 1 vial containing 75 ml (300 mg). The solution was then transferred into individual sterile borosilicate glass clear sample vial with fitted caps (Fisher Scientific UK Ltd), labelled with dose and concentration/ml. The labelled vials were flushed with nitrogen, sealed and stored at -20^{o}C to be used either with test meals (4 mg) or reference iron doses (3 mg).

2.2.3.1.4 Test meals

The test meal used in the study was Express Oat Porridge Original (Sainsbury's UK Ltd). Test meals were freshly prepared on the day of each clinic by adding 200 ml of boiling TraceSELECT Ultra, Fluka Analytical ultrapure water (Sigma-Aldrich UK Ltd) to 55 g of porridge. This test meal was used at all clinics using the same standardised method of preparation.

The nutritional content of the porridge is summarised in **Table 2.5**. To each test meal, 4 mg dose of $^{57}FeSO_4$ was extrinsically added before the consumption. The reference iron dose was extrinsically labelled with 3 mg dose of the $^{57}FeSO_4$ with 35 mg of ascorbic acid and administered in ultrapure water without any test meals.

Table 2.5 Nutrition information of test meal

Nutrient	Per 100 g	Per serving (1 pot = 55 g)
Energy (kJ/kcal)	1569/372	863/205
Fat (g)	6.2	3.4
Saturated (g)	1.1	0.6
MUFA (g)	2.4	1.3
PUFA (g)	2.3	1.3
Carbohydrate (g)	59.6	32.8
Sugars (g)	15.9	8.7
Starch (g)	43.7	24.0
Fibre (g)	7.2	3.9
Protein (g)	15.8	8.7
Salt (g)	0.25	0.14

2.2.3.1.5 Tea

Black tea (Yorkshire Tea, Bettys and Taylors Group Ltd, UK) was prepared using a standardised method at each clinic, by adding 200 ml of boiling TraceSELECT Ultra, Fluka Analytical ultrapure water (Sigma-Aldrich UK Ltd) to one tea bag (3 g) and the mixture was steeped and infused for 3 minutes before straining for consumption. In each cup of tea, 12 ml of homogenised semi skimmed milk UHT (Lakeland Dairies Co-op Society UK Ltd) was added and participants were offered white granulated sugar (Fairtrade International, Bonn, Germany) with the tea to their preference. The tea was prepared on the day of the clinic and kept warm in a flask before consumption. The tea was administered at specific times either simultaneously with test meal, or 1 hour post-meal.

2.2.3.2 Experimental Study 2 (Chapter 4)

The study was a placebo controlled, double-blind randomised controlled trial, designed to investigate the effect of vitamin D3 supplementation, consumed with iron-fortified breakfast cereal on iron absorption and hepcidin response in a cohort of iron deficient UK women. The study was registered at clinicaltrials.gov (ClinicalTrials.gov. ID: NCT 02714361). The study protocol was approved by the Faculty of Life Sciences Research Ethics Committee (FREC reference: 1078/15/SF/CSN) (Appendix G). The study was carried out for the period of 8 weeks in Chester, UK. The data was collected between September 2015 and April 2016.

2.2.3.2.1 Recruitment & screening

The prevalence of iron deficiency based on plasma ferritin concentration in women aged 19-64 years in the UK was reported to be approximately 16% (Bates et al., 2014). To achieve the required sample size, approximately 325 women would have to be screened. Participants were first recruited and screened from staff and students at the University of Chester, UK only using posters and flyers (Appendix H), emails sent to various departments within faculties (Appendix I), staff and student discussion boards, the university intranet, and university web page. However, due to the low number of women eligible for the study after the initial screening, recruitment was carried out on a larger scale, in the wider city of Chester. A press release was used as a medium of recruitment, in November 2015 and January 2016 to obtain the estimated sample size. The press release was placed in the local newspapers, The Chester Chronicle and The Standard (Appendix J). A total of 186 women attended the initial screening clinics and 62 were eligible based

on the plasma ferritin concentration threshold of < 20 µg/l to define iron deficiency and plasma 25(OH)D concentration of < 250 nmol/l. However, 12 of the eligible participants withdraw from participating in the study. The reasons were; fell sick (n=1), didn't respond to the invitation email (n=6), and refused to participate following the screening session (n=5). After screening, 50 participants were included and randomised. **Figure 2.2** outlines the study design which included 2 main phases. **Phase 1** was the recruitment and screening phase when potential participants were screened for iron deficiency and identified. **Phase 2** was the intervention phase where all participants consumed iron-fortified breakfast cereal, together with either vitamin D3 supplements containing 1500 IU (38 µg) (vitamin D group) or placebo (placebo group) daily for the duration of 8 weeks.

2.2.3.2.2 Phase 1: Screening

All participants were given a participant information sheet (PIS, Appendix K) and a date was arranged for the blood collection to ascertain the plasma ferritin concentration, which was used to indicate iron stores levels in the present study. The potential participants provided written informed consent (Appendix L) and were asked to complete a screening questionnaire to ensure eligibility criteria were met (Appendix M). During the screening clinic, 4 ml of blood was collected in a lithium heparin blood collection tube at the clinical laboratory within the Department of Clinical Sciences & Nutrition at the University of Chester by the researcher who is a trained phlebotomist. The plasma sample collected was used to determine the concentration of ferritin and 25(OH)D. Based on the blood analysis, participants were notified of their results via email. The eligible participants were then invited to continue to Phase 2 of the study. Participants who had abnormally high plasma ferritin concentrations from the screening (n=2) were advised to arrange a check-up with their registered general practitioner.

2.2.3.2.3 Phase 2: Intervention

A total of 50 participants who were eligible to continue with the study were randomised to receive either 1500 IU (38 µg) of vitamin D3 or placebo. Both groups were instructed to consume an iron-fortified breakfast cereals with either vitamin D3 or a placebo daily according to the group assigned for the duration of 8 weeks. The participants were also provided with semi-skimmed milk and were required to consume 60 grams of iron-fortified cereals in pre-weighed tubs with 200 ml of milk in the morning every day for the duration of the study. The participants were instructed to consume the vitamin D3 capsules or placebo capsules with 200 ml of water in the

evening, also daily, for the 8-week duration of the study. All the participants were reminded to not alter their dietary habits and physical activity, in addition, to abstaining from donating blood during the course of the study.

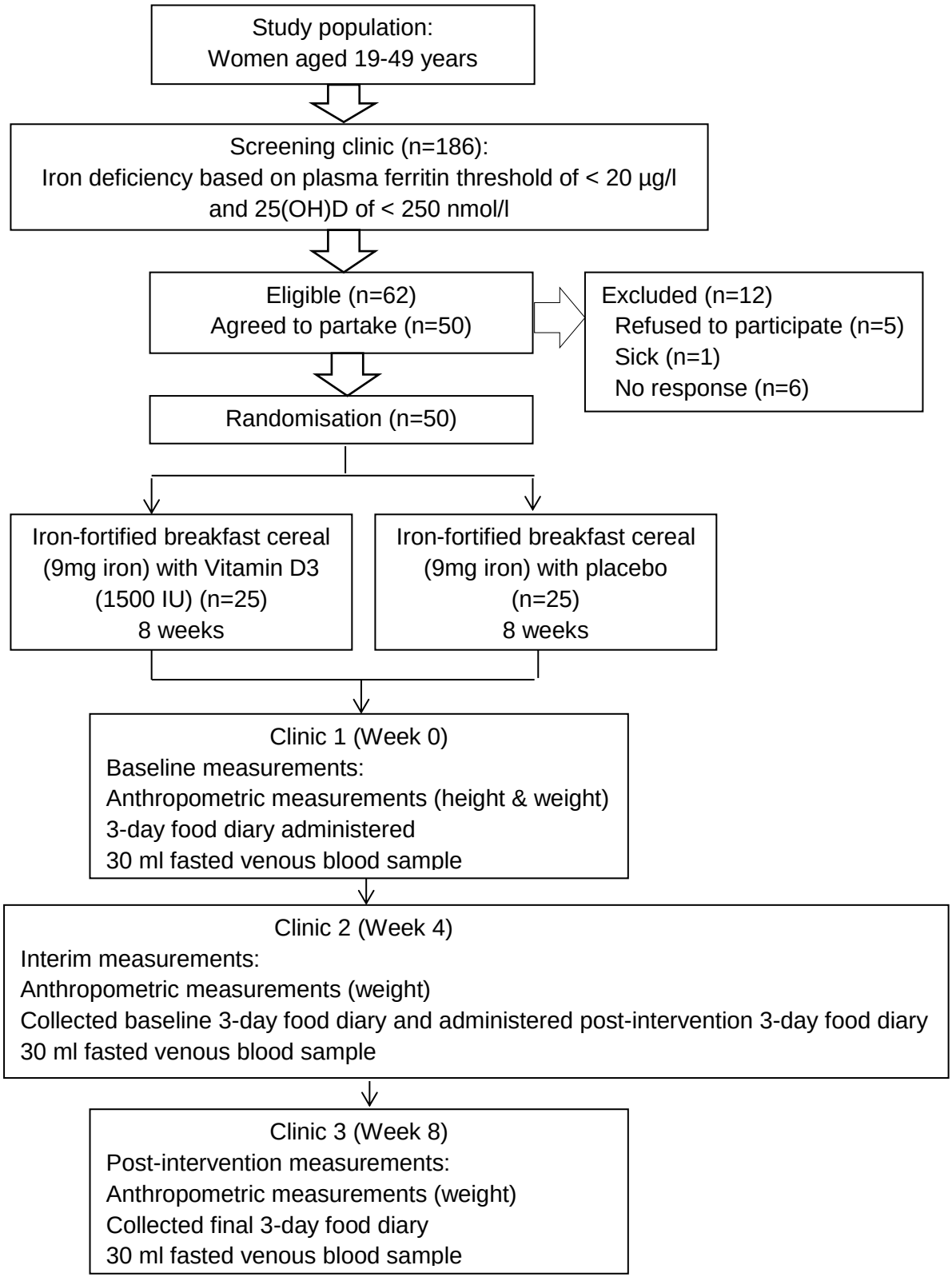


Figure 2.2 Study protocol

2.2.3.2.4 Study clinics

Participants were all required to attend clinics after overnight fasts of approximately 8 hours and were expected to attend 3 clinics in total. Participants were asked to consume only water during the overnight fast. Each clinic lasted approximately 30 minutes and took place between 8-10 am. Details of each clinic are as follows.

Clinic 1 (Week 0, baseline): Height (cm) was measured using digital stadiometer and weight (kg) using weighing scales, followed by collection of a 30 ml fasted venous blood sample collection. Participants were given a 3-day food diary to be completed before the next clinic. A 4-week supply of both iron-fortified breakfast cereals in individually packed containers and semi-skimmed milk were provided to the participants, together with a supplement pot, containing 8 weeks of supplements with assigned participant ID. The protocol was explained to participants and an email was sent as a reminder to the participants one day before the following clinic.

Clinic 2 (Week 4, interim): The same procedure was repeated except for measurement of height. Participants were given a further 3-day food diary to be completed before the next clinic and the previous diary was collected. A further 4-week supply of iron-fortified breakfast cereals and semi-skimmed milk were provided to the participants.

Clinic 3 (Week 8, post-intervention): Weight was measured and a 30ml fasted venous blood was taken. The final 3-day food diary and supplement pot (with any) of the remaining capsules to calculate the compliance was collected.

2.2.3.2.5 Randomisation and blinding of participants

The randomisation process was carried out using computer-generated software (www.randomization.com) by a third party, independent to the study. Participants (n=50) were randomised to 2 groups: vitamin D3 (vitamin D group) or placebo (placebo group). The third party allocated 62 capsules of vitamin D3 and placebo into each supplement pot according to the generated plan. An excess of 6 capsules in each pot enabled the researcher to estimate compliance once the study was completed. Each supplement pot was then sealed in a tamper proof supplement pot and numbered ready to be provided to participants. The participants and researcher were double-blinded as to which groups participants were assigned. The researcher administered the numbered supplement pot (i.e; 001) to the participants based on the sequence that participants attended their baseline clinic (Week 0).

The blinding was maintained throughout the study period of 8 weeks and allocation was not unlocked until the end of the data analysis.

2.2.3.2.6 Iron-fortified break cereals

The iron-fortified breakfast cereal used in the study was a commercially available whole grain rice and wheat flakes cereal (Weight Watchers UK Ltd). The supplies of both breakfast cereals and milk were provided to the participants at the first visit (Clinic 1). Participants were instructed to consume one pre-weighed tub of cereal (60g) containing approximately 9 mg of iron with 200 ml of semi-skimmed milk (Tesco PLC,UK), daily. Each participant was also given a measuring cup to measure approximately 200 ml of semi-skimmed milk to consume with the cereal, to ensure the standardisation of meal consumed by each participant. It was stressed during the first clinic that the participants need to consume the cereal daily. However, in the event of missing a meal, it was advised that they consumed the cereal immediately. If the cereal was consumed the following day, the specified cereal which was supposed to be consumed that day will have to be consumed as usual. Participants were asked about any problems regarding consumption of the provided cereal during the interim clinic (Week 4) and were requested to notify researcher if any problem surfaced e.g. adverse effects during the 8-week course of study. This cereal was selected as it is fortified with iron, and previous iron-fortified cereal studies have reported the use of between 7-18 mg of total iron. A total of 9 mg iron in the current study provides approximately 60% of the RNI/day for women aged 19-50 years (Department of Health, 1991). The nutritional content of the breakfast cereal is shown in **Table 2.6**.

Table 2.6 Nutritional information of iron-fortified breakfast cereal

Nutrient	Per 100 g	Per recommended serving (60 g)
Energy (kJ/kcal)	1566/370	940/222
Fat (g)	2.1	1.2
Saturated	0.4	0.2
Carbohydrates (g)	70	42
Sugars	16	9.6
Fibre (g)	7.5	4.6
Protein (g)	14	8.4
Salt (g)	0.6	0.4
Iron (mg)	15.0	9

2.2.3.2.7 Vitamin D supplement and placebo

The supplement used was vitamin D3 cholecalciferol (1500 IU, 38 µg, Pharma Nord ApS, Vejle, Denmark), liquefied in cold-pressed olive oil and encapsulated in a clear soft gel 7 mm capsule. Each capsule supplied was made of a combination of olive oil, gelatine, glycerol, and purified water at Pharma Nord ApS, Vejle, Denmark. The supplements were packaged in blister packs, and manufactured specific to the research requirement and according to pharmaceutical standards (EC and Scandinavia). Batch numbers and expiry dates were printed on each blister pack.

The matching placebo was also custom-produced to the requirement of the study by Pharma Nord ApS, Vejle, Denmark. Placebo was produced in the same manner, without the active ingredients. The placebo was matched with vitamin D3 in terms of appearance, size, colour and taste to achieve the double-blind design.

2.2.3.2.7.1 Assessment of compliance

Compliance to the supplementation (%) was calculated on the basis of (62 – remaining capsules in the pot)/56 x100. For the total duration of 8 weeks, participants were required to consume 56 capsules of vitamin D3 or placebo.

2.2.4 ANTHROPOMETRIC MEASUREMENTS

Instruments used were calibrated before every measurement.

2.2.4.1 Height

Height was measured to the nearest 0.1 cm with a wall mounted digital stadiometer (Model 264 SECA, Hamburg, Germany). Before the start of measurement, participants were checked to ensure they were wearing minimal clothing and barefoot to allow accurate positioning. With the head positioned at Frankfurt horizontal plane, participants were instructed to stand with the legs straight, heels closed together, shoulders relaxed, and arms at the side. The 4 points, which included back of the head, buttocks, heels and scapulae, were checked to make sure they were in line with the vertical surface of the stadiometer.

The reading was taken when the headboard was lowered to the highest point of participants' head. The measurement was performed in duplicate to ensure the accuracy (Lee & Nieman, 2010).

2.2.4.2 Body weight

Body weight was measured to the nearest 0.1 kg using an electronic scale (Model 875 SECA, Hamburg, Germany). The scale was set on a flat and hard surface to ensure stability when the measurement was made. Before the measurement, participants were asked to be barefoot, empty out the pockets and wear minimal clothing. To ensure even distribution of body weight on both feet, participants were instructed to stand still in the middle of the platform scale without touching anything. The measurement was performed in duplicate to ensure the accuracy (Lee & Nieman, 2010).

2.2.4.3 Body mass index (BMI)

The BMI was calculated using height and weight obtained from the anthropometric measurement of the participants. The equation used to obtain the BMI was: BMI = weight (kg)/height (m^2) and the cut off values to define BMI are summarised in **Table 2.7**.

Table 2.7 Classification of BMI (WHO, 1998)

Classification of BMI	BMI (kg/m^2)	Obesity class
Underweight	< 18.5	
Normal	18.5 – 24.9	
Overweight	25.0 – 29.9	
Obesity	30.0 – 34.9	I
	35.0 – 39.9	II
Extreme obesity	≥ 40.0	III

2.2.5 VENEPUNCTURE PROCEDURE

2.2.5.1 Venepuncture protocol

Venepuncture was performed by the researcher, a trained phlebotomist, to obtain 30 ml blood samples from participants during each clinic. The participant was positioned with the arm stretched straight from shoulder to wrist on the cushion of phlebotomy chair. After the suitable vein was identified and selected, tourniquet was applied approximately 3-4 inches above the selected puncture area. Using a 70% ethanol swab, the puncture site was cleaned by performing circular motion and the area was left to dry. The needle was then inserted to hub; cap removed and held with the bevel up.

With a thumb placed just below the puncture area, the needle was inserted smoothly into the vein by trained researcher. Holding the one-use holder firmly, the vacutainer tube was inserted and tourniquet was released once the blood started to flow. Once the blood flow stopped, the tube was removed from the needle and inverted several times to mix. A gauze pad was placed on the puncture site, and the needle was slowly removed and slight pressure was applied for at least 2 minutes. The needle was appropriately disposed in sharp bin container and a clean bandage or micropore tape was applied on puncture area.

2.2.5.2 Blood handling

Both whole blood and plasma obtained from the venepuncture were used in the study for analysis. Whole blood samples for iron absorption analysis were collected in trace element-free collection tubes with EDTA (BD Company, New Jersey, USA) for the first experimental study (Chapter 3) or lithium heparin and EDTA collection tubes for the second experimental study (Chapter 4). All the collected blood samples were aliquoted into micro centrifuge tubes, and stored at -80^{o}C prior to analysis. Whole blood samples were used immediately after each clinic to measure full blood counts (FBC). To obtain plasma samples, the blood tubes were centrifuged for 10 minutes at 1600 × g at 4^{o}C. The plasma was then aliquoted into micro centrifuge tubes and stored before being used for analysis. Whole blood and plasma samples were used for the analysis of iron status biomarkers, including full blood counts (red blood cell counts, haemoglobin concentration, haematocrit level, MCV, MCH, MCHC and white blood counts), plasma ferritin, plasma CRP and plasma hepcidin concentrations for the first experimental study (Chapter 3). Plasma samples were used to analyse iron status biomarkers (CRP, ferritin, soluble transferrin receptor, and hepcidin) and vitamin D metabolism biomarkers (25-OH D, PTH, and VDBP) concentrations for the second experimental study (Chapter 4).

2.2.6 ANALYSIS OF BLOOD SAMPLES

2.2.6.1 Determination of full blood counts (FBC)

The measurement of FBC was carried out using an automated Beckman Coulter Ac.T diff Haematology Analyser (Beckman Coulter Inc, California, USA). The analyser operated based on the coulter principle which involved counting and sizing, triplicate counting, extended platelet counting and cyanmethaemoglobin method. The analyser provided 18 parameters of full blood counts which include white cell differential. Once the sample mode was selected and ID number entered on the

interface screen, approximately 12 µl of whole blood was aspirated and the results were displayed. The normal values/ranges for the FBC used in the study are summarised in the following **Table 2.8**. The intra-assay CV for this measurement was 1.9%.

Table 2.8 Normal range of full blood count concentration for adult women

Full blood counts (FBC) indicators	Unit	Normal values
RBC count	$\times 10^{12}$/L	3.8 – 5.2
Haemoglobin concentration	g/dL	11.5 – 15.5
Haematocrit/packed cell volume (PCV)	L/L	0.37 – 0.47
Mean corpuscular volume (MCV)	fl	80 - 96
Mean corpuscular haemoglobin (MCH)	pg	27 – 32
Mean corpuscular haemoglobin concentration (MCHC)	g/dL	32 – 36
White blood count (WBC)	$\times 10^{9}$/L	4 - 11
Platelet	$\times 10^{9}$/L	150-400

(Data extracted from "Williams Haematology" (Kaushansky et al., 2015)

2.2.6.2 Determination of plasma ferritin concentrations

Plasma ferritin concentrations were measured using mini VIDAS Biomerieux automated immunoanalyser using enzyme linked fluorescent assay (ELFA) technique (Biomerieux, Marcy-l'Etoile, France). The procedure for measuring plasma ferritin involved ready-to-use and pre-dispensed sealed reagent strips and a solid phase receptacle (SPR). The SPR was coated with monoclonal anti-mouse ferritin immunoglobulins that act as the solid phase and pipetting device for the assay. During the assays, the SPR functioned as a pipetting device that aspirates the reagents in and out, and 4-Methyl-umbelliferyl phosphate the final substrate aspirated at the final stage of the assays. This substrate was then hydrolysed by a conjugate enzyme into 4-methyl-umbelliferone, a fluorescent product that can be detected at 450 nm wavelengths.

The necessary reagents required for the assay were removed from the refrigerator and allowed to reach room temperature for at least 30 minutes. The plasma sample was mixed using a vortex mixer and 100 µl of plasma sample was added to the first well of the ferritin strip and for each plasma sample, one ferritin strip and SPR was used. Both the ferritin strip and SPR were simultaneously inserted into the selected section of the instrument and assay was automatically initiated.

The assay procedure was completed in approximately 30 minutes. Once the result concentration was displayed, the ferritin strip and SPR were unloaded and appropriately disposed in a clinical waste container. The threshold concentration of >15 µg/l was used to define adequacy of plasma ferritin concentration for non-pregnant women aged above 15 years, as recommended by WHO/UNICEF/UNU (2001). The similar threshold was also used in assessing iron status of UK population in the NDNS, in combination with haemoglobin concentrations (SACN, 2010). The intra-assay CV for this assay was 4.69%.

However, some of the samples (n=3) in the experimental study 2 (Chapter 4) were analysed using a commercial human ferritin ELISA kit (Elabscience Biotechnology Co. Ltd), due to undetectable concentrations of below 1.5 µg/l when using mini VIDAS. The assay procedure is as follows.

The reagents were brought to room temperature before use. Concentrated wash buffer, a specific antibody, and conjugate were diluted to the required concentrations and cooled to room temperature prior to analysis. Using the serial dilution method, 8 tubes of standards were prepared 15 minutes prior to the procedure. The assay was started by adding 100 µl of standard, blank, and samples to a pre-coated 96 well plate, sealed and incubated for 90 minutes in an incubator at 37^{o}C. After 1.5 hours, wells were emptied and without washing, 100 µl of biotinylated detection antibody working solution was added to each well. The plate was sealed and incubated for a further 1 hour at 37^{o}C. Each well was then washed and aspirated with 350 µl of wash buffer three times. Following the washing step, 100 µl of HRP conjugate was added, the plate was sealed and incubated at 37^{o}C for 30 minutes. The washing step was repeated 5 times after the incubation period time. A total of 90 µl of substrate solution was then added to each well, plate was covered with a plate sealer, and incubated for a minimum of 15 minutes at 37^{o}C protected from light using aluminium foil. Once apparent gradient started to show in standard wells, 50 µl of stop solution was added to each well. The absorbance was read at a 450 nm wavelength using microplate reader.

2.2.6.3 Determination of plasma CRP concentrations

A commercial Quantikine Human CRP Immunoassay ELISA kit (R&D Systems Inc, Minneapolis, USA) was used to determine the plasma CRP concentrations of participants in the present study.

The reagents and plasma samples required for the assay were allowed to reach room temperature before the start of analysis. All the reagents were prepared: wash buffer and calibrator diluent RD5P (1X) was diluted, both colour reagents were mixed equally to form substrate solution, and 7 tubes of CRP standards were prepared by serial dilution process. A total of 100 µl of assay diluent RD1F was added to the wells followed by a 50 µl of each standard, control and sample added to the wells. An adhesive strip was used to cover the microplate which was allowed to incubate for 2 hours at room temperature. After the incubation period, each well was aspirated and washed 4 times using 400 µl of wash buffer. A total of 200 µl CRP conjugate was then added to the wells and microplate was covered with an adhesive strip for a further 2 hours incubation period time. Each well was then aspirated and washed again for 4 times using 400 µl of wash buffer. A total of 200 µl substrate solution was added to the wells and allowed to incubate for 30 minutes at room temperature. The microplate was protected from the light using aluminium foil. After the incubation period, 50 µl of stop solution was added to the wells. The assay was completed by determining the optical density of each well using a microplate reader that was set at 450 nm with a 570 nm secondary wavelength, within 30 minutes of adding the stop solution. A threshold of < 3-10 mg/l was used to define normal ranges of CRP concentrations (WHO/CDC, 2004). The intra-assay and inter-assay CV for this assay were 11.5% and 3.3%, respectively.

2.2.6.4 Determination of plasma hepcidin concentrations

Hepcidin concentrations were measured in plasma samples using a Human Hepcidin (Hepc) ELISA kit (Sincere Biotech Co Ltd, Beijing, China) for the first experimental study (Chapter 3) and using a human hepcidin immunoassay quantikine ELISA kit (R&D Systems Inc, Minneapolis, USA) for the second experimental study (Chapter 4).

2.2.6.4.1 Experimental Study 1 (Chapter 3)

The reagents and plasma samples required for the assay were brought to room temperature 30 minutes prior to the start of analysis. All the reagents were prepared: standards were reconstituted with deionised water in 1:2 serial dilutions. The wash buffer working solution was prepared by diluting wash buffer concentrate in deionised water at a ratio of 1:25. The three other reagents were prepared less than 30 minutes before the analysis. A total of 100 µl of standard, controls, and participants' diluted plasma samples were added to the wells and then allowed to incubate for 1.5 hours at 37^{o}C.

After the incubation period, the microplate was washed with 350 µl of wash buffer twice with 1 minute interval between each wash. A total of 100 µl biotinylated anti-human hepcidin working solution was then added to the wells and microplate was incubated for 1 hour at 37ºC. The microplate was then washed with 350 µl of wash buffer for 3 times with 1 minute interval between each wash. A total of 100 µl avidin-biotin-peroxidase complex (ABC) working solution was added to each well, except the control wells and microplate was incubated for 0.5 hours at 37ºC. After 30 minutes, the microplate was washed with 350 µl of wash buffer 5 times with 1 minute interval between each wash. A total of 100 µl substrate working solution was added to each well, except the control wells and microplate was incubated for ≤ 30 minutes at 37ºC away from light. A total of 100 µl stop solution was then added to the wells. The optical density was determined using a microplate reader set at 450 nm within 10 minutes of the stop solution added into each well. The normal threshold level of plasma hepcidin in healthy women used in the study was between 17 – 286 ng/ml (Ganz et al., 2008). The intra-assay and inter-assay CV for this assay were 5.7% and 10.8% respectively.

2.2.6.4.2 Experimental Study 2 (Chapter 4)

Hepcidin concentrations were measured in plasma samples using a human hepcidin immunoassay quantikine ELISA kit (R&D Systems Inc, Minneapolis, USA). Plasma samples to be analysed were first prepared with a 5-fold dilution using the calibrator diluent RD5-26 which was primarily diluted (1:4). All the reagents used in the assay were brought to room temperature prior to analysis. Wash buffer concentrate was diluted to the required volume. The human hepcidin standard was first reconstituted and used to make 8 standards of serial dilutions (0-1000 ng/ml). The assay was started by adding 50 µl of assay diluent RD1-21 into each well followed by 50 µl of standard, control, and samples into each well. The microplate was then sealed and incubated at room temperature for 2 hours. After the incubation period, the microplate was washed and aspirated 4 times. Following the wash steps, 200 µl of human hepcidin conjugate was added into each well, sealed and incubated for a further 2 hours at room temperature. Following this, the same wash step was repeated, followed by addition of substrate solution which was prepared 15 minutes before and the microplate was incubated protected from light using aluminium foil, for 30 minutes at room temperature. 50 µl of stop solution was then added and absorbance was measured at 450 nm (with a secondary wavelength of 570 nm) using a microplate reader within 30 minutes.

2.2.6.5 Determination of plasma soluble transferrin receptor (sTfR) concentration

Soluble transferrin receptor (sTfR) concentrations were measured in plasma samples using a human sTfR immunoassay Quantikine IVD ELISA kit (R&D Systems Inc, Minneapolis, USA). All reagents were allowed to reach room temperature before use. Wash buffer concentrate was then diluted to the required concentration, followed by reconstituting the vial of standards and allowing to sit for 30 minutes prior to analysis. The assay was started by adding 100 µl of sTfR assay diluent into each well, followed by 20 µl of standard, samples, and control. The microplate was then sealed and incubated at room temperature for 1 hour. After the incubation period, the microplate was washed and aspirated 4 times with 400 µl of wash buffer, followed the addition of 100 µl of the sTfR conjugate, microplate was sealed and incubated for a further 1 hour at room temperature. The wash step was repeated and 100 µl of the substrate was added to each well. The microplate then was incubated at room temperature for 30 minutes away from sources of light using aluminium foil. After 30 minutes, stop solution was added to the wells and the optical density was determined using microplate reader set at 450 nm, with a 570 nm of secondary wavelength.

2.2.6.6 Determination of plasma 25(OH)D concentration

Plasma 25(OH)D concentrations were measured using a mini VIDAS Biomerieux automated immunoanalyser using an enzyme-linked fluorescent assay (ELFA) technique (Biomerieux, Marcy-l'Etoile, France). The procedure for measuring 25(OH)D involved ready-to-use and pre-dispensed sealed reagent strips and a solid phase receptacle (SPR). The SPR was coated with vitamin D antigen that acts as the solid phase and pipetting device for the assay. During the assays, the SPR acts as a pipetting device that aspirates the reagents in and out, and 4-Methyl-umbelliferyl phosphate the final substrate aspirated at the final stage of the assays. This substrate was then hydrolysed by a conjugate enzyme into 4-methyl-umbelliferone, a fluorescent product that can be detected at a wavelength of 450 nm. The reagents required for the assay were allowed to reach room temperature for at least 30 minutes. The plasma sample was mixed using a vortex mixer and 100 µl of plasma sample was added to the first well of the vitamin D strip. For each plasma sample, one vitamin D strip and SPR was used. Both the vitamin D strip and SPR were simultaneously inserted into the instrument and the assay was automatically initiated.

The assay procedure was completed in approximately 40 minutes. Once the resulting concentration was displayed, the vitamin D strip and SPR were unloaded and appropriately disposed of into a clinical waste container.

Some samples in the second experimental study (Chapter 4) were re-analysed using 25(OH)D ELISA kit (Calbiotech Inc, California, USA) as the concentrations were <20.3 nmol/l which is below the detectable range of the mini VIDAS bio analyser. The assay procedure is as follows. All the reagents were allowed to reach room temperature before use. The wash buffer and biotinylated conjugate concentrates were diluted to the required concentrations before starting the assay. Standards, controls and samples (10 µl) were added to the wells of 96-well pre coated microplate. This was followed by the addition of 200 µl of biotinylated 25(OH) vitamin D reagent working solutions to the wells and the microplate was mixed using a microplate shaker at 400 rpm. After mixing, the microplate was sealed and incubated at room temperature for 1.5 hours. The microplate was then aspirated and washed 3 times using 300 µl of wash buffer, followed by the addition of 200 µl of streptavidin-HRP conjugate and the microplate was incubated at room temperature for 30 minutes. The same wash step was repeated, and 200 µl of the substrate was added to the wells. The microplate was then incubated for a further 30 minutes at room temperature, protected from the light using aluminium foil. After 30 minutes, 50 µl of stop solution was dispensed to each well and mixed using microplate shaker for a minimum 20 seconds. Following this, the absorbance was determined using microplate reader set at 450 nm.

2.2.6.7 Determination of plasma parathyroid hormone (PTH) concentration

Parathyroid hormone (PTH) concentrations in plasma samples were measured using an intact PTH ELISA kit (Calbiotech Inc, California, USA). Reagents were allowed to reach room temperature prior to analysis, and were ready to use except for wash buffer which was diluted using deionised water (1:25). Samples, standards, and controls (25 µl) were added to each well. This was followed by addition of 50 µl of anti-PTH-biotin reagents and 50 µl of the anti-PTH-HRP conjugate to each well. The microplate was then sealed, and incubated on a microplate shaker set at 500 rpm for 1.5 hours at room temperature. After the incubation period, the microplate was aspirated and washed 4 times using 300 µl of wash buffer. Substrate (100 µl) was added to each well and the microplate was incubated for 15 minutes at room temperature. After the incubation period, 50 µl of stop solution was added to each well, and the microplate was gently mixed.

Following this, the absorbance was determined using microplate reader set at 450 nm, with 620 nm as a secondary wavelength.

2.2.6.8 Determination of plasma vitamin D binding protein (VDBP) concentration

Vitamin D binding protein (VDBP) concentrations in the plasma samples were measured using a human vitamin D binding protein immunoassay Quantikine ELISA kit (R&D Systems Inc, Minneapolis, USA). The plasma samples were first diluted 2000-fold using calibrator diluent RD6-11. All reagents were brought to room temperature before use. Wash buffer was diluted into the desired concentration (1:20). Standard was first reconstituted with 1ml deionised water, followed by serial dilutions to produce 6 standards (0-250 ng/ml). Assay diluent RD1-19 (100 µl) was added to each well, followed by addition of samples, standards, and controls (50 µl) into the designated well. The microplate was then sealed and incubated at room temperature for 1 hour on a microplate shaker set at 500 rpm. After the incubation period, the microplate was aspirated and washed using 400 µl of wash buffer 4 times. VDBP conjugate (200 µl) was added into each well, sealed and incubated at room temperature for a further 2 hours on the microplate shaker at the same speed. The wash step was repeated with the same amount of wash buffer and wash times. After the wash step was completed, 200 µl of substrate solution was added to each well and the microplate was allowed to incubate for 30 minutes at room temperature protected from sources of light using aluminium foil. After 30 minutes, 50 µl of stop solution was added and when colour changes appeared uniform, the absorbance was determined at 450 nm, with 570 nm secondary wavelength.

2.2.6.9 Normal thresholds used for blood biomarkers using plasma samples

Table 2.9 shows the normal thresholds used to define normal ranges of blood biomarkers determined in Section 2.2.6.2 – 2.2.6.8.

Table 2.9 Normal thresholds of iron and vitamin D status biomarkers

Biomarkers	Normal thresholds	Reference
Ferritin	> 15 µg/l	WHO/UNICEF/UNU (2001)
CRP	<3-10 mg/l	WHO/CDC (2004)
Hepcidin	17-286 ng/ml	Ganz et al. (2008)
sTfR	<6 mg/l	*Manufacturer of kit (R&D Systems Inc, Minneapolis, United States)
25(OH)D	>50 nmol/l	IOM (Ross et al., 2011)
PTH	1.59-6.89 pmol/l	Blanco-Rojo et al. (2013)
VDBP	300-600 µg/ml	Gomme and Bertolini (2004)

* WHO (2014) suggested using manufacturer threshold as no internationally accepted threshold has been established

2.2.6.10 Coefficients of variations (CV): Inter and Intra-Assay

Table 2.10 shows both inter and intra-assay CV for each assay determined in Section 2.2.6.2 – 2.2.6.8. Samples of a standard calibration curves generated for each assay are shown in Appendix N. The automated immunoanalyser used was accredited by periodic quality control by the Vitamin D External Quality Assessment Scheme (DEQAS).

Table 2.10 Coefficients of variations (CV): inter and intra-assay

Assays	Inter-assay CV (%)	Intra-assay CV (%)
Ferritin (Mini VIDAS)	-	4.69
CRP	1.94	2.74
Hepcidin	1.92	11.45
sTfR	1.82	5.01
25(OH)D		
Mini VIDAS	-	1.99
ELISA kit	3.62	11.31
PTH	0.69	6.99
VDBP	2.70	11.29

2.2.7 IRON ABSORPTION DETERMINATION

2.2.7.1 Iron isotope ratio analysis

Whole blood samples were transported in dry ice to the MRC-Human Nutrition Research Unit, Cambridge, United Kingdom for the iron isotope ratio analysis. Analysis was carried out using triple quadrupole ICP-MS/MS 8800 (Agilent Technologies, Japan). Prior to analysis, the frozen samples were defrosted to room temperature and mixed by vortex mixer. A total of 100 µl whole blood sample was then mixed with 9.9 ml of 0.005% TMAH (v/v) (BioXtra grade, Sigma Aldrich Inc, Missouri, USA) ready for the isotopic analysis.

The ICP-MS used (Agilent 8800) has an octopole-based collision/reaction cell, positioned in the middle of two quadrupole analysers. The prepared samples, together with standards were then introduced into the ICP-MS with a MicroMist nebuliser and a Peltier-cooled (2^{o}C) Scott-type spray chamber.

Table 2.11 summarises the interferences that might occur during the ICP-MS analysis, which can be eliminated by the use of a mixture of 10% ammonia in helium as a reaction gas. This mixture which is introduced into the octopole cell also functioned to applicably reduce the signal at certain level in order to ensure a precise isotope ratio analysis. The isotopes were measured as a Fe ammonia cluster, $^{m}Fe\ (NH_3)_2$ at masses 88, 90, 91 and 92. An external standard correction for mass bias correction was performed using Iron (Fe) Pure Single Element Standard, 1,000 µg/mL, 2% HNO_3 (Perkin Elmer, Massachusetts, USA) as a baseline reference.

Table 2.11 Interferences affecting iron isotopes in ICP-MS analysis

Isotope	Natural abundance	Polyatomic and doubly charged interference	Isobaric interference
^{54}Fe	5.845	$^{40}Ar^{14}N$, $^{53}Cr^{1}H$, $^{108}Cd^{++}$, $^{108}Pd^{++}$	^{54}Cr
^{56}Fe	91.754	$^{40}Ar^{16}O$, $^{40}Ca^{16}O$, $^{55}Mn^{1}H$, $^{112}Cd^{++}$, $^{112}Sn^{++}$	
^{57}Fe	2.119	$^{40}Ar^{16}O^{1}H$, $^{40}Ca^{16}O^{1}H$, $^{56}Fe^{1}H$, $^{114}Cd^{++}$, $^{114}Sn^{++}$	
^{58}Fe	0.282	$^{40}Ar^{18}O$, $^{40}Ca^{18}O$, $^{42}Ca^{16}O$, $^{57}Fe^{1}H$, $^{116}Cd^{++,}$ $^{116}Sn^{++}$	^{58}Ni

2.2.7.2 Erythrocyte iron incorporation calculations

Fractional iron absorption (%) was estimated by the erythrocyte iron incorporation method, 14-days post-dosing of the isotope, which involved the use of published equations to establish the final % of iron absorption of each participant. The equations were used to determine the (a) circulating iron body pool (mg); (b) iron incorporation into red blood cells (RBCs), and subsequently estimate the (c) fractional iron absorption (%). The estimated % of iron absorption was then normalised to a fixed reference value of 40% to take into account inter-participant variability (International Atomic Energy Agency, 2012). All the equations used are described as follows and summarised in the **Table 2.12**.

Table 2.12 Equations to estimate fractional iron absorption (%)

Indication	Equations
[1]Blood volume (ml/kg)	(16.52 x height) + (38.46 x weight) – 1369
[2]Circulating body iron pool (mg)	Hb (g/l) x BV (ml) × 3.47 (mg iron)/ Hb (g) × 0.001 (ml)
[3]Iron incorporation into RBCs (mg)	$R^t - R^o/R^o$ × circulating iron (mg)/$W_{natural\ fe}$ × $NA^{57}Fe/100 \times m^{57}Fe$ R^t = ^{57}Fe: ^{58}Fe ratio at time t after dosing; R^o = ^{57}Fe: ^{58}Fe ratio at baseline; $W_{natural\ Fe}$ = average atomic weight of Fe (55.845mg/mmol); $NA^{57}Fe$ = natural abundance of ^{57}Fe (2.1191%); $m^{57}Fe$ = atomic mass of ^{57}Fe (56.935)
[4]Absorption of ^{57}Fe (%)	^{57}Fe incorporation/^{57}Fe dose × 1.25 x 100
[5]Normalised absorption (%)	Absorption observed × 40/Absorption from reference iron dose

[1]Shayeghi et al. (2005), [2]McKie et al. (2001), [3](Andrews, 2005), [4]Latunde-Dada et al. (2006), [5]Bjorn-Rasmussen, Hallberg, and Rossander (1977)

2.2.7.2.1 Blood volume

Blood volume value is required to calculate the circulating iron body pool, which can be estimated using published equations specific for women or men (Shayeghi et al., 2005). The current study used this published equation that is based on height and weight of participants. The estimation equation was developed based on women with 144-179 cm in height, weighing between 45-73 kg which fit the range of participants in the present study. However, for many of the previous bioavailability studies, a fixed 65 ml/kg body weight was typically used for healthy, non-pregnant women (Brown et al., 1962; Wennesland et al., 1959).

2.2.7.2.2 Circulating body iron pool

The published equation by McKie et al. (2001) was used to estimate each of participants' circulating body iron pool. The values of haemoglobin concentrations (Hb) and body weight used in the calculation were from Clinic 1. The value of 3.47 represents the approximate average amount of iron in 1 g of haemoglobin (International Atomic Energy Agency, 2012).

2.2.7.2.3 Iron incorporation into red blood cells

Based on the estimation of blood volume and circulating body iron pool for each participant, iron incorporation into RBCs was calculated using the equation in Table 2.12 (Andrews, 2005).

2.2.7.2.4 Percentage absorption of ^{57}Fe

Based on the previous bioavailability studies, a fixed 80% assumption of absorbed isotope into RBCs was used to estimate the percentage absorption from the equation in Table 2.12 (Latunde-Dada et al., 2006).

2.2.7.2.5 Normalisation to reference dose

To take into consideration inter-variability of iron status between participants, a reference iron dose containing 3 mg of $^{57}FeSO_4$ with 35 mg of ascorbic acid (Lynch et al., 2007) was used in Clinic 4, administered without any test meal. The dose given was to signify optimum conditions for iron absorption, which was fixed to a reference value of 40% (Bjorn-Rasmussen et al., 1977). The use of reference iron dose will correct the inter-variability between participants' iron status and enables comparison to be made with previous iron bioavailability studies.

2.2.8 ASSESSMENT OF DIETARY INTAKE

2.2.8.1 Experimental Study 1 (Chapter 3)

Two dietary assessment methods were utilised in the present study: (a) 3-day food diary and (b) 24-hour dietary recalls. Participants were required to complete a 3-day food diary at baseline and post-intervention (to include 2 weekdays and 1 weekend day) to estimate their habitual dietary intake. A 24-hour recall interview was completed at each clinic to assess dietary intake, a day prior to the test meal administration during which they were asked to recall food consumption, including drinks for the past 24 hours in detail to the researcher. All the dietary records were analysed for nutritional content by using computerised dietary analysis software (Microdiet for Windows software, Version 2.8.8, Downlee Systems UK Ltd). The food items used for the analysis were derived from McCance and Widdowson's The Composition of Foods 6th Summary Edition 2002 (Food Standards Agency, 2002). If there were food items not available in the specified database, McCance latest version of each food was used, or new food items added to database based on the available nutritional information. Dietary reference values from the Committee on the Medical Aspects of Food Policy (COMA) 1991 were used for comparison with the intake of energy, macronutrients and micronutrients of the participants (**Table 2.13**). Details on each dietary assessment method are as follows.

Table 2.13 RNI/day for females aged 19-50 years (COMA, 1999)

Nutrient/day	COMA RNI (1991)	Unit
Energy*	1940	kcal
Protein	45	gram
Carbohydrate**	33	gram
Fat**	47	gram
Iron	14.8	mg
Vitamin C	40	mg
Calcium	700	mg

*Estimated Average Requirements (EARs) for energy; **% of Energy;

2.2.8.1.1 3-day food diary

The dietary intake of the participants was first assessed using a 3-day food diary (Appendix O). The diary comprised of instructions on how to appropriately record participants' dietary intakes, including a guide to portion sizes, how to describe the foods/drinks in detail, together with a sample diary. In each section of diary, there were columns for the time, locations, description of food/drink consumed, brand and amount/quantity for the participants to complete. There was also a section for the participants to specify any physical activity performed for that particular day. If the foods/drinks consumed were homemade, there were also recipe sections provided for the participants to specifically note in the diary.

2.2.8.1.2 24-hour recall interview

This method was used at each clinic to assess participants' habitual food consumption, including drinks for the previous 24 hours. The researcher recorded intakes on a specific form (Appendix P), and the participants were asked to provide the description of the foods and drinks in as much detail as possible including brand, preparation methods/recipe or serving size. The participants were facilitated to estimate the portion size by demonstrating common household measurements such as table spoon, tea spoon, or cup. The participants were also asked about any physical activities performed on the previous day. Once the recall was completed, all the records were checked again with the participants to ensure there were no omission of foods and drinks.

2.2.8.2 Experimental Study 2 (Chapter 4)

Participants were required to complete a 3-day food diary (Appendix Q) at baseline and post intervention (to include 2 weekdays and 1 weekend day) to estimate their habitual dietary intake. The diary comprised of instructions on how to appropriately record participants' dietary intakes, including a guide to portion sizes, how to describe the foods/drinks in detail, together with a sample diary. In each section of the diary, there were columns for time of consumption, location, description of food/drink consumed, brand and amount/quantity for the participants to complete. There was also a section for the participants to specify any physical activity performed during that particular day. If the foods/drinks consumed were homemade, there were also recipe sections provided for the participants to specifically note in the diary.

Dietary records were analysed for nutritional content by using Nutritics Professional Nutrition Analysis Software (Nutritics Ltd, Dublin, Ireland). The food items used for the analysis were derived from McCance and Widdowson's The Composition of Foods (Food Standards Agency, 2002). The DRVs from the Committee on Medical Aspects of Food Policy (COMA) 1991 in combination with the Scientific Advisory Committee on Nutrition (2015) were used as a reference to compare the intake of energy, macro, and micronutrients of the participants. **Table 2.14** shows the RNI values used in the study to compare to the dietary intake of participants.

Table 2.14 RNI/day for females aged 19-50 years (SACN/COMA, 2015)

Nutrient/day	SACN/COMA RNI 2015
Energy	2175 kcal/ 9.1MJ (19-34 years)
	2103 kcal/ 8.8MJ (35-54 years)
Protein	0.75 gram/kg body weight
Total carbohydrate	50% daily food energy (g)
Total fat	< 35% daily food energy (g)
Iron	14.8 mg
Vitamin D	10 µg
Calcium	700 mg

*Estimated Average Requirements (EARs) for energy; **% of Energy;

2.2.9 STATISTICAL ANALYSIS

2.2.9.1 Experimental Study 1 (Chapter 3)

All statistical analyses were performed with IBM SPSS Statistic Data Editor Software (Version 21). The Shapiro-Wilks test was used to ascertain the normal/non-normal distribution for each parameter. The non-normally distributed data, including the fractional iron absorption (%) and total iron absorbed (mg) were log transformed for the purpose of performing statistical analyses, and results were re-transformed to be reported as mean and standard deviation. One way repeated measures Analysis of Variance (ANOVA) statistical tests were performed to compare the iron absorption from each pair of meals in comparison to control test meal (water). Pearson's correlation coefficient tests were performed to investigate the association between different iron status biomarkers and iron absorption (fractional absorption and total absorbed in mg). Differences were considered significant with a p-value ≤ 0.05.

2.2.9.1 Experimental Study 2 (Chapter 4)

All statistical analyses were performed using IBM SPSS Statistic Data Editor Software (Version 21). Shapiro-Wilks or Kolmogorov-Smirnov tests were used to determine the normal distribution of data as appropriate. Descriptive statistics were used to describe frequencies, means and standard deviations. Baseline characteristics comparisons between the groups (vitamin D and placebo groups) were carried out using independent t-test for normally distributed or Mann-Whitney test for non-normally distributed data. Mixed model repeated measures analysis of variance (ANOVA) was performed to determine the effect of intervention and the interaction with time points for all iron status and vitamin D status blood biomarkers. Post-hoc analyses were carried out when there were intervention x time point interactions observed to identify the differences within or between the groups. The changes in all iron status and vitamin D status blood biomarkers from baseline to post-intervention (week 8) between the two groups were compared and analysed using independent t-test for normally distributed or Mann-Whitney test for non-normally distributed data. Pearson's or Spearman's correlation coefficient tests were performed as appropriate, to investigate the relationship between different iron status biomarkers and vitamin D biomarkers. Differences were considered significant with a p-value ≤ 0.05.

CHAPTER 3

A one-hour time interval between a meal containing iron and tea consumption, attenuates the inhibitory effects on iron absorption. A controlled trial in a cohort of healthy UK women using a stable iron isotope (^{57}Fe).

- Findings from this chapter have been disseminated at the following conference: International Conference on Advance in Human Nutrition, Food Science & Technology 2016, 26-27th June, 2016, Toronto, Canada (Appendix R)
- An article based on this chapter has been submitted for peer review to the following journal: American Journal of Nutrition (AJCN) with a title “A one-hour time interval between meal containing iron and tea consumption, attenuates the inhibitory effect on iron absorption. A controlled trial in a cohort of healthy UK women using a stable iron isotope (^{57}Fe).

3.1 OBJECTIVES

3.1.1 General objective

To determine the inhibition effect of tea consumption on non-haem iron absorption from a porridge meal in a cohort of healthy UK women using of a single iron stable-isotope technique (^{57}Fe).

3.1.2 Specific Objectives

- To determine fractional non-haem iron absorption from an iron-containing isotope-labelled porridge meal (^{57}Fe) administered with tea and water (control).
- To assess the effect of variation in the time interval of tea consumption relative to a meal on non-haem iron absorption, following consumption of iron-containing isotope-labelled porridge meal (^{57}Fe).
- To investigate the potential role of hepcidin in regulating iron absorption by measuring plasma hepcidin concentrations following consumption of an iron-containing isotope-labelled test meal (^{57}Fe).

3.2 HYPOTHESES

H_1: There will be a significantly lower fractional iron absorption when a porridge meal is consumed with tea compared to water.
H_0: There will be no significantly lower fractional iron absorption when a porridge meal is consumed with tea compared to water.

H_1: Fractional iron absorption will be significantly lower when the tea is consumed simultaneously with a meal compared to when it is consumed 1-hour after the meal.
H_0: Fractional iron absorption will not be significantly lower when the tea is consumed simultaneously with a meal compared to when it is consumed 1-hour after the meal.

H_1: There will be a significant inverse association between fractional iron absorption and plasma hepcidin concentration.
H_0: There will be no significant inverse association between fractional iron absorption and plasma hepcidin concentration.

3.3 RESULTS

3.3.1 BASELINE PHYSICAL CHARACTERISTICS OF PARTICIPANTS

The participants were healthy women aged between 19-37 years, recruited from University of Chester with a mean (±S.D) age of 24.8 ± 6.9 years. The majority of the participants (67%) were in 19-29 years age group. The majority of participants were white (83.3%), with 1 Asian and 1 black participant (8.3%). Before the start of the study, the participants were required to complete a set of screening questionnaires to assess eligibility and one participant reported to be a vegetarian (8.3%). The BMI of participants ranged from 17.6-25.9 kg/m^2 with 67% in the normal category (<25 kg/m^2). There was no significant change in the participants' BMI from baseline to post-intervention (p=0.078). The baseline physical characteristics of participants are summarised in **Table 3.1**.

Table 3.1 Baseline characteristics of participants (n=12)

Variables	Mean (±S.D)	Range
Age	24.8 ± 6.9	19-37
Height (cm)	166.6 ± 6.5	154.7 – 176.2
Weight (kg)	63.0 ± 10.8	42.2 – 79.2
Body Mass Index (BMI) (kg/m^2)	22.6 ± 2.7	17.6 – 25.9

3.3.2 IRON STATUS

Table 3.2 shows the mean (±S.D) concentrations of all iron status biomarkers measured in the present study. The concentrations of participants' iron status biomarkers remained within the normal range at each points during the 56 days of trial. All participants had full blood count indices, plasma CRP, plasma ferritin, and plasma hepcidin concentrations within the normal ranges. There were no changes observed in participants' plasma CRP and plasma hepcidin concentrations between baseline (Day 0) and post-intervention (Day 56). The majority of the participants had plasma ferritin concentration within the normal range for healthy women, with no elevated plasma ferritin concentration due to acute phase reactions indicated by the plasma CRP measurement. Seven of the 12 participants (60%) were not iron deficient based on plasma ferritin concentration of < 15 µg/l, and only 2 participants were found to be anaemic (haemoglobin concentration < 12 g/dl) at baseline.

However, there was a significantly lower plasma ferritin concentration at post-intervention compared to baseline (p=0.026).

The mean (±S.D) concentrations of all parameters that comprise of FBC indices were within the normal range, apart from MCHC that were significantly lower (p=0.050) at Day 56, and MCV that was significantly higher (p=0.013) at post-intervention (Day 56) compared to baseline (Day 0). Mean (±S.D) plasma hepcidin concentration was similarly within the normal range (96.04 ± 119.58 ng/ml), apart from 3 participants who had plasma hepcidin concentrations of > 150 ng/ml, with the highest being 386 ng/ml at baseline, which explains the high standard deviation. In addition, one participant had a plasma hepcidin concentration below the detectable range of assay kit of <0.06 ng/ml at Day 56. A reference value of between 17-286 ng/ml from a study which reported 5-95% normal range for healthy women, sampled in the USA and Italy (Ganz et al., 2008) was used to compare the concentrations found in the current study, as there are no agreed universal normal threshold values for hepcidin to date.

Table 3.2 Mean (±S.D) concentration of iron status biomarkers (n=12)

Iron status biomarkers	Day 0	Day 56	p-value
Plasma ferritin (ug/l)	38.81 ± 42.71	29.39 ± 33.38	*0.026
Plasma CRP (mg/l)	0.62 ± 0.77	0.40 ± 0.46	0.084
Full blood counts			
- White blood cells (x10^9/l)	4.97 ± 1.12	5.08 ± 1.33	0.789
- Haemoglobin (g/dl)	12.33 ± 1.52	12.48 ± 1.12	0.683
- Haematocrit (%)	35.97 ± 4.49	38.46 ± 4.53	0.107
- Red blood cells (x10^{12}/l)	3.93 ± 0.55	4.10 ± 0.65	0.347
- Mean corpuscular volume (fl)	91.84 ± 6.93	94.23 ± 5.92	*0.013
- Mean corpuscular hb (MCH) (pg)	31.82 ± 4.81	31.03 ± 4.71	0.126
- MCH concentration (g/dl)	34.60 ± 4.63	32.91 ± 4.64	*0.050
- Platelet (x10^9/l)	260.67 ± 4.53	247.83 ± 55.03	0.132
Plasma hepcidin (ng/ml)	96.04 ± 119.58	75.90 ± 96.43	0.099

*Significant between time point (p<0.05)

3.3.3 DIETARY INTAKE

Dietary intake of the participants was measured using; (a) a 24-hour recall to ascertain participants didn't consume any food items containing high concentrations of iron, prior the clinics to attempt and eliminate confounding factors that may affect the proportion of iron absorption in participants; and (b) a 3-day food diary to monitor that participants maintained their usual dietary pattern and to establish their habitual food intake.

Table 3.3 shows the dietary intake of the participants measured using a 3-day food diary, with comparisons to population data reported in the 2008-2012 UK NDNS. There was no difference observed at baseline and post-intervention in the mean daily intake (±S.D) of energy, protein, fat, carbohydrate, vitamin C and calcium. Mean (±S.D) iron intake, however, was found to be significantly higher (8.4 ± 1.8 mg) at post-intervention, compared to baseline (6.7 ± 1.5 mg) ($p=0.035$). Analysis from the 24-hour recall showed that most of the foods consumed by the participants that contributed to the dietary iron intake of participants were from a haem source, (e.g. pork, beef, lamb and chicken) and the non-haem sources came generally from fortified food items (e.g. bread and breakfast cereals). This is consistent with figures reported in NDNS which showed that 38% of dietary iron intake of adults was from cereal/cereal products, followed by 19% from meat & meat products and 17% from vegetables and potatoes. Mean (±S.D) daily energy intake and macronutrient intake of participants were also comparable to the population data reported in the NDNS, despite the slightly lower intakes of iron, vitamin C, and calcium, which may be due to the low number of participants recruited in the present study compared to larger number in the national survey. The dietary analysis from the 24-hour recall from each clinic revealed no unusually high intake of iron, calcium or vitamin C, which may have confounded participants' iron absorption.

The participants' estimated dietary intakes were compared to Dietary Reference Values (DRV) recommended by the SACN, UK (**Table 3.4)**. Participant's energy intake was approximately 70% of the RNI, and 50% of the RNI for iron. No participant met the requirement of 14.8 mg of iron/day, indicating a poor dietary iron intake despite generally not being iron deficient or anaemic. Vitamin C, an enhancer of iron absorption, intake was 5-fold higher than the RNI. It was reported in the NDNS that women aged between 19-64 years consumed 204% (vitamin C) and 78% (iron) of the RNI.

Table 3.3 Estimated dietary intake of participants (n=12) at baseline and post-intervention

Nutrient	Day 0	Day 56	p-value	[a]2008-12 NDNS
	Mean ± S.D	Mean ± S.D		Mean (±S.D)
Energy (MJ)	6.746 ± 1.914	6.984 ± 1.670	0.523	6.78 ± 1.91
Energy (kcal)	1612 ± 458	1669 ± 399	0.523	1613 ± 455
Protein (g)	66.1 ± 22.9	67.0 ± 17.0	0.903	65.4 ± 18.3
Fat (g)	66.8 ± 32.1	62.4 ± 14.7	0.641	60.1 ± 22.7
Carbohydrate (g)	190.8 ± 47.1	220.5 ± 92.2	0.347	197 ± 61
Iron (mg)	6.7 ± 1.5	8.4 ± 1.8	*0.035	9.6 ± 3.0
Vitamin C (mg)	76.7 ± 68.3	77.5 ± 43.3	0.875	81.6 ± 59.8
Calcium (mg)	581.0 ± 186.2	610.7 ± 243.6	0.701	728 ± 260

[a]Bates et al. (2014)

Table 3.4 Comparison of participants' dietary intake to UK DRV

Nutrient	RNI (SACN 2015)	Day 0	Day 56
		[a]Percentage < RNI	
Energy (MJ/kcal)	9.1/2175 (19-34 years) 8.8/2103 (35-54 years)	92	92
Protein (g)	0.75 per kg	17	0
Fat (g)	<35%	42	58
Carbohydrate (g)	50%	50	50
Iron (mg)	14.8	100	100
Vitamin C (mg)	40	50	17
Calcium (mg)	700	75	67

[a]Percentage < RNI = Proportion of participants with mean daily intakes below the RNI

Figure 3.1 shows participants' dietary intakes of this study participant' compared to the RNI. Only protein and vitamin C exceeds the RNIs of women aged 19-50 years.

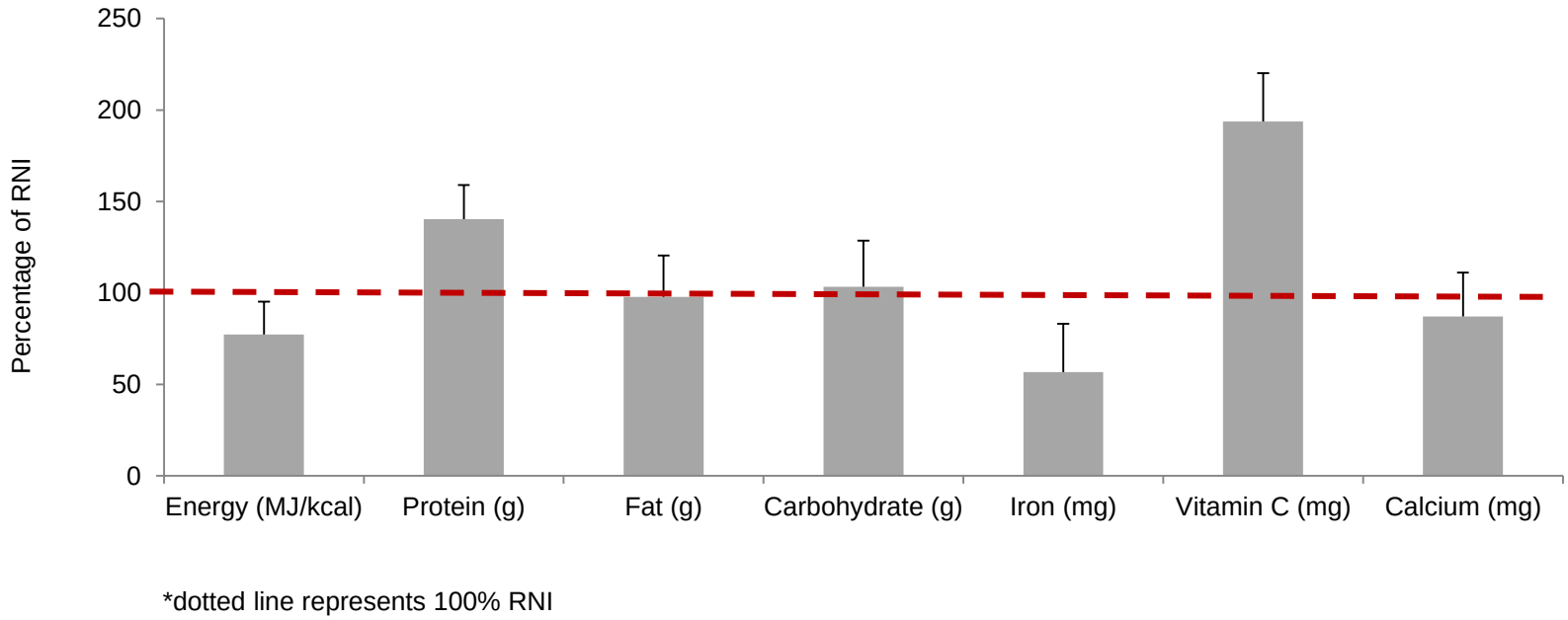


Figure 3.1 Participants' percentage mean daily intake of energy, protein, fat, carbohydrate, iron, vitamin C and calcium compared to UK DRVs

3.3.4 IRON ABSORPTION

Iron absorption in the current study was estimated using a standardised test meal, which was administered successively at 14-day intervals with either water (TM I), simultaneously with tea (TM II) and tea administered 1 hour post-test meal (TM III). Each test meal was extrinsically labelled with 4 mg $^{57}FeSO_4$, except for the reference iron dose which was labelled with 3 mg $^{57}FeSO_4$ and 35 mg of ascorbic acid. TM I (water) served as a reference meal and was used as the basis for comparison, to calculate the iron absorption ratio and inhibition effect of tea (%). **Table 3.5** shows (a) the fractional iron absorption percentage, normalised to the reference iron dose to correct inter-variability between iron status backgrounds of participants and (b) total iron absorbed in mg, without normalisation to the reference iron dose.

Table 3.5 Mean (±S.D) of iron absorption from test meals with specified beverages

	Test meals		
	Test meal I (with water)	Test meal II (with tea simultaneously)	Test meal III (with tea 1 hour post-meal)
Fractional iron absorption (%)	5.69 ± 8.5	3.57 ± 4.2	*5.73 ± 5.4
Iron absorption ratio		0.65 ± 0.67	1.18 ± 1.19
Total iron absorbed (mg)	0.226 ± 0.51	0.142 ± 0.26	*0.185 ± 0.28

*significantly higher than TM II ($p<0.05$)

3.3.4.1 Iron absorption from test meals

It was observed that mean (±S.D) iron absorption, both in terms of fractional absorption (%) or total absorbed (mg) was higher in TM I (meal + water) and TM III (meal + tea + 1 hour) compared to TM II (meal + tea), as anticipated. A marked reduction of 2.1% was observed in mean iron absorption when the test meal was administered simultaneously with tea (TM II), compared to the control test meal administered with water (TM I). However, the reduction was not statistically significant ($p=0.398$). Iron absorption was approximately 2.2% higher when tea was administered 1 hour (TM III) after the participants were administered their test meal ($p=0.046$), compared to when test meal was administered simultaneously with tea (TM II). Consistent with the percentage iron absorbed, the mean total iron absorbed (mg) was also significantly higher (0.043 mg) in TM III (meal + tea + 1 hour)

compared to TM II (meal + tea) (p=0.049). There was no significant difference between TM I (meal + water) and TM II (meal + tea) with regards to the total iron absorbed (p=0.530). No differences were observed between TM I (meal + water) and TM III (meal + tea + 1 hour) for both fractional iron absorption (p=0.335) and total iron absorbed (p=0.304). **Figure 3.2 (A)** shows the fractional iron absorption (%) and **(B)** shows the total amount of iron absorbed from all test meals including reference iron dose.

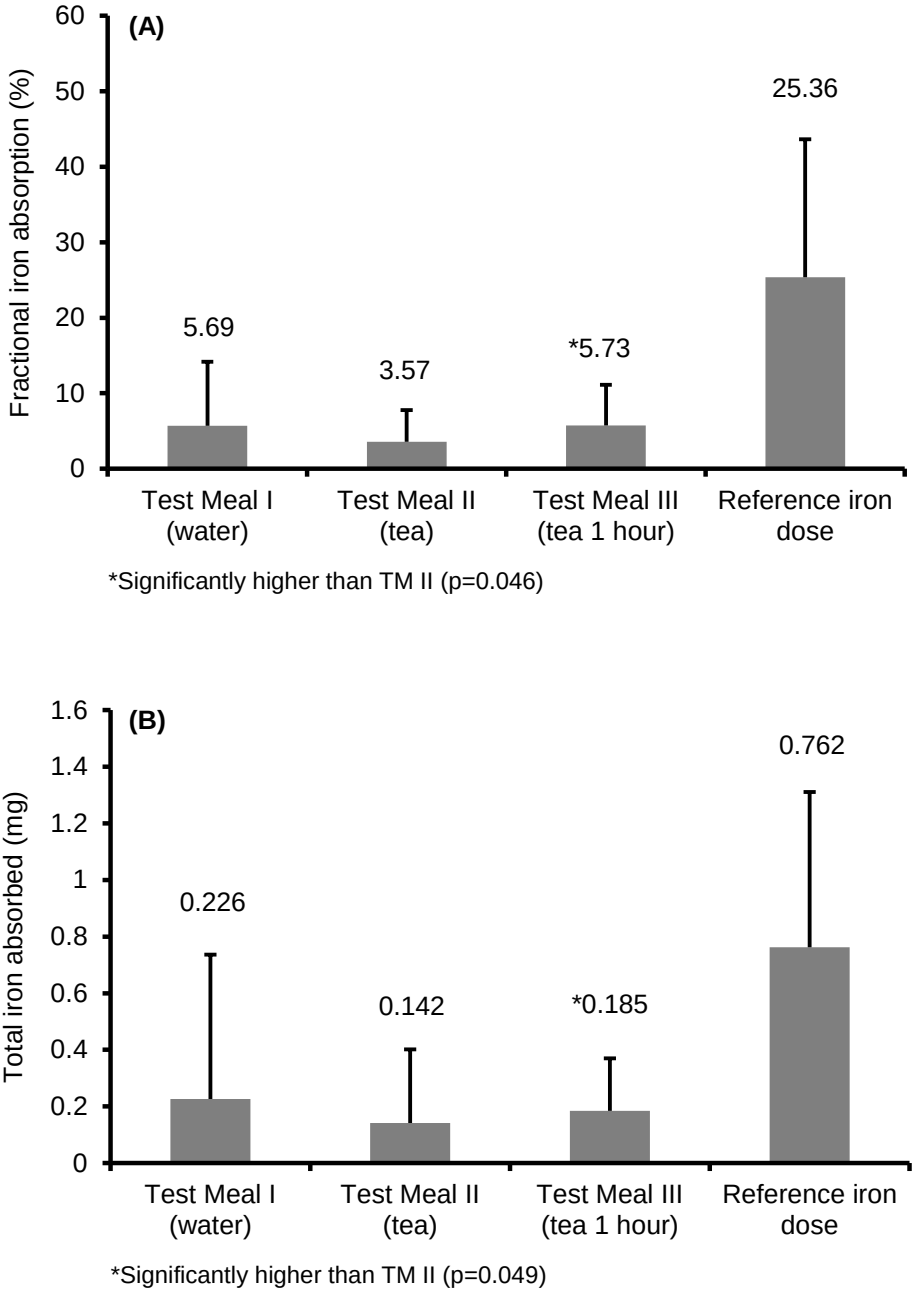


Figure 3.2 Iron absorption from test meals (A) fractional iron absorption (%) (B) total iron absorbed (mg)

Table 3.6 shows the participants' iron absorption, demonstrating individual variations. Due to variation in iron status of participants, which affects the iron absorption, the reported fractional iron absorption in this study has been normalised using a reference iron dose. The use of this reference dose is to account for inter-variability between participants, and enable the findings to be compared against the findings in previous studies. There was a wide range of percentage absorption, varying from undetectable (0%) to 31.1%, accounting for the large standard deviation.

Table 3.6 Individual iron absorption and absorption ratio compared to reference meal (water)

Participant	TM I	TM II		TM III		Reference iron dose
	%	%	*Ratio	%	*Ratio	%
1	31.1	15.5	0.5	17.0	0.6	58.8
2	5.0	2.9	0.6	2.2	0.4	41.2
3	0	3.0	0	0	0	6.5
4	0	0	0	0	0	10.4
5	2.4	4.6	1.9	5.9	2.4	23.0
6	8.7	2.1	0.2	7.6	0.9	12.6
7	4.5	2.6	0.6	6.7	1.5	9.9
8	3.6	5.5	1.6	4.9	1.4	55.1
9	1.5	1.5	1.0	3.3	2.3	36.9
10	7.9	0	0	6.5	0.8	16.6
11	0	0	0	0.5	0	10.8
12	3.7	5.2	1.4	14.3	3.9	22.4

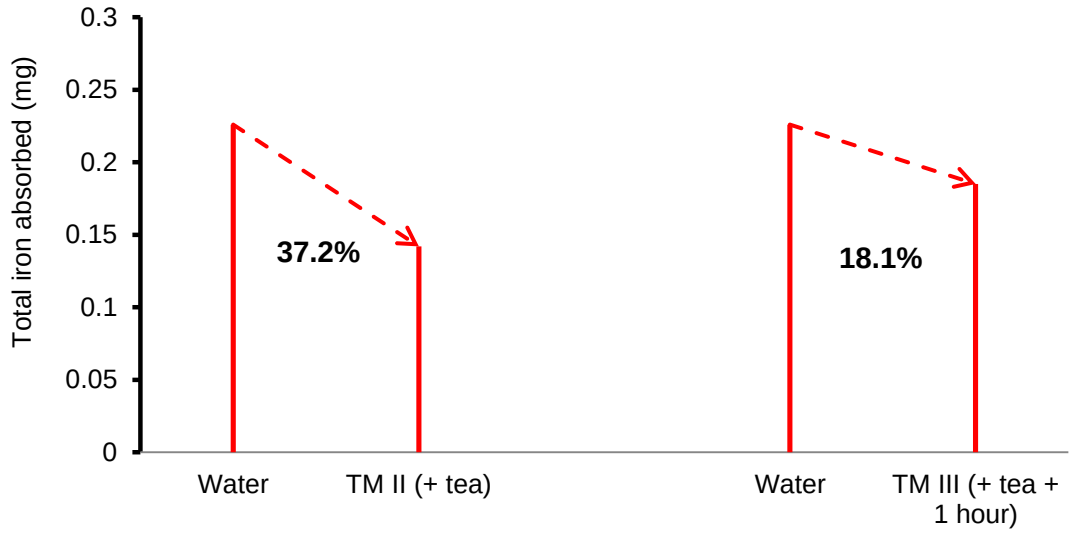
*Absorption ratio of less than 1 implied inhibition effect of tea, computed in relative to TM I (water) as reference test meal

A number of participants (n=4) were found to have not absorbed any iron for all the test meals except the reference iron dose. It was noted that their iron absorption from the reference iron dose were lower compared to others, indicating that they

were not iron deficient and had low iron demand physiologically. It was also observed that five participants had reduced iron absorption when the test meal was administered with tea, compared to when it was administered with water, however, the reduction was not significant. Eight participants had an increase in iron absorption when the tea was administered 1 hour after the test meal, as compared to when it was administered simultaneously with the meal. Based on the ratio data, an inhibition effect of tea when administered TM II (meal + tea) was observed in 8 participants, compared to smaller number (n=7) when administered TM III (meal + tea + 1 hour). This shows that the inhibition effect is reduced with a 1-hour time interval between administration of test meal and tea consumption.

3.3.4.2 Time interval effect of tea consumption on iron absorption

Inhibition effects (%) were calculated based on the mean total iron absorbed from the test meals administered with tea (TM II and TM III), in comparison to the test meal with water as a reference meal (TM I). This is to illustrate the inhibition effect related to the time intervals used, between administering the tea simultaneously with the meal, or a 1-hour time interval between the meal and the tea. A 2-fold reduction in inhibition effect was observed, from 37.2% in TM II (meal + tea) to 18.1% in TM III (meal + tea + 1 hour), indicating that allowing at least a 1-hour time interval between a meal and tea consumption will lead to increased iron absorption. **Figure 3.3** shows the impact of a 1-hour time interval on the inhibition effects of tea on iron absorption.



*dotted arrow indicates reduction in total iron absorbed from each test meal

Figure 3.3 Inhibition effects of TM II and TM III compared to reference meal

3.3.5 ASSOCIATION BETWEEN IRON ABSORPTION AND IRON STATUS BIOMARKERS (PLASMA FERRITIN AND HEPCIDIN CONCENTRATIONS)

Plasma ferritin concentration is the amount of total iron storage in body and it has been shown to be well correlated with iron absorption in previous studies. It was observed in the present study that there were inverse association between plasma ferritin concentration with both fractional iron absorption and total iron absorbed (mg) in the study **(Figure 3.4)**. It was found that there were low and modest, but significant inverse associations between plasma ferritin concentrations and iron absorption (r=-0.330, p=0.043) and total iron absorbed (r=-0.486, p=0.005), respectively.

Plasma hepcidin concentrations were measured to investigate the role of this peptide hormone in regulating iron absorption. Similar to the observation in plasma ferritin concentration, plasma hepcidin concentration was found to be moderate and significantly associated with both, fractional iron absorption (r=-0.475, p=0.007) and with total iron absorbed (r=-0.560, p=0.004) **(Figure 3.5)**.

A significant linear association between plasma ferritin and plasma hepcidin concentration (p=0.885, p=0.0001) was observed which indicates a very high association between these two key iron biomarkers that have a substantial role in the regulation of iron absorption **(Figure 3.6)**.

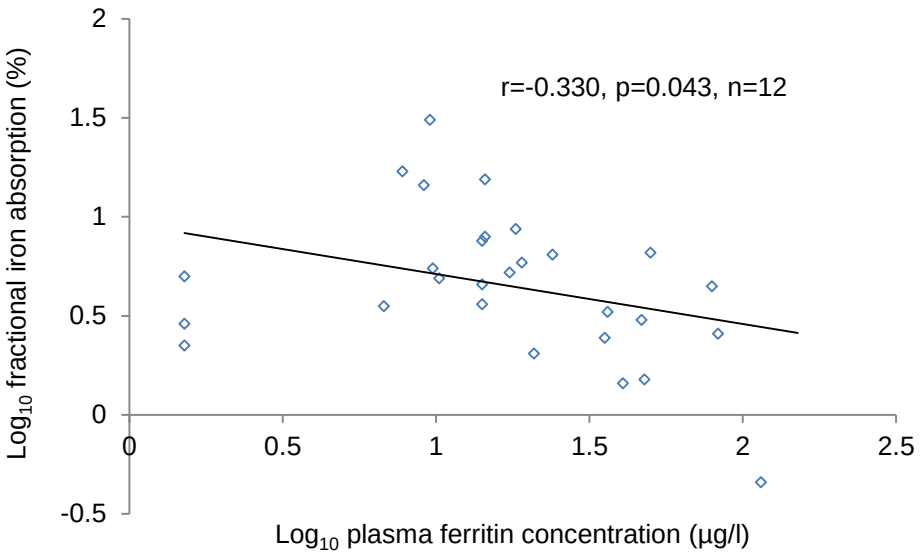


Figure 3.4 Association between plasma ferritin concentration & iron absorption

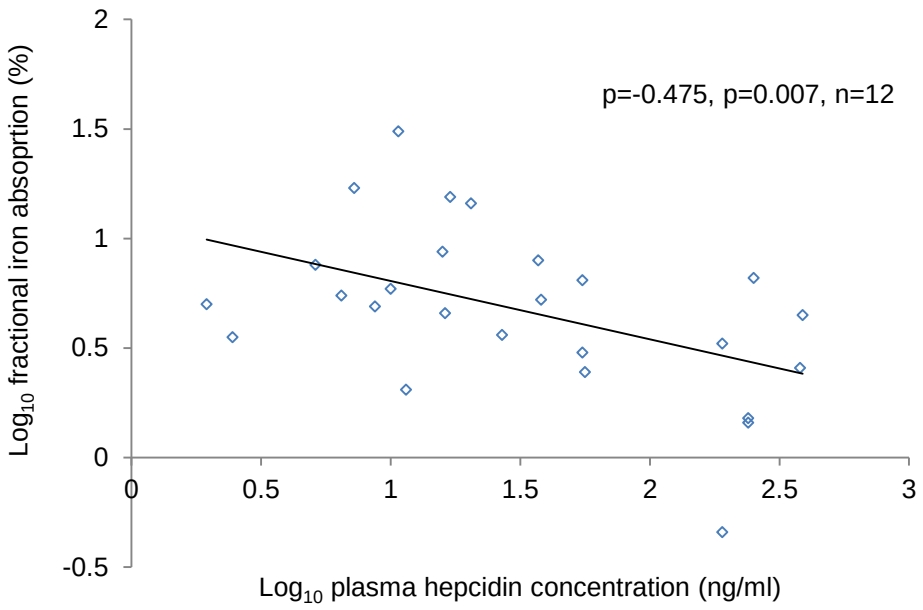


Figure 3.5 Association between plasma hepcidin concentration & iron absorption

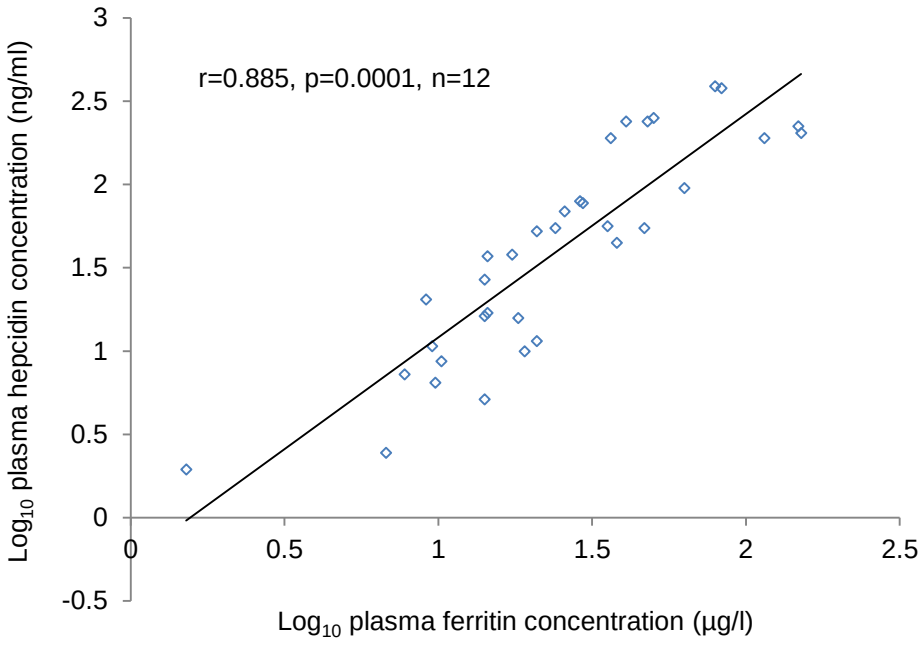


Figure 3.6 Association between plasma ferritin concentration (µg/l) and plasma hepcidin concentration (ng/ml)

A steeper slope is observed with TM II (meal + tea) (B) compared to TM I (water) (A) when comparing log iron absorption (%) against log ferritin concentrations. The steepest slope remained in TM II (B) when it was compared against TM III (meal + tea) (C), suggesting that higher inhibition effect is shown is B, when tea is consumed with test meal **(Figure 3.7)**.

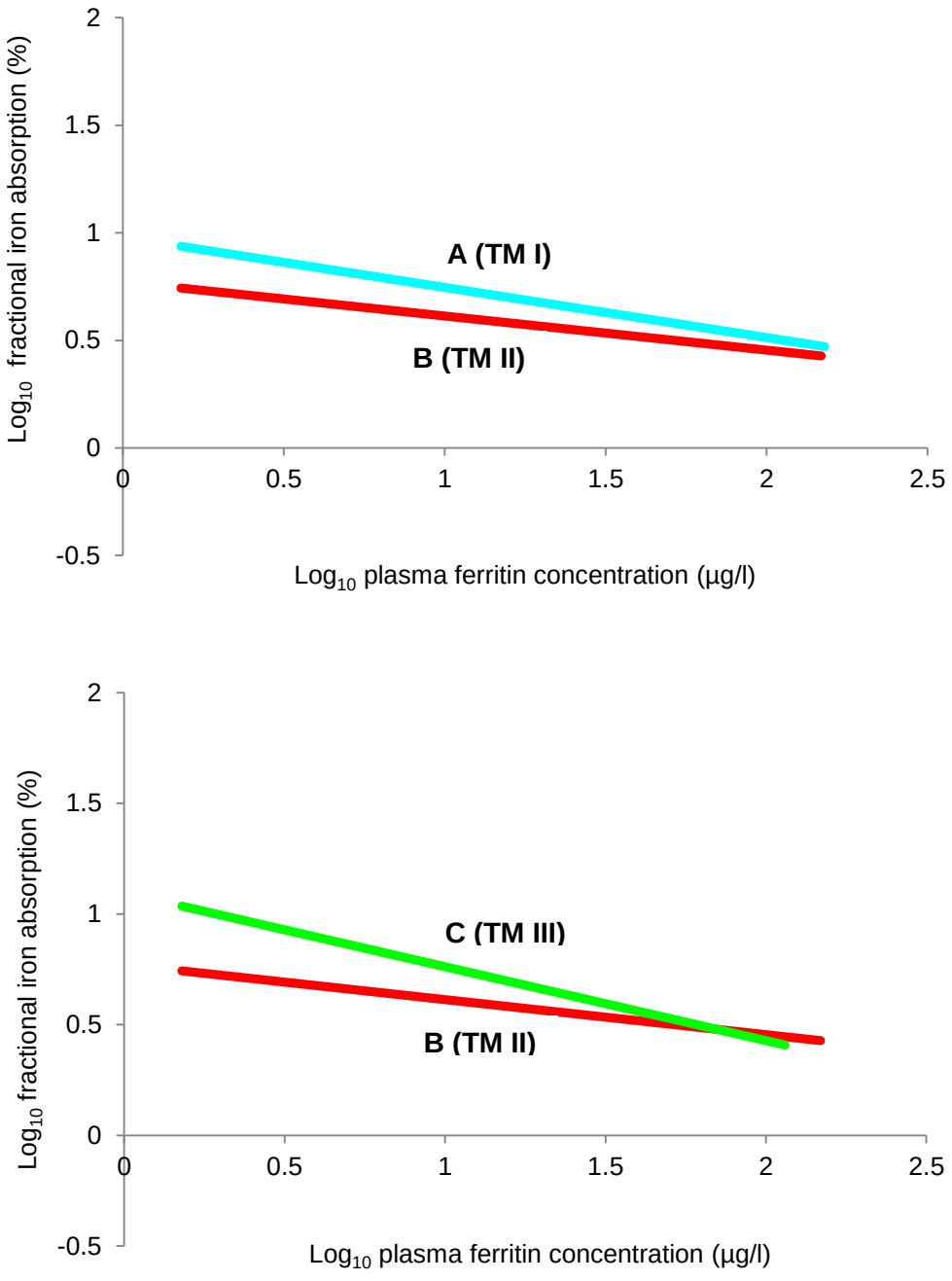


Figure 3.7 Linear association comparing log_{10} (fractional iron absorption) and log_{10} (plasma ferritin concentrations) in different test meals

CHAPTER 4

A double-blind randomised controlled trial investigating the effect of vitamin D3 supplementation on iron absorption and hepcidin response following the consumption of an iron-fortified breakfast cereal, in iron deficient women.

4.1 OBJECTIVES

4.1.1 General objective

To investigate the effect of an 8-week daily dose of vitamin D3 (1500 IU) consumed with 60 g of commercially available iron-fortified breakfast cereal containing 9 mg of iron, on haematological indicators in a cohort of marginally and iron deficient childbearing-aged UK women.

4.1.2 Specific objectives

- To examine the effect of vitamin D3 supplementation, consumed with the iron-fortified breakfast cereals on the recovery of iron status in women with marginal and low iron stores
- To assess the effectiveness of iron-fortified breakfast cereals consumption, as part of regular diet on the recovery of iron status in women with marginal and low iron stores
- To investigate the effect of vitamin D3 on plasma hepcidin concentration, to have better understanding of the potential role of vitamin D as an iron absorption enhancer

4.2 HYPOTHESES

H_1: There will be a significant improvement in haematological indicators following 8 weeks vitamin D3 supplementation consumed with iron-fortified breakfast cereals in the vitamin D group compared to placebo group.
H_0: There will be no significant improvement in haematological indicators following 8 weeks vitamin D3 supplementation consumed with iron-fortified breakfast cereals in the vitamin D group compared to placebo group.

H_1: There will be a significant improvement in haematological indicators following 8 weeks consumption of iron-fortified breakfast cereals in both groups.
H_0: There will be no significant improvement in haematological indicators following 8 weeks consumption of iron-fortified breakfast cereals in both groups.

H_1: Plasma hepcidin concentration will be reduced following 8 weeks vitamin D3 supplementation, and results in increased iron stores.
H_0: Plasma hepcidin concentration will not be reduced following 8 weeks vitamin D3 supplementation, and did not result in increased iron stores.

4.3 RESULTS

4.3.1 BASELINE CHARACTERISTICS OF PARTICIPANTS

A total of 50 women initially commenced the study, and 44 women completed the whole study at 8 weeks. Four participants dropped out after the first clinic due to feeling unwell (n=2) or without reason and did not respond to emails (n=2). The remaining two participants dropped out after interim clinics due to falling sick. The baseline characteristics are reported based on the data available for the total of 50 participants which included the drop-outs. **Figure 4.1** summarises the allocation of participants throughout the study. From the screening questionnaire, 11 participants considered themselves to be a vegetarian (22%). The participants recruited were aged between 19-49 years, with a mean (±S.D) age of 27.4 ± 9.4 years. More than half of the participants (56%) were in the 19-24 years age group, followed by 20% in the 25-34 years age group and the remaining 24% were in the 35-54 years age group. The majority of participants were white (80%), followed by Asian (12%) and others (8%).

Two blood biomarkers (plasma ferritin and 25(OH)D concentrations) were measured at the screening clinic to assess eligibility of the participants. The mean (±S.D) plasma ferritin concentration was 11.5 ± 5.6 µg/l and 25(OH)D concentration was 38.28 ± 21.40 nmol/l. This indicates that the participants were iron deficient (plasma ferritin < 15 µg/l) and vitamin D inadequate (plasma 25(OH)D between 30-50 nmol/l) at screening and eligible for the study. The mean (±S.D) plasma 25(OH)D concentration did not change from screening to when eligible participants commenced the study at baseline (p=0.205). On the contrary, it was observed that there was a significantly higher mean (±S.D) of plasma ferritin concentration at baseline (13.2 ± 7.8 µg/l) compared to during screening (11.5 ± 5.6 µg/l) (p=0.012).

The baseline characteristics of participants are summarised in **Table 4.1**. There were no significant differences in the participants' baseline physical characteristics (height, weight and BMI) between the vitamin D group and the placebo group. It was also observed that there were no significant differences in participants' iron and vitamin D status (haemoglobin, plasma ferritin, hepcidin and 25(OH)D concentrations) between the vitamin D group and the placebo group (p>0.05). In reference to dietary intake, there were no significant differences in nutrients (energy, protein, carbohydrate, fats, iron and vitamin D) between the vitamin D group and the placebo group, except for carbohydrate (% of energy).

Excluding drop-outs (n=44), there were no significant difference in physical characteristics, iron and vitamin D status, and dietary intakes at baseline between the two groups.

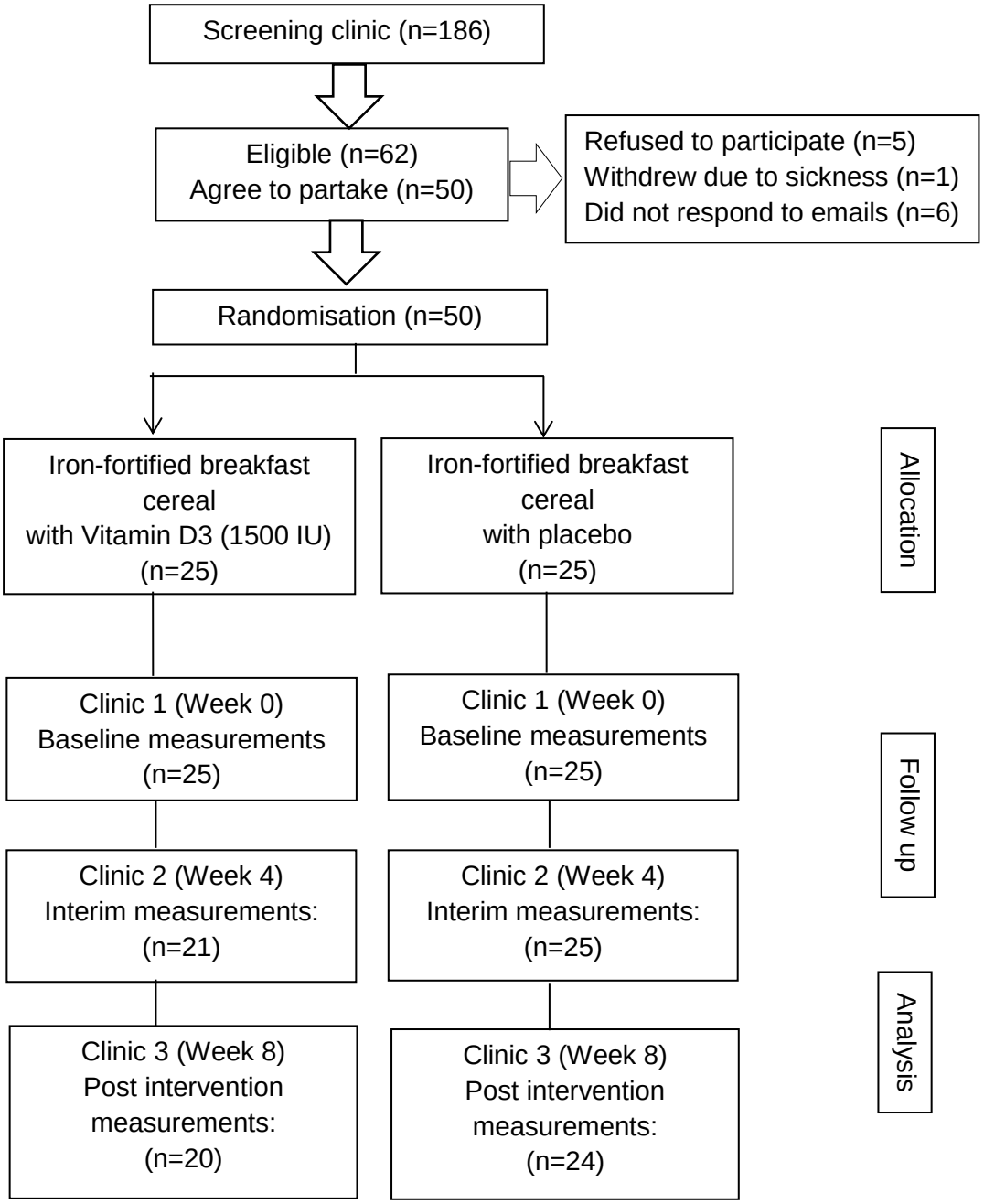


Figure 4.1 Allocation of participants throughout the 8-week intervention period

Table 4.1 Participant baseline physical characteristics, iron & vitamin D status and dietary intake (n=50)

Variables	All (n=50)	Vitamin D group (n=25)	Placebo group (n=25)	p-value
Physical characteristics				
Age (years)	27.4 ± 9.4	28.0 ± 9.0	26.9 ± 9.9	0.280
Height (cm)	165.6 ± 5.8	165.2 ± 6.4	166.0 ± 5.3	0.623
Weight (kg)	67.2 ± 14.8	68.3 ± 17.9	66.1 ± 11.1	0.930
BMI (kg/m^2)	24.4 ± 4.8	24.9 ± 5.7	24.0 ± 3.7	0.930
Iron & vitamin D status				
Haemoglobin (g/dl)	13.4 ± 1.4	13.6 ± 1.2	13.2 ± 1.5	0.210
Plasma ferritin (µg/l)	13.2 ± 7.8	14.1 ± 7.7	12.4 ± 8.0	0.467
Plasma hepcidin (ng/ml)	3.5 ± 4.3	4.2 ± 5.7	2.9 ± 2.2	0.968
Plasma 25(OH)D (nmol/l)	36.8 ± 23.6	35.0 ± 19.8	38.6 ± 27.2	0.992
Mean daily dietary intake				
Energy (kcal)	1671.3 ± 376.9	1722.5 ± 314.9	1628.3 ± 423.7	0.447
Energy (MJ)	6.9 ± 1.6	7.2 ± 1.3	6.8 ± 1.8	0.404
Protein (g)	70.0 ± 16.4	74.7 ± 16.4	66.1 ± 15.6	0.155
Carbohydrate (g)	224.2 ± 46.3	220.4 ± 30.6	227.4 ± 56.7	0.618
Carbohydrate (%)	51.8 ± 6.3	49.5 ± 6.3	53.6 ± 5.7	0.025
Fats (g)	57.5 ± 22.2	61.3 ± 23.7	54.2 ± 20.7	0.337
Fats (%)	30.6 ± 7.0	31.1 ± 6.8	30.1 ± 7.2	0.626
Iron (mg)	16.4 ± 2.3	16.5 ± 1.8	16.4 ± 2.6	0.108
Vitamin D (µg)	1.7 ± 1.6	1.6 ± 1.2	1.7 ± 1.9	0.604

All values are mean (±S.D) except for for carbohydrate & fats with additional % of total energy intake

p-value of > 0.05 indicates no significant difference at baseline between the 2 groups

4.3.1.1 Prevalence of anaemia, iron deficiency and vitamin D deficiency at baseline

Different thresholds were used to classify anaemia (haemoglobin < 11 g/dl), iron deficiency (plasma ferritin < 15 µg/l) and vitamin D deficiency (plasma 25(OH)D < 30 nmol/l). Prevalence of anaemia, iron deficiency and vitamin D deficiency at baseline were 12% (n=6), 64% (n=32) and 62% (n=31). **Figure 4.2** shows anaemia and iron deficiency proportions in participants based on the vitamin D status, at baseline.

It can be observed that 13% of the vitamin D deficient (VDD) participants were also anaemic and higher proportion of them was iron deficient (ID) (61%). This indicates the possible association between iron status and vitamin D status, which has been shown in previous observational studies.

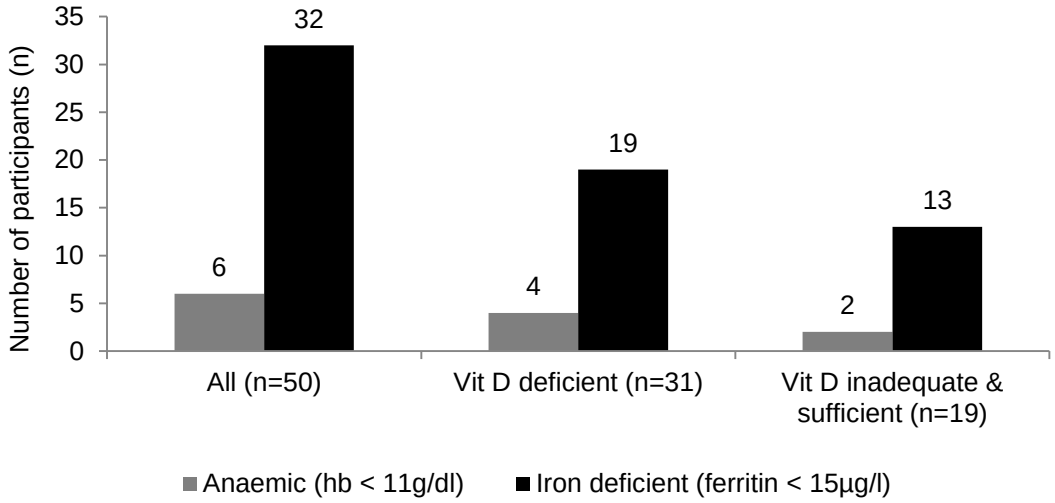


Figure 4.2 Anaemia and iron deficiency prevalence at baseline stratified by vitamin D status

4.3.2 COMPLIANCE & ADVERSE EVENTS OF VITAMIN D SUPPLEMENTATION

Compliance was measured based on the number of capsules remaining in the supplement pot returned by the participants at the final clinic. Overall mean (±S.D) compliance was 92.9 ± 8.0%, which indicates good compliance of the supplementation. Compliance was similar in both groups with 93.2% in vitamin D group compared to 92.6% in placebo group. No participants reported any adverse events associated with the consumption of supplements.

The consumption of iron-fortified breakfast cereal was examined using the diary record by the participants at baseline and before study completion. All participants recorded consumption of the supplied iron-fortified cereal at breakfast every day in both diaries. There were also no adverse events reported by the participants connected to cereal consumption.

4.3.3 EFFECT OF VITAMIN D SUPPLEMENTATION ON IRON STATUS

4.3.3.1 Improvement in iron biomarkers from baseline to post-intervention following vitamin D intervention

Table 4.2 shows the effect of intervention on iron status biomarkers. There was a significant increase in the two main iron status biomarkers: haemoglobin concentration and haematocrit % from baseline (Week 0) to post-intervention (Week 8) in the vitamin D group, compared to the placebo group. **Figure 4.3** shows the increase in haemoglobin concentration (0.36 g/dl) in the vitamin D group was significantly higher from the increase in the placebo group (-0.28 g/dl) (p=0.037). The increase was also significantly higher in haematocrit % in the vitamin D group (1.82%) compared to the placebo group (-0.51%) (p=0.032).

In the vitamin D group, consumption of vitamin D3 supplements with the iron fortified cereals however did not have impact on the change in other main iron status biomarkers including RBC, MCV, MCH, MCHC, plasma ferritin, plasma hepcidin and plasma sTfR, compared to the placebo group.

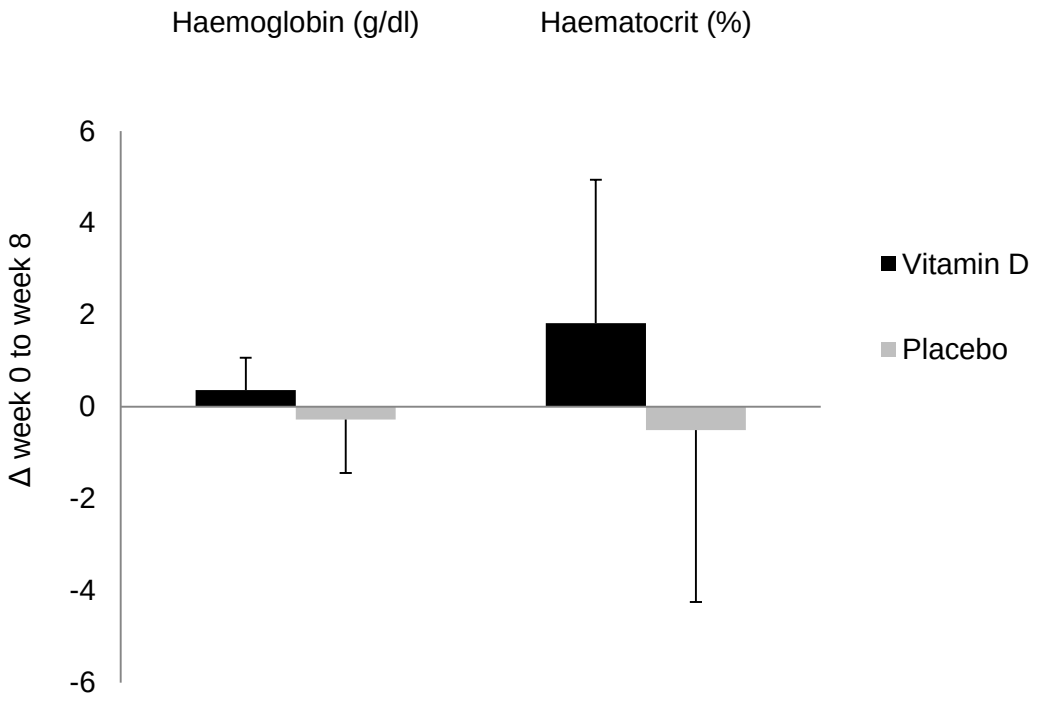


Figure 4.3 Effect of intervention on iron status biomarkers from baseline to post-intervention

Table 4.2 Effect of intervention on iron status biomarkers from baseline to post-intervention (n=44)

Biomarkers	Vitamin D group			Placebo group			p-value
	Baseline	Post-intervention	Change	Baseline	Post-intervention	Change	
Hb (g/dl)	13.48 ± 1.08	13.84 ± 0.98	0.36 ± 0.71	13.11 ± 1.53	12.83 ± 1.25	-0.28 ± 1.16	*0.037
Hct (%)	42.01 ± 2.95	43.83 ± 3.41	1.82 ± 3.12	41.16 ± 4.29	40.65 ± 3.56	-0.51 ± 3.74	*0.032
RBC ($x10^{12}$/l)	4.61 ± 0.25	4.77 ± 0.27	0.16 ± 0.24	4.60 ± 0.44	4.58 ± 0.33	-0.03 ± 0.37	0.055
MCV (fl)	91.25 ± 5.05	91.93 ± 5.70	0.68 ± 3.32	89.65 ± 7.39	89.03 ± 7.84	-0.61 ± 3.11	0.117
MCH (pg)	29.30 ± 2.00	29.06 ± 1.92	-0.24 ± 0.56	28.57 ± 2.96	28.13 ± 3.00	-0.44 ± 0.73	0.328
MCHC (g/dl)	32.10 ± 0.73	31.59 ± 0.98	-0.51 ± 0.96	31.82 ± 1.08	31.54 ± 1.14	-0.28 ± 0.91	0.425
Fer (µg/l)	14.08 ± 8.16	15.95 ± 10.80	1.87 ± 11.14	12.32 ± 8.12	13.76 ± 13.28	1.46 ± 9.14	0.540
Hep (ng/ml)	3.67 ± 4.92	4.12 ± 4.08	0.46 ± 5.79	2.87 ± 2.23	3.44 ± 5.57	0.57 ± 4.52	0.451
sTfR (mg/l)	1.61 ± 0.72	1.75 ± 0.96	0.15 ± 0.33	1.91 ± 1.17	2.01 ± 1.39	0.10 ± 0.29	0.724

All values are means (±S.D)

Changes are from baseline to post-intervention (week 0-week 8)

P-value represents the difference in changes observed between vitamin D and placebo groups, *= $p \leq 0.05$

Hb: haemoglobin; Hct: haematocrit; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; Fer: ferritin; Hep: hepcidin; sTfR: soluble transferrin receptor

4.3.3.2 Time and group interaction

No participants had elevated plasma CRP concentrations (<10 mg/l) at all time points. The mean (±S.D) plasma CRP concentration was below than 4 mg/l in both groups, at all time points. **Table 4.3** shows the mean (±S.D) concentrations of participants' iron status biomarkers at baseline, interim and post-intervention. All full blood count parameters measured (haemoglobin, haematocrit, RBC, MCV, MCH and MCHC) were within the normal range, in both groups, at all time points.

No significant difference was observed between the vitamin D group and the placebo group in plasma ferritin, plasma hepcidin, and plasma sTfR concentrations. No significant difference was also observed between the two groups in the principal iron status biomarkers, haemoglobin concentrations. There was no significant difference in all the other full blood count parameters including the haematocrit, RBC, MCV, MCH and MCHC.

3.3.3.3 Time effect within groups

In the vitamin D group, the mean (±S.D) haemoglobin concentration was significantly higher at post-intervention (13.84 ± 0.98 g/dl) compared to baseline (13.60 ± 1.15 g/dl) (p=0.035). No significant difference in haemoglobin concentration was observed within the placebo group, between each time point. In the vitamin D group, mean (±S.D) haematocrit % was significantly higher at post-intervention (43.83 ± 3.41%) compared to both; baseline (42.51 ± 3.21%, p=0.017) and 4-weeks (42.65 ± 3.15%, p=0.044). No significant difference in haematocrit % was observed within the placebo group, between each time point.

In the vitamin D group, mean (±S.D) RBC count was significantly higher at post-intervention (4.77 ± 0.27 $x10^{12}$/l) compared to baseline (4.63 ± 0.33 $x10^{12}$/l) (p=0.007). No significant difference in RBC count was observed within the placebo group, between each time point. In the vitamin D group, mean (±S.D) MCHC was significantly lower at post-intervention (31.59 ± 0.98 g/dl) compared to both baseline (32.00 ± 0.75 g/dl, p=0.028) and 4-weeks (32.04 ± 0.78, p=0.032). No significant difference in MCHC was observed within the placebo group, between each time point.

Difference within groups in MCH was observed in both groups. In the vitamin D group, mean (±S.D) MCH was significantly lower at post-intervention (29.06 ± 1.92 pg) compared to 4-weeks (29.27 ± 1.78 pg) (p=0.017). In the placebo group, mean (±S.D) MCH was significantly lower at post-intervention (28.13 ± 3.00 pg) compared

to both baseline (28.72 ± 3.00 pg, p=0.008) and 4-weeks (28.68 ± 2.99 pg, p=0.0001).

No significant difference was observed within both groups in MCV, plasma ferritin, plasma hepcidin and plasma sTfR concentrations, between each time point.

However, a trend of increased plasma ferritin concentration was observed within the vitamin D group, as mean (±S.D) plasma ferritin concentration was not within the normal range at baseline (14.07 ± 7.68 µg/l) and 4-weeks (13.85 ± 9.62 µg/l), but increased to normal range (15.95 ± 10.80 µg/l) at post-intervention. The similar non-significant increased trend was also observed in the placebo group, however, participants in the placebo group remained iron deficient (based on WHO threshold of <15 µg/l) at post intervention, indicated by the mean (±S.D) plasma ferritin concentration of 13.76 ± 13.28 µg/l.

A trend of reduced plasma hepcidin concentration was observed within the vitamin D group, as the mean (±S.D) reduced at post-intervention (4.12 ± 4.08 ng/ml), compared to baseline (4.20 ± 5.65 ng/ml) and 4-weeks (2.93 ± 2.63 ng/ml). In the placebo group, an increased non-significant trend was observed in plasma hepcidin concentration.

An increase was observed in plasma sTfR concentration in both groups (trend), but did not reached the normal range at post intervention, compared to baseline or interim time points.

Table 4.3 Effect of vitamin D supplementation on iron status biomarkers at 3 time points over 8-weeks intervention period

	Groups	Baseline (Week 0) (n=50)	Interim (Week 4) (n=46)	Post-intervention (Week 8) (n=44)	Time (p-value)	Time x Group (p-value)
Haemoglobin (g/dl)	Vitamin D	13.60 ± 1.15[a]	13.64 ± 1.08	13.84 ± 0.98[b]	<0.05	*0.037
	Placebo	13.16 ± 1.52	13.10 ± 1.12	12.83 ± 1.25	NS	
Haematocrit (%)	Vitamin D	42.51 ± 3.21[a]	42.65 ± 3.15[a]	43.83 ± 3.41[b]	<0.05	*0.032
	Placebo	41.27 ± 4.23	41.22 ± 2.98	40.65 ± 3.56	NS	
RBC (x10^{12}/l)	Vitamin D	4.63 ± 0.33[a]	4.67 ± 0.25	4.77 ± 0.27[b]	<0.001	0.055
	Placebo	4.60 ± 0.43	4.58 ± 0.33	4.58 ± 0.33	NS	
MCV (fl)	Vitamin D	91.96 ± 5.77	91.31 ± 4.26	91.93 ± 5.70	NS	0.190
	Placebo	90.02 ± 7.47	90.23 ± 7.81	89.03 ± 7.84	NS	
MCH (pg)	Vitamin D	29.42 ± 1.95	29.27 ± 1.78[a]	29.06 ± 1.92[b]	<0.05	0.328
	Placebo	28.72 ± 3.00[a]	28.68 ± 2.99[a]	28.13 ± 3.00[b]	<0.0001	
MCHC (g/dl)	Vitamin D	32.00 ± 0.75[a]	32.04 ± 0.78[a]	31.59 ± 0.98[b]	<0.05	0.425
	Placebo	31.86 ± 1.07	31.73 ± 0.99	31.54 ± 1.14	NS	
Plasma ferritin (µg/l)	Vitamin D	14.07 ± 7.68	13.85 ± 9.62	15.95 ± 10.80	NS	0.890
	Placebo	12.39 ± 7.96	12.64 ± 8.82	13.76 ± 13.28	NS	
Plasma hepcidin (ng/ml)	Vitamin D	4.20 ± 5.65	2.93 ± 2.63	4.12 ± 4.08	NS	0.941
	Placebo	2.93 ± 2.20	3.11 ± 2.78	3.44 ± 5.57	NS	
Plasma sTfR (mg/l)	Vitamin D	1.52 ± 0.67	1.68 ± 0.88	1.75 ± 0.96	NS	0.625
	Placebo	1.87 ± 1.16	1.89 ± 1.34	2.01 ± 1.39	NS	

Values are significantly different at specified time points if not sharing a common letter in the same row, *= p ≤ 0.05

4.3.3.4 Effect of vitamin D supplementation on iron status biomarkers when stratified for plasma ferritin concentration of <15 µg/l

Further analyses were carried out in participants who were iron deficient based on a plasma ferritin threshold of <15 µg/l at baseline (n=15 : vitamin D group, n=17: placebo group), to ascertain whether the severity of iron deficiency influenced participants' iron status following the consumption of vitamin D3 supplements and iron-fortified cereal. No significant effect of vitamin D3 supplementation on all biomarkers of iron status was observed in the two groups.

Table 4.4 shows the effect of vitamin D supplementation on all iron biomarkers within both groups when only participants with a ferritin threshold of <15 µg/l at baseline were included in analyses (n=32). The analyses were carried out to ascertain whether the severity of iron stores affected participants' response to the intervention. With the adjustment to the lower ID threshold, it was observed that there was a significant increase within the vitamin D group in mean (±S.D) RBC count from baseline (4.57 ± 0.25) to post-intervention (4.75 ± 0.26) (p=0.024). A significant increase within the vitamin D group was also observed in mean (±S.D) haematocrit % at post-intervention (43.53 ± 3.61%) from baseline (41.43 ± 3.15%, p=0.024) and 4-weeks (42.47 ± 3.26%, p=0.04).

It was also observed that there was a significant decrease within the vitamin D group in mean (±S.D) MCH from 4-weeks (29.21 ± 2.05 pg) to post-intervention (28.91 ± 2.19 pg) (p=0.04). In the vitamin D group, there was a significant decrease in mean (±S.D) MCHC at post-intervention (31.53 ± 1.05 g/dl) from baseline (32.19 ± 0.79, p=0.017) and 4-weeks (32.06 ± 0.90%, p=0.042). A significant decrease was observed in mean (±S.D) MCH at post-intervention (27.64 ± 3.43 pg) from baseline (28.11 ± 3.39 pg (p=0.036) and 4-weeks (28.00 ± 3.38 pg) (p=0.003) within the placebo group.

The improvement in the iron status biomarkers may be observed in both vitamin D and placebo groups, as both groups received iron-fortified breakfast cereals. No other significant improvement was observed within placebo group in any other iron status biomarkers.

Table 4.5 shows the effect of intervention on iron status biomarkers, when the lower threshold of plasma ferritin concentration is used in the analyses. There was no significant impact of intervention from baseline to post-intervention, on all of the iron status biomarkers.

Table 4.4 Effect of vitamin D supplementation on iron status biomarkers stratified by ferritin threshold of <15µg/l (n=32)

	Groups	Baseline (Week 0) (n=32)	Interim (Week 4) (n=32)	Post-intervention (Week 8) (n=32)	Time (p-value)	Time x Group (p-value)
Haemoglobin (g/dl)	Vitamin D	13.34 ± 1.21	13.60 ± 1.16	13.05 ± 2.66	NS	0.368
	Placebo	12.99 ± 1.67	12.86 ± 1.26	12.84 ± 1.39	NS	
Haematocrit (%)	Vitamin D	41.43 ± 3.15[a]	42.47 ± 3.26[a]	43.53 ± 3.61[b]	<0.05	0.160
	Placebo	40.83 ± 4.55	40.67 ± 3.26	40.72 ± 3.70	NS	
RBC (x10^{12}/l)	Vitamin D	4.57 ± 0.25[a]	4.67 ± 0.25	4.75 ± 0.26[b]	<0.05	0.354
	Placebo	4.64 ± 0.46	4.62 ± 0.39	4.67 ± 0.34	NS	
MCV (fl)	Vitamin D	90.65 ± 5.25	91.01 ± 4.87	91.67 ± 6.36	NS	0.187
	Placebo	88.19 ±8.35	88.49 ± 8.59	87.56 ± 8.64	NS	
MCH (pg)	Vitamin D	29.20 ± 2.25	29.21 ± 2.05[a]	28.91 ± 2.19[b]	<0.05	0.688
	Placebo	28.11 ± 3.39[a]	28.00 ± 3.38[a]	27.64 ± 3.43[b]	<0.05	
MCHC (g/dl)	Vitamin D	32.19 ± 0.79[a]	32.06 ± 0.90[a]	31.53 ± 1.05[b]	<0.05	0.338
	Placebo	31.81 ± 1.24	31.59 ± 1.14	31.47 ± 1.27	NS	
Plasma ferritin (µg/l)	Vitamin D	12.40 ± 8.55	10.20 ± 5.71	12.45 ± 6.94	NS	0.515
	Placebo	9.18 ± 6.69	9.71 ± 7.08	9.86 ± 9.22	NS	
Plasma hepcidin (ng/ml)	Vitamin D	3.10 ± 4.81	2.59 ± 2.69	3.00 ± 2.46	NS	0.758
	Placebo	2.03 ± 1.99	2.15 ± 2.47	2.87 ± 6.40	NS	
Plasma sTfR (mg/l)	Vitamin D	1.71 ± 0.80	1.85 ± 0.99	1.91 ± 1.05	NS	0.602
	Placebo	2.15 ± 1.32	2.18 ± 1.55	2.25 ± 1.59	NS	

Values are significantly different at specified time points if not sharing a common letter in the same row

Table 4.5 Effect of intervention on iron status biomarkers from baseline to post-intervention stratified by plasma ferritin <15 µg/l (n=32)

Biomarkers	Vitamin D group			Placebo group			p-value
	Baseline	Post-intervention	Change	Baseline	Post-intervention	Change	
Hb (g/dl)	13.34 ± 1.21	13.05 ± 2.66	0.37 ± 0.76	12.99 ± 1.67	12.84 ± 1.39	-0.16 ± 1.26	0.167
Hct (%)	41.43 ± 3.15	43.53 ± 3.61	2.09 ± 3.20	40.83 ± 4.55	40.72 ± 3.70	-0.11 ± 4.00	0.099
RBC ($x10^{12}$/l)	4.57 ± 0.25	4.75 ± 0.26	0.18 ± 0.27	4.64 ± 0.46	4.67 ± 0.34	0.02 ± 0.39	0.210
MCV (fl)	90.65 ± 5.25	91.67 ± 6.36	1.01 ± 3.24	88.19 ± 8.35	87.56 ± 8.64	-0.64 ± 3.27	0.164
MCH (pg)	29.20 ± 2.25	28.91 ± 2.19	-0.29 ± 0.58	28.11 ± 3.39	27.64 ± 3.43	-0.47 ± 0.85	0.486
MCHC (g/dl)	32.19 ± 0.79	31.53 ± 1.05	-0.67 ± 0.95	31.81 ± 1.24	31.47 ± 1.27	-0.34 ± 0.97	0.338
Fer (µg/l)	12.40 ± 8.55	12.45 ± 6.94	0.05 ± 8.65	9.18 ± 6.69	9.86 ± 9.22	0.70 ± 7.18	0.817
Hep (ng/ml)	3.10 ± 4.81	3.00 ± 2.46	-0.11 ± 4.17	2.03 ± 1.99	2.87 ± 6.40	0.85 ± 5.22	0.576
sTfR (mg/l)	1.71 ± 0.80	1.91 ± 1.05	0.20 ± 0.36	2.15 ± 1.32	2.25 ± 1.59	0.10 ± 0.33	0.418

All values are means (±S.D)

Changes are from baseline to post-intervention (week 0-week 8)

P-value represents the difference in changes observed between vitamin D and placebo groups

Hb: haemoglobin; Hct: haematocrit; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; Fer: ferritin; Hep: hepcidin; sTfR: soluble transferrin receptor

4.3.3.5 Effect of baseline iron status on changes in biomarkers of iron status

Figure 4.4 shows that baseline haemoglobin concentrations have a significant impact on the participants' response to iron-fortified breakfast cereal consumption. The more anaemic the participants were at baseline, the higher the improvement in RBC counts, haemoglobin concentrations and haematocrit %. It was observed that there were strong and moderate inverse correlations between baseline haemoglobin concentration and change of RBC, haemoglobin concentration and haematocrit levels. No significant association was observed between baseline plasma ferritin concentrations with any of the changes in iron status biomarkers.

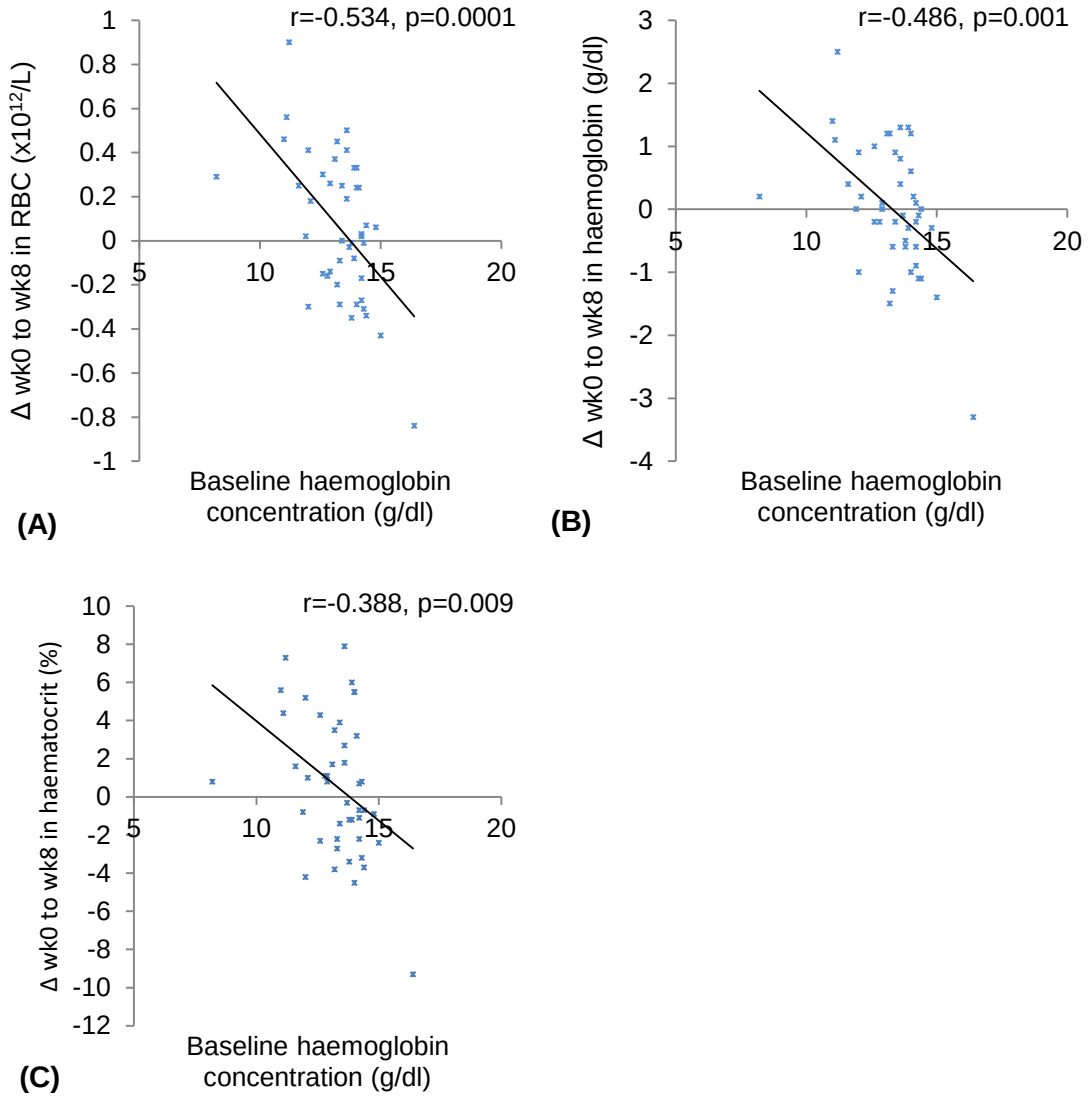


Figure 4.4 **Association between baseline haemoglobin concentration with change in (A) RBC count (B) haemoglobin concentration and (C) haematocrit % (n=44)**

4.3.3.6 Association between improvements in iron status biomarkers

All iron status biomarkers are hypothesised to be improved following iron-fortified breakfast cereal, impartial of intervention received in each group. Strong and linear associations were observed between several of the principal iron biomarkers **(Figure 4.5)**.

The RBC improvement was found to be positively and strongly associated with the improvement in haemoglobin concentrations (r=0.950, p=0.0001) and haematocrit % (r=0.910, p=0.0001), indicating the expansion in RBC counts lead to recovery of haemoglobin concentrations and haematocrit %.

A significant strong and positive correlation was observed between the recovery of haemoglobin concentration with the improvement in haematocrit % (r=0.933, p=0.0001). The increase in haemoglobin concentration was also found to be moderately but significantly associated with the MCV (r=0.359, p=0.017).

A strong and positive association was observed between plasma ferritin and plasma hepcidin concentrations (r=0.605, p=0.0001), indicating the role of hepcidin in suppressing the iron uptake once the participants' iron stores is replete.

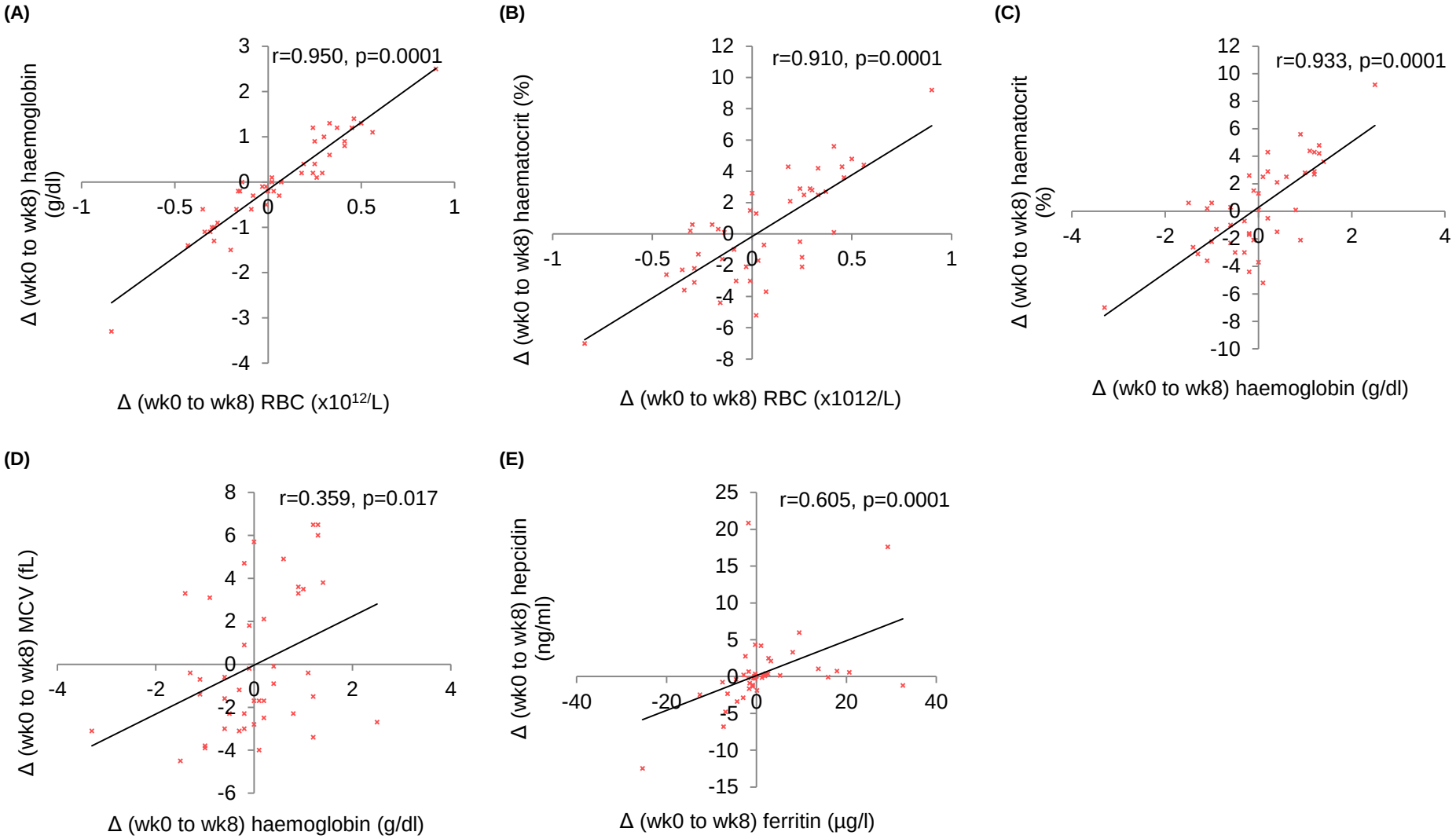


Figure 4.5 **Association between change in RBC with (A) Hb concentration and (B) Hct %; between change in Hb concentration with (C) Hct % and (D) MCV; and (E) between change in ferritin and hepcidin concentrations (n=44)**

4.3.4 EFFECT OF VITAMIN D SUPPLEMENTATION ON VITAMIN D STATUS

4.3.4.1 Time and group interaction

Table 4.6 shows the effect of intervention on participants' vitamin D status biomarkers at baseline, interim and post-intervention time points. Mean (±S.D) plasma 25(OH)D concentration was considered insufficient (30-50 nmol/l) at baseline, and was sufficient (>50 nmol/l) at 4-weeks and post-intervention in the vitamin D group. However, as expected, the mean (±S.D) plasma 25(OH)D concentration remained insufficient in the placebo group, at all time points. The mean (±SD) plasma PTH concentration was outside the normal range at all time points, in both groups. In addition, mean (±S.D) plasma VDBP concentration was within the normal range in the placebo group at all time points, but not in the vitamin D group. Mean (±S.D) plasma VDBP concentrations at 4-weeks and post-intervention were slightly lower than the normal range of plasma VDBP threshold (300-600 µg/ml) in the vitamin D group.

As expected, plasma 25(OH)D concentration was significantly higher in the vitamin D group, as opposed to the placebo group at 4-weeks and post-intervention, in relative to baseline. The increase from baseline to post-intervention in plasma 25(OH)D concentration in the vitamin D group (28.03 nmol/l) was significantly higher than the change in the placebo group (-5.65 nmol/l) (p=0.0001). The increase from baseline to 4-weeks in plasma 25(OH)D concentration in the vitamin D group (23.06 nmol/l) was also significantly higher than the change in the placebo group (0.22 nmol/l) (p=0.0001). In addition, the increase at post-intervention from 4-weeks in the vitamin D group (4.98 nmol/l) was also significantly higher than the change in the placebo group (-5.87 nmol/l) (p=0.003) **(Figure 4.6)**. However, no significant difference between vitamin D group and placebo group in plasma PTH and plasma VDBP concentrations. The intervention did not affected the plasma PTH and plasma VDBP concentrations **(Table 4.7)**.

Table 4.6 Effect of vitamin D supplementation on vitamin D status biomarkers at 3 time points over 8-weeks intervention period

	Groups	Baseline (Week 0) (n=50)	Interim (Week 4) (n=46)	Post-intervention (Week 8) (n=44)	Time (p-value)	Time x Group (p-value)
25(OH)D (nmol/l)	Vitamin D	35.01 ± 19.78$^{a\Psi}$	55.99 ± 11.02$^{b\Psi}$	62.16 ± 16.13$^{b\Psi}$	<0.0001	0.0001
	Placebo	38.57 ± 27.24$^{a\Psi}$	38.25 ± 27.10$^{a\Psi}$	34.15 ± 23.56$^{b\Psi}$		
PTH (pmol/l)	Vitamin D	7.77 ± 8.11^{a}	6.81 ± 7.03^{b}	7.24 ± 7.84^{b}	<0.05	0.198
	Placebo	7.61 ± 4.43	6.81 ± 3.08	7.63 ± 3.68	NS	
VDBP (µg/ml)	Vitamin D	307.82 ± 135.78	272.32 ± 90.12	289.07 ± 118.67	NS	0.413
	Placebo	367.39 ± 144.77	368.52 ± 131.58	382.65 ± 138.14	NS	

Values are significantly different at specified time points if not sharing a common letter in the same row

Values are significantly different between groups (vitamin d and placebo) if sharing a common symbol (Ψ) in the same column

Table 4.7 Effect of intervention on vitamin D status biomarkers from baseline to post-intervention (n=44)

Biomarkers	Vitamin D group			Placebo group			p-value
	Baseline	Post-intervention	Change	Baseline	Post-intervention	Change	
25(OH)D (nmol/l)	35.21 ± 18.43	62.16 ± 16.13	28.03 ± 28.01	39.33 ± 27.55	34.15 ± 23.56	-5.65 ± 9.73	*0.0001
PTH (pmol/l)	8.34 ± 8.94	7.24 ± 7.84	-1.11 ± 2.00	7.80 ± 4.42	7.63 ± 3.68	-0.17 ± 2.62	0.198
VDBP (µg/ml)	294.94 ± 131.20	289.07 ± 118.67	-5.87 ± 52.23	374.03 ± 143.95	382.65 ± 138.14	8.62 ± 62.16	0.413

All values are means (±SD), P-value represents the difference in changes observed between vitamin D and placebo groups, *= p ≤ 0.05

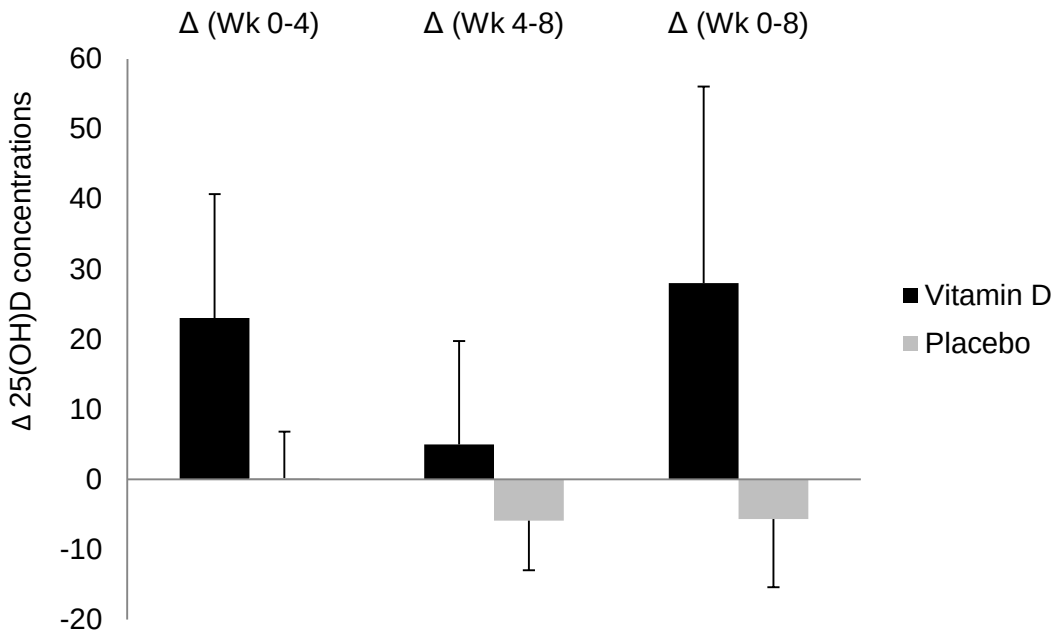


Figure 4.6 Effect of intervention on vitamin D status from baseline to post-intervention

4.3.4.2 Time effect within groups

In the vitamin D group, mean (±S.D) plasma 25(OH)D concentration was significantly higher at post-intervention (62.16 ± 16.13 nmol/l) compared to both; baseline (35.01 ± 19.78 nmol/l, p=0.0001) and 4-weeks (55.99 ± 11.02 nmol/l, p=0.0001). A significantly decrease in mean (±S.D) plasma 25(OH)D concentration was observed in the placebo group at post-intervention (34.15 ± 23.56 nmol/l) compared to both; baseline (38.57 ± 27.24 nmol/l, p=0.008) and 4-weeks (38.25 ± 27.10 nmol/l, p=0.001). In the vitamin D group, mean (±S.D) plasma PTH concentration was significantly lower at 4-weeks (6.81 ± 7.03 pmol/l, p=0.022) and post-intervention (7.24 ± 7.84 pmol/l, p=0.023) compared to baseline (7.77 ± 8.11 pmol/l). No significant difference in plasma PTH concentration was observed within the placebo group, between each time point. No significant difference in plasma VDBP concentration was observed within both vitamin D and placebo groups, at all time points.

4.3.4.3 Associations between baseline vitamin D status and iron status biomarkers

A weak but significant positive association was observed between baseline plasma 25(OH)D concentrations with the following iron status biomarkers: RBC count (r=0.339, p=0.0001), haemoglobin concentration (r=0.208, p=0.014) and haematocrit % (r=0.199, p=0.018). In addition, a weak but significant inverse association was observed between baseline plasma 25(OH)D concentrations and plasma PTH concentration (r=-0.229, p=0.006), as expected **(Figure 4.7)**. No significant association was observed between the baseline plasma 25(OH)D concentrations with the other iron status biomarkers including MCV, MCH, MCHC, plasma ferritin, plasma hepcidin, and plasma sTfR concentration. Baseline plasma 25(OH)D concentration was also not significantly associated with plasma VDBP concentrations.

In the vitamin D group (n=20), there was no significant association between baseline plasma 25(OH)D concentration and the mean changes (Δ wk0 - wk8) in plasma 25(OH)D concentration. To determine the potential effect of the improvement in vitamin D status in the vitamin D group on participants' iron status, a further correlation test was carried out between the mean changes (Δ wk0 - wk8) in plasma 25(OH)D concentration with mean changes (Δ wk0 - wk8) of all iron status biomarkers, however, no significant association was observed.

Significant associations were however, observed between mean changes (Δ wk0 - wk8) of plasma VDBP concentrations with RBC count, haemoglobin concentration, haematocrit, MCV and MCHC concentrations **(Figure 4.8).**

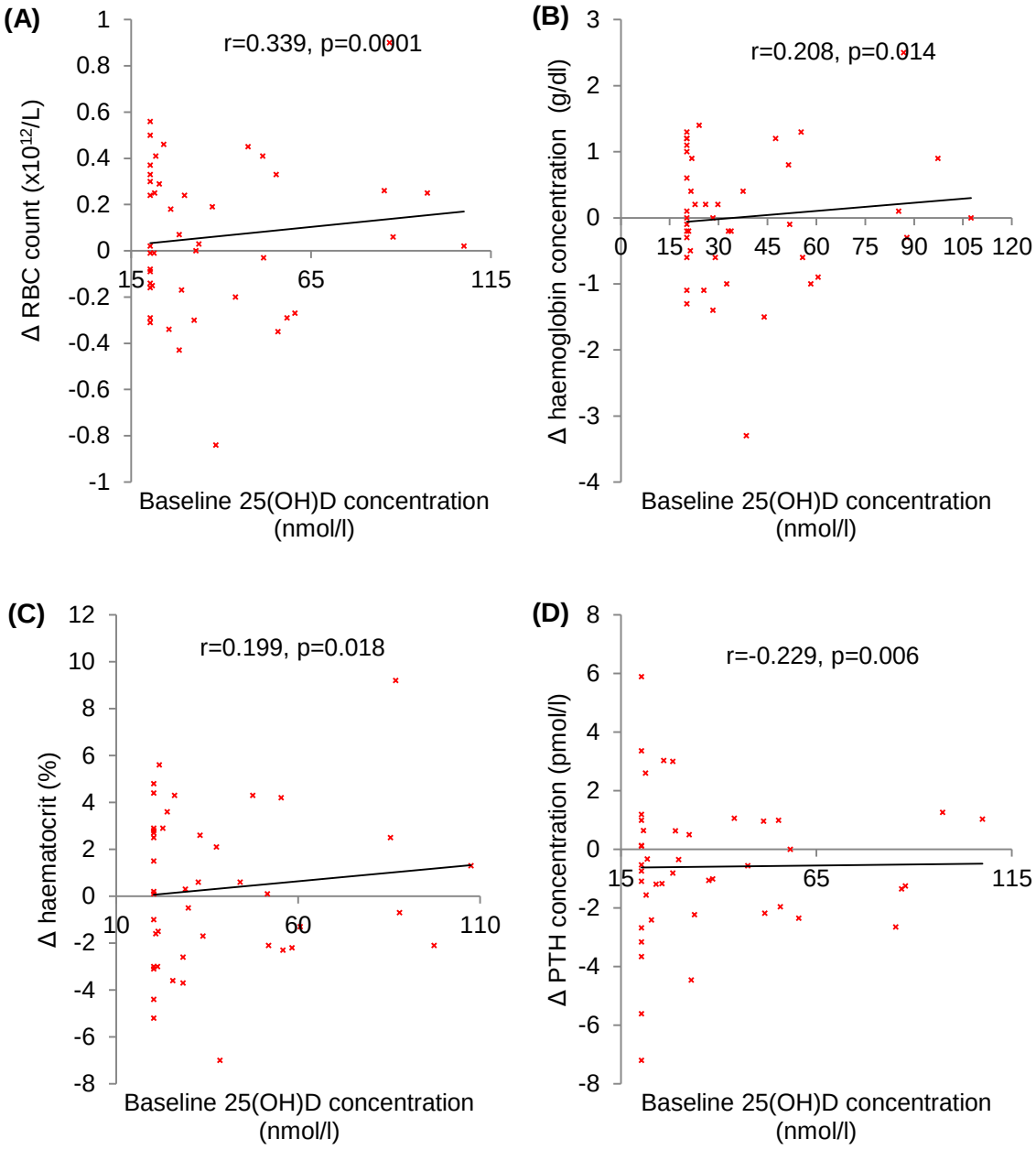


Figure 4.7 **Association between baseline plasma 25(OH)D concentrations with (A) RBC count (B) haemoglobin concentration (C) haematocrit % and (D) plasma PTH concentration in both groups combined (n=44)**

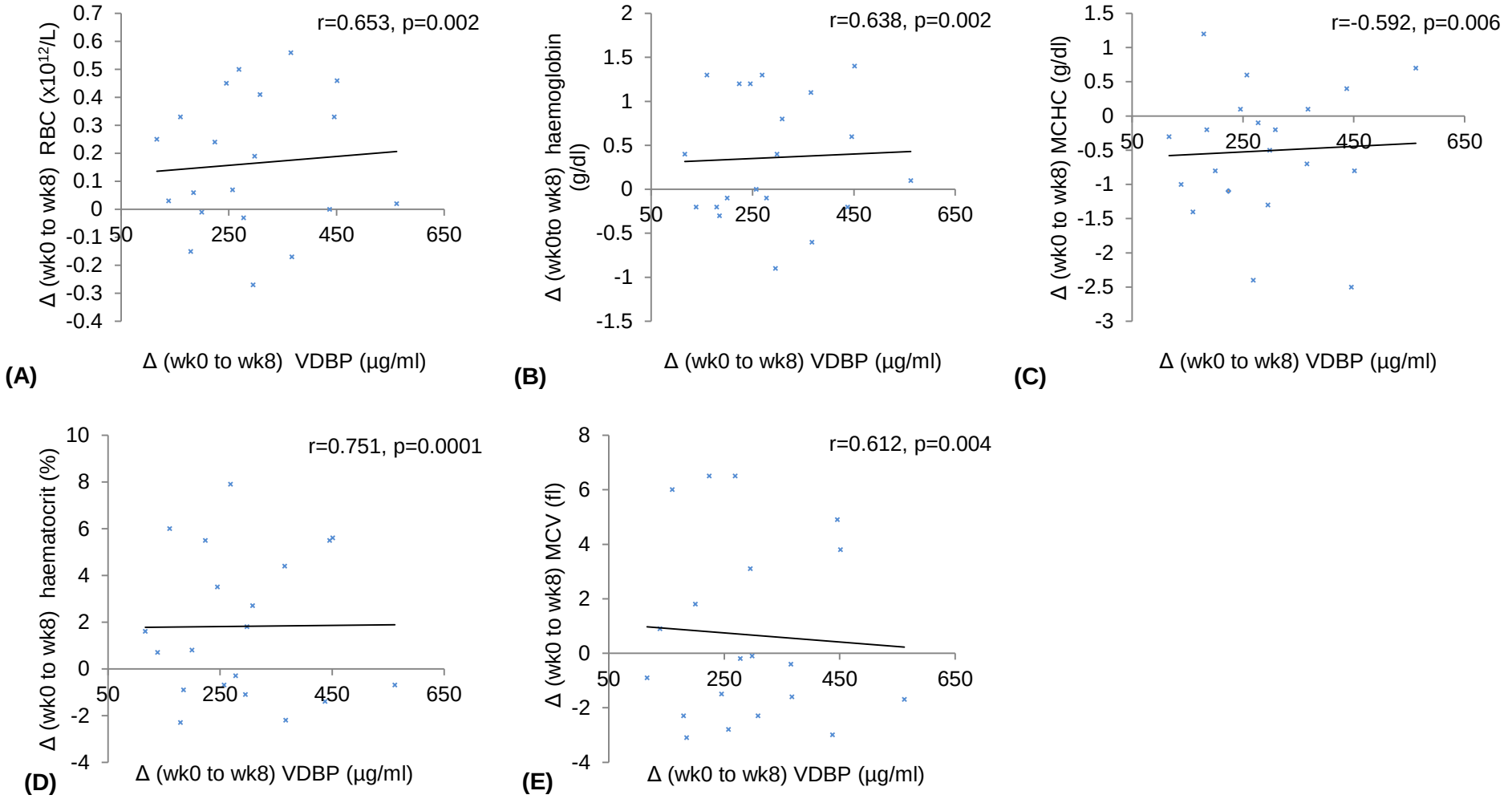


Figure 4.8 **Association between change in plasma VDBP concentrations with change in (A) RBC count (B) haemoglobin concentration (C) MCHC concentration (D) haematocrit % and (E) MCV in the vitamin D group (n=20)**

4.3.5 DIETARY INTAKE FOLLOWING VITAMIN D SUPPLEMENTATION AND IRON-FORTIFIED BREAKFAST CEREAL CONSUMPTION

Participants' dietary intake of the was measured twice using a 3-day food diary, provided after the baseline and interim clinics in order to monitor the dietary pattern of participants and to ascertain habitual food intake.

Table 4.8 summarises the energy and dietary intake of participants in both groups at 2 time points during the 8-weeks intervention period. At post-intervention, no significant differences were observed between the two groups in the energy, protein, carbohydrate, fat, iron, vitamin D, calcium and vitamin C daily intake.

Within each group, no significant difference was observed between baseline and post-intervention in the energy, protein, carbohydrate, fats, iron, vitamin D, calcium and vitamin C daily intake. Despite showing increase trend particularly in the iron intake, in both group, the changes were not significantly higher at post-intervention. In the vitamin D group, there was a non-significant change in mean (±S.D) dietary iron intake from 16.5 ± 1.8 mg (baseline) to 16.8 ± 2.3 (post-intervention) (+0.29 mg, p=0.6). The same non-significant trend was also observed in the placebo group, with mean (±S.D) dietary iron intake of 17.5 ± 2.9 mg (post-intervention) compared to 16.6 ± 2.7 mg (baseline) (+0.98 mg, p=0.134). Increase in the dietary iron intake was expected in both groups, as both groups received iron-fortified cereals.

The overall participants' estimated dietary intakes at post- intervention (week 8) (n=41) were compared to population data reported in the NDNS and also Dietary Reference Values (DRV) recommended by the UK SACN for women aged 19-64 years (British Nutrition Foundation, 1995) **(Table 4.9, Figure 4.9)**. Mean daily energy, carbohydrate (% of total energy intake), protein, iron, vitamin C and calcium daily intake was higher compared to the NDNS population data. However, mean fat (% of total energy intake) and vitamin D intake was lower compared to the NDNS population data. It was observed that the estimated dietary iron daily intake of participants was higher than the RNI of 14.8 mg recommended for women aged 19-50 years. Baseline dietary iron intake was estimated based on participants' 3-day dietary intake after they attended baseline clinic, which explains the higher estimated dietary iron intake (in comparison to typical intake reported by NDNS) due to consumption of the supplied iron-fortified breakfast cereals which contributed 9 mg iron if consumed.

Table 4.8 Daily dietary intakes of macronutrients and selected micronutrients following iron-fortified cereal intervention

Intake/day	Groups	Baseline	Post-intervention	Change
Energy (kcal)	Vitamin D	1738.4 ± 311.9	1729.7 ± 332.1	-8.61
	Placebo	1661.7 ± 435.4	1726.7 ± 470.5	65.04
Energy (MJ)	Vitamin D	7.3 ± 1.3	7.2 ± 1.4	-0.04
	Placebo	7.0 ± 1.8	7.2 ± 2.0	0.27
Protein (g)	Vitamin D	74.1 ± 16.0	73.2 ± 19.2	-8.69
	Placebo	67.4 ± 16.2	74.8 ± 21.2	7.34
Carbohydrate (g)	Vitamin D	222.8 ± 31.2	224.7 ± 51.8	1.88
	Placebo	231.0 ± 57.3	225.5 ± 60.4	-5.55
Carbohydrate (%)	Vitamin D	49.5 ± 5.5	50.1 ± 7.5	0.63
	Placebo	53.4 ± 5.7	50.3 ± 5.1	-3.09
Fat (g)	Vitamin D	61.9 ± 23.1	61.9 ± 17.5	0.03
	Placebo	55.9 ± 21.4	58.6 ± 22.4	2.70
Fat (%)	Vitamin D	31.3 ± 6.4	31.9 ± 4.9	0.63
	Placebo	30.5 ± 7.4	30.4 ± 6.3	-0.09
Iron (mg)	Vitamin D	16.5 ± 1.8	16.8 ± 2.3	0.29
	Placebo	16.6 ± 2.7	17.5 ± 2.9	0.98
Vitamin D (µg)	Vitamin D	1.4 ± 1.0	1.9 ±1.9	0.51
	Placebo	1.8 ± 2.0	2.0 ± 3.0	0.18
Calcium (mg)	Vitamin D	817.8 ± 170.1	834.5 ± 209.6	16.69
	Placebo	858.4 ± 300.8	875.0 ± 237.0	16.51
Vitamin C (mg)	Vitamin D	81.7 ± 42.6	103.0 ± 63.6	21.34
	Placebo	85.1 ± 39.9	82.8 ± 54.3	-2.37

All values are mean (±SD) except for for carbohydrate & fats with additional % of total energy intake

Table 4.9 Comparison of participants' mean (±S.D) daily intake to UK population means (NDNS) and RNIs

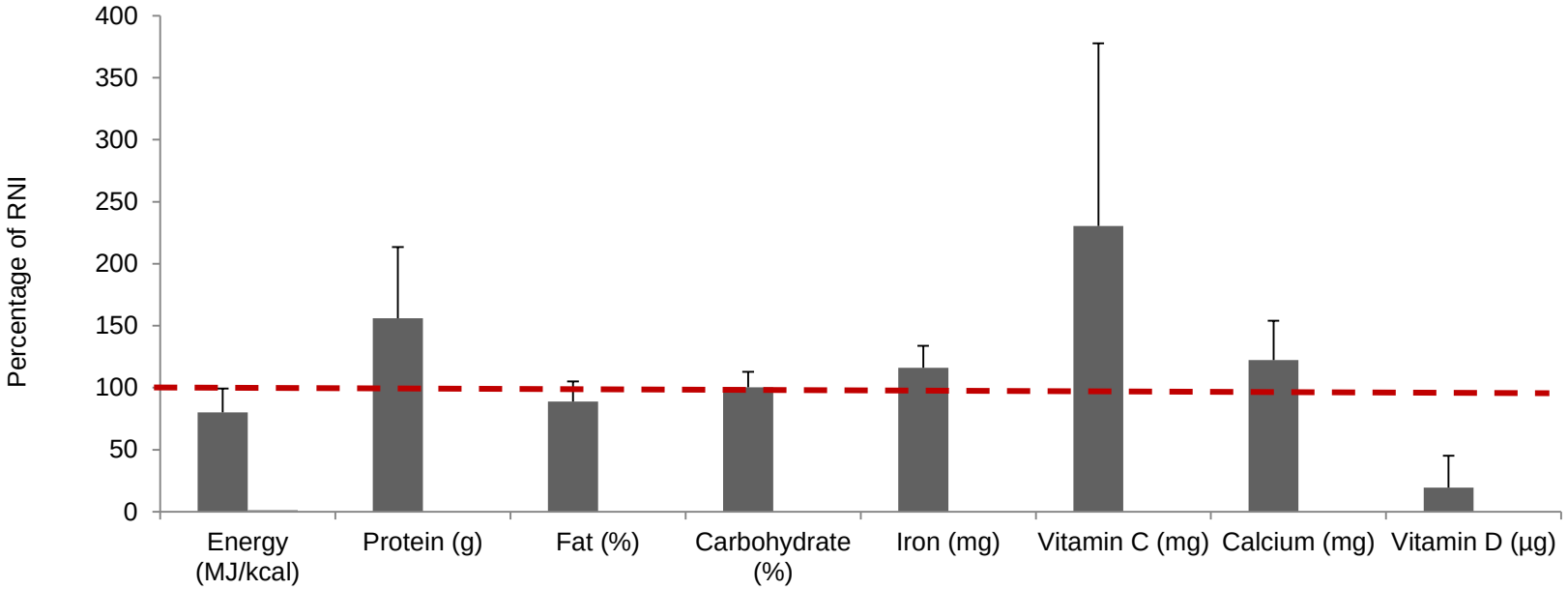
Intake/day	Post-intervention (n=41)	NDNS[a]	% RNI
Energy (kcal)	1728.1 ± 407.28	1613 ± 455	80.23
Energy (MJ)	7.2 ± 1.7	6.8 ± 1.9	
Protein (g)	74.1 ± 20.1	65.4 ± 18.3	156.08
Carbohydrate (g)	225.1 ± 55.9	197 ± 61	
Carbohydrate (%)	50.2 ± 6.2	46.3 ± 7.7	100.44
Fat (g)	60.1 ± 20.1	60.1 ± 22.7	
Fat (%)	31.1 ± 5.7	33.0 ± 6.6	88.85
Iron (mg)	17.2 ± 2.6	9.6 ± 3.0	116.01
Vitamin D (µg)	2.0 ±2.6	2.6 ± 1.9	19.52
Calcium (mg)	856.2 ± 222.9	728 ± 260	122.31
Vitamin C (mg)	92.2 ± 58.9	81.6 ± 59.8	230.41

[a]NDNS population data from Year 1-3 (Bates et al., 2014)

Further analysis was carried out to assess if there will be a difference between the mean daily iron intake if the baseline dietary intake estimation excluded the consumption of the specified study breakfast cereal. The mean (±S.D) baseline dietary iron intake was estimated to be 7.4 ± 2.3 mg (n=41). No significant difference was observed between the mean (±S.D) baseline dietary intake of vitamin D group (7.5 ± 1.8 mg) and placebo group (7.6 ± 2.7 mg). However, significantly higher dietary iron intake was observed between baseline and post-intervention, within both groups (p=0.0001), with the adjustment to the baseline dietary intake estimation (excluded study breakfast cereal).

3.3.5.1 Association between dietary intake and blood biomarkers

Baseline plasma 25(OH)D concentrations were not significantly associated with mean changes in dietary iron intake. In addition mean dietary iron intake was not associated with the change in plasma 25 (OH)D and any of the iron status parameter concentrations. However, non-significant trend of association was observed between changes in plasma VDBP concentration with the mean changes in dietary iron intake (p=0.092), indicating that upregulation in VDBP may have a role in the iron regulation, as demonstrated in blood parameters (Figure 4.8).



*dotted line represents 100% RNI

Figure 4.9 Participants' percentage mean daily intake of energy, protein, fat, carbohydrate, iron, vitamin C, calcium and vitamin D compared to UK DRVs

CHAPTER 5

General Discussion and Conclusion

5.1 DISCUSSION

Anaemia occurs predominantly in premenopausal women and affects approximately 25% of the general population in the world. A key aetiology of anaemia is ID, and dietary elements play a crucial role in the development of ID and IDA (Beck et al., 2014). One of the immediate determinants of IDA is dietary iron intake, which depends on several factors including (i) amount of iron in the diet; (ii) type of iron (haem or non-haem); (iii) inclusion of dietary iron inhibitors or enhancers in the diet and (iv) iron bioavailability (Pasricha et al., 2013). In the UK population, it is evident that the dietary iron intake is lower in women as opposed to men (Bates et al., 2014), and premenopausal women, in particular, are one of the at-risk groups susceptible to iron deficiency (WHO, 2008).

Ascorbic acid and muscle tissue from animal-based sources are the recognised iron absorption enhancers, whilst polyphenols, phytates, and calcium are classified as iron absorption inhibitors (Abbaspour et al., 2014). These dietary components have been shown in various intervention studies to be capable of either facilitating or impeding iron absorption, causing iron to be available or unavailable for utilisation, thus affecting individual's iron status. The upregulation of iron absorption is determined by the presence of ID and enhanced erythropoiesis, and downregulation occurs when infection is present or iron stores are at adequate levels. One of the many reasons that anaemia, ID or IDA are still prevalent across the world is due to technical challenges in the implementation of strategies to recover normal iron status in population (Lynch, 2011). The main strategies in the management of ID revolve around a (i) dietary approach that includes dietary modification and iron fortification or a (ii) therapeutic approach involving iron supplementation (WHO/UNICEF/UNU, 2001). Despite the implementation of these strategies, anaemia is still prevalent, especially in the at-risk groups such as women, young children and adolescents (Beck et al., 2014). A successful iron supplementation strategy is often limited to poor adherence due to classic gastrointestinal adverse events (Souza et al., 2009), therefore, the present research adopted the exploitation of a dietary approach by focussing on iron absorption inhibition (Chapter 3) and iron absorption enhancement (Chapter 4). The overall aims of the present research were to improve iron status of premenopausal women, by modulating the effect of tea containing polyphenols as iron inhibitor, and a potentially-novel iron enhancer, vitamin D supplements.

The first clinical trial as part of the overall research was carried out in the attempt to counteract the influence of tea containing polyphenols on iron absorption in healthy premenopausal women, using a single stable iron isotope technique (^{57}Fe) to assess iron absorption. The second clinical trial was carried out in iron deficient premenopausal women, to investigate the effect of vitamin D supplementation, as a potential novel iron absorption enhancer, consumed with iron-fortified breakfast cereal as part of daily diet, on the recovery of iron status.

The first part of the research was designed specifically to address the iron absorption aspect, by measuring both the fractional and absolute amount of iron absorbed, following administration of isotope labelled porridge meal with water and tea at different time intervals. Whilst a large number of studies have shown that tea containing polyphenols can render iron absorption, it remains uncertain whether the time interval of tea consumption relative to a meal has an impact on iron absorption, with a paucity of published evidence, especially in human interventions, investigating the subject matter.

The main research hypothesis of this first part of the research was to investigate the effect of employing 1-hour time interval between the consumption of porridge meals extrinsically-labelled with stable iron isotope ^{57}Fe and administration of tea on iron absorption in a healthy cohort of premenopausal women. Iron absorption can be predicted using series of algorithm (Hallberg & Hulthén, 2000), or estimated using a probability analysis based on ferritin concentration and dietary iron intake (Dainty, Berry, Lynch, Harvey, & Fairweather-Tait, 2014), however, the present study utilised a stable isotope technique which is the reference method to determine fractional iron absorption. The present study also was carried out to investigate the impact of tea consumption on non-haem iron absorption from a porridge meal using stable iron isotope (^{57}Fe).

5.2 EXPERIMENTAL STUDY 1 (CHAPTER 3)

In agreement with the previous studies, our findings suggest that tea consumption, reduces non-haem iron absorption, by at least 37% in comparison to water used as a control beverage. The study also demonstrates that a 1-hour time interval between tea consumption and a meal has a substantial impact on counteracting this inhibition effect, by at least 1.6 fold.

Iron absorption from the porridge meal was as low as 5.69% when consumed with water, and consuming the similar porridge simultaneously with tea aggravated the impact on iron absorption with a substantial reduction of iron absorption to 3.57%. The total amount of iron absorbed when porridge was administered with water was 0.23 mg, and the amount was reduced by 50% to 0.14 mg with consumption of tea. This shows that when the porridge meal is consumed with water, only 1.4% of RNI (14.8 mg) is achieved, and the percentage of meeting the RNI is even lesser when tea is consumed with the porridge meal.

The key finding in the present study is that with 1-hour interval between consuming the meal and tea will improve iron absorption by attenuating the inhibition effect of tannin component in tea. With 1-hour time interval, total iron absorbed is increased and found to be almost equal to when water is consumed with the meal (0.19 mg). Consistent with the extensive body of literature, this study also demonstrated that higher body iron storage (represented by plasma ferritin concentrations) is linked to low total iron absorbed. Interestingly, with little evidence available from the previous iron absorption studies, it was shown in the present study that higher plasma hepcidin concentrations are also associated to low total iron absorbed, strengthening the existing body of literature that demonstrated the role of hepcidin as an iron absorption regulator at systemic levels.

5.2.1 Inhibition effect of tea consumption on iron absorption

In agreement with the previous studies, the present study showed that tea plays a significant role in reducing non-haem iron absorption, by at least 37%. Despite the differences in test meal used, study population or isotopes used, with previously published studies, a similar outcome of reduced iron absorption with tea consumption was demonstrated. **Table 5.1** compares the iron absorption (%) reported in previous studies in which test meals were administered with either water (as a control) or tea (intervention) to illustrate the inhibition effect (%). The percentage of inhibition effect shown in the current study was relatively low (37%),

compared to a range of between 26-99% from the previous studies. The wide range of inhibition effects reported in these studies may be due to the fact that there were differences in tea brewing time, tea brands, and also total amount of tea used during the intervention which may have influence the phenolic concentration when tea is administered (Hallberg & Hulthén, 2000).

The present study also demonstrated that the iron absorption ratio was 0.65 when the test meal administered with tea, compared to when administered with water (control) which indicates a relatively low inhibition effect of tea. A lower absorption ratio of 0.28 was reported by Morck et al. (1983) when a standard meal of hamburger was administered with tea, compared to water, which amounts to a 64% inhibition effect. The same study found a much lower absorption ratio of 0.09 with 3 grams of coffee, compared to ratio of 0.17 when 1.5 grams coffee was used, indicating a lower percentage of inhibition effect. Thankachan, Walczyk, Muthayya, Kurpad, and Hurrell (2008) compared different concentrations of tea and also found a lower absorption ratio of 0.51 (150 ml) compared to 0.34 when using a higher concentration of tea (300 ml) and it was suggested that an absorption ratio of <1.0 implies an inhibition effect of tea. A similar absorption ratio (tea/water) was reported by other studies using different test meals with 0.3 (Kaltwasser et al., 1998), 0.03-0.53 (Hurrell et al., 1999) and 0.7 (Samman et al., 2001).

Table 5.1 Inhibition effect (%) of tea consumption on iron absorption (%) in comparison to water as control beverage

Study	Population / Mean age (±SD) or range (years)	Test meals	Absorption (%)			
			Meal (water)	Meal (tea)	Inhibition %	Reference iron dose
Present	Healthy women (n=12) / 24.8 ± 6.9	Porridge + 200 ml tea	5.7	3.6	37	25.36%
[1]	Healthy women (n=8) / 26-60	Bread + 200 ml black tea	10.4	3.3	68	35.8%.
		Rice & soup + 200 ml black tea	10.8	2.5	77	34.7%.
[2]	Healthy women (n=22) / 21-71	Maize porridge + 150 ml black tea	3.8	2.1	45	50.5%.
[3]	Thalassemia patients (n=5) / 11-23	Hamburgers + 240 ml tea	15.6	5.2	67	NA
[4]	Healthy adults (n=37) / 18-50	Beef hamburger + 200 ml tea	3.71	1.32	64	15.73%.
[5]	Haemochromatosis patients (n=18) / 47.4 ± 16.1	Homogenised rice & beef + 200 ml tea	22.1	6.9	69	NA
[6]	Healthy adults (n=77) / 19-40	Bread roll + 275 ml tea				
		275 ml tea (n=9)	12.9	0.74	94	NA
		275 ml tea (n=10)	5.63	0.89	84	
		275 ml tea (n=10)	4.46	0.92	79	
		275 ml tea (n=10)	8.64	0.83	90	
		100% strength (n=9)	6.58	0.59	91	
		50% strength		1.05	84	
		25% strength		0.06	99	
		25% strength (n=9)	4.33	1.18	73	
		10% strength		1.48	66	
		5% strength		1.47	66	

[7]	Healthy women (n=10) / 26 ± 4	Pasta + bread + 4ml green tea extract	12.1	8.9	26	38.6
[8]	IDA and healthy women (n=20)/ 18-35	Tomato rice + tea (different amount)				
	Iron deficient anaemia group (22.6 ± 3.5)	150 ml tea	18.2	7.1	61	NA
		300 ml tea	19.7	5.6	72	
	Iron replete group (24.3 ± 2.9)	150 ml tea	7.5	3.5	53	
		300 ml tea	5.2	1.6	69	

*[1] Disler et al., 1975 (India), [2] Derman et al.,1977 (India), [3] De Alarcon., 1979 (Italy), [4] Morck et al., 1983 (USA), [5] Kaltwasser et al., 1998 (Germany), [6] Hurrell et al., 1999 (USA), [7] Samman et al., 2001 (Australia), [8] Thankachan et., al 2008 (India)

*Inhibition effect (%) = $\frac{\text{[meal (water) – meal (tea)]}}{\text{meal (water)}} \times 100$

*All meals were extrinsically labelled with either stable or radio isotopes except the second sub-study of [1]

*[1 & 9]: stable iron isotopes; [2-8]: radio iron isotopes

To the best our knowledge, there is limited evidence to date on the mechanism of how tea may inhibit iron absorption, especially based on human trials, and the present study was not designed to investigate the mechanism behind the inhibition effect of the tea component *per se*. The only assumption proposed to date, based on human data, is that polyphenol-containing tannins in tea are responsible for the disruption of non-haem iron absorption (Disler et al., 1975a), as a result of the formation of insoluble iron-tannin complexes at luminal level that leads to interference of the absorption (Morck et al., 1983). The assumption was based on the inhibition effect observed when tea was administered with simple iron salt solutions, or the specific meal used in the study which is related to the Indian population. Our findings show similar outcomes, however, differ in the test meal used, which was specific to a typical UK breakfast meal, possibly explaining the difference in the percentages of iron absorption and the inhibition effect (Table 2.18). To further establish that tannin in tea causes the uptake interference, Disler et al. (1975b) carried out an animal study using 10 Sprague-Dawley male rats that were fed iron salts with either (a) tea; (b) tea tannin solution; (c) tannin-free tea; (d) tannic acid solution and (e) caffeine. It was shown that mean (±S.D) iron was absorbed approximately 3-fold more with tannin-free tea (49.6 ± 7.5%) compared to tea (13.8 ± 5.6%), verifying the effect of tannin content in tea on iron absorption. It was further suggested that apart from just tannin, the inhibition effect was due to high concentrations of galloyl esters (3 galloys) (Brune et al., 1989) in black tea, after Hurrell et al. (1999) observed a higher inhibition effect in black tea compared to herbal teas, wine or cocoa (Table 5.1).

However, it should be noted that these iron absorption studies that investigated the mechanism were carried out in cell lines or animal models, not humans, and findings cannot be extrapolated directly to humans.

5.2.2 One-hour time interval effect of tea consumption on iron absorption

In addition to investigating the effect of tea in diminishing iron absorption, the present study also aimed to explore the time interval effect between tea consumption and an iron-containing meal. To the best of our knowledge, the impact of time interval has not been investigated in human subjects, with respect to a specific UK diet, and the outcome may be particularly beneficial for the controlling of iron deficiency in at-risk groups such as reproductive-aged women. Findings in the present study that a 1-hour time interval between administering tea and a porridge meal substantially reduces the inhibition effect by increasing iron absorption by at least 1.6-fold supports the findings from previous studies carried out in rats (Disler et al., 1975b).

In the present study, it was primarily postulated that iron absorption would be highest in TM I (meal + water), followed by TM III (meal + tea + 1 hour) and lowest in TM II (meal + tea) as it was predicted that tea, consumed simultaneously with a meal would impede iron absorption the most.

Even though TM III (meal + tea + 1 hour) exhibited the highest percentage of absorption compared to the TM I (meal + water) which serves as a control, the difference in iron absorption between these 2 test meals was not significantly different ($p=0.335$). This is shown in the total iron absorbed, with 0.23 mg in TM I and 0.19 mg in TM III. As expected, it was demonstrated that the iron absorption (%) was significantly higher when a 1-hour time interval was allowed before tea was administered after the test meal (TM III), as compared to when tea was administered simultaneously with the meal (TM II), which amounts to a total of 0.04 mg more iron was absorbed with 1-hour time interval. However, few studies have been carried out, providing limited evidence to support findings on the time interval effect, particularly in human studies. In an animal study carried out by Disler et al. (1975b), it was demonstrated that the time interval between administration of tea and iron salt solutions had a significant impact on weakening the inhibition effect, when tea was administered at several interval times up to the maximum of 3 hours before (as opposed to ‘after’ in the present study) iron solutions were administered. A linear association between time intervals and iron absorption was observed, and iron absorption was found to be approximately 2-fold higher if the tea was administered 1 hour before. This is different in the present study because the tea was administered after the meal instead of before as in the Disler et al. (1975b) study, but iron absorption increased by a similar magnitude of 1.6-fold.

The findings from Disler's study, however, need cautious interpretation as the study was an animal study, which cannot be directly extrapolated to humans. In addition, the study utilised simple salt solutions instead of a complex test meal containing various iron enhancers or inhibitors, which may prompt nutrient interactions that may also affect iron uptake.

Another intervention employing same study design was carried out to investigate this time interval effect used coffee, another phenolic-containing beverage, administered with a hamburger as the test meal (Morck et al., 1983). However, coffee was found not to cause further inhibition if administered 1 hour later (4.55%) compared to when it was administered simultaneously with the hamburger (4.58%), even though iron absorption was reduced by at least 40% when water was administered with the hamburger meal (8.12%).

It was postulated that the same chelating mechanism occurred at a luminal level caused by coffee, similar to that suggested in tea. This mechanism is explained by the binding between a phenolic component and iron which is believed to have occurred via either catechol or galloyl group (Khokhar & Apenten, 2003), by forming an insoluble complex with metal cations in iron (Bravo, 1998).

There is no established mechanism relating to how a 1-hour time interval will aid in counteracting the inhibitory effect of tea on iron absorption, however, after the non-haem iron has been reduced to a ferrous state and pooled together with haem iron in the enterocyte, iron is expected to be chelated by other compounds to form insoluble complex, if not bound to ferritin (Steele et al., 2005). As the tea was administered only 1 hour after the meal, it could be stipulated that the non-haem iron is absorbed normally, as when it was administered with water, without forming complexes with any inhibitor, which in this case is tannin in tea, and transported directly to basolateral membrane, leading to a higher iron absorption. This is observed in the present study as the similar magnitude of absorption shown between TM I (meal + water) and TM III (meal + tea + 1 hour) at 5.69% and 5.73%. It was also suggested by Miret, Simpson, and McKie (2003) that as critical as it is to quantify the duration that iron is in contact with the duodenal surface to determine iron absorption rate, there is very scarce and contradictory evidence available.

5.2.3 Limiting factors of iron absorption

Depending on the conditions and chemical forms of iron itself (haem/non-haem), the overall fractional iron absorption (%) absorbed by individual is reported to be classically low, ranging from as low as 5% to as high as 35% (Abbaspour et al., 2014). It can be presumed that the nature of the cereal-based meal is the main grounds of the low iron absorption observed in the present study, as the porridge is low in iron content but also contains phytates which has been identified as an iron absorption inhibitor. It is also possible that as the participants were generally iron replete (60% not iron deficient), the body iron requirement was minimal, thus explaining the low iron absorption. The low iron absorption observed may be attributable to two key aspects: (a) meal-related factors and (b) host-related factors.

5.2.3.1 Meal-related factors

It was shown in the study that iron was poorly absorbed from a porridge meal, which is the most consumed breakfast (38% as reported in the latest NDNS) amongst women in the UK (Bates et al., 2014). Based on the nutritional information provided, the instant porridge used in the study did not have any known native iron or added iron as fortifiers, but it was reported in a study carried out by Bruggraber et al. (2012) which re-analysed iron content of selected UK plant-based foods using spectrometer, that iron content of instant porridge was 13.28 mg. This shows that the fractional iron absorbed from the porridge in the present study may be acceptable, considering the native iron in the oat porridge itself is already low and the dose of ^{57}Fe administered with each test meal was 4 mg. In addition, the lower iron absorption demonstrated in the present study is consistent with the amount iron absorbed (%) in previous studies that have used the cereal-based meals **(Table 5.2)**.

Previous studies have reported a wide range of fractional iron absorption values, between 0.56 – 18.8%. The lowest absorption of 0.33% was reported in a study carried out in the US by Hurrell, Reddy, Burri, and Cook (2002) that used 50 g of extruded oats, prepared by adding 300 ml boiling water to be consumed as oat porridge, which is analogous to the test meal in the present study, but reported lower iron absorption. The highest absorption of 18.8% was demonstrated in a study carried out using roller-dried cereal as a test meal made from wheat flour, enriched with erythorbic acid:iron at molar ratio of 4:1, which explains the high iron absorption as the same test meal without the addition of enhancer led to an absorption of 4.1% (Fidler et al., 2004).

Table 5.2 Previous studies that have used cereal-based test meal with native iron and phytate contents

Study/Country	Participant/age range	Test meals	Iron absorption (%)	Native iron content (mg)	Phytate content (mg)
Layrisse et al. (2000) Venezuela	Adults (n=130) 15-50 years	Corn flour meal (C) Corn flour meal (T)	5.1% 7.9 – 13.2%	1.5 mg	168 mg
Mendoza et al. (2001) USA	Women (n=14) 19-42 years	Wild porridge (C) Low phy porridge (T)	1.69 - 5.73% 1.91 - 5.40%	3.4 mg	817 mg 361 mg
Davidsson et al. (2001) Peru	Children (n=47) 6-7 years	Cereal drink (C) Cereal drink (T)	2.2 – 2.9% 3.5 – 3.8%	2.3 mg	122 mg
Hurrell et al. (2002) USA	Adults (n=39) 19-39 years	Rice flour Maize flour High extraction wheat Low extraction wheat	1.82% (C) to 1.76 - 5.49% (T) 3.52% (C) to 2.92 – 4.17% (T) 0.57% (C) to 0.56 – 0.99% (T) 7.44% (C) to 4.92 – 13.6% (T)	N/A	72.5 – 96 mg 150 - 168 mg 118 – 195 mg 60 – 61 mg
Fidler et al. (2004) Switzerland	Women (n=10) 20-26 years	Wheat cereal (C) Wheat cereal (T)	4.1% 10.8 – 18.8%	0.6 mg	84

(T): Test meal was treated to reduce phytate content

(C): Control test meal without any treatment or addition of fortifiers

Iron absorption (%) that is reported in range indicates more than one treatment were used for each test meal in the specified study

Layrisse et al. (2000); Mendoza et al. (2001); Davidsson et al. (2001); Hurrell et al. (2002); Fidler et al. (2004)

According to a recent review by Beck et al. (2014), there are inconsistencies in findings related to non-haem iron absorption and that iron absorption is affected by different elements in the diet itself. For example, mineral bioavailability has been shown to be impaired in the presence of phytates that are found in high proportion, particularly in oat products (Larsson, Rossander-Hulthén, Sandström, & Sandberg, 1996). The phosphate component in *Myo*-inositol (1,2,3,4,5,6)-hex*akis*- phosphoric acid, which is negatively charged under specific physiological conditions is reported to be capable of forming iron-phytate complexes with cations of minerals such as zinc, calcium or magnesium, including iron (Nielsen et al., 2013), which results in chelation of the cations, causing interference in iron absorption, especially in the intestines where the pH is neutral, compared to the acidic environment in the stomach where the complex can still be absorbed (Schlemmer, Frølich, Prieto, & Grases, 2009). As of the most potent inhibitors of iron absorption besides polyphenols (Siriporn, Kunchit, Christophe, & Emorn, 2006), phytates can be found widely in plant-based food sources such as cereals and legumes (Schlemmer et al., 2009). Varied in its amounts, phytates can be found at levels of between 0.05 – 3.29% in ready-made cereal products, except instant oat porridge, which was used as test meal in the present study. Instant oat porridge has been analysed to contain a lower amount of phytates at approximate levels of 0.5 – 0.68% (Reddy, 2002). It was proposed that a molar ratio of <1:1 of phytates: iron in a meal will counteract the inhibition effect of phytates on iron absorption, even though it was shown that at a ratio as low as 0.2:1, phytates can still interfere with the absorption of iron (Hurrell et al., 2002).

Cook, Reddy, Burri, Juillerat, and Hurrell (1997) also supported the link between phytate content and fractional iron absorption, by demonstrating a substantial inverse association between these 2 parameters from a cereal-based meal ($r=-0.801$ with $p<0.02$). It was also demonstrated in previous studies (Table 5.2) that fractional iron absorption (%) was generally increased when the test meals were either treated with a process to reduce phytate content, or by adding certain enhancers such as ascorbic acid to enhance the absorption. For instance, a study by Hurrell, Reddy, Juillerat, and Cook (2003) using different sources of cereal-based meals (oat, rice, maize, wheat, wheat-soy, sorghum) established that the removal of a phosphatase group by a process known as dephytinitisation that reduce phytate content aids in improving iron absorption by at least 2-fold to a maximum of 12-fold.

The fractional iron absorption reported between control and treated test meal were 1.73 to 5.34% (rice); 0.33 to 2.79% (oat); 1.8 to 8.92% (maize), 0.99 to 11.54% (wheat); 0.94-1.52% to 1.26-3.1% (different type of sorghums); and 1.15 to 3.75% (wheat-soy), showing significantly higher absorption ratios for all test meals except for one type of sorghum. It was also found that the highest iron absorption (11.5%) was exhibited with the lowest proportion of phytates from the treated wheat group compared to control (0.99%), establishing that reduced amount of phytates helps in increasing the fractional iron absorbed.

Most of the specified studies in Table 5.2 reported a small amount of native iron, ranging between 0.6 – 3.4 mg per test meal and it is observed that the treated test meals, either with degradation of phytates content or addition of other enhancers/fortifiers to offset the effect of phytates in cereal-based meals showed a higher fractional iron absorption compared to the control test meal. However, no means of measuring phytates nor native iron content were carried out in the present study, which future research should take into account so that a fair comparison can be made in terms of the effect of phytates or native iron in the test meal.

5.2.3.2 Host-related factors

According to a review by Gibson (2007), host-related factors could be branched into 2 different elements of (a) systemic aspects which include age, nutritional & physiological status; and (b) intestinal aspects which may have a substantial effect on iron digestion and uptake. The latter factor is controlled in the present study by excluding women who have a history of gastro-intestinal related illness, however, the individual's systemic requirement is a difficult aspect to control, except by using a reference iron dose. The use of a reference iron dose which corrects inter-variability between participants' iron status enables comparisons to be made between different studies. To reduce the confounding factors, fractional iron absorption in the current study was normalised to 40% using a published equation (Bjorn-Rasmussen et al., 1977), as participants with depleted iron storage have been shown to have an average iron absorption of 40% (Hallberg & Hulthén, 2000).

A potential explanation as to why a lower iron absorption was observed in the present study compared to other studies, based on host-related factors, with particular relevance to nutritional status, could be due to the fact that the majority of the participants (60%) in the present study were not anaemic nor iron deficient, based on haemoglobin and plasma ferritin concentrations.

This is supported by the low mean (±S.D) absorption of the reference iron dose (25.36 ± 18.27%) compared to previous studies that reported a higher absorption of iron from both test meals and reference dose (Table 5.1). The iron status of the participants may be one of the reasons that influence iron absorption, which is consistent with findings reported in previous studies. In a study carried out by Derman et al. (1977), it was reported that the mean absorption from an iron reference dose was 50.5%, indicating that majority of the participants were deficient, therefore the iron requirement was high (Kuhn, Monsen, Cook, & Finch, 1968), leading to a higher iron absorption. Thankachan et al. (2008) measured iron absorption from a rice meal, comparing iron deficiency anaemia (IDA) and iron replete participants, and demonstrated that iron absorption was, in fact, showing a magnitude towards a higher absorption in the IDA group (7.1%) compared to control group (3.5%) ($p=0.26$) suggesting that iron status may have an impact on the level of iron absorbed and is dependent on physiological requirements. This inverse association between body iron storage and iron absorption has also been demonstrated by a large number of previous iron absorption studies, implying that the need for iron depends on individuals' ferritin concentration.

However, unlike the previous studies, in addition to measuring absorption using iron stable isotopes and iron status biomarkers such as plasma ferritin concentration, the peptide hormone plasma hepcidin concentration was also measured in the present study in order to examine its role in regulating iron homeostasis. It has previously been suggested that hepcidin is associated with regulation of iron uptake, by internalising ferroportin, causing it to be unavailable to transport iron into the blood stream (Nemeth & Ganz, 2006). A significant inverse association between hepcidin concentrations and iron absorption has been reported in few studies carried out to date, in various settings, with different test meals used.

Our finding that iron absorption and plasma hepcidin concentrations are inversely associated is consistent with previous studies, even though a different test meal was used. Young et al. (2009) demonstrated similar associations (r value not provided) when sweet potato meal ($p=0.038$) and ferrous sulphate supplements ($p=0.0296$) were used. A study carried out by Zimmermann et al. (2009) to investigate the relationship between hepcidin and iron absorption using 3 different iron compounds labelled in a rice meal observed a significant inverse association between hepcidin and ferrous sulphate ($r=-0.46$, $p<0.001$) and ferrous fumarate ($r=-0.44$, $p<0.001$). An inverse association was also observed, in healthy male subjects who consumed fortified cereal products ($r=-0.55$, $p<0.001$) in a different study by Roe et al. (2009).

It should be noted that, to the best of our knowledge, there are limited studies that measure hepcidin concentration in iron absorption studies. The present study was the only iron absorption study carried out in the UK population investigating the effect of tea consumption, assessing two different time intervals, on iron absorption which measured plasma hepcidin concentration, hence limited comparison can be made with existing published evidence.

5.2.4 Clinical implications of findings

On the strength of the findings of the present study, it may be practical to advise the at-risk groups of iron deficiency to allow at least 1-hour time interval between tea and meal consumption. This is to avoid probability of complex formation between tannin in tea and iron in meal which may render iron absorption, and ultimately improves the iron uptake leading to better iron status overall. In a country where drinking tea is considered to be a norm, if not tradition, with 67% of the population older than 10 years reported to consume an average of 3 cups of tea daily (Khokhar & Magnusdottir, 2002), these findings should be critically addressed. In consequence, this findings, consistent with existing body of literature, may have major implication especially when tea is not only widely consumed in the UK (Nelson & Poulter, 2004), but extensively consumed worldwide.

The clinical aspects of the findings in the present study may be particularly applied to iron deficient at-risk groups or in areas where iron deficiency is prevalent and daily iron intake is predominantly from plant-based sources. Even though the western diet, or in particular, the UK diet, did not predominantly consist of plant-based sources as the main component, a European Prospective Investigation into Cancer and Nutrition (EPIC)-Oxford cohort study showed that 33% (n=15459) of the adults aged ≥ 20 years recruited in the study were vegetarian. A higher proportion of the vegetarians were women (n=11519) as opposed to men (n=3940), with 60% of the women vegetarian were in the 20-39 years age group and 41% had university degree or equivalent (Crowe, Appleby, Allen, & Key, 2011). Premenopausal women are known as one of the at-risk groups of iron deficiency, and it was showed that vegetarian trend is growing popular in the UK adults especially young premenopausal women, hence the importance of findings demonstrated in our study.

It is well-established that with iron deficiency, the iron demands are physiologically high, and the implication on the iron absorption, or iron status in general, would be even more significant as drinking tea with the meal will intensify the inhibition effect. The consumption of orange juice, which is known to be high in vitamin C with the meal may be one of the alternatives as a means of enhancing iron absorption in addition to allowing at least a 1-hour time interval.

The DRV of iron dietary intake for women was recommended as 14.8 mg/day taking into consideration the menstrual losses, but with the assumption that there will be 15% of iron absorbed from dietary-based sources (SACN, 2010). However, the present study demonstrated that the overall iron absorption from a porridge meal was no higher than 6%. Cereal and cereal-based products has been reported to be the most consumed breakfast by UK women in the NDNS population data (Bates et al., 2014). Geissler and Singh (2011) in the review on iron argued that the DRV proposed for dietary iron intake was on the higher side, and the recommendation was generated by not considering the adaptive response, and based on short-duration studies. The authors suggested that re-appraisals of the DRV are required (Geissler & Singh, 2011), and it would be practical for policy makers related to nutrition to take into account the strength of findings of the present study. The present study also demonstrated that a 1-hour time interval between meal and tea consumption is a successful strategy to counteract the tea inhibitory effect on iron absorption, therefore, the findings are beneficial and can be utilised as one of the ways to maximise iron absorption, in addition to the existing general advice recommended by the WHO in the report on the management of IDA (WHO/UNICEF/UNU, 2001). The finding may be crucially useful and can be incorporated as part of dietary advice at healthcare level to improve quality of diet in general, or in particular on how to ensure optimal iron absorption.

It will be particularly useful in population that is prone to iron deficiency, to avoid the possibility to advance into the use of iron supplements as means of controlling iron deficiency which is known to associate with various adverse events and low compliance. Additionally, the findings can be particularly beneficial in preventing the development of iron deficiency or anaemia, by alerting the healthy population on the effect of consuming tea simultaneously with meal.

Tea consumption, however, should not be completely omitted in individual's dietary intake as tea has been demonstrated in various studies to have health benefits, for instance, in the prevention of cardiovascular disease or cancer and also its potential role as antioxidants (Vuong, 2014). Additionally, tea has been found to be clinically useful in thalassemia patients, for the management of iron overload (de Alarcon, Donovan, Forbes, Landaw, & Stockman III, 1979), despite its potent inhibitory effect in iron absorption.

The links between iron absorption, iron stores (plasma ferritin concentrations) and iron regulator (plasma hepcidin concentrations) demonstrated in the first part of the research (Chapter 3), is connected to the hypothesis formulated in the second part of the research (Chapter 4).

The second part of the research was designed to address the iron utilisation aspect, by measuring iron status of iron deficient women including the concentration of plasma hepcidin that is known for its role as systemic iron regulator. It is evident in the body of literature that ascorbic acid is capable of increasing iron absorption, and vitamin D has recently been demonstrated to have a similar potential, but with an unclear underpinning mechanism. Hepcidin is known for its role in regulating iron metabolism, and the role of vitamin D is recently postulated to occur by suppression of hepcidin expression (Smith et al., 2016a), however, little evidence is available especially from human trials. The main research hypothesis of the second study was to investigate the effect of daily vitamin D3 supplementation, as a potential novel iron absorption enhancer, on the iron status recovery of women with low iron stores.

The study was an 8-week double-blind randomised controlled trial, in which participants were required to consume vitamin D3 or placebo with iron-fortified breakfast cereals and iron status was assessed at week 0, 4 and 8. The key findings of the present study support the study hypothesis and verify the role of vitamin D in iron regulation, by demonstrating a conclusive effect of vitamin D3 in improving participants' iron status, indicated by increased in haemoglobin concentrations and haematocrit levels. It was also demonstrated that the upregulation of vitamin D binding protein, which is the carrier of the circulating form of vitamin D, led to improvement in participants' iron status biomarkers. The supporting mechanism that connects the vitamin D, upregulation of vitamin D binding protein and improvement of iron status is unclear, but the information gathered is sufficient to warrant future investigations.

5.3 EXPERIMENTAL STUDY 2 (CHAPTER 4)

The present study investigated the effect of supplementation with a high daily dose of vitamin D3 (1500 IU), consumed with an iron-fortified breakfast cereal (9 mg/day) on iron status. The study was designed to determine the effect of vitamin D as a potential novel iron absorption enhancer, as well as to assess the efficacy of using an iron-fortified breakfast cereal as a tool for improving iron status in women with marginal and low iron stores.

The study used a combination of supplementation and fortification strategies to improve iron status of iron deficient women. Improvement in the iron status was presumed to occur in both, vitamin D and placebo groups, as both groups consumed the fortified cereal, though it was hypothesised that there would be a greater improvement in the vitamin D group. To the best of our knowledge, there is no evidence from RCTs investigating the effect of vitamin D, as a potential novel iron absorption enhancer, consumed in supplement form simultaneously with iron-fortified foods, on iron status. The present study found that daily consumption of vitamin D3, with iron-fortified breakfast cereal, led to improvement in haemoglobin concentrations and haematocrit levels in women with marginal and low iron stores.

5.3.1 Effect of vitamin D supplementation, consumed with an iron-fortified breakfast cereal on participants' iron status

Overall compliance of vitamin D3 supplementation was good (93%), and not significantly different between the groups. As expected, plasma 25(OH)D concentration increased at each time point in the vitamin D group, and was significantly higher compared to the placebo group. This shows that the vitamin D3 supplements used in the study were effective in improving participants' mean ±S.D vitamin D status, from insufficient levels at baseline (35.0 ± 19.8 nmol/l) to sufficient levels at post-intervention (62.2 ± 16.1 nmol/l) based on the threshold proposed by the IOM (Ross et al., 2011). Interestingly, there was a significantly lower mean (±S.D) plasma 25(OH)D concentration at post-intervention (34.15 ± 23.56 nmol/l) compared to 4-weeks (38.25 ± 27.10 nmol/l) in the placebo group. Recent NDNS population data reported that mean (±S.D) serum 25(OH)D concentrations in adults aged 19-64 years were 45.6 ± 22.7 (Oct-Dec) and 34.8 ± 22.9 nmol/l (Jan-Mar) (Bates et al., 2014). The plasma 25(OH)D concentrations observed in the placebo group at all time points were consistent with the NDNS data, as the study commenced in September, paused during the Christmas period, resumed in January and was complete by April.

The key finding of the present study was that vitamin D3 supplementation was found to be effective in the improvement of two key iron status indices; haemoglobin concentration and haematocrit level. Haemoglobin concentrations reflect the presence or absence of anaemia and oxygen-carrying capacity required for normal cell functions, whilst haematocrit levels indicates the proportion of circulating RBCs relative to whole blood total volume. The shifts (increase or decrease) in these two key indices of iron status are typically interrelated (George-Gay & Parker, 2003). The change in haemoglobin concentration and haematocrit levels were significantly higher in the intervention group (0.36 g/dl, 1.82%) as opposed to the placebo group (-0.28 g/dl, -0.51%). It can be observed that these two biomarkers were improved in the intervention group, whilst diminishing in the placebo group, suggesting that daily consumption of iron-fortified cereal with vitamin D supplements, compared to only iron-fortified cereal, exerts additional effect on iron absorption and iron status.

Additionally, it was observed in the present study that baseline concentrations of both haemoglobin and plasma 25(OH)D significantly influenced participants' response to the intervention. It was observed that there were larger and significant improvements in the RBC counts ($r=-0.534$, $p=0.0001$), haematocrit levels ($r=-0.388$, $p=0.009$), and haemoglobin concentrations ($r=-0.486$, $p=0.001$) if the participants were anaemic at baseline. The mean (±S.D) baseline concentration of haemoglobin was 13.4 ± 1.4 g/dl, and 1/8 of the participants were anaemic based on the WHO threshold of <12 g/dl (WHO/UNICEF/UNU, 2001). A greater improvement in these biomarkers may have been observed with a higher proportion of anaemic participants at baseline, however, it was evident in the present study that the baseline concentration of haemoglobin dictates the participants' response to the intervention. The mean (±S.D) baseline concentration of plasma 25(OH)D was also found to influence the response to intervention. Greater improvement was observed in the RBC counts ($r=0.339$, $p=0.0001$), haematocrit levels ($r=0.199$, $p=0.018$), and haemoglobin concentrations ($r=0.208$, $p=0.014$), if the participants were not vitamin D deficient at baseline. The mean (±S.D) baseline concentration of plasma 25(OH)D concentration was 36.8 ± 23.6 nmol/l, and 62% of the participants were vitamin D deficient based on the threshold of <30 nmol/l by the IOM (Ross et al., 2011). This shows that consumption of the iron-fortified breakfast cereal provided in the present study, with that additional vitamin D, may have led to the recovery of iron status, aided by improving participants' vitamin D status.

Interestingly, it was also observed in the present study that the improvement in plasma VDBP concentrations were strongly associated with the recovery of the RBC counts (r=0.653, p=0.002), MCV (r=0.612, p=0.004), haematocrit levels (r=0.751, p=0.0001), haemoglobin (r=0.638, p=0.002) and MCH concentrations (r=-0.592, p=0.006). This suggests a possible indirect connection of the potential role of vitamin D as an iron absorption enhancer, as VDBP is known as its role as the 25(OH)D transporter in the vitamin D metabolism. VDBP is a carrier protein that functions as the 25(OH)D transporter, and approximately 90% of 25(OH)D or 1,25(OH)D are reported to be bound to VDBP, with the remaining 10% normally bound to albumin, and 1% as the free form (Chun et al., 2014). The upregulation in the concentration of plasma VDBP may have contributed in the mechanism of action of plasma 25(OH)D in the enhanced participants' iron status observed in the current study.

In the present study, it was observed that anaemia (13%) and iron deficiency (61%) was present in the participants with plasma 25(OH)D concentrations <30 nmol/l at baseline, which is the threshold that the present study used to define VDD. As anticipated, the proportion of VDD participants, was halved at post-intervention. Supporting the existing literature from various observational studies that demonstrated concurrent incidence of both anaemia and vitamin D deficiency (VDD) in the same individuals (Jin et al., 2013; Sharma et al., 2015; Shin & Shim, 2013; Sim et al., 2010) (detail is discussed in Section 3.1.3.1, page 126), the reduction in VDD prevalence in the present study also led to slightly reduced in anaemia prevalence (12%). This observation may have also supported the role of vitamin D in improving iron status in participants in the present study. However, the mechanism of action of vitamin D on iron regulation is unclear.

It was hypothesised in the present study that the action of vitamin D on the recovery of iron status occurs via the mechanism by which vitamin D suppresses plasma hepcidin expression, which leads to increase in ferroportin availability for iron uptake and ultimately increases plasma ferritin concentration in participants with low iron stores. The hypothesis was based on a recent *in vivo* study by Bacchetta et al. (2013) which observed that single bolus oral ingestion of 100 000 IU vitamin D2 led to significant decrease in serum hepcidin concentrations at 24 (34%, p<0.05) and 72 hours (33%, p<0.01). It was also observed in the same study that vitamin D had a potent effect of on transcription of the *HAMP*, the gene that regulates hepcidin expression.

However, in the present study, no significant effect of vitamin D supplementation on hepcidin response was observed, despite the non-significant decrease observed in the vitamin D group at post-intervention. It was noted in the present study that plasma hepcidin concentrations measured were lower than the normal range suggested by Ganz et al. (2008) for a healthy population, however, this is expected as the participants recruited in the study were iron deficient. Serum hepcidin concentrations were reported to be undetectable in 18-19 iron deficient patients in the study carried out by Ganz et al. (2008) who developed and validated the first ELISA assay specific for hepcidin. The non-significant observation is postulated to contribute to the fact that insufficient dose of vitamin D3 was used in the present study. Bolus high dose of vitamin D2 used in the pilot study by Bacchetta et al. (2013) was probably sufficient to suppress the hepcidin expression, as opposed to 1500 IU dose utilised in the study, hence, the non-significant impact observed in the plasma hepcidin concentrations. A specific dose of 1500 IU vitamin D3 was used in the present study as the study carried out by Toxqui et al. (2013), which to the best of our knowledge, was the only study that was carried out using a similar study design have used a low vitamin D dose of 200 IU that was incorporated in the fortified foods, and did not include the measurement of plasma hepcidin concentration in the study.

The concentration of plasma ferritin, which is used to signify iron stores in the present study was found to be similar at post-intervention relative to baseline, following the intervention indicating no improvement was observed in recuperating participants' iron stores. Despite the non-significant recovery of iron stores, mean (±S.D) plasma ferritin concentration showed an increasing trend in the intervention group from baseline (14.07 ± 7.68 µg/l) to post-intervention (15.95 ± 10.80 µg/l), and participants were no longer deficient at the end of study period, if ferritin threshold of <15 µg/l by WHO is used (WHO/UNICEF/UNU, 2001). The improvement in plasma ferritin concentration was higher, but not significant in the intervention group (1.87 µg/l), as opposed to placebo group (1.46 µg/l). The present study included participants with a wide range of iron stores background (plasma ferritin concentration < 20 µg/l), which extends from marginally low to low iron stores, which may have contributed to the non-significant observation in the plasma ferritin concentration. The threshold used in the present study was justified based on studies that defined marginal ID as between 12-20 µg/l (Ahmed, Coyne, Dobson, & McClintock, 2008; DellaValle & Haas, 2011).

However, additional analysis carried out in sub-sample of participants who had plasma ferritin concentration of < 15 µg/l at baseline, revealed only a slight increase, (albeit non-significant) from baseline to post-intervention in both groups. This may indicate that the severity of ID did not affect the response of iron stores to the intervention.

Hepcidin and ferritin are the two principal iron biomarkers that are important in iron metabolism, and have been demonstrated to be well-associated (Ganz et al., 2008). Both of these biomarkers were not affected by the vitamin D intervention in the present study, however, it was observed that there was a significant and strong positive association between the increase in plasma ferritin and hepcidin concentrations (r=0.605, p=0.0001). This observation indicates that replenishment in the iron stores, signified by plasma ferritin concentration, is linear with the increase in plasma hepcidin concentration, to regulate intestinal iron uptake and avert the presence of iron overload. The non-significant observation in both biomarkers may be interrelated, and it may have not been sufficient for the vitamin D used in the present study to act on both biomarkers on a short study duration of 8-weeks, to allow the effect to be substantially observed.

Due to paucity in well-designed intervention studies, especially RCTs investigating a possible mechanism linking vitamin D and iron metabolism, it is still unclear whether the influence of vitamin D on iron regulation occurred as a result of suppressed *HAMP* gene expression which controls hepcidin expression, as postulated and demonstrated in a study by Bacchetta et al. (2013). As previously discussed in Section 1.8.2.5, the mechanism of action of vitamin D in exerting additional effect on the recovery on iron status was proposed to revolve around suppression of hepcidin expression, pro-inflammatory cytokine production, and rate of erythropoiesis.

Under normal circumstances, iron homeostasis involves the circulation of transferrin-iron complexes that move to the bone marrow to produce red blood cells in erythropoiesis. The senescent erythrocytes will degenerate and will be engulfed by macrophages. Iron is then reutilised and released back into the circulation to repeat the same erythropoiesis process (Smith et al., 2016a). Depending upon physiological demands, haem and non-haem dietary iron will enter the labile iron pool from intestinal iron uptake. When there are increased concentrations of pro-inflammatory cytokines, production of the RBCs in the bone marrow is suppressed. This will then lower half-life of RBCs as a result of elevated macrophages and phagocytic activity activation.

IL-6 and IL-1β are among the cytokines that are capable of stimulating the liver into increasing production of the *HAMP* gene, which leads to increased or decreased iron uptake (Smith et al., 2016a). Vitamin D has been demonstrated in previous studies to be capable of increasing proliferation of erythroid precursors in the bone marrow to support erythropoiesis by decreasing the expression of pro-inflammatory cytokines which cause the suppression of hepcidin. Decreased cytokines and suppressed hepcidin leads to higher iron bioavailability for RBC production and haemoglobin synthesis (Smith et al., 2016a). **Figure 5.1** illustrates the proposed mechanism on the effect of vitamin D supplementation on iron homeostasis.

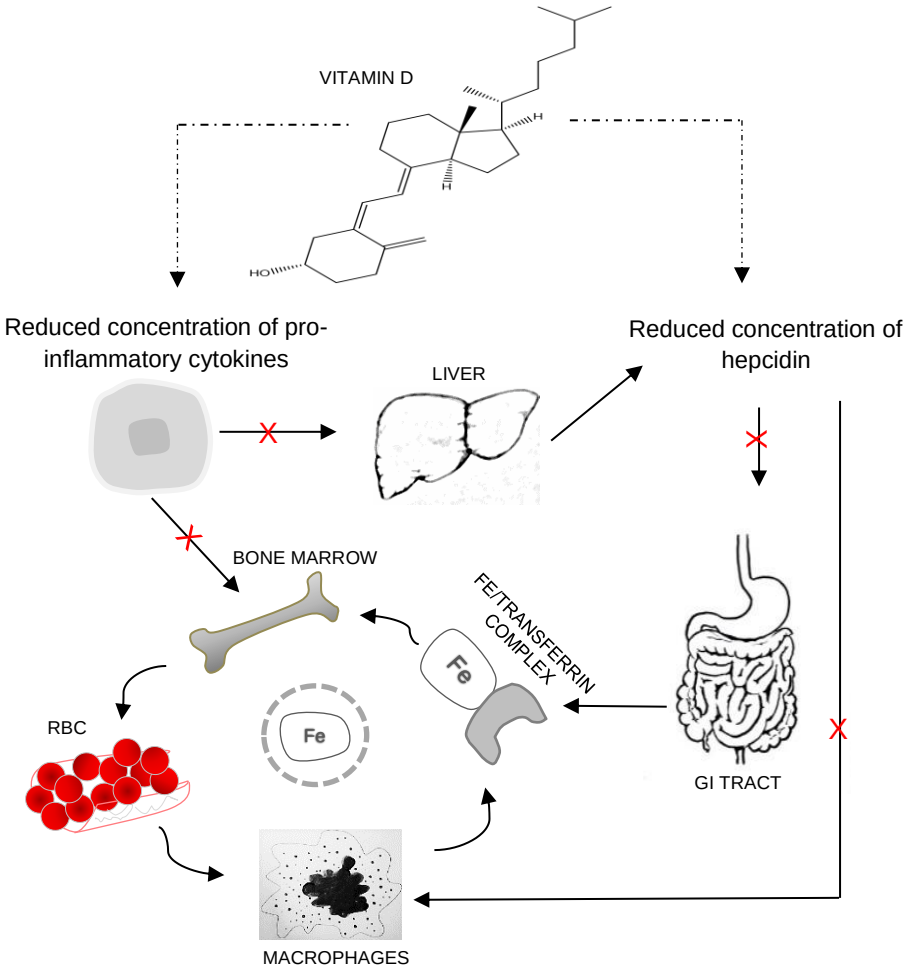


Figure 5.1 **Proposed mechanism of vitamin D action on iron metabolism - adapted from Smith et al. (2016a)**

In the present study, there was no change in the participants' energy, macronutrients, iron, and vitamin D intake at baseline and post-intervention, which shows that participants did not modify their daily intake during the intervention period to iron-rich or vitamin D-rich food selections that may have influenced the findings. The mean (±S.D) daily iron intake, in particular, was 1.7-fold higher at baseline (16.5 ± 2.3 mg), relative to typical iron intake of adult women reported in the NDNS (9.6 ± 3.0 mg), and remained similar at post-intervention (17.2 ± 2.6 mg), suggesting good compliance of iron-fortified breakfast consumption, as reflected in the food diary provided at the beginning and end of study period. Despite the non-significant increase in mean daily iron intake, it was observed that the overall iron intake at post-intervention, irrespective of groups, was 116% of the UK RNI. This implies that 9 mg added iron in the breakfast cereal provided in study may have improved participants' dietary iron intake, as the recent NDNS population data reported an iron intake of approximately 9.6 mg daily in adult women which approximates to only 65% of RNI.

Findings in the present study show that iron-fortified breakfast cereal consumption can lead to iron status recovery, by 5 components of full blood count indices, observed within the groups. The significant improvement in MCH was observed within both vitamin D and placebo groups, but the improvement in the haemoglobin, haematocrit, RBC, MCH and MCHC was observed only within the intervention group. However, none of the improvements observed in iron status biomarkers measure in the present study were associated with mean daily iron intake. Findings in the present study that showed significant improvement in haemoglobin concentrations and haematocrit levels following vitamin D supplementation is consistent with findings reported in a study by Toxqui et al. (2013) which investigated the effect of iron and vitamin D-fortified skimmed milk consumption on iron status in Spanish women with low iron status. The participants were randomised to receive 500 ml/day of iron/vitamin D fortified skimmed milk (Fe/D group) or iron-fortified skimmed milk (Fe group). No significant difference was observed in the increase of haemoglobin concentrations between the groups at post-intervention relative to baseline. However, at interim (week-8), mean (±S.D) haemoglobin concentration and haematocrit levels were significantly higher in the Fe/D group (13.3 ± 0.8 g/dl and 39.5 ± 2.3%) compared to the Fe group (13.0 ± 0.7 g/dl and 38.7 ± 2.2%) ($p<0.05$), respectively.

The study was a longer 16-week controlled trial, however, the significant increase in haemoglobin concentration and haematocrit levels in the Fe+D group were observed at the interim point of the study (8-week), which is total duration of the present study. The study found significantly higher mean (±S.D) RBC in the Fe+D group (4.41 ± 0.28 x 10^{12}/L) as opposed to the Fe group (4.28 ± 0.29 x 10^{12}/L), at interim point, however, the RBC increase in the present study observed in the vitamin D group showed distinct trend towards significance (p=0.055). Despite a longer study duration in comparison to the present study, the study did not show a significantly higher mean (±S.D) plasma ferritin concentration at post-intervention in the Fe+D group (23.8 ± 11.3 µg/l), compared to the Fe group (24.3 ± 12.0 µg/l) (Toxqui et al., 2013), which is also comparable to the findings in the present study. In spite of disparity in the iron and vitamin D amount used in the fortified food vehicle, study duration, and type of fortification food vehicle used in both studies, the findings were found to be comparable, particularly in relation to the fact that the significant impact of vitamin D was observed as early as at 8-week point, which was the interim point in the study which is equal to post-intervention in the present study.

A study by Madar et al. (2016) also carried out over period of 16 weeks observed no significant improvement in plasma ferritin or haemoglobin concentrations following vitamin D3 supplementation. The study was carried out in 251 adults, randomised into 3 groups of vitamin D3 (1000 IU), vitamin D3 (400 IU) and placebo. No significant effect of the intervention was observed between the vitamin D and placebo groups, on all iron status biomarkers including serum ferritin and haemoglobin concentrations. Similar non-significant observations were demonstrated when both groups of vitamin D3 were analysed as pooled (1000 and 400 IU) or separately in comparison to the placebo group (Madar et al., 2016). The study recruited healthy adults of both genders, as opposed to the present study which included ID participants who may have greater reaction to iron therapy, due to their high physiological demands of iron. The study also did not include any iron intervention as opposed to the present study, hence, the difference in the observation. However, despite the differences, the study demonstrated no significant association between the increase in plasma 25(OH)D concentrations with any improvement in iron status biomarkers, which is in agreement with the present study.

A study carried out by Smith et al. (2016a) applied a similar study design as Bacchetta et al. (2013) to investigate the potential role of vitamin D in iron homeostasis using a bolus dose of vitamin D3 (250 000 IU) in 28 adults, but included not only plasma hepcidin concentrations, but also the measure of plasma pro-inflammatory cytokines. It was observed that plasma hepcidin concentration (geometric mean, 95% CI) significantly reduced by 73% at post-intervention in the vitamin D group (2.4, 0.8-7.4 ng/ml) compared to the placebo group (9.0, 4.8-16.7 ng/ml) ($p=0.04$), consistent with the observation from Bacchetta et al. (2013) study. However, no significant increase in plasma ferritin or cytokine concentrations was observed between the groups (Smith et al., 2016a), indicating that vitamin D may directly affect hepcidin expression independent to the presence of inflammation (Smith et al., 2016a). Differences in the type of vitamin D supplements (daily vs bolus at 1 occasion), sample size (50 vs 7-28 participants), participants' iron status background (ID vs healthy), and the higher dose (1500 IU vs 100 000-250 000 IU) used in Smith et al. (2016a) and Bacchetta et al. (2013) studies as opposed to the present study may have contributed to the difference in observed rate of hepcidin response following vitamin D supplementation. In addition, both of the specified studies, compared to the present study that was carried out in a specific population of women with low iron stores, measured the acute effect of a very high dose of vitamin D supplementation in a set of healthy participants as a pilot study. Despite the promising observation on the action of vitamin D on hepcidin suppression, which supports the hypothesised mechanism, it was suggested by Smith et al. (2016a) that a long-term study should be carried out to further validate the findings.

The present study included the measurement of plasma PTH and VDBP concentrations as novel aspects of the study, aimed to investigate the potential underlying mechanism on vitamin D metabolism. Interestingly, the present study found that increased plasma VDBP concentrations were associated with improvement in iron status biomarkers. As yet, there is no existing evidence to support the findings and the link is still unclear. However, a cross-sectional study including data of 2073 male and female adults aged > 25 years who were recruited as part as population-based Canadian osteoporosis cohort study (Dastani et al., 2014) observed a weak, but significant association between plasma 25(OH)D and VDBP concentrations ($r=0.19$, $p<0.001$). The connection observed in the Dastani et al. (2014) study is expected as VDBP is the carrier protein of 25(OH)D which is the active metabolite of vitamin D.

Based on those findings, in combination with observations in the present study, it was hypothesised that a significant effect may have been observed if a longer duration was applied in the present study for the upregulation of plasma VDBP concentrations to have an effect on the circulating 25(OH)D concentrations, and ultimately lead to greater improvement in iron status biomarkers. PTH was the only vitamin D biomarker that showed significant within-group changes following vitamin D supplementation. The mean (±S.D) concentrations decreased to 6.81 ± 7.03 pmol/l (interim) and 7.24 ± 7.84 pmol/l (post-intervention) from 7.77 ± 8.11 (baseline) in the intervention group, indicating good response to the vitamin D supplementation. However, the change was not associated with any improvement in the iron status biomarkers, which may have suggested that PTH is not involved in iron status regulation, via vitamin D mechanism of action. The findings in the present study are consistent with the findings of Madar et al. (2016) who did not observe any association between plasma PTH concentrations with the baseline concentration of haemoglobin and plasma ferritin. It was postulated that a decrease in haemoglobin concentration which caused a lower prevalence of anaemia, will only occur via the action of vitamin D if there is a very high concentration of PTH, which will normally be found in patients with bone marrow fibrosis (Madar et al., 2016). It was noted in the present study that plasma PTH concentrations were only slightly higher than the normal threshold at baseline and post-intervention in both groups, and this may not be sufficiently high enough to affect participants' iron status biomarkers. Memon et al. (2013) carried out a cross-sectional study which included data of 10750 participants in the USA aged > 18 years with a history of diabetes and hypertension, to compare the association between serum PTH and haemoglobin concentrations in diabetic and non-diabetic patients. It was observed that higher concentrations of serum PTH were associated with higher concentration of haemoglobin. This improvement in haemoglobin concentration was however, significant only in participants with diabetes (0.1. g/dl, 95% CI: 0.01-0.14, $p<0.0001$) but not in non-diabetics (0.01 g/dl, 95 CI: -0.03-0.05, $p=0.83$) (Memon et al., 2013). This observation may be explained by the fact that production of the erythropoietin hormone is impaired in diabetic patients, proceeding to development of chronic kidney disease, hence the higher anaemia prevalence (Memon et al., 2013).

The significant finding observed within-group in the present study in the mean (±S.D) haemoglobin concentration from 13.60 ± 1.15 g/dl (baseline) to 13.84 ± 0.98 g/dl (post-intervention) in the vitamin D group, independent of intervention, following consumption of iron-fortified cereal is consistent with findings from a 6-month randomised controlled trial carried out in children by Barbosa, Taddei, Palma, Ancona-Lopez, and Braga (2012). The 24-week double-blind randomised controlled study was carried out in 324 Brazilian children aged 2-6 years, randomised into 2 groups to consume iron-fortified rolls with ferrous sulphate (FS group) or unfortified rolls (control group). It was observed in the study that the increase in haemoglobin concentration was significantly higher at post-intervention in both groups, but no effect of intervention on iron status was observed. Mean (±S.D) haemoglobin concentration was reported to increase to 12.6 ± 1.1 g/dl (FS group) and 12.3 ± 1.1 g/dl (control group) at post-intervention compared to baseline concentration of 11.7 ± 1.0 g/dl and 11.1 ± 1.1 g/dl in FS and control groups, respectively. The study also demonstrated that participants with lower baseline haemoglobin concentration (Hb < 11 g/dl), exhibited a greater improvement in haemoglobin concentration, and is similar to that demonstrated in the present study. The increase in haemoglobin concentration (2 g/dl) was greater in participants with low haemoglobin concentration, as opposed to participants with a higher haemoglobin concentration in the FS group (0.6 g/dl) (p=0.0001). A similar observation was demonstrated in the control group, as the increase was higher in in participants with low haemoglobin concentration (2.1 g/dl), as opposed to participants with a higher haemoglobin concentration (0.5 g/dl) (Barbosa et al., 2012). It should, however be noted that the study is not comparable with the present study in terms of the population used, study duration and methods used to measure haemoglobin concentrations as the study used capillary blood samples as opposed to venous blood samples in the present study.

It was previously demonstrated in a meta-analyses by Casgrain et al. (2012) that participants' response to iron intervention vastly depends on their initial iron status, and that the improvement is greater in the anaemic participants, as the iron requirement is higher due to physiological demands. Additionally, a meta-analysis carried out in pool of 18 trials that investigated the effect of iron-fortified foods on haemoglobin concentration in children concluded that a longer duration of children receiving the fortified foods may result in a higher haemoglobin concentration (Athe, Rao, & Nair, 2014).

Gera et al. (2012) demonstrated that overall iron status was improved following iron fortification intervention, analysed in a meta-analysis carried out with the inclusion of 60 iron fortification intervention studies. However, there was significant heterogeneity in the included trials, observed in the improvement of these following outcomes; serum ferritin (I^2=95.6, p<0.001), transferrin saturation (I^2=85, p<0.001), sTfR (I^2=98.4, p<0.001), and TIBC (I^2=81.4, p<0.001), except for serum iron (I^2=41.6, p=0.128). This indicates inconsistencies in the improvement of iron status biomarkers observed in those 60 trials included in the meta-analysis. The index of heterogeneity (I^2) of 75% is classified as high heterogeneity (Higgins, Thompson, Deeks, & Altman, 2003). Post-hoc sub analysis carried out in 33 of the included RCTs demonstrated that haemoglobin concentration had greater improvement in cereal-based studies, and is consistent with findings in the present study which used iron-fortified breakfast cereal. Lower response to haemoglobin concentration was however observed when the analyses included only adults (Gera et al., 2012).

Existing literature investigating the effect of iron-fortified foods on iron status has yielded inconsistent findings. **Table 5.3** summarises recent iron fortification studies that were carried out in various settings, reporting significant or non-significant improvement in iron status biomarkers, in comparison to the present study.

Table 5.3 Recent iron fortification studies using cereal and cereal-based products

Study	Population / Mean age (±SD) or range (years)	Intervention/Duration	Main findings	Remarks
[1]	ID women (n=50) / 27.4 ± 9.4 years	Iron-fortified cereal 8 weeks	Mean (±S.D) Hb concentration was significantly higher at post-intervention in the vitamin D group (13.84 ± 0.98 g/dl) compared to placebo group (12.83 ± 1.25 g/dl) ($p<0.05$).	Present study
[2]	Children (n=112) / 6-12 years	Iron-fortified biscuits (high or low iron) 4 months	Mean (±S.D) Hb concentration was significantly higher at post-intervention in the high iron group (10.52 ± 0.81 g/dl) compared to low iron group (10.03 ± 0.73 g/dl) ($p<0.01$).	The study showed Hb concentration can be increased in 4 months, but the present study showed that Hb concentration is increased at shorter duration of 8 weeks. The high iron group received 30 mg of iron/serving of biscuits, whilst iron-fortified cereal used in the present study consist of 9 mg iron/serving and the study included only measurement of Hb to evaluate iron status.
[3]	Children (n=47) / 3-6 years	A: Fortified biscuit (FS) B: Fortified biscuit (HIC) C: Placebo biscuit 10 weeks	Mean (±S.D) Hb concentration was significantly higher at post-intervention in A (14.9 ± 0.2 g/dl) and B (14.7 ± 0.3 g/dl) compared to placebo group (15.3 ± 0.3 g/dl) ($p<0.05$).	The study recruited the participants in group C as non-anaemic to make comparison with anaemic participants in group A and B, hence the higher Hb concentration at post-intervention. None of the other iron status biomarkers measured in the study (RBC, MCV, MCH, MCHC, and SF) was affected by the intervention, comparable to the present study. The study is limited with small sample size in each group.

[4]	Adolescents (n=71) / 16-19 12 weeks	Iron-fortified or unfortified breakfast cereals	Mean (±S.D) SF concentration was significantly higher at post-intervention in the intervention group (22.1 ± 16.7 µg/l) compared to unfortified group (18.4 ± 11.6 µg/l) ($p<0.001$).	The study found no effect of intervention on the other iron biomarkers including Hb, Hct and MCV, contrary to the present study. The participants in the study was not ID as opposed to the present study, but was recruited based on riboflavin status (EGRAC > 1.4 and Hb < 13.7 g/dl). The significant observation reported in the study may be due to a longer study duration, as opposed to 8 weeks in the present study.

*[1] Present study (Chester, UK), [2] Bal, Nagesh, Surendra, Chiradoni, and Gomathy (2015) (India), [3] Quintero-Gutiérrez, González-Rosendo, Pozo, and Villanueva-Sánchez (2016) (Mexico), [4] Powers, Stephens, Russell, and Hill (2016) (United Kingdom)

Hb: haemoglobin; SF: serum ferritin; RBC: red blood cell; MCV: mean corpuscular volume: MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; FS: ferrous sulphate; HIC: haem iron concentrate; EGRAC: erythrocyte glutathione reductase activation coefficient

5.3.2 Clinical implications of findings

Iron deficiency in the UK is currently 16% in premenopausal women with vitamin D deficiency prevalence of 8% during summer months and up to 39% during winter months in adults, therefore, these findings are clinically relevant. It was demonstrated in the present study that concurrent incidence of both VDD and ID exists in women with low iron stores, and vitamin D therapy (1500 IU) was shown to be effective in the recovery of both nutritional deficiencies to the normal levels. The recent UK SACN report on vitamin D proposed (i) a recommended threshold to define VDD (< 25 nmol/l) and (ii) reference nutrient intake of vitamin D (10 µg or 400 IU/day) for the general population aged > 4 years.

The present study demonstrated that with a 3.8-fold higher dose than the newly proposed RNI, vitamin D status of participants was improved from deficient to sufficient levels at the end of study, and with that improvement in vitamin D status, participants' iron status was also improved. This shows the necessity of vitamin D supplementation, in addition to a healthy balanced diet with iron-rich foods, in the general population, or specifically for at-risk groups of ID. On the strength of findings, a higher RNI for vitamin D deserves consideration, taking into account the prevalence of ID also. The findings in this present study are of great importance at the healthcare level as the utilisation of vitamin D supplements may lead to the recovery of iron status but also vitamin D status. These findings have wider implications, especially in the countries where the concurrent incidence of vitamin D and iron deficiencies are common, and it was demonstrated in the present study that there was a concurrent incidence of both nutritional deficiencies.

The present study was one of the few investigations carried out addressing the subject matter, and findings showed that not only iron status was improved following vitamin D supplementation, but also vitamin D status. In the UK, iron has been mandatorily added to white and brown flour since 1953 as a means of fortification (SACN, 2010), and with the novel insight of the role of vitamin D in upregulation of iron status, it may clinically useful if the most consumed foods by at-risk groups in the population are mandatorily or voluntarily fortified with both vitamin D and iron. This could be a potential alternative to therapeutic management of ID using oral supplements that will normally cause gastrointestinal discomfort and other adverse events, leading to unsuccessful iron status recovery. A longitudinal study carried out in 140 women age 20-55 years living in Surrey, UK showed that vitamin D deficiency is more prevalent in Asian women as opposed to Caucasian women across all 4

seasonal variations. The prevalence is particularly higher during winter season, with 80.8% in Asian women as opposed to 10% in Caucasian women (Darling et al., 2013), indicating that the magnitude of this nutritional problem is greater in specific population.

The clinical aspects of findings in the present study may be particularly applied to the recovery of iron status in iron deficient population, through the use of vitamin D supplementation as a novel iron absorption enhancer. The recently proposed RNI of 400 IU for vitamin D, however, was derived from RCTs that were focused on the prevention of risk to develop musculoskeletal health consequences. It was shown in the present study a higher dose of 1500 IU of vitamin D did not lead to replenished iron stores, thus, a higher RNI should be considered to take into account deficiency in iron. This is important not only for premenopausal women who require extra iron due to physiological demands and menstrual losses, but to young children and adolescents who needs iron for growth, cognitive and motor developments who are also prone to incidence of ID and VDD.

The findings from the current study showed that iron-fortified breakfast cereals, which have been reported in the NDNS to contribute to the highest proportion of mean daily iron intake, are efficient in improving participants' iron status. This could be a suitable alternative in the management of ID as opposed to the oral iron therapy that has poor adherence due to adverse gastrointestinal events, and vitamin D supplementation has not been shown to be associated with any adverse events in the present study or any existing body of literature. Vitamin D may be incorporated in the strategies of managing ID, and enriching diets with vitamin D, as an iron absorption enhancer, can be integrated in the dietary advice for general population at health care levels.

5.4 LIMITATIONS OF STUDY

5.4.1 Experimental study 1 (Chapter 3)

The present study was carried out in a cohort of healthy premenopausal women aged between 19-37 years, who were not regularly consuming nutritional supplements and did not report any known gastro-intestinal or metabolic illness which may interfere with iron absorption. Even though there was a small sample size in the present study, it was a controlled metabolic study and sufficiently powered. However, a higher number of participants may have yielded a better representation of the population. Women of childbearing-age were recruited for the present study as it has been suggested that this specific age group is an at-risk group that is susceptible to iron deficiency, due to combined factors of poor dietary intake, high physiological requirement, and menstrual losses (Murray-Kolb & Beard, 2007). Future studies should include an additional iron deficient (plasma ferritin concentration < 15 µg/l) group, as plasma ferritin concentration has been shown to affect the rate of iron absorption. In addition, measuring plasma hepcidin concentration will be useful to establish the mechanism of how iron is regulated.

The present study took place in controlled laboratory conditions and may not represent the actual dietary pattern of free-living participants. Future research could use a longer intervention period which includes specific frequency of tea consumption, consumed alongside typical dietary intake at household level, however, nutrient interactions may not be able be accounted for as a mixed diet contains combinations of potential enhancers or inhibitors. In addition, the present study included only 2 time intervals of tea consumption; simultaneously and 1-hour with the test meal, due to time and resource constraints. Future research should include more time intervals, possibly up to 2 or 3 hours, to investigate whether the iron absorption will continuously increase with longer time intervals. Despite its limitation, findings of the present study are suffice to initiate further investigations, particularly in the aspect of variability of time intervals.

5.4.2 Experimental study 2 (Chapter 4)

The initial power calculation yielded a sample size of 31 participants/group to show a significant effect of intervention on iron status recovery. Due to time constraint, only 50 eligible participants were recruited (out of 62 who were eligible but did not agree to partake) to enrol in the study. However, it should be noted that the screening phase involved 186 women carried out in short period of time. The study did not include a specific measure of compliance for the iron-fortified breakfast cereal consumption (e.g; returning empty cereal container), however, the 3-day food diary provided at baseline and post-intervention returned by participants showed that the provided cereal were all consumed at breakfast, as suggested. The present study did not utilise the gold method (liquid chromatography-tandem mass spectrometry) in the measurement of the vitamin D active metabolite, circulating 25(OH)D concentrations, due to time and resources constraints. An automated immunoanalyser with ready-to-use reagent strips and commercially available ELISA kits were used in the present study. However, the automated analyser used for the measurement of vitamin D is accredited with Vitamin D External Quality Assessment Scheme (DEQAS), to ensure that the measurement is corroborated with quality controls. In addition, findings in the present study may be not be able to be generalised to another study population, especially in the area where iron and vitamin D are not prevalent and cereal is not the most consumed breakfast to be suitable as a food fortification vehicle. However, the major strength of present study is the double-blind randomised controlled trial study design, carried out in a specific population of marginally low and low iron stores, which allows interpretation of findings to be extrapolated in clinical settings. The present study also measured vitamin D status biomarkers in order to provide information on the mechanism of action which was not measured in previous intervention studies, and included a measure of dietary intake to account for confounding factors that may affect the findings.

Future studies should be carried out on a larger scale with a longer study duration, focusing on the at-risk groups such as children, pregnant, non-pregnant women and using a higher vitamin D3 dose in supplement form, to be able to observe a substantial effect on iron stores and hepcidin response. The inclusion of more specific vitamin D status biomarkers such as calcitriol should be considered in designing future studies.

5.5 FUTURE PERSPECTIVES OF STUDY

Future studies should focus on investigations of iron absorption from the whole diet, administered for a longer duration to take into account adaptation responses and allowing a more accurate representative of absorption. Single meal studies have been demonstrated to have a pronounced effect on iron absorption (Armah, Carriquiry, Sullivan, Cook, & Reddy, 2013), however, the controlled laboratory environment method employed in the present study enabled monitoring of potential confounding factors that may affect iron absorption.

With respect to iron absorption studies, future work should not only focus on dietary elements, but also non-dietary elements that may affect absorption, such as infection, or host-related factors such as body fat in relation to hepcidin expression. The investigation from the second part of the research is one of the few studies carried out to the best of our knowledge investigating the potential role of vitamin D in iron status regulation. Future directions should involve establishing the underpinning mechanism on the action of vitamin D on iron homeostasis, and the inclusion of additional vitamin D status biomarkers may improve the understanding of the mechanism. The investigations should address all the at-risk groups that are susceptible to both ID and VDD, across the world as these nutritional deficiencies are not only prevalent in the UK, but worldwide despite different implementations of various strategies to manage the issues.

5.6 CONCLUSION

It can be concluded in the first part of the research that tea containing polyphenol can be distinctly regarded as a potent inhibitor of non-haem iron absorption, and it is also evident that a 1-hour time interval between a cereal-based meal and tea consumption will counteract the inhibitory effect of tea on iron absorption. It was demonstrated in the present study that plasma hepcidin concentration was associated with iron absorption, strengthening the findings from previous studies that demonstrated the role of hepcidin as an iron regulator in iron homeostasis.

The second part of the research concluded that breakfast cereal, used as a fortification vehicle, consumed daily as part of normal diet, is effective in the recovery of iron status indices. The key finding was that vitamin D, consumed daily in supplement form, at a dose of 1500 IU, was shown to be a novel potential iron absorption enhancer, lead to the recovery of haemoglobin concentrations and haematocrit levels in women with marginal and low iron stores. The finding in the

present study that shows baseline concentration of haemoglobin plays a role in dictating participants' response to intervention strengthens the existing published literature. Both studies carried out as part of the research have provided new insights on how manipulating dietary elements can lead to improved iron absorption and overall iron status in premenopausal women. **Figure 5.2** summarises both studies carried out, including outcomes and clinical implications of the research.

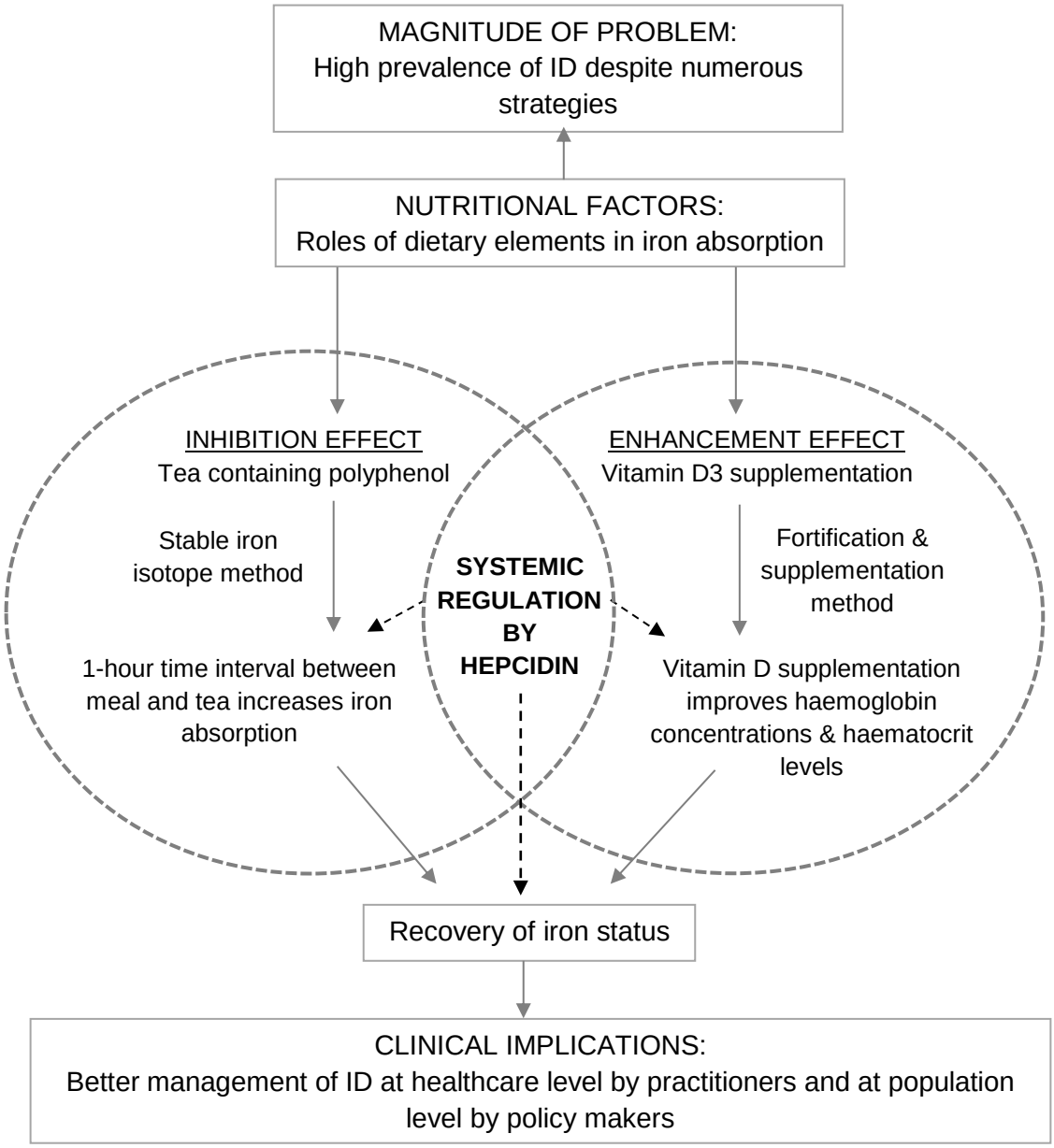


Figure 5.2 Overall overview, outcomes and clinical implications of the studies

Findings in both studies will be applicable for a nutrition-related practitioners or governing institutions for the purpose of:

(i) prevention: dissemination of information to the general population on how to maximise iron absorption from the diet by manipulating the specified dietary components, and to maintain normal iron status;

(ii) management and treatment: prescribing diet with vitamin D, to the individual with ID and VDD at healthcare levels in order to recover normal iron and vitamin D status; and

(iii) regulation: reconsidering the RNI for both iron and vitamin D, taking into account the bioavailability (iron) and not only musculoskeletal health consequences but also ID (vitamin D). Regulation also includes the implementation of fortified iron/vitamin D food items to address the concurrent incidence of these nutritional deficiencies.

CHAPTER 6

References

REFERENCES

Abbaspour, N., Hurrell, R., & Kelishadi, R. (2014). Review on iron and its importance for human health. *Journal of Research in Medical Sciences, 19*(2), 164-174.

Abizari, A.-R., Moretti, D., Schuth, S., Zimmermann, M. B., Armar-Klemesu, M., & Brouwer, I. D. (2012). Phytic acid-to-iron molar ratio rather than polyphenol concentration determines iron bioavailability in whole-cowpea meal among young women. *The Journal of Nutrition, 142*(11), 1950-1955. doi:10.3945/jn.112.164095

Adams, J. S., & Hewison, M. (2010). Update in vitamin D. *The Journal of Clinical Endocrinology & Metabolism, 95*(2), 471-478.

Ahmed, F., Coyne, T., Dobson, A., & McClintock, C. (2008). Iron status among Australian adults: findings of a population based study in Queensland, Australia. *Asia Pacific Journal of Clinical Nutrition, 17*(1), 40-47.

Aisen, P., Enns, C., & Wessling-Resnick, M. (2001). Chemistry and biology of eukaryotic iron metabolism. *The International Journal of Biochemistry & Cell Biology, 33*(10), 940-959.

Andersson, M., Theis, W., Zimmermann, M. B., Foman, J. T., Jäkel, M., Duchateau, G. S., . . . Hurrell, R. F. (2010). Random serial sampling to evaluate efficacy of iron fortification: a randomized controlled trial of margarine fortification with ferric pyrophosphate or sodium iron edetate. *The American Journal of Clinical Nutrition, 92*(5), 1094-1104.

Andrews, N. C. (1999). Disorders of iron metabolism. *New England Journal of Medicine, 341*(26), 1986-1995.

Andrews, N. C. (2005). Understanding Haem Transport. *The New England Journal of Medicine, 353*(23), 2508-2509. doi:10.1056/NEJMcibr053987

Andrews, N. C., & Schmidt, P. J. (2007). Iron homeostasis. *Annu. Rev. Physiol., 69*, 69-85.

Armah, S. M., Carriquiry, A., Sullivan, D., Cook, J. D., & Reddy, M. B. (2013). A complete diet-based algorithm for predicting non haem iron absorption in adults *The Journal of Nutrition, 143*(7), 1136-1140.

Aspuru, K., Villa, C., Bermejo, F., Herrero, P., & López, S. G. (2011). Optimal management of iron deficiency anemia due to poor dietary intake. *Int J Gen Med, 4*, 741-750.

Athe, R., Rao, M. V. V., & Nair, K. M. (2014). Impact of iron-fortified foods on haemoglobin concentration in children (< 10 years): a systematic review and meta-analysis of randomized controlled trials. *Public Health Nutrition, 17*(03), 579-586.

Bacchetta, J., Zaritsky, J. J., Sea, J. L., Chun, R. F., Lisse, T. S., Zavala, K., . . . Hewison, M. (2013). Suppression of iron-regulatory hepcidin by vitamin D. *Journal of the American Society of Nephrology, 25*, 564-572. doi:10.1681/asn.2013040355

Bæch, S. B., Hansen, M., Bukhave, K., Jensen, M., Sørensen, S. S., Kristensen, L., . . . Sandström, B. (2003). Nonheme iron absorption from a phytate-rich meal is increased by the addition of small amounts of pork meat. *The American Journal of Clinical Nutrition, 77*(1), 173-179.

Bal, D., Nagesh, K., Surendra, H., Chiradoni, D., & Gomathy, G. (2015). Effect of supplementation with iron fortified biscuits on the haemoglobin status of children in rural areas of Shimoga, Karnataka. *The Indian Journal of Pediatrics, 82*(3), 253-259.

Barbosa, T. N. N., Taddei, J. A. d. A. C., Palma, D., Ancona-Lopez, F., & Braga, J. A. P. (2012). Double-blind randomized controlled trial of rolls fortified with microencapsulated iron. *Rev Assoc Med Bras, 58*(1), 118-124.

Bates, B., Lennox, A., Prentice, A., Bates, C., Page, P., Nicholson, S., & Swan, G. (2014). *The National Diet and Nutrition Survey. Results from Years 1,2,3 and 4 (combined) of the Rolling Programme (2008/2009-2011/2012).* London, United Kingdom: TSO.

Beck, K., Conlon, C. A., Kruger, R., Coad, J., & Stonehouse, W. (2011). Gold kiwifruit consumed with an iron-fortified breakfast cereal meal improves iron status in women with low iron stores: a 16-week randomised controlled trial. *British Journal of Nutrition, 105*(1), 101-109.

Beck, K. L., Conlon, C. A., Kruger, R., & Coad, J. (2014). Dietary determinants of and possible solutions to iron deficiency for young women living in industrialized countries: a review. *Nutrients, 6*(9), 3747-3776.

Bendich, A. (2001). Calcium supplementation and iron status of females. *Nutrition, 17*(1), 46-51. doi:10.1016/S0899-9007(00)00482-2

Benito, P., & Miller, D. (1998). Iron absorption and bioavailability: An updated review. *Nutrition Research, 18*(3), 581-603. doi:dx.doi.org/10.1016/S0271-5317(98)00044-X

Berber, I., Diri, H., Erkurt, M. A., Aydogdu, I., Kaya, E., & Kuku, I. (2014). Evaluation of Ferric and Ferrous Iron Therapies in Women with Iron Deficiency Anaemia. *Advances in Hematology, 2014*, 1-6. doi:10.1155/2014/297057

Bikle, D. D. (2014). Vitamin D metabolism, mechanism of action, and clinical applications. *Chemistry & Biology, 21*(3), 319-329.

Bjorn-Rasmussen, E., Hallberg, L., & Rossander, L. (1977). Absorption of fortification iron. Bioavailability in man of different samples of reduced iron, and prediction of the effects of iron fortification. *Br. J. Nutr, 37*, 375-388.

Blanco-Rojo, R., Pérez-Granados, A. M., Toxqui, L., González-Vizcayno, C., Delgado, M. A., & Vaquero, M. P. (2011). Efficacy of a microencapsulated iron pyrophosphate-fortified fruit juice: a randomized, double-blind, placebo-controlled study in Spanish iron-deficient women. *The British Journal of Nutrition, 105*(11), 1652. doi:10.1017/S0007114510005490

Blanco-Rojo, R., Pérez-Granados, A. M., Toxqui, L., Zazo, P., de la Piedra, C., & Vaquero, M. P. (2013). Relationship between vitamin D deficiency, bone remodelling and iron status in iron-deficient young women consuming an iron-fortified food. *European Journal of Nutrition, 52*(2), 695-703.

Blanton, C. (2013). Improvements in iron status and cognitive function in young women consuming beef or non-beef lunches. *Nutrients, 6*(1), 90-110.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews, 56*(11), 317-333. doi:10.1111/j.1753-4887.1998.tb01670.x

Breet, P., Kruger, H. S., Jerling, J. C., & Oosthuizen, W. (2005). Actions of black tea and Rooibos on iron status of primary school children. *Nutrition Research, 25*(11), 983-994.

British Nutrition Foundation. (1995). *Iron: nutritional and physiological significance*. London, United Kingdom: Chapman & Hall.

Brouwer-Brolsma, E., Bischoff-Ferrari, H., Bouillon, R., Feskens, E., Gallagher, C., Hypponen, E., . . . Kies, A. (2013). Vitamin D: do we get enough? *Osteoporosis International, 24*(5), 1567-1577.

Brown, E., Hopper Jr, J., Hodges Jr, J., Bradley, B., Wennesland, R., & Yamauchi, H. (1962). Red cell, plasma, and blood volume in healthy women measured by radiochromium cell-labeling and hematocrit. *Journal of Clinical Investigation, 41*(12), 2182.

Bruggraber, S. F., Chapman, T. P., Thane, C. W., Olson, A., Jugdaohsingh, R., & Powell, J. J. (2012). A re-analysis of the iron content of plant-based foods in the United Kingdom. *British Journal of Nutrition, 108*(12), 2221-2228.

Brune, M., Rossander, L., & Hallberg, L. (1989). Iron absorption and phenolic compounds: importance of different phenolic structures. *European Journal of Clinical Nutrition, 43*(8), 547-557.

Camaschella, C. (2015). Iron-deficiency anemia. *N Engl J Med, 2015*(372), 1832-1843.

Canonne-Hergaux, F., Donovan, A., Delaby, C., Wang, H.-j., & Gros, P. (2006). Comparative studies of duodenal and macrophage ferroportin proteins. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 290*(1), G156-G163.

Casgrain, A., Collings, R., Harvey, L. J., Boza, J. J., & Fairweather-Tait, S. J. (2010). Micronutrient bioavailability research priorities. *The American Journal of Clinical Nutrition, 91*(5), 1423S-1429S. doi:10.3945/ajcn.2010.28674A

Casgrain, A., Collings, R., Harvey, L. J., Hooper, L., & Fairweather-Tait, S. J. (2012). Effect of iron intake on iron status: a systematic review and meta-analysis of randomised controlled trials. *The American Journal of Clinical Nutrition, 96*(4), 768-780.

Cercamondi, C. I., Egli, I. M., Zeder, C., & Hurrell, R. F. (2014). Sodium iron EDTA and ascorbic acid, but not polyphenol oxidase treatment, counteract the strong inhibitory effect of polyphenols from brown sorghum on the absorption of fortification iron in young women. *British Journal of Nutrition, 111*(3), 481-489. doi:10.1017/S0007114513002705

Christakos, S., Hewison, M., Gardner, D. G., Wagner, C. L., Sergeev, I. N., Rutten, E., . . . Bikle, D. D. (2013). Vitamin D: beyond bone. *Annals of the New York Academy of Sciences, 1287*(1), 45-58.

Chun, R. F., Peercy, B. E., Orwoll, E. S., Nielson, C. M., Adams, J. S., & Hewison, M. (2014). Vitamin D and DBP: the free hormone hypothesis revisited. *The Journal of Steroid Biochemistry and Molecular Biology, 144*, 132-137.

Clark, S. F. (2008). Iron deficiency anemia. *Nutrition in Clinical Practice, 23*(2), 128-141.

Conrad, M. E., & Umbreit, J. N. (1993). A concise review: Iron absorption—The mucin-mobilferrin-integrin pathway. A competitive pathway for metal absorption. *American journal of hematology, 42*(1), 67-73.

Cook, J. D., Reddy, M., Burri, J., Juillerat, M. A., & Hurrell, R. F. (1997). The influence of different cereal grains on iron absorption from infant cereal foods. *The American Journal of Clinical Nutrition, 65*(4), 964-969.

Cook, J. D., & Reddy, M. B. (2001). Effect of ascorbic acid intake on nonheme iron absorption from a complete diet. *The American Journal of Clinical Nutrition, 73*(1), 93-98.

Crowe, F. L., Appleby, P. N., Allen, N. E., & Key, T. J. (2011). Diet and risk of diverticular disease in Oxford cohort of European Prospective Investigation into Cancer and Nutrition (EPIC): prospective study of British vegetarians and non-vegetarians. *BMJ, 343*. doi:10.1136/bmj.d4131

Dainty, J. R., Berry, R., Lynch, S. R., Harvey, L. J., & Fairweather-Tait, S. J. (2014). Estimation of dietary iron bioavailability from food iron intake and iron status. *PLoS One, 9*(10), e111824.

Darling, A., Hart, K., Macdonald, H., Horton, K., Kang'ombe, A., Berry, J., & Lanham-New, S. (2013). Vitamin D deficiency in UK South Asian Women of childbearing age: a comparative longitudinal investigation with UK Caucasian women. *Osteoporosis International, 24*(2), 477-488.

Dastani, Z., Berger, C., Langsetmo, L., Fu, L., Wong, B. Y., Malik, S., . . . Richards, J. B. (2014). In healthy adults, biological activity of vitamin D, as assessed by serum PTH, is largely independent of DBP concentrations. *Journal of Bone and Mineral Research, 29*(2), 494-499.

Davidsson, L. (2003). Approaches to improve iron bioavailability from complementary foods. *The Journal of Nutrition, 133*(5 Suppl 1), 1560S-1562S.

Davidsson, L., Walczyk, T., Zavaleta, N., & Hurrell, R. (2001). Improving iron absorption from a Peruvian school breakfast meal by adding ascorbic acid or Na2EDTA. *The American Journal of Clinical Nutrition, 73*(2), 283.

de Alarcon, P. A., Donovan, M.-E., Forbes, G. B., Landaw, S. A., & Stockman III, J. A. (1979). Iron absorption in the thalassemia syndromes and its inhibition by tea. *New England Journal of Medicine, 300*(1), 5-8.

De Domenico, I., Ward, D. M., & Kaplan, J. (2009). Specific iron chelators determine the route of ferritin degradation. *Blood, 114*(20), 4546-4551.

DellaValle, D. M., & Haas, J. D. (2011). Impact of iron depletion without anemia on performance in trained endurance athletes at the beginning of a training season: a study of female collegiate rowers. *International Journal of Sport Nutrition and Exercise Metabolism, 21*(6), 501-506.

DeLuca, H. F. (2004). Overview of general physiologic features and functions of vitamin D. *The American Journal of Clinical Nutrition, 80*(6), 1689S-1696S.

Department of Health. (1991). *Dietary Reference Values for food energy and nutrients for the United Kingdom: report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy.* (Vol. 41). London, United Kingdom: H.M.S.O.

Derman, D., Sayers, M., Lynch, S. R., Charlton, R. W., Bothwell, T. H., & Mayet, F. (1977). Iron absorption from a cereal-based meal containing cane sugar fortified with ascorbic acid. *The British Journal of Nutrition, 38*(2), 261-269.

Detivaud, L., Nemeth, E., Boudjema, K., Turlin, B., Troadec, M. B., Leroyer, P., . . . Loreal, O. (2005). Hepcidin levels in humans are correlated with hepatic iron stores, hemoglobin levels, and hepatic function. *Blood, 106*(2), 746-748. doi:10.1182/blood-2004-12-4855

Disler, P. B., Lynch, S. R., Charlton, R. W., Torrance, J. D., Bothwell, T. H., Walker, R. B., & Mayet, F. (1975a). The effect of tea on iron absorption. *Gut, 16*(3), 193-200. doi:10.1136/gut.16.3.193

Disler, P. B., Lynch, S. R., Torrance, J. D., Sayers, M. H., Bothwell, T. H., & Charlton, R. W. (1975b). The mechanism of the inhibition of iron absorption by tea. *S Afr J Med Sci, 40*(4), 109-116.

Donovan, A., Lima, C. A., Pinkus, J. L., Pinkus, G. S., Zon, L. I., Robine, S., & Andrews, N. C. (2005). The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell Metabolism, 1*(3), 191-200.

Dufresne, C. J., & Farnworth, E. R. (2001). A review of latest research findings on the health promotion properties of tea. *The Journal of Nutritional Biochemistry, 12*(7), 404-421.

Ernst, J. B., Tomaschitz, A., Grübler, M. R., Gaksch, M., Kienreich, K., Verheyen, N., . . . Zittermann, A. (2016). Vitamin D supplementation and hemoglobin levels in hypertensive patients: a randomized controlled trial. *International Journal of Endocrinology, 2016*. doi:dx.doi.org/10.1155%2F2016%2F6836402

Fairweather-Tait, S. J. (2001). Iron. *The Journal of Nutrition, 131*(4 Suppl), 1383S-1386S.

Fairweather-Tait, S. J. (2004). Iron nutrition in the UK: getting the balance right. *Proceedings of the Nutrition Society, 63*(4), 519-528. doi:10.1079/PNS2004394

Fairweather-Tait, S. J., & Collings, R. (2010). Estimating the bioavailability factors needed for setting Dietary Reference Values. *International Journal for Vitamin and Nutrition Research, 80*(4), 249-256.

FAO/WHO. (2001). *Food based approaches to meeting vitamin and mineral needs. In:human vitamin and mineral requirements.* Rome, Italy: Food And Nutrition Division (FAO).

Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. (2007). G* Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods, 39*(2), 175-191.

Ferreira da Silva, L., Dutra-de-Oliveira, J. E., & Marchini, J. S. (2004). Serum iron analysis of adults receiving three different iron compounds. *Nutrition Research, 24*(8), 603-611. doi:10.1016/j.nutres.2003.10.013

Fidler, M. C., Davidsson, L., Zeder, C., & Hurrell, R. F. (2004). Erythorbic acid is a potent enhancer of nonheme iron absorption. *The American Journal of Clinical Nutrition, 79*(1), 99-102.

Finch, C. (1994). Regulators of iron balance in humans. *Blood, 84*(6), 1697-1702.

Fleming, M. D., Romano, M. A., Su, M. A., Garrick, L. M., Garrick, M. D., & Andrews, N. C. (1998). Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport. *Proceedings of the National Academy of Sciences, 95*(3), 1148-1153.

Food Standards Agency. (2002). *McCance and Widdowson's The Composition of Foods* (Sixth Summary Edition ed.). Royal Society of Chemistry, Cambridge, United Kingdom.

Fuqua, B. K., Vulpe, C. D., & Anderson, G. J. (2012). Intestinal iron absorption. *Journal of Trace Elements in Medicine and Biology, 26*(2), 115-119.

Galan, P., Yoon, H., Preziosi, P., Viteri, F., Valeix, P., Fieux, B., . . . Favier, A. (1998). Determining factors in the iron status of adult women in the SU. VI. MAX study. *European Journal of Clinical Nutrition, 52*(6), 383-388.

Ganz, T. (2011). Hepcidin and iron regulation, 10 years later. *Blood, 117*(17), 4425-4433.

Ganz, T. (2013). Systemic iron homeostasis. *Physiological Reviews, 93*(4), 1721-1741.

Ganz, T., Olbina, G., Girelli, D., Nemeth, E., & Westerman, M. (2008). Immunoassay for human serum hepcidin. *Blood, 112*(10), 4292-4297. doi:10.1182/blood-2008-02-139915

Gardner, E. J., Ruxton, C. H. S., & Leeds, A. R. (2006). Black tea - helpful or harmful? A review of the evidence. *Eur J Clin Nutr, 61*(1), 3-18.

Gavin, M. W., McCarthy, D. M., & Garry, P. J. (1994). Evidence that iron stores regulate iron absorption-a setpoint theory. *The American Journal of Clinical Nutrition, 59*(6), 1376-1380.

Geissler, C., & Singh, M. (2011). Iron, meat and health. *Nutrients, 3*(3), 283-316. doi:10.3390/nu3030283

George-Gay, B., & Parker, K. (2003). Understanding the complete blood count with differential. *Journal of Peri Anesthesia Nursing, 18*(2), 96-117.

Gera, T., Sachdev, H. S., & Boy, E. (2012). Effect of iron-fortified foods on hematologic and biological outcomes: systematic review of randomized controlled trials. *The American Journal of Clinical Nutrition, 96*(2), 309-324. doi:10.3945/ajcn.111.031500

Gibson, S. (2007). The role of diet and host-related factors in nutrient bioavailability and thus in nutrient-based dietary requirement estimates. *Food and Nutrition Bulletin, 28*(suppl 1), S77-S100.

Gibson, S., & Ashwell, M. (2003). The association between red and processed meat consumption and iron intakes and status among British adults. *Public Health Nutrition, 6*(4), 341-350. doi:10.1079/PHN2002442

Gomme, P. T., & Bertolini, J. (2004). Therapeutic potential of vitamin D binding protein. *Trends in Biotechnology, 22*(7), 340-345.

Green, R., Charlton, R., Seftel, H., Bothwell, T., Mayet, F., Adams, B., . . . Layrisse, M. (1968). Body iron excretion in man: a collaborative study. *The American Journal of Medicine, 45*(3), 336-353.

Gregory, J., Foster, K., Tyler, H., & Wiseman, M. (1990). *The Dietary and Nutritional Survey of British adults: a survey of the dietary behaviour, nutritional status and blood pressure of adults aged 16 to 64 living in Great Britain*. London, United Kingdom: HMSO.

Gulec, S., Anderson, G. J., & Collins, J. F. (2014). Mechanistic and regulatory aspects of intestinal iron absorption. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 307*, G397-G409.

Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., . . . Hediger, M. A. (1997). Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature, 388*(6641), 482-488.

Hallberg, L., & Hulthén, L. (2000). Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron. *The American Journal of Clinical Nutrition, 71*(5), 1147-1160.

Hallberg, L., Rossander-Hulthèn, L., Brune, M., & Gleerup, A. (1993). Inhibition of haem-iron absorption in man by calcium. *British Journal of Nutrition, 69*(02), 533-540.

Haslam, E. (2003). Thoughts on thearubigins. *Phytochemistry, 64*(1), 61-73.

Heath, A.-L. M., Skeaff, C. M., Williams, S., & Gibson, R. S. (2001). The role of blood loss and diet in the aetiology of mild iron deficiency in premenopausal adult New Zealand women. *Public Health Nutrition, 4*(2), 197-206. doi:10.1079/PHN200054

Heeney, M. M., & Finberg, K. E. (2014). Iron-refractory iron deficiency anemia (IRIDA). *Hematology/Oncology Clinics of North America, 28*(4), 637-652.

Henderson, L., Irving, K., Gregory, J., Bates, C. J., Prentice, A., Perks, J., . . . Farron, M. (2003). *The National Diet & Nutrition Survey: adults aged 19 to 64 years. Volume 3: Vitamin and mineral intake and urinary analytes.* London, United Kingdom: TSO.

Hennigar, S. R., Gaffney-Stomberg, E., Lutz, L. J., Cable, S. J., Pasiakos, S. M., Young, A. J., & McClung, J. P. (2016). Consumption of a calcium and vitamin D-fortified food product does not affect iron status during initial military training: a randomised, double-blind, placebo-controlled trial. *British Journal of Nutrition, 115*(04), 637-643.

Henry, H. L. (2011). Regulation of vitamin D metabolism. *Best Practice & Research Clinical Endocrinology & Metabolism, 25*(4), 531-541.

Hentze, M. W., Muckenthaler, M. U., & Andrews, N. C. (2004). Balancing acts: molecular control of mammalian iron metabolism. *Cell, 117*(3), 285-297.

Hentze, M. W., Muckenthaler, M. U., Galy, B., & Camaschella, C. (2010). Two to tango: regulation of mammalian iron metabolism. *Cell, 142*(1), 24-38.

Higgins, J. P., Thompson, S. G., Deeks, J. J., & Altman, D. G. (2003). Measuring inconsistency in meta-analyses. *BMJ, 327*(7414), 557-560.

Hilger, J., Friedel, A., Herr, R., Rausch, T., Roos, F., Wahl, D. A., . . . Hoffmann, K. (2014). A systematic review of vitamin D status in populations worldwide. *British Journal of Nutrition, 111*(01), 23-45.

Hogenkamp, P. S., Jerling, J. C., Hoekstra, T., Melse-Boonstra, A., & MacIntyre, U. E. (2008). Association between consumption of black tea and iron status in adult Africans in the North West Province: the THUSA study. *British Journal of Nutrition, 100*(2), 430-437. doi:10.1017/S000711450889441X

Holick, M. F. (2008). Vitamin D: a D-Lightful health perspective. *Nutrition Reviews, 66*(suppl 2), S182-S194.

Holick, M. F., Binkley, N. C., Bischoff-Ferrari, H. A., Gordon, C. M., Hanley, D. A., Heaney, R. P., . . . Weaver, C. M. (2011). Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *The Journal of Clinical Endocrinology & Metabolism, 96*(7), 1911-1930.

Hoppe, M., Brün, B., Larsson, M. P., Moraeus, L., & Hulthén, L. (2013). Heme iron-based dietary intervention for improvement of iron status in young women. *Nutrition, 29*(1), 89-95.

Hunter, H. N., Fulton, D. B., Ganz, T., & Vogel, H. J. (2002). The solution structure of human hepcidin, a peptide hormone with antimicrobial activity that is involved in iron uptake and hereditary hemochromatosis. *Journal of Biological Chemistry, 277*(40), 37597-37603.

Hurrell, R. (1997). Preventing iron deficiency through food fortification. *Nutrition Reviews, 55*(6), 210-222. doi:10.1111/j.1753-4887.1997.tb01608.x

Hurrell, R. (2002a). Fortification: overcoming technical and practical barriers. *The Journal of Nutrition, 132*(4 Suppl), 806S-812S.

Hurrell, R. (2002b). How to ensure adequate iron absorption from iron-fortified food. *Nutrition Reviews, 60*(7), 7-15. doi:10.1301/002966402320285137

Hurrell, R., & Egli, I. (2010). Iron bioavailability and Dietary Reference Values. *The American Journal of Clinical Nutrition, 91*(5), 1461S-1467S. doi:10.3945/ajcn.2010.28674F

Hurrell, R., Reddy, M., Burri, J., & Cook, J. (2002). Phytate degradation determines the effect of industrial processing and home cooking on iron absorption from cereal-based foods. *British Journal of Nutrition, 88*(02), 117-123.

Hurrell, R., Reddy, M., & Cook, J. D. (1999). Inhibition of non-haem iron absorption in man by polyphenolic-containing beverages. *British Journal of Nutrition, 81*(4), 289-295. doi:10.1017/S0007114599000537

Hurrell, R., Reddy, M. B., Juillerat, M.-A., & Cook, J. D. (2003). Degradation of phytic acid in cereal porridges improves iron absorption by human subjects. *The American Journal of Clinical Nutrition, 77*(5), 1213-1219.

International Atomic Energy Agency. (2012). *Assesment of iron bioavailability in human using stable iron isotopes techniques* (Vol. 21). Vienna, Austria: IAEA.

International Atomic Energy Agency. (2014). *A small part can reveal the whole: how isotope techniques help nutrition*. Vienna, Austria: IAEA.

Jackson, J., Williams, R., McEvoy, M., MacDonald-Wicks, L., & Patterson, A. (2016). Is higher consumption of animal flesh foods associated with better iron status among adults in developed countries? A systematic review. *Nutrients, 8*(2), 89.

Jin, H. J., Lee, J. H., & Kim, M. K. (2013). The prevalence of vitamin D deficiency in iron-deficient and normal children under the age of 24 months. *Blood Research, 48*(1), 40-45.

Kalasuramath, S., Kurpad, A. V., & Thankachan, P. (2013). Effect of iron status on iron absorption in different habitual meals in young south Indian women. *The Indian Journal of Medical Research, 137*(2), 324.

Kaltwasser, J. P., Werner, E., Schalk, K., Hansen, C., Gottschalk, R., & Seidl, C. (1998). Clinical trial on the effect of regular tea drinking on iron accumulation in genetic haemochromatosis. *Gut, 43*(5), 699-704. doi:10.1136/gut.43.5.699

Karl, J. P., Lieberman, H. R., Cable, S. J., Williams, K. W., Young, A. J., & McClung, J. P. (2010). Randomised, double-blind, placebo-controlled trial of an iron-fortified food product in female soldiers during military training: relations between iron status, serum hepcidin, and inflammation. *The American Journal of Clinical Nutrition, 92*(1), 93-100.

Kaushansky, K., Lichtman, M., Prchal, J., Levi, M., Press, O., Burns, L., & Caligiuri, M. (2015). *Williams Haematology* (9th ed.). New York, USA: McGraw Hill Education.

Keskin, Y., Moschonis, G., Dimitriou, M., Sur, H., Kocaoglu, B., Hayran, O., & Manios, Y. (2005). Prevalence of iron deficiency among schoolchildren of different socio-economic status in urban Turkey. *European Journal of Clinical Nutrition, 59*(1), 64-71.

Khokhar, S., & Apenten, R. K. O. (2003). Iron binding characteristics of phenolic compounds: some tentative structure–activity relations. *Food Chemistry, 81*(1), 133-140.

Khokhar, S., & Magnusdottir, S. (2002). Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. *Journal of Agricultural and Food Chemistry, 50*(3), 565-570.

Knutson, M., & Wessling-Resnick, M. (2003). Iron metabolism in the reticuloendothelial system. *Critical Reviews in Biochemistry and Molecular Biology, 38*(1), 61-88.

Kuhn, I. N., Monsen, E. R., Cook, J. D., & Finch, C. A. (1968). Iron absorption in man. *Journal of Laboratory and Clinical Medicine, 71*, 715-721.

Kumar, P. J., & Clark, M. L. (2009). *Kumar & Clark Clinical Medicine* (8 ed.). Edinburgh, United Kingdom: Saunders, Elsevier.

Larsson, M., Rossander-Hulthén, L., Sandström, B., & Sandberg, A.-S. (1996). Improved zinc and iron absorption from breakfast meals containing malted oats with reduced phytate content. *British Journal of Nutrition, 76*(05), 677-688.

Latunde-Dada, G. O., Simpson, R. J., & McKie, A. T. (2006). Recent advances in mammalian haem transport. *Trends in Biochemical Sciences, 31*(3), 182-188.

Layrisse, M., García-Casal, M. N., Solano, L., Barón, M. A., Arguello, F., Llovera, D., . . . Tropper, E. (2000). Iron bioavailability in humans from breakfasts enriched with iron bis-glycine chelate, phytates and polyphenols. *The Journal of Nutrition, 130*(9), 2195-2199.

Lee, J. A., Hwang, J. S., Hwang, I. T., Kim, D. H., Seo, J.-H., & Lim, J. S. (2015). Low vitamin D levels are associated with both iron deficiency and anemia in children and adolescents. *Pediatric hematology and oncology, 32*(2), 99-108.

Lee, R. D., & Nieman, D. C. (2010). *Nutritional Assessment* (5 ed.). New York, USA: McGraw-Hill Education.

Lin, L., Valore, E. V., Nemeth, E., Goodnough, J. B., Gabayan, V., & Ganz, T. (2007). Iron transferrin regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP2/4. *Blood, 110*(6), 2182-2189.

Lopez, A., Cacoub, P., Macdougall, I. C., & Peyrin-Biroulet, L. (2016). Iron deficiency anaemia. *The Lancet, 387*(10021), 907-916.

Łuczaj, W., & Skrzydlewska, E. (2005). Antioxidative properties of black tea. *Preventive Medicine, 40*(6), 910-918.

Lynch, M. F., Griffin, I. J., Hawthorne, K. M., Chen, Z., Hamzo, M. G., & Abrams, S. A. (2007). Iron absorption is more closely related to iron status than to daily iron intake in 12- to 48-mo-old children. *J Nutr, 137*(1), 88-92.

Lynch, S. R. (2011). Why nutritional iron deficiency persists as a worldwide problem. *The Journal of Nutrition, 141*(4), 763S-768S.

Madar, A. A., Stene, L. C., Meyer, H. E., Brekke, M., Lagerløv, P., & Knutsen, K. V. (2016). Effect of vitamin D3 supplementation on iron status: a randomized, double-blind, placebo-controlled trial among ethnic minorities living in Norway. *Nutrition Journal, 15*(1), 74-83.

McArthur, J. O., Petocz, P., Caterson, I. D., & Samman, S. (2012). A randomized controlled trial in young women of the effects of consuming pork meat or iron supplements on nutritional status and feeling of well-being. *Journal of the American College of Nutrition, 31*(3), 175-184.

McGillivray, G., Skull, S. A., Davie, G., Kofoed, S. E., Frydenberg, A., Rice, J., . . . Carapetis, J. R. (2007). High prevalence of asymptomatic vitamin D and iron deficiency in East African immigrant children and adolescents living in a temperate climate. *Archives of Disease in Childhood, 92*(12), 1088-1093.

McKie, A. T., Barrow, D., Latunde-Dada, G. O., Rolfs, A., Sager, G., Mudaly, E., . . . Simpson, R. J. (2001). An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science, 291*(5509), 1755-1759. doi:10.1126/science.1057206

Mehta, S. W., Pritchard, M. E., & Stegman, C. (1992). Contribution of coffee, and tea to anemia among NHANES II participants. *Nutrition Research, 12*(2), 209-222.

Memon, I., Norris, K. C., Bomback, A. S., Peralta, C., Li, S., Chen, S.-C., . . . Tamura, M. K. (2013). The association between parathyroid hormone levels and hemoglobin in diabetic and non diabetic participants in the National Kidney Foundation's Kidney Early Evaluation Program. *Cardiorenal Medicine, 3*(2), 120-127.

Mendoza, C., Viteri, F. E., Lönnerdal, B., Raboy, V., Young, K. A., & Brown, K. H. (2001). Absorption of iron from unmodified maize and genetically altered, low-phytate maize fortified with ferrous sulfate or sodium iron EDTA. *The American Journal of Clinical Nutrition, 73*(1), 80-85.

Mennen, L., Hirvonen, T., Arnault, N., Bertrais, S., Galan, P., & Hercberg, S. (2007). Consumption of black, green and herbal tea and iron status in French adults. *European Journal of Clinical Nutrition, 61*(10), 1174-1179. doi:10.1038/sj.ejcn.1602634

Milman, N. (2011). Anemia—Still a major health problem in many parts of the world! *Annals of Hematology, 90*(4), 369-377.

Milman, N., Pedersen, A. N., Ovesen, L., & Schroll, M. (2004). Iron status in 358 apparently healthy 80-year-old Danish men and women: relation to food composition and dietary and supplemental iron intake. *Annals of Haematology, 83*(7), 423-429. doi:10.1007/s00277-003-0830-y

Miret, S., Simpson, R. J., & McKie, A. T. (2003). Physiology and molecular biology of dietary iron absorption. *Annual Review of Nutrition, 23*, 283-301. doi:10.1146/annurev.nutr.23.011702.073139

Miskulin, D. C., Majchrzak, K., Tighiouart, H., Muther, R. S., Kapoian, T., Johnson, D. S., & Weiner, D. E. (2016). Ergocalciferol supplementation in hemodialysis patients with vitamin D deficiency: a randomized clinical trial. *Journal of the American Society of Nephrology, 27*(6), 1801-1810.

Morck, T. A., Lynch, S. R., & Cook, J. D. (1983). Inhibition of food iron absorption by coffee. *The American Journal of Clinical Nutrition, 37*(3), 416-420.

Murray-Kolb, L. E., & Beard, J. L. (2007). Iron treatment normalizes cognitive functioning in young women. *The American Journal of Clinical Nutrition, 85*(3), 778-787.

Naini, A. E., Hedaiati, Z. P., Gholami, D., Pezeshki, A. H., & Moinzadeh, F. (2015). The effect of Vitamin D administration on treatment of anemia in end-stage renal disease patients with Vitamin D deficiency on hemodialysis: A placebo-controlled, double-blind clinical trial. *Journal of Research in Medical Sciences, 20*(8), 745-750.

Nelson, M., & Poulter, J. (2004). Impact of tea drinking on iron status in the UK: a review. *Journal of Human Nutrition and Dietetics, 17*(1), 43-54. doi:10.1046/j.1365-277X.2003.00497.x

Nemeth, E., & Ganz, T. (2006). Regulation of iron metabolism by hepcidin. *Annu. Rev. Nutr., 26*, 323-342.

Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., Ward, D. M., . . . Kaplan, J. (2004). Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science, 306*(5704), 2090-2093. doi:10.1126/science.1104742

Nicolas, G., Chauvet, C., Viatte, L., Danan, J. L., Bigard, X., Devaux, I., . . . Vaulont, S. (2002). The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *The Journal of Clinical Investigation, 110*(7), 1037-1044.

Nielsen, A. V., Tetens, I., & Meyer, A. S. (2013). Potential of phytase-mediated iron release from cereal-based foods: a quantitative view. *Nutrients, 5*(8), 3074-3098.

Norman, A. W. (2008). From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *The American Journal of Clinical Nutrition, 88*(2), 491S-499S.

Ohgami, R. S., Campagna, D. R., Greer, E. L., Antiochos, B., McDonald, A., Chen, J., . . . Fleming, M. D. (2005). Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells. *Nature Genetics, 37*(11), 1264-1269.

Olivares, M., Pizarro, F., Hertrampf, E., Fuenmayor, G., & Estévez, E. (2007). Iron absorption from wheat flour: effects of lemonade and chamomile infusion. *Nutrition, 23*(4), 296-300. doi:10.1016/j.nut.2006.04.014

Ortiz, R., Toblli, J. E., Romero, J. D., Monterrosa, B., Frer, C., Macagno, E., & Breymann, C. (2011). Efficacy and safety of oral iron (III) polymaltose complex versus ferrous sulfate in pregnant women with iron-deficiency anemia: a multicenter, randomized, controlled study. *Journal of Maternal-Fetal and Neonatal Medicine, 24*(11), 1347-1352.

Palacios, C., Trak, M. A., Betancourt, J., Joshipura, K., & Tucker, K. L. (2015). Validation and reproducibility of a semi-quantitative FFQ as a measure of dietary intake in adults from Puerto Rico. *Public Health Nutrition, 18*(14), 2550-2558.

Pasricha, S.-R., Drakesmith, H., Black, J., Hipgrave, D., & Biggs, B.-A. (2013). Control of iron deficiency anemia in low- and middle-income countries. *Blood, 121*(14), 2607.

Pasricha, S.-R., Flecknoe-Brown, S. C., Allen, K. J., Gibson, P. R., McMahon, L. P., Olynyk, J. K., . . . Thomson, A. R. (2010). Diagnosis and management of iron deficiency anaemia: a clinical update. *Med J Aust, 193*(9), 525-532.

Pavord, S., Myers, B., Robinson, S., Allard, S., Strong, J., & Oppenheimer, C. (2012). UK guidelines on the management of iron deficiency in pregnancy. *British Journal of Haematology, 156*(5), 588-600.

Petry, N., Egli, I., Campion, B., Nielsen, E., & Hurrell, R. (2013). Genetic reduction of phytate in common bean (Phaseolus vulgaris L.) seeds increases iron absorption in young women. *The Journal of Nutrition, 143*(8), 1219-1224.

Petry, N., Ines, E., Christophe, Z., Thomas, W., & Richard, H. (2010). Polyphenols and phytic acid contribute to the low iron bioavailability from common beans in young women. *The Journal of Nutrition, 140*(11), 1977.

Petry, N., Rohner, F., Gahutu, J. B., Campion, B., Boy, E., Tugirimana, P. L., . . . Moretti, D. (2016). In Rwandese women with low iron status, iron absorption from low-phytic acid beans and biofortified beans is comparable, but low-phytic acid beans cause adverse gastrointestinal symptoms. *The Journal of Nutrition, 146*(5), 970-975.

Pinto, J. P., Ribeiro, S., Pontes, H., Thowfeequ, S., Tosh, D., Carvalho, F., & Porto, G. (2008). Erythropoietin mediates hepcidin expression in hepatocytes through EPOR signaling and regulation of C/EBPα. *Blood, 111*(12), 5727-5733.

Pizarro, F., Olivares, M., Hertrampf, E., Nuñez, S., Tapia, M., Cori, H., & Lopez de Romana, D. (2006). Ascorbyl palmitate enhances iron bioavailability in iron-fortified bread. *The American Journal of Clinical Nutrition, 84*(4), 830-834.

Powers, H. J., Stephens, M., Russell, J., & Hill, M. H. (2016). Fortified breakfast cereal consumed daily for 12 wk leads to a significant improvement in micronutrient intake and micronutrient status in adolescent girls: a randomised controlled trial. *Nutrition Journal, 15*(1), 69.

Prystai, E. A., Kies, C. V., & Driskell, J. A. (1999). Calcium, copper, iron, magnesium and zinc utilization of humans as affected by consumption of black, decaffeinated black and green teas. *Nutrition Research, 19*(2), 167-177. doi:10.1016/S0271-5317(98)00181-X

Public Health England. (2016). *The National Diet and Nutrition Survey. Results from Years 5 and 6 (combined) of the Rolling Programme (2012/2013-2013/2014).* London, United Kingdom: PHE.

Quintero-Gutiérrez, A. G., González-Rosendo, G., Pozo, J. P., & Villanueva-Sánchez, J. (2016). Haem iron concentrate and iron sulfate added to chocolate biscuits: effects on haematological indices of Mexican school children. *Journal of the American College of Nutrition, 35*(6), 544-551. doi:10.1080/07315724.2015.1060875

Ramey, G., Deschemin, J.-C., Durel, B., Canonne-Hergaux, F., Nicolas, G., & Vaulont, S. (2010). Hepcidin targets ferroportin for degradation in hepatocytes. *Haematologica, 95*(3), 501-504.

Reddy, N. (2002). Chapter 3: Occurrence, distribution, content, and dietary intake of phytate. In N. Reddy & S. Shridhar (Eds.), *Food phytates* (pp. 25-51). Florida, USA: CRC Press LLC.

Richardson, D. R., Lane, D. J., Becker, E. M., Huang, M. L.-H., Whitnall, M., Rahmanto, Y. S., . . . Ponka, P. (2010). Mitochondrial iron trafficking and the integration of iron metabolism between the mitochondrion and cytosol. *Proceedings of the National Academy of Sciences, 107*(24), 10775-10782.

Roe, M. A., Collings, R., Dainty, J. R., Swinkels, D. W., & Fairweather-Tait, S. J. (2009). Plasma hepcidin concentrations significantly predict interindividual variation in iron absorption in healthy men. *The American Journal of Clinical Nutrition, 89*(4), 1088-1091. doi:10.3945/ajcn.2008.27297

Ross, A. C., Taylor, C. L., Yaktine, A. L., & Del Valle, H. B. (2011). *Dietary Reference Intakes for calcium and vitamin D: Food and Nutrition Board*. Washington DC, USA: Institute of Medicine of the National Academies Press.

Ruxton, C., & Derbyshire, E. (2010). Women's diet quality in the UK. *Nutrition Bulletin, 35*(2), 126-137.

SACN. (2010). *Iron and Health*. London, United Kingdom: TSO.

SACN. (2016). *Vitamin D and Health*. London, United Kingdom: TSO.

Samman, S., Sandström, B., Toft, M. B., Bukhave, K., Jensen, M., Sørensen, S. S., & Hansen, M. (2001). Green tea or rosemary extract added to foods reduces nonheme iron absorption. *The American Journal of Clinical Nutrition, 73*(3), 607-612.

Santiago, P. (2012). Ferrous versus ferric oral iron formulations for the treatment of iron deficiency: a clinical overview. *The Scientific World Journal, 2012*, 846824. doi:10.1100/2012/846824

Santoro, D., Caccamo, D., Lucisano, S., Buemi, M., Sebekova, K., Teta, D., & De Nicola, L. (2015). Interplay of vitamin D, erythropoiesis, and the renin-angiotensin system. *BioMed Research International, 2015*, 145828.

Schlemmer, U., Frølich, W., Prieto, R. M., & Grases, F. (2009). Phytate in foods and significance for humans: food sources, intake, processing, bioavailability, protective role and analysis. *Molecular Nutrition & Food Research, 53*(S2), S330-S375.

Schlesier, K., Kühn, B., Kiehntopf, M., Winnefeld, K., Roskos, M., Bitsch, R., & Böhm, V. (2012). Comparative evaluation of green and black tea consumption on the iron status of omnivorous and vegetarian people. *Food Research International, 46*(2), 522-527. doi:10.1016/j.foodres.2011.06.019

Scholz-Ahrens, K. E., Schaafsma, G., Kip, P., Elbers, F., Boeing, H., & Schrezenmeir, J. (2004). Iron-fortified milk can improve iron status in young women with low iron stores. *Milk Science International, 59*(5/6), 253-257.

Sharma, S., Jain, R., & Dabla, P. K. (2015). The role of 25-hydroxy vitamin D deficiency in iron deficient children of North India. *Indian Journal of Clinical Biochemistry, 30*(3), 313-317.

Sharp, P., & Srai, S. K. (2007). Molecular mechanisms involved in intestinal iron absorption. *World Journal of Gastroenterology, 13*(35), 4716-4724.

Shaw, G. C., Cope, J. J., Li, L., Corson, K., Hersey, C., Ackermann, G. E., . . . Traver, D. (2006). Mitoferrin is essential for erythroid iron assimilation. *Nature, 440*(7080), 96-100.

Shayeghi, M., Latunde-Dada, G. O., Oakhill, J. S., Laftah, A. H., Takeuchi, K., Halliday, N., . . . Hider, R. C. (2005). Identification of an intestinal heme transporter. *Cell, 122*(5), 789-801.

Shi, H., Bencze, K. Z., Stemmler, T. L., & Philpott, C. C. (2008). A cytosolic iron chaperone that delivers iron to ferritin. *Science, 320*(5880), 1207-1210.

Shin, J. Y., & Shim, J. Y. (2013). Low vitamin D levels increase anemia risk in Korean women. *Clinica Chimica Acta, 421*, 177-180.

Silva, B., & Faustino, P. (2015). An overview of molecular basis of iron metabolism regulation and the associated pathologies. *Biochimica et Biophysica Acta, 1852*(7), 1347-1359.

Sim, J. J., Lac, P. T., Liu, I. L. A., Meguerditchian, S. O., Kumar, V. A., Kujubu, D. A., & Rasgon, S. A. (2010). Vitamin D deficiency and anemia: a cross-sectional study. *Annals of Hematology, 89*(5), 447-452.

Siriporn, T., Kunchit, J., Christophe, Z., & Emorn, W. (2006). Chili, but not turmeric, inhibits iron absorption in young women from an iron-fortified composite meal. *The Journal of Nutrition, 136*(12), 2970-2974.

Smith, E. M., Alvarez, J. A., Kearns, M. D., Hao, L., Sloan, J. H., Konrad, R. J., . . . Tangpricha, V. (2016a). High-dose vitamin D3 reduces circulating hepcidin concentrations: A pilot, randomized, double-blind, placebo-controlled trial in healthy adults. *Clinical Nutrition*. doi:dx.doi.org/10.1016/j.clnu.2016.06.015

Smith, E. M., Jones, J. L., Han, J. E., Alvarez, J. A., Sloan, J. H., Konrad, R. J., . . . Tangpricha, V. (2016b). High-dose vitamin D3 administration is associated with increases in hemoglobin concentrations in mechanically ventilated critically ill adults. A pilot double-blind, randomized, placebo-controlled trial. *Journal of Parenteral and Enteral Nutrition*. doi:dx.doi.org/10.1177/0148607116678197

Sooragonda, B., Bhadada, S. K., Shah, V. N., Malhotra, P., Ahluwalia, J., & Sachdeva, N. (2014). Effect of vitamin D replacement on hemoglobin concentration in subjects with concurrent iron-deficiency anemia and vitamin D deficiency: a randomized, single-blinded, placebo-controlled trial. *Acta hematologica, 133*(1), 31-35.

Souza, A. I. d., Batista Filho, M., Bresani, C. C., Ferreira, L. O. C., & Figueiroa, J. N. (2009). Adherence and side effects of three ferrous sulfate treatment regimens on anemic pregnant women in clinical trials. *Cadernos de Saúde Pública, 25*(6), 1225-1233.

Steele, T. M., Frazer, D. M., & Anderson, G. J. (2005). Systemic regulation of intestinal iron absorption. *International Union of Biochemistry and Molecular Biology, 57*(7), 499-503.

Tandara, L., & Salamunic, I. (2012). Iron metabolism: current facts and future directions. *Biochemia Medica, 22*(3), 311-328.

Tanno, T., Bhanu, N. V., Oneal, P. A., Goh, S.-H., Staker, P., Lee, Y. T., . . . Wang, R.-H. (2007). High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. *Nature Medicine, 13*(9), 1096-1101.

Tanno, T., Porayette, P., Sripichai, O., Noh, S.-J., Byrnes, C., Bhupatiraju, A., . . . Ganz, T. (2009). Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. *Blood, 114*(1), 181-186.

Teucher, B., Olivares, M., & Cori, H. (2004). Enhancers of iron absorption: ascorbic acid and other organic acids. *International Journal for Vitamin and Nutrition Research, 74*(6), 403-419.

Thacher, T. D., & Clarke, B. L. (2011). Vitamin D insufficiency. *Mayo Cli Proc, 86*(1), 50-60.

Thankachan, P., Muthayya, S., Walczyk, T., Kurpad, A. V., & Hurrell, R. F. (2007). An analysis of the etiology of anemia and iron deficiency in young women of low socioeconomic status in Bangalore, India. *Food and Nutrition Bulletin, 28*(3), 328-336.

Thankachan, P., Walczyk, T., Muthayya, S., Kurpad, A. V., & Hurrell, R. F. (2008). Iron absorption in young Indian women: the interaction of iron status with the influence of tea and ascorbic acid. *The American Journal of Clinical Nutrition, 87*(4), 881-886.

Toxqui, L., Pérez-Granados, A. M., Blanco-Rojo, R., Wright, I., González-Vizcayno, C., & Vaquero, M. P. (2013). Effects of an iron or iron and vitamin D–fortified flavoured skimmed milk on iron metabolism: a randomized controlled double-blind trial in iron-deficient women. *Journal of the American College of Nutrition, 32*(5), 312-320.

UNICEF/WHO. (1999). *Prevention and control of iron deficiency anemia in women and children*. Geneva, Switzerland: UNICEF Regional Office for Central and Eastern Europe/ WHO Regional Office for Europe.

van Schoor, N. M., & Lips, P. (2011). Worldwide vitamin D status. *Best Practice & Research Clinical Endocrinology & Metabolism, 25*(4), 671-680.

Vaucher, P., Druais, P.-L., Waldvogel, S., & Favrat, B. (2012). Effect of iron supplementation on fatigue in nonanemic menstruating women with low ferritin: a randomized controlled trial. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne, 184*(11), 1247-1254. doi:10.1503/cmaj.110950

Vieth, R. (2005). Chapter 61: The pharmacology of vitamin D, including fortification strategies. In D. Feldman & F. Glorieux (Eds.), *Vitamin D* (Vol. 2, pp. 995-1015): Elsevier Acad Press.

Vokurka, M., Krijt, J., Sulc, K., & Necas, E. (2006). Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. *Physiological Research, 55*(6), 667.

Vulpe, C. D., Kuo, Y.-M., Murphy, T. L., Cowley, L., Askwith, C., Libina, N., . . . Anderson, G. J. (1999). Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nature Genetics, 21*(2), 195-199.

Vuong, Q. V. (2014). Epidemiological evidence linking tea consumption to human health: a review. *Critical Reviews in Food Science and Nutrition, 54*(4), 523-536.

Vyoral, D., & Petrák, J. (2005). Hepcidin: a direct link between iron metabolism and immunity. *The International Journal of Biochemistry & Cell Biology, 37*(9), 1768-1773.

Wang, J., & Pantopoulos, K. (2011). Regulation of cellular iron metabolism. *Biochemical Journal, 434*(3), 365-381.

Wennesland, R., Brown, E., Hopper Jr, J., Hodges Jr, J., Guttentag, O., Scott, K., . . . Bradley, B. (1959). Red cell, plasma and blood volume in healthy men measured by radiochromium (Cr51) cell tagging and hematocrit: influence of age, somatotype and habits of physical activity on the variance after regression of volumes to height and weight combined. *Journal of Clinical Investigation, 38*(7), 1065.

WHO. (1998). *Obesity: preventing and managing the global epidemic : report of a WHO consultation on obesity*. Geneva, Switzerland: World Health Organization.

WHO. (2008). *Worldwide prevalence of anemia 1993-2005: WHO database on anemia*. Geneva, Switzerland: WHO Press, World Health Organization.

WHO. (2014). *Serum transferrin receptor levels for the assessment of iron status and iron deficiency in populations*. Geneva, Switzerland: World Health Organization.

WHO/CDC. (2004). *Assesing the iron status of populations : including literature reviews : report of a Joint World Health Organization/Centers for Disease Control and Prevention Technical Consultation on the Assessment of Iron Status at the Population Level*. Geneva, Switzerland: World Health Organization/Centers for Disease Control and Prevention.

WHO/UNICEF/UNU. (2001). *Iron deficiency anemia : assessment, prevention and control. A guide for programme managers.* . Geneva, Switzerland: World Health Organization.

Wienk, K., Marx, J., & Beynen, A. (1999). The concept of iron bioavailability and its assessment. *European Journal of Nutrition, 38*(2), 51-75.

Wrighting, D. M., & Andrews, N. C. (2006). Interleukin-6 induces hepcidin expression through STAT3. *Blood, 108*(9), 3204-3209.

Young, M. F., Glahn, R. P., Ariza-Nieto, M., Inglis, J., Olbina, G., Westerman, M., & O'Brien, K. O. (2009). Serum hepcidin is significantly associated with iron absorption from food and supplemental sources in healthy young women. *The American Journal of Clinical Nutrition, 89*(2), 533-538.

Zijp, I. M., Korver, O., & Tijburg, L. B. (2000). Effect of tea and other dietary factors on iron absorption. *Critical Reviews in Food Science and Nutrition, 40*(5), 371-398. doi:10.1080/10408690091189194

Zimmermann, M. B., Chaouki, N., & Hurrell, R. F. (2005). Iron deficiency due to consumption of a habitual diet low in bioavailable iron: a longitudinal cohort study in Moroccan children. *The American Journal of Clinical Nutrition, 81*(1), 115-121.

Zimmermann, M. B., Troesch, B., Biebinger, R., Egli, I., Zeder, C., & Hurrell, R. F. (2009). Plasma hepcidin is a modest predictor of dietary iron bioavailability in humans, whereas oral iron loading, measured by stable-isotope appearance curves, increases plasma hepcidin. *The American Journal of Clinical Nutrition, 90*(5), 1280-1287. doi:10.3945/ajcn.2009.28129

Zughaier, S. M., Alvarez, J. A., Sloan, J. H., Konrad, R. J., & Tangpricha, V. (2014). The role of vitamin D in regulating the iron-hepcidin-ferroportin axis in monocytes. *Journal of Clinical & Translational Endocrinology, 1*(1), e19-e25.

CHAPTER 7

Appendices

APPENDIX A

University of Chester

Iron Intake Study – We need your help!

We are looking for volunteers to take part in a PhD study investigating the effects of dietary components on iron absorption in healthy non-pregnant women aged between 19—40 years.

If you:

- ☆ Have no history of gastrointestinal and metabolic disorders
- ☆ Have not donated blood within the past 2-6 months
- ☆ Are not taking nutritional supplements such as vitamins

We would like to hear from you.

The study will involve consuming specially prepared breakfasts containing iron tracers to monitor iron absorption. Blood samples will also be collected.

For taking part in the study you will receive:

- a free dietary analysis
- information on your iron status

If you would like to take part, and for further information, please email Salma at:

ironstudy@chester.ac.uk

APPENDIX B

EMAIL OF INVITATION TO PARTICIPANTS

Dear Participants,

INVESTIGATION INTO THE INHIBITORY EFFECTS OF TEA CONSUMPTION ON IRON BIOAVAILABILITY IN A COHORT OF HEALTHY UK WOMEN, USING A STABLE IRON ISOTOPE

I am a PhD student from the Department of Clinical Sciences and Nutrition at University of Chester. I am inviting you to take part in my study which will investigate whether tea consumption effects how well we absorb iron, particularly in iron deficient "at risk" groups such as women. Iron tracers will be incorporated into meals in this study as they are one of the best methods for investigating how well iron is absorbed in human body and it is non-invasive. With the findings, I hope to use the information to help individuals with impaired iron status to maximize their iron absorption, which ultimately helps in the improvement of their iron status.

This invitation is especially for women between the ages of 19-40 years who: are healthy, non-pregnant or lactating, with no history of gastro-intestinal and metabolic disorders, have not donated blood in the past 2 to 6 months, and are not regularly taking nutritional supplements.

If you fulfil the inclusion criteria and you decide to take part in this study, you will be required to attend clinics on 6 different occasions. After an overnight fast, a baseline fasting blood sample will be drawn followed by the administration of the first test meal, in which 14 days post dosing, another blood sample will be drawn to measure the iron absorption. The same procedure will be repeated for the next clinics, where there will be another 2 test meals and a reference dose of iron.

Detailed information on the study procedure will be provided, should you be interested to take part in this study.

Yours sincerely,

Salma Ahmad
MPhil/PhD Student (Clinical Sciences and Nutrition)
ironstudy@chester.ac.uk

APPENDIX C

Participant information sheet

INVESTIGATION INTO THE INHIBITORY EFFECTS OF TEA CONSUMPTION ON IRON BIOAVAILABILITY IN A COHORT OF HEALTHY UK WOMEN, USING A STABLE IRON ISOTOPE

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being carried out and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study?

The study aims to find out whether tea consumption with or between meals will affect iron absorption in healthy childbearing-aged women. Iron tracers will be incorporated into meals in this study as they are one of the best methods for investigating how well iron is absorbed in human body and it is non-invasive. The study is being carried out to find out whether consuming tea with a meal and 1 hour after meal will decrease iron absorption among the participants.

Why have I been chosen?

You have been chosen because you satisfy the inclusion criteria of the study which includes the following : female; between the ages of 19-40 years; healthy; non-pregnant or lactating; with no history of gastro-intestinal and metabolic disorders; have not donated blood within the last 6 months and are not regularly taking nutritional supplements.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect you in any way. If you decide to withdraw at any time during the course of the study, samples collected so far may still be used in the study.

What will happen to me if I take part?

You will be asked to attend 6 clinics on 6 separate days where you will be expected to fast overnight for at least 10 hours prior to each session. There will be a time interval of 14 days between clinics, to allow the incorporation of the iron tracer into your system. Each clinic will last for approximately 45 minutes between 8 and 10 am in the morning, and participants will be asked not to consume any food or beverage 4 hours after the test meals.

Participants will attend each clinic after an overnight fast, and 30 ml blood samples (5 teaspoons) will be collected at each clinic.

Clinic 1 (Day 0): Participants will fill in a screening test questionnaire, followed by physical measurement of height and weight. If you satisfy the inclusion criteria, you will be invited to participate in the study and be asked to sign an informed consent form. Participants will be asked about their food consumption over the previous 24-hours and will be required to complete a 3-day food diary after this clinic to reflect habitual intake. Participants will be given explanation on how to record the dietary intake and be required to bring the completed 3-day diary during Clinic 2. A baseline blood sample will be drawn and this will be followed by consumption of the control test meal.

Clinic 2 (Day 14): Measurement of height and weight followed by a baseline blood sample and 24 hour food recall. This will be followed by consumption of the test meal II with tea given simultaneously with the test meal.

Clinic 3 (Day 28): Measurement of height and weight followed by a baseline blood sample and 24 hour food recall. This will be followed by consumption of the test meal III with tea given 1 hour after the test meal.

Clinic 4 (Day 42): Prior to the completion of the study, participants will be given another 3 days food diary which has to be completed before the clinic for another assessment of dietary intake. Measurement of height and weight followed by baseline blood sample and 24 hour food recall. This will be followed by consumption of the reference dose iron without any meal.

Clinic 5 (Day 56): Measurement of height and weight followed by a final blood sample drawn to measure the incorporation of iron from the reference dose and 24 hour food recall.

The test meals will be administered as breakfast, where you will be given a standardised meal consisting oat porridge with one cup of tea with semi-skimmed milk, with additional sugar and sweetener if requested.

What are the possible disadvantages and risks of taking part?
There are no disadvantages foreseen in taking part in the study, however, you may experience slight discomfort and bruising due to the blood sampling procedures. The study will take a total of 56 days which may cause you inconvenience by having to attend 6 separate clinics. Unfortunately, it will not be possible to reimburse any travel or other expenses incurred in taking part in the study.

What are the possible benefits of taking part?

There are no direct benefits from taking part in the study, however you will be provided with a full dietary analysis and an iron status assessment. Dietary analysis will be performed by the researcher on completion of the study using dietary software. The analysis will be based on the information you will have already provided us in your 3-day food diaries and 24 hour recalls during the course of the study and will thus require no further time input. We will provide information on your nutrient intake, in particular, iron intake, as well as information on your total energy, fat, carbohydrate and protein intake with a comparison to recommended values.

If the iron status assessment reveals a deficient iron status, with your consent, we will notify your GP of your involvement in the study and of any adverse findings. You will also be contributing to the development of knowledge on how to maximise iron absorption in the prevention of iron deficiency.

What if something goes wrong?

In the event of adverse findings during the course of the study, the researcher will contact your GP with permission. If you wish to complain or have any concerns about any aspect of the way you have been approached or treated during the course of this study, please contact Professor Sarah Andrew, Dean of the Faculty of Life Sciences, University of Chester, Parkgate Road, Chester, CH1 4BJ, 01244 513055.

Will my taking part in the study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential and anonymised so that only the researcher carrying out the research will have access to such information. An 8ml plasma sample will be anonymised and stored in freezer located in the Clinical Laboratory in the Department of Clinical Sciences & Nutrition at the University of Chester. The samples will be retained for further research purposes after the completion of study, which will be subject to further ethical approval.

What will happen to the results of the research study?

The results will be written up into a dissertation for my PhD thesis. Individuals who participate will not be identified in any subsequent report or publication. After the completion of the study, a sheet summarising the findings of the study will be sent to the participants via post or email.

Who is organising the research?

The research is conducted as part of a PhD research project in Human Nutrition within the Department of Clinical Sciences & Nutrition at the University of Chester. The study is organised with supervision from the department, by Salma Ahmad, a PhD student. The academic supervisor for the study is a Senior Lecturer from Department of Clinical Sciences and Nutrition, Dr Sohail Mushtaq and he can be contacted at 01244513367 or s.mushtaq@chester.ac.uk.

Who may I contact for further information?

If you would like more information about the research before you decide whether or not you would be willing to take part, please contact: Salma Ahmad at 01244511198 or email at ironstudy@chester.ac.uk.

Thank you for your interest in this research.

APPENDIX D

University of Chester

Title of Project: Investigation into the inhibitory effects of tea consumption on iron bioavailability in a cohort of healthy UK women, using a stable iron isotope.

Name of Researcher: Salma Ahmad

Please initial box

1. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my legal rights being affected. ☐

3. (If appropriate) I understand that relevant sections of my medical notes and data collected during the study may be looked by individuals from University of Chester, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. (If appropriate) I agree to my General Practitioner being informed of my participation in the study. ☐

5. I agree to the use of my blood samples in this research study. ☐

6. I agree to gift my blood samples (8 ml of plasma) to be stored and used for a period of 5 years in future similar studies which have been given a favourable ethical opinion by a Research Ethics Committee. ☐

7. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Researcher	Date	Signature

1 for participant; 1 for researcher

APPENDIX E

NHS
Health Research Authority
National Research Ethics Service

NRES Committee North West - Greater Manchester East
3rd Floor, Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7820

12 June 2014

Dr Sohail Mushtaq, Senior Lecturer
Department of Clinical Sciences and Nutrition
University of Chester
Parkgate Road
Chester
CH1 4BJ

Dear Dr Mushtaq

Study title:	**Investigation into the inhibitory effects of tea consumption on iron bioavailability in a cohort of healthy UK women, using a stable iron isotope**
REC reference:	**14/NW/0310**
Protocol number:	**S-LS17042014**
IRAS project ID:	**154775**

Thank you for your letter of 10 June 2014 responding to the Committee's request for further information on the above research and submitting revised documentation. The further information has been considered on behalf of the Committee by the Chair and Jacqueline Crowther.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Elaine Hutchings, nrescommittee.northwest-gmeast@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study:

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

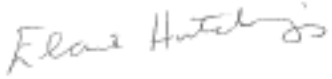
You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/NW/0310	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project.

Yours sincerely



Mr Francis Chan
Chair

Email:nrescommittee.northwest-gmeast@nhs.net

Enclosure: "After ethical review – guidance for researchers"

Copy to: *Mark Helsdon, University of Chester*

Salma Faeza Ahmad Fuzi

Trace Sciences International Corp.
40 Vogel Road, Suite 42
Richmond Hill, Ontario • L4B 3N6 CANADA
Tel: (905) 770-1100 • Fax: (905)770-1160
Email: sales@tracesciences.com

TRACE SCIENCES INTERNATIONAL

Trace Sciences International Inc.
PO Box 1496
Pilot Point, Texas • 76258 USA
Tel: (940) 324-3505 • Fax: (940) 324 3504
Website: www.tracesciences.com

CERTIFICATE OF ANALYSIS

#462.a

Name of Preparation: ^{57}Fe

Country of Destination: United States

Consignee: AnazaoHealth Corporation

CHARACTERISTICS OF ISOTOPE-ENRICHED PRODUCT

1. **Weight of enriched isotope:**

   Compound weight: 300.0 mg **Element weight: 300.0 mg**

   Form: Fe (Metal)

2. **Isotopic composition:**

Isotope	54	56	57	58
Enrichment (%)	-	0.57	95.93	3.5

3. **Chemical Impurities:**

Element	Symbol	Impurity Measurement(ppm)
Carbon	C	580
Aluminum	Al	
Chromium	Cr	
Copper	Cu	
Magnesium	Mg	700
Nickle	Ni	
Silicon	Si	
Zinc	Zn	

4. **Analytical method:** ICP-MS

Verified and signed by: Date: September 25, 2014
Crystal Richardson

Please note: This material is not approved for use in humans

APPENDIX G

University of Chester

Faculty of Life Sciences

Research Ethics Committee

frec@chester.ac.uk

24/06/2015

Salma Faeza Ahmed Fuzi
Sibell Street
Chester

Dear Salma

Study title: **A double blind randomised controlled trial to investigate the effect of vitamin D3 supplementation on iron absorption and hepcidin response in iron deficient women**
FREC reference: **1078/15/SF/CSN**
Version number: **1**

Thank you for sending your application to the Faculty of Life Sciences Research Ethics Committee for review.

I am pleased to confirm ethical approval for the above research, provided that you comply with the conditions set out in the attached document, and adhere to the processes described in your application form and supporting documentation.

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Application Form	1	May 2015
Appendix 1 – List of References	1	May 2015
Appendix 2 – Summary CV for Lead Researcher	1	May 2015
Appendix 3 – Email of invitation to participants	1	May 2015
Letter(s) of invitation to participants	1	May 2015
Appendix 4 – Participant Information Sheet [PIS]	2	May 2015
Appendix 5 – Participant Consent Form	1	May 2015
Appendix 6 – Risk Assessment	1	May 2015
Appendix 7 – Copies of advertising materials	2	May 2015
Appendix 8 – Questionnaire for screening test	1	May 2015
Appendix 9 – Flow chart of study protocol	1	May 2015
Appendix 10 – 3 day food diary dietary form	1	May 2015
Response to FREC request for further information or clarification		May 2015

Please note that this approval is given in accordance with the requirements of English law only. For research taking place wholly or partly within other jurisdictions (including Wales, Scotland and Northern Ireland), you should seek further advice from the Committee Chair / Secretary or the Research and Knowledge Transfer Office and may need additional approval from the appropriate agencies in the country (or countries) in which the research will take place.

With the Committee's best wishes for the success of this project.

Yours sincerely,

Dr. Stephen Fallows

Chair, Faculty Research Ethics Committee

Enclosures: Standard conditions of approval.

Cc. Supervisor/FREC Representative

University of Chester

Iron and Vitamin D Study – FEMALE VOLUNTEERS needed!

We are looking for volunteers to take part in a PhD study investigating the effects of vitamin D supplementation consumed with iron fortified breakfast cereals on iron absorption in IRON DEFICIENT non-pregnant women aged between 19—49 years.

If you:

- ☆ Have no history of gastrointestinal and metabolic disorders
- ☆ Have not donated blood within the past 6 months
- ☆ Are not taking nutritional supplements such as vitamins

We would like to hear from you.

Following a screening clinic, the study will involve consuming vitamin D supplements with iron fortified breakfast cereals for 8 weeks. Blood samples will also be collected at 3 different time points.

Upon completion the study you will receive:

- a free dietary analysis
- information on your iron and vitamin D status
- **£25 AMAZON VOUCHER**

If you would like to take part, and for further information, please email Salma Ahmad at: ironstudy@chester.ac.uk

APPENDIX I

EMAIL INVITATION TO PARTICIPANTS

Dear All,

A STUDY TO INVESTIGATE THE EFFECT OF VITAMIN D3 SUPPLEMENTATION ON IRON STATUS IN IRON DEFICIENT WOMEN

Would you like to know about how vitamin D can affect your iron status and how well your system is absorbing the iron food products that you consumed?

If you are female and aged between the ages of 19-49 years and are: healthy, non-pregnant or lactating, with no history of gastro-intestinal and metabolic disorders, have not donated blood in the past 6 months, and are not regularly taking nutritional supplements, I would like to invite you to take part in my PhD study which will investigate the effect of vitamin D supplementation consumed with iron fortified breakfast cereals to treat iron deficiency. Iron fortified breakfast cereals will be given to you to be consumed for 8 weeks, together with vitamin D supplements which have been shown to help increase iron status. With the findings, I hope to use the information to help individuals with impaired iron status to improve their iron status.

If you are interested in partaking in the study, you will be asked to attend a screening clinic to assess if you are iron deficient, and I will contact you to inform about your iron levels. If you then fulfil the inclusion criteria and you decide to take part in this study, you will be asked to attend clinics on 3 separate occasions. Detailed information on the study procedure is provided in the attached Participant Information Sheet (PIS), should you be interested to take part in this study.

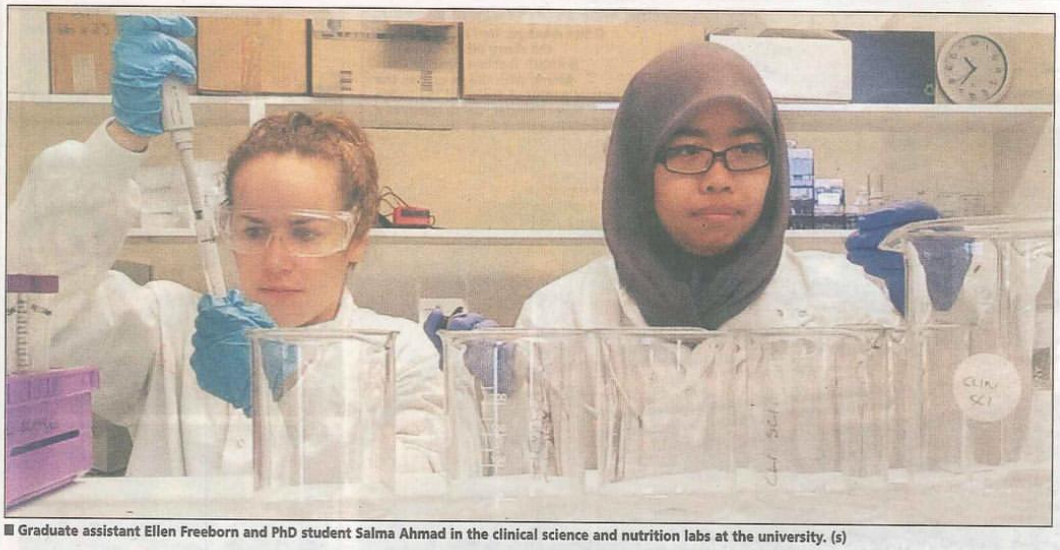
All clinics will take place at the University of Chester Main Campus and upon completion of the study, you will be provided with a full dietary analysis and an iron status and vitamin D assessment, a £25 Amazon voucher as well as contributing to increasing our knowledge in this area of nutrition and health.

Thank you for taking the time to read this email and if you would like to take part in the study or if you have any questions, I would very much like to hear from you. You can contact me at 01244511198 or email ironstudy@chester.ac.uk.

Yours sincerely,

Salma Ahmad
MPhil/PhD Student (Clinical Sciences and Nutrition)

Have you got a story? Email our news team at new



■ Graduate assistant Ellen Freeborn and PhD student Salma Ahmad in the clinical science and nutrition labs at the university. (s)

Nutrition experts seek women for vitamin D trial

NUTRITIONISTS investigating the effects of vitamin D supplements and iron absorption in iron deficient women are looking for women aged 19 to 40 to take part in their study.

Most of the body's vitamin D intake is from sunlight – which enables the vitamin to be made by the body under the skin.

Reserves of the vitamin tend to fall over the winter months, with less sunshine and fewer bright days.

With the autumn weather setting in and the clocks going back at the end of this month, this timely research is being carried out by the department of clinical sciences and nutrition at the University of Chester, and

is being led by Dr Sohail Mushtaq, who leads the micronutrient metabolism research group, and PhD student Salma Ahmad, with graduate assistant, Ellen Freeborn.

Salma said: "Deficiency in both vitamin D and iron is a significant public health issue in the UK. Recent studies have shown that taking vitamin D can help increase iron absorption and national dietary surveys show that between 25 per cenbt and 40 per cent of women of child-bearing age have lower than the recommended intake of iron in their food.

"We are interested to find out whether our study helps improve iron levels of the women taking part."

Interested participants have up until October 23 to register interest in taking part. If you think you could fit the bill and would like to advance research in this area, please email ironstudy@chester.ac.uk

More details about vitamin D can be found at nhs.uk/Conditions/vitamins-minerals/Pages/Vitamin-D.aspx.

APPENDIX K

Participant information sheet

A STUDY TO INVESTIGATE THE EFFECT OF VITAMIN D3 SUPPLEMENTATION ON IRON STATUS IN IRON DEFICIENT WOMEN

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being carried out and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study?

The study aims to investigate about the effect of vitamin D supplementation consumed with iron fortified breakfast cereals to treat iron deficiency. Iron fortified breakfast cereals will be given to you to be consumed for 8 weeks, together with vitamin D supplements which have been shown to help increase iron status where there will be 3 clinics for blood sampling to assess the iron status.

Why have I been chosen?

You have been chosen because you satisfy the inclusion criteria of the study which includes the following: iron deficient; female; between the ages of 19-49 years; healthy; non-pregnant or lactating; with no history of gastro-intestinal and metabolic disorders; have not donated blood within the last 6 months and are not regularly taking nutritional supplements.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect you in any way.

What will happen to me if I take part?

You will first be asked to attend a screening clinic to assess your eligibility to take part in the study. A 6 ml venous blood (1 teaspoon) will be drawn to assess full blood counts, including haemoglobin concentrations. A blood ferritin concentration, which is a measure of your iron status with a threshold of below 20.0 µg/L and vitamin D concentration of below 250 nmol/l will be used for inclusion. You will then have to fill in a screening test questionnaire that will provide information about your health status, drugs/medication/supplement usage, or recent occurrences of illness/trauma/injury, followed by physical measurement of height and weight.

If you satisfy the screening criteria, you will be randomly allocated into 2 groups;

a) Fe group (iron fortified breakfast cereals and placebo)

b) Vitamin D group (iron fortified breakfast cereals and vitamin D3)

You will be asked to consume 60 grams of iron fortified breakfast cereals containing a total of 9 mg of iron daily for 8 weeks with either placebo or vitamin D3 according to your assigned group. You will be asked to consume the iron fortified breakfast cereals in the morning with 200 ml of ultra-high temperature (UHT) semi-skimmed milk daily for a period of 8 weeks. Please consume the vitamin D or placebo in the evening with 200 ml of water. Please maintain your dietary habits and physical activity and not to donate blood during the course of study. You will be asked to return the remaining supplements at the final clinic (week 8), and compliance will be estimated.

You are required to attend 3 clinics on 3 separate days where you will be expected to fast overnight for at least 8 hours prior to each session. During the fasting period before each clinic, you are permitted to consume water only. There will be a time interval of 4 weeks between each clinic which each clinic will last for approximately 20-30 minutes between 8 and 10 am in the morning.

Clinic 1 (Week 0): Measurement of height and weight will be followed by a blood sample (30 ml). You will be required to complete a 3-day food diary after this clinic to reflect your habitual intake.

Clinic 2 (Week 4): Measurement of weight will be followed by a blood sample (30 ml).

Clinic 3 (Week 8): Measurement of weight followed by a final blood sample (30 ml). You will be required to complete a 3-day food diary after this clinic to reflect your habitual intake.

What are the possible disadvantages and risks of taking part?
There are no disadvantages foreseen in taking part in the study, however, you may experience slight discomforts and bruising due to the blood sampling procedures. Unfortunately, it will not be possible to reimburse any travel or other expenses incurred in taking part in the study.

What are the possible benefits of taking part?

You will be provided with a full dietary analysis, iron and vitamin D status. Dietary analysis will be performed by the researcher on completion of the study using dietary analysis software. The analysis will be based on the information you will have already provided us in your 3-day food diaries during the course of the study and will thus require no further time input. We will provide you information on your nutrient intake, in particular, iron intake, as well as information on your total energy, fat, carbohydrate and protein intake with a comparison to recommended values. Upon completion of the study, you will also be given a £25 Amazon voucher. You will also be contributing to the development of knowledge on how to maximise iron absorption in the prevention of iron deficiency.

What if something goes wrong?

If you wish to complain or have any concerns about any aspect of the way you have been approached or treated during the course of this study, please contact Professor Sarah Andrew, Dean of the Faculty of Life Sciences, University of Chester, Parkgate Road, Chester, CH1 4BJ, 01244 513055.

Will my taking part in the study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential and anonymised so that only the researcher carrying out the research will have access to such information. The blood sample will be anonymised and stored in freezer located in the Clinical Laboratory in the Department of Clinical Sciences & Nutrition at the University of Chester.

What will happen to the results of the research study?

The results will be written up into a dissertation for a PhD thesis and potential publications. Individuals who participate will not be identified in any subsequent report or publication. After the completion of the study, a sheet summarising the findings of the study will be sent to the participants via post or email.

Who is organising the research?

The research is conducted as part of a PhD research project in Human Nutrition within the Department of Clinical Sciences & Nutrition at the University of Chester. The study is organised with supervision from the department, by Salma Ahmad, a PhD student.

Who may I contact for further information?

If you would like more information about the research before you decide whether or not you would be willing to take part, please contact: Salma Ahmad at 01244511198 or email at *ironstudy@chester.ac.uk*.

Thank you for your interest in this research.

APPENDIX L

PARTICIPANT CONSENT FORM

TITLE OF PROJECT: A STUDY TO INVESTIGATE THE EFFECT OF VITAMIN D3 SUPPLEMENTATION ON IRON STATUS IN IRON DEFICIENT WOMEN

NAME OF RESEARCHER: SALMA AHMAD

Please initial box

1. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my legal rights being affected. ☐

3. I agree to take part in the above study. ☐

4. I agree to allow my blood samples to be stored for a period of 5 years for related nutritional studies. ☐

_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for participant; 1 for researcher

APPENDIX M

University of Chester

QUESTIONNAIRE FOR SCREENING TEST

TITLE OF PROJECT:

A STUDY TO INVESTIGATE THE EFFECT OF VITAMIN D3 SUPPLEMENTATION ON IRON STATUS IN IRON DEFICIENT WOMEN

NAME OF RESEARCHER: SALMA AHMAD

Participant ID :			
Date :			

SECTION 1 : PERSONAL DETAILS

1. Name : _____
2. Address : _____
3. Age : _____ years
4. Date of birth : ___ day ___ month _____ year
5. Ethnicity : Please (√) in the appropriate box.

White	Black	Asian	Other

6. Contact No : _____
7. Email : _____

SECTION 2 : SCREENING

Please tick where appropriate.

Question	YES	NO
1. Are you pregnant?		
2. Are you lactating?		
3. Are you a vegetarian?		
4. Have you ever donated blood within the past 6 months?		
5. Are you regularly taking nutritional supplements?		
If yes, please state :		
6. Do you have any gastro-intestinal or metabolic disorders?		
If yes, please state :		

SECTION 3 : IRON DEFICIENCY SCREENING TEST VALUE

1. Serum Ferritin : _____ µg/l
2. Vitamin D : _____ nmol/l

SAMPLE OF PLASMA CRP ELISA KIT CALIBRATION CURVE

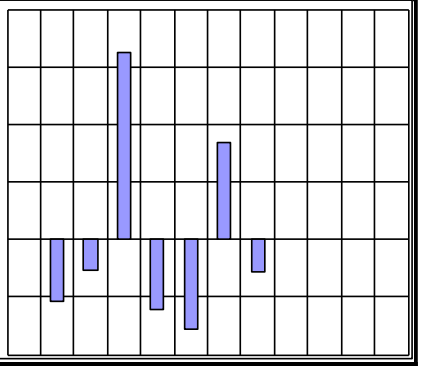
4- & 5-Parameters-Logistics Curve Fit

STEP 1	Set Extras/Macro/Security to medium (for SOLVER), close, reopen and activate		
STEP 2	Enter the standard concentrations in the blue column [std] in the order of increasing concentration, for 0 set 0,0000001		
STEP 3	Enter the corresponding standard readings in the yellow column		
STEP 4	Optional weighting: set value of B for weighting. For no weighting set B = 0.	**0**	<-- enter value here
STEP 5	start optimizing by clicking first the 4-PL button. Note the goodness of fit: MAE and the distribution of error in the plot. Click the 5-PL button and compare the goodness of fit.		

STANDARDS

[std]	Reading	Fit	Recalc. Conc.	% Recovery	WSE
0	0.006				
0.78	0.1105	0.12115047	0.648513306	83.1427316	0.00011343
1.56	0.184	0.18929757	1.501713526	96.2636876	2.8064E-05
3.12	0.3685	0.33615788	3.458691296	110.85549	0.00104601
6.25	0.616	0.62807822	6.1152105	97.843368	0.00014588
12.5	1.1045	1.12003617	12.27287368	98.1829894	0.00024137
25	1.779	1.76223052	25.44458844	101.778354	0.00028122
50	2.363	2.36854917	49.63605648	99.272113	3.0793E-05
8	8	SUM OF WEIGHTED SQUARED ERRORS			0.00188677

ERROR PLOT



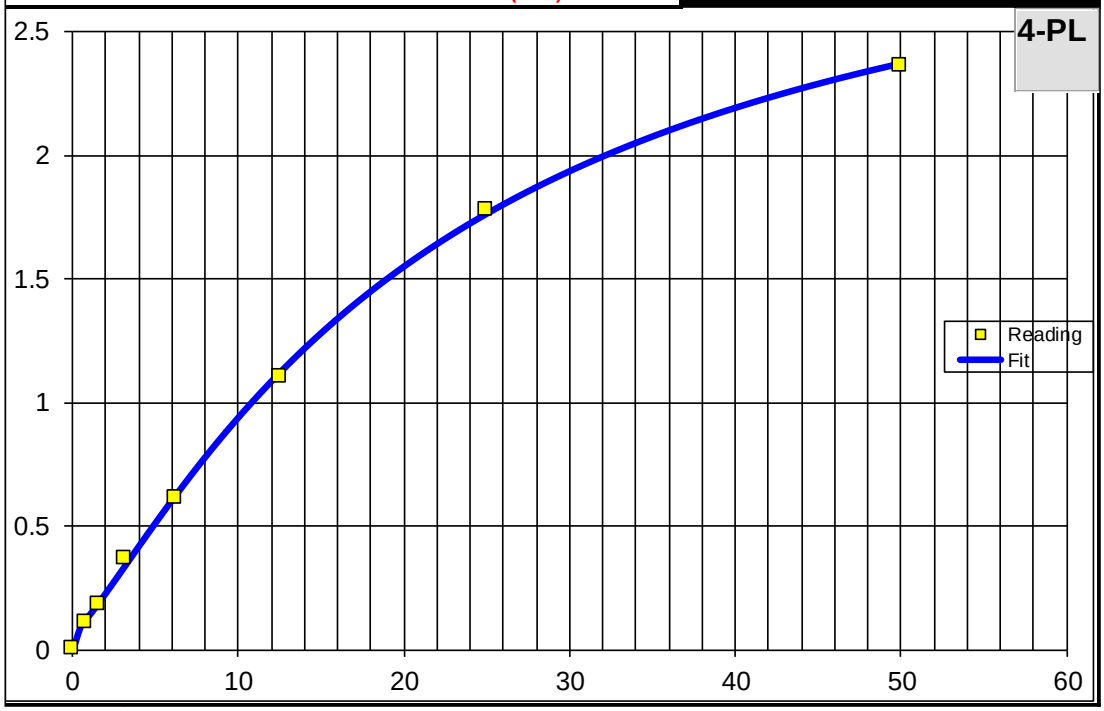
PARAMETERS

3.228255676	(max) **a**	rdg. of asymptotic maximum
-1.21117521	(slope) **b**	slope at inversion point
22.17719949	(IC50) **c**	concentration at inversion point (4-PL)
0.067257173	(min) **d**	rdg. of asymptotic minimum
1	(symf) **s**	asymmetry factor (if s = 1, fit is 4-PL)

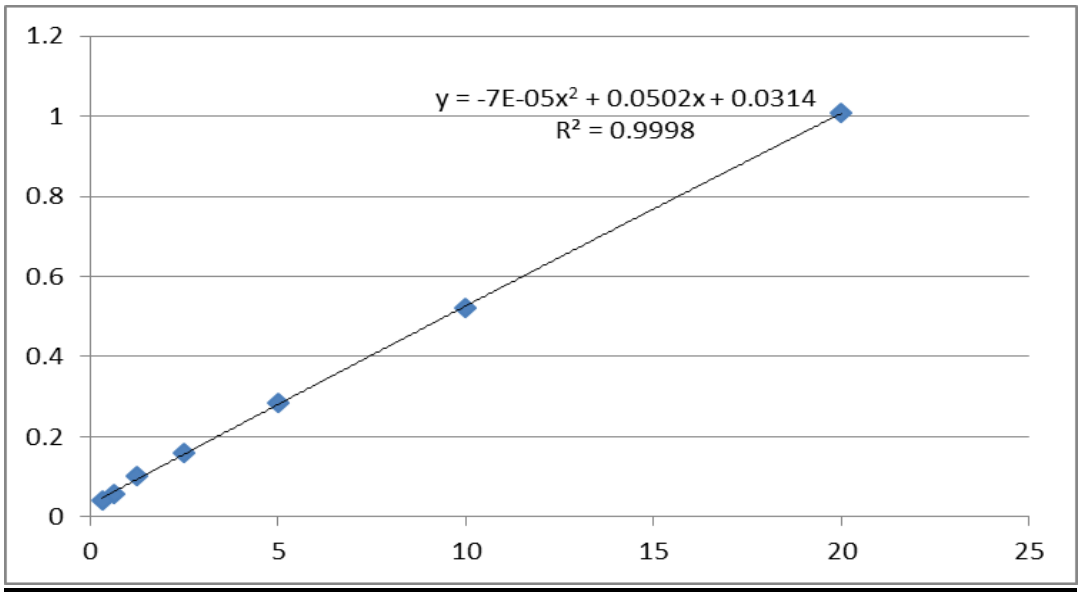
R^2 0.98079095 MEAN ABSOLUTE ERROR (MAE) 0.01403189

4-PL

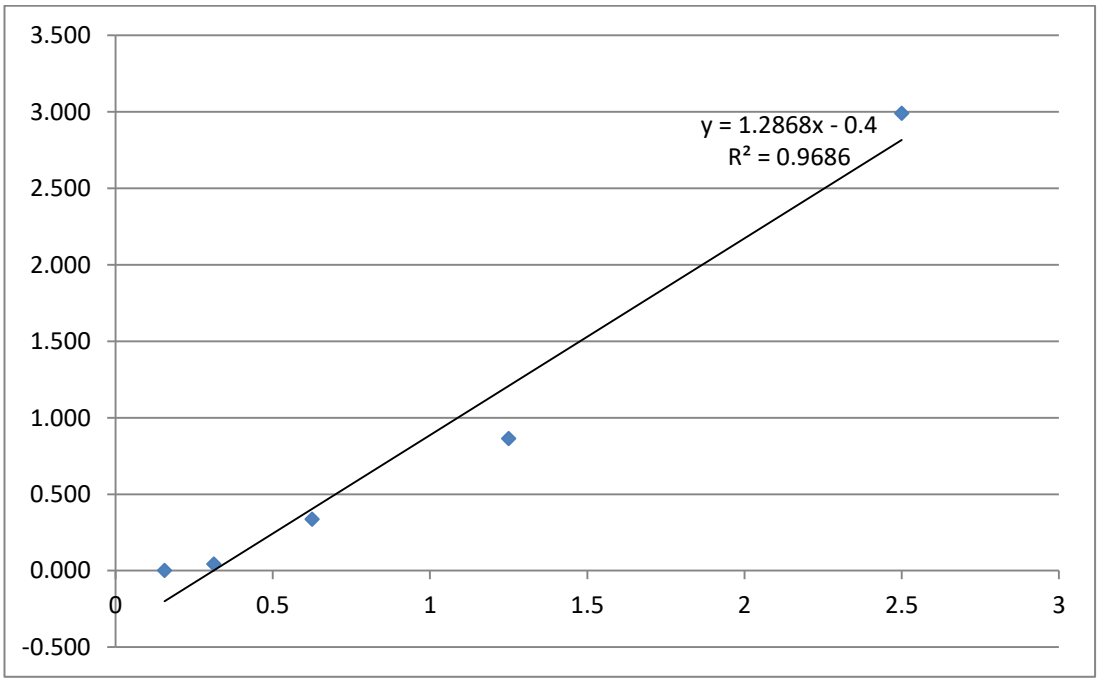
5-PL



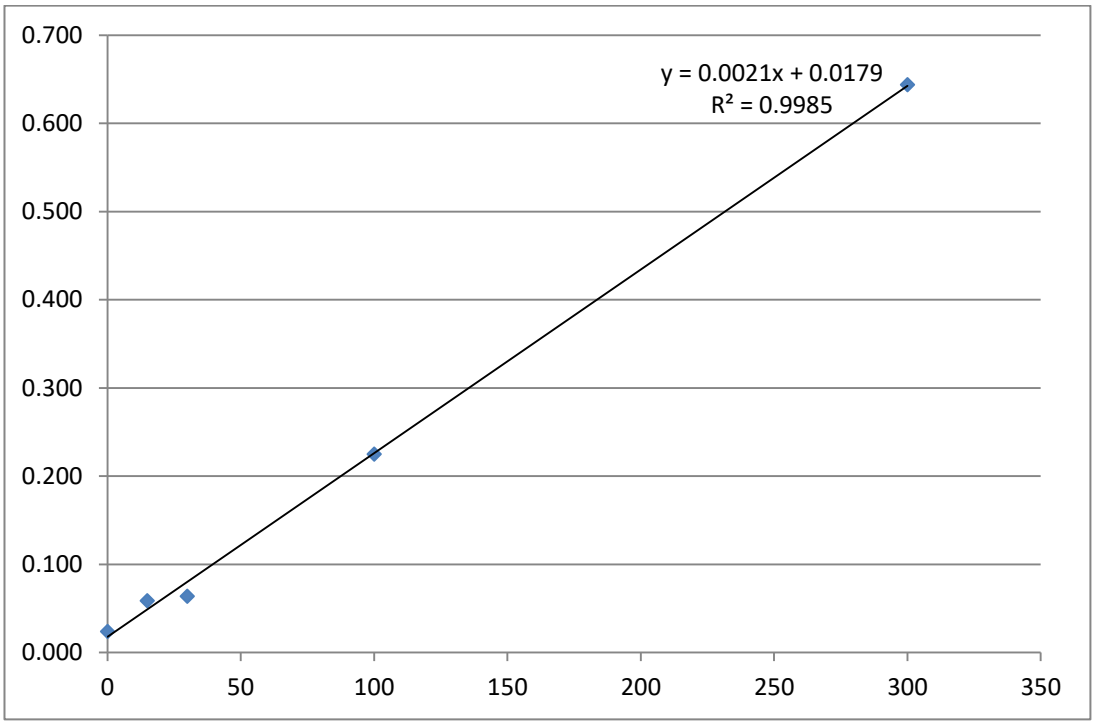
SAMPLE OF PLASMA HEPCIDIN ELISA KIT CALIBRATION CURVE



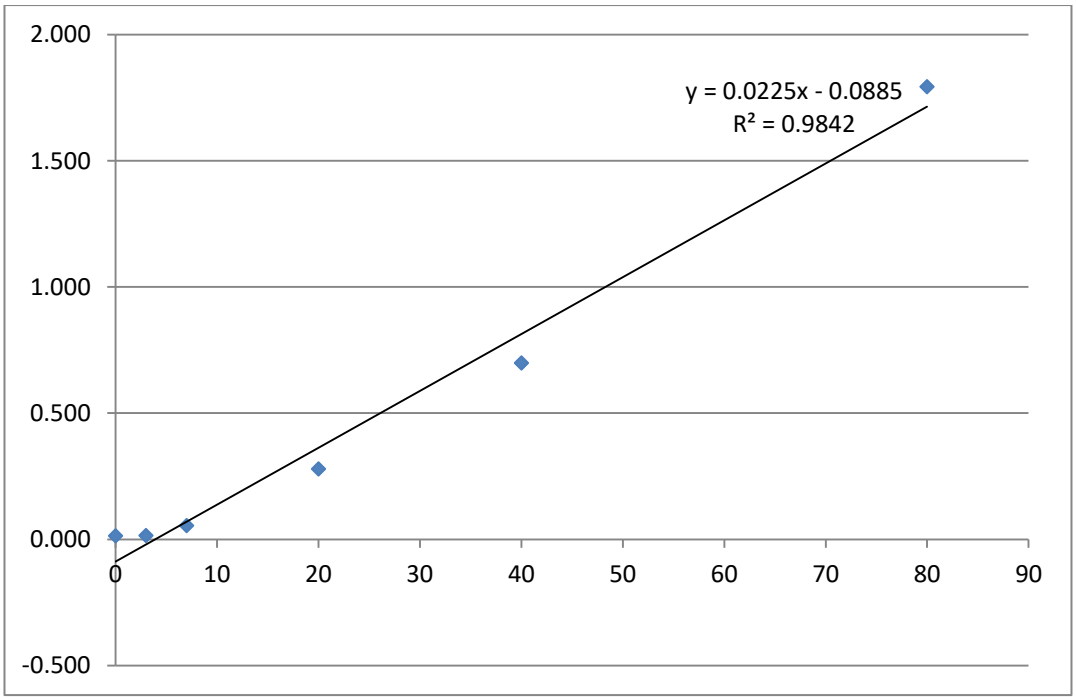
SAMPLE OF PLASMA FERRITIN ELISA KIT CALIBRATION CURVE



SAMPLE OF PLASMA PTH ELISA KIT CALIBRATION CURVE



SAMPLE OF PLASMA STFR ELISA KIT CALIBRATION CURVE



SAMPLE OF PLASMA CRP ELISA KIT CALIBRATION CURVE

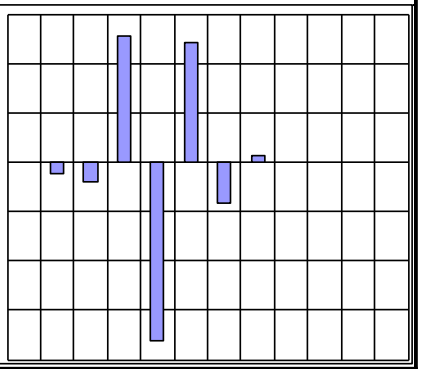
4- & 5-Parameters-Logistics Curve Fit

STEP 1	Set Extras/Macro/Security to medium (for SOLVER), close, reopen and activate
STEP 2	Enter the standard concentrations in the blue column [std] in the order of increasing concentration, for 0 set 0,0000001
STEP 3	Enter the corresponding standard readings in the yellow column
STEP 4	Optional weighting: set value of B for weighting. For no weighting set B = 0. **0** <-- enter value here
STEP 5	start optimizing by clicking first the 4-PL button. Note the goodness of fit: MAE and the distribution of error in the plot. Click the 5-PL button and compare the goodness of fit.

STANDARDS

[std]	Reading	Fit	Recalc. Conc.	% Recovery	WSE
0	0.007		0.102206879		
0.78	0.091	0.09215082	0.769544489	98.6595498	1.3244E-06
1.56	0.170	0.17151969	1.538847366	98.6440619	4.0792E-06
3.12	0.321	0.30780017	3.277749425	105.056071	0.00016129
6.25	0.518	0.53611968	5.979333526	95.6693364	0.00032832
12.5	0.912	0.89985911	12.73635945	101.890876	0.0001474
25	1.436	1.44016768	24.88649743	99.5459897	1.737E-05
50	2.164	2.16288283	50.02676109	100.053522	3.809E-07
8	8	SUM OF WEIGHTED SQUARED ERRORS			0.00066016

ERROR PLOT



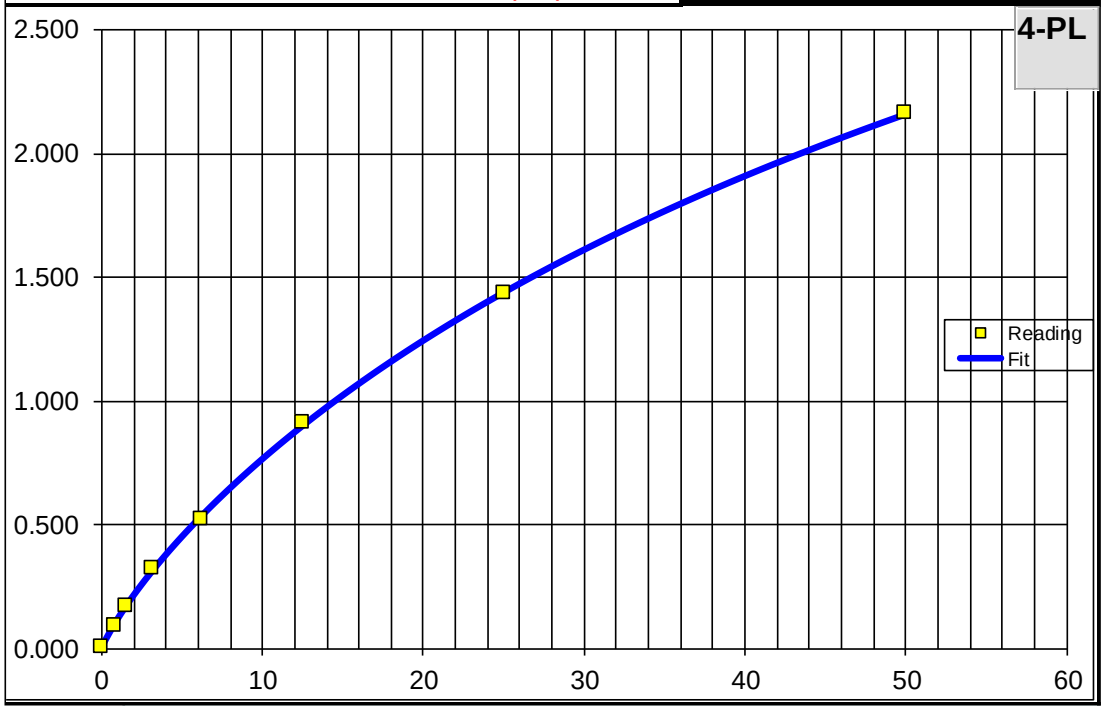
PARAMETERS

5.959574321	(max) **a**	rdg. of asymptotic maximum
-0.83395697	(slope) **b**	slope at inversion point
97.49846412	(IC50) **c**	concentration at inversion point (4-PL)
-0.01249464	(min) **d**	rdg. of asymptotic minimum
1	(sym f) **s**	asymmetry factor (if s = 1, fit is 4-PL)

R^2 0.98703420 MEAN ABSOLUTE ERROR (MAE) 0.00727368

4-PL

5-PL



SAMPLE OF PLASMA HEPCIDIN ELISA KIT CALIBRATION CURVE

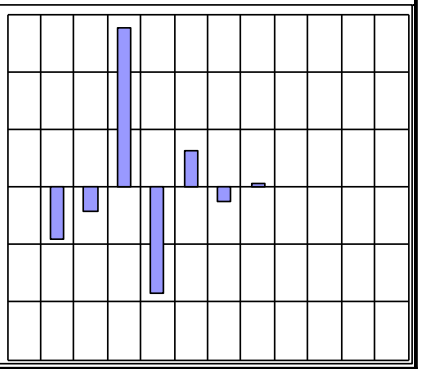
4- & 5-Parameters-Logistics Curve Fit

STEP 1	Set Extras/Macro/Security to medium (for SOLVER), close, reopen and activate	
STEP 2	Enter the standard concentrations in the blue column [std] in the order of increasing concentration, for 0 set 0,0000001	
STEP 3	Enter the corresponding standard readings in the yellow column	
STEP 4	Optional weighting: set value of B for weighting. For no weighting set B = 0.	**0** <-- enter value here
STEP 5	start optimizing by clicking first the 4-PL button. Note the goodness of fit: MAE and the distribution of error in the plot. Click the 5-PL button and compare the goodness of fit.	

STANDARDS

[std]	Reading	Fit	Recalc. Conc.	% Recovery	WSE
0	0.014		1.012576899		
15.6	0.028	0.02934311	14.04606525	90.0388798	3.3971E-06
31.3	0.049	0.04937731	30.64496379	97.9072326	7.6967E-07
62.5	0.100	0.09396465	66.17174837	105.874797	3.064E-05
125	0.190	0.1936688	122.8024281	98.2419425	1.346E-05
250	0.416	0.41474956	250.67779	100.271116	1.5636E-06
500	0.897	0.89751925	499.7394405	99.9478881	2.6962E-07
1000	1.918	1.91737732	1000.059613	100.005961	1.505E-08
8	8	**SUM OF WEIGHTED SQUARED ERRORS**			**0.00005012**

ERROR PLOT



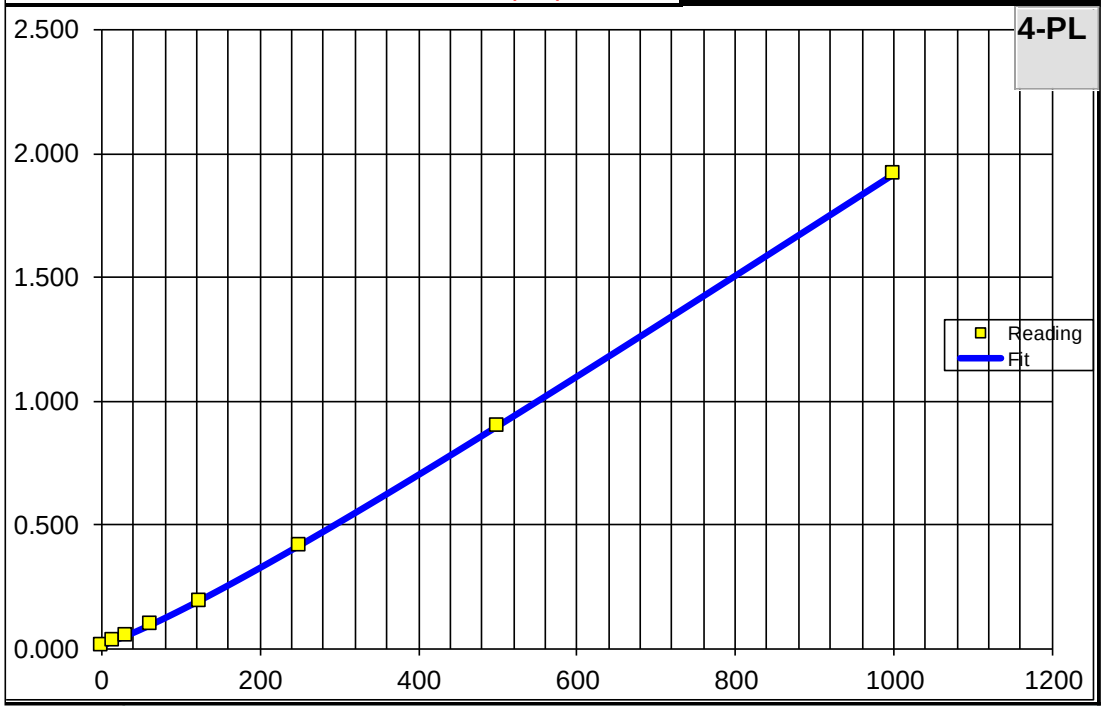
PARAMETERS

25.80904625	(max) **a**	rdg. of asymptotic maximum
-1.16695828	(slope) **b**	slope at inversion point
8737.704141	(IC50) **c**	concentration at inversion point (4-PL)
0.013341699	(min) **d**	rdg. of asymptotic minimum
1	(sym f) **s**	asymmetry factor (if s = 1, fit is 4-PL)

R^2 **0.99637292** **MEAN ABSOLUTE ERROR (MAE)** **0.00197385**

4-PL

5-PL



SAMPLE OF PLASMA VDBP ELISA KIT CALIBRATION CURVE

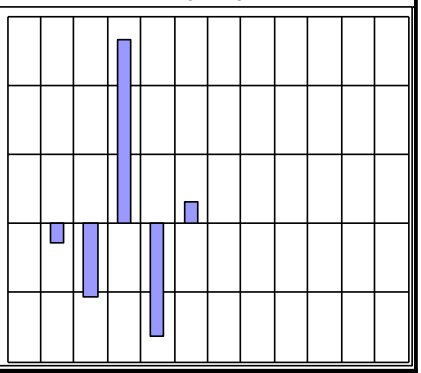
4- & 5-Parameters-Logistics Curve Fit

STEP 1	Set Extras/Macro/Security to medium (for SOLVER), close, reopen and activate	
STEP 2	Enter the standard concentrations in the blue column [std] in the order of increasing concentration, for 0 set 0,0000001	
STEP 3	Enter the corresponding standard readings in the yellow column	
STEP 4	Optional weighting: set value of B for weighting. For no weighting set B = 0.	**0** <-- enter value here
STEP 5	start optimizing by clicking first the 4-PL button. Note the goodness of fit: MAE and the distribution of error in the plot. Click the 5-PL button and compare the goodness of fit.	

STANDARDS

[std]	Reading	Fit	Recalc. Conc.	% Recovery	WSE
0	0.004		5.264749467		
15.6	0.054	0.05489397	15.3382294	98.3219833	1.9431E-06
31.3	0.140	0.14479055	30.42329003	97.1990097	2.799E-05
62.5	0.361	0.34717069	64.44176323	103.106821	0.00017767
125	0.797	0.80516612	123.9415457	99.1532365	6.6686E-05
250	1.836	1.83397867	250.1761483	100.070459	2.3144E-06
6	6	**SUM OF WEIGHTED SQUARED ERRORS**			**0.00027660**

ERROR PLOT



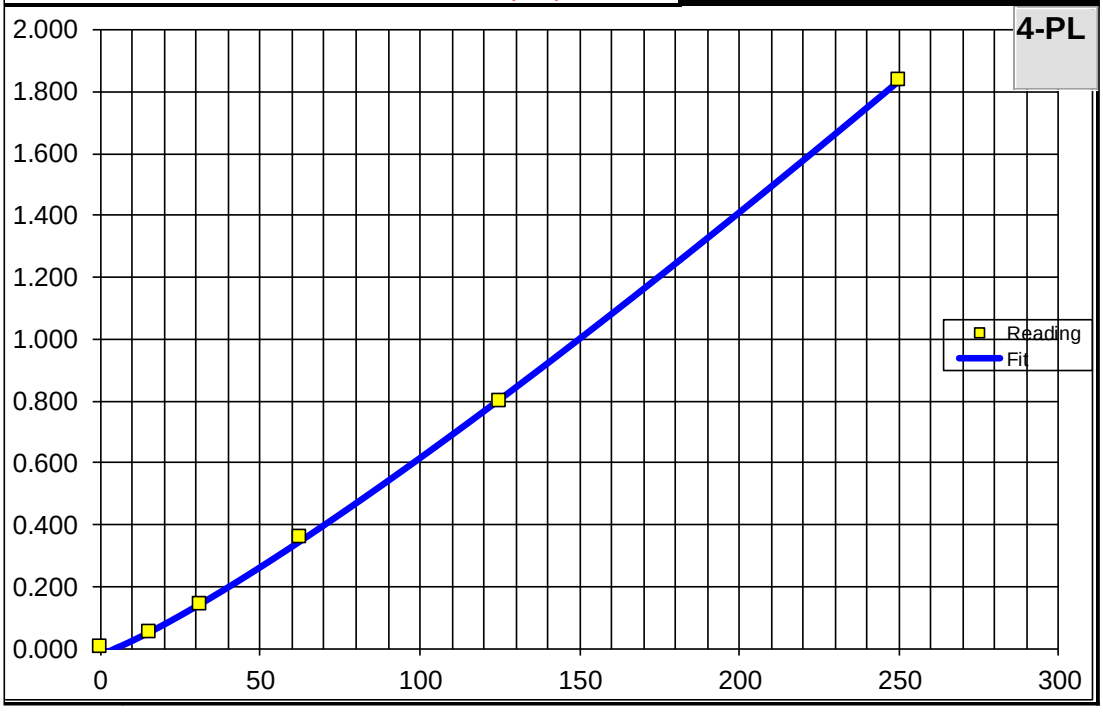
PARAMETERS

141.2072717	(max) **a**	rdg. of asymptotic maximum
-1.18288391	(slope) **b**	slope at inversion point
9657.271296	(IC50) **c**	concentration at inversion point (4-PL)
-0.01547738	(min) **d**	rdg. of asymptotic minimum
1	(sym f) **s**	asymmetry factor (if s = 1, fit is 4-PL)

R^2 **0.99068715** **MEAN ABSOLUTE ERROR (MAE)** **0.00594026**

4-PL

5-PL



SAMPLE OF PLASMA 25(OH)D ELISA KIT CALIBRATION CURVE

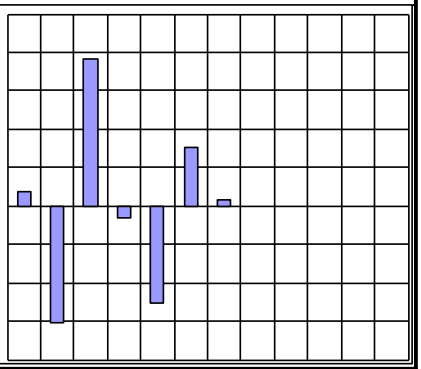
4- & 5-Parameters-Logistics Curve Fit

STEP 1	Set Extras/Macro/Security to medium (for SOLVER), close, reopen and activate		
STEP 2	Enter the standard concentrations in the blue column [std] in the order of increasing concentration, for 0 set 0,0000001		
STEP 3	Enter the corresponding standard readings in the yellow column		
STEP 4	Optional weighting: set value of B for weighting. For no weighting set B = 0.	**0**	<-- enter value here
STEP 5	start optimizing by clicking first the 4-PL button. Note the goodness of fit: MAE and the distribution of error in the plot. Click the 5-PL button and compare the goodness of fit.		

STANDARDS

[std]	Reading	Fit	Recalc. Conc.	% Recovery	WSE
0	2.159	2.15539905			1.2967E-05
2.5	1.549	1.57924467	2.725566408	109.022656	0.00091474
5	1.352	1.31356386	4.554135124	91.0827025	0.00147734
15	0.846	0.84906719	15.11482554	100.765504	9.4076E-06
35	0.522	0.54722164	38.10340258	108.866865	0.00063613
70	0.385	0.3699375	65.25720245	93.2245749	0.00022688
150	0.244	0.2425661	148.3418635	98.8945757	2.0561E-06
7	7	**SUM OF WEIGHTED SQUARED ERRORS**			**0.00327952**

ERROR PLOT



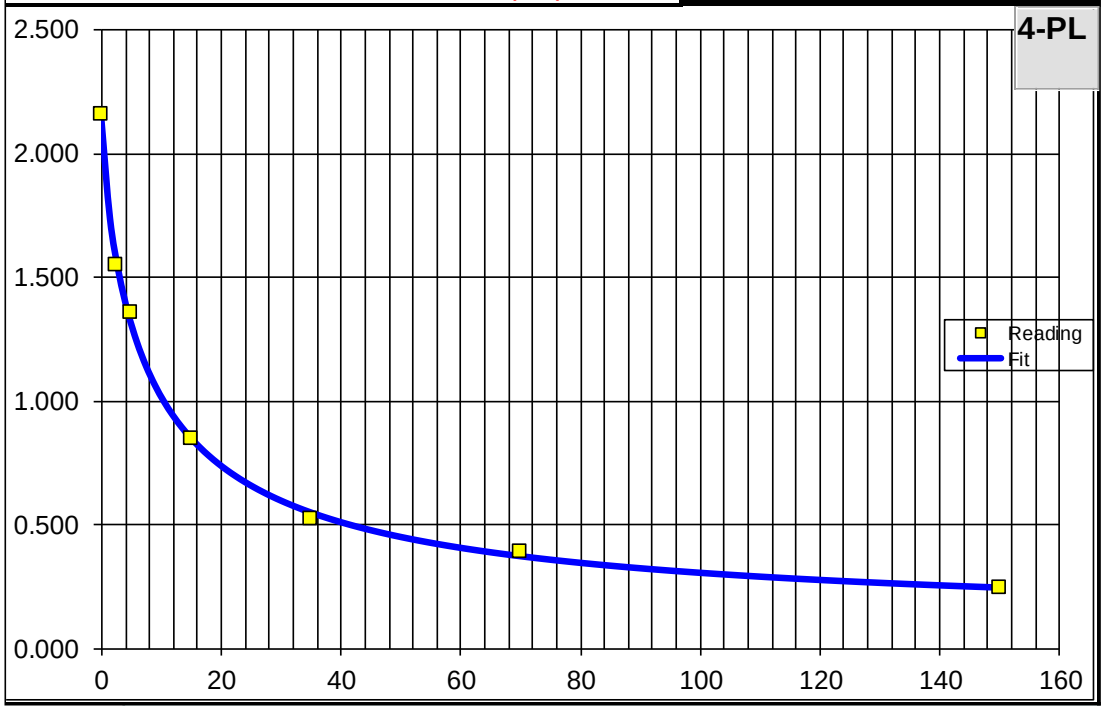
PARAMETERS

2.15539905	(max) **a**	rdg. of asymptotic maximum
0.827140397	(slope) **b**	slope at inversion point
7.986407072	(IC50) **c**	concentration at inversion point (4-PL)
0.073477083	(min) **d**	rdg. of asymptotic minimum
1	(sym f) **s**	asymmetry factor (if s = 1, fit is 4-PL)

R^2 **0.97198598** **MEAN ABSOLUTE ERROR (MAE)** **0.01672386**

4-PL

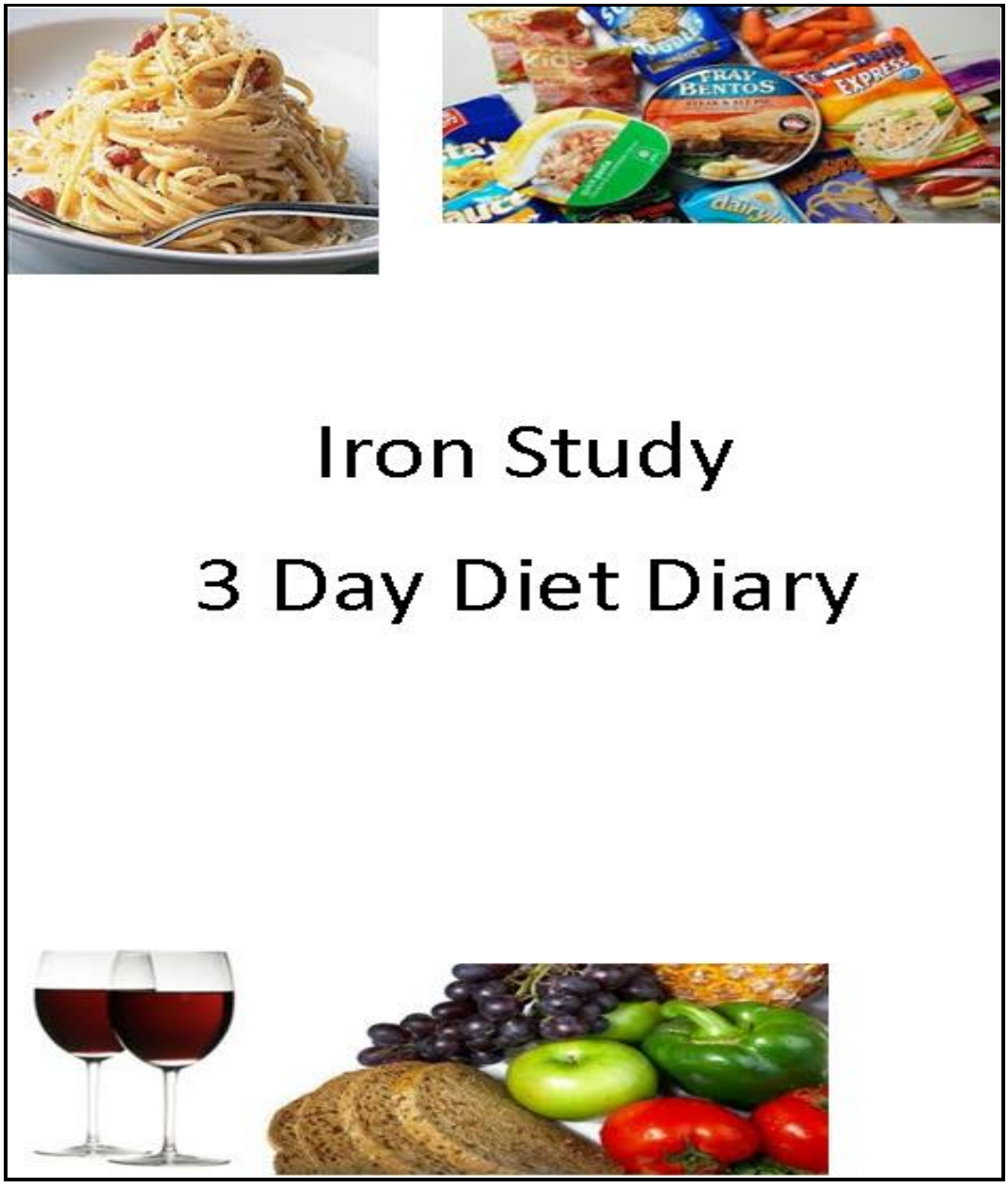
5-PL



APPENDIX O

University of Chester

TITLE OF PROJECT: INVESTIGATION INTO THE INHIBITORY EFFECTS OF TEA CONSUMPTION ON IRON BIOAVAILABILITY IN A COHORT OF HEALTHY UK WOMEN, USING A STABLE IRON ISOTOPE



We would like you to keep his diary of everything you eat and drink over 3 consecutive days. Please do not change your habits because you are keeping this record.

Instructions:

- Record your eating & drinking as you go, not from memory. Remember to include foods/drinks consumed between meals or at night.
- Ensure you write the day, time, description of meal or drink, amount and preparation method as well as brand of product or recipe as appropriate.
- Homemade dishes: Record the name of the recipe, ingredients with the amounts & cooking method. These can be recorded on the page at the back of the diary. Take-aways/restaurant: Including as much information as you can eg; vegetable curry with chickpeas, onions, aubergine.
- Please ensure you record the portion size of the food you eat—there is a guide overleaf to aid you with this.
- Remember to include any added sugar in drinks/food, salt during cooking or at table and table sauces.
- Ensure you note details of cooking method—grilled, fried (in oil/butter), steamed, boiled etc.
- If foods come with added vitamins or minerals please write this down.
- Do write down any exercise participated in during each day. Include the nature of exercise (eg swimming) and length of time.
- Make a note in the margin if there is any wastage and the quantity.

Physical Activity: 20mins household chores (mopping, hovering) 20min slow jog

Guide to Portion Sizes:

For foods, quantity can be described using:

Household measures: eg 1 tsp of sugar, 2 thick slices of bread, 1/2 cup gravy.

Weights from labels: eg 125g pot yoghurt, 420g tin baked beans

Number of items: 4 fish fingers, 1 regular sized doughnut

For drinks, quantity can be described using:

The size of the glass (eg large glass) or the volume (300ml)

Use the example below to assist you with completing your food diary

Day: Monday Date: 21st October

Time	**Location**	**Description of food/drink consumed**	**Brand**	**Amount/ Portion Size**
7.50am	Home	Bran flakes with semi skimmed milk. Banana	Tesco Bran Flakes.	1 cup cereal, 1/2 cup milk 1/2 banana
12.30pm	Work	Sandwich Tuna Light mayo Tomato Flapjack	Hovis wholemeal, Tesco tuna Tesco mayo, Homemade flapjack (See recipe, serves 15)	2 medium slices. 1 can tuna 2 tbsp. mayo 1 tomato 2 servings flapjack

Guide to help with information required to complete your food diary.

Food/Drink	**Description/Preparation**	**Portion Size/Quantity**
Bacon	Back, middle, streaky, smoked, un-smoked. Fried in oil/ grilled	Number of rashers
Beefburger	Homemade (ingredients), from packet (brand name), with or without bread roll	Number, large or small, grams
Butter, Margerine	Give full product name	Thick/average/thin spread, spoons
Cheese	Name, brand & type eg cheddar, low fat	Number of slices, thick or thin cut
Milk	Type semi, skimmed, UHT, Soya	Pints, glass (size of volume), cup. For on cereal: little/normal/drowned. For in tea/coffee: little/some/lot
Salad	Ingredients, what sort of dressing (oil, mayonnaise)	Amount of each component (eg number of tomatoes, slices cucumber, tablespoons of dressing)
Savoury snacks in packets	What sort; Cheddars, walkers, give brand name	Size, packet weight, number
Soup	What sort, brand name, canned, packet, instant, homemade	Tablespoons, mug, bowl (small, medium, large)

Pasta	Type (eg fresh, dried, white, wholemeal, canned, in sauce, type of filling)	Tablespoons (or how much dry pasta used per portion)
Vegetables	What sort, how cooked, added butter/oil/sauce	Tablespoons, number of florets, weight from tins

Typical quantities of drinks in various containers measured in ml

	Small glass	**Average glass**	**Large glass**	**Vending cup**	**Cup**	**Mug**
Soft drinks	150	200	300			
Wine	125	175	250			
Hot drinks				170	190	260

Day: **Date :**

Time	Location	Description of food / drink consumed	Brand	Amount/Quantity

Day: **Date :**

Time	Location	Description of food / drink consumed	Brand	Amount/Quantity

Day: **Date :**

Time	Location	Description of food / drink consumed	Brand	Amount/Quantity

Recipes

For any homemade meals/snacks please write down the ingredients used, quantities and preparation method in the space provided.

APPENDIX P

IRON STUDY
24 HOUR RECALL FORM

Participant ID	
Clinic	
Date	

Which day of the week does this record?

Sun ☐ Mon ☐ Tues ☐ Weds ☐ Thurs ☐ Fri ☐ Sat ☐

Is this a typical day? Yes ☐ No ☐

Time	**Location**	**Description of food / drink consumed**	**Brand**	**Amount/Quantity**

APPENDIX Q

University of Chester

TITLE OF PROJECT: A STUDY TO INVESTIGATE THE EFFECT OF VITAMIN D3 SUPPLEMENTATION ON IRON STATUS IN IRON DEFICIENT WOMEN

Vitamin D and Iron Study

3 Day Diet Diary

We would like you to keep his diary of everything you eat and drink over 3 consecutive days. Please do not change your habits because you are keeping this record.

Instructions:

- Record your eating & drinking as you go, not from memory. Remember to include foods/drinks consumed between meals or at night.
- Ensure you write the day, time, description of meal or drink, amount and preparation method as well as brand of product or recipe as appropriate.
- Homemade dishes: Record the name of the recipe, ingredients with the amounts & cooking method. These can be recorded on the page at the back of the diary. Take-aways/restaurant: Including as much information as you can eg; vegetable curry with chickpeas, onions, aubergine.
- Please ensure you record the portion size of the food you eat—there is a guide overleaf to aid you with this.
- Remember to include any added sugar in drinks/food, salt during cooking or at table and table sauces.
- Ensure you note details of cooking method—grilled, fried (in oil/butter), steamed, boiled etc.
- If foods come with added vitamins or minerals please write this down.
- Do write down any exercise participated in during each day. Include the nature of exercise (eg swimming) and length of time.
- Make a note in the margin if there is any wastage and the quantity.

Physical Activity: 20mins household chores (mopping, hovering) 20min slow jog

Guide to Portion Sizes:

For foods, quantity can be described using:

Household measures: eg 1 tsp of sugar, 2 thick slices of bread, 1/2 cup gravy.

Weights from labels: eg 125g pot yoghurt, 420g tin baked beans

Number of items: 4 fish fingers, 1 regular sized doughnut

For drinks, quantity can be described using:

The size of the glass (eg large glass) or the volume (300ml)

Use the example below to assist you with completing your food diary

Day: Monday Date: 21st October

Time	Location	Description of food/drink consumed	Brand	Amount/ Portion Size
7.50am	Home	Bran flakes with semi skimmed milk. Banana	Tesco Bran Flakes.	1 cup cereal, 1/2 cup milk 1/2 banana
12.30pm	Work	Sandwich Tuna Light mayo Tomato Flapjack	Hovis wholemeal, Tesco tuna Tesco mayo, Homemade flapjack (See recipe, serves 15)	2 medium slices. 1 can tuna 2 tbsp. mayo 1 tomato 2 servings flapjack

Guide to help with information required to complete your food diary.

Food/Drink	Description/Preparation	Portion Size/Quantity
Bacon	Back, middle, streaky, smoked, un-smoked. Fried in oil/ grilled	Number of rashers
Beefburger	Homemade (ingredients), from packet (brand name), with or without bread roll	Number, large or small, grams
Butter, Margerine	Give full product name	Thick/average/thin spread, spoons
Cheese	Name, brand & type eg cheddar, low fat	Number of slices, thick or thin cut
Milk	Type semi, skimmed, UHT, Soya	Pints, glass (size of volume), cup. For on cereal: little/normal/drowned. For in tea/coffee: little/some/lot
Salad	Ingredients, what sort of dressing (oil, mayonnaise)	Amount of each component (eg number of tomatoes, slices cucumber, tablespoons of dressing)
Savoury snacks in packets	What sort; Cheddars, walkers, give brand name	Size, packet weight, number
Soup	What sort, brand name, canned, packet, instant, homemade	Tablespoons, mug, bowl (small, medium, large)

Pasta	Type (eg fresh, dried, white, wholemeal, canned, in sauce, type of filling)	Tablespoons (or how much dry pasta used per portion)
Vegetables	What sort, how cooked, added butter/oil/sauce	Tablespoons, number of florets, weight from tins

Typical quantities of drinks in various containers measured in ml

	Small glass	**Average glass**	**Large glass**	**Vending cup**	**Cup**	**Mug**
Soft drinks	150	200	300			
Wine	125	175	250			
Hot drinks				170	190	260

Day: **Date :**

Time	Location	Description of food / drink consumed	Brand	Amount/Quantity

Day: **Date :**

Time	Location	Description of food / drink consumed	Brand	Amount/Quantity

Day: **Date :**

Time	Location	Description of food / drink consumed	Brand	Amount/Quantity

Recipes

For any homemade meals/snacks please write down the ingredients used, quantities and preparation method in the space provided.

APPENDIX R

Investigation into the inhibitory effects of tea consumption on iron bioavailability in a cohort of healthy UK women, using a stable iron isotope (^{57}Fe).

Salma Faeza, **AHMAD FUZI**[1 2]; **Sohail**, **MUSHTAQ**[1]

[1]Department of Clinical Sciences & Nutrition, University of Chester, United Kingdom
[2]Department of Nutrition & Dietetics, Universiti Putra Malaysia, Malaysia

Tea can inhibit iron absorption but it is unclear whether time of consumption in relation to a meal influences bioavailability. The present study assessed the effect of time variability of tea consumption on iron absorption from a test meal in non-pregnant women using a ^{57}Fe tracer. Twelve women (mean age (± SD): 24.8 ± 6.9 years) were given a standardised breakfast extrinsically labelled with 4mg ^{57}Fe on 3 occasions with a 14 day time interval between each test meal. The isotope was administered with: (a) water (b) tea simultaneously (c) tea 1 hour after the meal. A reference dose was also administered (3mg ^{57}Fe with 35mg ascorbate) without a test meal. Fasted blood samples were collected and iron absorption was estimated by the erythrocyte iron incorporation method, assuming 80% absorption. Mean iron absorption (± SD) was 5.69 ± 8.5%, 3.57 ± 4.2% and 5.73 ± 5.4% (test meals a,b and c, respectively). Mean iron absorption was found to be 2.2% higher when tea was consumed 1 hour after test meals ($p = 0.05$). Mean total iron absorbed was also found to be 0.043mg higher when tea was consumed 1 hour after the meal ($p= 0.049$). This study demonstrates that consuming tea separately from a meal may attenuate the inhibition effect thus increasing iron absorption. This may have implications for those who are iron deficient.

KEYWORDS: Iron deficiency; Iron bioavailability; Tea