Strategies to improve non-haem iron absorption

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A thesis submitted for the Degree of Doctor of Philosophy

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December 2013

ABSTRACT

Iron deficiency anaemia is one of the most prevalent nutritional deficiency disorders in the world. Food iron fortification is a widely used strategy to reduce the risk of deficiency but presents a major challenge to the food industry. The more bioavailable forms of iron, such as ferrous gluconate, cause adverse organoleptic changes when added to foods. The primary aim of the work described in this thesis was to test whether alginate would bind soluble forms of iron and thereby maintain its bioavailability. Initial *in vitro* studies demonstrated that alginate solutions and beads loaded with ferrous gluconate delivered iron in an available form for uptake into Caco-2 cells (measured by ferritin formation). A human study was undertaken to assess the bioavailability of ferrous gluconate in alginate beads, and it was found to be significantly lower than ferrous gluconate on its own, so further *in vitro* studies were undertaken to examine possible reasons for the inhibitory effect of the beads. It was concluded that alginate beads, containing calcium as a gelling agent, are not an effective delivery vehicle for soluble iron compounds. However, these findings should not rule out the potential use of alginates as a delivery system for iron, especially in diets containing high levels of phytate. Other related work reported in this thesis includes studies of iron availability from two wheat cultivars with varying phytate and iron concentrations, potential use of nicotianamine and 2'-deoxymugineic acid as iron enhancers, and investigations into calcium-iron interactions in a Caco-2 cell model, with the use of live cell imaging techniques and confocal microscopy.

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification at this or any other university or other institute of learning.

STATEMENT OF ORIGINALITY

I certify that this thesis, and the research to which it refers, is my own work, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged.

LIST OF PUBLICATIONS

- **Wawer A.A**., Sharp P.A., Perez-Moral N., Fairweather-Tait, S. J.: Evidence for an enhancing effect of alginate on iron availability in Caco-2 cells. *J. Agric. Food Chem.*, 2012, *60* (45), pp 11318–11322.

- **Wawer A.A**., Harvey L.J., Dainty J.R., Perez-Moral N., Sharp P.A., Fairweather-Tait, S. J: Alginate inhibits iron absorption from ferrous gluconate. *J Nutr. Submitted November 2013*

Other publications:

- Siyame E. W.P.,Hurst R. , **Wawer A.A**. , Young S.D., Broadley M.R., Chilimba A.D.C., Ander E.L., Watts M.J., Chilima B., Gondwe J., Kang'ombe D., Kalimbira A., Fairweather- Tait S.J., Bailey K.B., Gibson R.S.,: A High Prevalence of Zinc- but not Iron-Deficiency among Women in Rural Malawi: a Cross-Sectional Study. *Int. J. Vitam. Nutr. Res. Accepted, August 2013.*

- Fairweather-Tait S.J., **Wawer A.A**., Gillings R., Jennings A., Myint P.K,: Iron status in the elderly. *Mechanisms of Ageing and Development. Accepted November 2013.*

- Fairweather-Tait S.J., Guile G.R., Valdes A.M., **Wawer A.A** Hurst R., Skinner J., Macgregor A.J.: The contribution of diet and genotype to iron status in women: a classical twin study. *Accepted, PLOS ONE, November 2013*

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ACKNOWLEDGEMENTS

First and foremost I would like to thank my primary Supervisor Professor Susan Fairweather-Tait. I am extremely grateful for all her contributions, ideas and time spent towards this PhD thesis. Sue's enthusiasm, patience, support and trust make her an inspirational mentor. It is a privilege to be her PhD student. I am extremely grateful to Dr Linda Harvey for her invaluable help and support especially in the low moments. I could not have done all this work without her expertise. I am also thankful for her perpetual battles with my academic writing, especially the articles.

I would like to thank Dr Paul Sharp for his knowledge and support in times when the cells were not exhibiting any willingness to cooperate. Furthermore I would like to thank Dr Ben Thompson and Dr Rachel Hurst for their time and effort spent to carve a worthy lab researcher out of me.

I am also grateful to the collaborators on this project: Dr Natalia Perez-Moral, Dr Jack Dainty and Professor Peter Wilde for their professional guidance, help and expertise whenever I needed it. Furthermore I would like to thank my lab mates Sandy Bednar, Michael Edwards and Wei Wang for having been great friends as well as for sharing the good and the bad moments during my PhD.

I would also like to thank Professors Jane Coad, Kevin Pedley and their PhD student Bob Stuart for unforgettable down-under lab and life experience and for allowing me to become a part of their team for a short while.

On a personal level I would like to thank my parents for their encouragement to learn foreign languages and thus opening up an opportunity for me to undertake this PhD. Finally, I would like to thank my partner Rad Suchecki for his unreserved love, for being my strength in everyday struggles and for his much needed unconditional faith that I will complete this PhD.

LIST OF ABBREVIATIONS

AA- L-ascorbic acid

AAS- atomic absorption spectroscopy

AGP- alpha acid 1 glycoprotein

A1- A6- different types of alginates with varying mannuronic to guluronic acid ratio

BMP6- bone morphogenetic protein

B1-B18- different alginate beads formulations

Ca- Calcium

CRP- C reactive protein

CRTU- Clinical Research and Trials Unit

DcytB- duodenal cytochrome b

DI water- Deionized Water

DMA- 2'-deoxymugineic acid

DMEM- Dulbecco’s modified Eagle’s media

DMSO- dimethyl sulfoxide

DMT 1- divalent metal transporter 1

DPD- 2,2′-Bipyridyl

EH media- Earles/HEPES buffer

FAC- ferric ammonium citrate

G- L-guluronic acid

GLUT5- fructose transporter

GLUT1, GLUT3- glucose transporters

G/O/W- gel in oil in water emulsion

Hb- Haemoglobin

HBBS- Hanks' Balanced Salt solution

HCP1- haem iron protein 1

HCT- haematocrit

HFE- haemochromatosis protein

HJV- haemojuvelin

HNU- Human Nutrition Unit

Hp- Hephaestin

HRP- horseradish peroxidase

IFR- Institute of Food Research

IP2, IP3- inositol phosphatases

IP5- inositol pentaphosphate

IP6- inositol hexakisphosphate

Fe- Iron

FeG- iron (II) gluconate

IDA- iron deficiency anaemia

IRE- iron responsive element

IRP1 and IRP2- iron regulatory proteins

M- D-mannuronic acid

MCH- mean corpuscular haemoglobin

MCV- mean corpuscular volume

MEM- minimum essential media

MFP- monoferric phytate

MTP1/ Ferroportin 1/IREG-1metal transporter protein

NA- nicotianamine

NDNS- National Diet and Nutrition Survey

NHANES- National Health and Nutrition Examination Survey

NNUH- Norfolk and Norwich University Hospitals

NRP- Norwich Research Park

NU-AGE- New dietary strategies addressing the specific needs of elderly populations for healthy ageing in Europe

PG- Phen GreenTM SK, diacetate

PIS- participant information sheet

RBC- red blood cells

RRI- Rothamsted Research Institute

SN14, SN 15- different starch types loaded with iron

sTfR- Soluble transferrin receptor

TA- tannic acid

Tf- transferrin

TfR1- transferrin receptor 1

TfR2- transferrin receptor 2

UEA- University of East Anglia

UTR- untranslated region

WBC- white blood cells

WHO- World Health Organisation

W/O/W- water/oil/water emulsions

w/v- weight by volume

1. INTRODUCTION

1.1 Iron

Iron (Fe) is one of the most abundant trace elements in the Earth's crust. It can be found in ten different oxidative states (from –II to +VIII). However in aqueous solutions it is only present in either of the two following forms:

- Fe^{2+}, ferrous form
- Fe^{3+}, ferric form

Iron can readily transfer an electron and switch between these two oxidation states, thus it is an ideal element to catalyse biochemical reactions, including redox reactions[1].

Under aerobic conditions ferrous iron is oxidised to the ferric form. Fe^{3+} is insoluble at physiological pH and therefore not available for mammalian cells[2]. The problem can be overcome with the use of reducing agents or proteins which have evolved to reduce iron from the ferric to ferrous form, thus ensuring that iron is soluble and bioavailable. The latter are essential to allow iron uptake[3, 4, 5], storage[6] or haem synthesis[7]. Consequently, reduction reactions of iron are crucial in iron metabolism.

However, when iron is present in excess it can catalyse reactions in which harmful oxygen radicals[2] are generated. Reduction of one electron of dioxygen by ferrous iron leads to formation of superoxide, which reacts further leading to the sequence of reactions known as Haber-Weiss-Fenton chemistry in which hydroxyl radicals[7] are produced from superoxide and hydrogen peroxide. Hydroxyl radicals are toxic within biological systems, oxidising proteins, nucleic acid and carbohydrates which can result in peroxidation of lipid membranes[7]. Consequently, oxidative stress (elevated levels of reactive oxygen and nitrogen which exceed the antioxidant capacity of the organism) occurs in the presence of raised levels of redox-active iron leading to enhanced deterioration of tissues[2]. Therefore, in order for biological systems to function optimally, it is crucial that iron concentrations are tightly regulated.

1.1.1 Importance of iron homeostasis in mammalian metabolism

Unlike other essential metals, there is no specific mechanism for iron excretion from the body[8]. The only loss of iron from the body takes place through shedding epithelial cells from the intestine, urinary tract, skin (including hair and nails), via blood loss (menstruation or other), urine, bile, semen and sweat[9]. Nearly 90% of

daily iron requirements are covered by recycling from the breakdown of senescent red blood cells[10]. The remaining 10%, which is approximately 1-2mg iron[12], needs to be acquired from the diet via intestinal iron absorption.

Nonetheless, according to the World Health Organisation (WHO) around 2 billion people worldwide are affected by anaemia[12], the reduced capacity of red blood cells to carry oxygen. It is caused by impaired or decreased haemoglobin or red blood cell concentrations in the blood. Progressive anaemia can lead to hypoxia. By contrast, the iron overload disorder, haemochromatosis, affects approximately 1 in 150 people in populations of Northern European origin[10]. Thus, tight regulation of iron homeostasis (Figure 1.1.1) is fundamental for maintaining health.

Iron in the ferrous state is a component of haemoglobin and myoglobin. Haemoglobin in red blood cells is needed in order to transport oxygen around the body. Myoglobin is an oxygen storage and utilisation protein within the muscle tissue. Iron is vital for many biochemical processes within the body, such as synthesis of DNA, mitochondrial respiration and other crucial metabolic reactions[11].

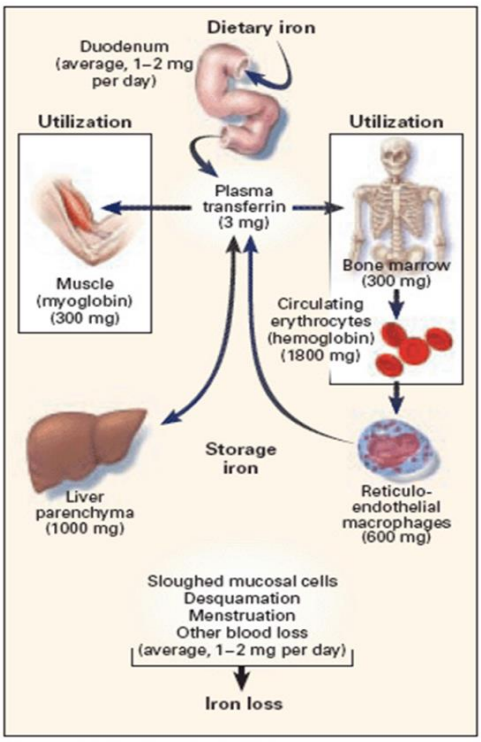

Figure 1.1.1 Schematic diagram showing major pathways of iron homeostasis with approximate values of iron content in tissues. From: http://ahdc.vet.cornell.edu/clinpath/modules/chem/femetb.htm. Copyright © Cornell University- accessed on 02.02.2011.

1.1.2 Iron deficiency in humans

Iron deficiency anaemia (IDA) accounts for 50% of all anaemias[12] with infants, children and pre-menopausal women at most risk. Both in developing and developed[14] countries the consequences of anaemia result in significant health problems and economic cost.

Nutritional iron deficiency is a state of imbalance when the iron content of the diet is insufficient to meet physiological needs[15]. Iron is present in a wide variety of foods, so dietary intake usually correlates positively with energy intake[16]. Depending on the presence of dietary enhancers and inhibitors, non-haem (inorganic) iron absorption from the diet ranges between 1% and 15%[17]. Therefore the cause of nutritional deficiency is often related to low bioavailability rather than inadequate intake of iron. However if body iron requirements exceed intake, the risk of iron deficiency is elevated.

In developing countries instances of iron deficiency are 2.5 times that of anaemia. The WHO defines iron deficiency as “a condition in which there are no mobilizable iron stores and in which signs of a compromised supply of iron to tissues, including the erythron, are noted. The more severe stages of iron deficiency are associated with anaemia”[14].

In the United Kingdom according to the National Diet and Nutrition Survey (NDNS) iron deficiency affects ≈29% of children below 4.5 years of age, 23.8% of female teenagers, 16% of young woman and 14.3% of free-living elderly females (85 years and over) with serum ferritin levels in above mentioned groups below 15µg/L[14]. IDA occurs at some stage of pregnancy in 25% of woman and in 33.3% of woman in the postpartum period[19]. Furthermore children below 6 years of age, females aged 15-18 and 25-34 are also reported to have IDA with a prevalence of 11.5% and 9.1% respectively[14]. According to the NDNS IDA is very common in the elderly, with haemoglobin (Hb) concentrations below 130g/L in 37.5% of free-living males and below 120g/L in 16.0% of females aged 85 years and over. IDA is even more pronounced in institutionalised elderly individuals aged 65 and over with 52.2% of males and 38.6% of females being affected. The main reasons for IDA are:

- Diet with low iron content
- Diet rich in iron inhibitors thus reducing iron bioavailability
- High iron demand due to physiological state

Other causes of anaemia include:

- Acute and chronic infections (malaria, HIV, tuberculosis, cancer)
- *Helicobacter pylori* infection[20]
- Heavy blood loss
- Parasite infections
- Other micronutrient deficiencies (riboflavin, folate, copper, vitamin A, B12)
- Haemoglobinopathies affecting African, Southeast Asia and Mediterranean populations.
- Impaired iron absorption

Often more than one factor contributes to the development of anaemia. Although it is continuously being addressed through food fortification programmes across the world, eradication of the condition remains challenging.

The physiological demands for iron determine how efficiently iron is absorbed, but the iron must be present in the gut lumen in a form that is available for absorption, therefore dietary factors play a key role in delivering the required amount of iron to maintain balance. Requirements are increased in the following situations:

- Pregnancy
- Infants and young children (rapid growth)
- Menstruating teenagers (growth plus iron losses)
- Menstruating woman (iron losses)

The symptoms of anaemia are varied. In adults iron deficiency decreases the capacity for physical activity, affects work performance and causes tiredness and lethargy[12,18]. IDA adversely affects the immune system, increasing the possibility of upper respiratory tract infections in children, and leads to prolonged recovery times[21]. The health consequences of not meeting body iron requirements depend on the physiological state:

- During pregnancy, due to placental growth and the increase in red blood cell mass, iron requirements increase three fold[22]. IDA during pregnancy can result in preterm labour, low birth weight, and increases the risk of anaemia in infants after 4 months of age. In severe instances it can lead to maternal and infant mortality[23,24].
- Very rapid growth during infancy quickly drains iron reserves accumulated by the fetus during gestation, frequently resulting in iron deficiency[15]. At

birth, a significant proportion of iron is in the form of fetal haemoglobin; however this is depleted by approximately 6 months of age with adult haemoglobin being produced from around 10-12 weeks onwards[25]. This can lead to compromised cognitive and motor development, which might not be reversible. However, reported findings are conflicting; with a lack of convincing evidence that IDA causes any impairment in children less than 2 years old[26]. Nonetheless, there are data demonstrating that cognition, motor activity, social attention and overall school performance in school age children are affected in a state of IDA[27].

- Imbalance between energy intake and increased iron needs occurs during female adolescence due to rapid growth and the onset of menstrual iron loss. Instances of iron deficiency occur more frequently in individuals with heavy blood losses during menstruation[28].

As the lifespan of the population on the whole is increasing the elderly are another group at risk of IDA (as supported by the above NDNS figures[14]). Reasons for anaemia in the elderly may be due to number of following factors:

- Poor and monotonous diet
- Loss of appetite associated with lower physical activity
- Chronic diseases and inflammation[29,30]
- Impaired efficiency of iron absorption[31]
- Decreased functionality of erythrocytes[32]
- Regular intake of medications (such as aspirin)[30]
- Occult blood loss[31]
- Institutionalisation[31]

Anaemia in the elderly is related to several health implications, including a decline in physical performance and strength, cognitive impairment, increased susceptibility to falling, frailty, and mortality[33,34,35] (for more detailed review please refer to Appendix- A).

Finally there is evidence in the literature (for details please see Appendix- B) that genotype is a major determinant of body iron content. Latest information in twins demonstrate that the genetic contribution to iron status is around 50%[36,37].

1.2 Mechanisms of iron absorption and influence of dietary factors

Dietary iron can be present as haem (organic) and non-haem (inorganic) iron. Animal meat is a rich source of haem iron due to the presence of the iron-containing proteins, haemoglobin and myoglobin. Non-haem iron can be found mainly in plant-based food and meat[11]. Haem iron is better absorbed than non-haem iron, partly due to metalloporphyrin (in which haem iron is incorporated) being less susceptible to inhibitors than non-haem iron. In addition, haem iron is taken up by the enterocyte via a pathway distinct from inorganic iron[38]. To date, only the haem iron transporter, haem carrier protein 1 (HCP1) has been identified as being involved in haem uptake, but the exact process of haem iron uptake remains unclear[39,40]. It is reported that HCP1 is upregulated in situations of iron deficiency or hypoxia, and that it is also transports folate[39].

The absorption of haem iron from the diet is estimated to be between 15% to 40% in populations where meat is regularly consumed[41,42]. In comparison, non-haem iron absorption ranges between 1% and 15%[17], depending on the iron status of the individual and on the presence of dietary enhancers and inhibitors to iron absorption.

The mechanism of inorganic iron uptake and metabolism is much better characterised and it will be described in a greater detail later in this Chapter.

1.2.1 Iron inhibitors

1.2.1.1 Phytate

Phytic acid (myo-inositiol hexakisphosphate (IP6), Figure 1.2.1) or phytate when in salt form is the main storage form of phosphate and inositol, which accumulates within plant grains and seeds during maturation[43]. The chemical structure of phytic acid allows it to chelate with calcium, iron, magnesium, potassium and zinc cations.

Consequently, phytate is a significant inhibitor of inorganic iron absorption in plant-based foods such as legumes or cereals.

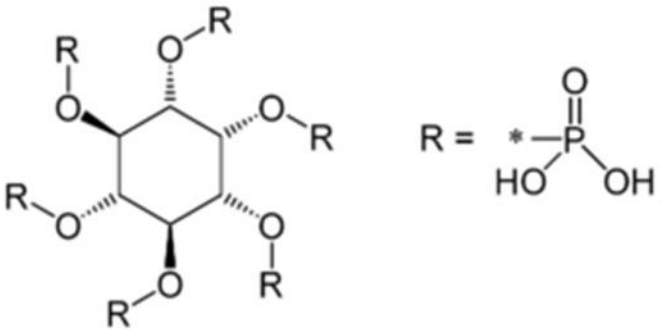

Figure 1.2.1 Phytic acid structure, (Kumar *et al*[43]).

The basis of the inhibitory interaction with mineral ions such as iron is through formation of insoluble phytate-mineral complexes[43] which are not freely available for absorption, thus reducing the availability of iron or other minerals. The human gut lacks the enzyme phytase, which if not present in consumed foods or if inactivated by food processing (such as heating, or extrusion) results in phytate degradation in the upper GI tract of around 0-28%[44]. However if dietary phytate is consumed with foods rich in plant food phytases, phytate can be, at least in part (37-66%), degraded in the stomach and small intestine[44,45,46]. Furthermore, animal research (in pigs) showed that phytate hydrolysis reaches a plateau at around 66% and cannot be improved further by increasing the phytase content of foods[47]. This may be due to the incomplete release of phytate from foods[44,48]. In addition pH plays an important role in phytate solubility; with a pH increase from ≈2 in the stomach to ≈5-7 in the small intestine, phosphates, particularly IP6, precipitate and adversely affect availability of metal ions such as iron[49]. On the contrary, less phosphorylated inositol phosphatases (IP3, IP2) remain soluble at duodenal pH thus keeping iron in solution[49]. However, the major site for phosphate hydrolysis is the large intestine where microbial phytase plays a major role[50].

The effect of phytate on iron absorption is apparent at concentrations as low as 2-10mg/meal and is dose dependant. However the inhibiting effect of phytate on iron absorption can be diminished by addition of ascorbic acid[51,52]. The molar ratio of phytate to iron needs to be below 0.4:1 to prevent inhibition of iron uptake[53,10].

1.2.1.2 Calcium

Calcium (Ca) is an unusual inhibitor of iron absorption because it is able to influence both non-haem and haem iron uptake in a dose-dependent manner[54]. It has been proposed that the inhibition of iron uptake occurs at the basolateral membrane[10] of the enterocyte. Research suggests that calcium down-regulates iron uptake by translocating divalent metal transporter 1 (DMT 1) at the apical membrane[55]. For more details please refer to Chapter 6.

1.2.1.3 Polyphenols

Polyphenols can be found in plant-based foods such as vegetables, fruits and cereals as well as in tea, coffee and wine. However, their inhibiting effect on non-haem iron absorption is greater when originating from black tea rather than from herb tea or wine[56]. The inhibitory effect of polyphenols and phytate is cumulative if present together in the diet[41].

1.2.1.4 Milk, egg and soy proteins

Bovine milk proteins including casein (mainly β-casein A1 and β-casein A2) and whey (β-Lactoglobulin and α-Lactalbumin), egg white (albumen) and the conglycinin fraction in soy are the main protein groups which are reported to inhibit inorganic iron absorption[57,58].

1.2.2 Enhancers of iron absorption

1.2.2.1 Ascorbic acid

Ascorbic acid (AA) is one of the best described enhancers of non-haem iron uptake. Its effect is attributed to its abilities to both chelate iron and reduce ferric iron, thus increasing intestinal absorption[59]. It has also been shown that AA has the potential to diminish the effects of iron inhibitors such as phytates and polyphenols[51]. However, during food processing the positive influence of AA on iron absorption is decreased, mainly due to the losses resulting from heat treatment and food storage. Erythrobic acid, an epimer of L- ascorbic acid, has an enhancing effect and is able to withstand cooking and is used as an antioxidant in food processing[60,61]. It has been reported that erythrobic acid has 1.6 fold the capacity of ascorbic acid to enhance iron uptake at 4:1 (erythrobic acid to iron) ratio[61].

1.2.2.2 Meat

There is evidence in the literature that addition of meat, poultry or fish to meals has an enhancing effect on non-haem iron absorption (2 to 4 fold increase in iron absorption)[62,63]. The enhancing effect of muscle tissue on iron absorption may be due to the many small peptides present in the tissue rather than to a single peptide fraction[64] and is often referred to as the 'meat factor'.

1.2.2.3 Nicotianamine.

Nicotianamine (NA) is involved in intra- and intercellular transport of metal cations in the plant. When it is present at elevated levels in the grain it has been reported to enhance iron uptake in both cell and murine models[65,66]. Please refer to Chapter 7 for more details.

There is a well-justified use of iron fortification as a strategy to prevent iron deficiency anaemia. Food fortification with iron is recognised as a sustainable and realistic way to reduce the occurrence of iron deficiency[67]. Mainly because when iron is added to foods that are commonly consumed, iron status of the whole population can be improved.

1.2.3 Iron fortification strategies

Iron compounds commonly used in food fortification programmes can be divided into the following categories[68]:

- Water soluble (ferrous sulphate, ferrous gluconate, ferrous lactate, ferric ammonium citrate)
- Poorly water soluble but soluble in dilute acid (ferrous furmarate, ferrous succinate, ferrous saccharate)
- Water insoluble and poorly soluble in dilute acid (ferric orthophosphate, ferric pyrophosphate, elemental iron powders)
- Iron compounds that are protected (sodium-iron EDTA complex)[68]

Food fortification can be challenging to the food industry, mainly due to organoleptic changes that may occur during storage or preparation[69]. Water soluble forms of iron are considered highly bioavailable. However they often cause sensory problems when added to foods (undesirable colour and flavour changes)[68,69]. Forms of iron that are poorly water soluble but soluble in gastric juice are also well absorbed[14]. Water insoluble and poorly acid soluble forms of iron have the lowest bioavailability

but cause fewer organoleptic problems. However because they are generally not well absorbed they are therefore ineffective as fortificants[68,69].The main advantage of using sodium EDTA is that when iron is bound to sodium EDTA it will no longer form complexes with phytate present in cereal based foods[68].

In the UK, mandatory enrichment of white and brown wheat flour was introduced in 1953[70] in order to restore it to the levels found in wholemeal flour. Since then, other foods such as wheat bread, breakfast cereals, infant food formulas, infant cereals and other low moisture what products such as pasta and noodles[70] have been fortified with iron on a voluntary basis. In developing countries fortification of sugar, soy sauce, curry powder and fish sauce is also quite common[14].

Recently, more challenging strategies to fortify foods in iron were employed, such as fortification of fresh cheese with glycine stabilised ferrous gluconate[71]; finger millet flour fortification with iron EDTA and folic acid[72]; addition of ferrous sulphate to germinating brown rice[73] and dephytinisation of West African cereal (fonio) with intrinsic wheat phytase coupled with addition of ferrous sulphate[74], all with promising potential in relation to preventing iron deficiency.

1.2.4 Intestinal iron absorption

Iron absorption in humans takes place mainly in the enterocytes of the duodenum and proximal jejunum. Intestinal enterocytes have a unique pathway of iron uptake which is not found in other tissues. Prior to uptake into the enterocyte via DMT1[75], iron, if present in the ferric form, needs to be reduced to the ferrous state by duodenal cytochrome b (DcytB)[3]. Once within the enterocyte, iron can either be transported through the basolateral membrane (in the presence of hephaestin), via ferroportin 1 into the bloodstream[76], or it can be stored within the labile iron pool as the protein ferritin[77]. When iron is exported from the enterocyte, it binds to transferrin and is transported to the sites of iron utilization,[15] where it is taken up by cells via either transferrin receptor 1 (TfR1) or the less expressed transferrin receptor 2 (TfR2). As iron is liberated from the transferrin-transferrin receptor complex, it is reduced from the ferric to ferrous form, and DMT1 transports iron from endosomes to the cytosol[9]. Further information regarding each of the key proteins involved in iron homeostasis is given below.

1.2.4.1 Iron transport (uptake and export) proteins

- **Duodenal cytochrome b (DcytB)**

  The role of DcytB is to reduce iron from the ferric to the ferrous form thus enabling it to be taken up by DMT1 into the enterocyte[3]. In addition, Wyman *et al.* showed in Madin-Darby canine kidney (MDCK) cells that DcytB is not only a ferric reductase, but also a cupric reductase[78]. DcytB is located in mature enterocytes of the duodenal brush border and uses intracellular reducing cofactors to undertake reduction[3]. However, research carried out in genetically modified mice, where DcytB was not expressed, revealed that uptake of iron from the duodenum was reduced, but not entirely blocked[79]. These findings suggested that in addition to DcytB, other reducing mediators could be present. As a result, two further mammalian ferrireductases have been identified: cytochrome *b* $_{561}$ (Dcytb is a member of this family)[80,81], and Steap metalloreductase[4]. An additional candidate for the role of a reducing agent as well as an electron donor is AA[8]. AA has also been shown to enhance iron absorption in humans[59, 82].

- **Divalent metal transporter 1 (DMT1)**

  In the duodenum, inorganic iron, in the ferrous form, enters intestinal absorptive cells from the intestinal mucosa[75] via DMT1. DMT1, which was first identified in rats[83], is a natural resistance associated macrophage (Nramp2)[75] situated in microvilli on the brush border of mature enterocytes[8].

  The slightly acidic environment on the mucosal surface of the duodenum[84] is thought to provide the potential energy for the proton electrochemical gradient[85]. DMT1 is able to use the energy to transport ferrous iron through the membrane into the duodenal enterocyte in addition to other divalent metal ions (Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+})[75]. However, other research suggests that whilst DMT1 shows reactivity with these metals, further investigation is needed to characterize the affinity and specific nature of the transport processes of each metal[86]. DMT1 is also expressed in the proximal intestine, thymus, kidneys, brain and other organs[75]. Moreover, this twelve transmembrane domain protein not only mediates iron transport from the duodenal lumen, but is also involved in cellular uptake of iron. When iron is liberated from the transferrin-transferrin receptor complex (see 'Transferrin receptor' Section below for more details) and reduced from the ferric to ferrous form, DMT1 transports iron from endosomes to the cytosol[9].

- **Metal transporter protein Ferroportin 1 (MTP1/IREG1)**

  Once iron enters the enterocyte it enters the labile iron pool and can either be stored as the protein, ferritin (discussed in further detail in Section 1.2.4.2 below), or it can be directed through the basolateral membrane into the circulation via metal transporter protein (MTP1)/ Ferroportin 1/IREG1 in the presence of ferroxidase[87]. Ferroportin 1 is an iron regulated, multidomain transmembrane protein[88] localized at the basolateral membrane of polarised epithelial cells. Its mRNA contains an iron responsive element (IRE) (refer to Section 1.2.5.1 for further details). It is involved in exporting iron from macrophages[89] and is expressed in kidney, liver and testis[87]. Once iron is exported from enterocytes via ferroportin, it needs to be oxidised to the ferric state in order to bind to transferrin (primary iron transport protein) and enter the circulation[90].

- **Hephaestin**

  Hephaestin (Hp), a transmembrane multicopper ferroxidase oxidises ferrous iron at the intracellular basolateral membrane of the enterocyte[91], and facilitates iron efflux into the blood stream. Studies carried out in sex-linked (sla) mice, where expression of hephaestin was disrupted, showed that despite unaffected uptake of iron and a consequent overload in the enterocyte, mice developed moderate to severe anaemia due to the impaired egress of iron from intestinal enterocytes into the circulation[5]. Hp is also a caeruloplasmin homologue, which is a 'multicopper ferroxidase found in plasma[92]. Caeruloplasmin is involved in the oxidation of iron, which is distributed from the mononuclear phagocyte system into the plasma[93].

- **Transferrin (Tf)**

  Once iron is exported from the enterocyte via ferroportin 1 and hephaestin, it is subsequently bound to plasma transferrin (Tf) and either distributed to the erythroid cells[15] or other tissues. Serum transferrin is a glycoprotein consisting of a single polypeptide chain, which is synthesised in the liver and from there is released into the bloodstream[54] (Figure 1.2.2)

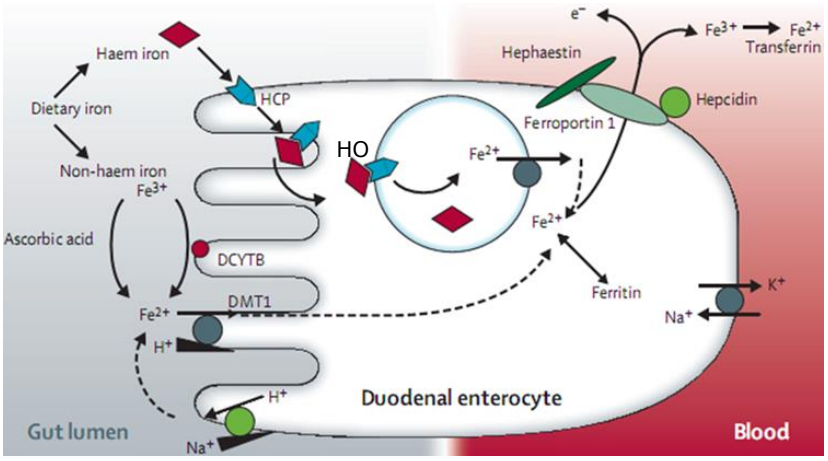

Figure 1.2.2 Schematic diagram showing intestinal iron uptake through the enterocyte. Adapted from Zimmermann *et al.*[15].

This metal ion binding protein can bind two ferric ions in the presence of other anions, such as carbonate. The function of these anions may be to prevent water from binding thus "locking the metal firmly to the protein and avoiding hydrolysis"[85] enabling transport of iron to the cells in depletion[64,65].

1.2.4.2 Iron storage proteins

- **Ferritin**

  Following uptake into the enterocyte, iron which is not exported through the basolateral membrane into the circulation is stored as ferritin, mainly in the cytosol. A small proportion of ferritin can also be found in serum, thus making it a useful marker of iron storage[6]. Ferritin is anoligomeric protein composed of 24 chains (4 helix bundle) containing light (L) and heavy (H) subunits (Figure 1.2.3). Due to the presence of H subunits, the protein is capable of oxidising ferrous iron to the ferric form, which occurs when ferrous iron binds to the ferroxidase centre where it is oxidised and subsequently moved to the central cavity. An increased number of L subunits allows the cell to expand its iron storage capacity within the ferritin molecule. L subunits also facilitate iron nucleation in the presence of acidic residues at the cavity surface, which increases ferroxidase activity [6].

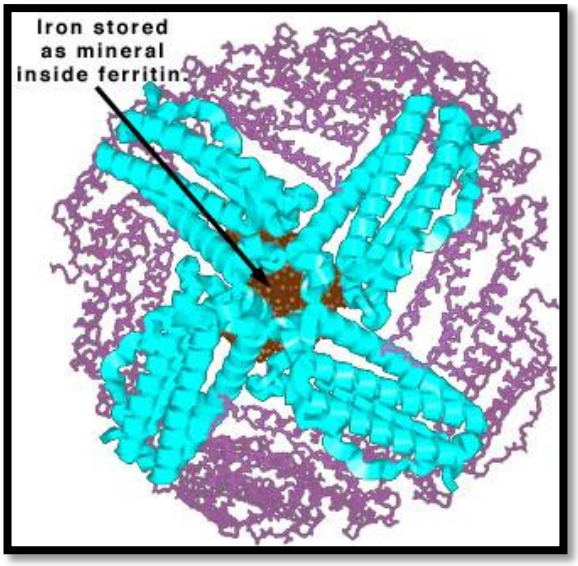

Figure 1.2.3. Diagram showing spherical shape of ferritin and iron stored as a mineral within it. From: Iron in Biology: Study of the Iron Content in Ferritin, The Iron-Storage Protein, Rachel Casiday and Regina Frey Department of Chemistry, Washington University, St. Louis, MO 63130-accessed on 27.02.2011.

On average, ferritin is able to store 2500-3000 atoms of ferric iron in the core of the molecule. However, the number can be as high as 4500 of ferrihydrite [85, 96]. Higher numbers of L subunits are present in ferritin in storage tissues, such as liver or spleen. Thus, they contain greater amounts of iron than ferritins with a higher proportion of H subunits which are expressed in heart and brain[77].

How iron is released from ferritin is the subject of ongoing research. Based on *in vitro* studies, there are two main hypotheses. One is that due to chelation or reduction, iron release from ferritin occurs preceding its degradation[97]. The other suggestion is that iron is released when cytosolic ferritin undergoes degradation within the lysosome[98]. In a more recent study, De Domenico *et al.* showed that overexpressed ferroportin mediates iron release from cytosolic ferritin prior to its degradation by proteasome[99]. Degradation of iron deplete ferritin is performed by ubiquitin which disassembles ferritin nanocages[99]. Nonetheless the precise mechanism of iron release from ferritin in humans needs further investigation.

- **Haemosiderin**

  Haemosiderin accumulates mainly in liver lysosomes. It is a water-insoluble iron storage protein, which is derived from ferritin degradation products. Thus, it has greater iron content than the latter[85]. However attempts to produce haemosiderin *in vitro,* using purified lysosomes, failed, suggesting that additional reactions occur during its formation [100].

  Storage of iron in the haemosiderin complex only occurs in states of iron excess, and consequently it can be found in iron overload conditions such as haemochromatosis[85,101]. This may be a response to the fact that iron locked within haemosiderin, unlike iron in ferritin, is water insoluble, thus limiting its availability[102]. Moreover, iron stored in haemosiderin does not participate in lipid peroxidation[103] or the formation of free oxygen radicals to the same extent as iron in the form of ferritin[104]. In addition, due to much greater variability in the phosphate content and mineralised structure of the iron core in comparison to ferritin, it was shown *in vitro* that haemosiderin releases iron from its core twice as slowly as H-ferritin[105, 106]. However, this does not fully explain the differences in the rate of iron release between haemosiderin and ferritin, and it has been suggested that differences in the structure of the mineralised iron core between haemosiderin and ferritin have an impact on iron release[107].

1.2.5 Iron uptake into non-intestinal mammalian cells

As described in the above section iron acquisition by enterocytes is a unique mechanism. The uptake of iron in other cell types is briefly described below.

Having entered the bloodstream, iron is acquired from transferrin by cells via either transferrin receptor 1 (TfR1) or the less expressed transferrin receptor 2 (TfR2). The abundance and affinity of TfR1 to bind diferric transferrin is much greater than that of TfR2[108]. Moreover, TfR2 mRNA has been shown not to contain iron responsive elements (IRE), as opposed to TfR1 and as a result, TfR2 cannot be down-regulated in response to iron overload[109]. However, other research demonstrated that TfR2 is unable to maintain sufficient intracellular iron in the absence of TfR1[110].

Both TfR1 and TfR2 are cell surface glycoproteins consisting of two polypeptide chains[85]. The transferrin-transferrin receptor complex is taken up by the cell via clathrin coated endocytic vesicles. Within the endosomes a proton pumping ATPase

lowers the pH and in the presence of STEAP metalloreductases, ferric iron is reduced to the ferrous form and transported by DMT1 into the cytosol[4,111]. TfR and apotransferrin, assisted by the trafficking protein Sec15l1, migrate to the cell surface where apotransferrin is released into the circulation awaiting iron, whilst TfR is bound to the cell membrane. The concentration of TfR at the cell surface reflects cellular iron requirement[9, 111] (Figure 1.2.4).

Transferrins play a vitally important role in the erythroid cycle as they are required to fulfil the demand for iron needed by erythroid precursors in bone marrow in order to produce red blood cells. Iron resources are tightly regulated, thus when aged red blood cells are utilized by macrophages, iron is recycled and returned to the system ready for transport by transferrin[15].

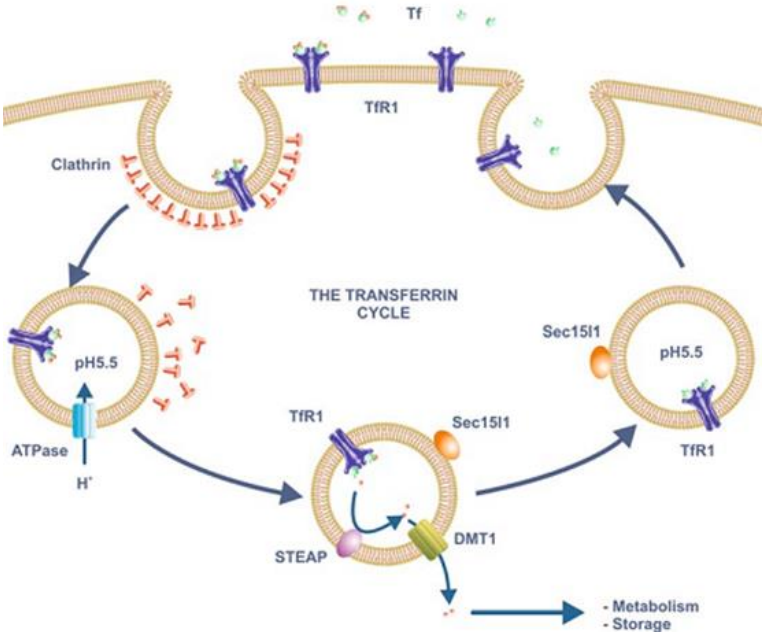

Figure 1.2.4 Schematic diagram of the transferrin cycle. From: Anderson *et al.*[112].

TfR1-mediated uptake of differic transferrin (high affinity uptake) applies to the majority of mammalian cells. Apart from TfR1 mediated uptake, non-receptor mediated uptake by which cells can acquire diferric trancferrin has also been described[113]. Hepatocytes, for example, can acquire iron via low and high affinity uptake processes. The proposed route for low affinity uptake is via TfR2 receptors, which are expressed in high numbers in the liver[114]. Research conducted by Trinder and Morgan in hepatocyte cells suggest that uptake of diferric transferrin occurs via proteoglycans or fluid phase endocytosis[115]. Also macrophages are capable of taking up diferric transferrin as well as phagocytosing senescent red blood cells in order to recycle the iron[112]

However, when uncontrollably high dietary iron uptake takes place (in iron overload disorders) there are elevated levels of iron entering the plasma. When the binding capacity of circulating transferrin is exceeded, non-transferrin-bound iron is found in the plasma and it can be taken up by the cells[116]. The exact mechanism by which non-transferrin-bound iron enters the cell is uncertain[112]. Also in particular pathological conditions iron from free haemoglobin or haem can be taken up by hepatocytes and other cells via a haem/haemopexin complex[117] through both low and high affinity pathways[112].

1.2.5.1 Systemic iron regulation protein

- **Hepcidin**

  Hepcidin is an antimicrobial, liver-derived, 25 amino acid peptide. It is secreted by hepatocytes into the plasma and excreted in urine[118]. In the mature form, hepcidin coordinates iron efflux from the basolateral membrane of intestinal enterocytes into the bloodstream. Hepcidin regulates iron absorption by direct influence on ferroportin (present in enterocytes or in the plasma membrane of macrophages), the only iron protein exporter identified to date[85]. When body iron levels are high, or in a situation of inflammation, hepcidin binds to iron loaded ferroportin as it exits the enterocyte or macrophages, causing its internalisation and degradation. Consequently iron efflux into the circulation is abolished[119]. Conversely, in a situation where the body is depleted, for example during pregnancy, hypoxia, and elevated erythropoiesis, hepcidin synthesis is reduced, thus allowing ferroportin to transport iron from the enterocyte into the bloodstream[119] allowing iron to become available for sites of iron utilization (Figure 1.2.5).

  Regulation of hepcidin expression is coordinated by the following hepatocyte plasma membrane proteins: TfR2, haemojuvelin (HJV) and haemochromatosis protein (HFE)[112]. It has been shown that HFE binds to TfR1, thus making it impossible for transferrin to bind to the receptor, whereas TfR2 can simultaneously bind HFE and transferrin. The TfR2-HFE complex stimulates hepcidin expression[120,121]. However further investigation is required in order to establish the extracellular signals by which these proteins trigger hepcidin synthesis[122]. Nonetheless, the mechanism by which HJV stimulates hepcidin is better understood. HJV acts through the bone

morphogenetic protein (BMP6)/SMAD pathway, which is thought to be the main route of hepcidin regulation[123,124].

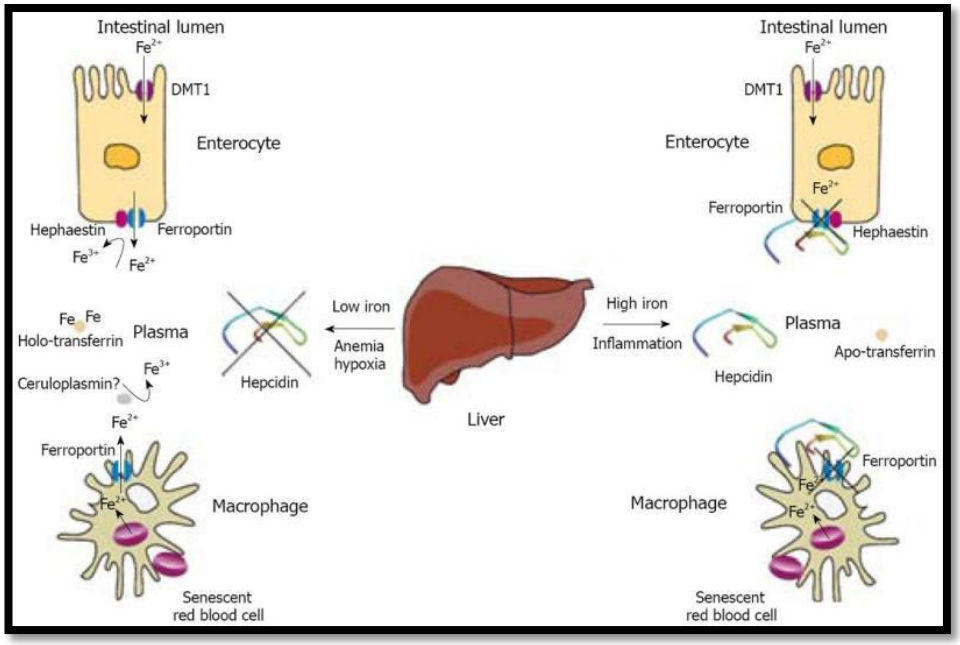

Figure 1.2.5. Schematic diagram of hepcidin regulation of iron efflux. From: Pantopoulos K.[125].

1.2.5.1 Summary of systemic and cellular regulation of iron

Cellular iron homeostasis is post-transcriptionally mediated by the RNA binding iron regulatory proteins (IRP1 and IRP2) and iron responsive elements (IRE)[126]. In instances of iron deficiency, IRP1 and IRP2 bind to the iron responsive elements found in mRNA encoding proteins involved in iron metabolism, namely DMT1, TfR1, ferroportin and ferritin. Depending where IREs are located in the untranslated region (UTR) of mRNA (in the 5' or 3' of UTR), the binding of IRPs can either block or upregulate the translation [127,112]. Ferritin mRNA contains one IRE in the 5' UTR, while mRNA of TfR1 contains many IREs in 3' UTR[128]. Therefore the process by which IRP/IRE orchestrates ferritin and TfR1 in a situation of increased iron demand is as follows: the translation of the message to collect iron for ferritin storage is blocked by IRP, and simultaneously the TfR1 is upregulated causing increased iron uptake. Similarly to TfR1, in a situation of iron deficiency the mRNA of DMT1 is regulated by the IRE/IRP system[75]. Interestingly, ferroportin is controlled by both cellular and systemic iron regulation. Intracellular regulation of ferroportin is performed via the 5' UTR IRE of its mRNA, whereas systemic regulation is

maintained by hepcidin as described earlier. This cross talk between cellular and systemic iron metabolism is crucial for controlled iron efflux[9,129].

1.3 Methods of estimating iron status

Biomarkers by which ID or anaemia are usually measured in serum/plasma samples are:

- Haemoglobin (Hb) concentration. Hb lacks sensitivity and specificity and therefore is only useful for diagnosing severe iron deficiency (anaemia)[130], but due to its relatively low cost and diagnostic availability it is often used[12]. An important factor which needs to be considered in establishing appropriate cut-offs for Hb are ethnic and gender differences. Whilst the latter is well-characterised the former is a controversial issue, despite the fact that it has been identified in the literature since 1975[131,132,133]. On the other hand there is also evidence in the literature that ethnic difference in Hb between Blacks and Whites was not significant[134]. More recent reports suggest applying a reduction in the Hb cut-off from 12.0g/dL to 11.5g/dL for black populations. The reduced cut-off was established in response to observing no change in the relationship between haemoglobin concentration and transferrin saturation, whilst still observing different distributions in Hb concentrations[135]. Another factor which needs to be considered in establishing appropriate Hb cut-offs is living at high altitude. In order to retain oxygen supply during hypoxemia, Andean highlanders (at high altitude) have higher Hb levels compared to those at sea level [136]. But, this is not necessarily a trait found in all populations living at high altitudes (3200-4000m)[137]; another adaptation is reduction in affinity between Hb and oxygen to ensure that Hb bound oxygen is more accessible for tissues[138].

- Serum/plasma ferritin concentration is a relatively good estimate of iron status[139] as there is a quantitative relationship between circulating ferritin in the plasma and iron stores in the liver[140]. Phlebotomy studies have shown that a circulating ferritin value of 30µg/L is equivalent to 300mg of storage iron[141,142]. Nevertheless there are some limitations in the use of ferritin as an iron biomarker. It may not be appropriate in the presence of acute and chronic inflammation or infections because ferritin is an acute-phase

protein[143] and increases in response to infection/inflammation, no longer reflecting body iron stores. In this case the usual ferritin cut-off to estimate ID of <15µg/L would no longer be relevant[144].

- Soluble transferrin receptor (sTfR) has also been used as an early indicator of iron deficiency[145,146] as it is increased when the supply of iron is insufficient for optimal erythropoiesis, and this increase is observed before Hb levels start to fall[18,111]. sTfR is not considered to be an acute phase protein therefore its fluctuations in inflammatory states are considerably smaller than those of ferritin[147]. However there is evidence that in the presence of malaria sTfR levels may increase considerably[148]. Furthermore in HIV positive populations sTfR may be lacking sensitivity[145]. Therefore, both ferritin and sTfR should be interpreted with caution in populations where malaria or HIV are prevalent.

- More recently, the body iron method presented by Cook *et al.*[149] has been introduced as a more accurate measure of iron status. Body iron takes into account two biomarkers of iron status, ferritin and sTfR, and combines them into the following equation:

  Body iron(mg/kg)=- [log(sTfR/Ferritin ratio)-2.8229]/0.1207. However, this equation is only valid when the method for sTfR is the same one as that used by Cook.

  The relationship between ferritin and sTfR was obtained through repeated phlebotomy of 14 healthy subjects[150]. The body iron method was further evaluated by examining iron status in three studies[149]. Later this approach was evaluated to measure iron status in the US population in the National Health and Nutrition Examination Survey (NHANES)[151]. This method is more informative than Hb, ferritin or sTfR as it allows individuals to be placed at a specific point in the iron status spectrum, ranging from positive values representing iron sufficiency to negative values indicating iron deficiency, rather than using a cut-off value. However the adjustment for inflammation status is not considered in this method. Another limitation has been the use of different assays without any standardised samples (although the WHO reference reagent has recently become available[152]), to determine levels of sTfR, thus making results from the assays incomparable[153,154].

In addition, in developing countries the main difficulties in estimating iron status are subclinical, acute and chronic infections (such as malaria, HIV, tuberculosis, helicobacter pylori infection, parasite infections, cancer)[155,156,20]; other coexisting micronutrient deficiencies (folate, vitamin A, B12)[130, 157] and genetic disorders (haemoglobin disorders such as sickle-cell disease or thalassemia affecting mainly African, Southeast Asia and Mediterranean populations)[158, 159].

Inaccurate diagnosis of IDA could result in the underestimation of iron deficiency in a population or inappropriate provision of iron therapy in individuals whose underlying cause of anaemia is not iron deficiency.

1.3.1 Approaches used for addressing the confounding effects of inflammation when measuring iron status

Three different strategies have been suggested in order to estimate iron status in populations with widespread presence of inflammation[160]. These are:

- Determination of the presence of inflammation with the use of inflammation biomarkers: C-reactive protein (CRP) and alpha acid 1 glycoprotein(AGP) and exclusion of the data obtained from participants with elevated inflammation biomarkers.
- An increased ferritin cut-off for identifying ID from the commonly used 12-15μg/L[111] to 30 or even 70μg/L in the presence of HIV[161].
- Utilisation of inflammation biomarkers (CRP and AGP) to adjust the data to allow for the degree of inflammation[162].

Exclusion of data is not a desired solution as a large number of samples may be lost (which could cause studies to be underpowered). Ferritin is an acute phase protein and its values increase with inflammation, therefore increasing ferritin cut-offs to identify individuals with ID is a possible solution. However application of an increased cut-off for one population may not necessarily be appropriate for another due to potential population differences and variances in analytical techniques[144].Therefore the use of inflammation biomarkers to adjust ferritin values is potentially the most appropriate approach and will be described in greater detail below.

Thurnham *et al.*[162] suggested adjusting ferritin values with the use of two biomarkers of inflammation: CRP and AGP. The rationale for using these inflammation biomarkers is as follows: at the start of an infection there is an observed sharp increase in CRP levels with the peak occurring at approximately 24-48 hours. After that time CRP levels fall, however they stay elevated compared to levels prior to the infection for up to 7 days. Ferritin increases during inflammation and has similar characteristics to CRP[163] although it is not as rapid and it remains elevated for longer. On the other hand, AGP levels start to increase after 1 day of acquiring an infection, peaking at day 3 and staying at that level for at least a further 4 days[162, 163]. Analysis and use of the above 2 inflammation biomarkers in populations where infections are common could be the tool to capture individuals with current or very recent infections, thereby allowing for either exclusion of the individuals in question from the dataset or adjustment for inflammatory state (please refer to Appendix-C where the above correction for inflammation was used).

There is a clear need to employ the most appropriate methods available to estimate the true extent of ID in the most vulnerable population groups, such as those from developing countries, as well as pregnant woman, toddlers and woman of childbearing age.

1.4 The Caco-2 cell model

The development of a reproducible model of the small intestine that can be used to predict iron availability has been the goal of researchers for decades. Trials to culture epithelial cells of rodent origin were not successful, mainly due to the short lifespan of cultured cells and morphological changes that occur in early passages[164].

Human gastrointestinal tumour cells isolated from a colon adenocarcinoma in 1974[165] were found to have a much longer viability under culture conditions. One cell line in particular, Caco-2, has been shown to share many characteristics of normal small intestine absorptive cells[166,167]. Due to these properties Caco-2 cells have been used in various nutritional studies as a model for the human small intestine.

As reported by Vachon and Beaulieu[168] cultured Caco-2 cells are present in 3 states:

- Homogeneously undifferentiated (at sub-confluence).

- Heterogeneously polarized and differentiated (from day 0 to day 20 after confluence).
- Homogeneously polarized and differentiated (more than 30 days after confluence).

Once differentiated, Caco-2 cells demonstrate several biochemical and morphological properties of small intestine enterocytes[169]. Stierum *et al.*[102] showed by proteome examination that once Caco-2 cells undergo differentiation, their protein phenotype modifies from adenocarcinoma towards the cells of small intestine phenotype[170]. Under ideal culture conditions the cells grow to form a polarized monolayer, display cylindrical morphology with tight junctions between adjacent cells, and the development of microvilli on the apical side. They also express hydrolase enzymes (i.e. sucrase-isomaltase, lactase, aminopeptidase and alkaline phoshatases[167]). However, these are associated with transient human fetal[171] small intestine enzymes and are not expressed in adult human colon.

To highlight the usefulness of the Caco-2 cell model as a tool for investigating iron availability and uptake, it is important to mention that once those cells spontaneously differentiate[172], among the features of mature small intestinal enterocytes which they express as mammalian cells, are: DMT1 [173], DcytB[174], Hp[171], IREG1[175], transferrin receptor[171] and ferritin[173]. As discussed in the previous Section 1.2.3 these proteins are crucial for iron uptake and storage to take place in enterocytes. Due to changes occurring in Caco-2 cells upon differentiation, the following proteins have been shown to be up-regulated:

- Ferric reductase activity was found to be increased in differentiated cells in comparison with proliferating cells[176].
- DMT1 has been showed to be upregulated at 15 days post seeding[177,178]. However in Caco-2/TC7 subclones this up-regulation occurred between day 7 and 21 from seeding[179].
- Hephaestin mRNA has been shown to increase nearly 3 fold upon differentiation on day 15 from seeding[177].
- TfR mRNA was also reported to progressively increase with the maximum value on day 15 from seeding[177].
- Ferroportin has been shown to increase 3 fold in days 8 to 18 postconfuence[175].

Furthermore Caco-2 cells have also been used as an intestine model to explore calcium transport (transcellular- across cell membranes, and paracellular-

through intercellular spaces) into and across the enterocyte under various conditions[180,181]. Once differentiated, Caco-2 cells express vitamin D receptors[182]; with the active form of vitamin D coordinating the efficacy of intestinal calcium absorption (in animals and in humans)[183]. Additionally Caco-2 cells also express low levels of epithelial calcium channel mRNA (CaTI, mediators of calcium influx when expressed in *Xsemopus* oocytes) which has been shown to be upregulated by vitamin D[183]. Hence it is also possible to investigate vitamin D dependant transcellular uptake of calcium by the cells[184]. It has been reported by Chirayath *et al*.[185] that this upregulating effect of vitamin D in the cell model, at least in part, may be due to a rise in paracellular ion permeability. However as noted by Artursson *et al*.[186] the transmembrane resistance of the small intestine epithelium is quite low in comparison to the resistance of colon epithelium (≈60ohms/cm^2 and ≈280ohms/cm^2 respectively). Nonetheless differentiated Caco-2 cells possess many fundamental features to study calcium transport[187].

1.4.1 Advantages and limitations of the Caco-2 cell model

The main advantage of using Caco-2 cells to investigate transport of micronutrients and their bioavailability is that they are derived from a human source unlike other techniques using rodent models[164]. However, the fact that they originate from a tumour means that they express glucose transporters (GLUT1, GLUT3), as observed in colonic or cancer cells[188] rather than in normal duodenum enterocytes. Conversely, peptidase activity in Caco-2 cells is more similar to small intestinal epithelia than colonic epithelia[166].

A number of Caco-2 characteristics are inconsistent and they are observed to transform with passage of the cells. It has been reported that expression of sucrase-isomaltase, fructose transporter GLUT5, trans-epithelial electrical resistance[189] as well as proliferation rate increase in line with the increase of cell passage number[190]. In contrast, alkaline phosphatase activity decreases with increasing passage number[190].

Additional factors which have been demonstrated to influence Caco-2 cells growth and biology are the culture conditions such as choice of seeding surface (plastic surfaces versus porous membranes[191]), cell seeding density and alteration of the media composition[171]. In order to exploit the potential of the cell system and to establish valuable and comparable results it is important to use passages of Caco-2

cells which have the desired properties and to use modifications of culture conditions as an additional tool if required.

1.4.2 Incorporation of simulated digestion phase

The purpose of using a simulated digestion phase before applying the food matrix to the dialysis membrane[192] in the Caco-2 cell model is to simulate the enzymatic phase of human digestion in the stomach and duodenum and associated changes in the pH[193].

In this technique (Figure 1.4.1) samples under investigation need to be first exposed to pepsin digestion for 1 hour in an acidic environment (pH 2) at 37ºC on a rolling table to represent stomach conditions. Once the first phase is completed, gradual adjustments of pH need to be made to reach a value of 6.7[194]. Then, to mimic duodenal conditions, pancreatin bile solution is added to the samples. Prepared digest is applied onto dialysis membranes which are placed above the Caco-2 monolayer and the intestinal model is ready for incubation for 2 hours at 37ºC. The role of the membrane (12.000-14.000 molecular weight cut off) is to imitate the mucus layer[107] present in the digestive tract and to protect cells from the destructive activity of enzymes[192]. After 2 hours the digestate is removed from the upper compartment and the Caco-2 cells are incubated for a further 22 hours prior to harvesting and analysis of ferritin levels[193].

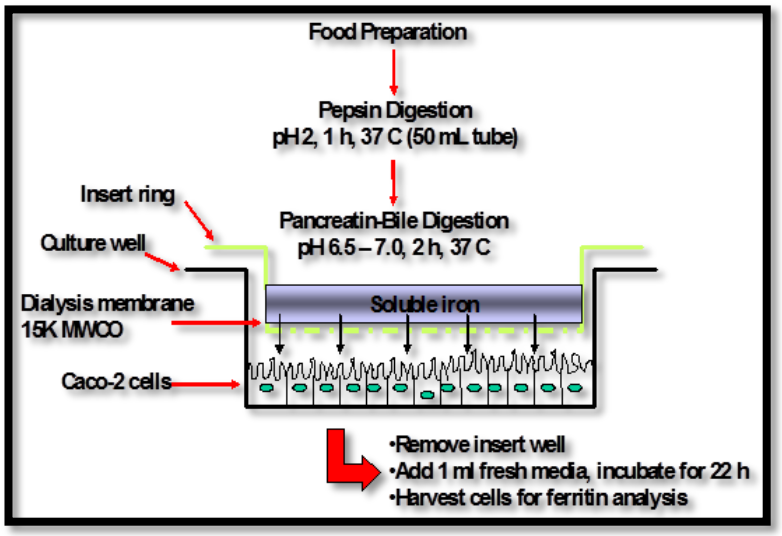

Figure 1.4.1. Schematic diagram of *in vitro* digestion, a Caco-2 cell model adopted from Glahn *et al*.[192].

It should be emphasised that the Caco-2 cell system, even when combined with simulated digestion cannot be considered an ideal duodenal model. In addition to the limitations stated in Section 1.4.1 there are other weaknesses: absence of peristaltic movements, mucus, micro-flora and digestive secretions. Furthermore, the influence of food composition on transit time and enzyme release, as well as interactions with other food components cannot be investigated using this model system[196].

Nevertheless, Caco-2 cells combined with simulated digestion is a useful model system that attempts to imitate small intestinal conditions and is a valuable tool for screening different iron sources in order to rank them for potential bioavailability and for investigating mechanisms of nutrient absorption in humans[197,198].

The simulated digestion process involves increasing the pH associated with gastric conditions (pH2) to that found in the duodenum (pH7). The increase in pH is a major determinant of iron solubility in solutions in the presence of oxygen. With the increase in pH both ferrous and ferric iron hydrolyse forming polymers and these will ultimately precipitate as insoluble complexes, thereby reducing the proportion of iron that is available to cells/humans[194,199]. Ferric iron forms insoluble hydroxides more readily than ferrous iron[69]. In order to avoid the formation of insoluble hydroxides iron chelators such as ascorbic acid is normally added prior the start of the digestion.

The iron compounds used in experiments reported in the thesis are summarised in Table 1.4.1. All of the iron compounds used in this study are freely soluble in water[68,200]. FeG and FAC are also used as food fortificants and are classified as highly bioavailable[15,68,69,201]

Studies conducted by Thompson[202] using atomic absorption spectroscopy (AAS) revealed that the solubility in water of FAC and $FeCl_3$ decreased from 92.6% and 95.4% at pH 2 to 10.5% and 4.4% pH 6.5 respectively.

The quantification of % free iron in post experimental digests (which underwent simulated digestion as described in Chapter 2, section 2.2.2) was quantified using bathophenanthroline colorimetric assay (also described in detail in Chapter 2, Section 2.2.2). The % of free ferrous iron quantified after the simulated digestion was 1.7% for ferrous gluconate and ferric ammonium citrate and 1.2% for ferric chloride and not 4.4% as quantified by Thompson[202] with the use of AAS. Low iron solubility and the discrepancy between the 2 methods might be due to the use of

hydroxylamine solution in the colorimetric assay which probably further increases the pH of tested samples, thus potentially reducing the solubility of iron. It is also worth mentioning that only pre-frozen digest were analysed and the effect of freezing on iron solubility was not investigated.

Table 1.4.1 Characteristics of iron compounds

Iron compound	Molecular weight	Chemical formula	Solubility in water
Ferrous gluconate (FeG)	482.2	$C_{12}H_{22}FeO_{14} * 2H_2O$	10g in 100g
Ferric ammonium citrate (FAC)	265.0	$C_6H_{11}FeNO_7$	100g in 100g
Ferric chloride ($FeCl_3$)	162.2	$FeCl_3$	92g in 100g

.

Kapsokefalou *et. al*.[203] investigated % total soluble iron in water digests and reported that it was significantly higher when ferrous gluconate was used than when ferrous lactate, ferrous bis-glycinate or iron EDTA were used. Ferrous gluconate has been reported to be a successful food fortificant of specific foods (in that it is not associated with adverse organoleptic changes) in combatting iron deficiency anaemia[68,201,204].

Different iron compounds with various chemical properties have differing solubility and complexation at pH7 or higher, as reported by Minihane when comparing iron ascorbate and iron citrate complexes[205].

As mentioned earlier, when using the Caco-2 cell model ferritin is measured as a surrogate of iron availability. Therefore if during the simulated digestion all of the iron present in solution forms insoluble hydroxides or if it precipitates at

physiological pH in the presence of oxygen, then there would be no iron available for the cells and there would be no ferritin response.

The general methods Chapter (Chapter 2) contains a more detailed experimental protocol based on the method described by Glahn *et al.*[193] which is a 3 day procedure that starts 12 days post seeding of Caco-2 cells in collagen coated 6 well plates.

1.5 Characteristics of Alginate

Alginates (sodium alginate, E401) are natural copolymers present in the cell walls of brown seaweed as sodium, potassium, calcium and magnesium salts of alginic acid. Through the process of extraction (which is comprised of ion exchange in an alkaline medium, precipitation, purification and extraction of alginic acid) dry sodium alginate powder is obtained[206]. Alginates are comprised of varying ratios of two different acids: D-mannuronic (M) and L-guluronic acid (G), and due to the variable length of the polymer chains they exhibit differing physiochemical properties[207] (Figure 1.5.1).

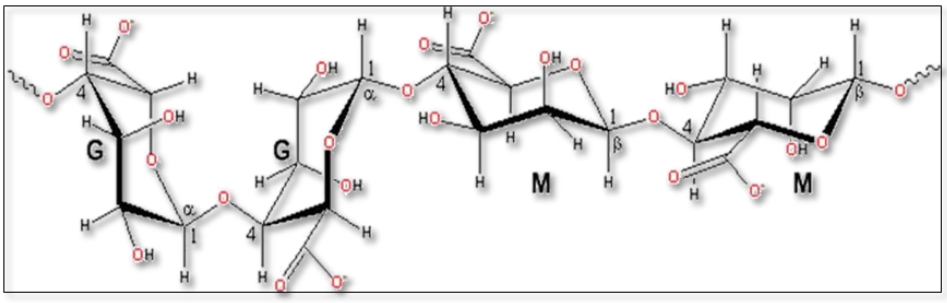

Figure 1.5.1 Schematic diagram of D-mannuronic (M) and L-guluronic acid (G).From http://www.btinternet.com/~martin.chaplin/hyalg.html- accessed on 12.09.2010.

Alginates are used in a wide variety of applications, including drug delivery systems (anti-reflux preparations)[208] and the food industry (as thickening, gelling, emulsifying and stabilising agents in food products (used in ice cream, sauces, fruit pies, onion rings etc.)[206]. They have been shown to bind divalent and trivalent cations[209,210], with better iron binding capacity at lower alginate concentrations (when calculated as mg Fe/g alginate)[211], and therefore might be a useful vehicle for soluble iron compounds used to fortify foods.

1.5.1 Alginate gelation

The best described gelling mechanism of alginate solutions is through addition of calcium ions (Figure 1.5.2 and Figure 1.5.3). Grant *et al.*[212] and Morris *et al.*[213] proposed a Ca-alginate junction zone model called the 'egg box' model which forms through calcium ion induction of chain-chain association (2/1 zigzag helix conformation) with a 1:4 ratio between Ca^{2+} and G units in alginate gel.

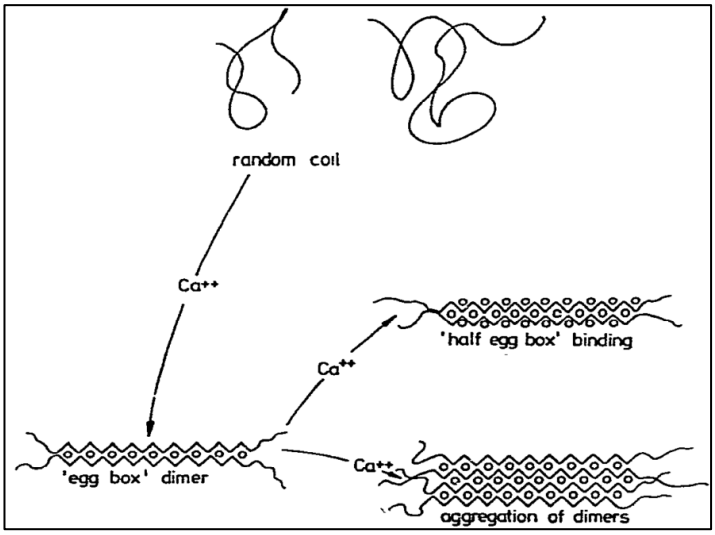

Figure 1.5.2 Schematic formation of the 'egg box' by calcium ion chelation to poly-L-guluronate sequences (Morris *et al.*[213]).

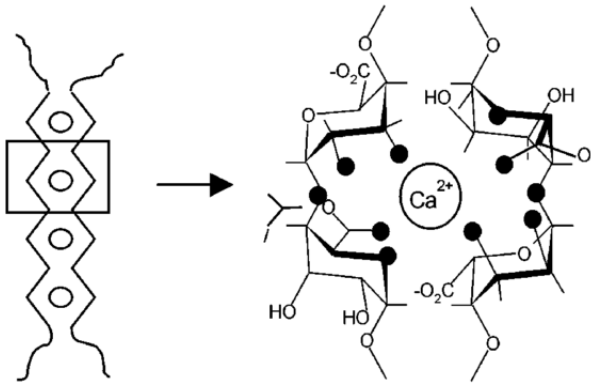

Figure 1.5.3 Schematic formation of the 'egg box' model sourced from Braccini *et al.*[214]. Dark dots are oxygen atoms taking part in the coordination of calcium ion.

However, more recent investigations with advanced X-ray scattering techniques suggested that the 'egg box' dimer is not the only possible conformation. It has been suggested that not only GG blocks (and their length), but also MG blocks[215] (Figure

1.5.4) contribute to the junction zone through Ca^{2+} binding[216] supporting the theory of 3/1 and 2/1 helix conformation coexistence. Moreover, the Ca-alginate gelation rate has a direct influence on junction zone conformation. According to Li *et al.*[216] a fast gelation (such as spinning fibers in calcium chloride solution) process promotes 2/1 helix conformation, whereas slow gelation (through diffusion) results in 3/1 helix conformation.

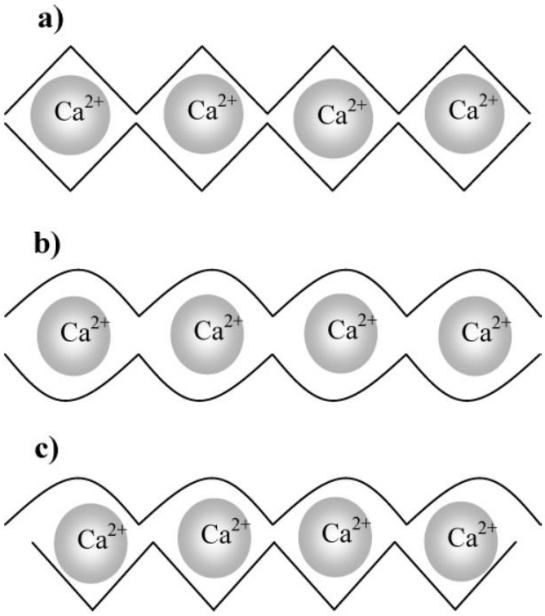

Figure 1.5.4 Schematic representation for three possible junctions in alginate gels : a)GG/GG; b) MG/MG and c) GG/MG junctions as described by Donati *et al*.[215].

The aforementioned variations in alginate structure and calcium binding properties ultimately underline why alginates are so versatile and commonplace in multiple industrial processes and are of valuable use both in medicines and the food industry.

1.5.1.1 Alginate gelation in presence of iron

It has been demonstrated that alginates can form a stable complex with ferric and ferrous iron over a range of different pH values[210, 217]. Nonetheless, it has been reported that binding of iron by alginates increases with decrease in pH[211]. This increase should not occur if the mechanism of binding solely has an ionic basis, because then binding of iron should increase with increase in pH[211]. A potential explanation for this phenomena may be that alginates form gels at low pH and the

aggregation of polymers may then trap iron[211]. Brenel *et al.*[211] also showed that pH changes during *in vitro* digestion interrupt binding between alginate and iron.

Conversely, if alginates bind iron in the lumen of the large bowel they may offer positive health benefits by restricting iron supply to cells in individuals with polyps who are at increased risk of colorectal cancer[218,219]. In support of this hypothesis, studies carried out in rodents[220,221] and humans[222] have shown that alginates can inhibit iron absorption by up to 40%.

The overall aim of the research project was to develop potential delivery systems for fortification iron in order to contribute to strategies for preventing iron deficiency. Consequently, it was essential that food grade materials were used that could potentially protect the iron through digestion in the stomach and deliver it in soluble form to the site of absorption.

Investigations were undertaken to determine whether alginates could be used as a delivery system for iron. Specifically, Caco-2 cell studies were carried out to determine the effects of varying ratios of mannuronic and guluronic acids within alginate on iron uptake from ferric ammonium citrate (FAC) and ferrous gluconate (FeG) in the presence of an iron enhancer, ascorbic acid (AA), and iron inhibitors, namely tannic acid (TA) and calcium. The results of the *in vitro* investigations were used to inform the design of a human study investigating the effectiveness of using an alginate barrier to deliver FeG to the duodenum, thus maximising its availability for absorption.

2. GENERAL METHODS

2.1 Routine Caco-2 cell culture procedures

Unless otherwise stated all reagents were purchased from Sigma Aldrich, UK. Caco-2 cells (HTB-37®) were obtained from American Type Culture Collection (Manassas, VA) at passage 20 and stored in liquid or vapour nitrogen (at a temperature of -196^{0}C). When resuscitated, the cells were grown in Dulbecco's modified Eagle's media (DMEM) from Gibco UK, supplemented with 10% fetal bovine serum (FBS), 25mM HEPES solution, 4mM L-glutamine and 5ml 5000u/ml Penicillin/Streptomycillin solution (Gibco, UK). The cells were maintained at 37^{0}C in an incubator with humidified atmosphere consisting of 5% carbon dioxide and 95% air. Routinely, cells were resuscitated (frozen Caco-2 cells were allowed to thaw at room temperature for one minute followed by re-suspension in DMEM supplemented media) at passage 26 in 25cm^2 cell culture flasks (Nunc, UK) and sub-cultured at 80% confluence into 75cm^2 cell culture flasks (Nunc, UK) with media changes every three days.

2.1.1 Cell resuscitation

Ampoules of Caco-2 cells, stored in liquid or vapour nitrogen were allowed to thaw at room temperature for one minute. The cells were then placed in a water bath at 37°C for 1-3 minutes until fully defrosted. The ampoule of cells was wiped with 70% ethanol and the cell suspension transferred to a 25 cm^2 cell culture flask (Nunc, UK) containing DMEM supplemented media.

2.1.2 Growing and sub-culturing procedures

Once resuscitated, the cell media was replaced 24h following resuscitation and every 2 days thereafter. Cells were sub-cultured at 80% confluence in 75cm^2 culture flasks. Briefly, the media from sub-confluent cells was removed and the cells washed with 6ml of Dulbecco's Phosphate Buffered Saline (DPBS). Cells were then detached with the addition of 1ml of trypsin (incubated at 37^{0}C for 4-6 minutes). The trypsin was neutralised with the addition of 4ml DMEM supplemented media. The cells were transferred to 75cm^2 culture flasks, suspended in an additional 8ml of DMEM supplemented media, and grown (with media changes every 2 days) until 80% confluence. The cells were grown (with media changing procedure every 2 days) in 75cm^2 flasks until they reached 80% of confluence, then they were sub-cultured (as above). The cell suspension was transferred into 10x75cm^2 flasks in a

volume of 1ml per flask. Each flask was topped up with 11ml of DMEM supplemented media. Caco-2 cells were grown (with a media change every 2 days) in 75cm^2 flasks until they reached 80% confluence, and then sub-cultured (as above), counted using a microscope and haemocymeter and finally seeded in 6 well collagen plates at density of 4.75x10^5 per well.

However, due to issues associated with cell adherence the culturing and sub-culturing techniques of cells grown in flasks were modified as follows: when harvested cells were initially centrifuged at 600rpm for 5min (Eppendorf, UK) in order to remove trypsin residues, counted using a haemocytometer, then a constant number of cells (7.5x10^5 or 2.25x10^6) was seeded into 25cm^2 or 75cm^2 culture flasks respectively and cells were grown for 7 days to full confluence. The change in culturing techniques avoided the pre-selection of faster growing cells within the Caco-2 monolayer and assured more uniformity between cell passages. This modified cell culture technique was used to grow cells for experiments reported in Chapters 5, 6 and 7.

2.1.3 Freezing procedure

Cells were harvested, counted, transferred to a 50ml tube and centrifuged for 3 minutes at 1000g. Cells were then re-suspended in 90% fetal bovine serum and 10% Dimethyl sulfoxide (DMSO) to give a final concentration of 2-4x10^{6}cells/ml. The cells were then aliquoted into cryovials and frozen at a cooling rate of 1-3^{0}C/min using a 'Mr. Frosty' (Nalgene, UK), which contained isopropanol. After 24 hours the ampoules were stored at -80^{0}C until use.

2.1.4 Seeding of 6 well plates

The Caco-2 cells were seeded onto collagen coated 6 well plates (Greiner, UK) at a density of 4.75x10^5, and suspended in 2 ml of supplemented DMEM, which was replaced every 2 days. Cells were used at 13 days post seeding between passages 28-40 for all experiments. Supplemented DMEM was removed from cell monolayers in 6 well plates after 24 hours prior to experimentation and replaced with serum free media consisting of DMEM (Gibco, UK) supplemented with 25mM HEPES solution, 4mM L-glutamine and 5ml 5000U/ml Penicillin/Streptomycillin solution (Gibco, UK).

2.1.5 Harvesting of Caco-2 cells

After 24 hours post experiment, the treatment media was aspirated from the 6 well plates and the cells were gently washed twice with 2 ml DPBS (Gibco, UK). However, if the experiment was carried out following a simulated digestion procedure, then the cells were washed with 130mM sodium chloride, 5mM potassium chloride and 5mM PIPES buffer at pH6.7 instead of DPBS. Subsequently, deionised water was applied to each well and the Caco-2 cells were scraped off using an inverted 10µl pipette tip. The cell suspension from each well was then transferred into 5ml sample tubes and stored at -20^{0}C.

2.1.6 Sonication

Sonication in order to lyse the cells was carried out 24 hours after cell freezing. Briefly, 5ml tubes containing cell lysates were defrosted at room temperature and subsequently kept on ice. Cell suspensions were sonicated 3 times for 5 seconds on ice using a probe sonicator. Following sonication, cell suspensions were transferred to pre-labelled 2ml sample tubes and stored at -20^{0}C.

2.2 Simulated digestion experimental protocol

2.2.1 Procedures prior to experiment day (12 days post seeding)

Preparation of media: 1 litre of minimum essential media (MEM) was prepared by dissolving the following compounds in 700ml of MilliQ water: 3.5g D-glucose, 3.6684g PIPES, 2.2g sodium bicarbonate, 1 packet of MEM (Gibco, UK), 4mg hydrocortisone. 10ml of antimicrobial/antibiotic solution was added, followed by the addition of 5mg of insulin dissolved in 2ml of acetic-dH2O (pH dH2O to pH2 with acetic acid) at pH2. 10µl of 0.5mg/ml sodium selenite solution, adjusted to pH7, was then added and the volume brought up to 1000ml with dH_2O. The media was completed with the addition of 1.7ml of 20µg/ml tri-iodothyronine solution and 1ml of Epidermal Growth Factor solution. The resulting solution was filter sterilized using filters with cellulose nitrate membrane 0.2µm filters, (Millipore, UK) and aliquoted into 50ml sterile tubes and refrigerated at 4^{o}C until use. Prior to the application onto Caco-2 cell monolayers, the MEM was warmed to 37ºC in a water bath. 24 hours

prior to the experiment all wells were washed once with 2ml of MEM to remove residual amounts of DMEM (containing fetal bovine serum) and replaced with a further 2ml of MEM. All plates were incubated for a further 24 hours.

Preparation of inserts: Dialysis membrane (15kDa MWCO, Fisher, UK) was cut into pieces approximately 5cm in length and soaked in 18.2MΩ (MilliQ) water for a few minutes and subsequently rinsed several times. The membrane was mounted onto inserts by cutting along one of the folds and fixed using silicone O-rings. Excess membrane was carefully trimmed using scissors. Inserts were immersed in 18.2MΩ water and refrigerated until use.

2.2.2 Experiment day (13 days post seeding)

Experiments were undertaken at 13 days post seeding. In all plates, MEM was replaced with 1ml of fresh media and inserts with mounted membranes were incorporated onto wells. Enzyme solutions: pepsin to imitate the gastric phase and pancreatin and bile solution to imitate the duodenal phase were prepared. A suitable number of 20ml tubes (Sterilin, UK) (for control samples, positive controls and treatment samples) were labelled and their weight without the lids noted. The appropriate amount of alginate beads or other samples (to match the required iron concentration) were weighed into the tubes. 10ml of sodium chloride and potassium chloride solution at pH 2 was added to each tube, and the pH adjusted to 2 to provide an optimal environment for pepsin to work; 0.5ml of pepsin solution was added to each tube and the tubes were incubated for 1 hour on a rotating table at 37ºC. Once the gastric phase was completed, the pH was adjusted to 5.5-6 using 1.0M sodium bicarbonate to inactivate pepsin digestion and 2.5ml of pancreatin/bile solution was added to each sample. The pH was further adjusted to 6.7 with sodium bicarbonate and the volume (by weight) of each tube readjusted to tube weight plus 15g using sodium chloride and potassium chloride solution at pH 6.7.

Each tube was well mixed using a vortex during sampling and 1.5ml of sample from each tube was transferred onto appropriate inserts on the plates. Plates were then moved onto the plate shaker (slow setting, 20oscilations/min) and incubated at 37ºC for 2 hours to imitate the duodenal phase. After 2 hours the inserts were removed, and each well supplemented with an additional 1ml of MEM and the plates incubated for a further 22 hours.

The iron content of the digests was quantified using a bathophenanthroline colorimetric assay. Briefly, 1.5ml of sample digestates from the *in vitro* digestion was centrifuged at 10000g for 5 min, then 380µl of the resulting supernatant was transferred to a fresh tube, and 20µl of hydroxylamine hydrochloride solution was added. Ferrous standards ranging from 0 to 2.0 µg/ml were prepared by diluting a 5µg/ml stock solution of iron (High-Purity Standards,USA) in 0.1M HCl in the presence of 20µl of 0.1g/ml hydroxylamine hydrochloride (Sigma, UK) dissolved in 10M HCl, then 100µl of each standard and sample was added to a 96-well plate, in triplicate, and 50µl of chromagen solution (0.0156g of bathophenanthroline disulfonic acid disodium salt (Sigma, UK) dissolved in 50ml of 2.0M sodium acetate) was added. The plate was incubated on a plate shaker at 200 oscillations per minute at room temperature for 10min. Once incubation was completed the absorbance was measured at 562 nm using a microplate reader (Omega, BMG Labtech, UK).

2.2.3 Post experiment day procedures (14 days post seeding)

24 hours after the start of the duodenal phase, MEM was removed from the cells, each well was washed twice with sodium chloride and potassium chloride buffer, and cells from each well were suspended in 2ml of MilliQ water, frozen and kept at -20ºC until sonication. Further analysis was performed as described in Sections 2.1.6 and 2.3.

2.3 Analysis of Caco-2 cell lysates

2.3.1 Spectroferritin ELISA assay

Sonicated cell lysates were defrosted at room temperature and kept on ice and a spectroferritin ELISA assay (Ramco, USA) was carried out 24 hours post-sonication, according to the manufacturer's instructions. The spectroferritin kit was left to equilibrate at room temperature for a minimum of 20 minutes and the microplate wells were dried and placed on a 96 well grid. Standard solutions containing 0.3ml human spleen ferritin calibrated to concentrations of 6, 20, 60, 200, 600 and 2000ng/ml were applied in duplicate to obtain a standard curve. Test samples were applied in duplicate at a volume of 10µl. Once all the samples were applied to the microplate wells, 200µl of conjugated anti-human ferritin was applied to each well. The plates were then incubated for 2 hours at room temperature with agitation. After 2 hours, all wells were washed three times with deionised water. The plate was then incubated for a further 30min at room temperature in the presence of 200µl substrate. Following incubation, 100µl of potassium ferricyanide was added and mixed for one minute on a rotating plate shaker set at 180rpm. Absorbance was measured using an absorbance microplate reader (Omega, BMG Labtech, UK) at 490, 570 and 630nm. Absorbance at 570-630nm was subtracted from absorbance at 490nm.

2.3.2 BCA protein assay

In order to adjust for well-to-well cell number variability (which results in variations in ferritin) the ferritin measurements were standardised to allow comparisons within and between experiments. This was achieved by measuring the protein content and expressing the ferritin content per mg of total protein. Total protein was quantified using a bicinchoninic acid (BCA) protein assay (Pierce, USA), carried out in parallel with the spectroferritin assay. A BCA working solution and standard solutions (0, 125, 250, 500, 1000 and 2000µg/ml Bovine Serum Albumin (BSA)) were prepared according to the manufacturer's instructions. The assay was performed with the addition of 10µl protein standard (in duplicate) or 10µl experimental samples (in duplicate) to 200µl of BCA working solution in a 96-well microplates. The plates were mixed on a rotating plate shaker for 1 minute at 180 rpm and then incubated at 37^0C for 30min. Absorbance was read at 562 nm using a microplate reader (Omega, BMG Labtech, UK)

2.3.3 Statistical analysis

Unless otherwise stated, all statistical analysis was performed using SPSS Inc, USA, (version 16.0.0). ANOVA general linear model with Tukey post-hoc testing was used to examine pair wise differences. Data are presented as mean ±SD.

2.4 Analysis of iron and calcium content of alginate beads

Atomic absorption spectroscopy (AAS) was used to determine the iron content of the alginate beads or the filtered liquid resulting from the digestion. Briefly, alginate beads were first separated from the liquid in which they are dispersed by filtration using a filter paper (Whatman 4). Then, a minimum of 0.5 g of wet beads or liquid were transferred to a crucible and dried overnight in an oven at 60ºC before being placed in a muffle furnace (Vulcan Box Furnace, NEY Dental International, USA) and heated with a two-step temperature program (20ºC - 250ºC for 2h followed by a fast ramp and hold for 24h at 550ºC). Once cooled, a known volume of 20% (v/v) nitric acid was added to each crucible to dissolve the ashes including the metal ions. The acidic solution was then transferred to a tube and the amount of iron and/or calcium measured by AAS using a Perkin Elmer Atomic Absorption Spectrophotometer 3300 (Perkin Elmer Ltd., Beaconsfield, UK).

3. *IN VITRO* STUDIES OF ALGINATE

3.1 Introduction

As a part of a joint project between the IFR, Norwich, Norfolk, UK and the University of East Anglia (UEA), funded by Biotechnology and Biological Sciences Research Council, and Diet and Health Research Industry Club (BBSRC, DRINC, Grant Reference Number BB/G53015X), a food grade sodium alginate was selected as a potential delivery system for iron and was the subject of further investigation using the Caco-2 cell model.

The use of iron fortification as a strategy to prevent IDA is widespread as it is recognised as a sustainable and realistic way to reduce the occurrence of iron deficiency[67]. Nonetheless, fortification is challenging to the food industry, mainly due to organoleptic changes during food storage or preparation[69] brought about by the addition of iron. Water soluble forms of iron are generally more bioavailable than non-soluble iron compounds but they often cause adverse sensory changes when added to foods. A potential strategy to overcome this problem is the use of water soluble iron compounds protected by a water resistant barrier which will prevent any organoleptic changes in the fortified food products. When combined with AA FeG is an iron compound that has been reported to be an effective food fortificant[223], which may reduce the prevalence of anaemia.[15] In order to maximise iron absorption *in vivo*, it would be advantageous to employ a delivery system that will reduce organoleptic problems in food and also protect the iron from adverse interactions in the gut lumen and deliver iron directly to the duodenum where it is absorbed.

Physico-chemical studies of alginate beads (with the use of different alginates), starch, water-in-oil-in water emulsions (W/O/W) and gel in water in oil emulsions (G/O/W) were performed at IFR. Four iron compounds (divalent salts: iron (II) gluconate and iron (II) sulphate and trivalent salts ammonium (III) citrate and iron (III) chloride) were used for the preparation of iron containing alginate beads. Once optimal conditions for the preparation were selected, the most promising materials were tested using Caco-2 cells as a screening tool.

The hypothesis tested in this chapter was that iron-containing alginate beads would remain intact during the gastric digestion phase and that iron would be released under duodenal conditions, thus delivering soluble iron for cell uptake.

The primary objective of these experiments was to determine whether alginates would interfere with iron uptake into Caco-2 cells, to assess whether AA would demonstrate its usual enhancing effect on iron uptake in the presence of alginate

and if alginate beads subjected to simulated digestion would deliver available iron to be taken up by the cells.

3.2 Methods

3.2.1 Development of iron containing materials

Iron containing materials were developed by scientists at IFR, with lab work undertaken by Dr Natalia Perez-Moral.

3.2.1.1 Preparation of alginate beads

As empirically tested at the Institute of Food Research (IFR) by Dr Natalia Perez-Moral alginate gelation in the presence of FeG with the use of Encapsulator (EncapBioSystemS Inc, Switzerland) resulted in amorphous gel and not bead formation. Thus, in order to obtain reproducible morphology and uniformity in bead shape, calcium had to be introduced to the system. For experimental details please see the details further down this Section.

Perez-Moral *et al.*[217] used four alginates differing in M/G acid ratio (characterised in

Table 3.2.1) and investigated their cross-linking capacity with FeG in the presence of calcium chloride (Figure 3.2.1). The researchers wanted to test the hypothesis that there is competition between calcium and iron for binding sites of alginate gel[224]. Their results showed that the highest amount of FeG incorporated into alginate beads was observed at the lowest calcium chloride concentration (5mM) used (Figure 3.2.1; graph a) across all alginates used. However, in alginate with the highest guluronic acid content (35/65 M/G ratio) iron loading was approximately 2 fold higher than in the alginate with the lowest guluronic acid ratio (60/40 M/G).

In fact, increasing iron content of alginate beads was found to be consistent with increasing guluronate content of alginates. This observation suggests a potentially distinct guluronate and iron interaction. Nevertheless, as calcium concentrations were increased, the differences between iron loading in alginate beads containing varying guluronate content was negligible. The latter supports the hypothesis of competitive binding between Fe^{2+} and Ca^{2+} in alginate gel beads. Interestingly, when only calcium (5mM) was used, alginate with the highest guluronic acid content resulted in the highest calcium concentration (Figure 3.2.1;graph b). When a range

of calcium concentrations were used (between 5 and 50mM), calcium content of alginate beads was increased until it reached a plateau at a concentration of 40-50mM of calcium.

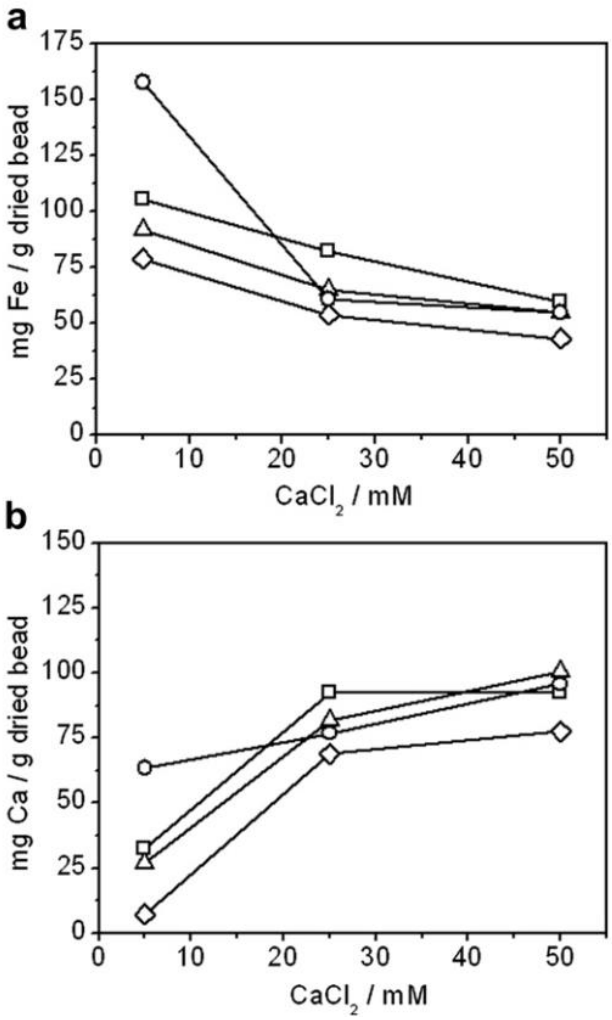

Figure 3.2.1 Graphs of 0.5% (w/v) alginate beads with following M/G ratios: ◊ ((60/40); Δ(50/50); □ (45/55); ○(35/65) with FeG (a) or without (b) in presence of increasing calcium concentrations as described by Perez-Moral *et al*.[217].

Iron containing alginate beads (Figure 3.2.2) were prepared from 4 different alginates (Danisco, DK;

Table 3.2.1) using an encapsulator. Three different routes of bead synthesis were evaluated:

- Route 1: by mixing iron and alginate solutions (0.5% (w/v)), which were then transferred to an encapsulator (to form drops) and collection in a calcium bath where crosslinking (with calcium) took place[225].

- Route 2: use of 0.5% (w/v) alginate solution to form beads with an encapsulator with collection of the beads into a calcium and iron bath for crosslinking (calcium and iron ions competition; approximately 20 hours of loading).

- Route 3: use of 0.5% (w/v) alginate solution to form beads with an encapsulator, which were crosslinked in a calcium bath and subsequently soaked in an iron bath (exchange process, 35 days of loading).

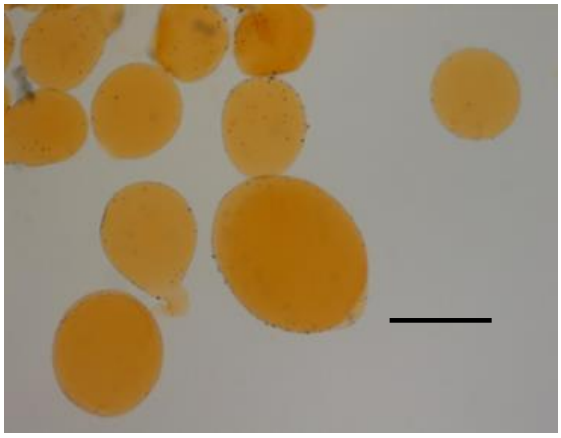

Figure 3.2.2 Microscopic photograph of beads containing 178mg Fe/g dried beads prepared via route 2 after 31 days of loading. Black bar represents 100µm.

Table 3.2.1 Composition (M/G) ratio, molecular weight (MW) and the solution viscosity of the alginates. Data supplied by manufacturer.

Alginate	M/G	MW(kDa)	Viscosity of 1%w/w solution (mPa s)
A1	60/40	350	520
A2	50/50	350	520
A3	45/55	320	370
A4	35/65	315	350

Preparation of the beads via route 1 caused technical issues regardless of the type of alginate and iron compound used. Due to an increase in viscosity of the iron and alginate solution (caused by the interaction of iron with alginate) the flow in the encapsulator was often disrupted resulting in faulty bead shapes. Therefore, formation of beads via route 1 was discontinued.

The best iron loading of the beads using route 2 was obtained with FAC regardless of type of alginate used. Ferric chloride loading was the most successful when alginates A1 and A4 were used, whereas loading with divalent salts was not as effective. FeG loading was not dependent on the type of alginate used.

Conversely, FeG combined with alginate A2, A3 or A4 led to the best loading when route 3 was applied; followed by FAC when combined with alginate A1 or A4. Differences in iron loading with routes 2 and 3 could have been due to different incorporation processes. In route 2, loading with iron occurred through a competitive process between iron and calcium ions whereas in route 3 iron loading arose through the exchange process[217].

From an analysis of the bead morphology and iron content the most effective methods to prepare the beads were shown to be routes 2 and 3. Microscopic examination revealed that bead uniformity in shape, lack of adhesiveness or aggregation was higher with increased guluronic acid content of the alginates. Once loading time and iron content of the beads was taken into account the following 2 bead preparations were investigated further: alginate A4 loaded with FeG or FAC prepared via route 2; and alginate A2 loaded with FeG and FAC prepared via route 3[217].

Application of a simplified simulated *in vitro* digestion procedure described by Perez-Moral *et al.*[217] demonstrated that approximately 4.7g of wet beads prepared via route 2 and route 3 survived gastric digestion (Figure 3.2.3), and would deliver nutritionally relevant amounts of iron (approximately 10mg of iron) into the duodenum. However beads loaded with FeG via route 3 showed a progressive decrease in iron content (starting at day 5 and continuing until the end of the experiment on day 30) which was not the case when FAC was used. Because FeG is considered to be more bioavailable than FAC, beads prepared via route 2 with FeG were selected for further screening using Caco-2 cells and simulated digestion as a model to predict iron bioavailability.

More specific details of the bead preparation process are given in Perez-Moral *et al.*[217].

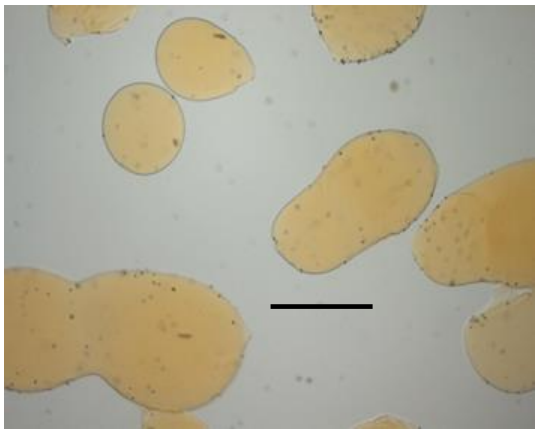

Figure 3.2.3 Microscopic photograph of beads after simplified simulated digestion which contain 39mg Fe/g dried beads prepared via route 2. Black bar represents 100µm.

3.2.1.2 Preparation of emulsions and starch particles

In addition to alginate beads other materials tested were: W/O/W emulsions; gel in oil in water (G/O/W) emulsions and starch particles.

- **G/O/W emulsions**

G/O/W are multiple emulsions were oil globules containing small alginate droplets are dispersed in an aqueous continuous phase[226]. G/O/W emulsions are prepared as follows: the inner aqueous phase consisted of 10ml of 1% sodium alginate solution mixed with 2ml of FeG 0.2M and 0.34ml of a 5% slurry of calcium tartrate. Prior to that, the oil phase was prepared in a separate beaker by melting 48g of inter-esterified palm kernel oil, chocotan (donation by Macphie, Glenbervie, Scotland) at 40ºC and mixing it with 1g of PGPR (the oil soluble emulsifier poliglycerol polyricinoleate PGPR, Palsgaard 4125, Healy Group, UK). The aqueous inner phase was added to the oil phase in a high speed blender and mixed at a low shear position (up to 18000rpm) for 30 seconds, then 1ml of acetic acid was added and after 30 seconds the emulsion was mixed again for 30 seconds at high shear position (up to 22000rpm). After 1 min 200ml of a 2% whey protein isolate (Bipro, Danisco, UK) aqueous solution was added before starting another mixing cycle consisting of 30 seconds at low speed, 30 seconds rest and another 30 seconds at low speed.

- **W/O/W emulsions**

W/O/W emulsions (Figure 3.2.4) were prepared using chocotan. Briefly, 12ml of water containing the iron compound (ferrous sulphate or FAC) was blended at low power (up to 18000rpm) with 48g chocotan and 1g PGPR for 30 seconds at 44ºC, rested for a further 30 seconds and blended again at high power (up to 22000rpm) for 30 seconds to establish water in oil droplets. These droplets were then suspended in water containing 200ml of 2% whey protein isolate by high power blending for 30 seconds.

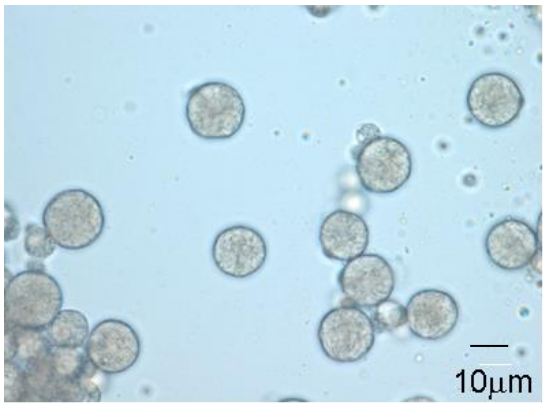

Figure 3.2.4 Microscopic photograph of W/O/W emulsion.

- **Preparation of starch particles**

20g of starch (Sigma, UK) was mixed with 180ml of deionised (DI) water in a round bottomed flask equipped with a mechanical stirrer and immersed in an oil bath to control the temperature at 85ºC. Then a solution containing 9.44g of FeG in 25ml of water was added and the mix was stirred at approximately 500rpm. After 30 minutes the temperature was increased to 85ºC and stirred for a further two hours. The resulting mixture was poured on two plastic Petri dishes (13.5cm diameter) and placed in an oven at 50ºC. Once the starch film was dried, it was grounded and sieved in order to get a range of different sized particles.

3.2.2 Preparation of alginate solutions

Four alginate formulations with different ratios of mannuronic (M) to guluronic (G) acid were used in this study: A1 (M:G ratio 60:40), A4 (M:G 35:65) from Danisco, Copenhagen, Denmark (Table 3.2.2); A5 (Manucol®DH,M:G ratio 60-70:30-40) and

A6 (Manugel®GHB, M:G ratio 30-40:60-70) from FMC BioPolymer, Haugesund, Norway.

Table 3.2.2 Characteristics of alginates used to make up alginate solutions.

Alginate	M/G ratio	Supplier
A1	60/40	Danisco, Denmark
A4	35/65	Danisco, Denmark
A5 (MANUCOL® DH)	60-70/30-40	FMC Biopolymer, Norway
A6 (MANUGEL® GHB)	30-40/60-70	FMC Biopolymer, Norway

All glassware and magnetic stirrers were washed in 10% hydrochloric acid (HCl) and rinsed with MilliQ water prior to use to eliminate iron contamination. The alginate solutions were prepared by dissolving 3g of alginate powder in 300ml in serum free media to obtain 1% (w/v) stock solutions. The solution was covered with sterilized para-film and left overnight at room temperature with stirring. If needed, the stock solutions were diluted further in serum free media in order to obtain working solutions of 0.5% (w/v) or 0.1% (w/v) alginate concentrations which were applied to the Caco-2 cells in media containing FAC (30µmol/L), with or without L-ascorbic acid (AA) (600µmol/L) or tannic acid (TA) (molar ratio of 1:0.1 FAC:TA).

3.2.3 Preparation of ascorbic acid and ferric ammonium citrate solutions

Shortly before use, AA was prepared at a concentration of 0.06M (1/100 dilution of concentrated stock). A working solution of 600µM was prepared in treatment medium. FAC was prepared at a concentration of 3mM (1/100 dilution of concentrated stock) in 0.1M HCl. A working solution of 30µM was prepared in relevant treatment media. Both solutions were filter sterilised using hydrophilic 0.2µm syringe filters (Sartorius Stedim Biotech, Germany).

3.2.4 Preparation of treatment media

Shortly before use, 1% (w/v) alginate solution and serum free media were heated to 37^0C. Subsequently, treatment media were prepared as described below (Table 3.2.3) in 50ml tubes under sterile conditions.

Shortly before the start of an experiment, serum free media was aspirated from the Caco-2 cell monolayer and 2ml of required treatment media was applied to each well and incubated (37°C, 5% CO_2) for 24 hours. After that time the cells were harvested and sonicated according to the procedure in Chapter 2, Section 2.2.3.

Table 3.2.3 Example of treatment media description.

Tube (50ml)	Serum free DMEM (ml)	1% alginate solution in serum free DMEM (ml)	FAC 3mM (ml)	AA 0.06M (ml)	Total vol (ml)
1. Control (Blank)	50		----	----	50
2. 1% alginate	----	50	----	----	50
3. 1% alginate +AA	----	49.5	----	0.5	50
4. FAC	49.5	---	0.5	---	50
5. FAC +AA	49	---	0.5	0.5	50
6. FAC + 0.1% alginate	44.5	5	0.5	---	50
7. FAC + 0.1% alginate+AA	44	5	0.5	0.5	50
8. FAC + 0.5% alginate	24.5	25	0.5	---	50
9. FAC + 0.5% alginate+AA	24	25	0.5	0.5	50
10.FAC + 1% alginate	---	49.5	0.5	---	50
11.FAC + 1% alginate +AA	---	49	0.5	0.5	50

Uptake of FAC by Caco-2 cells was measured from the ferritin content 24h post-exposure (ng/mg total protein), as this reflects the quantity of iron entering the cell, and is therefore a surrogate index of iron availability.

3.2.5 Statistical analysis

Unless otherwise stated, all statistical analyses were performed using SPSS Inc, USA, (version 16.0.0). 2-factor ANOVA with Tukey's post-hoc or Dunnett (2-sided) t-test were conducted to examine pairwise differences on log-transformed data. Data are presented as mean ±SD. Differences were considered significant at $p<0.05$. Each table or figure represents data from one experiment. The number of replicates in each experiment performed is indicated and is provided in figures/tables legends.

3.3 Results

3.3.1 Iron in presence of alginate solutions

As described earlier four types of alginate (A1; A4 from Danisco, UK and A5 (Manucol); A6 (Manugel) from FMC bioPolymer, Norway) with different ratios of mannuronic to guluronic acid (Table 3.2.2), were applied to the Caco-2 cells at various concentrations in media solution containing FAC.

There was no effect of 0.1% (w/v) A1 (Figure 3.3.1), A5 and A6 with the exception of A4 (Figure 3.3.2) alginate on FAC-induced ferritin formation in Caco-2 cells exposed to the same dose of iron. Higher concentrations of alginates resulted in a significantly higher ferritin expression ($p=0.002$, $p<0.0005$ and $p<0.0005$ for 0.5% (w/v) alginates A4, A5 or A6 respectively; $p=0.004$ for 1% (w/v) A1 alginate and $p<0.0005$ in case of remaining 1% (w/v) alginates used) together with FAC compared to cells treated with FAC alone. In cells exposed to FAC (30 µmol/L) there was a 10-fold increase in cell ferritin concentrations when compared to untreated control cells (data not shown, ferritin concentration 4.7ng/mg protein). These data demonstrate that alginates can increase iron bioavailability in this *in vitro* system. There were no major differences between the effects of 4 different alginates tested on ferritin formation in Caco-2 cells. There was a similar trend in cell response (Figure 3.3.1; Figure 3.3.2), when treated with 4 different types of alginate (A1, A5 and A6), with the exception of A4 alginate at 0.1% concentration in the presence of FAC, (however when the experiment was repeated (Figure 3.3.3), the results were

different, suggesting that it was not a reproducible effect) indicating that the different M:G ratios in the alginates tested had a similar effect on ferritin formation.

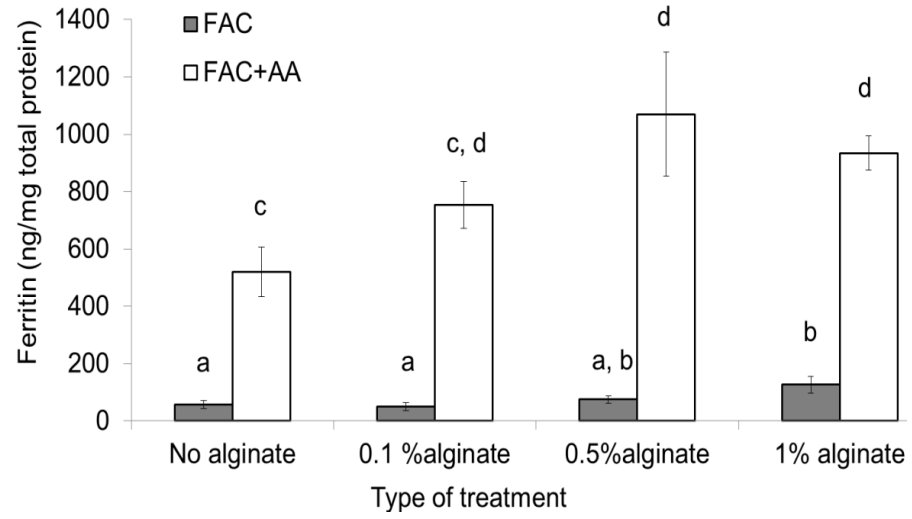

Figure 3.3.1. Ferritin concentration in Caco-2 cells exposed to FAC and different alginate concentrations and AA. Ferritin concentration (ng/mg total protein) after treatment with 30μmol/L FAC (n=6), FAC plus A1 at 3 concentrations (0.1, 0.5 and 1% w/v) (n=4 for each alginate concentration used, black bars), FAC and AA (at 600μmol/L) (n=3), or FAC and A1 and AA (n=4 for each alginate concentration used, white bars). Cell ferritin response to control (blank) was 1.4±SD 0.2. Data represent mean ±SD. Bars without a common letter (a, b, c, d) are significantly different, $p<0.05$.

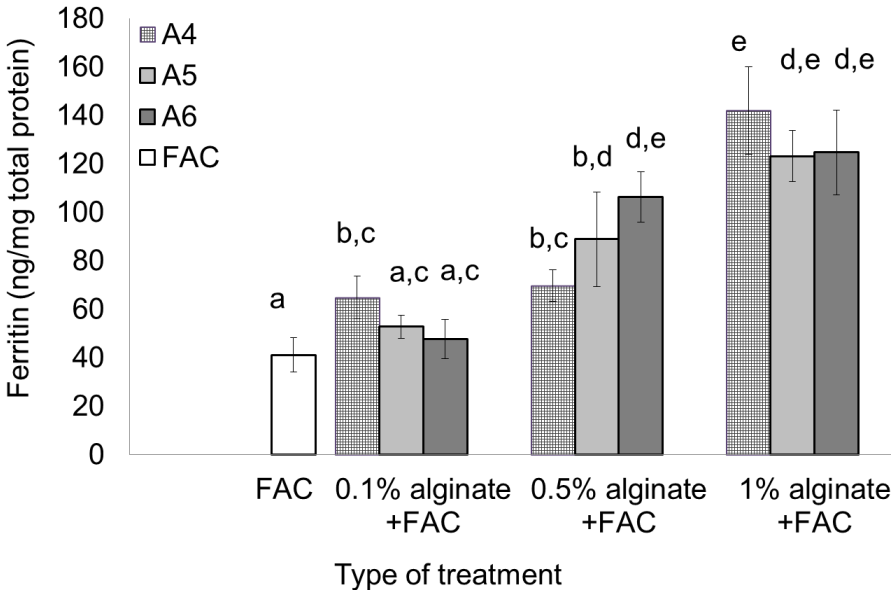

Figure 3.3.2 Ferritin concentration in Caco-2 cells exposed to FAC and different alginates. Data combined from 2 separate experiments. Ferritin concentration (ng/mg total protein) after treatment with 30μmol/L of FAC, FAC plus A4 or A5 or A6 at 0.1%; 0.5% and 1% (w/v) concentration. Data represent mean ±SD (n=4, except FAC where n=6). Bars without a common letter (a, b, c, d, e) are significantly different, $p<0.05$**.**

3.3.2 Iron and ascorbic acid in the presence of alginate solutions

As predicted, cells exposed to FAC plus AA had a significantly higher ferritin concentration than cells incubated with FAC alone (p<0.0005 Figure 3.3.1; Figure 3.3.3). Interestingly, ferritin formation was increased further in the presence of 0.5% and 1% A1 alginate when compared to AA alone (p=0.01 and p=0.041, respectively), (Figure 3.3.1). However this was not the case when A4 alginate was used (Figure 3.3.3).

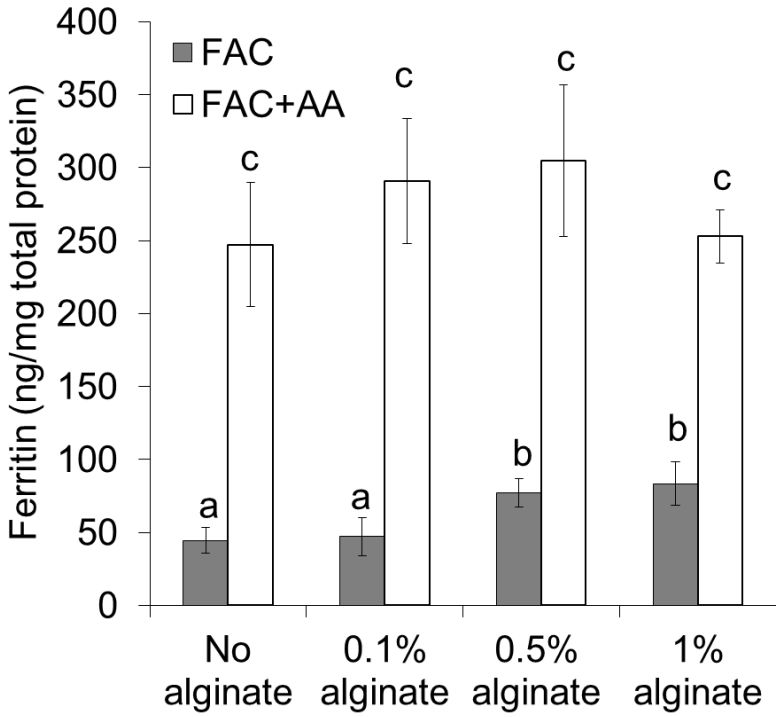

Figure 3.3.3 Ferritin concentration in Caco-2 cells exposed to FAC and different alginate concentrations and AA. Ferritin concentration (ng/mg total protein) after treatment with 30µmol/L FAC (n=6), FAC plus A4 at 3 concentrations (0.1, 0.5 and 1% w/v) (n=4 for each alginate concentration used, black bars), FAC and AA (at 600µmol/L) (n=3), or FAC and A4 and AA (n=4 for each alginate concentration used, white bars). Cell ferritin response to control (blank) was 2.0±SD 0.9. Data represent mean ±SD. Bars without a common letter (a, b, c) are significantly different, p<0.05**.**

3.3.3 Iron and tannic acid in presence of alginate solutions

It has previously been reported that at 1:0.1 (Fe:TA molar ratio) TA inhibits iron uptake into Caco-2 cells by 92%[227]. Co-addition of TA significantly inhibited ferritin formation induced by FAC at 30µmol/L or at 100µmol/L (p=0.044, Figure 3.3.4; and p=0.002; Figure 3.3.5 respectively). When 0.5% A5 alginate was added with iron (30µmol/L or at 100µmol/L) and TA, the ferritin formation was restored to levels obtained in samples treated with iron alone, resulting in significantly higher ferritin

concentrations than in cells treated with iron and TA alone (p=0.042 and p<0.0005 respectively). However the inhibitory effect of TA was not blunted at low or high (0.1% or 1% w/v) alginate concentrations (Figure 3.3.4). These results suggest that an alginate concentration of 0.5% (w/v) was the most favorable in terms of negating the inhibitory effect of TA.

It is also worth commenting that as in previous experiments a dose-response effect was noted in samples treated with increasing amounts of alginate in the presence of iron, with a significant increase in ferritin formation in samples at 0.5% (w/v) alginate concentration in comparison with a lower (0.1% w/v) concentration (p=0.013 in experiment where 100µmol/L FAC was used).

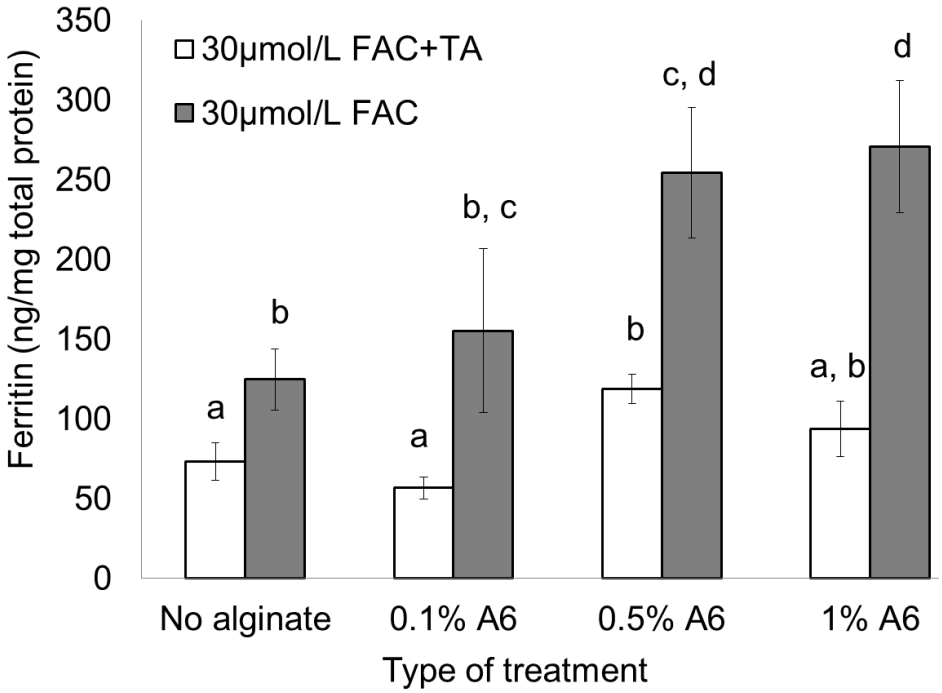

Figure 3.3.4. Ferritin concentration in Caco-2 cells exposed to FAC, alginate and TA. Ferritin concentration (ng/mg total protein) after treatment with 30µmol/L FAC, or FAC and A6 at 0.1%; 0.5% and 1% (w/v) concentration, with or without TA at iron: TA molar ratio 1:0.1. Cell ferritin response to control (blank) was 1.5±SD 1.3. Data represent mean ±SD (n=4). Bars without a common letter (a, b, c, d) are significantly different, p<0.05.

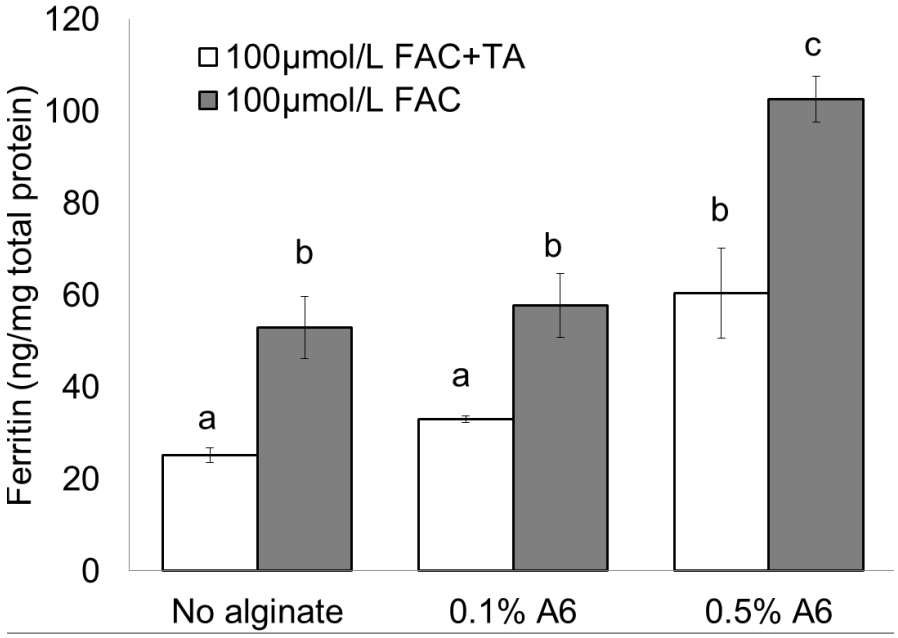

Figure 3.3.5. Ferritin concentration in Caco-2 cells exposed to FAC, alginate and TA. Ferritin concentration (ng/mg total protein) after treatment with 100µmol/L FAC, or FAC and A6 at 0.1% and 0.5% (w/v) concentration, with or without TA at iron: TA molar ratio 1:0.1. Cell ferritin response to control (blank) was 5.2±SD 0.5. Data represent mean ±SD (n=4). Bars without a common letter (a, b, c) are significantly different, $p<0.05$.

Based on the above results, alginate solutions at concentrations more than 1% (w/v) were prepared to investigate if the ameliorating effect of alginate on TA was greater at higher concentrations of alginate. However, due to very high viscosity of the alginate solutions it was impossible to undertake the planned experiments to test the above hypothesis.

3.3.4 Iron containing alginate beads

Experiments were performed to examine the stability of alginate beads containing varying levels of alginate (Table 3.3.1) but a constant amount of FeG at the level of 8.38µg in each treatment (apart from 'blank' control) when subjected to simulated digestion. Iron (III) chloride in the presence of AA was used as a positive control (Fe:AA ratio 1:10). The beads were loaded with two iron compounds: FeG and FAC.

Table 3.3.1. Composition of the beads.

Beads	Iron compound added	Weight of beads, *g*	Iron: AA ratio	Total iron content, *µg*	Total calcium content *µg*
---	$FeCl_3$+AA	---	1:2	8.38	---
B1	FAC[1]	0.049	---	8.38	Data not available
B2	FeG[2]	0.044	---	8.38	Data not available
B3	FeG	0.174	---	8.38	Data not available
B4	FeG	0.419	---	8.38	Data not available
---	$FeCl_3$+AA	---	1:10	8.38	---
B5	FeG	0.044	---	8.38	16.7
B6	FeG	0.093	---	8.38	65.1
B7	FeG	0.175	---	8.38	85.8
B8	FeG	0.419	---	8.38	289.1
B9	FeG	0.209	1:10	8.38	108.7
B10	FeG	0.419	1:10	8.38	217.9

[1]FAC -ferric ammonium citrate; [2]FeG- ferrous gluconate

To ensure identical iron concentrations for each treatment, varying weights of alginate beads were used. The results showed that FeG was more effective in inducing a ferritin response in the Caco-2 cells than FAC (Figure 3.3.6) with

significantly higher ferritin values for beads B3 and B4 (p=0.04 and p=0.024 respectively).

In addition, the design of an experiment presented in Figure 3.3.7 also enabled the evaluation of ferritin formation in Caco-2 cells in response to increasing weight of alginate beads but to a constant amount of iron (Table 3.3.1). However, with higher weights of alginate beads the calcium content per treatment also increased.

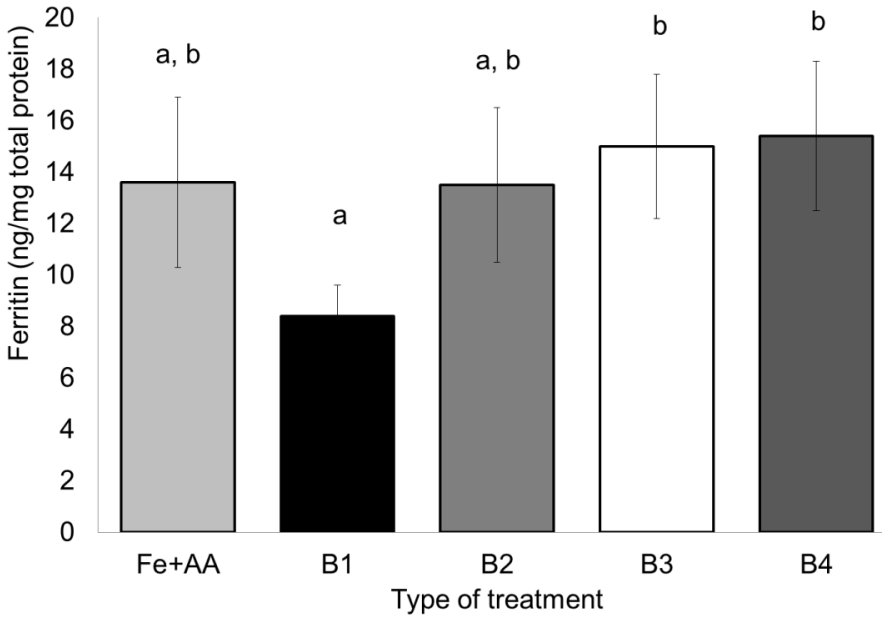

Figure 3.3.6 Ferritin concentration in Caco-2 cells treated with iron (III) chloride in presence of AA, alginate bead formulations (B1-B4) containing either FAC (B1) or FeG (B2, B3 or B4). Cell ferritin response to control (blank) was 5.9 ±SD 0.7. Data represent the mean ±SD (n=4). Means without a common letters (a, b) are significantly different (p<0.05).

Ferritin formation in cells treated with digestates of B6 beads (containing 0.093 g alginate) was similar to the positive control ($FeCl_3$:AA) (Figure 3.3.7). Increasing the alginate content of the beads (B7, B8) inevitably increased the calcium content, and this resulted in a significantly lower ferritin concentration. However, addition of AA during bead formation (i.e. B9, B10) counteracted the inhibitory effects observed in B7 and B8 (p<0.0005 for both comparisons, Figure 3.3.7; Table 3.3.1). Furthermore, AA containing beads B10 produced a significantly greater ferritin response than AA alone (p=0.013), suggesting a synergistic effect between AA and alginate.

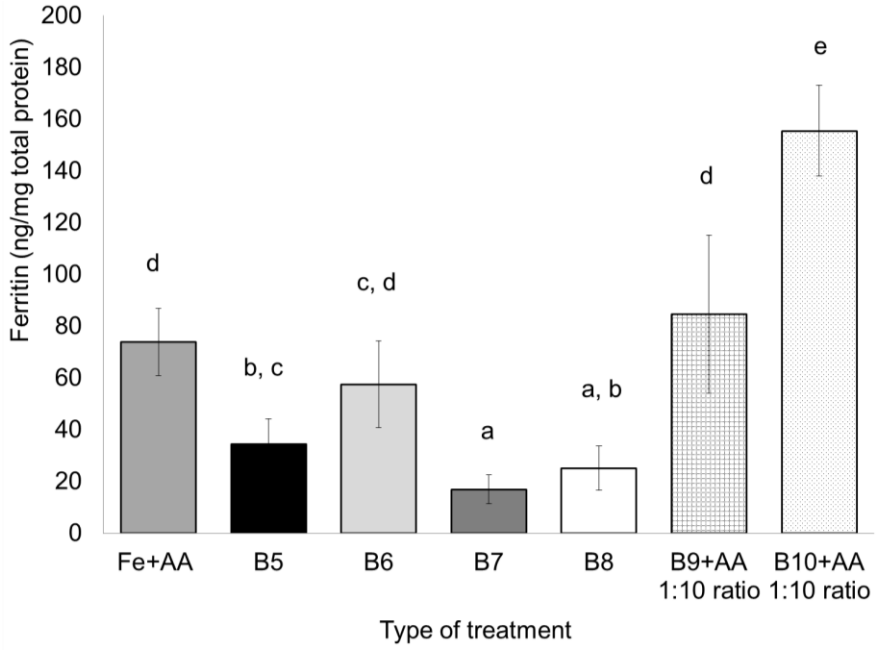

Figure 3.3.7 Ferritin concentration in Caco-2 cells treated with iron (III) chloride in presence of AA (at 1:10 ratio and alginate bead formulations (B5-B10) containing either FeG (B5- B8) or FeG in presence of AA at 1:10 Fe to AA ratio (B9-B10). Cell ferritin response to control (blank) was 1.7 ±SD 2.0. Data represent the mean ±SD (n=4). Means without a common letters (a, b, c, d) are significantly different (p<0.05).

3.3.5 Iron containing starch and emulsions

W/O/W) described earlier in Section 3.2.1 loaded with 16µM, 33µM, 80µM or 160µM of FAC and subjected to simulated digestion (described in paragraph 2.2) did not deliver iron in an available form and therefore there was no ferritin response in Caco-2 cells (experiment was performed twice with the same findings, data not shown).

As a result of these experiments, further analysis was undertaken at the IFR. More detailed microscopic examination revealed that although emulsion droplets remained intact at physiological temperatures (37ºC) for several hours, they did not withstand the addition of 0.15M sodium chloride solution. Osmotic pressure caused the diffusion of iron from the W/O/W particles; hence the emulsions did not provide a protective barrier. Based on the above evidence, no further experiments using W/O/W emulsions were undertaken.

3.3.6 Comparison of iron availability from iron (III) chloride, iron (II) gluconate, iron containing alginate beads, starch and G/O/W

In previous cell experiments iron (III) chloride was used as a positive control but since FeG was being used to load alginate beads it was appropriate to use FeG as a positive control in order to investigate the effect of alginate beads on FeG uptake in cells. In addition, use of FeG and ferric chloride in one experiment allowed for comparison between these two forms of iron in the Caco-2 cell model. An experiment was undertaken to examine the effect of simulated digestion on FeG, iron (III) chloride (Figure 3.3.8) as well as dried beads, wet beads, G/O/W emulsion and 2 types of starch. The exact quantities of iron-loaded materials are shown in Table 3.3.2

Table 3.3.2**.** Composition of iron loaded materials.

Iron loaded material	Iron compound added	Weight of iron loaded materials used (*g)*	Iron content per well (*µg)*
----	Iron (III) chloride	----	8.5
---	FeG	---	8.5
Wet beads (B11)	FeG	0.0945	8.5
Dry beads (B11)	FeG	0.00289	8.5
Wet beads (B12)	FAC	0.00233	8.5
G/O/W	FeG	4.83g	23.1*
Starch SN14	FeG	0.00289	8.5
Starch SN 15	FeG	0.00289	8.5

*Iron content of G/O/W was 2.5 fold higher than other materials used because (as revealed by the IFR team) G/O/W's in their *in vitro* test showed a small release of iron.

Results presented in Figure 3.3.8 suggest that iron as FeG is more available to the cells than iron delivered as iron (III) chloride when assessed by ferritin formation (p=0.001). However, when the cell ferritin response to wet beads B11 and dried beads B11 was compared with the cell ferritin response to ferric chloride and FeG there were no significant differences between the beads and the two iron compounds.

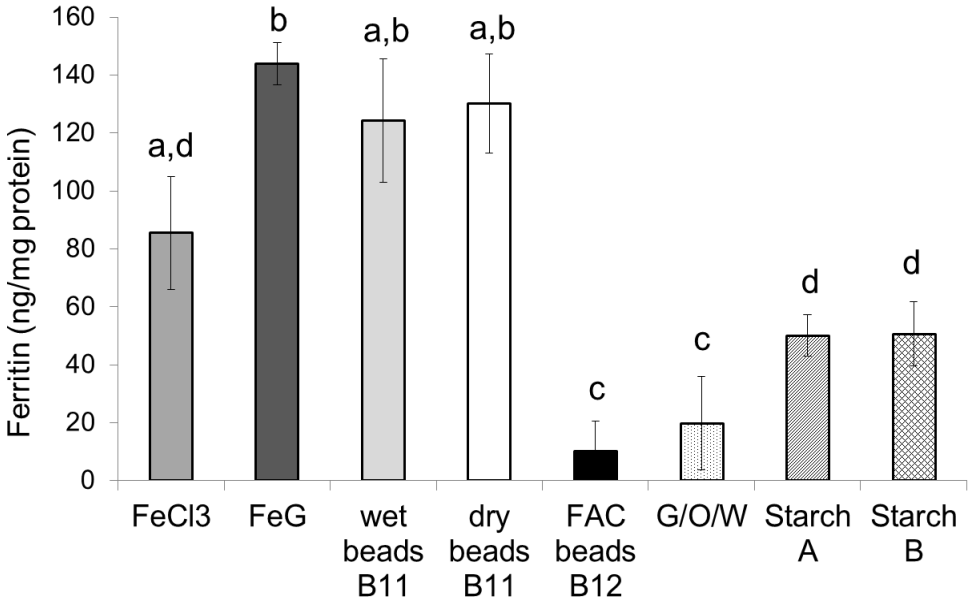

Figure 3.3.8 Ferritin concentration in Caco-2 cells treated with iron (III) chloride, FeG, alginate beads containing either FeG (B11 wet or B11 dried beads) or FAC (B12); G/O/W containing FeG and 2 starch samples also containing FeG. There was no ferritin response in untreated cells control (blank). Data represent the mean ±SD (n=4) except control, $FeCl_3$ and FeG treatment where n=6. Means without a common letters (a, b, c, d) are significantly different (p<0.05).

Beads loaded with FAC (B12), G/O/W as well as starch SN14 and SN15 gave a significantly lower ferritin response than unprotected FeG (p<0.0005 in all instances). These results suggest that alginate beads loaded with FeG are the most promising (wet or dried) vehicle. However, ferritin produced by the cells when treated with beads containing FeG was at a similar level to unprotected FeG.

3.4 Discussion

Caco-2 cell models have been developed as a rapid tool for investigating iron availability, and when combined with a simulated digestion procedure they are considered to mimic more closely the *in vivo* situation. However, seeding the cells at very high density and culturing them for 12 days post-confluence in 6 well plates is technically challenging. There may be non-uniform distribution of cells which can reduce the cell monolayer by 30 to 40% in some parts of the well and across many plates which will result in a low cell response for iron treatments. For example the cell ferritin response to ferric chloride treatment in the presence of AA (Figure 3.3.6) was six fold lower than the cell ferritin response to ferric chloride treatment presented in Figure 3.3.8. These problems were encountered on several occasions and a significant amount of time was spent trying to determine the cause of the growth problems and changing the culturing and sub-culturing conditions. These issues illustrated the importance of accurate seeding procedures for obtaining uniform cell distribution, and this was more difficult to achieve in a round culture dish.

In experiments performed using W/O/W emulsions as a carrier of iron, there was no ferritin response, indicating that either iron was not released from W/O/W emulsions or G/O/W emulsions did not provide an adequate barrier to protect iron from precipitation as ferric hydroxide at high (duodenal) pH, therefore preventing it from being in an available form for uptake by Caco-2 cells.

The mechanism by which alginates enhance iron uptake into Caco-2 cells remains uncertain. Based on the fact that alginates bind calcium cations[209,228] and that alginates have a higher affinity with calcium in the presence of iron[211], one of potential hypotheses is that when alginates are added to the culture media they form a complex with calcium, a recognized inhibitor of iron absorption[54], which is present in the media as calcium chloride (at a concentration of 200mg/L). High concentrations of calcium can act as a non-competitive inhibitor of iron absorption via DMT1[229] and, in addition, can induce translocation of DMT1 away from the cell surface of Caco-2 cells[55]. A limitation of our model system is that calcium (an essential component of cell culture media) cannot be removed without deleterious effects to the cell monolayer. Furthermore, attempts at increasing the calcium content in culture media in the presence of alginate failed due to the immediate formation of a thick gel.

Additionally, alginate beads loaded with FeG resulted in a greater response than FAC in terms of ferritin formation. Thus, FeG was used in the subsequent human study (Chapter 4) as this compound appears to be the more promising than FAC and has the benefit of already being used in food fortification.

The experimental procedures employed in these studies had some weaknesses. All the ferritin results (ng/mg total protein) presented Figure 3.3.6 are relatively low (ferritin values are low across all treatments) which suggests that the cells were not responding sensitively to the iron treatments. Thus, these results should be treated with caution. Furthermore the ELISA assay used to quantify ferritin (S-22, Ramco, USA procedure described in detail in Chapter 2) measured ferritin in the range between 6 and 2000ng/ml, therefore results presented in Figure 3.3.6 are at the lower end of the standard curve.

The simulated digestion procedure (described in Chapter 2) was designed to investigate food samples which have lower iron concentrations (w/w) than the iron containing alginate beads and therefore higher weights (e.g. 0.5g) could be tested in each treatment. The Caco-2 cell system works best with solutions of iron ranging from 10-100µmol/L[230]. At higher values the response is no longer linear, and at lower values, the ferritin response is low and the sensitivity of the assay is greatly reduced[230]. The weight of alginate beads that contained iron within this range was relatively low, increasing the possibility of errors when weighing of the samples. To minimize these errors the iron loading conditions and consequently the iron content of the beads was reduced, but this inevitably increased the calcium content of the beads.

In addition, enzymes that are involved in the digestion of fat (lipase and lecithin) and starch (duodenal amylase) were not included in the standard simulated digestion procedure (designed specifically for screening iron availability, Glahn *et al.*[230]). Therefore the low release of iron from starch or G/O/W may be the result of incomplete digestion. This was tested by adding egg lecithin enzyme to the gastric phase of digestion and duodenal amylase to the duodenal phase of digestion. However, despite the use of a membrane during the digestion step, the Caco-2 cells did not survive the experimental conditions.

In conclusion, the results from the *in vitro* experiments demonstrate that alginates increased the uptake of iron into Caco-2 cells, whilst not modifying the effect of AA; thus having a positive effect on iron bioavailability. Alginates also protected iron from the inhibitory effects of TA under the conditions tested. Furthermore, from all the

materials tested (W/O/W, starch, G/O/W, alginate beads) the alginate beads provided the highest ferritin response in Caco-2 cells suggesting that these may be a promising vehicle for iron fortification. However it has to be noted that cell response to FeG containing alginate beads was at similar level to unprotected FeG. The efficiency of iron uptake from alginate beads into Caco-2 cells was dependent on the calcium content and chemical form of iron in the alginate beads. Overall, the results therefore suggest that alginate beads could be considered as a suitable food delivery vehicle for iron in food fortification programs. The performance of the iron-loaded alginate beads was subsequently examined in a human study (Chapter 4).

4. *IN VIVO* STUDIES OF ALGINATE

4.1 Introduction

The aim of the human study was to investigate the effectiveness of using an alginate barrier to deliver FeG to the duodenum, thus maximising its availability for absorption. The technique employed was to give the test material to volunteers who had fasted overnight and measure iron appearance in the serum over 6 hours[231] and to use compartmental modelling to calculate percentage iron absorption[232]. Based on previous reports in the literature, measurements of serum iron increase were in good agreement with iron absorption measured by whole-body counting[233].

The hypothesis tested in this study was that the absorption of iron from FeG loaded alginate beads (Figure 4.1.1) would be higher than from unprotected FeG (administered in a capsule). This hypothesis was based on the results of the *in vitro* experiments, and the proposed mechanism was that the alginate provided protection for the water-soluble FeG by keeping it in a bioavailable form.

Figure 4.1.1 Iron containing alginate beads.

The primary objective of this human study was to determine if iron absorption from FeG incorporated into alginate beads was greater than from unprotected FeG. The secondary objective was to investigate the modulating effect of calcium by comparing iron absorption from FeG incorporated into alginate beads with that from unprotected FeG when both were given with calcium.

4.2 Methods

A human study entitled: 'Study to measure the absorption of iron from FeG incorporated into alginate beads' was approved by the UEA, Faculty of Medicine and Health Research Ethics Committee on 31st November 2011 (reference number 2011/2012-05). The study was also registered on a publicly accessible clinical trials database (www.clinicaltrials.gov), registration number NCT01528644

4.2.1 Subjects

A total of fifteen apparently healthy subjects completed the study (Figure 4.2.1, for consort checklist please see Appendix-D). Participants were selected from populations with a higher prevalence of low iron stores; these were women of child-bearing age (18-45y) and men who were regular blood donors (aged 18-65y). Underweight and obese individuals were excluded and the BMI inclusion range was >18.5< 30kg/m^2**.** Iron absorption is up-regulated when iron stores are low to moderate (ferritin stores <60µg/L[6]), thus selecting individuals with a ferritin <60µg/L increases the sensitivity of the absorption test. Volunteers were recruited from the Norwich Research Park (NRP) and the surrounding Norwich area. Advertisements were placed on the UEA website, NRP newsletters, and distributed via email to UEA staff and students.

Volunteers who expressed an interest in taking part in the study, and who met the basic inclusion criteria (listed below) were sent participant information sheet (PIS) and invited to attend a meeting where full details of the study were explained. A 10ml screening blood sample was taken to exclude subjects whose serum ferritin was ≤15 or ≥60µg/L.

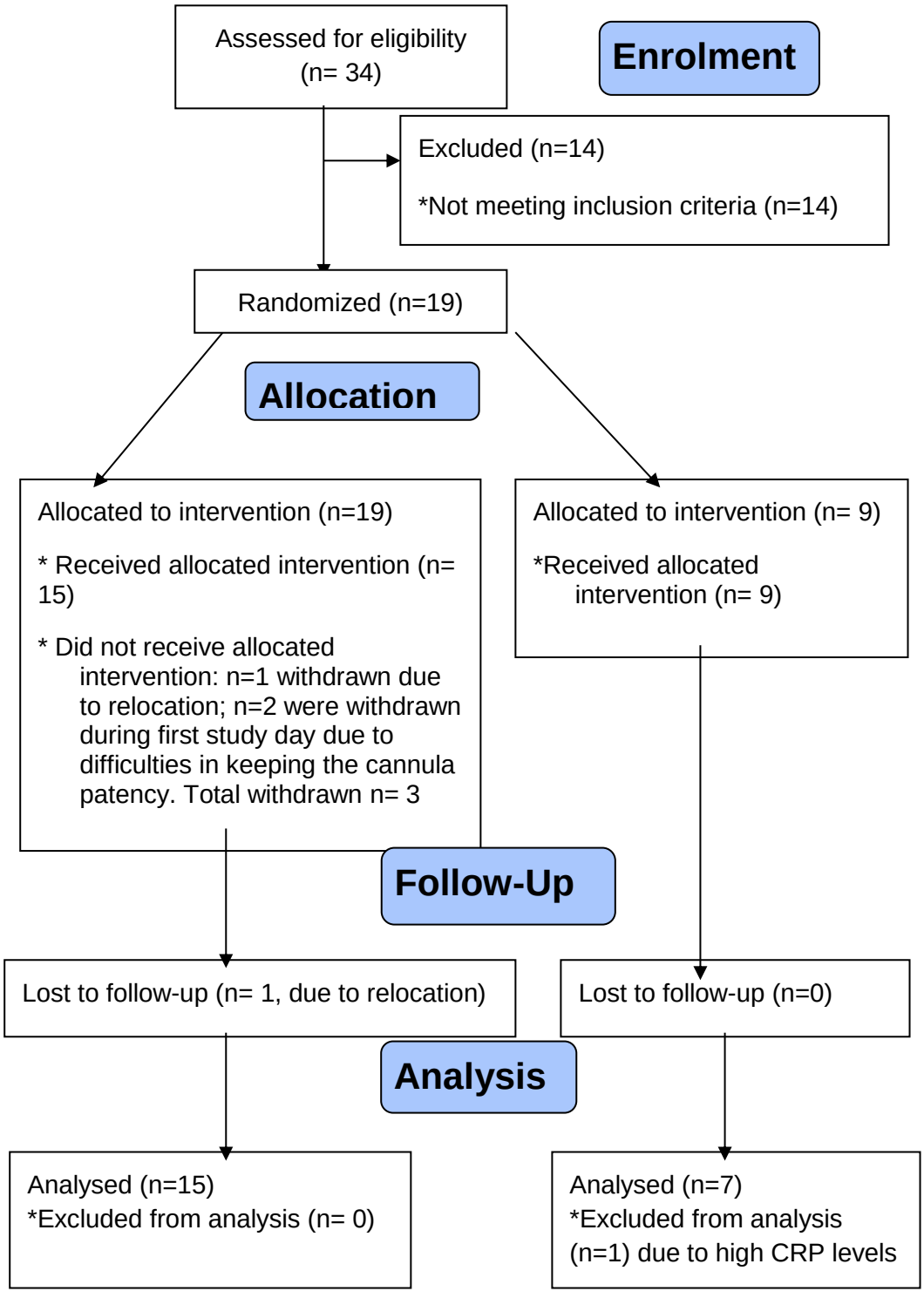

Figure 4.2.1 CONSORT 2010 flow diagram describing stages of the study with number of participants at every stage.

The exclusion criteria for study were:

- Serum ferritin concentration ≤15µ/L or ≥60µg/L.
- Blood pressure (measured in the Clinical Research and Trials Unit (CRTU) at the screening visit) >160/100mmHg
- Previous diagnosis with a long-term illness requiring active treatment, e.g. diabetes, cancer, cardiovascular disease
- Currently smoking or stopped smoking in the last 12 months
- Previous or current gastrointestinal disease
- Any significant co-morbidity
- Previous gastrointestinal surgery
- Blood donation within the previous 3 months assuming that volume taken is above 500ml in a 4-month period
- Blood transfusion within the last 3 months
- Relation of someone in the study team i.e. spouse, partner or immediate family member
- Regularly prescribed medication that may interfere with iron metabolism
- Regular use of antacids and laxatives (at least once a week)
- Women who are pregnant or less than 12 months since giving birth
- Women who are breast feeding
- Vitamin supplements with or without minerals if taken more than once a week, and unwillingness to discontinue occasional use for the duration of study
- Unwillingness to discontinue use of herbal supplements for the duration of study
- Unwillingness to consume jelly prepared with animal source gelatine
- Use of antibiotics within four weeks prior to study start

- Parallel participation in another study which involves dietary interventions or sampling of blood that may increase the volume taken above 500ml in a 4-month period

- Asthma requiring treatment within the last two years

4.2.2 Study design

The study design and outline are presented in Figure 4.2.2. The study was a randomised, single blinded, cross-over trial with participants randomly allocated into two groups: A (n=8 volunteers) and B (n=15 volunteers). Group A underwent 4 tests, whereas group B underwent 2 out of the 4 tests. For each test meal (cola jelly) an oral dose of iron (on its own or incorporated into alginate beads) of approximately 21mg, sufficient to perturb normal serum iron concentration[2], with or without calcium was given to apparently healthy adult volunteers after a 10 hour (overnight) fast.

All appointments (initial interview, screening visit and study days) were carried out by the study scientist and took place in the CRTU based in the UEA Norwich Medical School. The CRTU provided all the required facilities and resources to undertake the study. A trained research nurse based in the CRTU undertook all study-related clinical procedures, details of which are described in the following Sections.

4.2.1 Study day procedures

4.2.1.1 Initial Interview

Interested individuals who responded positively to the advertisements and the PIS were asked to visit the CRTU for an individual informal meeting to receive further information about the study. During the meeting the study scientist explained the purpose of the study and encouraged the potential volunteer to ask questions to ensure that he/she understood the requirements of study participation. Volunteers who met the basic inclusion and exclusion criteria (outlined in Section 4.2.1) and expressed an interest to take part in the study were given a minimum of 72 hours (3 days) to further consider whether they wanted to participate in the study.

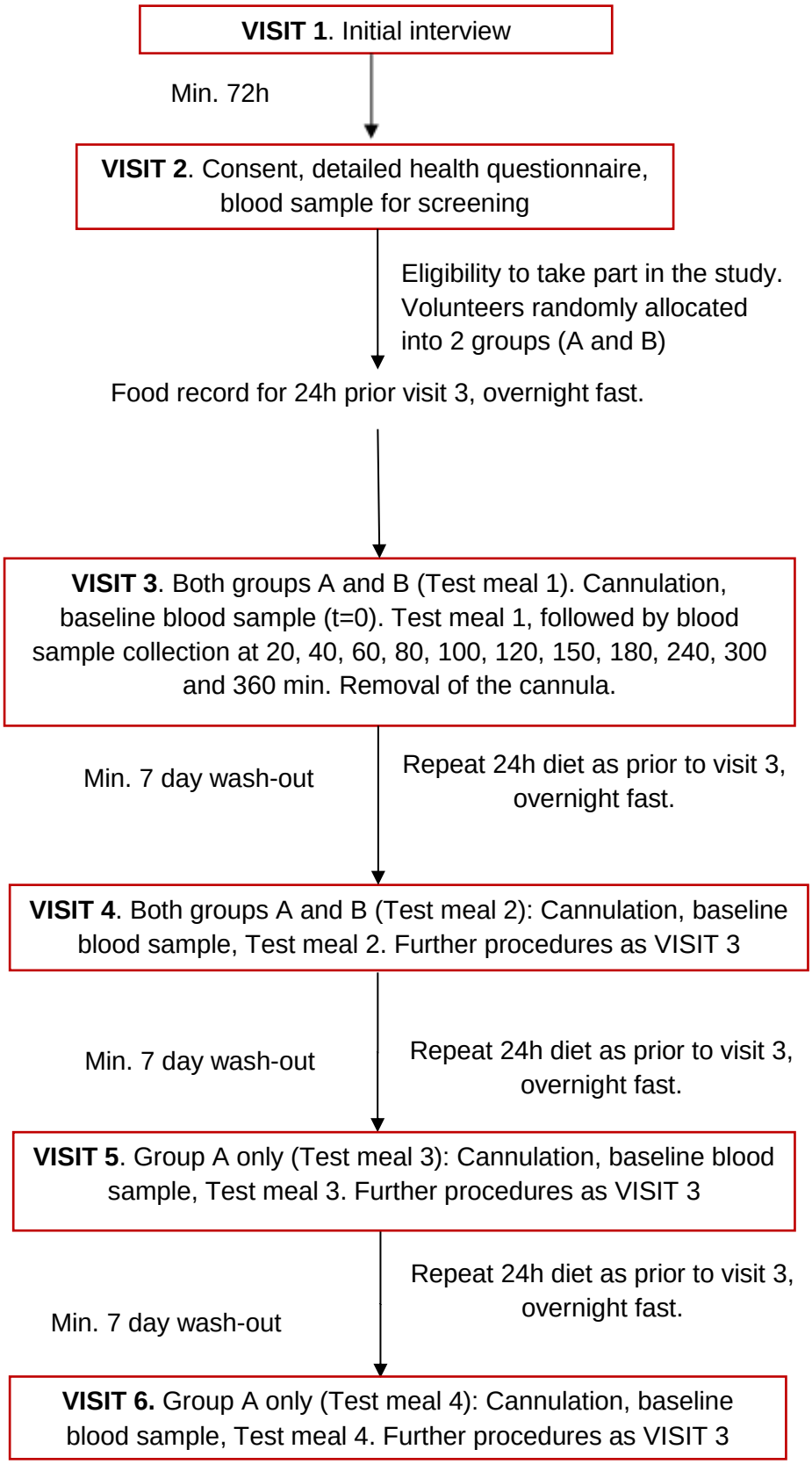

Figure 4.2.2 Study design.

During this period the volunteers were not contacted by any member of the research team. If, following the period of consideration, the volunteer expressed an interest in participating in the study, a clinical screening visit at the CRTU was arranged.

4.2.2.1 Consent and screening

Volunteers were invited to attend a clinical screening visit at the CRTU after an overnight fast of at least 8 hours. On arrival, the study scientist presented the consent form and encouraged the volunteer to ask any questions they had at this stage. Volunteers were then asked to sign a consent form agreeing to participate in the study. A copy of this form was given to the volunteer to keep. Subsequently, a researcher along with an experienced CRTU nurse completed a basic health questionnaire and collected information about weight and height. The CRTU nurse obtained and recorded blood pressure measurements, pulse and BMI. A single venous blood sample (10ml) was also taken by a CRTU nurse to measure full blood count: WBC, RBC, Hb, HCT, MCV, MCH, platelets, neutrophils, lymphocytes, monocytes, eosinophils, basophils, fasting glucose and ferritin. The blood was analysed at an accredited pathology laboratory at the Norfolk and Norwich University Hospital (NNUH), Norwich, UK. Once all clinical procedures were completed, the volunteers were offered and encouraged to have a meal provided by the study team.

Blood screening results were examined by an experienced CRTU nurse and study medical advisor (Dr Phyo Myint, Norwich Medical School, UEA) who informed the study scientist if all screening parameters were within the normal range for sex and age, and therefore whether the participant could take part in the study. If any of the parameters (apart from ferritin values of ≤15 ≥ 60µg/L, study specific requirement) fell outside the normal range the study medical advisor was contacted by a study scientist and asked whether the results could affect the study data or have implications for the health of the volunteer, and therefore whether inclusion, exclusion or re-screening was appropriate.

During the screening interview the study scientist also provided instructions on keeping a 1-day food diary to record the “pre-test diet” in preparation for the first experimental day (visit 3 on Study flow diagram, Figure 4.2.2). Volunteers were asked to repeat the same pre-test diet prior to each experimental day. Where there were any deviations, volunteers were asked to note them down in the Record of Differences Diary. During the meeting participants also received instructions with

regard to undertaking a 10 hour overnight fast. They were asked to have a meal no later than 9pm on the night prior to each experimental day and to arrive at the CRTU at around 8:00 am the next morning.

4.2.2.2 Confidentiality

Personal data was handled in line with the regulations of the Data Protection Act 1998. Once recruited onto the study, volunteers were assigned a three digit code number with only the named study scientists approved by the Ethics Committee having access to allow them to link codes to volunteers. All personal data and biological samples were coded with this number to ensure confidentiality. All personal information was kept confidential and the information linking codes to participants had restricted access known only to the chief investigator, academic supervisors, CRTU research nurses, and the volunteer's GP. It was not possible for an individual to be identified solely from their code number.

4.2.2.3 Randomization

Randomization was undertaken by a third party unconnected with the study using the randomization generator available at www.randomization.com website. The study was single-blinded so participants were unaware to which group they were allocated and which test meal they received on each experimental day. Volunteers were assigned randomly to group A or B and were asked to complete 4 study days in group A and 2 study days in group B. Volunteers were unaware (until the completion of the first 2 study days) to which group they had been assigned. After completing 2 study days, volunteers from group B were thanked for their involvement and informed that their participation in the study was complete. At the same time participants from group A were asked to continue their participation for 2 further study days.

Thirty four volunteers were screened, of which 20 were eligible to take part in the study (based on health questionnaire and screening results). Out of 20 eligible participants one did not respond to the study invitation, one withdrew prior to the first experimental day (due to relocation), one withdrew after the first study day (due to relocation) and two were withdrawn during the first study day due to difficulties in finding an appropriate peripheral vein and maintaining cannula patency. In addition, one participant had elevated CRP levels on the 4th study day; therefore data from

that day were excluded from the final analysis. The remaining 15 participants completed all study days as planned (Figure 4.2.1).

4.2.3 Iron Dose Administration

For each test meal an oral dose of iron (mean=21.6mg ±SD 2.1mg of iron as FeG), sufficient to perturb the normal serum iron concentration[235], was given, either incorporated into alginate beads or weighed into a gelatine capsule (Distinctive Medical, UK).

4.2.4 Test meal composition and preparation

All test meals were prepared in the CRTU kitchen at the University of East Anglia.

The composition of each of the four test meals was as follows:

- Test meal 1 (study day 1) for all volunteers (groups A and B): 200ml cola jelly (as described below) into which alginate beads were mixed (mean=21.77g ±SD 0.14g)) containing iron (mean=21.6mg ±SD 2.1mg) as FeG (mean=0.187g ±SD 0.019g) followed by 3 placebo capsules (each filled with 50mg commercially available dextrose powder, 'Glucose Dextrose Powder', (Pharmacy Near U, Huddersfield, UK) acting as placebos for calcium, an iron capsule and 200ml of diet cola drink.
- Test meal 2 (study day 2) for all volunteers (group A and B) consisted of 200ml cola jelly. The jelly was stirred with a plastic spoon prior to administration. The test meal also included administration of one FeG capsule (prepared exclusively for each volunteer to match the iron content in the alginate beads administered in test meal 1), two dextrose (approximately 50mg of dextrose in each capsule) placebo capsules and 200ml of a diet cola drink.
- Test meal 3 (study day 3) for 8 volunteers only (group A): 200ml cola jelly with the addition of FeG in alginate beads (mean=21.77g ±SD0.14g) followed by 200ml of a diet cola drink and two capsules containing calcium phosphate, (Freeda vitamins, USA) (300mg of calcium in each capsule)[3] and one placebo capsule (50mg of dextrose).
- Test meal 4 (study day 4) was prepared in exactly the same way as previously with the exception of the alginate beads which were replaced by 1 FeG capsule (prepared exclusively for each volunteer to match the iron

content in the alginate beads administered in test meal 3) and 2 calcium phosphate capsules (each containing 300mg of calcium phosphate) followed by 200ml diet cola drink.

Results of previous human intervention studies have shown that the consumption of a cola drink has no effect on iron absorption and study participants find it a more palatable way of consuming a solution of iron than in water alone[237].

4.2.5 Preparation of iron-containing alginate beads

Iron-containing alginate beads were prepared in the kitchen of the Human Nutrition Unit (HNU) at IFR using a specially purchased encapsulator (Figure 4.2.3) designated for the sole preparation of beads for human consumption. Beads were prepared by either the study scientist or a scientist from the IFR, Natalia Perez-Moral.

Alginate (Flavicans HV; 315KDalton, M/G 35/65 and viscosity 1% 350 cp; Danisco, DK) solution was prepared by dissolving alginate in distilled water for 12 hours to obtain a concentration of 0.5% (w/v). Once dissolved, the solution was put through the encapsulator in order to produce beads. The beads were collected into an FeG (0.1M) and calcium chloride (0.1M) (Cat. No. 1.72570.1000; VWR International LTD, UK) solution at 50/50 ratio and left to soak for 20h at 4ºC in order to load them with iron. Once the beads were fully saturated with iron they were washed with distilled water and then filtered for 5 minutes with Whatman (grade 4) filter papers.

Appropriate quantities of sample (≈22g for an experimental day and ≈5g for atomic absorption analysis) were weighed and stored in a fridge in the HNU (at 4ºC). The beads were subsequently transported to the CRTU at the UEA ready to be used in an experiment on the same day.

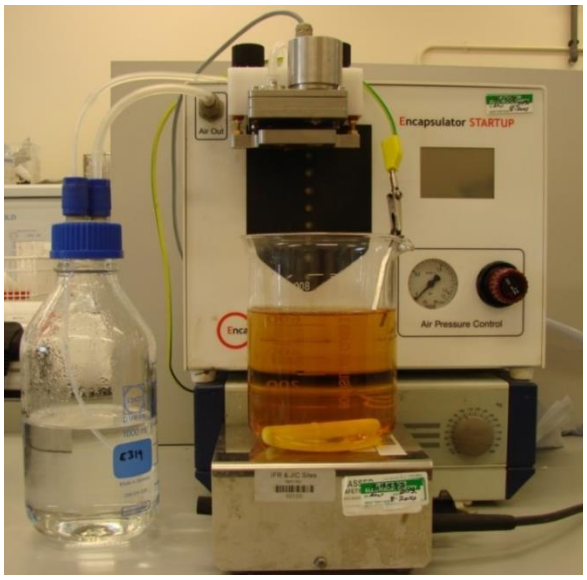

Figure 4.2.3 Encapsulator.

The preparation of iron-containing alginate beads was a long process taking approximately 32h, including 12h for dissolving the alginate, followed by a further 20h for loading with iron in the iron and calcium bath. Preparation of the beads was timed such that the beads were ready for consumption by the participants on the morning of the experimental day. Sub-samples of every batch of beads administered to each volunteer were analysed for iron content using AAS (see Chapter 2, Section 2.4 for method details). During the study, the development of the iron-containing beads was on-going research, and consequently their preparation imposed certain limitations on the study design. Iron incorporation into the alginate beads was generally found to be within 15% of that predicted (with average iron content of 1.03mg/g wet bead ±SD 0.11 and average calcium content 1.96mg/g wet bead ±SD 0.12 as analysed by AAS; however, analysis of the iron content of the alginate beads was a two day procedure. This latter time constraint, coupled with the absence of an established long term storage method for the beads, meant that the sequence of test meal administration could not be randomised. Consequently, every batch of the beads needed to be analysed in order to obtain an accurate measure of the iron content. Once the exact iron content of the beads administered to each individual volunteer in test meals 1 and 3 was known (and recorded), exactly the same amount of FeG was weighed into a coloured gelatine capsule (Distinctive Medical, UK) and administered to that volunteer in a subsequent test meal (test meals 2 and/or 4 respectively) after a minimum 7 day wash-out period.

4.2.6 Capsule preparation

Preparation of the FeG, placebo and calcium capsules was undertaken in the kitchen of the HNU at the IFR.

- FeG capsule preparation: approximately 0.187g (±SD 0.019g) of food grade FeG powder (Cat.No. 1.03868.5000; VWR International LTD, UK) was weighed into empty gelatine red and white coloured capsules, size '00' (Distinctive Medical, UK).

- Placebo capsule preparation: approximately 50mg of 'Glucose Dextrose Powder', (Pharmacy Near U, UK was weighed into empty gelatine red and white coloured capsules, size '00' (Distinctive Medical, UK).

- Calcium capsule preparation: approximately 300mg of calcium phosphate (Freeda Vitamins, USA) was weighed into empty gelatine red and white coloured capsules (Distinctive Medical, UK).

4.2.7 Preparation of cola jelly

The cola jelly was prepared in the CRTU kitchen the day before each study day. 200ml of Coca-Cola® was heated in a microwave, 10ml of Diet Cola flavoured drink concentrate (Sodastream, Lakeland) and 5g of gelatine (Dr. Oetker, UK) were added and mixed until the gelatine was fully dissolved. Once cooled, 200ml of the liquid jelly was poured into a disposable plastic cup and allowed to set in a refrigerator at 4ºC.

On the morning of the experimental day and depending on the test meal (with or without alginate beads) an average of 21.77g (±SD 0.14g, approximately 5 teaspoons) of iron-containing alginate beads were stirred into the jelly in an attempt to mask the presence of the beads, or if the test meal was without alginate beads, the jelly was just stirred to look similar to the bead-containing jelly. In addition to the jelly, participants were given 200ml coca cola® drink (poured directly from a newly opened 330ml can) in a disposable plastic cup along with 3 capsules (depending on the test meal: placebo or/and calcium or/and iron capsule).

4.2.8 Experimental day procedures

On the day before the first study day, participants recorded all food and drink consumed (pre-test diet) in a food diary provided by the study team. On the same evening volunteers began a 10 hour overnight fast from 10:00pm, and subsequently attended the CRTU at approximately 8:00am the following morning. During the fasting period volunteers were advised to drink water. All clinical procedures were carried out by a fully trained nurse in the CRTU.

Prior to cannulation the volunteer's blood pressure was measured and if it was <90/50mmHg, or <95/55mmHg if symptomatic, or >160/100mmHg three further measurements were taken and recorded at 5 minute intervals. The approved protocol stated that if the blood pressure measurement remained outside these ranges after the third measurement, the volunteer would be excluded from the study, referred to their GP and the study day cancelled. However, there were no exclusions from the study based on blood pressure measurements.

If the blood pressure was within the acceptable range a CRTU nurse inserted an intravenous (i.v.) cannula into an appropriate peripheral vein in one of the volunteer's arms. No more than two attempts were made to insert the cannula on any one experimental day. After the cannula was appropriately sited and the first blood sample taken, the volunteers received test meal 1 (as described in Section 4.2.4).

When there were problems with the cannula during the 6 hour sampling period either it was removed and a second cannula inserted or, if there were no more than two blood samples remaining, they were taken by venepuncture. The cannula was flushed with sterile normal saline between blood samples to maintain its patency. During the 6 hour cannulation period volunteers had access to a drinking water dispenser (Aquarius, UK). Any adverse events were recorded by the nurse on the experimental day record sheet and dealt with in accordance with Good Clinical Practice and National Research Ethics Service guidelines.

After the final blood sample was collected, volunteers were offered and encouraged to consume lunch which they had pre-selected from a menu during their screening visit. Lunch was prepared by Catering Direct at UEA and consisted of: a sandwich filled with ham, cheese or tuna mayonnaise, 1 piece of fresh fruit, 1 cake bar, 1 packet of crisps, and a fruit juice drink.

Following a minimum seven day “wash-out” period, the volunteers were asked to repeat their experimental day 1 pre-test diet, prior to the second experimental day. All remaining experimental days: day 2 and if required day 3 and 4 were carried out in exactly the same manner as experimental day one with the exception of the administered test meal.

For taking part in the study participants received inconvenience payments (£85 per study day) and travel expenses were reimbursed on production of a receipt for public transport or at 40p per mile rate for private cars. If the volunteer withdrew from the study before completion inconvenience payments were paid *pro rata*.

Data Storage

Electronic study data stored at UEA was account password protected and only study scientists and appointed nurses had access to the coded information.

4.2.9 Analytical methods

4.2.9.1 Serum sample preparation

Serum samples were prepared for total iron, ferritin and soluble transferrin receptor analysis. Following collection into trace element and EDTA free tubes (BD Vacutainer, UK) blood samples were allowed to stand at room temperature for a period of 30-45 minutes prior to centrifugation at 500g. The serum (approx. 2ml supernatant) was removed and aliquoted into pre labelled screw-top micro tubes (Sarstedt, DE, product code: 72.694.006) and stored at -80ºC prior to analysis.

4.2.9.2 Serum sample analysis

Total serum iron

Total serum iron was measured in duplicate using the Quantichrom^{TM} (BioAssay Systems, USA) iron assay kit, a colorimetric assay which utilizes a chromogen that forms a blue coloured complex with iron (II). Iron (III) is reduced during the incubation of the assay. Briefly, Fe (III) is converted to Fe (II) using AA. Then, Fe (II) forms a colored complex with TPTZ (2,4,6-tripyridyl-s-triazine). The change in absorbance is directly proportional to the concentration of Fe in the sample, therefore the intensity of the colour at 590nm is proportional to the total iron content of the analysed sample.

Serum ferritin receptor analysis

Ferritin measurements were performed using a Spectro ferritin enzyme immunoassay (Cat. No. S-22), Ramco, USA following the assay procedure provided by the supplier (described in detail in Chapter 2). Duplicate measurements of ferritin resulted in average %CV 10%.

Soluble transferrin receptor analysis

Soluble transferrin receptor (sTfR) concentration in plasma samples was performed in duplicate using T*f*R enzyme immunoassay (Cat. No. TFC-94, Ramco, USA), following the assay procedure provided by the supplier. Briefly, samples and controls were diluted with sample diluent (1/100) then 50µl of samples, standards, controls and WHO Reference reagent (Recombinant rsTfR, 303nmol/L; NIBSC code:07/202) were loaded into pre-coated (with polyclonal antibody to sTfR) micro wells of 96 well plates, then 150µl of horseradish peroxidase (HRP) conjugated murine monoclonal antibody (specific for sTfR) was added to the wells and the plates were incubated for 2 hours at room temperature. During the 2 hour incubation, the sTfR binds to the polyclonal antibodies adsorbed to the wells and the HRP-conjugated second antibodies bind to the sTfR adsorbed to the wells. After 2 hours the content of the wells was removed (removing any unbound sTfR and HRP conjugate), plates were washed three times with washing buffer and 200µl of enzyme substrate (chromogen tetramethylbenzidine) was added to the wells and incubated for 30min. A blue product is formed through the action of HRP enzyme substrate. In order to stop the colour reaction 50µl of an acid stop solution (2.5M sulphuric acid) was added converting the product from a blue to a yellow colour. Yellow colour intensity was measured in a plate reader set at 450nm.

In addition to controls provided in the TfR kit WHO Reference reagent was reconstituted (according to the supplier procedure) and run with each plate in duplicate and at 2 different concentrations. The values obtained were transformed with the conversion equation published by WHO in 2009[238] with a mean value of 306.7mol/L (11 measurements in total, ±SD 45.5). Average %CV for serum sTfR was 4%.

C-reactive protein (CRP)

CRP concentration was performed on each experimental day in all baseline samples in order to establish whether the iron concentration might be elevated due to

inflammation/infection. CRP measurements were performed at an accredited pathology laboratory at the Pathology Department of the NNUH, Norwich, UK. According to the NNUH the normal CRP range is defined as 0-10mg/L however for the purpose of this work if values of CRP were above 6mg/L, samples were excluded from the analysis in case there was any low grade inflammation that might reduce iron absorption and elevate serum ferritin concentrations.

Visual examination of serum samples revealed that 8.8% (52 out of the total of 588 of all collected samples) were haemolysed. In order to assure high quality of the analysis and to eliminate the possibility that haemolysed samples would be included in the analysis (providing unreliable results due to haemoglobin interfering with the Quantichrom iron assay) the optical densities of all serum samples were compared at the following haemoglobin peak wavelengths 415; 417; 540; 570; 575nm[239,240] with a standard serum (normal mixed pool) sample obtained from TCS Biosciences Ltd, UK, product code:CR100.

In addition, any interference of haemoglobin with the Quantichrom assay was assessed by constructing a ten point standard curve ranging from 0.03125 to 16.0mg/ml with the use of haemoglobin from human red blood cells (product code: 338-10, Lee Biosolutions, USA), reconstituted in phosphate buffered saline according to the manufacturer's instructions. A separate analysis was also conducted in which haemoglobin was reconstituted in standard serum.

4.2.10 Statistics

Statistical power

The initial assumption made for the power calculation in order to estimate the number of required study participants was that a difference of 2.5% in iron absorption (primary outcome measure) between the 2 tests (test meal 1 and test meal 2) would be nutritionally significant.

Since absorption is related to iron stores it would be expected that there would be a good correlation between iron absorption from the iron loaded alginate beads and FeG in its native form i.e. volunteers who absorb higher amounts of one form will also absorb higher amounts of the other form. With each volunteer acting as his/her own control, this reduced the number of subjects required. Consequently, a minimum of 12 volunteers was required to complete the study to detect a difference of 2.5% at a significance level of 0.05 for 80% power.

For the secondary objective, a minimum of 8 volunteers was required to detect a significant difference in iron absorption of 3.3% between a test meal containing 20mg of iron as FeG with calcium, and a test meal containing 20mg of iron as FeG with calcium and alginate beads (test meals 4 and 3). This assumes a power of 80%, a level of significance of 0.05 and standard deviation of differences (within pairs) of 2.8%

Statistical analysis

The primary outcome measure for the study was percentage iron absorption. Estimating the difference in iron (FeG) absorption when consumed in a form of iron incorporated into alginate beads compared with the same amount of iron in an unprotected form i.e. not in alginate beads was the main objective of the study. The secondary objective of the study was to establish the difference in iron (FeG) absorption when consumed in the presence of calcium in a form of iron incorporated into alginate beads compared with the same amount of iron not in alginate beads, also in the presence of calcium.

Paired, two-tailed Student's t test was used to assess any significant differences in iron absorption. This method was found to be appropriate when assessing iron absorption in previous studies conducted at the IFR[231]. In addition, a mixed effects model was used for Group A to determine whether there was an effect of treatment on iron absorption. Pairwise comparisons (with multiple test correction) were then carried out using contrasts. Repeated measures ANOVA analysis was carried out in R version 3.0.

Data analysis

Dainty *et al.*[231] have previously shown that the measurement of serum iron concentrations for a period of 6 hours following an oral iron dose is a simple and effective method for estimating iron absorption which correlates well with stable isotope techniques for measuring iron absorption. From the serum iron appearance, it is possible to calculate percentage iron absorption from the administered dose using the following method:

Given a time of between 3 and 6 hours post administration of an oral dose ($dose_{oral}$) the rate of infusion R can be calculated from the serum iron concentration

(1) $R = \frac{mass\ of\ dose\ that\ is\ absorbed}{time\ period\ for\ absorption} = \frac{M}{T}$

Given a volume of distribution V, and a rate constant of elimination from the compartment k, the concentration in the compartment can be approximated to:

(2) $C = \frac{M}{V.T.k} \times (1 - e^{-kt})$ (0<t<T)

(3) $C = \frac{M}{V.T.k} \times \left(1 - e^{-kT}\right).e^{-k(t-T)}$ (t<T)

By fitting the equations to the concentration data, M, T and k can be calculated.

The fractional absorption from the oral dose is then:

(4) $Fractional\ absorption = \frac{M}{dose\ oral}$

This study was single-blinded (the volunteers were blinded as to the treatment). In order to ensure that the analysis was blinded, all sample tubes were relabelled by someone unrelated to the study prior to the analysis. Once all the samples were analysed and the data summarised by the statistician; the code was broken.

4.3 Results

Most of participants who took part in the study were young adult females aged 20-30y, with the exception of one male participant who was aged 49y at the time of the intervention. Table 4.3.1 provides details of the characteristics of the study population.

There was no relationship between body iron values, ferritin values or sTfR and % of iron dose absorbed on study day 1 or 2. This is most likely due to the small sample size and the fact that the ferritin range was relatively narrow.

One participant had a raised CRP concentration of 23mg/L suggesting the presence of infection/inflammation which could potentially interfere with iron absorption,

therefore the whole set of data for this one study day was excluded from the final analysis.

Table 4.3.1 Characteristics of population studied (n=15; 14 females, 1 male). Data are means ±SD.

	Screening	Study day 1 (beads+ Fe)	Study day 2 (Fe)	Study day 3 (beads+ Fe+Ca)	Study day 4 (Fe+Ca)
Age (years)					
A (n=9)	24.9±9.5	---	----	---	---
B (n=15)	23.6±7.5	---	----	---	---
BMI					
A (n=9)	22.2±2.0	---	----	---	---
B (n=15)	22.1±1.8	---	----	---	---
Ferritin* (µg/L)					
A (n=9)	28.8±10.3	22.7±9.9	18.7±9.2	22.3±16.2	24.0 ±17.9$^{\Delta}$
B (n=15)	27.1±11.7	24.6±10.2	19.8±9.3	---	---
sTfR** (mg/L)					
A (n=9)	---	6.1±2.8	6.3±2.7	6.2±2.2	6.3±1.7$^{\Delta}$
B (n=15)	---	5.6±2.5	5.9±1.9	---	---
BI (mg/kg body)					
A (n=9)	---	3.5±2.4	2.4±2.2	2.5±3.1	2.6±3.4$^{\Delta}$
B (n=15)	---	3.5±2.4	2.5±2.3	---	---
CRP (mg/L)					
A (n=9)	---	2.0±1.7	1.9±1.5	2.1±1.3	2.3±1.8$^{\Delta}$
B (n=15)	---	1.7±1.3	1.7±1.4	---	---

*Mean ferritin CV between duplicates on the sample plates was 8.2%. Plate to plate inter-assay CV was 17.4 %. The high CV difference is most likely due to the fact that most samples were at the lower end of the standard curve hence the lower sensitivity of the assay.

** Average sTfR CV between the duplicates on the same plate was 4.3%. WHO reference reagent was run on each plate (used at 2 different concentrations: 30µg/L and 90.21µg/L run on 2 plates) and its value of 319.5nmol/L; ±SD 28 was calculated using the following WHO conversion[241]: mean of 2 readings x dilution x 3.8685+1.3965

$^{\Delta}$(n=8) one data point from one participant removed due to high CRP

Analysis of serum samples for potential contamination by haem iron from haemolysed red blood cells revealed that haemoglobin in PBS adversely affected the Quantichrom total iron assay at the lowest concentrations tested (0.03mg/ml). Furthermore, haemoglobin dissolved in the standard serum sample (at constant serum volume) started to interfere with the Quantichrom assay readings at a concentration of 0.125mg/ml, giving markedly increased readings at a concentration of 1mg/ml. From the above results the percentage of haemolysed samples estimated visually to be 8.8% increased slightly to 9.5%. All of these samples were excluded from the subsequent modelling.

Absorption of FeG (Day 2) ranged from 5.7% to 17.9%, (Table 4.3.2), with a mean value of 12.6% (n=13), and this was significantly higher (p=0.0025) than absorption from FeG incorporated into alginate beads (Day 1), which ranged from 4.6% to 14.6%, with a mean value of 8.5% (n=13).

The dose of calcium (600mg calcium as calcium phosphate) administered with either FeG (Day 4; n=5) or FeG in alginate beads (Day 3; n=5) significantly reduced the % of iron absorbed (from 11.4% to 5.9%, p<0.001 and from 8.4% to 5.0%, p=0.0213, respectively) compared to the administration of iron alone. In addition, the lower absorption of iron when incorporated into alginate beads in the presence of calcium was also significant compared with iron incorporated into alginate beads with no additional calcium (p=0.023).

There was no significant difference in iron absorption between FeG and iron-containing alginate beads when given with calcium. Therefore, at the doses given, alginate did not modify the inhibitory effect of calcium on iron absorption. There was also no effect of time on serum ferritin and soluble transferrin receptor concentrations.

Table 4.3.2 Absorption of FeG (% of dose absorbed) with and without alginate

	% of iron dose absorbed			
	Day 1	Day 2	Day 3	Day 4
Recoded Volunteer number	Fe in alginate beads	Fe	Fe in alginate beads +Ca	Fe+Ca
ALG1	5.4	14.2	---	---
ALG2	8.2	15.7	---	---
ALG3	12.7	16.1	---	---
ALG4	6.3	13.1	---	---
ALG5	6.6	15.7	---	---
ALG6	---□	16	---	---
ALG7	6.3	11.9	3.5	4.9
ALG8	5.7	7.5	5.4	---*
ALG9	8.4	8.7	3.2	5.3
ALG10	---□	5.7	---□	5.6
ALG11	10.5	6.3	6.1	---□
ALG12	8.1	8.8	4.8	6.3
ALG13	4.6	11.4	4.4	6.3
ALG14	14.6	16.4	9.3	6.6
ALG15	13.0	17.9	---□	4.2
Mean % of dose absorbed for complete pairs	$8.5^{\lozenge}$(n=13) 8.4^{a}(n=5)	12.6^{Δ}(n=13) 11.4^{b}(n=5)	5.0^{c}(n=5)	$5.9^{a, c}$(n=5)
±SD (%) (n=13) ±SD (%) (n=5)	3.2 (n=13) 3.8 (n=5)	4.1(n=13) 3.1 (n=5)	2.5 (n=5)	0.7 (n=5)

$^{\Delta; \lozenge}$Different symbols denote statistically significant differences between day 1 and day 2 test meals. Different letters denote statistically significant differences between day iron treatments Differences were considered to be significant at $p<0.05$. *Data point removed due to high CRP. □Sub-standard, not modelled data

Due to the constraints associated with the bead preparation outlined in Section 4.2.6, their iron content varied from 0.9 to 1.27mg iron per gram wet bead and the content of calcium varied from 1.79 to 2.29mg of calcium per gram wet bead. On average each participant consumed 21.8g (±SD 0.14g) of beads containing on average 21.6mg (±SD 2.1mg) of iron and 42.82mg (±SD 2.71mg) of calcium chloride on test days involving consumption of alginate beads. Test meals involving the consumption of an iron capsule contained an amount of iron that was equivalent to the dose provided in the beads. It did not however contain the quantity of calcium that was present in the beads. The absence of calcium phosphate on test days not involving beads was intentional; otherwise it would not have been possible to investigate the potential protective effect of the beads.

4.4 Discussion

There were two main findings from this human study. Firstly, alginate beads significantly reduced iron absorption from FeG. Secondly, the beads did not protect the iron from the inhibitory effect of calcium (at a calcium dose of 600mg).

The observed lack of correlation between body iron, ferritin or sTfR and % of iron dose absorbed may be due to the fact that participants recruited onto the study had relatively low iron stores (in order to maximise iron absorption from the test meal) thus the sample did not include individuals with ferritin above 60ng/ml. In addition the sample size was low (n=15).

One of the potential reasons for the beads not demonstrating an enhancing effect on iron absorption (as observed in the Caco-2 cell model system[242]) may have been the presence of high levels of calcium in the beads, which was an essential part of the production process to maintain their integrity and shape[217]. Another possibility is that alginates, (as reported in earlier Chapter 1, Section 1.5) have the potential to bind divalent and trivalent cations[210,209], and are able to bind iron ions in the human gut, thus limiting iron absorption. Research supporting the latter hypothesis was reported by Sandberg *et al.*[222]. In their study 6 ileostomy participants were subjected to a diet low in fibre, with or without 7.5g sodium alginate (M to G acid ratio: 60:40), dissolved in a milk shake and also given as a jam. Mineral absorption was calculated from the difference between dietary intake and the quantity excreted in ileostomy fluid. There was a reported decrease in apparent absorption of iron and manganese in 5 out of 6 volunteers. However, as the authors point out, the sample size was too small to determine if the effect was statistically significant. Comparison of our study in

healthy subjects with participants from the ileostomy trial may not be appropriate, not only due to physiological differences but also due to differences in the design of the two studies, alginates used (different M:G ratios), test meals consumed, and analyses performed. In addition, considerably less alginate was used in our iron absorption study (0.5% alginate solution; approximately 0.58g ±SD 0.11g of alginate powder per person per test meal) than in the study involving ileostomy participants (7.5g of alginate per volunteer per test meal).

In contrast, Bosher *et al.*[243] reported that the availability of iron and zinc from infant formulas increased with the addition of 2g of alginic acid when investigated with the use of an *in vitro* dialysis model with a preliminary intraluminal digestive phase. These observations support the *in vitro* findings reported earlier in this thesis (Chapter 3).

Differences between the *in vivo* and *in vitro* studies reported are as follows: the *in vitro* study did not include a test meal consisting of cola-flavoured jelly (described in Section 4.2.7 of this Chapter), the bead preparation was slightly different, and, possibly the most important difference was that a rinsing step was introduced to make the test meal (in the human study) more palatable, which removed any unbound iron from the surface of the beads. All of the above discrepancies between the *in vitro* and *in vivo* studies have been addressed in the final set of *in vitro* experiments conducted after completion of the human study (see Chapter 5)

As described in an earlier Chapter 1 alginates are natural polysaccharides occurring in brown seaweed which form gels with ions[244] producing ‘egg-box’-like structures. In industrial use the most common gelling ion is calcium[245,246] and to a large extent gel rigidity depends on the M:G ratio (in particular guluronic acid residues) and stoichiometry of alginate[245]. Alginate beads used in this human study were prepared by soaking in a calcium chloride (0.1M) and FeG (0.1M) bath (50/50) and the final content of calcium per gram of wet bead was roughly 2 fold higher than that of iron, thus illustrating a higher binding affinity of alginate for calcium than for iron. As reported by Perez-Moral *et al.*[217] 57% of iron incorporated into alginate beads (prepared using the same technique as the beads in this study) was released during simulated digestion (43% during gastric and 12% during duodenal phase of digestion) leaving the remaining 47% of iron within the beads. It is therefore possible that due to the high guluronic acid content of the alginate used, which results in the firm structure of the beads when bound with calcium[247], and due to the indigestibility of alginate in the small intestine[248,249], iron remains trapped within the beads

throughout the *in vivo* digestion process and is thus unavailable for absorption. The above hypothesis could very well explain the results reported within this study, however further research is required to confirm this.

Since alginates have a strong affinity to bind calcium[248] we wanted to test if alginate beads would bind calcium consumed with the test meal and thereby ameliorate its inhibitory effect on the absorption of iron. The form and dose of calcium selected from a previous publication[236] was predicted to reduce iron absorption from FeG. The Inhibitory effect of calcium phosphate was significant with and without the addition of alginate to the test meal. Thus it was concluded that alginate beads did not diminish the inhibitory effect of calcium on iron absorption. One potential explanation could be the fact that the beads, during their production process, were soaked in a calcium and iron bath for approximately 20 hours which could have caused saturation with calcium leaving no further capacity for binding with the additional calcium administered in the test meal. This hypothesis warrants further investigation.

The human study had the following limitations: The study scientist was not blinded when administering the test meals (due to the requirements for alginate bead preparation and analysis, as described earlier). However, in order to minimise bias, serum samples were relabelled prior the analysis, by a third party not involved in the study. The study would have benefitted from more detailed observation of other potentially confounding factors which could have influenced iron absorption, such as plasma hepcidin concentration. Additionally, it would have been preferable if subsequent test days for all participants were at the same, fixed time intervals, but this was not feasible. Also, to further increase sensitivity of the total serum iron assay future studies may benefit from using a slightly higher dose of iron.

One of the limitations of measuring changes in serum iron concentration in conjunction with compartmental modelling is diurnal differences in serum iron concentration. However our study days commenced at approximately the same time of the day (±30min) thus minimising the diurnal effect.

This study has several strengths. The crossover design of the study permitted each subject to act as his/her own control and enabled us to use a smaller sample size. The self-selected pre-test diet ensured high compliance, maintained the habitual diet, and removed any effect of pre-test meal diet on efficiency of iron absorption. Furthermore, the number of blood samples collected on the test days resulted in good quality data (frequent data points) and allowed us to use single compartment

mathematical modelling[232] to estimate % absorption of the iron dose. The results clearly demonstrate that iron absorption from FeG, administered to participants in capsules, was significantly higher than FeG incorporated into alginate beads. Alginate beads did not reduce the inhibitory effect of calcium on iron absorption, as observed in earlier *in vitro* Caco-2 cell studies.

5. HUMAN STUDY FOLLOW UP- *IN VITRO* INVESTIGATIONS

5.1 Rationale for undertaking further *in vitro* investigations.

As described in Chapter 1, Section 1.4 the use of Caco-2 cells in combination with a simulated digestion procedure has been shown to be a valuable tool for screening different iron sources in order to rank them for potential bioavailability of iron and to advance our understanding of intestinal absorption and metabolism[192,193,197,227,250–262]. The findings of the *in vivo* study (Chapter 4) did not support those of the *in vitro* experiments (Chapter 3) in that the iron absorption results were different from what was predicted. Consequently, it was decided to conduct follow-up experiments using the Caco-2 cell model to try to find out the reason(s) for the differences.

One important factor that warranted further investigation was the discrepancy between test meals administered to participants in the human study and treatments which were applied to the cells. The differences were that the beads used for the *in vitro* treatments were not washed with water as part of the production process and were not accompanied by cola jelly given as part of the test meal.

Also, as specified in Chapter 3, small quantities of beads (mostly between 0.002 to 0.095g) had to be weighed out for the simulated digestion because of the relatively high iron content of the beads. Thus any small weighing errors could have contributed to variations in response between replicates. The beads had to be suspended in a final volume of 15ml and then 10% of this suspension used to treat the cells. In order to investigate the possibility of errors in sample weighing and application onto the cells, beads B13-B17 (described below in methods Section) were prepared in modified conditions to ensure lower iron loading per gram of wet beads and by this means, the weight of beads required for each treatment was greater and therefore weighing errors were less important.

The hypothesis tested in this chapter was that when washed and mixed in with cola jelly the iron-containing alginate beads would deliver less iron than FeG on its own, thus confirming the findings of the *in vivo* study.

The aims of the experiments described in this Chapter were to investigate whether:

- the cola jelly present in the test meal and washing step in the bead production could affect iron uptake by Caco-2 cells;
- FeG is a more bioavailable form of iron than FAC when incorporated into alginate beads (confirmation of previous findings);

- the use of higher weights of beads would reduce potential weighing errors occurring when lower quantities of beads were used; thus reducing the variability of results obtained (Chapter 3);
- alginate beads protected iron during the simulated digestion process, thus delivering more bioavailable iron than unprotected iron for the Caco-2 cell model;
- the enhancing effect of AA on iron absorption was unaffected by the presence of alginate beads;
- the calcium present within the beads played a role in decreasing iron absorption *in vivo* compared with the administration of unprotected iron

5.2 Methods

5.2.1 Cell culture and experimental procedures

Caco-2 cells were cultured as described earlier in Chapter 2 and for experimental purposes they were combined with the simulated digestion procedure described in Chapter 2, Section 2.2. However, the following modification to the simulated digestion methodology was introduced in some of the experiments described below: the duodenal phase of digestion was continued for 2 hours in test tubes (rather than over the cells) at 37ºC on a rotating table and once completed all samples were centrifuged at 3184g for 10min in order to remove any potential jelly residue, which could have resulted in physical blockage of the dialysis membrane. Once centrifugation was completed, 10% of the resulting supernatant from each treatment was applied onto the dialysis membranes and placed over appropriate wells. Cells were incubated with the supernatant for an hour at 37ºC on a rotating table at a speed of 20 oscillations per minute.

5.2.2 Preparation of jelly treatments

Cola-flavoured jelly was prepared in exactly the same way as in the human study, as reported in Chapter 4, Section 4.2.7. The amount of cola jelly used in each *in vitro* treatment was calculated based on the ratio of beads to jelly used in the human study, which was 1:6.77 grams of beads to grams of jelly.

5.2.3 Preparation of iron containing alginate beads

5.2.3.1 Beads prepared in altered conditions

Beads B13-B16 were prepared under different conditions to those of the beads previously used in *in vitro* or *in vivo* experiments in order to assure lower loading with iron. To achieve this, the calcium and iron bath in which the beads were loaded contained 0.1M calcium chloride (Fluka, UK) and iron at following concentrations: 0.1M FeG; 0.015M FeG; 0.01M FeG and 0.005M FeG resulting in the production of beads B13, B14, B15, B16 respectively. Beads B17 were prepared with the use of FAC instead of FeG at the concentration of 0.01M. The above conditions resulted in a range of iron content per gram of wet beads, for details see Table 5.2.1

5.2.3.2 Conditions for preparation of beads B18

Alginate beads (Table 5.2.1) used in experiments presented in Figure 5.3.2; Figure 5.3.3 and Figure 5.3.4 were prepared using exactly the same parameters (dissolving time, nozzle size, flow speed, loading time and rinsing step) as those made for the human study, and using the same batch of alginate powder, calcium chloride solution and FeG powder. However, the beads produced for these *in vitro* experiments contained 1.712mgFe/g and 1.618mg Ca/g wet bead. Whereas 1g of wet alginate beads prepared for the human study contained 1.04mgFe/g wet bead (±SD 0.08) and 1.96mgCa/g wet bead (±SD 0.12). For more details please refer to Chapter 4.

The final concentration of iron per well was 102.3µM (once accounted for dilution. In order to obtain the required amount of iron per well, an appropriate amount of iron-containing alginate beads (to obtain a concentration of 238.76µM of elemental iron) was weighed into relevant digest tubes.

FeG (VWR International LTD), UK, from the same batch as the one used in human study) solution was prepared at a concentration of 0.00716mol/L in 0.1M HCl, and an appropriate volume was used to obtain a concentration of 238.76µM of elemental iron (in order to match the same concentration of iron which was incorporated into the alginate beads) in a 15ml total volume of digest. One tenth of each digest solution was used to treat each well containing cells, resulting in a final concentration of 102.3µM per well.

Table 5.2.1 Composition of iron containing beads (B13-B18).

Iron loaded material	Iron compound added	Weight of alginate beads used (*g)*	Iron content per well (*µg)*
B13	FeG	0.12	15
B14	FeG	0.39	15
B15	FeG	0.53	15
B16	FeG	0.72	15
B17	FAC	0.46	15
B18	FeG	0.117	20

5.2.4 Preparation of experimental solutions and treatments for beads (to replicate the test meals used in the human study)

In order to replicate the calcium content of the test meals in the human study, calcium phosphate (Freeda Vitamins, USA), from the same batch as the one used in human study) was applied to the cells. The molar ratio of FeG and calcium phosphate was calculated based on the average values used in the human study: on average 21.8g of wet beads were administered to each participant with approximately 21.6mg iron served together with 150g cola jelly and, where appropriate, 600mg of calcium phosphate were added to the test meals. Therefore, the FeG to Ca phosphate molar ratio used in the human study was 1 : 11.813. Furthermore, the calcium chloride content of the beads was also quantified using atomic absorption spectroscopy (described in Chapter 2, Section 2.4) in order to add the same amount of calcium chloride (Sigma, UK) to the appropriate FeG positive controls.

5.2.5 Preparation of experimental solutions and treatments for beads B13-B17

Iron solutions were prepared and treatments administered in exactly the same way as described in the above Section, the only exception being that the final concentration of FeG or FAC applied to the cells was 76.9µM per well.

AA was dissolved in MilliQ water and the final concentration was 1:10 Fe:AA molar ratio per well.

5.2.6 Optimisation of simulated digestion procedure

In order to eliminate the possibility of dialysis membrane blockage by the jelly, two experiments were performed with the duodenal phase conducted in test tubes, followed by sample (digestate) centrifugation at 3,184g for 10 minutes prior to application of the supernatant onto the dialysis membrane (Figure 5.2.1.) Caco-2 cell monolayers were incubated with the supernatants from the digestate for one hour at the same conditions as described in Chapter 2, Section 2.2. Further digestion procedure steps together with cell harvesting procedures, sonication and analysis were also performed as in the standard method described in Chapter 2, Section 2.2.3.

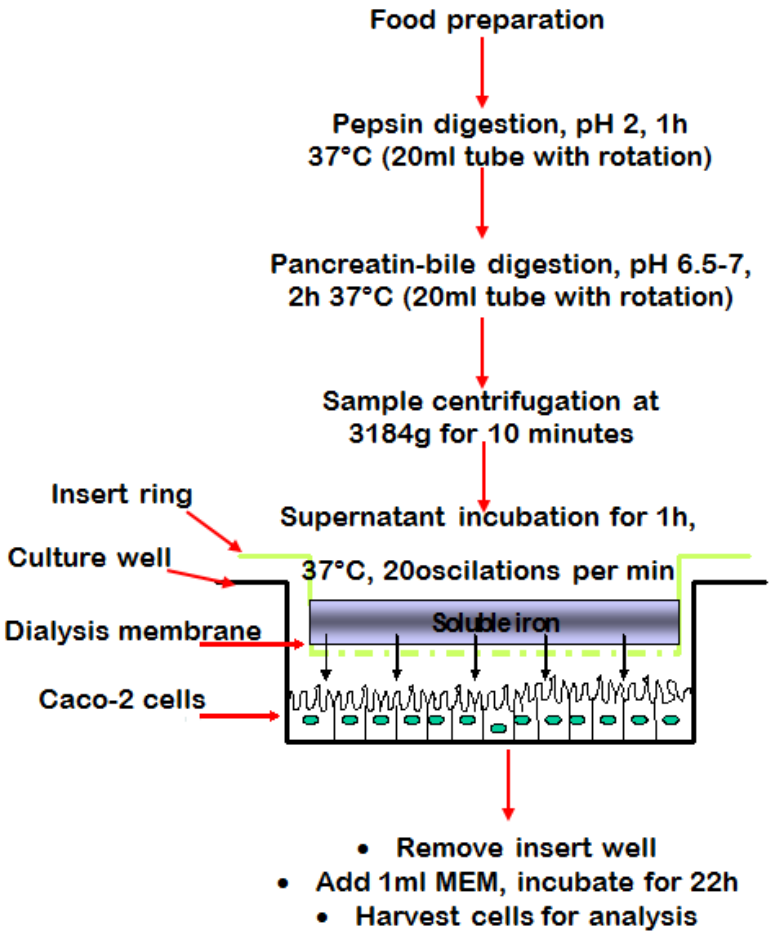

Figure 5.2.1 Schematic diagram of *in vitro* digestion, a Caco-2 cell model adopted from Glahn *et al.*[192].

5.2.7 Statistical analysis

Unless otherwise stated, all statistical analyses were performed using SPSS Inc, USA, (version 16.0.0). 2-factor ANOVA with Tukey HSD post-hoc tests were conducted to examine pairwise differences on power-transformed data. Data are presented as mean ±SD. Differences were considered significant at $p<0.05$.

5.3 Results

5.3.1 Beads B13-B17 with and without ascorbic acid.

Beads B13-B16 (detailed specification in Table 5.2.1) delivered available iron to the cells at a similar level to unprotected FeG (Figure 5.3.1). The only exception were beads B17 which contained FAC; these delivered significantly less available iron to the cells than unprotected FeG (p <0.0005) or any of the other beads. When cells were treated with any of the beads (B13-B17) they produced significantly less ferritin than when they were treated with FeG in the presence of AA (p<0.0005 in all instances). Cells treated with iron-containing alginate beads in the presence of AA (at 1:10 iron to AA ratio) had significantly higher ferritin concentrations than cells treated with iron- containing alginate beads only (p<0.0005 in all instances). The enhancing effect of AA was maintained in the presence of iron-containing alginate beads and was as potent as with unprotected FeG with the exception of beads B16 (where the highest weight of beads was used) and beads B17 (where FAC was used instead of FeG), which resulted in a significantly lower cell ferritin response compared to FeG in the presence of AA (p=0.03 and p=0.02 respectively).

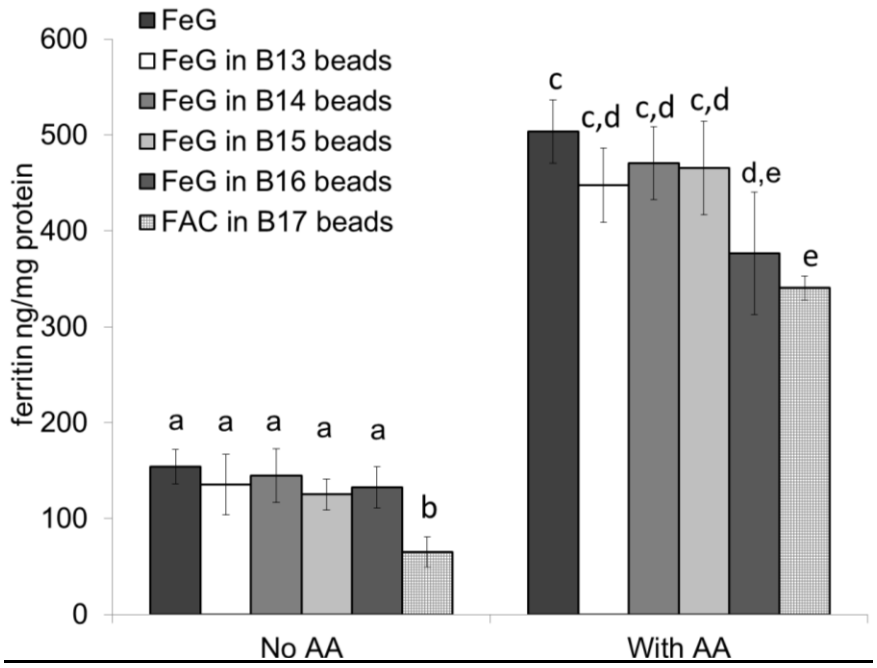

Figure 5.3.1 Ferritin concentration in Caco-2 cells exposed to unprotected FeG or in alginate beads (B13-B16) or FAC containing beads (B17) with or without AA at 1:10 Fe:AA molar ratio. Ferritin concentration (ng/mg total protein) after exposure to 107.7µmol/L FeG across all treatments. Data represent mean ±SD. Bars without a common letter (a, b, c, d, e) are significantly different, $p<0.05$. Ferritin concentration in cells not exposed to any iron (no treatment) was 4.0±SD 2.4.

5.3.2 Iron uptake from iron-containing alginate beads in the presence of cola-flavoured jelly.

Also, to replicate the human study test meals 3 and 4, Caco-2 cells were treated with FeG together with cola-flavoured jelly and calcium phosphate or with FeG-containing alginate beads together with cola-flavoured jelly and calcium phosphate (Figure 5.3.2; Figure 5.3.3; Figure 5.3.4).

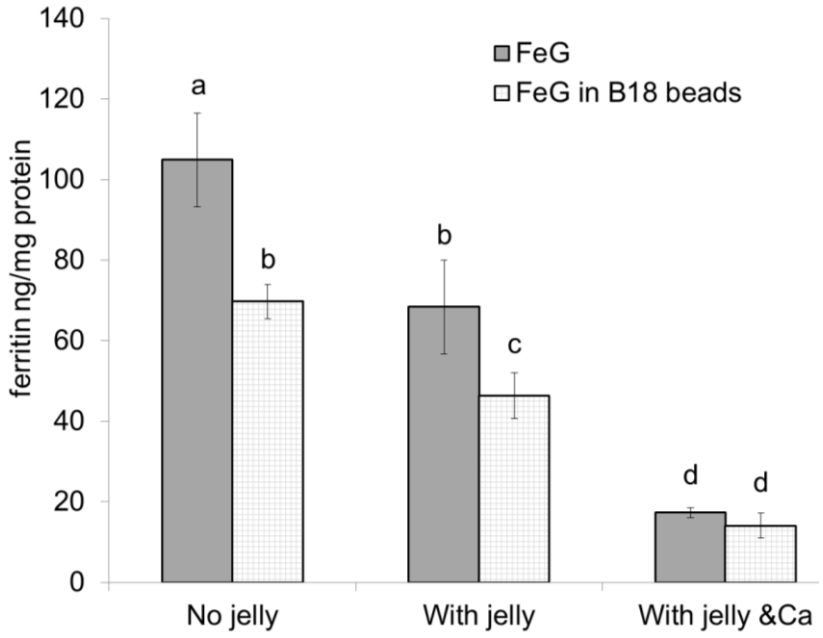

Figure 5.3.2. Ferritin concentration in Caco-2 cells exposed to FeG unprotected or in alginate beads with or without cola jelly test meal or with cola jelly test meal in presence of calcium phosphate. Ferritin concentration (ng/mg total protein) after treatment with 102.3µmol/L FeG (n=6). Data represent mean ±SD. Bars without a common letter (a, b, c, d) are significantly different, $p<0.05$. Ferritin concentration in cells not exposed to any iron (no treatment) was 7.6±SD 1.0, cells exposed to test meal only or beads not containing iron did not produce any ferritin.

Additionally, to investigate the hypothesis that calcium chloride present within the beads (calcium acquired during the production of the beads, for details see Chapter 3) was at a high enough level to inhibit iron uptake into the cells (which limits the production of ferritin), FeG in the presence of exactly the same amount of calcium chloride was applied to the cells with (Figure 5.3.4) and without the cola jelly test meal (Figure 5.3.3; Figure 5.3.4).

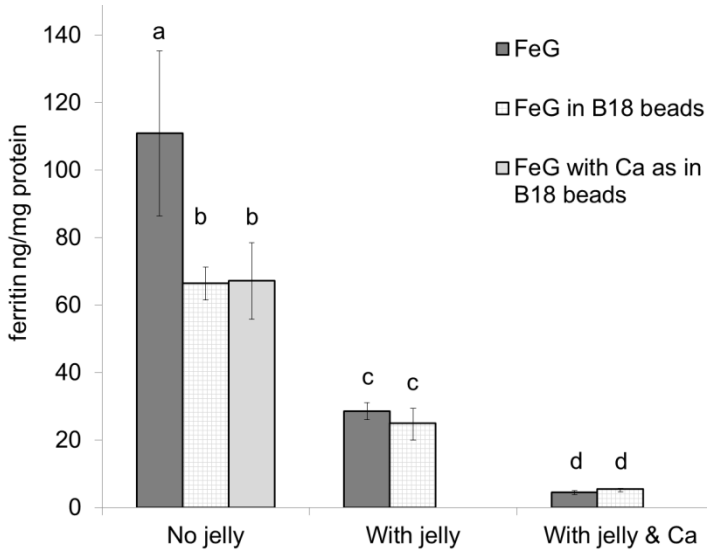

Figure 5.3.3. Ferritin concentration in Caco-2 cells exposed to centrifuged digests with FeG unprotected or in alginate beads with or without cola jelly test meal or with cola jelly test meal in presence of calcium phosphate. Ferritin concentration (ng/mg total protein) after treatment with 102.3µmol/L FeG (n=6). Data represent mean ±SD. Bars without a common letter (a, b, c, d) are significantly different, p<0.005. Ferritin concentration in cells not exposed to any iron (no treatment), cells exposed to test meal only or beads not containing iron was 3.8±SD 0.7; 6.8±SD 0.2 and 6.5±SD 0.2 respectively.

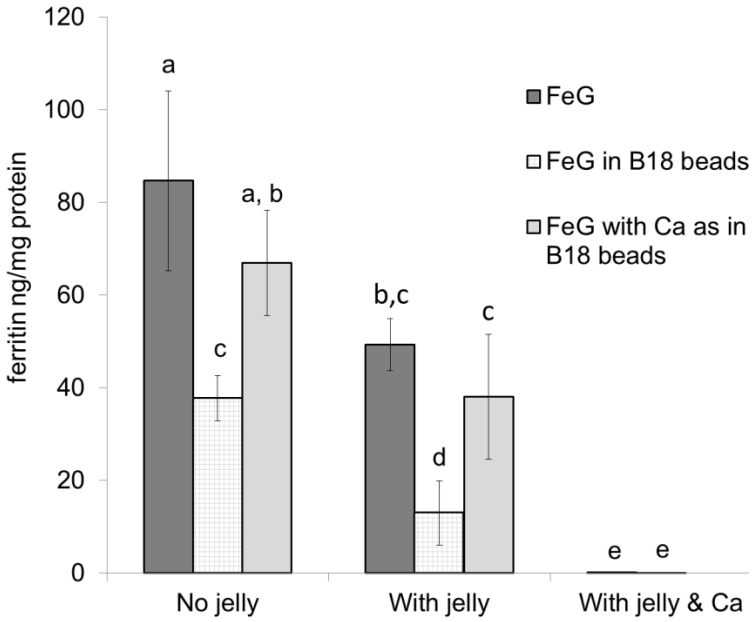

Figure 5.3.4 Ferritin concentration in Caco-2 cells exposed to centrifuged digests with FeG unprotected or in alginate beads with or without cola jelly test meal; with cola jelly test meal in presence of calcium phosphate or with FeG with calcium chloride at the concentration present in the beads. Ferritin concentration (ng/mg total protein) after treatment with 102.3µmol/L FeG (n=6). Data represent mean ±SD. Bars without a common letter (a, b, c, d, e) are significantly different, p<0.006. Cells which were not exposed to any iron (no treatment) did not produce any ferritin.

All of the above experiments have the following findings in common:

- FeG on its own triggered a significantly higher ferritin concentration in cells than iron-containing alginate beads (p=0.008; p<0.0005 and p<0.0005; presented in Figure 5.3.2; Figure 5.3.3; Figure 5.3.4 respectively);
- cells treated with FeG together with jelly had a significantly lower ferritin concentration than cells treated with FeG only (p=0.003; p<0.0005 and p=0.006; presented in Figure 5.3.2; Figure 5.3.3 and Figure 5.3.4 respectively);
- calcium phosphate provided to the cells with unprotected FeG or with FeG-containing alginate beads significantly reduced the amount of iron that was available to the cells compared with corresponding treatments without calcium phosphate (p<0.0005 in all instances) (similar to the results reported from the human study);
- there was no significant difference (results presented in Figure 5.3.2; Figure 5.3.3 and Figure 5.3.4) between ferritin concentrations in cells treated with FeG in the presence of calcium phosphate in the cola jelly test meal and iron-containing alginate beads in the presence of calcium phosphate in the cola jelly test meal (similar to the results reported from the human study).
- cells treated with FeG-containing alginate beads together with the cola jelly test meal had significantly lower ferritin concentrations than cells treated with FeG-containing alginate beads only (p=0.003; p<0.0005 and p<0.0005; presented in Figure 5.3.2; Figure 5.3.3 and Figure 5.3.4 respectively).

The results relating to the comparison of iron-containing alginate beads and FeG in the presence of calcium chloride (at the same concentration as in the beads) proved to be inconclusive. In both experiments there was a lower ferritin concentration in cells treated with FeG in the presence of calcium chloride (equal to the calcium chloride concentration in beads) when compared with cells treated with FeG. However, the reduction was statistically significant in only one of the 2 experiments performed; results presented in Figure 5.3.3 (p<0.0005) and Figure 5.3.4;

Similar inconsistencies were observed in relation to ferritin concentrations in cells treated with FeG together with cola jelly and iron-containing alginate beads together with cola jelly. The observed difference between the two treatments was either significant, with lower ferritin formation in cells treated with iron-containing alginate

beads in the presence of cola jelly (Figure 5.3.2 and Figure 5.3.4; p=0.009 and p<0.0005 respectively) or not significant (Figure 5.3.3).

5.4 Discussion

The use of iron containing alginate beads prepared using the technology described in Chapter 3 is a novel approach. No *in vitro* or *in vivo* studies were found in the literature apart from Wawer *et. al.*[242] report which was based on Chapter 3 of this thesis.

Results obtained with beads B13-B18 reported in this Chapter consistently demonstrate that alginate beads do not exhibit the expected protective/enhancing effect of iron, similar to that conferred by AA, as reported earlier[242]. There are two potential explanations for this observation:

- increasing the amount of beads used in this series of experiments reduced the potential error in weighing of the sample and its application onto the cells, however at the same time it inevitably increased the concentration of calcium which could have inhibited iron uptake by the cells[19].
- not all the iron present within the beads was being released during the simulated digestion process, as reported by Perez-Moral *et al.*[217], hence there was a lower quantity of iron available to be taken up into cells.

Alginate beads (B18) used for the cell experiments were prepared in an identical manner to those administered in the human study. However, despite this, there were differences in both the calcium and iron loading of the beads prepared for the *in vivo* and *in vitro* experiments. Beads used for the human study contained on average: 1.96mgCa/g wet bead (±SD 0.12) and 1.04mgFe/g wet bead (±SD 0.08), whereas beads B18 used for the above cell experiments contained 1.618mg Ca/g wet bead and 1.712mg Fe/g wet bead. The discrepancies in the iron and calcium content of the beads, despite the use of the same protocol and reagents, illustrate limitations in the preparation technique used for iron containing alginate beads which should be addressed in future work.

When FeG and washed FeG-containing alginate beads B18 were presented to the cells, there was a significantly lower cell ferritin response from beads B18 than from

unprotected FeG. Furthermore, the cell ferritin concentrations were significantly lower when the iron treatments were carried out in the presence of the cola jelly test meal, suggesting an interaction between the cola jelly and iron. When washed beads B18 and FeG accompanied by the jelly test meal were compared, their effect on cell ferritin formation was inconsistent.

Centrifugation of the digests prior to application onto the dialysis membrane placed above the cells did not reduce the apparently inhibitory effect of the cola jelly indicating that the pores in the dialysis membrane were not blocked by the jelly. Additionally, the effect of the jelly may be more pronounced in the *in vitro* cell model than in the human study, where a small amount of jelly enters the relatively large volume of the stomach. However, the possibility of interaction between cola jelly and iron occurring *in vivo* cannot be ruled out. Collings *et. al.*[237] reported that cola beverage had no effect on iron absorption *in vivo*, suggesting that the inhibitory effect of cola jelly observed in experiments reported in this Chapter may be due to gelatine jelly. However research carried out in rodents suggest that gelatine can actually protect iron from the inhibitory effect of tea[263] which makes this an unlikely explanation. Due to limited time further investigations on the effect of the jelly on iron uptake by the cells could not be carried out.

The inhibitory effect of calcium phosphate was consistent across all experiments and agreed with the *in vivo* findings reported in Chapter 4 as well as with findings reported by others[236]. However, it was not possible to isolate the effects of calcium present in the beads from other potential confounders (such as the jelly or alginate itself).

The findings reported in this Chapter consistently demonstrate that when washed, FeG- containing alginate beads result in significantly lower ferritin concentrations in cells than unprotected FeG.

6. CALCIUM - IRON INTERACTIONS

6.1 Introduction

Work discussed in this Chapter is a result of a collaborative project undertaken in the lab of Professors Kevin Pedley and Jane Coad at the Institute of Food, Nutrition & Human Health at Massey University in New Zealand. The research was funded by Marie-Curie IRSES (International Research Staff Exchange Scheme) grant funding ("REINFORCE" FP7-PEOPLE-2010-IRSES) awarded to UEA.

Experiments using live cell imaging techniques were performed together with immunocytochemistry experiments investigating the localisation of DMT1 and tight junctions. However, the latter investigations are still on-going and therefore only live cell imaging data are presented here.

In contrast to other dietary inhibitors of iron absorption (described in Chapter 1, Section 1.2.1) calcium affects uptake of both non-haem and haem iron[264,54] which suggests that the effect is not just luminal (the site where modulators of iron absorption exert their effect).

Effects of calcium supplementation on iron absorption in humans has been the subject of research for decades[42,265,266].Seligman *et al.*[265] compared iron absorption from prenatal multivitamin-mineral supplements with equivalent amounts of iron administered as iron supplements only and reported that iron from the latter was significantly better absorbed than the former. Decreased absorption of iron from multivitamin-mineral supplements was attributed to the presence of calcium carbonate, magnesium oxide and in some instances to ineffective iron release.

Cook *et al.*[236] investigated the effect of different forms of calcium supplements (calcium carbonate, calcium citrate and calcium phosphate) at doses of 300mg and 600mg on ferrous sulphate absorption used at doses of 37mg or 18mg of iron respectively. The results demonstrated that when taken with no food matrix, higher doses of calcium phosphate or calcium citrate significantly inhibited iron absorption whereas calcium carbonate did not. Moreover, when taken with single meals all the above mentioned calcium forms significantly reduced iron absorption from supplements as well as dietary iron (present in the test meal). Similar findings were reported by Dawson-Hughes *et al.*[266] when 500mg of calcium carbonate or hydroxyapatite was administered together with a test meal containing ≈3.6mg of iron; thereby, highlighting the issue of potentially ineffective iron supplementation (or uptake from meals) with simultaneous administration of calcium supplements. On the contrary, Snedeker *et al.*[267] showed no effect of calcium gluconate and

phosphorus at three different supplementation levels on biomarkers of iron status. However, this lack of effect could be attributed to a small sample size and imprecise analytical methods.

In a study performed by Hallberg *et al.*[54] wheat rolls were supplemented with increasing calcium chloride concentrations (40 to 600mg) and the strongest inhibiting effect (50-60%) was reported at higher doses in the range 300-600mg. A similar effect was also present when the calcium chloride supplement was replaced with an equivalent dose of dietary calcium (≈165mg) which inhibited both haem and non-haem iron. Interestingly, addition of as little as 40mg of calcium to the dough (80g of flour) significantly decreased iron absorption. This observation was attributed to the protective effect of calcium on phytate degradation during the baking process, thus indirectly impacting on iron availability from the test meal. This hypothesis was confirmed later by addition of calcium to wheat rolls after the baking process was completed. Another study[268] reported an inhibiting effect of calcium carbonate (400mg per meal, total daily intake of 1200mg) on iron absorption from a reasonably rich iron diet (15mg/day). This inhibiting effect was not present at lower doses of calcium (<320mg/day) confirming previously reported findings where calcium chloride (178mg dose) did not significantly inhibit iron (4.1mg) absorption from a single meal[269]. Moreover, the long term effect of calcium (6 months, taken daily with meals at the dose of 1200mg of calcium per day) was also investigated by Minihane *et al*.[268] and no changes in iron status parameters, such as plasma ferritin, haemoglobin, zinc protoporphyrin and haematocrit were reported. These findings may suggest that some adaptive mechanism of iron absorption to prolonged calcium exposure may be present, or perhaps the study was not continued long enough to affect its endpoint (measures of iron status).

Reallocating the intake of high calcium products (such as cheese or milk) from lunch or dinner meals (which are most likely to contain higher percentage of iron) to breakfast and /or supper significantly increased total iron absorption (non-haem and haem) from reduced calcium meals[270] compared with calcium intake spread across all main meals during the day. These findings suggest that redistribution and separation of calcium and iron rich products into different meals during the day may be a way forward to ensure sufficient absorption of both these vital nutrients.

More recently, calcium chloride (200 to 1500mg) was given to participants together with 5mg of haem or non-haem iron with no test meal and an inhibiting effect was reported for calcium doses exceeding 800mg (non-haem and haem iron absorption

inhibited by ≈50% and ≈38% respectively)[271]. However this study has been criticised for assessing iron absorption on one occasion from a single meal, thus not accounting for day to day variations in iron absorption[272]. Nonetheless contradicting results of the effect of calcium from single meals or complete diets on iron absorption reported in the literature warrant further investigation[273,274].

In summary, based on the above literature review the inhibiting effect of calcium on relative iron absorption may be dependent on:

- dose of calcium and iron
- source of calcium and iron (dietary or supplemental),
- pattern of consumption (single meal-acute studies or studies over several months)
- distribution of calcium (and iron) across different meals/times in the day[275]

Caco-2 cell (as reported earlier in Chapter 1, Section 1.4) are commonly used as an *in vitro* model to study iron bioavailability and/or uptake from different iron compounds[227], foods[193,257] and their composition[18,19]. Overall, published reports suggest good agreement between *in vitro* findings (using this particular cell line) and *in vivo* research estimating iron bioavailability from food in the presence of iron enhancers and inhibitors[252,258] e.g. AA. In addition it has also been reported that calcium inhibits iron uptake in Caco-2 cell line[55,254] therefore this model can be used to investigate the mechanisms by which calcium inhibits iron uptake.

As previously discussed (Chapter 1) DMT1 is the only non-haem iron transporter identified to date. DMT1 is expressed by Caco-2 cells at levels allowing study of its properties and optimal conditions to transport iron (II). One of the most intriguing processes in the intestinal epithelium is the occurrence of the 'mucosal block' when treated with high, prolonged or frequently reoccurring iron doses. Recent literature reports of investigations in a murine model[276] suggest that this phenomena takes place at the apical side of the enterocyte by reduction in expression of Dcytb and DMT1(IRE+) mRNAs, thus decreasing iron uptake. Interestingly, in Caco-2 cells iron induced (24 hours incubation) translocation of DMT1 into the cytosol occurred prior to reduction of DMT1 mRNA levels[179,277]. It is also worth mentioning that Sharp *et al.*[179] demonstrated that total levels of DMT1 within the cells remained unaltered supporting the translocation hypothesis. In addition 72 hours of incubation were required to reduce DMT1 mRNA by 50%[179].

The hypothesis tested in this chapter was that the presence of calcium would reduce iron uptake into the cell thus confirming previously reports of an inhibitory effect of calcium on iron absorption at the apical side.

The aim of the research reported in this Chapter was to further the understanding of complex calcium-iron interactions. Cell imaging techniques using a confocal microscope were used in conjunction with the Caco-2 cell line model as an investigation tool.

6.2 Methods

6.2.1 Cell culture procedures

Cell culture procedures (harvesting and seeding) were identical to the ones reported in Chapter 2 with the exception of media composition. For experiments reported in this Chapter DMEM media was supplemented with 10% fetal bovine serum, 4mM L-glutamine, 5ml of 5000u/ml Penicillin/Streptomycillin solution (Gibco, UK) and 5ml of 100x non-essential amino acids (Sigma, UK). Cells used for these experiments were at passages: 30 to 35.

Cells were seeded onto flame sterilised and collagen coated round glass coverslips (25mm, Warner instruments, USA). Prior to seeding coverslips were washed in 0.1% Decon solution (Decon Labolatories, UK), washed 10 times in MilliQ water, immersed in 96% ethanol, flamed in sterile conditions and placed in 35mm petri dish. Collagen coating solution was prepared by mixing 0.02M acetic acid (Sigma, UK) and collagen from rat tail (Fisher, UK) to obtain a final concentration of 50µg/ml of collagen. Glass coverslips were then covered in collagen coating solution and left for 1 hour in sterile conditions. After 1 hour coverslips were washed twice with Hank's balanced salt solution (HBSS, Sigma, UK) and immediately after that the cells were seeded. Experiments took place 3 weeks post seeding.

6.2.2 Preparation of experimental solutions and treatments

Ferric chloride and calcium chloride (both from Sigma, UK) were dissolved in 0.1M HCl to obtain the following stock solutions: 0.125M; 0.25M and 0.5M $CaCl_2$ solutions and 0.01M and 0.003M $FeCl_3$ solutions. On experimental days the above solutions were diluted further in experimental media to obtain the required concentrations.

Earles/HEPES buffer (EH media) composition: 124.0mM of sodium chloride; 5.4mM of potassium chloride; 0.8mM of magnesium sulphate; 1.0mM of monosodium phosphate; 14.3mM of sodium bicarbonate; 10.0mM HEPES; pH 7.4, 1.2mM of calcium chloride and 5.55mM D-(+)-glucose.

Experimental media was prepared using phenol red free, serum free DMEM (Gibco, UK). Media pH was adjusted to 6.5 and HEPES buffer added at a final concentration of 25mM in order to stabilise the pH.

DPD solution was used at the end of the incubation period with iron chloride. DPD (2,2′-Bipyridyl, Sigma, UK) powder was dissolved in dimethyl sulfoxide (DMSO, Sigma, UK) to obtain 1M stock solution, which was further diluted in experimental media to obtain a final concentration of 5mM.

6.2.3 Phen green loading- optimisation of the technique

Phen Green™ SK, diacetate ((PG); Invitrogen, UK)) powder was dissolved in DMSO to obtain 1mM and stored at -20ºC in 50µl aliquots. Investigations were undertaken to optimise loading conditions into Caco-2 cells. PG concentration, loading time and temperature were tested. The best loading conditions for cells (21 days post seeding, Figure 6.2.1) were: rinsing coverslip with EH media, 20min loading at room temperature with 10µM of PG in EH media and following incubation rinsing the coverslip twice with EH media.

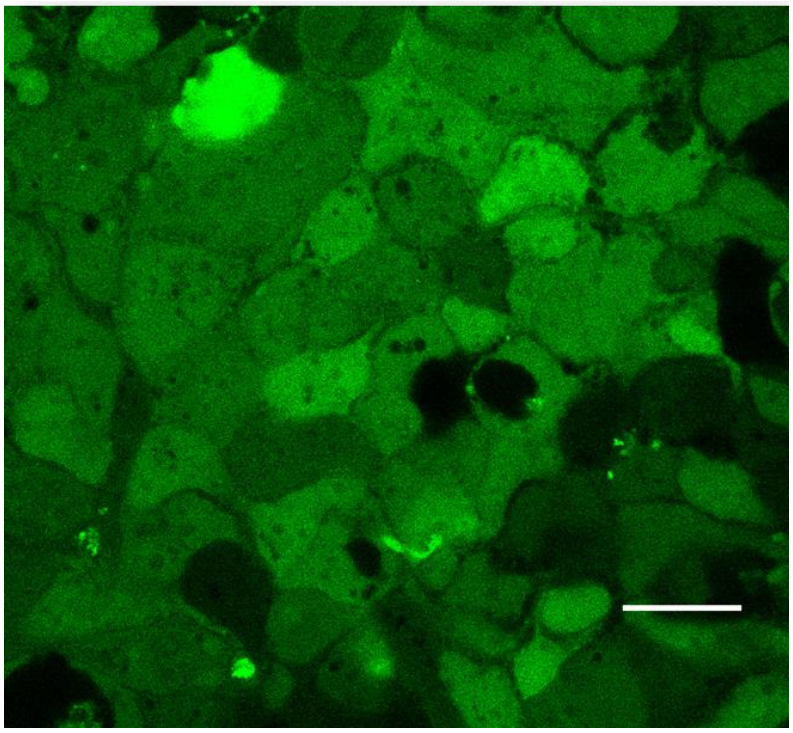

Figure 6.2.1 PG loaded Caco-2 cells. The length of white bar is 40microns.

6.2.4 Confocal microscopy

Images were collected using a Nikon eclipse TE2000-U confocal microscope, lens 60x1.2, with Nikon EZ-C1 FreeViewer 3.80 software. Fluorescence of PG was excited at 488nm (blue excitation) and collected through a 488 nm detector (green emission). Images were collected for 100min (at 5min intervals) at 512 resolution. The first image collection phase comprised of baseline image collection: 2 images collected 5min apart of cells in EH media followed by 2 images collected 5min apart (4 images in total) with experimental media (pH 6.5). The second image collection phase comprised of images taken of cells exposed to iron alone or in the presence of calcium (30 or 100μM $FeCl_3$ with or without 1.25 or 5mM $CaCl_2$). This latter collection phase (in which a decrease in fluorescence was expected to be observed as iron entered the cells) took 60min with images collected at 5min intervals (13 images in total). In the final phase which lasted 20min, images were again collected at 5min intervals (5 images in total) with iron dequenched from PG using a 5mM solution of DPD, which resulted in an observed increase in fluorescence.

Collected stacks of images were combined using the ImageJ 1.46r; Java program and fluorescence values (in arbitrary units) were obtained.

6.2.5 Statistical analysis

Prior to statistical analysis the data were normalised to baseline readings (the time point at which iron was added onto the cells). Every experiment considered 10 cells (areas; n=10). Data from 2 (Figure 6.3.1 and Figure 6.3.5) or 3 (Figure 6.3.3) separate experiments was combined.

Statistical analysis was performed using SPSS Inc, USA, (version 16.0.0). One way ANOVA with Tukey HSD or Games-Howel post-hoc tests were conducted to examine pairwise differences on normalized data. Data are presented as mean ±SD. Differences were considered significant at $p<0.05$.

6.3 Results

6.3.1 Cell exposure to 100µM ferric chloride, live cell imaging data

Three time-points were selected for visual representation of the results: baseline (t=0), when iron was applied onto the cells (control); t=60 (incubation with iron for 60min) and the final time-point (t=80) being 20min incubation with DPD following incubation with iron. In total cells were observed for 80min.

After the Caco-2 cells were loaded with PG they were exposed to either 100µM of ferric chloride or 100µM ferric chloride in the presence of 1.25 or 5mM calcium chloride (Figure 6.3.1A). A significant decrease ($p<0.0005$ in all instances) in fluorescence was observed after 60min incubation with iron in all 3 treatments when compared with baseline (t=0) suggesting that iron was entering the cells despite the presence of calcium. There were no significant differences in the rate of fluorescence decrease between the three treatments investigated (Figure 6.3.1B). An explanation for iron entering Caco-2 cells in the presence of calcium at a similar rate to iron alone is the possibility that a 1h exposure to iron may not be long enough to observe changes in the rate of iron uptake into the cells. Therefore, in the experiment presented in Figure 6.3.3A cells exposed to iron after pre-incubation for 24 hours with 2.5mM were compared with cells exposed to iron only.

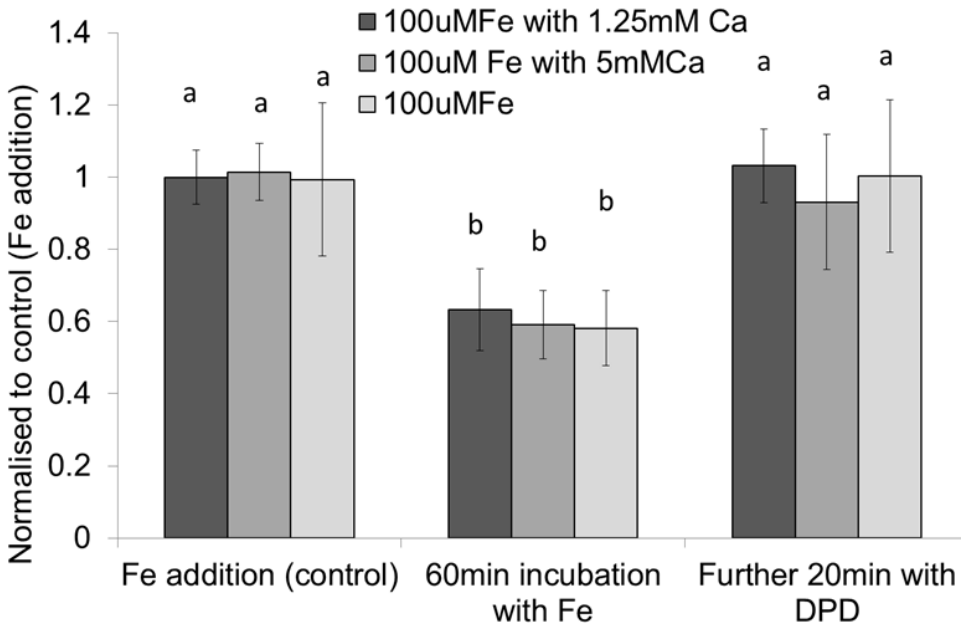

Figure 6.3.1A Change in fluorescence in cells exposed to 100µM ferric chloride with or without presence of 1.25 or 5mM calcium chloride. Combined data from 2 experiments (n=20 in each treatment), normalised to control. Data represents mean ±SD. Bars without a common letter (a, b, c) are significantly different, $p<0.05$.

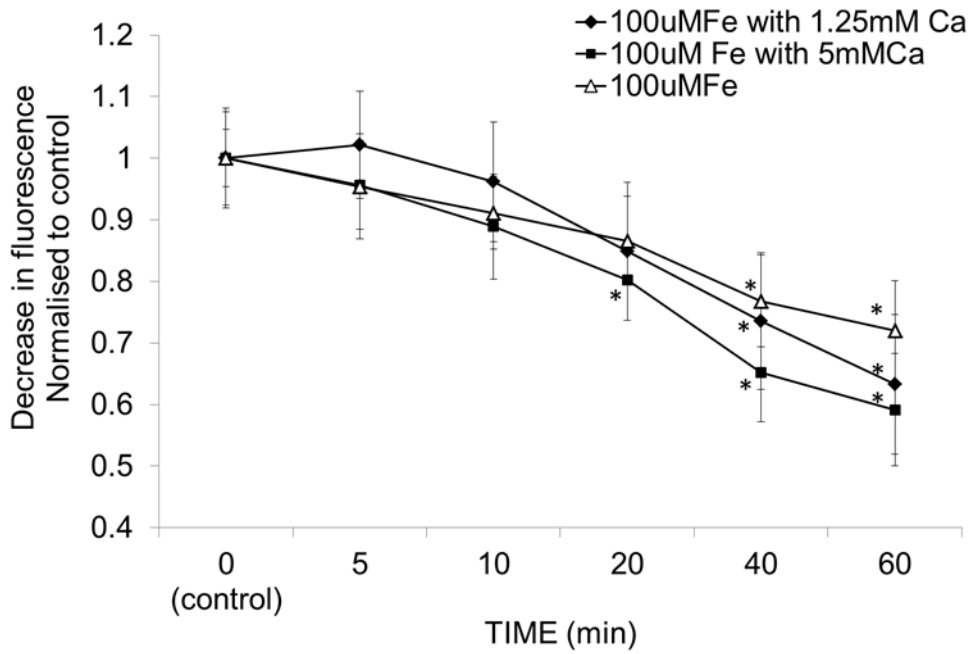

Figure 6.3.2B Data as in figure 6.3.1A with additional time points in order to investigate decrease in fluorescence (normalized to control) over 60min incubation time. Data represents mean ±SD. Star (*) represent points with significantly lower fluorescence than the control ($p<0.05$).

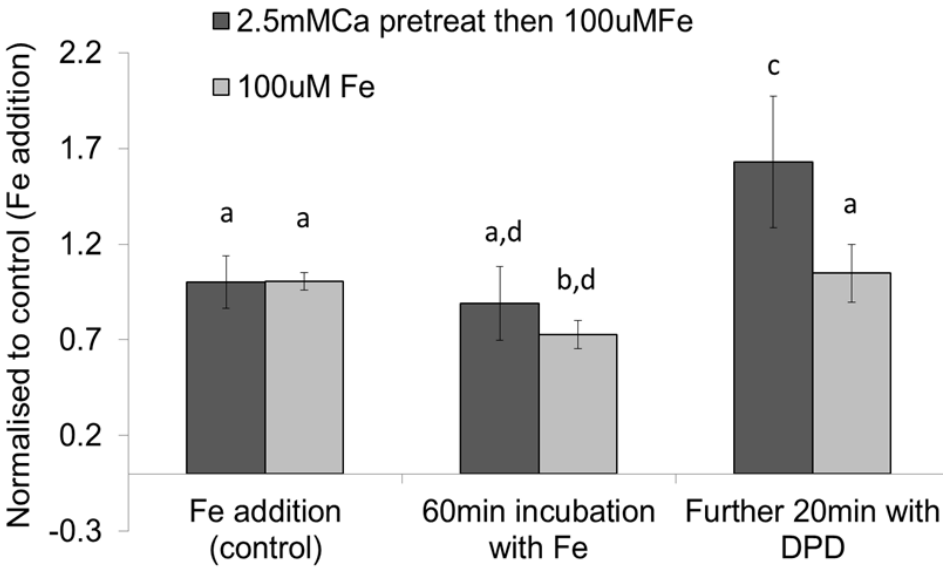

Figure 6.3.3A Change in fluorescence in cells exposed to 100µM ferric chloride or pre-treated (24h) with 2.5mM calcium and then exposed to 100µM ferric chloride. Combined data from 3 experiments (n=30), normalised to control. Data represents mean ±SD. Bars without a common letter (a, b, c, d) are significantly different, $p<0.05$.

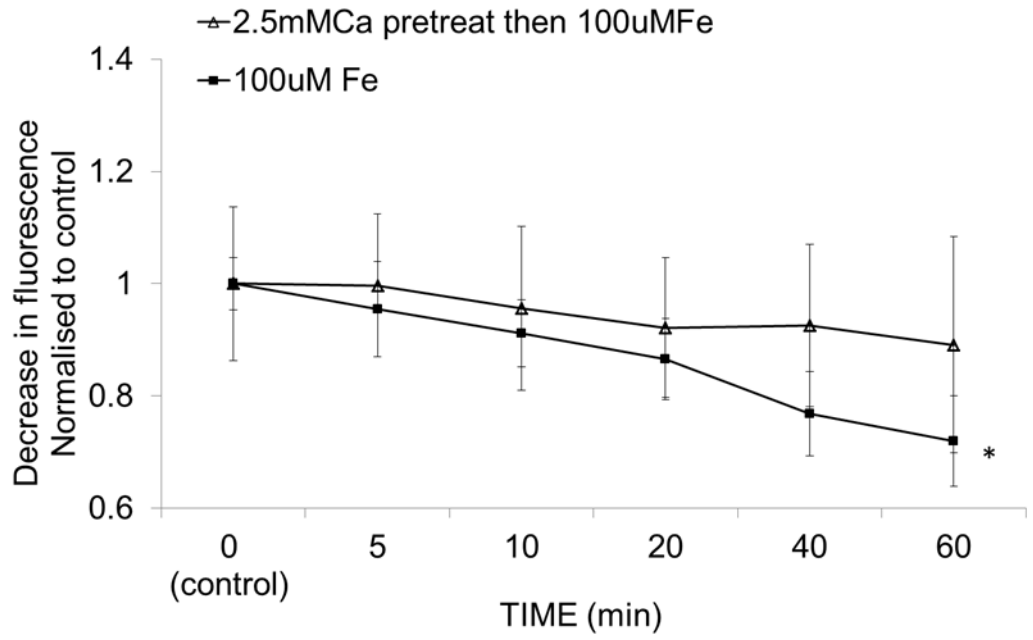

Figure 6.3.4B Data as in figure 6.3.2A with additional time points in order to investigate decrease in fluorescence (normalized to control) over 60min incubation time. Data represents mean ±SD. Star (*) represent points with significantly lower fluorescence than the control ($p<0.05$).

The decrease in fluorescence in cells pre-treated with calcium (Figure 6.3.3A) after 60min incubation with iron was not statistically significant when compared with baseline, but the decrease in fluorescence in cells exposed to iron was significant

when compared to baseline (p=0.001). However, the decrease in fluorescence between calcium pre-treated and non-pre-treated cells after 60min of incubation with iron was not statistically significant. There were also no significant differences in the rate of fluorescence decrease between the two treatments investigated (Figure 6.3.2B).

6.3.2 Cell exposure to 30µM ferric chloride, live cell imaging data

In order to investigate if similar trends would be observed in the presence of lower iron concentrations, experiments with 30µM ferric chloride were also performed (Figure 6.3.5A). There was a decrease in fluorescence after incubation for 60min with 30µM iron in both the presence and absence of 2.5mM calcium when compared with baseline (p<0.0005 in both cases). These findings are also in agreement with findings when 100µM of iron was used.

When cells were pre-treated with calcium prior to exposure with 30µM of iron a significant decrease in fluorescence was also noted when compared with baseline (t=0); (p<0.0005). This finding is not in agreement with calcium pre-treated data when 100µM of iron was used.

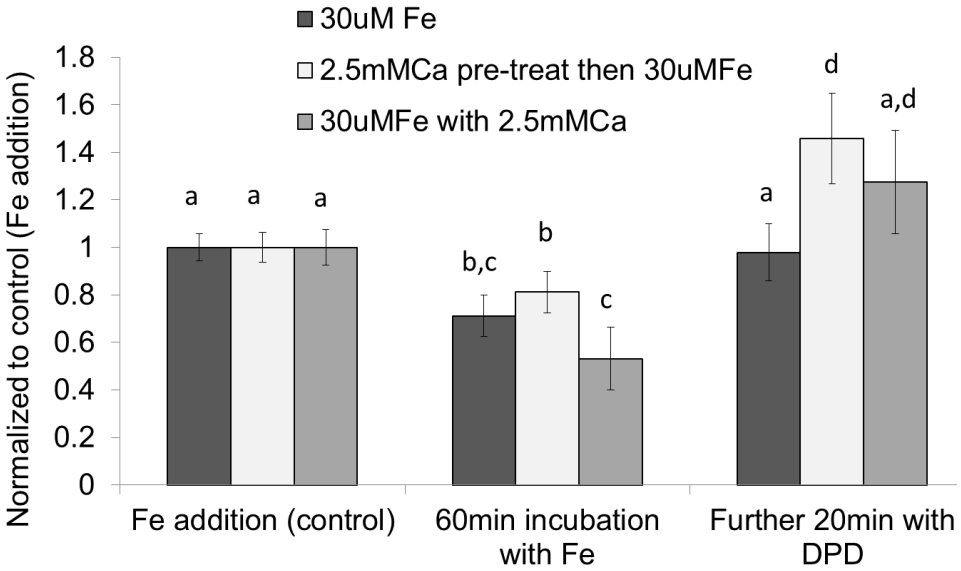

Figure 6.3.5A Change in fluorescence in cells exposed to 30µM ferric chloride or 30µM ferric chloride in presence of 2.5mM Ca or pre-treated (24h) with 2.5mM calcium and then exposed to 30µM ferric chloride. Combined data from 2 experiments (n=20), normalised to control. Data represents mean ±SD. Bars without a common letter (a, b, c, d) are significantly different, p<0.05.

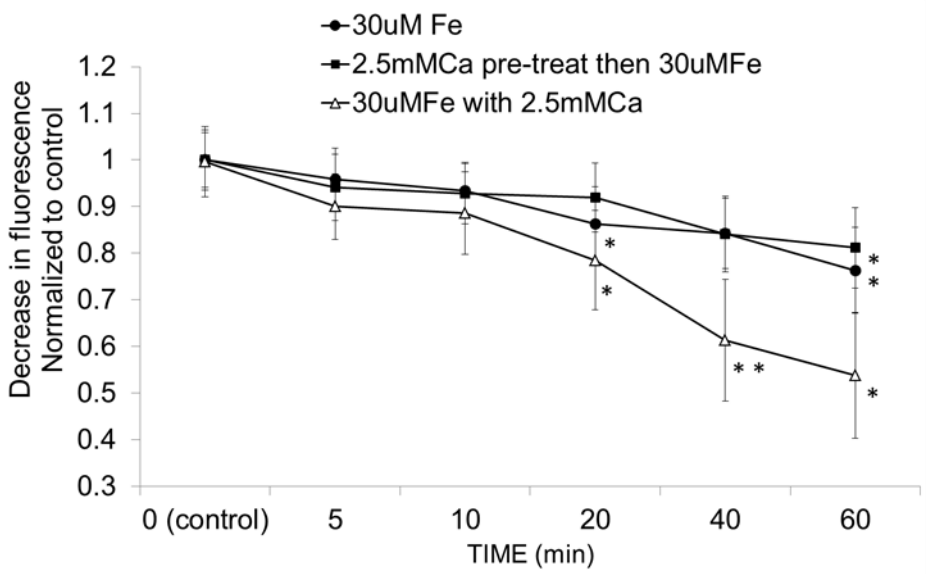

Figure 6.3.6B Data as in figure 6.3.3A with additional time points in order to investigate decrease in fluorescence (normalized to control) over 60min incubation time. Data represents mean ±SD. Star (*) represent points with significantly lower fluorescence than the control, two stars (**) represent the treatment with significantly lower fluorescence at a given time point (p<0.05).

Also there was a significant difference in the rate of fluorescence decrease ($p<0.05$) between the treatment with calcium added at the same time as iron at the 40min time point and the two remaining treatments (Figure 6.3.3B). Overall, the rate of decrease of phen green fluorescence was more rapid when the cells were exposed to 30µM ferric chloride in the presence of 2.5mM Ca than with other treatments.

6.4 Discussion

As mentioned earlier investigations into calcium and iron interactions reported in this Chapter are still on-going. Complementary images using immunocytochemistry techniques to stain and fix DMT1 and tight junction proteins (following iron, iron with calcium or calcium only exposure for 1, 2 and 4 hours) have yet to be analysed. Thus interpretation of the results reported in this Chapter is currently incomplete.

Thompson *et al.*[55] suggested that the mechanism by which calcium affects non-haem iron uptake is through translocation of DMT1. The authors used ferritin as a surrogate index of iron availability, therefore incubation times with iron and calcium solutions were relatively long (16 or 24 hours) and as expected calcium inhibited iron uptake into the cell. Thompson *et al.*[55] also showed that DMT1 levels in the cell membrane fraction were reduced after a 4 hour exposure to iron, calcium or a

combination of both. Similarly, results reported by Johnson *et al.*[277] showed that maximal reduction in membrane expression of DMT1 (as well as decrease in plasma membrane DMT1(+IRE)) occurred 4 hours post exposure to 100μM of iron, whilst there was no difference after a 1hour exposure. Johnson *et al.*[277] also reported that DMT1 mRNA (+IRE) expression was unchanged after 4h exposure to iron. In addition, a significant reduction in the IRE containing DMT1 was observed after 24h[179,277].

Published data indicate that the inhibitory effect of calcium on iron uptake in the Caco-2 cell model is observed between the second and fourth hour post exposure to calcium and/or a high dose of iron. The observations made in this Chapter were only collected for one hour post exposure. Interestingly, when cells were pre-incubated with calcium for 24 hours and then exposed to 100μM of iron, the decrease in fluorescence after 60min was not significantly lower when compared to the control cells. However, when a lower iron concentration was used (30μM) after pre-incubation with calcium no inhibitory effect was detected. A possible explanation for this observed inconsistency may be the confounding effect of calcium which may be more pronounced with lower iron concentration (Ca:Fe ratio of 83:1). The possibility of calcium binding PG within the cell, thus reducing PG fluorescence, is supported by the rate of fluorescence decrease presented in Figure 6.3.3B. This observation warrants further investigation.

One of the limitations of work presented in this Chapter is the lack of images of cells exposed to calcium only (1.25, 2.5 and 5mM). These data are required in order to investigate whether calcium alone (especially at higher concentrations) can induce a reduction in PG fluorescence to distinguish between the effect of iron and that of calcium.

Another limitation which needs to be noted is that the experimental media contained 1.8mM of calcium, a standard concentration of calcium in DMEM media. Calcium is essential for cells to maintain their integrity; therefore complete elimination of calcium from experimental procedures is not possible. PG loading however was undertaken in EH media which contained a reduced calcium concentration of 1.2mM.

Finally, this investigation would have benefitted from extending the incubation time from 1h to 4-5h. However due to technical issues (such as PG photo bleaching and the inability to keep the cells in 5% CO_2 while image collection was taking place) ideal experimental optimisation could not take place. Future plans should include 1h,

2h 3h and 4h cell pre-incubation with calcium prior to exposure to iron and then collection of images. Furthermore, the analysis of experiments undertaken using immunocytochemistry techniques would elucidate some of the above uncertainties.

Nonetheless, despite some experimental limitations, this research permitted a better understanding of the kinetics of iron uptake and the inhibitory effect of calcium on pre-incubated cells, and further analysis of the data collected (on-going) will add further information.

7. USE OF CACO-2 CELL MODEL AS A TOOL TO INVESTIGATE IRON AVAILABILITY FROM TWO WHEAT CULTIVARS

7.1 Introduction

The investigations reported in this Chapter are a result of collaboration between a PhD student, Tristan Eagling, based at Rothamsted Research Institute (RRI) and myself. Preparation of all samples and speciation of iron within the wheat samples used for the experiments was carried out by Tristan Eagling at RRI, whereas screening of the samples using the Caco-2 cell line model, coupled with the simulated digestion technique described in Chapter 2, was carried out by myself at the UEA.

One of the strategies for addressing iron deficiency is biofortification of food crops. By means of selective plant breeding or genetic engineering techniques new varieties of staple foods (such as rice, wheat, maize, millet and legumes)[278–281] cultivars are being developed with higher nutritional value. This approach of increasing the micronutrient density of foods is useful in settings (remote rural areas) where food fortification or supplementation is not feasible[281,5].

If a successful programme of staple food biofortification can be developed in a given region, nutrient-rich foods will then be widely available to the majority of the population[282]. In addition, mineral-rich seeds may be more resilient to diseases and thus provide higher yields[282].

There are, however, potential issues with implementation of biofortified varieties of plants for everyday use by farmers. Namely, breeding must be effective and provide high yields, efficacy must be verified in the most commonly consumed foods produced from biofortified crops, and, finally, new seeds need to be accepted and widely used by both farmers and consumers[283]. All the aforementioned aspects may slow down the introduction of biofortified crops worldwide.

During the milling process of wheat flour the outer layer of the grain (bran) and embryo (germ) is removed leaving endosperm as the main component of white flour[284] (Figure 7.1.1). However, it is the bran that contains higher concentrations of iron, not the endosperm[285]. Therefore the control (by means of biofortification) of iron accumulation at sites within the grain is also of importance[286].

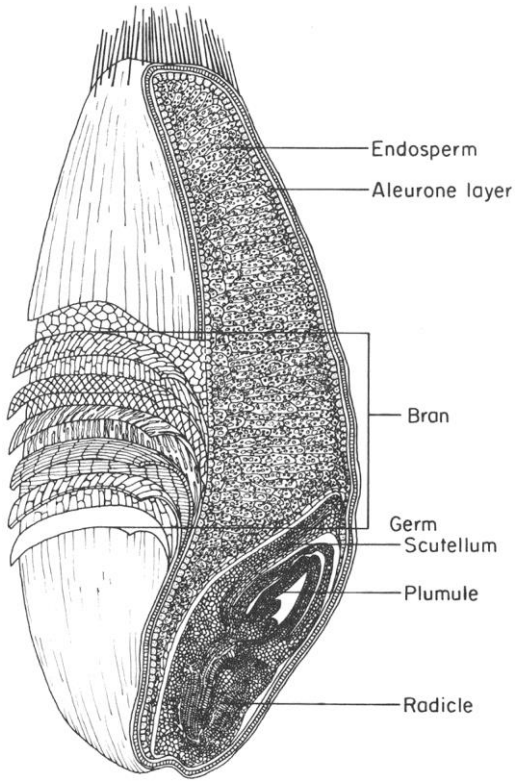

Figure 7.1.1 Diagram of wheat grain (sourced from Wheat Flour Institute:https://dl.sciencesocieties.org/publications/books/abstracts/acsesspublicati/cropqualitystor/).

The form in which iron is present within the grain and the presence of iron-binding complexes such as phytate can influence iron availability. Most of the iron present in cereal grain is bound to inositol hexa*kis*phosphate (IP_6) or pentaphosphate (IP_5) and forms phytate salts[287]. Minerals bound to phytate are not accessible to iron transporters in the human gut due to the lack of specific enzymes[49], but there is evidence that phytates, depending of the nature of the bonding, may be insoluble or soluble. Soluble, and thus available for the uptake, salts in the form of monoferric phytate (Fe-IP_6 or Fe-IP_5[287,288]) may be a potential source of available iron[52]. Therefore, potential bioavailability of monoferric phytate (MFP) was also the subject of investigations reported in this Chapter.

Metal chelator[65]- nicotianamine (NA, Figure 7.1.2) formed by NA synthase from three molecules of S- adenosylmethionine[289], is involved in intra- and intercellular transport of metal cations in the plant. When it is present at elevated levels in the grain it has been reported to enhance iron uptake in both cell and murine models[65,66].

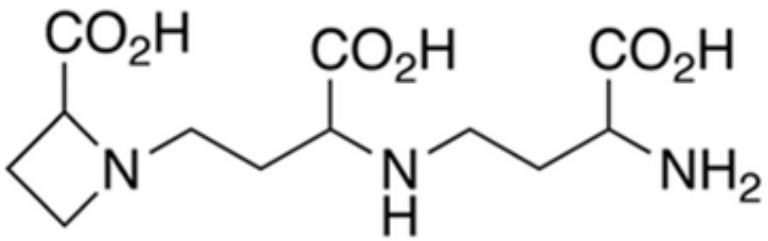

Figure 7.1.2 Chemical structure of NA adopted from Von Viren *et al*.[290].

Another chelator and potential enhancer of iron uptake is 2'-deoxymugineic acid (DMA, Figure 7.1.3) which is synthesised via NA aminotransferase in graminaceous plants[291,292]. DMA is a phytosiderophore and thus has a high affinity with ferric iron, and plays an important role in the solubilisation and acquisition of the iron (III)-phytosiderophore complex by the plant from the rhizosphere[292,293] . Therefore, an increase in both NA and/or DMA is another potential avenue of improvement with the use of transgenic technologies.

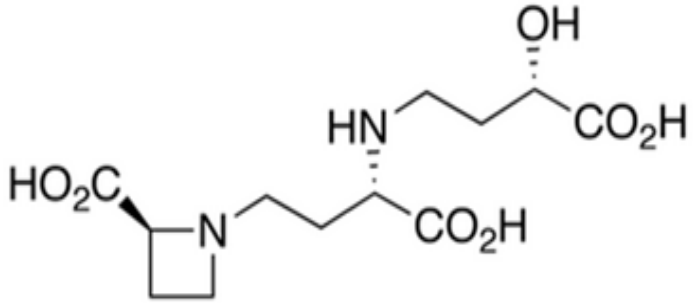

Figure 7.1.3 Chemical structure DMA adopted from Von Viren *et al*.[290].

Finally, there are large natural genetic variations between (commercially used) cultivars of wheat grain with respect to total iron concentration, ranging from 19-58mg/kg[294–296], suggesting that biofortification as a method of producing nutrient rich crops has the potential for success. However, it is not known if similar variations in iron content are present when white flours are produced from grains that are low and high in iron.

The hypothesis tested in this chapter was that the cell ferritin response would be the highest in cells treated with chapattis made of the white flour that had the highest iron content; that DMA (similarly to NA) would enhance iron uptake into the cell, and that MFP would not prevent iron from being taken up by the cells, compared with the strongly inhibitory effect of phytate in the inositol hexakisphosphate form.

The main aim of this collaboration was to investigate if there are differences in iron availability between wholegrain and white flours made out of two commercially available wheat cultivars selected by Rothamsted team: Rialto (high in iron) and Riband (low in iron), with and without AA and additional iron (II) sulphate. Iron availability was tested in the Caco-2 cell model combined with a simulated digestion procedure. In addition, the availability of iron from MFP and the effect of NA, DMA (the two iron transporters present in graminaceous plants) and AA on iron uptake by the cells was also investigated.

7.2 Methods

7.2.1 Sample preparation

7.2.1.1 Grain materials and unleavened flatbread preparation

Analyses were performed on two wheat cultivars selected to represent high (Rialto) and low iron (Riband) cultivars[296]. Growing conditions (soil and climate) were described by Shewry *et al.*[297]. Based on these analyses the two cultivars were grown in field trials at Rothamsted Research in 2010.

Milling, extraction, iron and phytate analysis in flour samples and unleavened flatbread (chapattis, from Rialto and Riband wholemeal and white flours) was undertaken by Tristan Eagling and are described in detail in his thesis entitled 'Understanding the amount and availability of iron in bread wheat cultivars to prevent global iron deficiency' submitted in September 2013. Characteristics of flour and flatbread samples are summarised in Table 7.2.1

Table 7.2.1 Specification of flours and chapattis.

Flour type	Total Fe[1] (mg/kg) in flours	Total Fe[1] (mg/kg) in chapattis	Phytic acid[2] (g/kg dw) in chapattis
Rialto White	16.98	11.85	1.0
Rialto W-meal	46.7	46.7	6.4
Riband White	6.83	6.65	0.4
Riband W-meal	30.3	30.25	4.5

[1] Total Fe determined by ICP-MS

[2]Analysis of phytic acid was performed using a commercially available kit (K-PHYT 12/12 Megazyme, Ireland), as per the manufacturer's instructions. Briefly, either 1g or 0.5g of samples were extracted with 10ml of HCl followed by an enzymatic dephosphorylation step with phytase and alkaline phosphatase and precipitation using a colour reagent prepared from AA in sulphuric and ammonium molybdate. The absorbance (655 nm) of free phosphorous and total phosphorous were then compared to phosphorus standards using a Varioskan spectrometer (Thermo, Finland).

7.2.1.2 Preparation of experimental solutions- AA, NA, DMA, MFP (work undertaken by Tristan Eagling)

Ascorbic acid (AA).

The usual practice is to add AA to obtain an AA:Fe molar ratio in the range of 10-20:1. Therefore the AA final concentration is determined by the amount of iron in the samples[257,298]. However, there were large variations in total Fe in the chapatti samples (6.65-46.7mg/kg) and an identical amount of 132µM AA solution (AA dissolved in MilliQ water) was added per gram of flatbread used. Although this meant that some samples would have a higher AA:Fe ratio than the others (Table 7.2.2), the amount of AA chosen was sufficiently high to ensure that it achieved maximal effect as observed in previous studies[299] .

Mono-ferric phytate (MFP).

A solution of 100ml of 0.5M acetic acid with 10g of sodium phytate and 1g $FeCl_3$-$6H_2O$ was stirred for 2 hours until all the sodium phytate was completely dissolved. The resulting solution was allowed to stand briefly, then 100ml of 95% ethanol was added and the solution left overnight. The precipitate was then collected by centrifugation (5000*g* for 10min) (Medowrose scientific LTD, Oxford , UK), washed three times with acetone, allowed to air dry, and stored in a desiccator. The final product underwent inductively coupled plasma atomic emission spectroscopy analysis to verify the expected structure[300].

Table 7.2.2 Iron and AA concentrations of treatments containing 3g of Chapatti's.

Chapatti treatments containing 3g of:	Fe content (µg) per digest	Fe (µM) per digest	AA (µM) per digest	Fe:AA molar ratio
Riband White	19.95	23.82	396	1 : 16.6
Rialto W-meal	35.55	42.44	396	1 : 9.3
Riband W-meal	90.75	108.34	396	1 : 3.7
Rialto W-meal	140.10	167.25	396	1 : 2.4

Fe-DMA and Fe-NA.

Both DMA and NA form 1:1 molar complexes with iron. Fe (II)-NA was prepared from ferrous sulphate and Fe (III)-DMA was prepared from ferric chloride (both from Sigma Aldrich, Austria). DMA/NA were dissolved in MilliQ water and iron was dissolved separately also in MilliQ water. Appropriate solutions were then mixed immediately to avoid oxidation. Both NA and DMA were purchased from Toronto Research Chemicals Inc.

7.2.2 Cell culture procedures

All cell culture procedures with Caco-2 cells were identical to those reported in Chapter 2 with the exception of media composition and supplier. For experiments reported in this Chapter DMEM media (0.5L) was sourced from LGC, UK and supplemented with 10% fetal bovine serum, 2mM L-glutamine, 5ml of 5000u/ml Penicillin/Streptomycillin solution (Gibco, UK) and 5ml of 100x non-essential amino

acids (Sigma, UK). Caco-2 cells used for these experiments were at passages: 27 to 34

7.2.2.1 Simulated digestion procedures

Initially the simulated digestion procedures were identical to those reported in Chapter 2, Section 2.2. However, due to the low iron content of the flour samples the following changes were made to the simulated digestion: sample volume was increased from 0.5g to 1g and subsequently to 3g which produced a thick digestate, so the duodenal phase of the simulated digestion was performed in a test tube for 2 hours and not above the Caco-2 monolayer (usual procedure). Once completed, the tubes with the digestates were centrifuged at 21,000*g* for 10min (as described in Chapter 5, Figure 5.2.1, with the exception of centrifuge force) and the resulting supernatant was flash frozen in liquid nitrogen and stored ready for the Caco-2 cell experiments. The supernatant was warmed to 37°C, then applied to the inserts with dialysis membranes and placed above the wells containing Caco-2 cells for 2 hours. After that time the inserts with dialysis membranes were removed and the cells were incubated for a further 22 hours. In order to increase the cell response further AA and/or iron (II) sulphate was added to all treatments.

7.2.3 Experimental procedures without a simulated digestion phase with the use of MFP, NA, DMA and AA

Experiments performed with the use of NA, DMA and MFP were performed without a simulated digestion phase due to the limited quantity of material available for the experiments.

Solutions containing NA, DMA and MPF were prepared by Tristan Eagling in MilliQ water (at approximately 1:9 Fe to AA molar ratio across all treatments), the pH was adjusted to 2 and the samples were incubated for 1 hour at 37 ºC. After that time the samples were frozen and sent to UEA where they were stored at -18 ºC.

On the experimental day samples were thawed, diluted with MEM media (Gibco, UK, complete composition is described in Chapter 2) to the desired iron concentrations (1.6 or 3.11µg Fe/ml) and kept in water bath at 37ºC for an hour. Because small concentrated volumes of NA, DMA or MFP solutions were used (600µl were diluted in 6ml) it did not markedly affect the pH of solutions (final pH

was in a range of 6.9-7). Before adding onto the cell monolayer all treatment solutions were filtered with 0.22µm syringe filters (Merck Millipore, UK). The cells were incubated with the experimental solutions for 24h and after that time they were harvested, sonicated and analysed (as described in Chapter 2).

7.2.4 Protein and ferritin analysis

Protein and ferritin analyses were performed as described in Chapter 2, Section 2.3.

7.2.5 Statistical analysis

Unless otherwise stated, all statistical analyses were performed using SPSS Inc, USA, (version 16.0.0). 2-factor ANOVA with Tukey post-hoc to examine pairwise differences on power-transformed data. Data are presented as mean ±SD. Differences were considered significant at $p<0.05$.

7.3 Results

7.3.1 Caco-2 ferritin response to unleavened flatbread (chapatti) samples-simulated digestion experiments

Results obtained with 1g of flatbread (Figure 7.3.1) showed that there was significantly more ferritin produced by cells treated with Riband white flour bread in the presence of AA than from Riband or Rialto wholemeal flours ($p<0.0005$ and $p=0.001$ respectively). There were no significant differences between the two wholemeal or white flour cultivars.

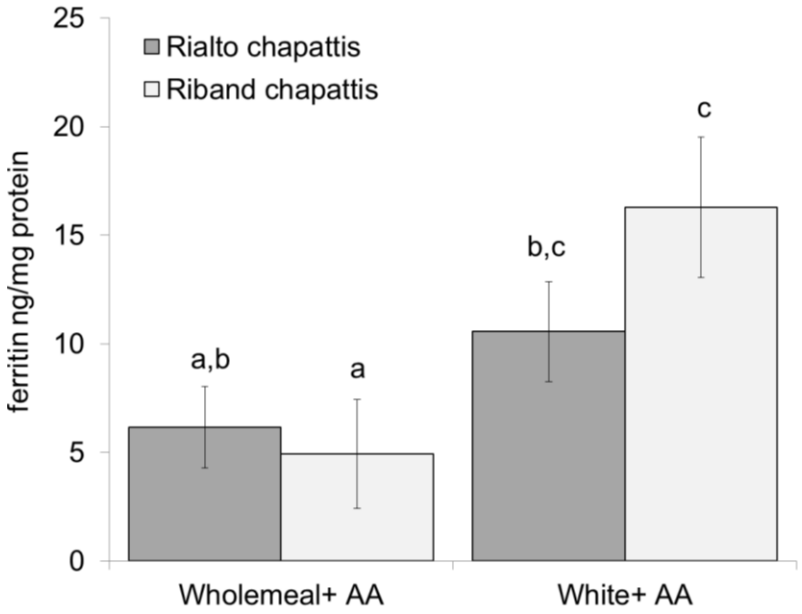

Figure 7.3.1 Ferritin concentration in Caco-2 cells exposed to digests prepared from unleavened flatbread made out of Rialto and Riband white and wholegrain flours in the presence of 132µM AA, expressed as ferritin concentration (ng/mg total protein). Cell ferritin response to control (blank) was 6.2 ±SD 1.8. Data represent mean ±SD (n=6). Bars without a common letter (a, b, c) are significantly different, $p<0.05$.

The cell ferritin response (143.1 ±SD 18.5 and 106.0 ±SD 12.7ng/mg total protein) to positive control treatments (8µg Fe as $FeCl_3$+AA or MFP+AA respectively) was significantly higher ($p<0.0005$ for all comparisons) than any other treatment. Initially, no ferritin response was observed from the wholemeal flour breads (Rialto or Riband) therefore a further increase from 1g to 3g in sample quantity was implemented (Figure 7.3.2).

When 3g of sample were used (Figure 7.3.2) cells exposed to white flatbreads (Rialto and Riband) produced significantly more ferritin than cells exposed to wholemeal products ($p<0.0005$ in both instances). Cells exposed to Riband white flatbread produced significantly more ferritin than cells exposed to any other flatbreads ($p<0.0005$ in all instances).

Rialto or Riband wholemeal flour breads again resulted in very low cell ferritin formation (significantly lower than blank (7.5 ±SD 0.6ng/mg total protein; $p=0.001$ and $p<0.0005$ respectively). One potential explanation for this observation may be that phytate present in wholemeal flours is inhibiting residual/contaminant iron present in the samples resulting in lower ferritin levels than in the blanks. To ensure that the cells were responsive positive controls (8ug $FeCl_3$+AA and MFP+AA both in

presence of 132µM AA) were also included in this experiment and resulted in a high ferritin response (437.9 ±SD 33.5 and 166.9 ±SD 19.4ng/mg total protein).

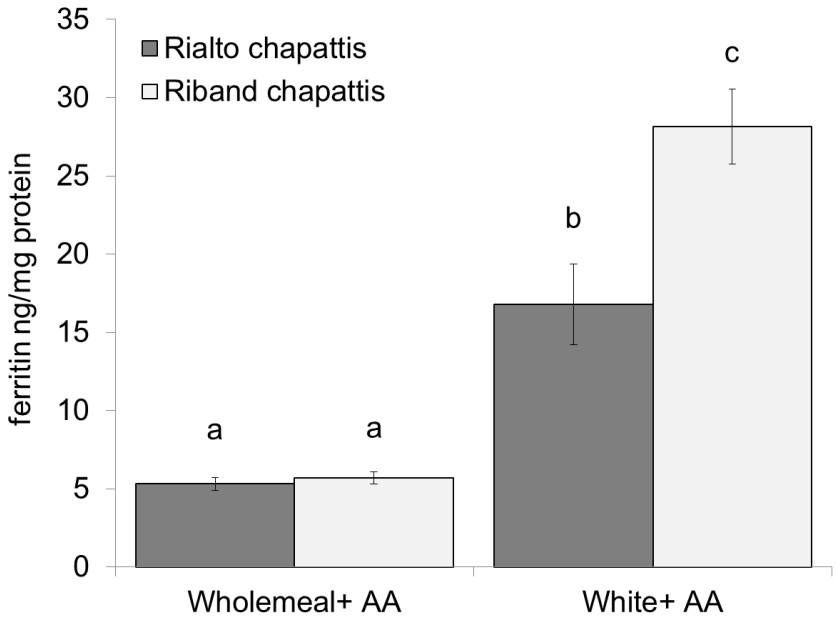

Figure 7.3.2 Ferritin concentration in Caco-2 cells exposed to digests prepared from unleavened bread made out of Rialto and Riband white and wholegrain flours in the presence of 132µM AA expressed as ferritin concentration (ng/mg total protein). Data represent mean ±SD (n=6). Bars without a common letter (a, b, c) are significantly different, $p<0.05$.

In order to increase the cell response further all flour samples were supplemented with ferrous sulphate with 30mg Fe/kg prior to the production of flatbread (Figure 7.3.3). There was a significantly higher cell ferritin response to samples from Rialto or Riband white flatbreads than wholemeal flours ($p<0.0005$ for all comparisons). Cells exposed to Riband wholemeal flatbread produced significantly more ferritin than cells exposed to Rialto wholemeal flatbread ($p=0.001$). Cell ferritin response to positive control (53.54µM $FeSO_4$) was not significantly different from cell response to Riband white bread, but it was significantly higher than the response to Rialto white, wholemeal and Riband wholemeal ($p=0.049$; $p<0.0005$ and $p<0.0005$ respectively). It is also worth mentioning that the cell response to flatbread made out of Rialto wholegrain flour was not significantly different to the blank (17.7 ±SD 2.5ng/mg total protein).

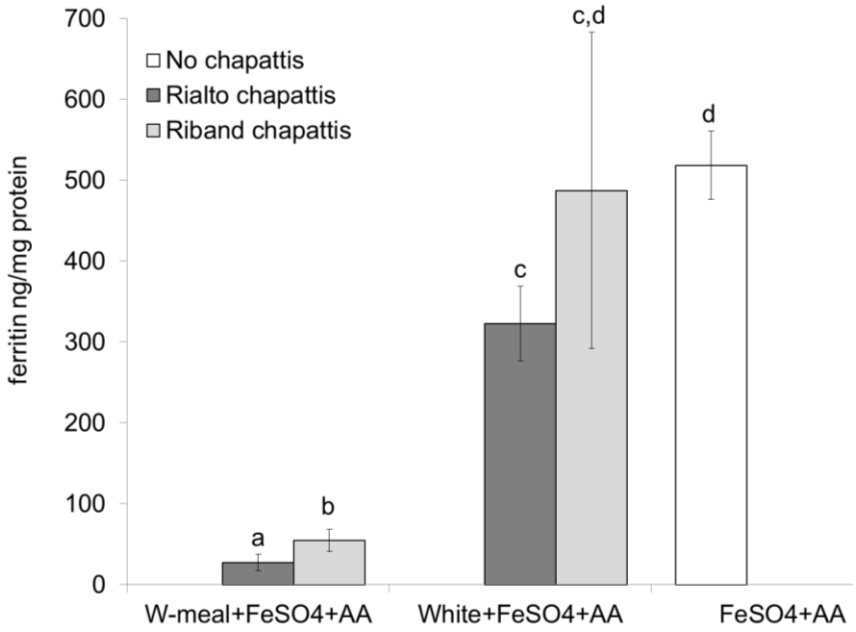

Figure 7.3.3 Ferritin concentration in Caco-2 cells exposed to digests prepared from unleavened bread made out of Rialto and Riband white and wholegrain flours in presence of 132µM AA and 53.54µM $FeSO_4$ expressed in ferritin concentration (ng/mg total protein). Data represent mean ±SD (n=6). Bars without a common letter (a, b, c, d) are significantly different, $p<0.05$.

When the above experiment was repeated (data not shown) similar results were observed, the only difference being that cells exposed to $FeSO_4$+AA produced significantly more ferritin that cells treated with Riband white flour ($p=0.001$).

7.3.2 Cell ferritin response to iron (II), iron (III) or MFP treatments with NA, AA, DMA or NA and AA

The results presented in this Section focus on iron availability in the presence of NA, DMA and/or AA.

It has to be noted that AA was present in the treatments (Figure 7.3.4) at a ratio of ≈1:9 (Fe:AA) whereas DMA and/or NA were present at a ratio of 1:1 (Fe:NA and/or DMA).

Cell ferritin response to iron in the presence of NA (Figure 7.3.4) was significantly higher than to iron in the presence of DMA ($p<0.0005$) suggesting that NA is a more potent enhancer than DMA (at the tested molar ratio). Cell ferritin response significantly increased when AA was added to iron and DMA treatment ($p<0.0005$), but further addition of NA had no extra effect. However due to the absence of an

'iron only' treatment it is not possible to evaluate the enhancing effect of DMA on iron uptake by Caco-2 cells. The evidence indicates that DMA at 1:1 Fe to DMA molar ratio is not as effective as AA at 1:9 Fe to AA molar ratio.

Interestingly, the cell ferritin response to MFP+AA was similar to ferrous chloride+AA, suggesting similar iron availability of both compounds. Also, further addition of NA to MFP+AA or to iron (II) chloride+AA significantly increased ferritin formation in cells (p<0.0005 in both instances).

When the above experiment was repeated a lower ferritin response was obtained with MFP+AA.

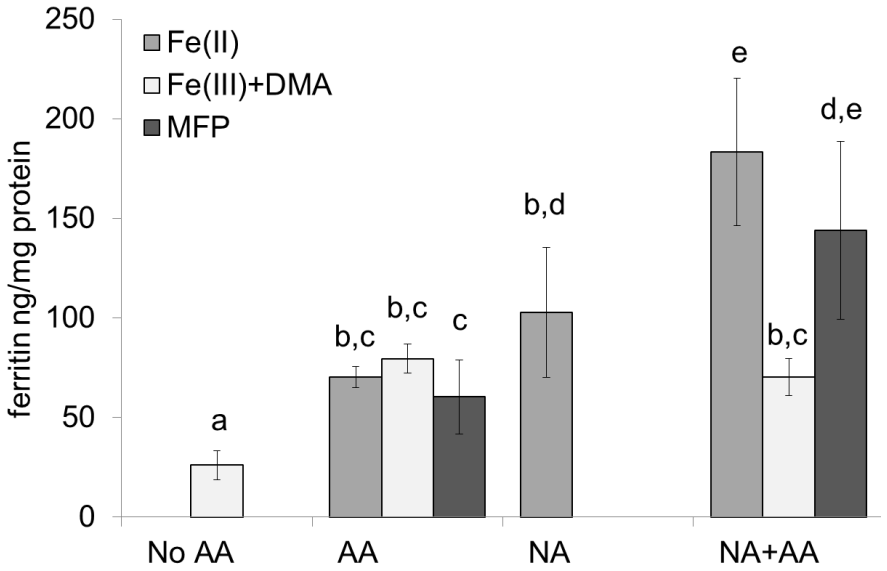

Figure 7.3.4 Ferritin concentration in Caco-2 cells exposed to 1.6µg of Fe(II)Cl, Fe(III)-DMA or MFP in presence of NA (at 1:1 Fe to NA ratio) and/or AA (at 1:9 Fe to AA ratio). Cell response expressed in ferritin concentration (ng/mg total protein). Cell ferritin response to control (blank) was 1.9±SD 1.3. Data represent mean ±SD (n=6). Bars without a common letter (a, b, c, d) are significantly different, p<0.05.

The final experiment (Figure 7.3.5) was designed to investigate whether there was a dose-response effect of NA and to compare it with the most potent enhancer of iron uptake, namely AA.

Cells exposed to iron in the presence of increasing NA concentrations (from 1:0.25 Fe:NA molar ratio through 1:0.5 to 1:1 molar ratio) produced significantly more ferritin with every increase in NA (p<0.0005; p=0.001 and p=0.004 respectively). When a higher Fe:NA 1:4 molar ratio was used ferritin levels were not significantly different to those in cells exposed to 1:0.5 Fe:NA molar ratio.

The cell ferritin response was positively related to AA concentration (from 1:0.25 Fe:AA molar ratio through 1:4 and 1:10 molar ratio, p=0.002; p<0.0005 and p<0.0005 respectively). Cells exposed to the highest 1:10 Fe to AA molar ratio produced ferritin at similar levels as cells exposed to the lowest 1:0.25 Fe to NA molar ratio suggesting that NA is a more potent iron enhancer than AA.

When the experiment was repeated (data not shown), the only difference observed was that cells exposed to 1:4 Fe:NA molar ratio produced ferritin at significantly higher (p=0.019) levels than cells exposed to 1:1 Fe:NA molar ratio, indicating that NA continued to have an enhancing effect with increasing dose.

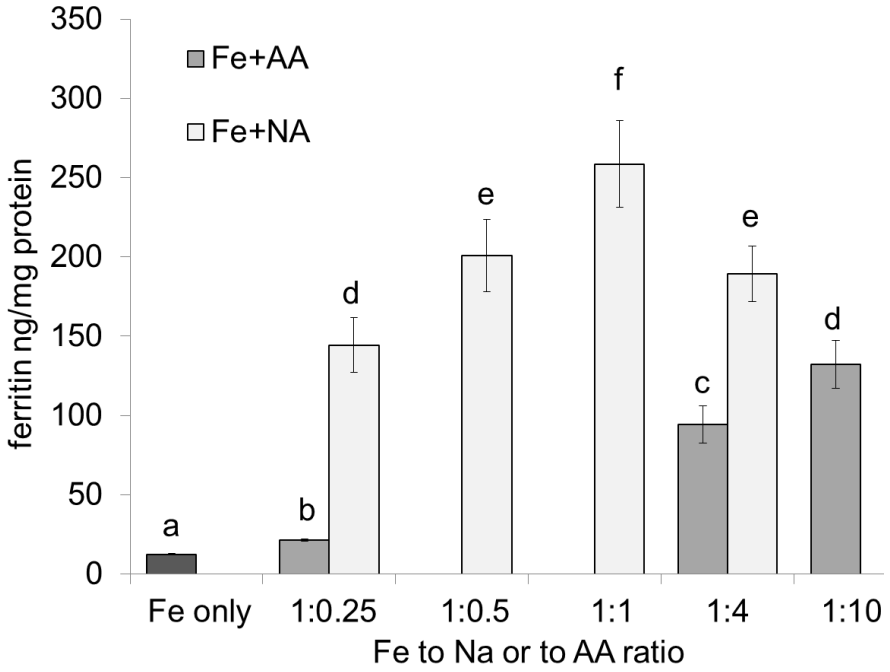

Figure 7.3.5 Ferritin concentration in Caco-2 cells exposed to 3.11µg (13.88µM $FeSO_4$) without or with NA (at following Fe:NA ratios: 1:0.25; 1:0.5; 1:1; 1:4) or AA (at following Fe:AA ratios: 1:0.25;1:4 and 1:10). Cell response expressed as ferritin concentration (ng/mg total protein). Cell ferritin response to control (blank) was 4.0±SD 0.9. Data represent mean ±SD (n=6). Bars without a common letter (a, b, c, d, e, f) are significantly different, p<0.05.

7.4 Discussion

Riband white flatbread delivered more available iron (in the presence of AA) than any other flatbread investigated despite having the lowest iron content of all samples (Figure 7.3.1; Figure 7.3.2 and Figure 7.3.3) when tested in the *in vitro* model. One potential explanation for this phenomenon may be the fact that Riband white flatbread also contained the lowest phytate content of all flatbreads tested (Table 7.2.1). The results suggested that the cell ferritin response increased with the fall in phytate rather than the increased iron content. This observation suggests that phytate and not the iron content of the flours tested was the main determinant of iron bioavailability.

When flatbreads were supplemented with additional iron at 30µg/g together with AA, (Figure 7.3.3) the same trend was observed. The cell ferritin response was inversely proportional to the concentration of phytate and iron in flatbreads tested. However it has to be noted that because the iron content of all samples differed, the AA:Fe molar ratios in the samples were not the same (Table 7.2.2), with the highest AA:Fe ratio found in the sample with the lowest iron (Riband white flatbread). The rationale for using equal amounts of AA across all samples provided by Tristan Eagling (who prepared the samples) is that when AA is used in excess it should have a similar effect across all iron concentrations[299].

Since 1953, it has been mandatory in the UK to add iron to white and brown flour to restore it to the levels found in wholemeal flour (16.5mg kg^{-1})[301, 70] The results reported in this Chapter suggest that most of the iron present in wholemeal products is not available, almost certainly due to the presence of phytate.

The enhancing effect of DMA requires further investigation. The design of the experiment reported in Figure 7.3.4 had its limitations (including the lack of appropriate comparison; which would have been an iron only treatment) and has not explained the effect of DMA on iron uptake in the Caco-2 cell model. However, the enhancing effect of NA on iron availability was clear. NA is a more potent iron enhancer than DMA (Figure 7.3.4) or AA (Figure 7.3.5), as also reported by Zheng *et al.*[66]. NA at the lowest 1:0.25 Fe:NA molar ratio tested was similar to AA at a 1:10 Fe:AA molar ratio (Figure 7.3.5).The most effective Fe:NA molar ratio observed in experiments reported in this work seems to be 1:1 or 1:4 (data not shown). Zheng *et al.*[66] also specified a 1:1 Fe:NA molar ratio was the most effective when used with wild type rice and that a 1:4 Fe:NA molar ratio proved to be the most effective in a

transgenic rice grain that was tested. However these findings cannot be directly compared with those presented here as iron compounds were tested without a food matrix and the test materials were not subjected to a simulated digestion procedure.

These results also indicate that MPF with AA may provoke a similar cell ferritin response to ferrous chloride with AA (when the experiment was repeated MFP with AA did not provide as much iron as ferrous chloride with AA, data not shown). Furthermore, when ferrous chloride was applied to the cells in the presence of AA and NA the ferritin response was significantly higher than when ferrous chloride was applied to the cells in the presence of AA or NA ($p<0.0005$ in both instances). Similarly, when MFP was applied to the cells in the presence of NA and AA, the cell ferritin response was significantly higher ($p<0.0005$) than when MFP was accompanied by AA alone. The above observations suggest an additive effect of NA and AA, which requires further investigation.

Future research should include studies of iron availability from flour products (or flour samples) in the presence of NA in order to confirm that the positive effect of NA on iron uptake from rice[65,66,302] and from iron compounds reported in this work is also present in food products made from wheat flours.

8. FINAL DISCUSSION AND FUTURE PLANS

The overall aim of the research presented in this thesis was to investigate potential strategies for increasing iron availability in order to help address global issues of iron deficiency.

When studying the effects of diet on iron bioavailability it is essential to take into account the iron status of study participants as this has a major impact on the efficiency of iron absorption. It is important to have tools to identify individuals most at risk of IDA who would benefit from iron supplementation or other strategies. The assessment of body iron, calculated from serum ferritin and soluble transferrin receptor concentrations, is the most informative as it allows individuals to be placed at a specific point in the iron status spectrum, rather than using a cut-off value. However, the body iron method is not valid in the presence of inflammation as it relies on ferritin measurements, and ferritin is an acute phase protein (see Section 1.3 for more details).

The presence of underlying conditions such as malaria, HIV, and obesity cause an inflammatory response and confound biomarkers of iron status (serum ferritin and in some cases soluble transferrin receptor) thus making it difficult to determine whether or not the anaemia is caused by insufficient iron supply. Probably one of the best approaches to deal with the confounding effect of inflammation on iron status is to include the use of inflammatory biomarkers in the analysis, namely CRP and AGP and to adjust the data accordingly (see Section 1.3.1 for more details).

As mentioned at the beginning of this Chapter, research reported in this thesis focused on methods of delivering bioavailable iron to reduce the risk of iron deficiency. First, the use of food constituents (such as alginates, Chapter 3, 4, 5) was investigated to test if they could be used to protect a soluble form of iron through the digestion process and thus deliver iron in a more available form into the duodenum. For our preliminary investigations an *in vitro* Caco-2 cell line model was used. The Caco-2 system is recognised to be a valuable screening tool for ranking different iron sources in terms of potential bioavailability, and for investigating mechanisms of nutrient absorption in humans[197,198]. Initial experiments with alginate solutions and alginate beads were promising[242] and provided justification for a human study. As reported in Chapter 4, contrary to previous *in vitro* findings (Chapter 3), alginate beads reduced iron absorption (measured by the plasma appearance method) when tested in human volunteers. Therefore, investigations into potential reasons for this discrepancy were undertaken and resulted in

identifying the following differences between the beads produced for *in vitro* and *in vivo* research:

- Varying conditions of the loading bath in which alginate beads were soaked. The beads for *in vitro* investigations were prepared to minimise technical (weighing) errors, hence they contained less iron per 1g of beads, whereas for the human study investigations the aim was to load the beads with a maximal amount of iron in order to reduce the quantity of beads that the volunteers had to consume.

- An additional washing step was introduced into the production of beads prepared for human consumption. In-house organoleptic tests of beads revealed a strong metallic taste, therefore in order to increase the palatability of the beads and to keep the volunteers blinded to treatments the additional washing step was added to the protocol.

- Administration of beads in a test meal (cola jelly) in the human study. Again, after in-house organoleptic tests cola jelly was selected as a test meal in order to blind participants to the treatment and to increase palatability of the beads.

All the above differences can be considered as limitations when trying to compare *in vivo* and *in vitro* experiments. Therefore, follow-up *in vitro* investigations were undertaken in order to address these limitations. Interestingly, when cola jelly was used in the cell model it inhibited iron uptake from both alginate bead and iron only treatments (Chapter 5). The possibility of jelly limiting iron absorption *in vivo* cannot be ruled out, but since it was added to all treatments, the differences in iron absorption reported in Chapter 5 were likely to be due to other test meal components (FeG alone or alginate beads). Since the beads used in the human study contained a considerable amount of calcium (Fe:Ca ratio 1 : 1.9), on the basis of published evidence (refer to Chapter 6 for details), the possibility of calcium being the main contributor to the observed inhibitory effect of alginate beads *in vivo* and in the *in vitro* model cannot be ruled out. It is also worth mentioning that beads containing iron and AA produced a greater ferritin response than iron alone, suggesting a synergistic effect between AA and alginate which warrants further investigation.

In order to understand better the observed inhibitory effect of calcium on iron absorption in the human study, preliminary *in vitro* work investigating calcium and

iron interactions with the use of live cell imaging was undertaken (Chapter 6). If live cell imaging methods prove to be useful (i.e. sufficiently sensitive) for evaluating calcium and iron interactions, then further investigations could be undertaken with alginate solutions in the presence of iron and/or calcium, thus providing direct answers to the following issues:

- to confirm (via a different empirical method) that iron enters Caco-2 cells in the presence of alginate,
- to investigate if calcium, present in the media at standard concentrations (1.8mM calcium chloride), binds to alginate
- to test whether additional calcium introduced into the experimental media binds to alginate.

In addition, it would be beneficial to quantify inflammation proteins such as interleukin 6 or 8 in cell lysates in order to rule out the (unlikely) possibility that alginates induce an inflammatory state in cells, thus increasing ferritin concentrations unrelated to iron uptake. It also has to be emphasised that the use of ferritin as a surrogate marker of iron availability needs to be treated with caution due the fact that a single ferritin molecule can contain up to 4500 atoms of ferric iron[85,96] and to date there is no simple method to quantify the iron content of ferritin in Caco-2 cells.

In addition to exploring the possibility of using alginates to protect iron and provide a bioavailable food fortificant (as discussed above) a collaborative project with Rothamsted Research Institute was undertaken, in which iron availability from 2 different wheat cultivars (Chapter 7) with varying iron and phytate content was investigated. This research revealed that the cell ferritin response increased as the concentration of phytate decreased and was not related to the iron content of the flatbreads. These findings suggest that not only iron, but also the phytate content of staple foods, should be taken into consideration when food fortification policies are introduced.

Furthermore, the research revealed that monoferric phytate in the presence of AA is (at least, in part) available to be taken up by the cells.

Finally, there was the interesting observation that NA is a more potent iron enhancer than AA (at much lower iron : NA ratio) and that DMA may also enhance iron uptake into the Caco-2 cell model, but its effect is not as strong as that of AA at 1:10 iron to

AA ratio. Future work should include dose response experiments with increasing DMA : iron and AA : iron ratios in order to elucidate the potential enhancing effect of DMA on iron uptake. These should also include studies of iron availability from flour products (or flour samples) in the presence of NA or DMA in order to confirm that the positive effect of NA/DMA on iron uptake from iron compounds reported in this work is also the case for food products made from wheat flours.

The use of iron containing alginate beads as an iron fortificant in foods with high water activity was a novel approach and ultimately a lot of technical problems had to be overcome. The conclusion from the work on alginates was that alginate beads with the use of calcium as a gelling agent is not an effective delivery vehicle for soluble iron compounds. However, these findings should not rule out the potential use of alginates. Building on findings reported herein future work should focus on the use of alginates with different gelling agents such as zinc, or with the addition of AA, as a delivery system for iron. Use of alginates in diets containing high levels of tannic acid and phytate where alginates may prevent binding of iron to tannic acid or phytate should also be investigated in future.

Another tool for tackling iron deficiency is biofortification, and increasing the iron availability of staple crops, especially focussing on increasing NA and or DMA content of different varieties/cultivars appears to be a promising strategy.

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10. APPENDICES

Appendix A. Iron status in the elderly

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Abstract
IDA is prevalent in older age, particularly after the age of 80. Serum ferritin concentrations also decline, although there is no evidence to suggest that changes in iron stores are an inevitable consequence of ageing. Chronic inflammation is a common condition in older people, making the measurement of iron status difficult, and it is likely that elevated levels of circulating hepcidin are responsible for changes in iron metabolism that result in systemic iron depletion. Other contributory factors are poor diet and some medications, such as aspirin. Anaemia in older age has undesirable health outcomes, including increased susceptibility to falling and depression. However, there are concerns about possible adverse effects of iron supplements, either in relation to pro-inflammatory effects in the gut or inappropriate tissue iron deposition. Brain iron levels are increased with age-related degenerative diseases, but it is not known if this is the cause or a consequence of the disease, and genetic factors are likely to play a role. In order to maintain body iron within the normal range a personalised approach is required, taking into account all of the factors that may affect iron metabolism and the available strategies for preventing iron deficiency or overload.

Aim of this review
The objective of this narrative review was to summarise the latest information on changes in iron metabolism and status in the elderly population and consequent effects on health in order to provide the framework for studies on iron in an ECfunded project, NU-AGE (New dietary strategies addressing the specific needs of the elderly population for healthy ageing in Europe). Serum ferritin and soluble transferrin receptor will be measured in 1,250 male and female volunteers between the ages of 65-79 years from five different European centres (UK, Italy, France, Netherlands, and Poland). Half of the volunteers have been randomly assigned to a one-year 'whole diet' intervention centred on dietary guidelines specifically tailored for elderly people with the aim of reducing age-related inflammation. The other half have been asked to maintain their usual diet for the year. Iron status is being measured at the beginning and end of the intervention to determine if a reduction in inflammatory status, resulting from the dietary changes, has an impact on iron metabolism. In addition, dietary intake data will be analysed to identify factors that explain the variance in iron status in elderly men and women, using both crosssectional and longitudinal data.

Background
The adult human body contains 3-4 g of iron, approximately 70% of which is present in haemoglobin (Hb) in red blood cells and myoglobin in muscle. Iron is instrumental for the transport of oxygen around the body and is an essential component of many enzymes and cytochromes where it plays a role in electron transport, respiration and hormone synthesis. As a result of these multiple functions, iron is important for physical performance, immunity, cognitive development and function, thermoregulation, and thyroid metabolism. The body

efficiently recycles iron from degraded red blood cells so the daily requirement to replace endogenous losses from the gastrointestinal tract, skin, hair, sweat and menstrual blood loss in women is relatively low, at about 1-1.5 mg/d.

Iron deficiency (ID) is the most common nutritional deficiency disorder in the world, defined as a lack of body iron stores, and usually caused by inadequate absorption and/or excessive iron losses. It is the result of an imbalance between iron supply and iron requirements of the erythroid bone marrow. The next stage of deficiency is iron deficient erythropoiesis, characterised by reduced transferrin saturation. Finally, Hb concentrations fall and hypochromic, microcytic anaemia (IDA) is observed; this affects over 1 billion people worldwide (WHO 2008).

Measurement of iron status

There are a number of biomarkers that reflect different aspects of iron metabolism and can be used singly or collectively to assess body iron status. An in-depth review of biomarkers of iron status is available on the Biomarkers of Nutrition for Development (BOND) website (www.nichd.nih.gov/global_nutrition/programs/bond).

1. Bone marrow grading is the gold standard method of assessing iron deficiency but is highly invasive so rarely used.
2. Serum iron concentration and transferrin saturation indicate the adequacy of the iron supply to developing red blood cells. Serum iron is less reliable as it is subject to diurnal rhythms and increases after the ingestion of iron-containing foods. A transferrin saturation of <15% generally indicates iron deficiency.
3. Zinc protoporphyrin (ZPP). When there is an inadequate supply of iron, zinc is incorporated into the protoporphyrin ring of the haem structure. An elevated ZPP is characteristic of iron deficient erythropoiesis.
4. Soluble serum/plasma transferrin receptor (sTfR). This binds diferric transferrin (Tf) on the cell surface. The main source of serum sTfR is bone marrow erythroid precursors (Flowers *et al* 1989). When intracellular iron supply is reduced, cell surface TfR1 expression is up-regulated in order to acquire more iron, and it is down-regulated when there is sufficient iron. An elevated sTfR is a marker of tissue ID and increased bone marrow
erythropoietic activity. The sTfR concentration increases in parallel with the severity of iron depletion and treatment of individuals with IDA results in a progressive fall in sTfR values (Skikne *et al* 1990).
5. Serum/plasma ferritin concentration correlates closely with body iron stores, and values of <12 ug/L indicate depletion of liver iron stores. However it is an acute phase protein and is elevated in people with infection or inflammation (see below). In order to identify raised ferritin values that do not accurately reflect body iron stores, C-reactive protein (CRP) or alpha-acid-glycoprotein (AGP) are determined and if these are above the normal cut-off, ferritin concentration is not used as a biomarker of iron status.
6. Body iron is not a quantitative measure of iron in the body but is a sensitive index that is useful for monitoring changes in iron status, for example resulting from interventions. It is the ratio of serum transferrin receptor to serum ferritin concentration. It is a relatively new epidemiological technique for monitoring iron status in population groups susceptible to iron deficiency in which inflammation is uncommon or has been excluded by laboratory screening.
7. Hb concentration is a commonly measured biomarker but is not a specific measure of IDA as there are other causes of anaemia e.g. folate and B12 deficiency, and also the anaemia of chronic disease. It is not sensitive as the cut-off values and normal ranges vary according to sex, age, and ethnicity. Hypochromic microcytic appearance of red cells in blood film examination is used by clinician as suggestive of iron deficiency but requires a second biomarker (e.g. ferritin) to confirm the diagnosis of IDA.

Factors that affect biomarkers of status

Hb concentration has been reported to decline with advancing age, even in the absence of demonstrable disorders. In one report this was calculated to be 0.53 g/L/y in men and 0.05 g/L/y in women between the ages of 70 and 88 years (Nilsson-Ehle *et al* 2000) and in another the decline was 0.1 g/L/y in men and 0.09 g/L/y in women between the ages of 70 and 80 years (Milman *et al* 2008). The decline appears to increase after the age of 80, particularly in men. It has been shown that growth hormone and/or insulin-like growth factor-1 are positively and erythropoietin negatively correlated with Hb in elderly people (Nilsson-Ehle *et al* 2005). Erythrocytes released from the bone marrow are less functional and partially damaged in aged individuals and as they are less able to protect themselves against stress which results in their early sequestration (Gershon & Gershon 1988).

Differences in Hb concentration and ferritin levels have been noted between ethnicities (Patel *et al* 2007). For example, anaemia is reported to be more common amongst black American compared to white American adults. However, there is evidence that the Hb distribution curve is shifted towards lower values in blacks (Perry *et al* 1992) which has led to the debate about race-specific criteria for defining anaemia (Beutler & Waalen 2006). Cross-sectional data from the second National Health and Nutrition Examination Survey show that serum ferritin concentrations increase with age until the sixth decade of life at which time they reach a plateau (Yip 1994). In a cross-sectional study of 441 men and women aged 60-93 y, serum ferritin concentration was reported to be positively associated with increasing age in women ($p=0.0223$) but not in men.

However, in a longitudinal study undertaken in a sub-set of 125 people there was no significant change in iron stores over the 10 y monitoring period (Garry *et al* 2000), suggesting that changes in serum ferritin (and iron stores) are not an inevitable consequence of ageing. Chronic inflammation, a common condition in older people, alters iron metabolism and haematopoiesis and can lead to anaemia (Lee 1983), but it is difficult to determine whether or not the cause of anaemia is insufficient iron supply because indices of iron status (notably serum iron, ferritin and transferrin) are modified by the inflammatory state. It has been observed that malnutrition, not uncommon in the elderly, can exacerbate the effect of inflammation on biomarkers of iron status. Nevertheless, it is possible to differentiate between pure IDA, anaemia of chronic disease, and anaemia of chronic disease with co-existing iron deficiency using the sTfR and sTfR/log serum ferritin index (Jain *et al* 2010; Hanif *et al* 2005). Rimon *et al* (2002) undertook tests for anaemia in consecutive patients admitted to an acute geriatric ward who were older than 80 years. Bone marrow examination confirmed IDA in 49 individuals but the routine laboratory tests identified only 8, whereas the transferrin receptor-ferritin index identified 35, demonstrating that this is a more sensitive and specific method for diagnosing IDA in the elderly when bone marrow aspirates are not feasible. Karlsson *et al* (2010) compared bone marrow iron status with various biomarkers of iron status in 50 elderly patients. The sTfR assay correctly identified 87% of iron deficient individuals; the specificity was 74%. With ferritin cut-offs of 20 ug/L for men and 7 ug/L for women this biomarker was 100% specific for iron deficiency but only 35% sensitive. When a ferritin cut-off point of 40 ug/L was used, the specificity fell to 88% but the sensitivity increased to 100%. The sTfR-ferritin index with a cut-off point of 3.0 gave a sensitivity of 100% and specificity of 43%. Acute inflammation is another condition that affects iron metabolism. Cunietti *et al* (2004) monitored changes in biomarkers through an acute inflammation episode identified by raised CRP (≥ 30 mg/L) in 39 older hospitalised patients (median age 79 years). All haematological indices measured, except for MCV and % transferrin saturation, were rapidly disrupted by the acute inflammation and followed differing time courses. ZPP and sTfR were not measured. The effects of a short period of inflammation (□ 20 days) due to infection were similar to those observed in states of chronic inflammation.

The regular intake of aspirin, a commonly used antiplatelet agent in both primary and secondary prevention of cardiovascular diseases, is associated with lower serum ferritin. In 916 elderly people (aged 67-96 y) participating in the Framingham Heart Study, those who took >7 aspirins/week has a significantly lower serum ferritin (p=0.004), and the effect was more marked in diseased than healthy subjects (Fleming *et al* 2001). The authors suggested that the effect could be related to increased occult blood loss and possibly a cytokine-mediated effect on serum ferritin in individuals with inflammation, infection or liver disease. Although samples with a CRP >6 mg/L were excluded, it is possible that mild inflammatory states were not detected due to the low sensitivity of the CRP assay employed.

Obesity may complicate the interpretation of biomarkers of iron status. The observed hypoferraemia of obesity may be due to both true iron deficiency and inflammatory mediated functional iron deficiency. For example, in a cross-sectional study examining the iron status of 234 obese adults compared with 172 non-obese adults attending an outpatient clinic, the obese patients had a higher prevalence of iron deficiency defined by sTfR and serum iron but not by ferritin (Yanoff *et al* 2007). An increase in sTfR was reported to be associated with central obesity in men with hyperferritinemia (Freixenet *et al* 2009). Tussing-Humphreys *et al* (2010) measured serum hepcidin in obese premenopausal women to investigate the reason for iron depletion in obesity. They proposed that hepcidin was expressed in response to inflammation rather than changes in iron status, and concluded that the iron deficiency of obesity is true body iron deficit caused by a reduction in iron absorption rather than maldistribution of iron due to inflammation. If this is confirmed, sTfR may serve as an accurate biomarker of iron status in obese and overweight individuals.

Dietary intake

Good food sources of iron include meat and meat products which contain haem iron, especially red meat and offal and also dark poultry meat; oily fish such as tuna and sardines; cereal products such as fortified breakfast cereals; eggs; pulses; and dark green vegetables. Vegetarians avoid meat so do not consume any haem iron unless they eat fish; their main sources of iron are fortified cereals, soybeans, tofu, lentils, kidney beans, chickpeas, baked beans, dark green vegetables. Bread, potatoes and dried fruit are also a useful source of iron. In elderly people, obtaining an adequate supply of iron may be a challenge due to impaired absorption, reduced food intake associated with lower physical activity, and changes in dietary patterns that result in a more limited diet.

There are special challenges associated with collecting dietary information from elderly people, including fading memory and poor eyesight. A systematic review (Ortiz-Andrellucchi *et al* 2009) selected 33 papers and categorised the studies into short-term (<7 day) or long-term (>7 day) intakes. The quality of each validation study was assessed using a scoring system, which provides a measure of accuracy of the method, and hence give some confidence to the evaluation that was undertaken.

The correlations between the different methods are summarised below, where *very good* is >0.70, *good* is 0.51-0.70, and *acceptable* is 0.30-0.50. It should be noted that the degree of correlation is dependent on the accuracy of the two methods being assessed

- FFQ vs. diet history: very good
- FFQ vs. weighed record: good
- Diet history vs. estimated record: good
- Diet history vs. weighed record: good
- FFQ vs. 24-h recall: acceptable
- Videotaped assessment vs. 24-h recall: acceptable

Declining short-term memory makes the 24-hour recall method less appropriate for this age group. Diet history is a good method but extended diet history interviews should be avoided in the very old as they can take an excessively long time and concentration levels may fall. Instead these should be replaced with modified (shorter) questionnaires. Similarly, FFQs

with a large number of items should be refined to reduce the number of foods. The relationship between iron intake and iron status is complicated by variations in the efficiency of iron absorption, but a systematic review of randomised clinical trials show a positive time-dependent association between iron intake from supplemental iron and serum ferritin (Casgrain *et al* 2012).

In the Framingham Heart Study, cross-sectional data of recognised modifiers of iron bioavailability were analysed in 1,401 elderly men and women aged 67-95 y and positive associations with serum ferritin were observed for haem iron, supplemental iron, vitamin C and alcohol, whereas coffee intake had a negative association (Fleming *et al* 1998). A study in 358 elderly Danish men and women evaluating iron status and its relationship with diet and supplement use (Milman *et al* 2004) reported a positive correlation between serum ferritin and intakes of dietary iron ($p=0.03$), meat ($p=0.013$), alcohol ($p<0.001$), and BMI (in men only, $p=0.025$), and a negative correlation with tea consumption ($p=0.017$), but no association between supplement use and iron status. Although individual dietary enhancers and inhibitors identified from single meal absorption studies (Hurrell & Egli 2010) may have a significant impact on iron absorption and hence status, it is more important to use data on iron absorption from whole diets to predict iron status (Collings *et al*, AJCN, accepted).

Anaemia in the elderly

In the US, it has been estimated that approximately 11% of men and 10% of women aged 65 and above are anaemic, and that these figures double at the age of 85, with prevalence rates reaching 50-60% in residential/nursing homes (Price *et al* 2011). Although one of the conclusions from the Framingham Heart Study, initiated in 1948- 1950, with a cohort of ~6000 adults living in Framingham MA, was that free-living elderly white American eating a Western diet are more likely to have high iron stores, not iron deficiency (Fleming *et al* 2001a), iron supplement use and high intakes of vitamin C and red meat were shown to be important determinants of iron status. The English Longitudinal Study of Ageing, a prospective study of 3,816 community dwelling men and women (mean age 65.4 ± 9.0 y), reported that 5.2% were anaemic, and these individuals were older, less likely to drink alcohol, had a higher prevalence of morbidity, higher CRP (indicating anaemia of chronic disease) and demonstrated poorer performance on cognitive and physical function tests (Hamer & Molloy 2009).

Large epidemiological studies report that in 30% of older people with anaemia the aetiology is unknown and the patient is diagnosed as having ‘unexplained anaemia of aging’ (Guralnik *et al* 2004). An investigation into reasons for anaemia in 190 patients aged 65 and over recruited from a haematology clinic, reported that 12% had IDA, and half of these individuals normalised Hb in response to therapeutic iron (Price *et al*
2011).The ones who did not respond had malignancies (12%), renal disease (4%), or unexplained anaemia (35%). The latter had significantly higher inflammatory markers (hepcidin and ferritin, but not IL-6) than the non-anaemic controls.

Causes of IDA

Anaemia in the elderly may be caused by a number of individual or combined factors, including poor diet, reduced efficiency of iron absorption, occult blood loss, medications, and chronic disease (Lopez-Contreras *et al* 2010). Results from crosssectional studies measuring the prevalence of ID and IDA in elderly people are therefore very variable. Annibale *et al* (2001) examined 668 outpatients with IDA, aged 21-94 y, and were able to identify the underlying reason for anaemia in 85% of patients. Diseases associated with bleeding were found in 37%, including colon cancer, gastric cancer, peptic ulcer,hiatus hernia with linear erosions, colonic vascular ectasia, colonic polyps and Crohn’s disease. Causes not associated with bleeding were found in 51%, including atrophic gastritis, celiac disease, and *H. pylori*. The authors concluded that gastrointestinal diseases not associated

with bleeding are frequently associated with IDA in patients without gastrointestinal symptoms or other potential causes of gastrointestinal bleeding. The diseases observed in older patients were hiatus hernia, gastric cancer, colon cancer, and colonic vascular ectasia. *Helicobacter pylori* infection has been implicated as a risk factor for iron deficiency, but this may be a strain-related effect (Yokata *et al* 2012). A study in 220 Australian men and women aged 65 years or older reported no difference in median serum ferritin concentrations (or Hb) between *H. pylori* infected and uninfected individuals but serum ferritin concentrations were significantly lower in infected women who took low-dose aspirin (p-0.04) (Kaffes *et al* 2003). The authors conclude that it may the combination of *H. pylori* infection and use of low-dose aspirin that impact on iron stores.

Institutionalisation is another known risk factor. In 252 institutionalised elderly Spanish men and women, aged 65-96 years, 4-day weighed food records were collected and iron status measured (Lopez-Contreras *et al* 2010). The prevalence of anaemia was 25.4% using a Hb cut-off of <130 g/L for men and <120 g/L for women (WHO 2001), but only 3.6% had serum ferritin below the cut-off of 15 ug/L. There was a high prevalence of inflammation/infection as illustrated by the fact that 41% of individuals had raised CRP values (>5 mg/L), and there was a significant correlation between CRP and ferritin (p=0.023) but not with Hb. Diet was not one of the principal causes of anaemia in their study, except for folate intake, but it appears that infection/inflammation was a key component. In another study in Spain (Vaquero *et al* 2004) in which elderly people living in retirement homes were consuming an Atlantic-Mediterranean diet (rich in meat products, fish, vegetables, fruit, olive oil and dairy products, but poor in cereals), the prevalence of anaemia was only 6.7%, but there were no measurements of infection/inflammation.

Consequences of iron deficiency

Anaemia is associated with numerous health implications, including a decline in physical performance, cognitive impairment, increased susceptibility to falling, frailty, and mortality (reviewed by Price *et al* 2011).

A study in 1,156 community-dwelling Italian people aged 65 years and older showed a clear association between anaemia and disability, poorer physical performance and lower muscle strength (Penninx *et al* 2004) but there is no proof of causality in crosssectional epidemiological studies. In a 4-year prospective study in men (~30%) and women (~70%) aged 71 y and older (Penninx *et al* 2003) various physical performance tests were undertaken at baseline and after 4 y and the individuals classified for anaemia using Hb and MCV values. The number of volunteers who undertook both sets of tests was 1, 146. At baseline 5.9% had anaemia (Hb<130 g/L men, <120g g/L women), and 15% had borderline anaemia (Hb 130-140 g/L men, 120-130 g/L women). Those with anaemia were older and performed significantly worse on the baseline physical performance battery. There was a predicted fall in the physical performance score (p for trend 0.002), which was most marked in the anaemic group (p=0.003) followed by the borderline anaemic group (p=0.02). The results of this study suggest that anaemia in old age is an independent risk factor for decline in physical performance.

The association between serum iron status, cardiovascular disease and all-cause mortality was examined in 336 elderly Taiwanese men and women (aged >65 y) living in long-term care facilities (Hsu *et al* 2013). The degree of iron deficiency was defined according to serum iron, but it should be noted that serum iron is affected by inflammation, as commonly present in cardiovascular disease, and it is not a good 1 measure of iron deficiency. Although there was a positive association between low serum iron, cardiovascular disease and all-cause mortality, causality cannot be inferred. The authors propose several mechanisms for the link between iron deficiency and cardiovascular disease. Firstly, iron deficiency is associated with cancer, renal failure and chronic inflammation, which are generally accompanied by higher mortality; an iron deficient state may reflect undiagnosed conditions. Secondly, iron

deficiency may be a surrogate marker for malnutrition, for which there is a link with mortality. Thirdly, iron deficiency may promote oxidative damage, for example, as illustrated by elevated serum malonyldialdehyde production in iron deficiency anemia (Coghetto Baccin *et al* 2009). Mørkedal *et al* (2011) assessed sex-specific associations of iron status with ischaemic heart disease (IHD) mortality in a prospective study of 640,798 healthy Norwegian adults. Low iron status, particularly in the early stages of follow-up, was associated with increased risk of death from IHD, but the limited range of biomarkers of iron status (serum iron, transferrin saturation, and total iron binding capacity) were measured in non-fasting blood samples, and it is possible that low iron status was a late sign in the pathogenesis of IHD or that underlying disease influenced the results. A smaller prospective study was undertaken in Finland in which 361 men and 394 women aged 65-99 years were followed for up to 10 years (Marniemi *et al* 2005), Individuals in the highest tertile for baseline serum iron had a reduced risk of acute myocardial infarction (AMI) (RR 0.544, 95% CI 0.35, 0.88) and those in the middle tertile had a reduced risk of stroke (RR 0.474, 95% CI 0.25, 0.89). There was an increased risk of stroke in those in the highest tertile of serum transferrin (RR 1.67, 95% CI 1.07, 2.61), but no effect of blood Hb on either AMI or stroke. When the relationship with serum iron was analysed taking into account additional coronary heart disease risk factors the RR of AMI and stroke were still significant (p=0.016 and 0.047 respectively). In contrast, however, when the relationship between nutritional status and all-cause mortality was investigated in a prospective study of 405 community-dwelling Scottish men and women aged 75 y and older, no association was found between iron status and mortality (Jia *et al* 2007). Racial variation in the relationship of anaemia with mortality and mobility disability among older adults has been observed. In a group of 1,018 black and 1,583 white US adults, aged 71-82 years, anaemia was associated with higher mortality in white but not black people. Anaemia was significantly associated with increased risk of death and mobility disability in community-dwelling older whites, whereas older blacks were not at risk of adverse events. The authors suggest that the criteria for defining anaemia may need revising (Patel *et al* 2007).

A cross-sectional study in 1,875 men and women aged 65 y and older (enrolled in the 2005 Health Survey for England) was undertaken to examine the relationship between iron status (defined according to biochemical criteria) and symptoms of depression (Stewart & Hirani 2012). Anemia (Hb <130 g/L in men, <120 g/L in women) was present in 10.8% of 1,833 samples analysed, low ferritin (< 45 ug/L) in 21.6% of 1,851 samples analysed, moderately raised sTfR (>2.3 g/L) was present in 7.6% of the

1,875 samples analysed. Depressive symptoms were significantly higher in participants with anaemia, low ferritin and high sTfR, after adjustment for age, sex, social class, multivitamin intake, smoking status and BMI, but the association was reduced substantially after further adjustment for physical health status (chronic illness). There was a significant association between higher number of depressive symptoms and lower Hb and higher sTfR but not with ferritin, which suggests that the association with anaemia is accounted for by physical health status and thus may primarily reflect anaemia of chronic disease. Onder *et al* (2005) also found an association between anaemia and depression in 986 older adults from the InCHIANTI study, a prospective population-based study of community-dwelling men and women (mean age 75 y). Anaemia was present in 15% of the participants with depression and in 8% of the participants without depression (p<0.001), and the risk of anaemia progressively and significantly increased with severity of depression.

Adverse effects of iron

Concern has been expressed about potential adverse effects of moderately elevated iron stores in middle-aged and older people in that they may be associated with increased risk of several chronic diseases, such as heart disease, cancer and type 2 diabetes mellitus. Increased tissue iron stores have been implicated in risk of diabetes mellitus and decrease in

insulin sensitivity, but the mechanism is uncertain (Sung *et al* 2012). Iron has well-described pro-oxidant effects *in vitro,* and may have proinflammatory effects. For example, Sung *et al* (2012) reported that in 12,033 Korean men (mean age 41.2 y in the group with no coronary artery calcium and 47.7 y in those with coronary artery calcium) ferritin was independently associated with the presence of coronary artery calcium, a biomarker of preclinical atherosclerosis. Those in the highest quartile for ferritin (>257 ug/L) had a higher score for coronary artery calcium than the lowest quartile (<128 ug/L). However, the relationship with transferrin saturation was weaker, suggesting that there may be another contributory factor, unrelated to high iron status, e.g. inflammation or metabolic stress.

The explanation for the presence of high iron stores was sought in a study of 614 elderly Americans participating in the Framingham Heart Study. After excluding individuals with raised CRP (>6 mg/L, n=45), infection (diagnosed from white blood cell count, n=40) and other confounding factors (n=69), 11.4% of the remaining 460 participants were reported to have elevated iron stores (serum ferritin >300 ug/L in men, and >200 ug/L in women). Dietary factors associated with high iron stores included use of iron supplements (≥30 mg/d), >3 servings of fruit or fruit juice/d, and >4 servings of red meat/week. High intakes of wholegrain (>7 servings/week) were inversely associated with risk of having high iron stores (Fleming *et al* 2002). Three prospective studies reported an association between moderately elevated serum ferritin concentration (>200 ug/L) and risk of acute myocardial infarction (Salonen *et al* 1992, Tuomainen *et al* 1998, Klipstein-Grobusch *et al* 1999), but the association was not observed in other prospective studies (Manttari *et al* 1994, Frey *et al* 1994, Magnusson *et al* 1994). Furthermore, in a matched, nested case-control study (252 cases and 499 controls) drawn from the Copenhagen City Heart Study and the Copenhagen General Population Study, markers of iron overload (high serum iron and transferrin saturation) were associated with *reduced* risk of a near-term (4 years onset) myocardial infarction; conversely there was an association between low serum iron and transferrin saturation and increased risk of a near-term myocardial infarction in this apparently healthy population (Nordestgaard *et al* 2010).

A few early epidemiological studies indicated a weak positive association between very high body iron levels and risk of cancer e.g. colorectal cancer (Knedt *et al* 1994), possibly related to free radical tissue damage caused by iron released from degradation of tissue ferritin. The UK Scientific Advisory Committee on Nutrition has concluded that there are insufficient data to reach a clear conclusion about high iron levels and risk of cancer (Scientific Advisory Committee on Nutrition 2010). There may, however, be an interaction between high body iron levels and lipids (either high VLDL-cholesterol or low HDL-cholesterol) that promotes oxidative stress (Mainous 3rd *et al* 2005). There is emerging evidence to suggest that luminal iron in the gut may be pro-inflammatory and iron supplements given to treat IDA, common in irritable bowel disease and other gut disorders associated with faecal blood loss, may exacerbate the inflammatory processes by stimulating the production of reactive oxygen species and inflammatory cytokines (Weiss 2011). The form of iron appears to be important, for example chelated forms of iron are better tolerated than ferrous sulphate, presumably because it generates a lower concentration of soluble iron in the lumen (Liquori 1993). In healthy individuals brain iron levels increase with age (Bartzokis *et al* 1994) and abnormally high brain iron levels are observed in age-related degenerative diseases. For example, in Alzheimer's Disease hippocampal iron is increased beyond levels of non-demented controls (Pankhurst *et al* 2008). The hippocampus is the key region in memory function that is severely affected in ageing and dementing disorders.

Although there is no proof of a causal relationship, the fact that elevated levels have been observed in preclinical disease (Smith *et al* 2010) has generated the hypothesis that an accelerated trajectory of brain iron accumulation, and the associated oxidative damage, may occur during the transition from healthy ageing to dementia. Bartzokis *et al* (2011) measured hippocampal iron in healthy older people (aged 55 to 76 y) of mixed racial origin and found

that in men, but not women, there was a significant decrease in memory function with increase hippocampal ferritin iron (p=0.003), and independent of gender, worse verbal working memory performance was associated with higher basal ganglia iron (p=0.005) in individuals without the H63D and TfC2 gene variants. The authors suggest a combination of genetic and MTI biomarkers may be useful for identifying high risk groups for primary prevention clinical trials.

Iron requirements in the elderly

Although physiological iron requirements do not differ between adult and elderly men and post-menopausal and elderly women there is growing evidence that iron metabolism is affected by the ageing process. Chronic low-grade inflammation leads to less efficient absorption through hepcidin regulation. Identifying iron deficiency becomes more of a problem because of age-related changes in Hb, effects of medication prescribed for age-related disorders and diseases, and increased ferritin concentrations associated with inflammatory states. The sTfR-ferritin index appears to be the most useful method for detecting iron deficiency in older people. There is sufficient evidence linking iron deficiency with adverse health effects to justify correcting it through diet or iron therapy, but at the same time it is important to ensure that the risk of high body iron stores is not increased as this may have detrimental effects on the brain.

Future work

One of the most challenging problems with studies of iron metabolism in older people is measuring iron status in the presence of inflammatory conditions, such as obesity and age-related chronic and degenerative diseases. Therefore the development of improved biomarkers of iron status must be given high priority. This would enable the relationships between anaemia and iron deficiency and chronic diseases to be characterised more accurately and causality clarified, and would also facilitate research on the potential links between iron and dementia. There is still some uncertainty about iron requirements in the elderly, confounded by effects of inflammation on iron status, which better biomarkers would help address. Finally, there is virtually no information about how ageing of the GI tract affects iron absorption, and this is urgently required for calculating average requirements for iron (using the factorial method) to derive dietary reference values and develop dietary recommendations for elderly populations.

Acknowledgements

The research leading to these results has received funding from the Seventh Framework Programme (FP7/2007-2013) under grant agreement number 266486 (NU-AGE project entitled "New dietary strategies addressing the specific needs of elderly population for a healthy ageing in Europe"). The clinical trial is registered as NCT01754012 at http://ClinicalTrials.gov/.

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Appendix B. The contribution of diet and genotype to iron status in women: a classical twin study

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Abstract

This is the first published report examining the combined effect of diet and genotype on body iron content using a classical twin study design. The aim of this study was to determine the relative contribution of genetic and environmental factors in determining iron status. The population was comprised of 200 BMI- and age-matched pairs of MZ and DZ healthy twins, characterised for habitual diet and 15 iron-related candidate genetic markers. Variance components analysis demonstrated that the heritability of serum ferritin (SF) and soluble transferrin receptor was 44% and 54% respectively. Measured single nucleotide polymorphisms explained 5% and selected dietary factors 6% of the variance in iron status; there was a negative association between calcium intake and body iron (p=0.02) and SF (p=0.04).

Introduction

Levels of iron in the body are tightly regulated through changes in the efficiency of absorption to ensure sufficient iron is available for vital functions but at the same time to avoid the accumulation of excessive iron. It is well established that genetics plays a significant role in iron overload, the most common example being hereditary hemochromatosis which, in spite of its low penetrance, is attributable to mutations in the hemochromatosis gene, *HFE* (Feder *et al* 1996), but the detailed role of genetics in determining body iron status remains largely unexplained. Iron absorption is also dependent on dietary composition as a number of food constituents render luminal iron more or less available for duodenal uptake by the iron transport protein, DMT1 (Hurrell & Egli 2010). Estimates of the relative contribution of diet and genotype to iron status vary widely according to data from different studies, but they have not yet been studied simultaneously in the same population group. The aim of the present study was to assess the role of dietary and genetic factors in determining iron levels using a classical twin study design, and to quantify the relative contribution of each by including specific nutritional factors and candidate genes.

Materials and methods

A sample of 100 MZ and 100 DZ female healthy twin pairs was selected from a group of around 3,500 twins enlisted in the TwinsUK registry. The key inclusion criteria were data on body mass index (BMI), dietary intake and genotype, together with a serum blood sample. The sample size was chosen to provide power of 90% and type-I error rate of 0.05 (Visscher, 2004) in detecting a heritability of approximately 30%. The sample was selected at random but weighted in favour of premenopausal twins as their iron requirements are higher and hence they are more sensitive to environmental modulators. MZ and DZ pairs were also age- and BMI-matched in the selection process to achieve balance between the zygosity groups. All participants gave written, informed consent, and the Guy's and St Thomas' Hospital ethics committee approved the study (REC ref. EC04/015).

Dietary information was collected from a validated 131-item food frequency questionnaire (FFQ), previously developed for the European Prospective Investigation into Cancer and Nutrition Study (Bingham SA *et al*, 2001), from which nutrient intakes were determined using an established database (Teucher *et* al, 2007). Individuals completed questionnaires that included information on menopausal history. Intakes of dietary constituents that might influence iron status, by modifying the quantity of iron absorbed, were calculated: iron, calcium, dietary fibre (non-starch polysaccharide), vitamin C, and frequency of red meat (beef, pork, lamb) intake.

Blood samples were collected at approximately the same time as the FFQ and other questionnaires and stored at -80ºC. As part of other on-going research, some of the samples had been analysed for C-reactive protein, (data shown in Table 1). Analysis of serum ferritin (SF) and soluble transferrin receptor (sTfR) were performed using enzyme immunoassays (Spectro ferritin and TfR respectively, Ramco USA). Two additional standards (10ng and 100ng) were included in addition to the standards provided in the SF kit to increase the assay accuracy. A WHO reference reagent (recombinant soluble transferrin receptor, NIBSC code: 07/202) was run in duplicate on every sTfR plate at 30µg and 90µg/L to standardise the immunoassay (average CV 5.7%). The sTfR value was calculated from the WHO conversion (Thorpe *et al*, 2009). Duplicates were analysed in 30% of SF samples (average CV 6.5%) and in 20% of sTfR samples (average CV 5.2%). Body iron, widely accepted as a good measure of iron status, was calculated from the sTfR/log SF index (Cook *et al*, 2003).

Single nucleotide polymorphisms (SNPs) selected for this analysis were from genes known to be involved in iron metabolism (http://www.micronutrientgenomics.org/index.php/Iron). The list was filtered to include only SNPs with a minor allele frequency (MAF) >5%. The pairwise linkage disequilibrium was examined and where r^2 >0.2, the SNP with the greater MAF was retained. A total of 15 iron related SNPs were included in the analysis.

The twin model partitions variance in a trait into genetic and environmental components. The contribution of genetic and environmental factors was assessed through variance component analysis implemented in SOLAR v4 (Almasy & Blangero 1998). The quantitative age- and BMI-adjusted phenotype (y) was modelled as a linear function of a polygenetic genetic effect (g) and a random environmental component (e) (Model 1). This was extended to examine the influence of 5 individual nutrients (Model 2), and the 15 candidate SNPs (Model 3)

Model 1: $y = \mu + g + e$

Model 2: $y = \mu + g + \beta_i(nutr_i) + e$

Model 3: $y = \mu + g + \beta_i(snp_i) + e$

SOLAR models the covariance among phenotype values(Ω) using the kinship coefficient matrix (Φ) derived from the family structure, $\Omega = 2\Phi\sigma_g^2 + I\sigma_e^2$

where σ_g^2 and σ_e^2 are the variances of the polygenic and environmental effects and I is the identity matrix. The trait heritability was estimated from $\frac{\sigma_g^2}{\sigma_g^2+\sigma_e^2}$ in Model 1. The significance of individual covariates was assessed by examining the change in model likelihood after the covariates were omitted. Measures of genotypic association were tested under an additive model.

To account for multiple testing, the Benjamini-Hochberg adjustment was applied to p values using the p.adjust function in R, assuming a 10% false discovery rate. The proportional contribution of measured nutritional factors and measured SNPs to overall variance was estimated from the variance of $\beta_i(nutr_i)$ and $\beta_i(snp_i)$ in Models 2 and 3 respectively.

Results and discussion

Phenotypic data for the twins and the SF and TfR concentrations are shown in Table 1. There were no significant differences between MZ and DZ twins for any of the measured variables, including factors that might affect iron metabolism such as smoking status (assessed in all individuals) and C-reactive protein, a measure of inflammatory status (measured in some of the twins). Parity was similar in MZ and DZ twins, with an average of 1.6 pregnancies. Iron supplements were reported to have been used by only 5 MZ and 4 DZ individual twins, all of whom were premenopausal, and had SF concentrations that were below the overall mean (mean 39.2µg/L). These individuals were retained in the analysis. SF concentrations <12µg/L (depleted iron stores) were present in 18% of the total sample; the proportion was similar in MZ and DZ groups. However, none of the twins had been clinically diagnosed as iron deficient.

The heritability of iron status (Model 1) was calculated to be 46 (95% CI 28, 65)% for SF, 54 (95% CI 40, 67)% for sTfR, and 44 (95% CI 26, 61)% for body iron.

Table 1. Characteristics of the twins. Values are means ± SD (min, max)

	All	MZ twins	DZ twins
No of individuals	400	200	200
Post-menopausal (%)	64 (16)	32 (16)	32 (16)
Age (y)	43.3 (25.6, 69.8)	44.6 (25.6, 69.5)	45.1 (30.5, 69.8)
BMI (kg/m^2)	24.9 ± 24.8 (16.5, 44.4)	24.8 ± 4.6 (17, 44.4)	24.9 ± 5.2 (16.5, 43. 5)
Smoking status: Never (%) Former (%) Current (%)	 249 (62.3) 107 (26.8) 44 (11.0)	 136 (68.0) 45 (22.5) 19 (9.5)	 113 (56.5) 62 (31.0) 25 (12.5)
C-reactive protein (mg/l) (n=136 MZ, 140 DZ)	2.83 ± 4.37 (0.16, 34)	2.34 ± 2.93 (0.16, 20.9)	3.31 ± 5.39 (0.16, 34.0)
Dietary calcium (mg/day)	1074 ± 328 (232, 2119)	1070 ± 324 (232, 2010)	1078 ± 333 (232, 2119)
Dietary fibre (g/day)	19.1 ± 7.1 (5.3, 51.0)	19.3 ± 6.9 (5.3, 51.0)	18.9 ± 7.2 (5.4, 50.5)
Dietary vitamin C (mg/day)	154.9 ± 77.9 (31.0, 561)	160.4 ± 77.1 (41.2, 515)	149.4 ± 78.5 (31.0, 561)
Meat consumption (times/week)	2 ± 1.72 (0, 11.5)	1.9 ± 1.5 (0, 8.75)	2.2 ± 1.9 (0, 11.5)

The overall contribution of the selected dietary factors to variance in iron status was 6% (Model 2). There was a negative association between calcium intake and body iron (p=0.004) and SF (p=0.008) which remained significant (p=0.02 and 0.04 respectively) after taking multiple testing into account. The contribution of the 15 SNPs examined to variance in iron status was 5% (Model 3). Three were significant for one or more measures of iron status (rs960748 *CYBRD1*, rs9366637 *HFE,* rs4434553 *TFR2*) (Table 2) although none remained significant after taking into account multiple testing.

Table 2. Contribution from SNPs to body iron, serum ferritin (SF) and soluble transferrin receptor (sTfR): *unadjusted p values

SNP	Gene	Gene name	Body iron	SF	sTfR
rs960748	*CYBRD1*	cytochrome b reductase 1	0.008	0.079	0.168
rs2304704	*SLC40A1*	solute carrier family 40 (iron-regulated transporter),	0.783	0.566	0.623
rs8177190	*TF*	transferrin	0.565	0.558	0.294
rs1799852	*TF*	transferrin	0.420	0.174	0.228
rs3811647	*TF*	transferrin	0.990	0.753	0.237
rs1049296	*TF*	transferrin	0.805	0.796	0.466
rs1115219	*TF*	transferrin	0.367	0.948	0.424
rs3817672	*TFRC*	transferrin receptor (p90, CD71)	0.892	0.949	0.105
rs9366637	*HFE*	human hemochromatosis protein	0.063	0.028	0.516
rs1572982	*HFE*	human hemochromatosis protein	0.281	0.167	0.115
rs4727457	*TFR2*	transferrin receptor protein 2	0.627	0.191	0.863
rs4434553	*TFR2*	transferrin receptor protein 2	0.465	0.945	0.034
rs829021	*SLC11A2*	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	0.093	0.613	0.154
rs149411	*SLC11A2*	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	0.718	0.686	0.976
rs7251432	*HAMP*	hepcidin antimicrobial peptide	0.507	0.927	0.174

*Adjusted p values were all >0.05

This is the first published report examining the combined effect of diet and genotype on body iron content using a classical twin study design. The results demonstrate that there is a substantial genetic contribution to iron status, which agrees with Whitfield *et al* (2000) who reported that 47% of the variance in SF in twins was explained by additive genetic factors. However, in their study sTfR was not measured, therefore body iron could not be calculated, nor was there any assessment of the dietary impact on iron stores.

Our study provides evidence to support a contribution for genes known to be involved in iron metabolism. In particular, three (rs960748 *CYBRD1*, rs9366637 *HFE*, rs4434553 *TFR2*) are worthy of further investigation. These genes have also emerged in other studies. In a multi-ethnic population of iron-deficient and normal iron status adults SNPs in the *TR* and *HFE* genes were identified that were associated with at least one iron phenotypic measure (McLaren *et al*, 2011). In a Genome Wide Association Study (GWAS) in MZ twins and their siblings 40% of the genetic variation in serum transferrin concentration was explained by a combination of three SNPs in the transferrin (*TF*) gene and the *HFE* C282Y mutation, but the proportion of phenotypic variance in SF and other measures of iron status could not be explained by SNPs other than C282Y (Benyamin *et al*, 2009). In a meta-analysis of 5 GWAS Oexle *et al* (2011) found that sTfR concentration was associated with *HFE* and *TMPRSS6* genes and the PCSK7 locus. The involvement of these genes suggests that they might have a role in screening for susceptibility to iron deficiency. However, a substantial proportion of genetic variation remains unexplained, justifying the pursuit of more detailed genetic analysis in large scale genome wide studies.

Among the potential environmental factors influencing iron status, this study identified calcium as being negatively associated with iron status, which agrees with its reported inhibitory effect on iron absorption in single meal studies, for example, Minihane & Fairweather-Tait (1998). Data from calcium supplementation studies are inconsistent (Lonnerdal, 2010), and there are conflicting reports about the threshold dose of calcium that affects absorption from a 5 mg dose of iron, ranging from as low as 165 mg (Hallberg *et al* 1991) up to 800 mg (Gaitán *et al* 2011). One of the proposed mechanisms of action for the acute effect of calcium is that it internalises DMT1, thereby limiting iron transport across the apical membrane of the mucosal cell (Thompson *et al*, 2010). It could be argued that the consequence of this would be a compensatory increase in DMT1 expression when the cells are next exposed to digestive chyme, but the degree to which this is achievable is dependent on the dietary supply of available iron. It was notable that the selected dietary factors accounted for only 6% of the overall variation in iron status in this group. This may in part be a reflection of the relatively narrow range of dietary intakes in this group of healthy female twins. Nevertheless, as they are typical of the adult UK population, our findings suggest that modifications in a Western diet are not required to maintain iron status, with the possible exception of calcium intakes in individuals at high risk of iron deficiency, although it is possible that calcium intake in the twins was a proxy measure of differences in dietary patterns and/or intakes of other dietary modulators of iron absorption.

Whitfield *et al* (2003) reported that the combined effect of age, menopausal status and magnitude of blood loss accounted for up to 18% of the variance in SF, and Harvey *et al* (2005) found that menstrual loss accounted for 11.5% in the variance in SF. They reported that diet was responsible for a further 6.7%, a figure that agrees well with our estimate of 6%. Our analysis took age into account but we could not examine the effect of menstrual blood loss, although this is known to have a high degree of heritability (Rybo & Hallberg, 1966). It is also conceivable that interactions between diet and genes might account for part of the unexplained variation. However, gene environment interactions cannot be addressed in these data and would require a substantially larger sample size.

The results of our study quantify heritability of iron status using the most appropriate measures of body iron. With the classical twin study design we have been able to gain a unique insight into the effect of environmental factors and genotype on iron status, and demonstrated that they make an approximately equal contribution.

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Appendix C. A High Prevalence of Zinc- but not Iron-Deficiency among Women in Rural Malawi: a Cross-Sectional Study

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Abstract: Background: Zinc deficiency is often associated with nutritional iron deficiency (ID), and may be exacerbated by low selenium status. Aim: To investigate risk of iron and zinc deficiency in women with contrasting selenium status.

Methods: In a cross-sectional study, 1-day diet composites and blood samples were collected from self-selected Malawian women aged 18−50 y from low (Zombwe) (n=60) and high-plant-available soil selenium (Mikalango) (n=60) districts. Diets were analyzed for trace elements and blood for biomarkers. Results: Zinc deficiency (>90%) was greater than ID anemia (6%), or ID (5%), attributed to diets low in zinc (median 5.7 mg/d) with high phytate:zinc molar ratios (20.0), but high in iron (21.0 mg/d) from soil contaminant iron. Zombwe compared to Mikalango women had lower ($p<0.05$) intakes of selenium (6.5 vs. 55.3 µg/d), zinc (4.8 vs. 6.4 mg/d), iron (16.6 vs. 29.6 mg/d), lower plasma selenium (0.72 vs. 1.60 µmol/L), higher body iron (5.3 vs.3.8 mg/kg), although plasma zinc was similar (8.60 vs. 8.87 µmol/L). Body iron and plasma zinc were positive determinants of hemoglobin. Conclusion: Risk of zinc deficiency was higher than ID and not shown to be associated with selenium status. Plasma zinc was almost as important as body iron as a hemoglobin determinant.

Introduction

Iron deficiency is recognized as the most common micronutrient deficiency in low income countries. Women of child bearing age are especially at risk because pregnancy entails a high demand for iron. A major factor contributing to iron deficiency in these settings is inadequate intakes of bioavailable iron. Such deficits arise because diets are predominately plant-based, and intakes of readily available heme iron from flesh foods are generally low. These dietary patterns are also likely to be inadequate in absorbable zinc because zinc and

iron have a similar distribution in the food supply and are affected by many of the same dietary absorption modifiers [1]. Indeed, low iron stores have been identified as a risk factor for suboptimal zinc status [2]. Furthermore, a compromised zinc status may have a negative impact on hemoglobin concentrations, independent of iron status, as has been reported previously among pregnant women in Ethiopia [3]. Deficiencies of iron and zinc can have numerous adverse effects on women's health, including impairments in physical work capacity [4], cognitive function [5], poor pregnancy outcomes [6,7], and increased risk of morbidity and mortality [8].

An additional factor with the potential to compromise the zinc status of Malawian women is a low selenium status. Our earlier cross-sectional study reported low levels of selenium in the diets and plasma of rural Malawian women living in a region where plant-available selenium content of soils and concentrations in staple foods are low [9]. There is accumulating evidence that selenium interacts with zinc by several mechanisms [10-12]. Therefore in this study, we have determined the risk of zinc deficiency in two groups of rural Malawian women of child-bearing age living in district with contrasting plant-available selenium concentrations in the soil. We used two recommended indicators for assessing population zinc status: the prevalence of inadequate zinc intakes and low plasma zinc concentrations [13]. In addition, we have examined the risk of iron deficiency in these women, using hemoglobin, mean cell volume, and total body iron calculated from plasma ferritin and soluble transferrin receptor in an effort to provide a more accurate reflection of the prevalence and severity of nutritional iron deficiency among these rural Malawian women**.** We also explored possible determinants of plasma ferritin, total body iron, hemoglobin, and plasma zinc.

Subjects and Methods

Study site and subjects.

The women were a convenience sample recruited in March 2011 from six rural villages situated in a district with low available selenium acidic soils in the Extension Planning Area (EPA) of Zombwe (n=60) in Mzuzu Agricultural Development District in the north, and six villages in Mikalango EPA (n=60), a district with high available selenium calcareous soils in the Shire Valley in the south of Malawi. December to the end of March is the preharvest and rainy season in Malawi, when most of the farming activities take place and food intakes are lowest. Women (non- pregnant) aged 18 to 50 years, apparently healthy with no evidence of acute or chronic illnesses, and with an initial hemoglobin > 80 g/L, were eligible to participate. Only one woman per household was recruited. The sample size was sufficient to detect a difference in the prevalence of zinc deficiency of 15% between the women in the two EPAs with a confidence level of 95% [14].

The study protocol was approved by the National Health Sciences Research and Ethics Committee, Malawi. Verbal informed consent was obtained from the traditional authorities in the villages and from the participants. After recruitment, the women were interviewed in their homes by trained research assistants using a pretested questionnaire to obtain demographic and socio-economic data, and information on the health and family characteristics of the women. From these data, a socio-economic status (SES) index was developed based on a total possible maximum score of 14 [15].

Anthropometry

Weight and height were measured using standardized techniques and calibrated equipment with participants wearing light clothing and no shoes, from which body mass index (BMI) (wt/ht^2), and the proportions with height-for-age Z scores <−2SD and height <145cm were calculated.

Collection of duplicate diet composites

Trained research assistants collected 1-d weighed duplicate diet composites (including drinking water) from the women in their homes using digital scales accurate to ±1g (Salter, UK); details are described in Hurst et al. [9]. Research assistants also recorded the types of foods consumed. Weekend and weekdays were proportionately represented to account for any day-of-the-week effects on food and/or nutrient intakes in the group. Diet composites were transported chilled to a central laboratory in Lilongwe for processing. Women were instructed not to change their normal dietary pattern during the diet-composite day, and were reimbursed for the cost of the food.

Analyses of diet composites

The weighed diet composites were blended to a homogenous slurry, after which aliquots were withdrawn into trace-element free polyethylene containers, frozen at –20°C, and then shipped on dry ice to the University of Nottingham, where they were freeze-dried to constant weight. The diet composites were microwave digested, and the digests analyzed for Fe, Zn, Se, Ca, Al, Ti by inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Fisher X Series $^{(II)}$ model) in the laboratory of SY, as described elsewhere [9]. Analysis of phytate (as inositol penta-(IP5) and hexa-(IP6) phosphates) was performed by high performance liquid chromatography in the laboratory of RSG using the method of Lehrfeld [16], as modified by Hotz and Gibson [17]. Molar ratios of phytate:zinc were calculated for each diet composite from the analyzed values. Accuracy and precision of the diet composite assays were established using certified reference materials for the minerals [9] and an inter-laboratory comparison for phytate [17].

Calculation of the prevalence of inadequate intakes of zinc

The distribution of observed intakes of zinc based on the analyzed 1-d diet composites were adjusted to usual intakes by using PC-SIDE (PC software for intake distribution estimation) in conjunction with the intake, monitoring, assessment and planning program (IMAPP)[18]. An external variance component ratio for zinc (0.6993) calculated from dietary data for pregnant Malawian women [19] was used for this step because only 1-d diet composites were collected. The prevalence of inadequate intakes of zinc, a recommended indicator for population risk of zinc deficiency [13] was calculated using the Estimated Average Requirement (EAR) cut-point method and the EAR for an unrefined cereal-based diet set by the International Zinc Consultative Group [20]. The prevalence of inadequate intakes of iron was not estimated in view of the uncertainty about the bioavailability of iron in these diets [21].

Laboratory assessment

Fasting morning venipuncture blood samples were drawn into trace-element free heparinized evacuated tubes (Becton Dickinson, UK Ltd, Plymouth, UK) from all the women in the sitting position. Blood samples were stored in cooler boxes with ice packs as soon as possible after collection, and transported to the laboratories of the nearby district hospitals, where one aliquot of whole blood was analyzed for a complete blood cell count performed by an electronic counter. A second aliquot was separated by centrifugation using trace-element-free techniques based on the recommended procedures of the International Zinc Nutrition Consultative Group (IZiNCG) [20], and the plasma stored frozen at –80^{o} C in trace-element-free polypropylene vials. The frozen plasma samples were shipped to the UK on dry ice for analysis.

Plasma ferritin, soluble transferrin receptor (sTfR), α-1-acid glycoprotein (AGP), and C-reactive protein (CRP) were analyzed in duplicate in the laboratory of SFT by enzyme immunoassays using commercial kits manufactured by Ramco Laboratories (Houston, TX) for ferritin and sTfR, and R&D Systems (Minneapolis, MN, USA) for the inflammatory biomarkers. Plasma zinc and selenium were analyzed using ICP-MS (Thermo Fisher X Series $^{(II)}$ model) in the laboratory of SY, as described previously [9]. A pooled plasma sample together with manufacturer's controls (for Hb, ferritin, AGP and CRP) or certified reference materials (for plasma zinc, and selenium) [8] were used to check the precision and accuracy of the analytical methods. The WHO international reference material (Recombinant sTfR, ANIBSC code: 07/202) was used for the sTfR assay [22]. The between-assay CVs for serum ferritin, sTfR, CRP, AGP, zinc and selenium were 10 %, 4 %, 7 %, 6%, 1.3%, and 1.9%, respectively. Values for the controls fell within the certified ranges for plasma ferritin, sTfR, AGP, CRP, zinc and selenium.

Plasma concentrations of AGP > 1.0 g/L and CRP > 5 mg/L were used to indicate the presence of chronic and acute inflammation, respectively [23] and to adjust the ferritin values for subclinical inflammation [24]. Anemia was assessed by Hb <120 g/L [23], microcytic anemia by mean cell volume (< 82 fL), depleted iron stores as plasma ferritin <12 µg/L (adjusted for inflammation) in the absence of anemia [23], and iron deficiency (ID) as total body iron < 0 mg/kg, adjusted for inflammation [25]. The algorithm of Cook et al. [25] was used to calculate total body iron because the ratio sTfR to ferritin was measured using the Ramco assays [26]. Iron deficiency anemia (IDA) was defined as iron deficiency concurrent with anemia. Zinc deficiency was defined as plasma zinc <10.7 µmol/L [20, 27]. No universal interpretive criteria are available for plasma selenium because they vary markedly with geographic location [28]. In this study, plasma selenium concentrations were considered low when below the level of optimal activity of at least one of the selenoproteins (i.e., < 0.82 µmol/L) [29].

Statistical Analysis

All p values are 2-sided and not adjusted for multiple testing. Distribution of data was checked for normality using the Shapiro-Wilkes test. For consistency and comparability with earlier data, intakes of iron and zinc are reported as medians (1st, 3rd quartiles). Values for ferritin (adjusted for inflammation), sTfR, CRP, and AGP were log transformed to normalize

their distributions and expressed as geometric means (95th Confidence Interval). Differences in median intakes, arithmetic or geometric mean (ferritin, CRP and AGP) biomarker concentrations in the two EPA's were assessed using the Mann Whitney-U test and Student's two sample t tests, respectively, whereas differences between the two EPAs for the proportion with storage iron depletion, ID, IDA, iron sufficiency, non-iron deficiency anemia, and zinc deficiency were examined using Fisher's exact test. Spearman's rank correlation coefficients were used to explore associations between socio-demographic variables, hemoglobin, and the plasma biomarkers.

Multiple regression analysis was used to examine the independent predictors of adjusted log ferritin, adjusted total body iron, hemoglobin, and plasma zinc. The explanatory variables investigated in the regression models were those that were known or suspected [13] to be biologically important. Dietary intake data could not be included in the models because they were based on one day intakes per person [30]. There was no evidence in the multiple regression models of multiple colinearity for the independent variables. Statistically significant differences are indicated by $p < 0.05$. All statistical analyses were carried out using STATA-11.0 (Stata Corp, College Station, TX).

Results

Socio-demographic characteristics of study population

All women approached agreed to participate in the study and were predominantly subsistence farmers. Significantly more women from the high selenium EPA (i.e., Mikalango) had little or no primary education, were classified into the two lowest SES categories (i.e., SES score 1-3 and 4-7), and had a lower mean age and height than the women from the low selenium EPA (i.e., Zombwe) ($p <0.05$). In contrast, there were no significant differences in the mean number of persons per household, body weight, and BMI of the women in the two districts, with <15% in each district with a BMI below 18.5 (termed "mild underweight") (Table 1). Of the women, less than 10% were stunted, with very few ($\leq$ 5%) with a height below 145 cm, the critical height to limit the risk of delivery complications as a result of cephalo-pelvic disproportions [31].

Table 1: Selected socio-demographic and anthropometric characteristics of Malawian women with a low-plasma selenium (Zombwe) and high-plasma selenium (Mikalango) status

	Zombwe		Mikalango		p
Household are subsistence farmers [2] (%)	53/60	88	46/60	77	0.249
Respondents with no or only primary education [2](%)	6/60	10	19/60	32	0.049
Household SES score 5 or less on scale of 1-14[2] (%)	16/60	27	35/60	58	0.002
Number of persons in household[1,3]	4.8	1.6	4.7	1.6	0.610

Age (y) [1,3]	39.0	11.0	34.8	9.6	0.026
Height, cm[1,3]	156.5	5.5	152.8	5.9	0.001
Weight, kg[1,3]	55.4	11.6	52.8	8.0	0.173
BMI, kg/m^2 [1,3]	22.5	4.1	22.5	3.3	0.977
BMI less than 18.5[2] (%)	6/53	11	4/55	7	0.523
Stunted (HAZ < −2.0)[2] (%)	4/53	8	5/55	9	0.524
Height less than145 cm[2] (%)	2/53	4	3/55	5	0.518

[1] Values are mean and standard deviation

[2] Fisher's exact test

[3] Students t-test

Intakes from analyzed diet composites

Maize was the dominant cereal in Zombwe whereas in Mikalango, sorghum and millet replaced maize. Consumption of both cellular animal protein and dairy products were low in both groups, with the exception of a small amount of milk consumed with tea. Indeed, during the diet composite collection day, milk was consumed in tea by the women in Mikalango on only one of the nine tea-drinking- occasions compared to eight of the 51 tea-drinking-occasions in Zombwe. Median (1^{st}, 3^{rd} quartile) analyzed intakes (Table 2) were significantly higher ($p<0.05$) for iron, zinc, selenium, titanium, phytate, and molar ratios of phytate:zinc in Mikalango compared to Zombwe, although median intakes of aluminium were not significantly different in the two EPAs.

Biochemical status

In those women with elevated CRP levels indicative of acute inflammation (CRP >5 mg/L, 17.5%; 21/120), higher concentrations of log plasma ferritin ($p=0.048$) and a tendency for lower values for plasma zinc ($p=0.061$) were observed; hemoglobin, plasma sTfR and plasma selenium did not differ. Elevated AGP concentrations had no significant association with plasma ferritin or zinc.

In Zombwe, the mean plasma selenium was lower ($p < 0.001$) as noted earlier [9], whereas the mean cell volume, geometric mean ferritin, mean total body iron (mg/kg) (both adjusted for inflammation), and the geometric mean for CRP (but not AGP) were higher ($p < 0.05$) in Zombwe, although the proportions of women with elevated CRP and AGP levels in the two districts were not significantly different (Table 3).

The overall prevalence of anemia and iron deficiency anemia was 21% (25/119) and 6% (7/115), respectively, with no significant differences between the two districts (Tables 3 & 4). Of the anemic women, 14% (17/119) had a low mean cell volume (i.e., < 82 fL). Both storage iron depletion and iron deficiency (in the absence of anemia) were low in both districts (Table 4). Low plasma zinc concentrations were observed in 92% and 95% of the women Zombwe and Mikalango, respectively, with no significant differences between the two districts. As expected, 82% of women in Zombwe compared to none in Mikalango had plasma selenium concentrations < 0.82 µmol/L.

Table 2: Intakes of iron, zinc, selenium, phytate, molar ratios of phytate: zinc, aluminium, and titanium based on analyzed diet composites for Malawian women with a low- plasma selenium (Zombwe) and a high-plasma selenium (Mikalango) status

	Zombwe (n=55) Median (1,3rd Q)	Mikalango (n=58) Median (1,3rd Q)	P[1]
Iron (mg/d)	16.6 (10.9, 26.0)	29.6 (15.9, 49.3)	<0.001
Zinc (mg/d)	4.8 (3.5, 6.4)	6.4 (4.4, 8.5)	0.003
Selenium (µg/d)	6.6 (4.5, 10.1)	55.3 (31.4, 89.8)	<0.001
Phytate (mg/d)	846 (525, 1197)	1564 (1094, 2236)	<0.001
Phytate:zinc[2]	17 (14,20)	24 (20,31)	<0.001
Aluminium (mg/d)	15.6 (11.3, 27.6)	16.7 (10.0, 29.3)	0.827
Titanium (mg/d)	0.18 (0.12, 0.31)	0.32 (0.22,0.44)	<0.001

(1,3rd Q): 1st, 3rd Quartile

[1]Mann Whitney-U test

[2]Molar ratiovery complications as a result of cephalo-pelvic disproportions [31

The prevalence of inadequate intakes of zinc was 54% in Mikalango EPA and 89% in Zombwe EPA, based on an EAR set for unrefined cereal-based diets [20].

Table 3: Plasma biomarkers, hemoglobin and mean cell volume for Malawian women with a low-plasma selenium (Zombwe) and a high-plasma selenium (Mikalango) status

	Zombwe	Mikalango	
	Mean (95% CI)	Mean (95% CI)	p
Zinc, µmol/L[1,4]	8.60 (8.22, 8.99)	8.87 (8.55, 9.19)	0.289[1]
Zinc < 10.7 µmol/L, n (%)[5]	55/60 (92%)	57/60 (95%)	0.464
Selenium, µmol/L[1,4]	0.72 (0.68, 0.75)	1.60 (1.52, 1.68)	<0.001
Selenium < 0.82 µmol/L[5], n (%)	49/60 (82%)	0/60 (0%)	<0.001
Hemoglobin, g/L[1,4]	138.9 (132.4,145.4)	132.8 (128.6, 137.0)	0.119
Hemoglobin < 120 g/L[5], n (%)	14/60 (23%)	11/59 (19%)	0.530
MCV, fL[1,4]	90.2 (87.6, 92.9	85.3 (82.3, 87.9)	0.009
MCV < 82 fL[5], n (%)	14/60 (23%)	24/59 (41%)	0.042
Ferritin, µg/L[2,3]	38.6 (30.6, 48.7)	26.3 (20.5, 33.8)	0.027
Ferritin < 12.0 µg/L[5], n (%)	5/60 (8%)	11/56 (20%)	0.078
Body iron (mg/kg)[1,4]	5.3 (4.3, 6.4)	3.8 (2.7, 4.9)	0.046
Body iron<0 mg/kg[5], n (%)	6/60 (10%)	8/56 (14%)	0.479
CRP, mg/L[2,4]	1.6 (1.1, 2.4)	0.7 (0.5, 1.1)	0.004
CRP > 5 mg/L[5], n (%)	13/60 (22%)	8/60 (13%)	0.230
AGP, g/L[2,4]	0.5 (0.5, 0.6)	0.6 (0.5, 0.6)	0.326

AGP >1 g/L[5], n (%)	6/60 (10%)	4/59 (7%)	0.527

MCV: Mean cell volume; CRP: C-reactive protein; AGP: α-1-acid glycoprotein

[1]Values are arithmetic means

[2]Values are geometric means

[3]Ferritin values are adjusted for inflammation [24]

[4]Students t-test

[5]Fisher's exact test

Table 4: Prevalence of depleted iron stores, iron deficiency, iron deficiency anemia, iron sufficiency, and non-iron deficiency anemia for Malawian women with a low- plasma selenium (Zombwe) and a high-plasma selenium (Mikalango) status

Stages of iron deficiency	Zombwe n (%)	Mikalango n (%)	p[2]
Storage iron depletion[1,2]			
Ferritin < 12 ug/L+ Hb ≥ 120 g/L	2/60 (3%)	5/55 (9%)	0.269
Iron deficiency [1,2]			
Body iron<0 mg/kg + Hb ≥ 120 g/L	4/60 (7%)	2/55 (4%)	0.683
Iron deficiency anemia[1,2]			
Body iron<0 mg/kg + Hb < 120 g/L	2/60 (3%)	5/55 (9%)	0.269
Iron sufficient (non-anemic)[1,2]			
Body iron >0 mg/kg + Hb > 120g/L	42/60 (70%)	43/55 (78%)	0.699
Non-iron deficiency anemia[1,2]			
Body iron >0 mg/kg + Hb < 120g/L	12/60 (20%)	5/55 (9%)	0.196

Hb: Hemoglobin

[1]Ferritin values are adjusted for inflammation [24]

[2]Fisher's exact test

Interrelations among biomarkers and socio-demographic variables

Significant Spearman's rank correlations were observed between values for all the women for adjusted log ferritin and hemoglobin (r=0.245, p=0.008), log sTfR and hemoglobin (r=−0.320; p=0.005), adjusted total body iron and hemoglobin (r=0.388, p < 0.001), adjusted log ferritin and log sTfR (r=−0.554, p<0.001), and plasma zinc and hemoglobin (r=0.291, p=001). No significant relationships were observed between plasma biomarkers and the socio-demographic or anthropometric characteristics of the women

In view of the very large differences in the plasma selenium concentrations in the two EPAs [9], associations between plasma selenium and other biomarkers were examined separately for each EPA. In the low-selenium Zombwe EPA, the Spearman's rank correlation between plasma selenium and zinc was positive (r=0.253, p=0.052, n=60), whereas in the high-selenium Mikalango EPA the correlation was negative (r=–0.273; p=0.035, n=60). Plasma selenium was not associated with any of the other biomarkers or the socio-demographic variables of the women in each EPA.

Determinants of plasma ferritin, total body iron, plasma zinc and hemoglobin

Plasma CRP (but not AGP) was a significant and positive determinant of unadjusted log ferritin (p=0.004; R^2=0.10) based on linear regression analysis. Neither age, household socio-economic status, education level of the respondent, BMI, or district were significant determinants of adjusted log ferritin or adjusted total body iron, and for plasma zinc when log CRP or log AGP was also included as an additional independent variable in the regression analysis. In the multiple regression model for hemoglobin, total body iron adjusted for inflammation had the largest standardized beta coefficient, followed closely by plasma zinc; neither log AGP, household SES score, age or district (high or low selenium status area) was significant (Table 5).

Table 5: Multiple linear regression analysis with hemoglobin as the dependent variable

Independent variable	β coefficient (95% CI)	Standardized β	p>t
Total body iron, mg/kg[1]	0.183 (0.095, 0.272)	0.354	0.000
Plasma zinc, µmol/L	0.007 (0.003, 0.011)	0.293	0.001
Log AGP, g/L	−0.642 (−1.478, 0.195)	−0.126	0.131
Household SES score	−0.090 (−0.325, 0.145)	−0.069	0.448
Age, y	−0.026 (−0.061, 0.009)	−0.126	0.148
District code	−0.682 (−1.482, 0.118)	−0.159	0.094

n=115, R^2 = 0.252

[1]Plasma ferritin adjusted for inflammation [24]

Discussion

A striking feature of the results is the very high prevalence of zinc deficiency which was independent of their selenium status, and the contrasting much lower prevalence of iron deficiency among these Malawian women. This result was unexpected in view of the low intakes of readily available heme iron from cellular animal protein and the high phytate : iron molar ratios in the diets. Furthermore, the multiple regression results with hemoglobin as the dependent variable emphasized that the zinc status of these women was almost as important as total body iron (mg/kg body weight) as a determinant of hemoglobin.

Zinc status

In this study the prevalence of inadequate intakes of zinc was high, especially among the women from Zombwe, suggesting that dietary-induced zinc deficiency most likely contributed at least in part to the high prevalence of zinc deficiency noted here, a finding consistent with earlier reports of rural Malawian pregnant women [15]. This finding is not unexpected as the diets of the women in both EPAs were low in zinc with high phytate-to-zinc molar ratios, and the inhibitory effect of phytate on zinc absorption in adults is now thought to be much larger than previously estimated [32]. Moreover, there is no evidence of an adaptive response to habitual high phytate intakes on zinc absorption [33]. Nevertheless, the predicted risk of zinc deficiency based on low plasma zinc concentrations was higher than predicted from zinc intakes. Several factors may account for this lack of concordance, including the existence of tropical enteropathy, which has been reported earlier to exacerbate risk of zinc deficiency among Malawian children [34].

The prevalence of low plasma zinc concentrations among the women was unexpectedly high in the two districts, with a difference of only 3% , despite their contrasting plant-available soil selenium concentrations. Our sample size was too small to detect whether this was a significant difference in the prevalence of zinc deficiency.

Iron status

Less than 15% of the women had iron deficiency (defined by total body iron <0 mg/kg), despite the high prevalence of zinc deficiency (i.e., > 90%) in both EPAs. Indeed, total body iron for the Zombwe women, unlike the women in Mikalango EPA, was very comparable to that for US women of child bearing age (20 to 49 y) in the US National Health and Nutrition Examination Survey 2003−2006 [35]. A lack of concordance between the prevalence of iron and zinc deficiency has also been reported earlier among pregnant women in Ethiopia whose diets, like those reported here, contained negligible amounts of readily absorbable heme iron but high levels of non-heme iron [3]. Contaminant iron from soil was probably responsible, at least in part, for the high iron content of the Malawian diets, as reported earlier in Ethiopia [36, 37]. This suggestion is supported by the high concentrations of aluminum and titanium in the analyzed diet composites, well recognized markers of soil contamination [38], and their positive correlation ($p<0.05$) with the iron concentration of the diet composites. These findings highlight the importance of assessing iron intakes from analyzed duplicate diet composites in this setting. In Malawi, iron contamination from soil may be from cereals being threshed under the hooves of cattle. During threshing, soil adheres to the outer surface of the cereal grains, especially those with small grains such as sorghum and millet, the major cereals consumed in the Mikalango EPA .

The low prevalence of depleted iron stores, especially among the Zombwe women (i.e., 3%), even after adjusting ferritin for inflammation, suggests that at least some of the contaminant iron may have joined the common nonheme iron pool (i.e. is exchangeable) and thus been available for absorption as postulated in Ethiopia [36], although we recognize that predicting the exchangeability of contaminant iron is difficult [39]. Moreover, the low prevalence of iron deficiency anemia overall (i.e., 6 %) suggests that the few women identified with low iron

stores may have upregulated iron absorption from their predominantly cereal-based diets to preserve hemoglobin mass.

The women in the high selenium EPA – Mikalango – had significantly higher intakes of total iron compared to those in the low selenium EPA, yet significantly lower ($P < 0.05$) adjusted values for plasma ferritin and total body iron (Tables 2 and 3). Several factors may account for this discrepancy, including the significantly higher phytate (Table 2), and probably higher polyphenol content of the finger millet and brown sorghum consumed in Mikalango, compared to the maize in Zombwe, which has a low polyphenol content [40]. Both phytate and polyphenols inhibit absorption of the non-heme iron intrinsic to food as well as any exchangeable contaminant iron [1, 39]. Consumption of polyphenol-containing tea was not an additional inhibitory factor because the women in Mikalango consumed less tea than those in Zombwe.

It is also possible that the proportion of exchangeable contaminant iron was lower in the diets in Mikalango than Zombwe due to differences in soil mineralogy. Mikalango EPA is characterized by areas of calcareous Eutric Vertisols (median pH = 7.8), whereas in Zombwe EPA soils have a low pH (median 5.2) [9]. Certainly, the proportion of soil contaminant iron that is available for absorption is said to vary widely [39]. Reduction in iron absorption as a consequence of inflammation [41] is unlikely to be responsible for the discrepancy because there were no differences in the prevalence of elevated concentrations of CRP and AGP in the two EPAs.

Determinants of plasma ferritin, total body iron, hemoglobin, and plasma zinc

We were unable to explore the role of the dietary intakes as determinants of iron, hemoglobin, or zinc status of the women in our multiple regression models because only one-day diet composites were collected. Of the dependent variables investigated, only significant determinants of hemoglobin were identified. These were adjusted total body iron and plasma zinc, with plasma zinc being almost as important as total body iron (based on the absolute values of the standardized ß-coefficients). Furthermore, there was no significant correlation linking these two independent variables (i.e., total body iron and plasma zinc) (data not shown), confirming that a comparable distribution of iron and zinc in the food supply was not responsible for the positive association between zinc and hemoglobin noted here.

Both dietary and non-dietary factors probably contributed to the positive relationship observed here between adjusted total body iron and hemoglobin. As emphasized earlier, total iron intakes were unexpectedly high, in part due to contaminant iron from soil, although the extent to which the contaminant iron is exchangeable is uncertain. Consequently, we did not calculate the prevalence of inadequate intakes of iron.

It is of interest that 15% of the cases of anemia overall among these Malawian women were not attributable to iron deficiency (Table 4). Other factors, not investigated in this study, that have been reported in earlier studies to play a role in anemia in Malawi include deficiencies

of vitamin B-12 and vitamin A [42], as well as malaria, glucose-6-phosphate dehydrogenase deficiency (G6PD), α-thalassemia, and sickle cell disease [43]. Most of these factors were not investigated in this study with the exception of macrocytosis (i.e., mean cell volume > 99 fL) [44], a characteristic of vitamin B-12 deficiency, which was seen in 8% of the women.

Plasma zinc was found to be an important determinant of hemoglobin in an earlier study of pregnant Ethiopian women [3]. Several mechanisms exist whereby zinc has a role in hemoglobin concentrations. For example, hemoglobin synthesis depends on several zinc-dependent enzyme systems, specifically aminolevulinic acid dehydrase [45] and thymidine kinase and DNA polymerase [46], whereas a zinc finger transcription factor-- GATA-1-- is essential for normal hematopoiesis [47]. Zinc may also play a role in stabilizing red cell membranes and in increasing plasma IGF-1 levels, which may in turn stimulate erythropoiesis [48].

Despite the tendency for plasma zinc and selenium to be positively correlated among women in the low selenium district of Zombwe and negatively correlated in the high selenium district of Mikalango, district was not a significant determinant of plasma zinc in our multiple regression model. The reason for these findings is uncertain.

Strengths and weaknesses of study

We believe this study has several strengths. The analysis of the duplicate diet composites improved the estimates of trace element and phytate intakes, and confirmed the presence of contaminant iron from soil, although because the diet composites were based on only one day, the data could not be included in the multiple regression models. Furthermore, our estimates for the prevalence of inadequate zinc intakes were calculated from the distribution of usual intakes, whereas that of iron deficiency was based on total body iron adjusted for inflammation to provide a more accurate reflection of the true prevalence and severity of nutritional iron deficiency [49]. Finally, analysis of plasma zinc and selenium as well as multiple iron biomarkers allowed the assessment of inter-relationships among these micronutrients in Malawian women, which has not been possible in earlier reports.

Nevertheless, we were not able to assess the presence of malaria parasitemia or genetic hemoglobin disorders, all of which also have been implicated in anemia in Malawi [43] and also have the potential to confound total body iron assessment by elevating sTfR in response to an increased rate of erythropoiesis [49,50]. Further, we used the correction factors of Thurnham et al. [24] to adjust for the effect of subclinical inflammation on ferritin and body iron because our sample size was too small to yield reliable estimates based on internally generated correction factors.

We also recognize that our results are based on a convenience sample of women and therefore not representative of women of child-bearing age living in Zombwe and Mikalango EPAs of Malawi. However, in an effort to minimize bias, women from six villages in Zombwe EPA and six villages in Mikalango were approached, all of whom were eligible and agreed to participate in the study. A further limitation is that our small sample size may have accounted

for our inability to both detect any difference in the prevalence of low plasma zinc among the women in the two districts and explain none of the variance in ferritin, total body iron, and plasma zinc concentrations. Nonetheless, some of the nutritional status variables reported here were comparable to those reported in the same geographical areas in the 2010 Malawian Demographic and Health Survey [51]. For example, in Zombwe and Mikalango EPAs, both the mean BMI (Zombwe, 22.4 vs. 22.5; Mikalango, 21.6 vs. 22.5) and the proportion with BMI <18.5 (Zombwe, 5.6 vs. 10%; Mikalango, 7.8 vs. 15% were reasonably comparable to those reported here. Likewise, for Zombwe, the prevalence of anemia in the DHS survey was similar (26 vs. 23%), although higher for the DHS survey in Mikalango (37%) than that reported in this study (19%). Finally, because our study was observational, we cannot exclude the possibility of unmeasured confounding.

Conclusion

Risk of zinc deficiency among these Malawian women was much higher than that of iron deficiency in both districts. This finding was attributed to diets low in absorbable zinc but with a high total iron content due in part to contaminant iron from soil. We were unable to show any differences in the prevalence of zinc deficiency among the women living in districts with low and high plant-available soil selenium concentrations. Plasma zinc was almost as important as total body iron as a determinant of hemoglobin. Our findings suggest that at least some of contaminant iron from soil was exchangeable and thus available for absorption, and highlight the utility of analyzing diet composites to assess total iron intakes in this setting.

Acknowledgements

We wish to thank all the participants, the staff of the Ministry of Health and the Ministry of Agriculture and Food Security in Malawi, and the Malawian research assistants for their assistance. Author contributions to this study were funded primarily by a Partnership and Project Development Award (NE/1003347/1) from the UK National Environment Research Council (NERC), the UK Department for International Development (DFID), and the Economic and Social Research Countil (ESRC) under the Ecosystems Services for Poverty Alleviation (ESPA) scheme. Additional funding was provided by the Universities of Otago, East Anglia, and Nottingham, and the British Geological Survey.

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Appendix D. Consort checklist

	CONSORT 2010 checklist of information to include when reporting a randomised trial*		
Section/Topic	**Item No**	**Checklist item**	**Reported on page No**
Title and abstract			
	1a	Identification as a randomised trial in the title	n/a
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	n/a
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	72-101
	2b	Specific objectives or hypotheses	102
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	103; 105-106
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	n/a
Participants	4a	Eligibility criteria for participants	104-105
	4b	Settings and locations where the data were collected	107
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	110-111 115-116
Outcomes	6a	Completely defined pre specified primary and secondary outcome measures, including how and when they were assessed	117-120
	6b	Any changes to trial outcomes after the trial commenced, with reasons	n/a
Sample size	7a	How sample size was determined	120
	7b	When applicable, explanation of any interim analyses and stopping guidelines	n/a
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	109
	8b	Type of randomisation; details of any restriction such as blocking and block size)	n/a
Allocation Concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered	109

		containers), describing any steps taken to conceal the sequence until interventions were assigned	
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	107-109
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	105
	11b	If relevant, description of the similarity of interventions	106
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	120-121
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	n/a
Results			
Participant flow (a diagram is Strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	103
	13b	For each group, losses and exclusions after randomisation, together with reasons	103
Recruitment	14a	Dates defining the periods of recruitment and follow-up	106
	14b	Why the trial ended or was stopped	n/a
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	123
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	129
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	120-121; 129
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	129
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	129
Harms	19	All important harms or	n/a

		unintended effects in each group (for specific guidance see CONSORT for harms)	
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	131; 133-134
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	134
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	131-134
Other			
Registration	23	Registration number and name of trial registry	102
Protocol	24	Where the full trial protocol can be accessed, if available	102
Funding	25	Sources of funding and other support (such as supply of drugs)	73

Appendix E. List of presentations

Oral presentations given:

- 'Enhancing the availability of minerals used to fortify food products', Department of Nutrition Department Meeting, University of East Anglia, 26.11.2010
- 'Exploring iron availability using an in vitro Caco-2 cell model' Kellogg's Iron Day, Manchester, 17.08.2011
- 'Estimation of iron status in Malawi', Seventh DRINC Dissemination Event 12-13.10.2011

Poster presentations given:

- Joint poster with IFR 'Enhancing delivery of minerals using multifunctional carriers – II' prepared for Forth DRINC dissemination event, 27-28th April 2010.
- Poster 'Interactions between iron and alginates in Caco-2 cell model' presented at Bioavailability 2010 conference 26-30th September and Fifth DRINC dissemination event 20-21st October 2010.
- Joint poster with IFR 'Preparation of multifunctional carriers for the site directed delivery of iron' (co-author) prepared for Sixth DRINC Dissemination Event, 13-14th April.2011.
- Poster 'Challenges of estimating iron status in Malawi' PGR student conference, University of East Anglia, Norwich, 29th March 2012
- Poster 'Study to measure the absorption of iron from ferrous gluconate incorporated into alginate beads' prepared for clinical trials day at NNUH, 20th May 2012
- Joint poster with IFR 'Release and absorption characteristics of multifunctional carriers for the site-directed delivery of iron' prepared for 8th DRINC Dissemination event, 15-16th May 2012.