EFFECTS OF CARBOHYDRATES ON IRON METABOLISM IN INTESTINAL AND LIVER CELLS

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Declaration

"I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of the PhD being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised another's work."

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Abstract

EFFECTS OF CARBOHYDRATES ON IRON METABOLISM IN INTESTINAL AND LIVER CELLS

Iron deficiency and excess are worldwide public health problems. Studies suggest that carbohydrates such as fructose, and oligo-and polysaccharides (prebiotics), increase iron bioavailability, but results are inconclusive. There is also interest in whether iron and fructose contribute to the pathogenesis of colorectal cancer (CRC), hepatocellular carcinoma (HCC) and non-alcoholic fatty liver disease (NAFLD). Intake of sugars has increased in the past three decades, as has the prevalence and incidence of HCC and NAFLD, while CRC remains one of the top three occurring malignancies in the developed world, making these questions particularly relevant at this time. The aims of this thesis were to study iron-carbohydrate effects on: non-haem iron bioavailability, and on the expression of genes and proteins related to CRC, HCC and NAFLD. The principal carbohydrates investigated were fructose, the related sweetener high fructose corn syrup (HFCS), and fructo- and galacto-oligosaccharide prebiotics (FOS&GOS, respectively).

It was hypothesised that fructose, HFCS, and FOS&GOS would increase non-haem iron bioavailability, and that iron and fructose would alter expression of genes and proteins related to the pathogenesis of CRC, HCC and NAFLD.

Two human in vitro cell lines were used to investigate these questions: Caco-2 and HepG2 cells, models of the small intestine and liver, respectively. The Caco-2 in vitro digestion model was used to examine fructose, HFCS, and FOS&GOS effects on iron bioavailability with ferritin formation as a surrogate marker for iron uptake. Ferritin formation was also utilised to assess iron uptake in HepG2 cells. Expression of iron homeostasis proteins was analysed by Western Blot and Polymerase Chain Reaction (PCR) to explore biological mechanisms underlying observed effects on iron bioavailability. Lastly, Caco-2 and HepG2 cells were treated with iron and fructose, and the effects on selected genes and proteins involved in CRC, HCC and NAFLD development were evaluated through microarray, PCR and Western Blot analysis.

Results indicated that fructose in a water-based matrix with added ferric iron ($FeCl_3$) significantly increased ferritin formation in Caco-2 and HepG2 cells by 40 and 35 %, respectively, in comparison with iron alone treated cells; this effect was negated in the Caco-2 cell line by phytates and polyphenols at 1:5 and 1:1 iron:inhibitor molar ratios, respectively. Fructose treatment alone did not significantly increase ferritin formation in either cell line. Fructose combined with $FeCl_3$ in a pH 7 water-based matrix significantly increased ferrozine-chelatable ferrous iron levels by 320 % in comparison with $FeCl_3$ alone. Two liquid ferrous iron supplements with added fructose had significantly higher ferritin compared with dissolved ferrous sulphate tablets; Spatone Apple®, the supplement that had the highest fructose to iron molar ratio (62:1), had the highest iron

bioavailability with ferritin levels 610 % higher compared with ferrous sulphate alone, although it is important to note that it also had the highest concentration of ascorbic acid. Fructose added to ascorbic acid and $FeCl_3$ in a water-based matrix had an additive effect on ferritin formation in Caco-2 cells, significantly increasing ferritin two-fold compared with $FeCl_3$ and ascorbic acid alone. A high-fructose containing sweet potato (SP) infant complementary food with 240 % more fructose compared with a low fructose SP food had significantly higher ferritin levels by 30 %, but ferritin was still relatively low compared to a commercial weaning food (50 % less), possibly secondary to high levels of phytates and polyphenols in the SP-based foods. Lastly, a mixture of exogenous FOS&GOS prebiotics added to Young Child Formulae (YCF, milk-based products for toddlers) increased ferritin formation by approximately 25 %, and eliminated significant differences in ferritin levels in comparison to YCF with manufacturer added FOS&GOS. Gene and protein expression analysis did not support the hypothesis that the observed increase in fructose-induced ferritin was due to changes to iron transporter or homeostasis molecules. Caco-2 and HepG2 cell gene and protein analysis demonstrated significant changes in pathways related to CRC, HCC and NAFLD. Key significant findings of iron and fructose cell treatments were: 1.5 fold or greater changes in HepG2 gene expression of SMADs 2 and 3, STAT3 and NF-κβ -- signalling proteins implicated in development of inflammatory liver disease; increased HepG2 mRNA expression of cell cycle related genes, Cyclin D1 and Cyclin D2, and the proto-oncogene Skp2; decreased mRNA expression of HNF4A, a key liver transcription factor, but with increased HNF4A protein expression; increased HepG2 mRNA and protein expression of the "cancer chaperone" Heat Shock Protein 90; and significantly decreased mRNA expression of the tumour suppressor gene APC by 1.3 fold in iron alone treated Caco-2 cells.

In conclusion, fructose, HFCS, and a mixture of prebiotics increased iron bioavailability as assessed by ferritin formation in an in vitro intestinal cell model. The observed effects were negated by phytates and polyphenols in a water-based matrix, and also in a complex food matrix (sweet potato-based) containing high levels of these inhibitors, indicating that these carbohydrates would not increase gut iron uptake when eaten as part of a mixed diet containing polyphenols and phytates. Iron/mineral supplements with added fructose and ascorbic acid in a water-based matrix were associated with higher available iron in comparison with ferrous sulphate alone; fructose had an additive effect with ascorbic acid suggesting that iron supplement bioavailability could be improved with the addition of fructose. In a milk-based food matrix added prebiotics FOS&GOS significantly improved available iron suggesting that a mixture of FOS&GOS prebiotics could improve the nutritional benefits of YCF in relation to iron deficiency. Fructose added to iron solutions also increased ferritin in an in vitro hepatocyte cell model. Fructose-induced ferritin increases did not appear to be secondary to altered expression of iron homeostasis molecules, but rather to changed iron oxidation state to the more bioavailable ferrous form. Changes observed in genes and proteins associated with CRC, HCC and NAFLD with iron and fructose treatments related to multiple pathways implicated in these diseases, and require further study. All of the above were obtained in vitro, therefore *in*

vivo confirmation of these findings is required for further evaluation of their impact on human health and disease.

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List of Key Abbreviations

AA	Ascorbic Acid
ACD	Anaemia of Chronic Disease
APC	Adenomatous Polyposis Coli
a.u.	Arbitrary unit
CCAAT	Cytidine-cytidine-adenosine-adenosine-thymidine
cDNA	Complementary Deoxyribonucleic acid
CRC	Colorectal Cancer
CREBH	cAMP Responsive Element-Binding Protein
CP	Caeruloplasmin
CFSP	Cream Flesh Sweet Potato
Dcytb	Duodenal cytochrome b
DFO	Deferoxamine
DMEM	Dulbecco's modified eagles minimal essential medium
DMT1	Divalent metal transporter 1
EPO	Erythropoietin
ERS	endoplasmic Reticulum Stress
FAC	Ferric ammonium citrate
FBS	Foetal Bovine Serum
Fe	Iron
FOS	Fructo-oligosaccharides
FPN	Ferroportin
GO	Gene ontology
GOS	Galacto-oligosaccharides
Hb	Haemoglobin
HCC	Hepatocellular Carcinoma
HFE	Gene mutated in Hereditary Haemochromatosis
HFE2	Alternative name for gene for HJV
HH	Hereditary Haemochromatosis
HIF	Hypoxia-inducible transcription factor
HJV	Hemojuvelin
HNF4A	Hepatocyte Nuclear Factor 4, Alpha
HO	Haem oxygenase
HP	Hephaestin
HRE	Hypoxia responsive element
HRP	Horseradish peroxidase
HSP90AB	Heat Shock Protein 90
ID	Iron deficiency
IDA	Iron Deficiency Anaemia
IL6	Interleukin-6
IRE	Iron responsive element
IRP	Iron regulatory protein
IP	Inositol Phosphate
JAK2	Janus Kinase 2
JH	Juvenile haemochromatosis
LIP	Labile iron pool
LPS	Lipopolysaccharide

MFP	Meat, Fish, Poultry
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAFLD	Nonalcoholic Fatty Liver Disease
NTBI	Non-transferrin bound iron
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells /Nuclear Factor κ Beta
PA	Phytic Acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
rt-PCR	reverse transcription-polymerase chain reaction
PVDF	Polyvinylidene difluoride
OFSP	Orange Flesh Sweet Potato
RNA	Ribonucleic acid
RPM	Revolutions per minute
RBC	Red blood cell
RES	Reticuloendothelial system
ROS	Reactive Oxygen Species
SDS	Sodium dodecyl sulfate
SF	Serum Ferritin
SNP	Single Nucleotide Polymorphism
sTfR	Soluble or Serum Transferrin Receptor
SLC11A	Solute carrier family 11 member
SMADs	Mothers against decapentaplegic homologs
STAT3	Signal transducers and activators of transcription
STEAP	Six-transmembrane epithelial antigen of the prostate
TA	Tannic Acid
TCF7L2	Transcription Factor Seven Like 2
Tf	Transferrin
TfR1	Transferrin receptor one
TfR1	Transferrin receptor two
TGFβ	Transforming growth factor beta
TGFβR2	Transforming growth factor beta receptor 2
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
T2DM	Type Two Diabetes Mellitus
UPR	Unfolded Protein Response
UTR	Untranslated region
WNT	Combination Wingless and Int
WHO	World Health Organization

Publications and Conference Presentations

Original Research Articles

Christides, Ganis and Sharp. In vitro assessment of iron availability from commercial Young Child Formulae supplemented with prebiotics**.** Manuscript accepted for publication to the *European Journal of Nutrition*.

Loughrill, **Christides** and Zand (2016). Vitamin D and the Calcium to Phosphorus ratio of infant foods in the UK: Possible implications on bone development in children. Manuscript accepted for publication in *Maternal and Child Health Journal.*

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Christides and Sharp (2013). Action of Iron and Fructose on gene expression in Human Liver Cells. Presented (Christides) at the 5th International Bioiron Annual Meeting, London, UK.

Milligan, Bridges and **Christides** (2012). Audit of vitamin D intake in a nursing home (NH) for the elderly mentally infirm (EMI). Presented (Milligan) at the 2012 Nutrition Society Winter Meeting jointly with the Royal Society of Medicine. Published in the *Proceedings of the Nutrition Society* 71 (OCE3), E233.

Christides and Sharp (2012). Sugars increase non-heme iron bioavailability in human intestinal epithelial cells. Presented (Christides) at the European Iron Club Annual meeting 2012, University of Leuven, Leuven, Belgium.

Allgrove, Chapman, **Christides**, Tanner, and Smith (2010). The impact of upper body exercise on immunoendocrine responses in physically disabled athletes. Presented (Allgrove) at the European College of Sport Science in Atalaya, Spain, 2010.

CHAPTER ONE: INTRODUCTION

Iron is an essential nutrient required for oxygen transport, cellular energy generation and over 200 enzyme reactions in human beings, however excess iron is toxic; iron homeostasis is consequently highly regulated. Despite this, a significant number of people suffer from either iron deficiency or overload, and both these conditions have significant health sequelae.

Iron deficiency is a global problem even though iron is one of the most plentiful elements on earth. This paradox arises, in part, because in its most abundant forms iron is poorly bioavailable - bioavailability is the proportion of a nutrient from the diet that can be digested, absorbed and metabolised (Wienk, Marx et al. 1999).

Poor iron bioavailability may partly explain another iron paradox – that iron overload disorders are also a global problem (Fleming and Ponka 2012). The most common primary cause of iron overload in the Western world is Classic Hereditary Haemochromatosis (HH), and it has been suggested that in the early reproductive years HH confers a selective advantage because it produces resistance to dietary iron deficiency (Pietrangelo 2004, Ye, Cao et al. 2015), which may explain HH's persistence in the population. However, long-term excess iron causes cellular and end organ damage and malfunction. Even in the absence of absolute overload, disordered regulation or compartmentalization of intracellular iron causes pathology. For example, in the disease Friedreich's ataxia, a mutation in the iron handling protein frataxin that causes increased mitochondrial iron but without elevating total cellular iron levels, leads to progressive neurological and cardiac degeneration (Pandolfo and Pastore 2009). Total iron levels, location, and duration of overload determine toxicity.

The body has a complex system of proteins and enzymes that regulate iron balance. There is no active form of body iron excretion; iron homeostasis is primarily maintained by regulation of the proteins involved in iron absorption (Miret, Simpson et al. 2003). However, the amount of iron available in the gut to be absorbed by those proteins can affect iron status. Various dietary constituents interact biochemically with iron, and either enhance or decrease its bioavailability (via mechanisms that will be discussed later in this thesis). In addition, recent research suggests that dietary factors may also alter the expression of gastrointestinal iron transport/absorption proteins, and therefore directly change iron uptake flux (Thomas, Gaffney-Stomberg et al. 2013). Iron absorption and iron stores reflect the balance between systemic regulation of absorption and iron bioavailability

In summary, iron-related disorders are prevalent and have serious health implications, and diet impacts on iron status; it is therefore important to identify nutrients that affect iron bioavailability and consequently iron status in humans.

1.1: Iron biochemistry

Iron, a transition metal, typically has a coordination number - the number of ligands to which an element can bind - of six (Crichton 2009).

Iron may exist in several oxidation states, but the most physiologically relevant states are Fe^{2+} (ferrous iron, the reduced form) and Fe^{3+} (ferric iron, the oxidized form); under physiological conditions iron redox state can rapidly and reversibly change depending on pH, redox potential, and ligand binding (Crichton 2009). Iron's coordination chemistry and redox flexibility enable it to form complexes with many organic molecules and generate metallo-iron proteins involved in a wide range of metabolic reactions.

Iron's ability to easily alter its oxidation state is key to both its functionality in multiple redox reactions as an electron transporter, and it's potential toxicity through Fenton chemistry generated free radicals (Pierre and Fontecave 1999).The Fenton reaction occurs when hydrogen peroxide (H_2O_2), a common byproduct of cellular processes, reacts with Fe^{2+} to form Fe^{3+} and the highly reactive hydroxyl radical; hydroxyl radicals may attack and damage membrane lipids, cellular proteins and DNA (Crichton, Wilmet et al. 2002). Furthermore, the generated Fe^{3+} can then go on to react with superoxide radicals (common byproducts of cellular metabolic processes) in the cell to regenerate Fe^{2+} and perpetuate the cycle in a reaction known as the Haber-Weiss cycle (Pierre and Fontecave 1999) (Figure 1.1).

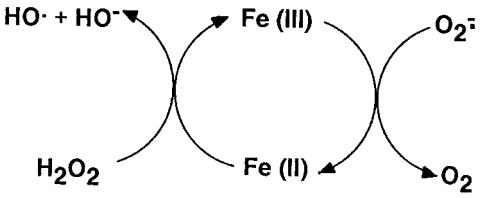

Figure 1.1 The Haber-Weiss Cycle including the Fenton Reaction.
The Fenton Reaction is the left side of the reaction. Small amounts of Fe^{2+} react with hydrogen peroxide and produce Fe^{3+} and the highly reactive hydroxyl free radical (·OH); Fe^{3+} in turn can react with the superoxide radical ($\cdot O_2^-$) and regenerate Fe^{2+} and the cycle is perpetuated (adapted from Pierre and Fontecave 1999).

Cells maintain "free" Fe^{2+} and Fe^{3+} at very low levels in order to protect against reactive oxygen species toxicity, but disturbed intracellular homeostasis or absolute iron excess may overload or over-ride these mechanisms leading to cell death and disease.

1.2: Dietary iron

Iron in the diet is mainly found in two forms – haem and non-haem iron. The iron storage protein ferritin may also be a dietary source of iron (especially from plants), although there is currently little data available about what percent of iron in the human diet is derived from this source.

1.2.1: Haem iron

Haem iron is iron complexed with porphyrin (a naturally occurring aromatic compound consisting of four modified pyrrole rings) and is derived predominantly from the metallo-iron proteins haemoglobin and myoglobin found in meat, poultry and fish (MFP).

1.2.2: Ferritin

Ferritin is the main iron storage protein in plants and mammals. It is a large protein consisting of peptide subunits arranged in a cage like structure around a central core of iron; ferritin can hold up to ≈ 4000 Fe^{3+} as iron hydroxide-phosphate complexes (Koorts and Viljoen 2007). The actual iron content of plant ferritin may vary depending on soil iron levels (Oikeh, Menkir et al. 2003, Zielinska-Dawidziak 2015). Mammalian ferritin iron content differs depending on host iron status, and cellular/organ location of the protein; human ferritin will be further discussed in section 1.4.1 of this thesis.

1.2.3: Non-haem iron

Non-haem iron consists of inorganic ferric or ferrous iron, bound to peptides, salts or organic acids (Miret, Simpson et al. 2003); it is the predominant dietary form of the mineral, and accounts for 70-90 % of dietary iron intake throughout the world – higher in developing countries and less in the developed world (Carpenter and Mahoney 1992). Non-haem iron is found in cereals, nuts, pulses, dark leafy green vegetables, and also in MFP (Hazell 1985).

1.3: Physiological roles of iron

Iron is a functional component in over 200 metallo-enzymes or cofactors; these molecules are amongst the most diverse assortment of metallo-complexes in biological systems (Frey and Outten 2011). Functions of iron include its role in: reversible oxygen transport and storage as part of haemoglobin and myoglobin, respectively; energy generation through electron transfer in the electron transport chain via the haem proteins cytochromes a and c and the iron-sulphur cluster enzyme succinate dehydrogenase; cell replication as a cofactor for the enzyme ribonucleotide reductase (RR); central nervous function as the co-factor for several enzymes used in the generation of neurotransmitters; and the innate immune system in the generation of hypochloric acid by the haem enzyme myeloperoxidase (Gkouvatsos, Papanikolaou et al. 2011). Table 1.1 lists some examples of iron-based proteins.

Table 1.1 Examples of Metallo-proteins and their functions (Geissler and Singh 2011).

Iron-containing protein	Function	Location	Iron content (mg)
Heme proteins			
Hemoglobin	Oxygen transport	Red blood cells	3,000
Myoglobin	Oxygen storage	Muscle	400
Heme enzymes		All tissues	c 30
Cytochromes a, b, c	Electron transfer Transfer of electrons to molecular oxygen at end of respiratory chain (also requires copper)		
Cytochrome C oxidase	Microsomal mixed function oxidases		
Cytochrome P450 + b_5	Phase I biotransformation of xenobiotics		
Dcytb	Ferrireductase (duodenal enterocytes)		
Catalase	Hydrogen peroxide breakdown		
Peroxidases	Peroxide breakdown		
Myeloperoxidase	Neutrophil bacteriocide		
Sulfite oxidase	Sulfites to sulfates		
Tryptophan 2,3-dioxygenase	Pyridine metabolism		
Iodase (iodoperoxidase)	Iodide to iodate		
Non-heme iron enzymes		All tissues	c 30
Ribonucleotide reductase	Ribonucleotides → 2'-deoxyribnucleotides Synthetic phase of cell division		
(Iron-sulfur proteins)			
Aconitase Isocitrate dehydrogenase Succinate dehydrogenase NADH dehydrogenase	Citric acid cycle and initial steps of oxidative phosphorylation		
Aldehyde oxidase	Aldehydes to carboxylic acids		
Xanthine oxidase	Hypoxanthine-uric acid		
Phenylalanine hydroxylase Tyrosine hydroxylase Tryptophan hydroxylase	Catecholamine, neurotransmitters, and melanin synthesis		
Prolyl hydroxylase Lysyl hydroxylase	Collagen synthesis, both dependent on ascorbic acid		

1.4: Iron metabolism

Iron metabolism is tightly regulated as a consequence of the metal's potential toxicity. In addition, iron use/re-use is efficient because iron is difficult to absorb, and therefore a scarce resource within the body.

1.4.1: Body Iron: levels, distribution and storage

Body iron is distributed amongst four conceptual compartments: absorption, transport, functional, and storage. Total body iron levels are on average 40 mg/kg in women and 50 mg/kg in men (Andrews and Schmidt 2007).

The majority of iron is in the functional compartment with approximately 70 % of body iron found within red blood cells and haemoglobin, and a smaller fraction in iron metallo-enzymes and myoglobin (Andrews and Schmidt 2007).

25 % of iron is in the storage compartment within the liver in the iron storage proteins ferritin and haemosiderin. Mammalian cytosolic ferritin consists of 24 subunits made up of light chains (L chains, important in maintaining protein structure), and Heavy chains (H chains) that are the site of the catalytic oxidase site that converts ferrous iron to ferric-oxohydroxide complexes (Arosio, Ingrassia et al. 2009). Exactly how iron is released from intracellular ferritin stores in response to iron needs is not clear and an area of active research.

A small percentage of ferritin is also found in the blood. The functional significance of serum ferritin, typically an iron-poor form of the protein, is unknown, although there is a constant mathematical relationship between serum and liver ferritin levels that allows serum ferritin levels it to be used, under normal physiological conditions, as a proxy measure of body iron stores (Wang, Knovich et al. 2010). Haemosiderin, the other iron storage protein, is a breakdown product of ferritin normally found in only small amounts in the liver and reticuloendothelial system (RES); in iron overload conditions haemosiderin level increase dramatically (Miyazaki, Kato et al. 2002).The function of haemosiderin is unknown, and it is not clear if iron can be retrieved from haemosiderin although some older studies suggest that it is an available iron form (Shoden, Gabrio et al. 1953).

The liver is defined as the main storage organ of iron, but this is only true in the passive/inert sense of storage; recycling macrophages of the RES of the liver and spleen phagocytize senescent red blood cells (RBCs), degrade their haemoglobin, and subsequently store the derived iron, to be released as needed (Knutson and Wessling-Resnick 2003). Recycling macrophages provide the majority of iron for body needs exceeding both gut and liver contributions (Ganz 2011). This is a dynamic part of the storage compartment with rapid fluxes; at any specific time point absolute amounts of stored iron in the RES are relatively small, but some 20 mg/day of iron are recycled and

used through this system, in contrast with the 1-2 mg/day absorbed through the gut (Ganz 2011).

The iron transport compartment primarily consists of transferrin – the major blood iron transporter (Gkouvatsos, Papanikolaou et al. 2011). Transferrin is a glycoprotein composed of one polypeptide chain folded into a bilobal structure; each "lobe" of the protein is able to bind one molecule of ferric iron - thus the whole molecule can carry two iron atoms ((Aisen, Leibman et al. 1978). Holotransferrin refers to the fully iron loaded form; apotransferrin is iron-free. Absolute levels of transferrin bound iron are low (approximately 30 μmol/L), but like the RES this is a high flux system and a large amount of iron goes through the transferrin cycle (Gkouvatsos, Papanikolaou et al. 2011).

The body passively loses 1-2 mg of iron/day through sweat, hair loss, and epithelial cell sloughing primarily in the gut and urinary systems and, in women, menstrual blood losses. All the above losses are replenished through gut absorption. Figure 1.2 shows the iron cycle through the different compartments.

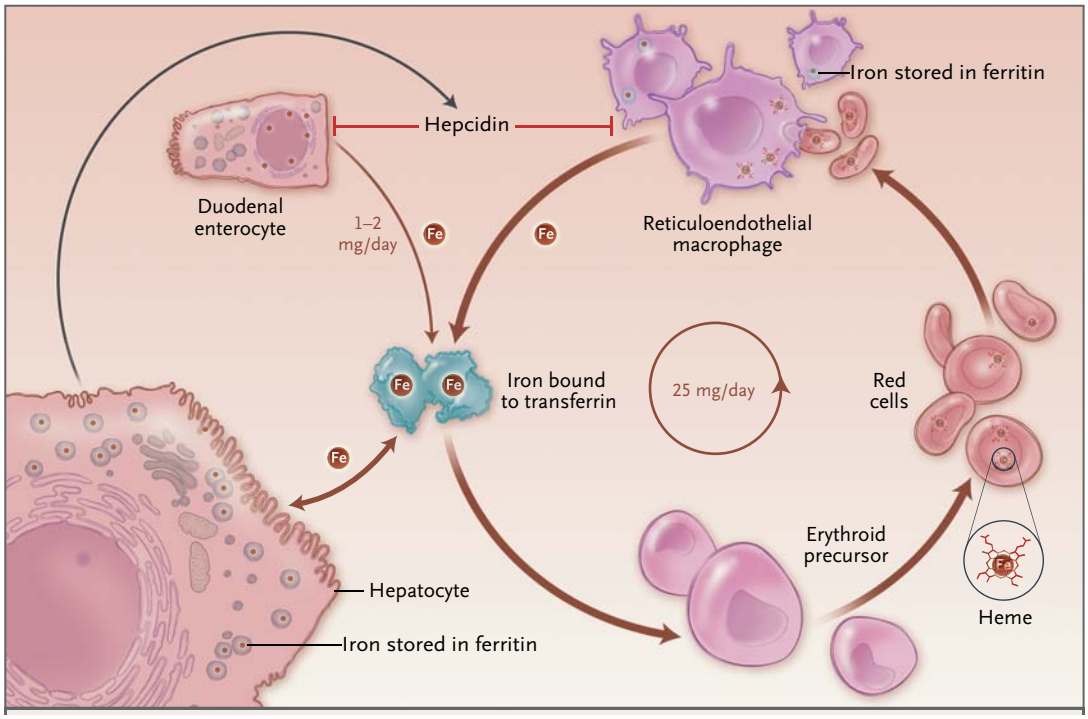

Figure 1.2 The iron cycle.
1-2 mg of iron is absorbed by enterocytes to replenish daily losses. This iron is transported by transferrin through the blood, and taken up primarily by the erythroid cells where it is incorporated into haem, and secondarily into storage cells of the reticuloendothelial system (RES) and liver. Storage iron can be released from RES or liver cells as needed for diverse metabolic functions -figure from (Fleming and Ponka 2012).

Iron deficiency (ID) increases gut iron absorption, but there is considerable genetic variation in the amount of adaptation possible and absolute absorption does not exceed approximately 40 % of available iron at best (Mathers, Meplan et al. 2010); absorption

therefore may become the limiting factor in maintaining adequate body iron status. Conversely, if regulation of absorption is disordered and iron absorbed despite normal stores/need, overload may result. It is within the absorption compartment that dietary factors can affect iron homeostasis.

1.4.2: Absorption

The duodenum is the primary site of dietary iron absorption (Wheby and Crosby 1963). Iron is taken up through enterocytes, the absorptive epithelial cells of the gastrointestinal system. Iron absorption is favored in a low pH environment (for reasons which will be discussed further on); the duodenum has the lowest pH of the small intestine, which facilitates iron absorption.

1.4.2.1: Haem iron

Haem and non-haem iron are absorbed through different pathways; mechanisms and regulation of haem iron absorption have not been fully characterized (Andrews and Schmidt 2007). Haem iron is postulated to enter the enterocyte either via endocytosis or its own specific carrier, or possibly both (Cremonesi, Acebron et al. 2002, West and Oates 2008). Haem Carrier Protein 1 or HCP1, an intestinal membrane transporter that transports haem, was identified in 2005 (Shayeghi, Latunde-Dada et al. 2005). Further work, however, has found that this protein primarily transports folate (Qiu, Jansen et al. 2006), and research continues on identifying other enterocyte haem transporters. Haem absorption is less tightly regulated compared with non-haem iron (Hunt and Roughead 2000, Roughead and Hunt 2000). Calcium is the only known dietary compound that decreases haem iron bioavailability (Hallberg, Brune et al. 1991, Hallberg, Rossander-Hulten et al. 1992, Hallberg, Rossander-Hulthen et al. 1993) although two recent in vitro studies suggest that polyphenols may decrease haem-iron absorption by forming intracellular iron-polyphenol complexes that subsequently can't egress from the enterocyte (Ma, Kim et al. 2010, Ma, Kim, et al. 2011).

Once haem has entered the enterocyte it is broken down within cell endosomes by haem oxygenase, and the iron released from the protein enters the cell to join the non-haem iron pool (Andrews and Schmidt 2007). The recent discovery that Feline Leukemia Virus C1 Receptor-related protein (FLVCR1), a haem-iron exporter, is expressed on the basolateral/blood surface of duodenal enterocytes, raises the possibility that some haem-iron may be transferred intact into the circulation, although at this time the physiological significance of this pathway is unknown (Keel, Doty et al. 2008).

1.4.2.2: Non-haem iron

In comparison with haem iron, more is known about the absorption of inorganic iron. Non-haem iron absorption is dependent on iron oxidation state and solubility; inorganic iron must be soluble and in the Fe^{2+} form to be absorbed by enterocytes (Miret, Simpson

et al. 2003). Non-haem iron in food is mainly in the Fe^{3+} form; reduction of Fe^{3+} may therefore be considered the first step of absorption. Fe^{3+} reduction is enhanced by low pH, gastric acid, and gut ascorbic acid derived from co-ingested foods. In addition, there is a luminal membrane-bound ferric reductase, Duodenal cytochrome b (Dcytb)(McKie, Barrow et al. 2001) that reduces iron to the bioavailable Fe^{2+} form. Dcytb levels increase in response to iron deficiency, hypoxia and increased erythropoiesis, and increased expression of Dcytb is associated with increased iron absorption in cell culture models (McKie 2008); Dcytb's essentiality to human iron absorption, however, is not established. No human Dcytb mutations with associated disease phenotype have been documented in healthy human beings, and mouse targeted deletion studies of Dcytb don't lead to disordered iron status (Gunshin, Starr et al. 2005). However, the extrapolation of results from mouse studies - animals that can synthesise ascorbic acid- to human beings for whom ascorbic acid is an essential nutrient, requires careful interpretation. Interestingly, a recently identified single nuclear polymorphism (SNP) Dcytb variant, with lower baseline enzyme activity, was found to be associated with lower iron stores in subjects with the iron overload disorder HH (Constantine, Anderson et al. 2009). Taken together, these studies suggest that human Dcytb essentiality may depend on levels of reducing agents versus oxidizing inhibitors in habitually consumed diets, and the individual's genetic make-up in relation to other iron homeostasis-related proteins.

Divalent Metal Transporter 1 (DMT1), also known as DCT1, NRAMP2 and SLC11A2, actively transports reduced Fe^{2+} across the apical enterocyte membrane and is the only currently identified gut transporter of non-haem iron (Gruenheid, Cellier et al. 1995, Fleming, Trenor et al. 1997, Gunshin, Mackenzie et al. 1997). DMT1 iron transport is an active proton coupled process and functions best in a low pH environment such as that found in the duodenum (Gunshin, Mackenzie et al. 1997, Tandy, Williams et al. 2000). A large body of experimental evidence supports the role of DMT1 as the major apical non-haem iron transporter of the gut: spontaneous mutations affecting DMT1 in two different mice strains are associated with iron deficiency anaemia (IDA) (Fleming, Feng et al. 2011); the organ targeted ablation of the Slc11a2 gene in the gut of a mouse model was lethal secondary to profound iron deficiency (Gunshin, Fujiwara et al. 2005); and several human SLC11A2 mutations are associated with severe iron deficiency anaemia (Mims, Guan et al. 2005, Beaumont, Delaunay et al. 2006, Iolascon, d'Apolito et al. 2006). In contrast with DMT1 mutations in mice, DMT1 mutations in humans thus far are associated with hepatic iron overload despite IDA, an example of species-specific manifestation of disease.

1.4.2.3: Ferritin

How dietary ferritin iron is absorbed is an area of current research. Recent work from several laboratory groups with in vitro cell lines, and human studies, suggests that ferritin is an available source of dietary iron (Davila-Hicks, Theil et al. 2004, Kalgaonkar and Lonnerdal 2008). A significant amount of plant iron is in ferritin; in plant-based diets

ferritin may be an especially important dietary source of the mineral(Lonnerdal 2009). It is proposed that a proportion of the iron in ferritin is released as non-haem iron during digestion and taken up via the non-haem iron pathway, while the remaining intact ferritin is endocytosed and the iron then released intra-cellularly (Kalgaonkar and Lonnerdal 2009). How this pathway of iron uptake is regulated is also unknown. These questions, and the significance of this form of dietary iron in the diet, require further study.

1.4.2.4: Intracellular iron transport

How iron traverses the enterocyte to exit from the basolateral surface is unknown. Work using Caco-2 cells found that DMT1 was internalized after cell treatment with iron and co-localized with intracellular apotransferrin vesicles, which suggested that iron was transferred from DMT1 to transferrin to then be transported out of the cell (Ma, Specian et al. 2002). Two other studies also found DMT1 internalization after iron treatment, however this was interpreted as a regulatory response to decrease further iron uptake, rather than an intracellular transport mechanism (Sharp, Tandy et al. 2002, Johnson, Yamaji et al. 2005).

Research continues on identification of intracellular chaperone proteins that transport iron through the enterocyte cytosol, analogous to the known chaperone proteins for other metals such as copper. Work in a yeast model found evidence that poly (rC)-binding protein 1 (PCB1) is an iron chaperone protein that delivers iron to ferritin (Shi, Bencze et al. 2008), and recent work from the same lab has found that its paralog PCB2 may be another iron chaperone (Nandal, Ruiz et al. 2011). These results remain to be confirmed in vivo.

1.4.2.5: Iron egress

Iron exits the enterocyte via ferroportin, also known as IREG1, MTP1 and SLC40A1, the only identified non-haem iron efflux transporter(Abboud and Haile 2000, Donovan, Brownlie et al. 2000, McKie, Marciani et al. 2000).

Studies have localized ferroportin to the basolateral membrane of polarized intestinal cells, and shown that ferroportin expression in the gut is highest in the duodenum, the major site of iron absorption (McKie, Marciani et al. 2000). Ferroportin is also expressed throughout the body including the liver and reticuloendothelial system. The exact functional effects of ferroportin expression vary depending on the organ system in question. This is illustrated in iron deficiency in which hepatic ferroportin is down regulated, whereas duodenal levels increase in order to facilitate iron absorption from the gut (Abboud and Haile 2000). The mechanism behind these different responses will be discussed later in this thesis. The essentiality of ferroportin is demonstrated by the fact that embryonic deletion is non-viable (Donovan, Lima et al. 2005); mutations in ferroportin classically lead to iron overload disorders, (Mayr, Janecke et al. 2010).

Ferroportin transports iron through the enterocyte basolateral membrane as Fe^{2+}, but release of iron into the circulation requires oxidation of Fe^{2+} to Fe^{3}.. In enterocytes it is believed that this is carried out by hephaestin, a membrane bound, copper dependent ferro-oxidase first identified in the sex linked anaemia (sla) mouse (Vulpe, Kuo et al. 1999). Mice with this mutation are able to absorb iron from the gut lumen, however egress from the enterocyte is diminished; the mutated protein has reduced ferro-oxidase activity (Chen, Attieh et al. 2004). Further in vitro work has demonstrated that hephaestin and ferroportin co-localise on the basolateral membrane of intestinal cells suggesting that hephaestin and ferroportin act together (Han and Kim 2007). Caeruloplasmin, a circulating homolog of hephaestin, oxidises iron released through ferroportin from other cells in the body for subsequent transport by transferrin (Hellman and Gitlin 2002).

In conclusion, luminal non-haem iron is reduced to Fe^{+2}, taken up by DMT1, traverses the cell via an unidentified mechanism, and effluxes from the cell through the coordinated actions of ferroportin and hephaestin.

Absorption of the three dietary forms of iron –non-haem, haem, and ferritin – is by different mechanisms. The pathways and regulation of absorption of the latter two forms of iron require further elucidation, and although much is known about non-haem iron absorption there are still significant knowledge gaps such as how iron traverses the enterocyte. A summary schematic of haem and non-haem iron absorption is shown Figure 1.3.

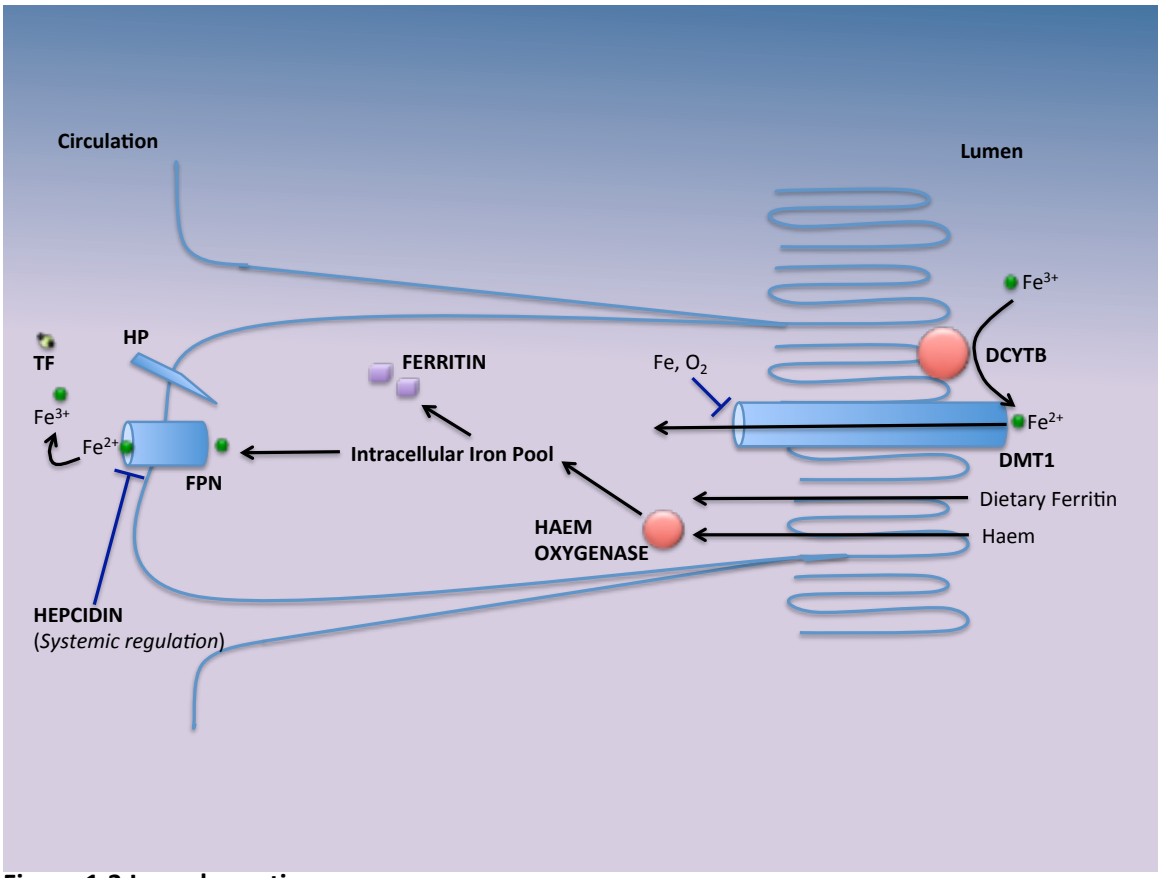

Figure 1.3 Iron absorption.

Dietary iron in the form of non-haem iron, ferritin or haem iron is present in the gut lumen. If non-haem iron is in the Fe^{3+} form it must be reduced to Fe^{2+} either by dietary factors or Duodenal cytochrome b (Dcytb) in order to be taken up into the enterocyte by Divalent Metal Transporter 1 (DMT1) the main (if not only) non-haem iron importer. Haem is taken up by Haem Carrier protein 1(HCP1), and an unidentified transporter or alternative mechanism; haem iron is released into the cell by haem oxygenase. Ferritin uptake is proposed to occur via endocytosis; it is broken down with release of iron intracellularly. Once in the enterocyte iron may: enter the intracellular iron pool (also known as the labile iron pool, LIP); form ferritin; be used for synthesis of intracellular metabolic proteins; or be exported out of the gut via the coordinated actions of ferroportin and the membrane bound ferro-oxidase hephaestin. Systemic control is exerted by hepcidin, a hormone made by the liver that degrades ferroportin and thus blocks iron egress. Released Fe^{3+} is transported through the body by transferrin. Diagram adapted from (Fuqua, Vulpe et al. 2012).

1.4.3: Iron transport and cellular uptake

Once iron has been absorbed transferrin transports the oxidised metal (Fe^{3+}) through the portal circulation to the liver. Circulating transferrin may have only one attached iron molecule (mono-ferric Tf), two (differic-Tf) or no iron (previously defined), and all three forms are found in the circulation. Circulating transferrin concentration in healthy individuals is in the range of 30 μM, and approximately 30 % of the molecules are saturated with iron (Gkouvatsos, Papanikolaou et al. 2012). Transferrin binding of iron serves three functions: it solubilises Fe^{3+} which under physiological pH conditions would otherwise precipitate; it prevents Fenton chemistry induced free radical toxicity; and it facilitates iron transport into cells. Transferrin is taken up into cells by transferrin receptors 1(TfR1) and 2 (TfR2) (Gkouvatsos, Papanikolaou et al. 2012). All cells express TfR1, and it is the major route for iron uptake throughout the body; TfR2 is primarily expressed in the liver and is part of the iron homeostasis regulatory system and will be discussed further on in this thesis. A robust body of evidence in in vitro and animal and human studies has demonstrated that cellular iron deficiency causes an upregulation of TfR1 both transcriptionally and translationally resulting in increased cellular iron uptake and improved iron status; conversely high cellular iron decreases TfR1 levels (Ponka and Lok 1999, Gkouvatsos, Papanikolaou et al. 2012). Interestingly, TfR2 levels appear to increase in high iron states (Johnson and Enns 2004, Robb and Wessling-Resnick 2004, Rapisarda, Puppi et al. 2010) consistent with its proposed role as a regulatory rather than primarily absorptive receptor.

Transferrin binds to TfR1 and enters the cell through clathrin-mediated endocytosis into the early endosome compartment. Transferrin iron binding is facilitated by high pH; within the endosome the pH drops via the action of a proton pump ATPase, transferrin then undergoes a conformational change and Fe^{3+} is released (Giannetti, Halbrooks et al. 2005). Fe^{3+} is reduced by six-transmembrane epithelial antigen of the prostate 3 (STEAP 3), an intra-endosomal reductase (Ohgami, Campagna et al. 2005), and exits the endosome via DMT1. DMT1 is thus necessary not only for gut iron absorption, but also for release of iron from the endosomal to the cytoplasmic compartment in cells throughout the body (Gunshin, Fujiwara et al. 2005). The apotransferrin/TfR1 complex remains within the endosome and is recycled to the cell membrane where TfR1 is reinserted and transferrin released into the general circulation (Mayle, Le et al. 2012). The transferrin cycle is shown in Figure 1.4.

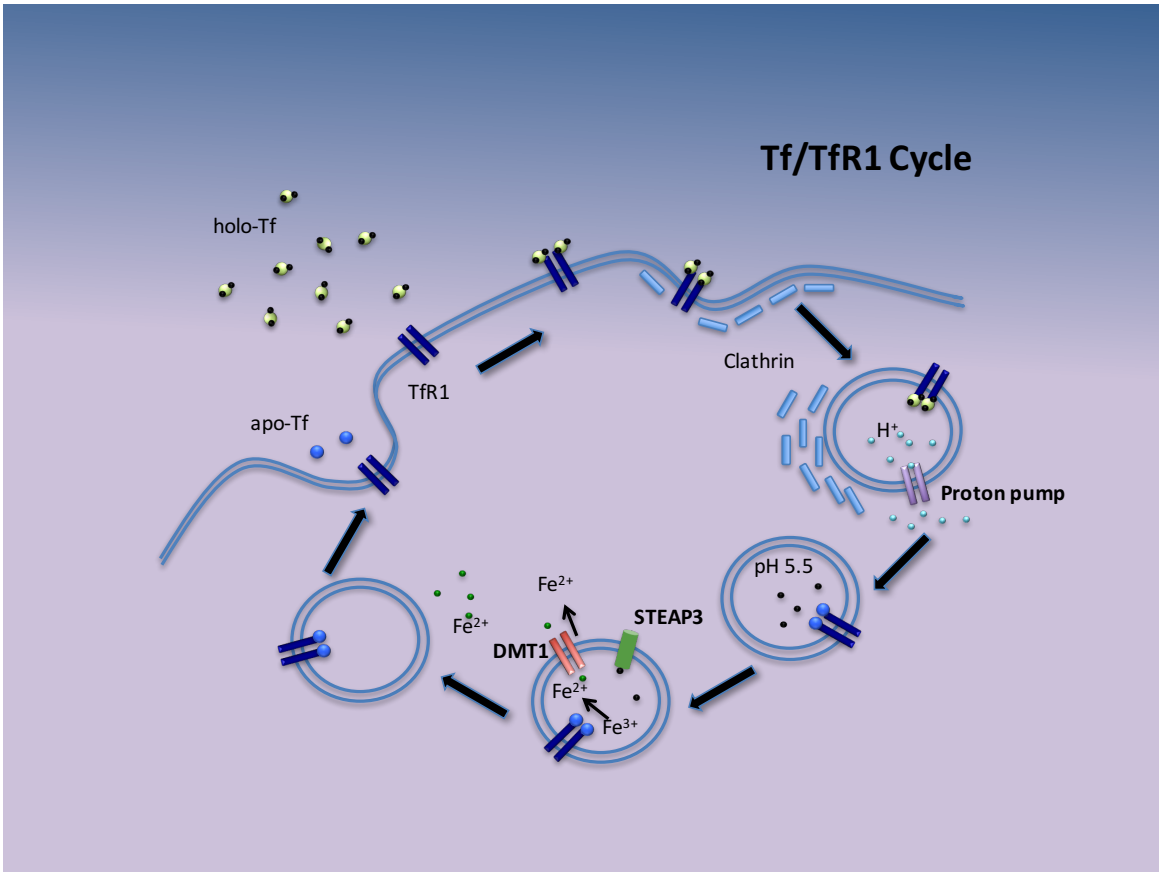

Figure 1.4 The transferrin cycle.
Circulating holotransferrin binds to TfR1 and is endocytosed through clathrin-coated pits to form an early endosome. The endosomal pH is decreased through the action of a membrane bound proton pump ATPase, and with the change in pH transferrin undergoes a transformational reconfiguration with the subsequent release of iron. Fe3+ is reduced to Fe2+ and released into the cytoplasm through DMT1; the apotransferrin/TfR1 complex is recycled back to the cell surface membrane and transferrin released into the circulation; the TfR1 reinserted into the plasma membrane. Diagram adapted from (Gkouvatsos, Papanikolaou et al. 2012).

The transferrin cycle is the main, but not the only, mode of cellular iron uptake. A small percentage of iron in the circulation is in the form of non-transferrin bound iron (NTBI); the chemical form of NTBI is unknown but evidence suggests iron is bound to both small anions such as citrate, and also the plasma protein albumin (Grootveld, Bell et al. 1989, Hider 2002). Under normal conditions blood levels of NTBI are 1 μmolar or less, but in certain pathological conditions associated with iron overload levels may reach up to 20 μmolar (Brissot, Ropert et al. 2012). The precise chemical structure and cellular uptake mechanisms of NTBI are unknown, and uptake may differ between cell types. Research with in vitro liver cell lines and mouse models found that in the liver both zinc ion transporter 14 (ZIP14) and DMT1 can take up NTBI, although a recent paper showed that mice with DMT1 liver-specific deletions demonstrate normal liver iron metabolism – at least in the short time frames used in the study (weeks) (Shindo, Torimoto et al. 2006, Pinilla-Tenas, Sparkman et al. 2011, Nam, Wang et al. 2013). Hepatocyte uptake of NTBI does not appear to be inhibited by increasing levels of liver iron when tested in vitro with

rat hepatocytes and in vivo in rats (Brissot, Wright et al. 1985, Wright, Brissot et al. 1986). This is consistent with the role of the liver as a buffer against excess iron, and also explains why many iron overload disorders are characterized by excess liver iron and disease (Deugnier, Brissot et al. 2008).

1.4.4: Regulation of iron homeostasis

Iron metabolism is regulated at multiple levels, systemically and intracellularly, in order to balance local cellular need with overall body requirements, while avoiding iron overload toxicity.

1.4.4.1: Systemic iron regulation - hepcidin

Hepcidin, a 25 amino acid peptide made by the liver, is the major systemic iron regulatory molecule (Park, Valore et al. 2001). It was initially identified as part of the innate immune system – infection and inflammation markedly increase expression - and hepcidin has direct antimicrobial activities (Krause, Neitz et al. 2000). Further work in mouse hepcidin knock out (KO) models has demonstrated hepcidin's role in iron regulation; mice with Hamp (the hepcidin gene) deletions developed massive iron overload (Nicolas, Bennoun et al. 2001), and excessive Hamp expression caused iron deficiency (Pigeon, Ilyin et al. 2001). Since its original discovery, the centrality of hepcidin to human iron metabolism has been confirmed by identification of many different human mutations involving either the HAMP gene, or other genes in HAMP expression pathways, leading to disordered iron homeostasis; the majority of primary iron overload disorders involve altered hepcidin expression (Pietrangelo and Trautwein 2004).

Hepcidin regulates iron homeostasis by binding to the iron exporter ferroportin; this leads to the transporter's internalization, ubiquitination and degradation, and subsequent diminished release of iron from absorption and storage cells (Nemeth, Tuttle et al. 2004). Hepcidin's role in both iron metabolism and immunity is explained by pathogens' need for iron, and iron sequestration as an adaptive immune response.

Hepcidin is a negative iron regulator - under normal conditions hepcidin levels are inversely related to body iron levels, dietary iron load, and iron need. Infection and inflammation increase hepcidin levels relatively unrelated to iron status. This latter response appears to relate to hepcidin's function as part of the innate immune system.

Hepcidin levels change in response to iron load, iron need, hypoxia, erythropoiesis, and inflammation (Figure 1.5). Levels are adjusted through signalling pathways involving multiple molecules; there are some points of convergence between pathways. The different regulatory hepcidin pathways will now be considered individually.

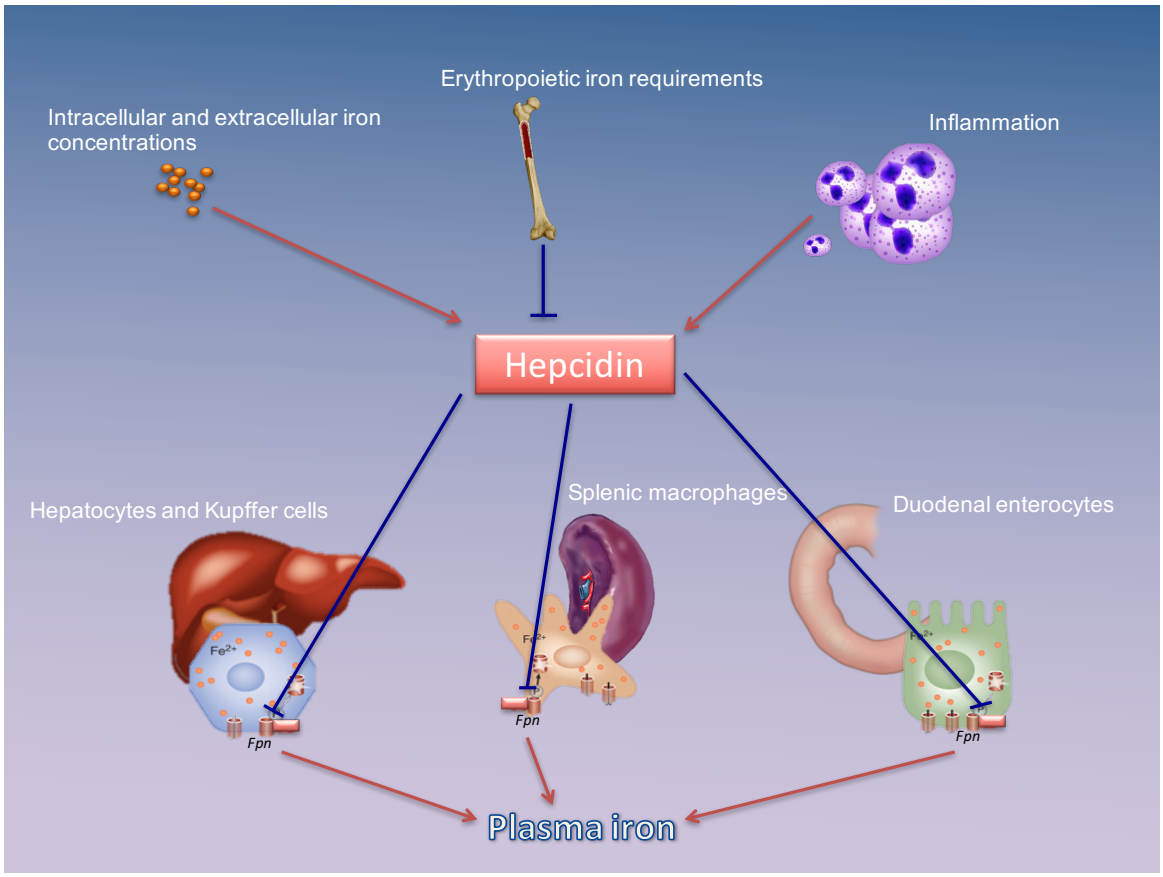

Figure 1.5 Hepcidin and iron homeostasis.
In response to liver iron, circulating iron, erythropoietic needs, or inflammation, hepcidin levels adjust to either decrease or increase the uptake and release of iron from gut and storage cells through the "opening" or "closing" of ferroportin iron export. Diagram adapted from (Ganz and Nemeth 2012).

1.4.4.1.1: Hepcidin & inflammation – IL6 & STAT3

Inflammation increases hepcidin expression - this is, in part, the molecular basis for the clinically observed anemia of chronic disease (ACD), a disorder documented in the medical literature for decades. Inflammation primarily induces hepcidin expression via the inflammatory cytokine interleukin 6 (IL6) (Nemeth, Rivera et al. 2004). Other inflammatory cytokines including interleukin 1(IL1) and Tumour Necrosis Factor α (TNFα), also contribute to ACD, but it is unclear whether this is a hepcidin-dependent effect.

1.4.4.1.2: Hepcidin & liver iron – BMPs & SMADs

Hepcidin expression changes in response to liver iron stores. This is mediated through activation of Bone Morphogenic Proteins (BMPs) - a group of proteins belonging to the Transforming Growth Factor Beta (TGFβ) superfamily; BMP6 is a key protein in hepcidin induction (Hentze, Muckenthaler et al. 2010). BMP6 interacts with its

receptor(s) causing phosphorylation of SMAD proteins 1, 5 and 8; this phosphorylated complex binds with co-factor SMAD4, and the entire complex translocates to the nucleus, binds to the hepcidin promoter and increases hepcidin expression (Hentze, Muckenthaler et al. 2010). SMAD 7, an inhibitory SMAD protein, is activated by low hepatic iron stores and suppresses hepcidin expression (Mleczko-Sanecka, Casanovas et al. 2010). BMP6 itself is not an iron sensor, and the initiating signal in this pathway is unknown.

1.4.4.1.3: Hepcidin & plasma iron – HFE, HJV, TfR1, TfR2, TMPRSS6

Hepcidin levels adjust in response to changes in circulating iron levels (Hentze, Muckenthaler et al. 2010). The main signal to the liver of circulating iron load is diferric –Tf - a large body of evidence has shown that as diferric-Tf levels increase so does release of hepcidin; low levels of diferric-Tf suppress hepcidin (Gkouvatsos, Papanikolaou et al. 2012). The pathway(s) initiated by this signal, are incompletely understood. Mutations in human genes coding for haemochromatosis protein (HFE), haemojuvelin (HJV), TfR1, TfR2, and Matriptase 2 (TMPRSS6) result in abnormal iron homeostasis. All these proteins appear to be essential for the liver to sense systemic iron levels and appropriately adjust hepcidin production and release. What is incompletely defined is how these proteins interact to achieve this.

HFE was the first protein involved in iron homeostasis to be identified; mutations in the gene for HFE cause Classic Hereditary Haemochromatosis (HH), a disorder of low hepcidin levels and excessive body iron absorption and stores (Feder, Gnirke et al. 1996). HFE is a membrane protein that can bind to both TfR1 and TfR2; it has a higher affinity for TfR1 and under basal or low iron conditions is primarily bound to TfR1 (Gkouvatsos, Papanikolaou et al. 2012). The transferrin receptor site on TfR1 overlaps with the HFE binding site, and research supports that high diferric-Tf levels saturate TfR1 causing it to dissociate from HFE; it is purposed that HFE is then freed to bind to TfR2, and the HFE-TfR2 complex starts a signalling cascade that induces hepcidin (Gkouvatsos, Papanikolaou et al. 2012).

HJV, coded for by HFE2, is another trans-membrane bound protein critical in iron homeostasis; people with HFE2 mutations develop massive body iron overload at an early age (Papanikolaou, Samuels et al. 2004). HJV is a BMP co-receptor and homolog of the Repulsive Guidance Molecule (RGM) family; it interacts with BMP6 and induces hepcidin expression through SMAD signalling (Hentze, Muckenthaler et al. 2010). As with activation of the BMP6 pathway by intracellular liver iron levels, the actual mechanism by which systemic iron levels activate this pathway is unknown.

Matriptase or transmembrane serine protease matriptase-2 (TMPRSS6) is a proteolytic enzyme expressed on liver cell membranes that is a negative hepcidin regulator; when iron levels are low it cleaves HJV from the membrane thus disrupting this hepcidin activating pathway (Lee 2009). Mutations of TMPRSS6 in people lead to iron resistant

refractory anaemia associated with inappropriately high hepcidin levels (Lee, Yong Song et al. 2005). How TMPRSS6 senses iron levels is unknown.

Figure 1.6 provides a summary schematic of the molecular regulation of hepcidin by the above named pathways.

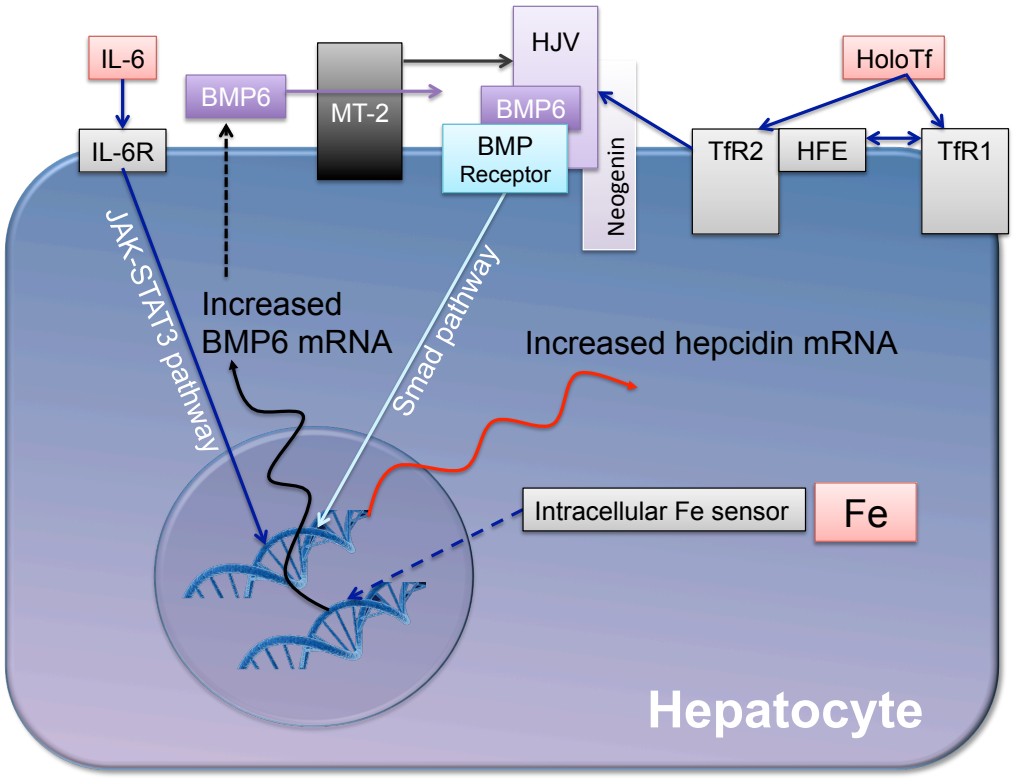

Figure 1.6 Molecular control of hepcidin by circulating iron, liver iron, and systemic inflammation.
Holotransferrin is postulated to signal circulating iron levels, and alter hepcidin expression by activating BMPs/SMAD signalling pathways. Liver iron levels, sensed by an as yet undetermined iron sensor, also activate the BMPs/SMAD signalling pathways and may also initiate other undetermined pathways altering hepcidin expression. Inflammation is a strong inducer of hepcidin through IL6/STAT3 signalling. Diagram adapted from (Ganz and Nemeth 2012).

1.4.4.1.4: Hepcidin & erythropoiesis

Erythropoiesis suppresses hepcidin, even when total body iron is elevated. The mechanisms and pathways involved are currently being elucidated with recent work from the Ganz lab suggesting that a peptide hormone produced by differentiating erythroblasts (initially named as Fam132B but now also identified as erythroferrone) may be a key molecule in this signalling pathway (Kautz, Jung et al. 2014).

1.4.4.1.5: Hepcidin & hypoxia – HIFs, PHD, HREs

Hypoxia (low oxygen levels) decreases hepcidin expression. Iron metabolism is directly linked to cellular oxygen status, because iron is essential for oxygen transport and storage - deficiency leads to hypoxia. Cellular hypoxia increases levels of Hypoxia-inducible factors (HIFs), a family of transcription factors essential to cellular adaptation to altered oxygen availability that act by binding to hypoxia responsive elements (HREs) in target genes (Wenger 2002). HIFs are dimers composed of two protein subunits: the constitutively expressed HIF-β, and the hypoxia regulated HIF-α's. Three HIF-α's have been described: HIF-1α, HIF-2α and HIF-3α; HIF-α's are degraded under normal oxygen conditions, but stabilised by hypoxia - the active transcription factor is formed when HIF-α binds with HIF-β, and HIF-2 α appears to be the primary HIF associated with iron homeostasis (Chepelev and Willmore 2011).

The hepcidin gene, HAMP, has HREs in its promoter, and there is evidence in murine models that HIF-HRE binding to the HAMP promoter decreases hepcidin production (Chepelev and Willmore 2011).

1.4.4.2: Cellular iron regulation

Intracellular iron is found in iron storage proteins, as a functional component of metallo-proteins, and as "free iron" in the labile iron pool (LIP) (Kakhlon and Cabantchik 2002). The LIP is a small fraction of total cellular iron, and not well characterized. Research suggests that iron in the LIP is in the reduced (ferrous) form complexed with glutathionine with an iron concentration of approximately 10^{-7} to 10^{-6} M (Hider and Kong 2011), and that this is a rapid flux pool (Zanninelli, Loreal et al. 2002).

Regulation of intracellular iron levels is complex –there must be enough iron for cellular needs whilst avoiding excess, and at the same time absorptive and storage cell iron homeostasis needs to be able to adjust to provide enough iron for whole body metabolism. Regulation occurs at transcriptional, translational and post- translational levels. Final cellular iron reflects the balance between local and systemic requirements within the context of habitual dietary intake.

1.4.4.2.1: Transcriptional control

HIFs are important transcriptional regulators of cellular and systemic iron levels beyond their effects on hepcidin – expression of several iron related proteins changes in response to HIF regulation. Evidence supports that the increased expression of gut Dcytb, DMT1 and ferroportin seen in iron deficiency is induced by HIF-2α; hypoxia also strongly stimulates erythropoiesis through HIF mediated increased expression of erythropoietin (the major hormone that stimulates red blood cell production), and expression of the related iron proteins transferrin and TfR1 to provide the iron for haemoglobin synthesis (Chepelev and Willmore 2011).

Another iron-oxygen connection exists because iron is a powerful pro-oxidant, and a number of iron-related genes have anti-oxidant response elements (AREs), which form part of the cellular defense system against oxidative stress (Chepelev and Willmore 2011). Figure 1.7 illustrates the relationship between iron and oxygen.

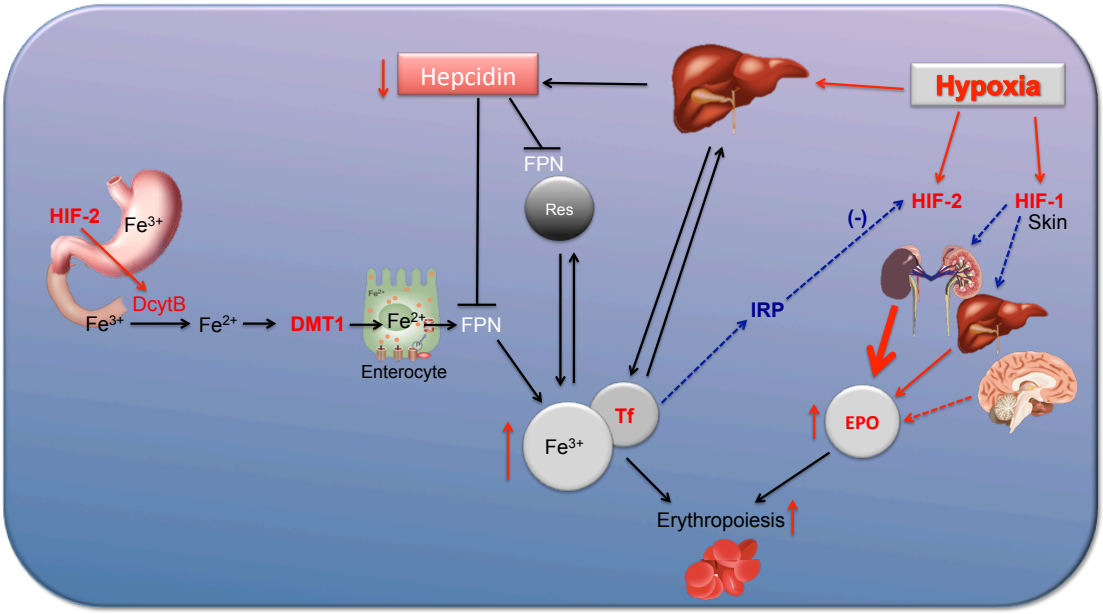

Figure 1.7 Hypoxia and iron homeostasis.
Hypoxia induces HIF associated erythropoiesis – this increases the need for iron for haem synthesis. HIFs increase Dcytb and DMT1 levels increasing the absorption of iron from the gut. Transferrin also has an HRE and therefore HIFs increase transferrin levels to transport iron. Hepcidin production is suppressed by erythropoiesis, low iron and hypoxia ensuring ferroportin levels are maintained to release iron from the gut and storage cells. If intracellular iron levels fall too low the IRP/IRE system inhibits the HIF system. Diagram adapted from (Haase 2010).

1.4.4.2.2: Translational control

The main control point of intracellular iron homeostasis is at the translational level, primarily via the Iron Responsive (IRE) and Iron Regulatory Protein (IRP) system. IRPs are RNA-binding proteins that bind to conserved RNA motifs on messenger RNA (mRNA) known as IREs; there are two known IRPs – IRP1 and IRP2 (Muckenthaler, Galy et al. 2008). IRP1 functions as an aconitase when iron levels are adequate, but when iron is low the Fe-S cluster of the enzyme is degraded opening up the IRE binding pocket; IRP2 is degraded by the proteasome when cellular iron is high (Anderson, Shen et al. 2012). Thus, the net effect of low iron levels is increased intracellular availability of both IRPs. The binding of IRPs to different iron-related mRNAs alters the mRNA stability, increasing or decreasing protein translation depending on the location of the IRE: IRP binding to 5' IREs destabilizes the mRNA and thus decreases protein synthesis, whereas IRP binding to the 3' IREs stabilizes mRNA leading to increased protein

synthesis (Muckenthaler, Galy et al. 2008). With the exception of Dcytb, all the major iron-related proteins have IRE isoforms differentially expressed depending on the tissue/organ. This provides a fine tuned system to adjust cellular iron requirements specific to organ need. DMT1 and TfR1 have 3' IREs and in cells expressing the IRE + isoform of the protein levels are increased in iron insufficiency secondary to IRP-IRE binding; conversely, ferritin and ferroportin protein levels decrease with iron insufficiency because of 5' IRP-IRE binding and subsequent mRNA degradation - thus favoring both cellular iron retention and availability of "free iron" for cellular needs (Anderson, Shen et al. 2012). The puzzle of why in iron deficiency gut ferroportin levels increase is explained both by the discovery of a primarily non-IRE splice variant isoform of ferroportin expressed in the gut (Zhang, Hughes et al. 2009), and evidence that gut ferroportin has an HRE activated by HIF-2α secondary to low iron status associated hypoxia (Taylor, Qu et al. 2011). In contrast, when hepatocyte iron concentration falls, levels of the predominantly 5' IRE ferroportin found in hepatocytes decreases; iron egress from the cell is decreased and thus conserved for use by the metabolically active liver cells. Similarly, erythroid cells need high iron levels in order to make haemoglobin; the predominant ferroportin form in mature cells has a 5' IRE such that intracellular iron levels are maintained to ensure adequate supply for haemoglobin synthesis. Interestingly, immature erythroid cells – those with the highest iron needs- have a non-IRE ferroportin form and low levels of basal ferroportin expression to further maximise retention of intracellular iron for haem synthesis (Cianetti, Gabbianelli et al. 2010).

IRP control of iron metabolism is further refined by differential IRP binding to IREs – two mRNAs both with 5' IREs, but coding for different proteins, may be suppressed to differing degrees by the IRP system depending on the strength of IRP-IRE binding (Anderson, Shen et al. 2012)

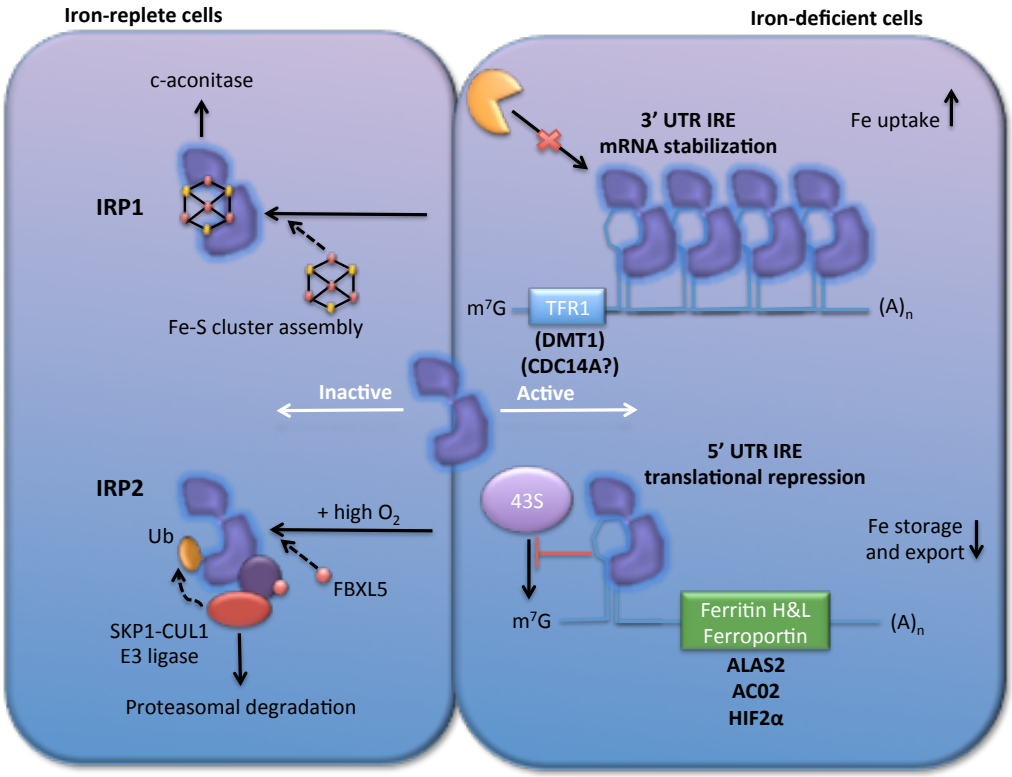

Figure 1.8 Cellular iron translational control: IRP/IRE system.
Under cellular iron replete conditions non-aconitase IRP1 and IRP2 levels are low. Translation of proteins with an mRNA 5' IRE, such as ferritin and HIF2α, are not suppressed by IRP/IRE binding. Conversely, under low intracellular iron conditions available IRP levels increase and there is IRP-IRE binding. Translation of proteins with an mRNA 3' IRE, such as transferrin receptor 1 (TfR1), is enhanced, while ferritin and HIF2α translation is suppressed. If increased TfR1 results in increased iron uptake, IRP levels re-adjust to altered cellular iron levels. Diagram adapted from (Hentze, Muckenthaler et al. 2010).

1.4.4.2.3: Post-translational control

Cellular and systemic regulation of iron metabolism meet at the ferroportin/hepcidin axis, the major post-translational control point in iron homeostasis. As previously described, hepcidin binding to ferroportin decreases ferroportin levels by inducing post-translational degradation of the hepcidin-ferroportin complex. When body iron levels are high, hepcidin levels increase. Release of absorbed gut and stored iron is suppressed; subsequently intracellular iron levels increase in these cells, and interact with the IRE/IRP and HIF/HRE systems. In the gut, DMTI and Dcytb levels fall so less iron is taken up into the enterocyte, and ferritin levels increase (as it has a 5' IRE) to protect the cells from iron toxicity. In addition, as enterocytes are shed every two-three days this sequence of events is also a means of passive iron excretion from the body, and important in the prevention of body iron overload (Sharp and Srai 2007).

1.5: Iron disorders

Iron related disorders are prevalent worldwide; both iron deficiency and iron excess negatively affect growth, development and health.

1.5.1: Iron deficiency

The World Health Organization (WHO) estimates that some two billion people suffer from anaemia, and that iron deficiency (ID) is the major cause of this disorder accounting for at least 50 % of cases (World Health Organization 2008). The highest incidence and prevalence of iron deficiency anaemia (IDA) is in the developing world, but IDA also exists in the developed world (World Health Organization 2008). In the United Kingdom 3 % of men and 8 % of women have haemoglobin levels consistent with iron deficiency anaemia and, in certain vulnerable subgroups, the estimated incidence is considerably higher (Hoare, Henderson et al. 2004, Swan 2004).

The most common cause of iron deficiency is nutritional inadequacy; nutritional iron deficiency may be a result of: increased iron needs relative to energy intake, as occurs during periods of rapid growth and pregnancy; low absolute dietary iron levels; low iron bioavailability; or a combination of all these factors (Zimmermann and Hurrell 2007).

Iron deficiency has a wide clinical spectrum. The functional significance of depleted iron stores without frank anaemia, technically a state of iron deficiency, is unknown. Iron deficiency severe enough to produce anaemia is the other end of this spectrum and is associated with significant morbidity and mortality including fatigue, decreased work ability, impaired immunity, impaired fertility, complications in pregnancy and at its most severe high output congestive heart failure and death (Zimmermann and Hurrell 2007, Scientific Advisory Committee on Nutrition 2010). In between these two extremes exist a range of clinical and biochemical findings such as impaired cognition possibly related to depletion of central nervous system iron dependent enzymes, and delayed psychomotor and cognitive development in infancy that may not improve even if iron is replenished later in life (Grantham-McGregor and Ani 2001, Grantham-McGregor 2003).

Toddlers, teenagers - especially teenage girls - pregnant women, premenopausal women, and the elderly are all high-risk groups for iron deficiency (Scientific Advisory Committee on Nutrition 2010).

Public health strategies to improve iron status include: supplementation, fortification, and biofortification (the breeding of nutrient enriched plants). In particular, there is great interest in plant breeding to increase plant bioavailable iron as a sustainable way to reduce iron deficiency worldwide (Mayer, Pfeiffer et al. 2008, Hoekenga, Lung'aho et al. 2011).

1.5.2: Iron overload

Iron overload may be primary or secondary. Secondary iron overload is caused by disorders of increased but ineffective erythropoiesis. This most commonly occurs either directly as a result of inappropriately low hepcidin and high iron absorption in the face of excess total body iron secondary to erythropoietic signals, or indirectly because of iron received through blood transfusions for patients' anaemia; or a combination of the two factors. The most common diseases associated with secondary iron overload are the haemoglobinopathies β thalassemia and sickle cell anaemia (Taher 2005).

Primary iron overload disorders arise from mutations in one of the iron homeostasis-related proteins, and are grouped under the name haemochromatosis. The most common primary iron excess syndrome is HH, in which HFE is mutated (Feder, Gnirke et al. 1996); 90 % of HH mutations in Europeans are caused by a single base change from cysteine to tyrosine (C282Y) (Adams and Barton 2007). This recessive disorder is the most frequently occurring single point mutation amongst white northern Europeans, affecting one in 200 people (Merryweather-Clarke, Pointon et al. 1997). Other more rare causes of haemochromatosis result from loss of function mutations in TfR2, HJV and HAMP; these conditions cause iron overload of increasing severity with an earlier age of onset compared with HH (Pietrangelo 2005, Pietrangelo 2006). Ferroportin mutations lead to an interesting array of outcomes depending on the exact functional consequence of the mutation, and which organ systems/cells are iron overloaded (Detivaud, Island et al. 2013).

HH has incomplete penetrance; not all people with HH will develop overt disease. The phenotypic penetrance of HH is a matter of debate - estimates range from 1-60 % (!) depending on the criteria used to define disease, and probably also on which population is screened. The question is complicated by the fact that currently treatment for HH is typically started early in the patient's life, because of greater awareness of the disease and also familial screening after proband identification. Penetrance varies even within families; men are at higher risk for disease, and women's' risk increases after menopause (Fleming and Ponka 2012). An incompletely answered question is to what extent does diet affects phenotypic expression? Alcohol is the only undisputed dietary risk factor for disease progression; some studies have found that ascorbic acid (vitamin C, a known enhancer of iron uptake) and red meat also increase risk, but this is not a consistent result across all studies (McCune 2008). A recent systematic review concluded that iron bioavailability of the usually consumed diet did affect disease expression, however lack of prospective randomized trials available for analysis limit the implications of these findings (Moretti, van Doorn et al. 2013).

Whether carbohydrates alter HH phenotypic expression is unknown – no published observational studies have identified sugars as a risk factor, although one study did find that non-citrus fruit (and therefore perhaps fructose) protected against HH disease expression (Milward, Baines et al. 2008).

Current dietary recommendations for people with HH are to avoid iron supplements and limit vitamin C supplements to ≤ 500mg/day, and to have only moderate amounts of red meat and alcohol (Adams and Barton 2010, Bacon, Adams et al. 2011). Although HH can't be treated by dietary therapy alone, the suggestion is that the above modifications may decrease the need for phlebotomy, the mainstay of therapy, and possibly delay/decrease phenotypic disease expression.

1.5.3: High iron intake & status, and disease

There is interest in whether elevated intracellular iron levels (though still within normal physiological parameters), high dietary iron intake, and disordered intracellular iron regulation, are involved in the pathogenesis of several non-communicable diseases including colorectal carcinoma (CRC), hepatocellular carcinoma (HCC) and non-alcoholic fatty liver disease (NAFLD) (World Cancer Research Fund 2007, Dongiovanni, Fracanzani et al. 2011, Fujita and Takei 2011, Torti and Torti 2013, Kew 2014).

1.5.3.1: Iron and NAFLD

NAFLD is a disorder with a wide clinical spectrum ranging from uncomplicated hepatosteatosis to non-alcoholic steatohepatitus (NASH) that in 10-20 % of cases progresses to cirrhosis, liver failure or hepatocellular carcinoma (HCC) (Starley, Calcagno et al. 2010). It is considered to be the liver expression of the metabolic syndrome— a cluster of conditions related to insulin resistance, central/truncal obesity, hyperglycemia and hyperlipidemia. NAFLD is the leading cause of liver disease in the developed world, and its incidence is increasing worldwide (Williams, Stengel et al. 2011). Type two diabetes mellitus (T2DM) and obesity are major risk factor for development of NAFLD, but why NAFLD progresses in only a certain percentage of people is unknown (Williamson, Price et al. 2011).

Elevated iron stores as measured by serum ferritin and transferrin saturation have been frequently described in patients with NAFLD, with approximately 35 % of patients with the condition found to have histologically confirmed elevated liver iron (Nelson, Wilson et al. 2011). The term dysmetabolic iron overload syndrome (DIOS) was coined to describe the condition of excess liver iron (documented by either MRI or liver biopsy) with the above mentioned biochemical indices in the setting of insulin resistance (Fargion 1999, Jezequel, Laine et al. 2015). The question is whether the iron is actually causal in disease progression to NASH and cirrhosis (Corradini and Pietrangelo 2012). It is postulated that iron-generated reactive oxygen species (ROS) interact with hepatic lipids and oxidise them, which in turn causes inflammation and liver disease progression (Mouzaki and Allard 2012). Iron may also interfere with insulin signalling in the liver leading to hepatic insulin resistance (IR) (Fargion, Dongiovanni et al. 2005); IR then contributes to worsening hepatosteatosis. Sharp has proposed a model that connects iron metabolism and NAFLD. He suggests that both hepatic steatosis, and increased free fatty acid flux as seen with excess energy intake and insulin resistance, increase inflammation

and endoplasmic reticulum stress, which in turn increase hepcidin expression; consequently, hepatic ferroportin decreases and traps iron within hepatocytes leading to increased liver iron stores that cause oxidative stress, and the cycle is perpetuated (Sharp 2010)(Figure 1.9).

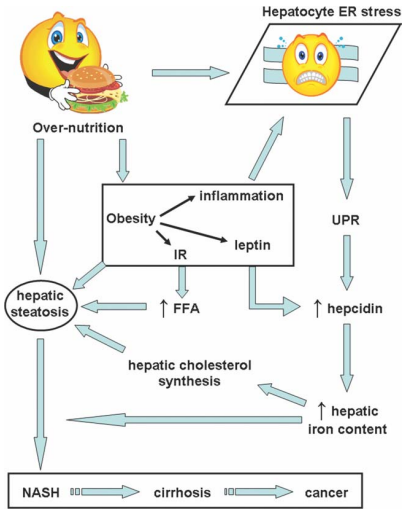

Figure 1.9 Proposed role of iron in NAFLD.
Increased inflammation within the liver, and increased leptin levels from adipocytes, leads to release of hepcidin. Increased hepcidin decreases ferroportin levels and iron is trapped within liver cells; this causes increased production of ROS increasing inflammation within the liver and perpetuating the inflammatory cycle. Diagram used with kind permission (Sharp 2010).

A recent, small phase II clinical trial in patients with NAFLD using phlebotomy as a therapeutic intervention suggested improved liver pathology, although the effects were modest (Beaton, Chakrabarti et al. 2013). Two larger randomised control intervention trials evaluating therapeutic phlebotomy in the management of NAFLD, however, demonstrated opposite findings. (Valenti, Fracanzani et al. 2014, Adams, Crawford et al. 2015). The trial conducted by Valenti et al demonstrated improved hepatosteatosis documented by biopsy after phlebotomy that correlated with decreased stainable hepatocyte iron as well as decreased ferritin and transferrin saturation. Patients who had undergone phlebotomy also had lower levels of liver damage as assessed by liver enzyme measurements. In contrast, Adams et al found no benefit from phlebotomy as evaluated by Magnetic Resonance Imaging (MRI) measurement of hepatic steatosis, and blood levels of liver enzymes, nor was insulin resistance improved. Of note, the studies were conducted in different populations (the Adams study was carried out in Australia, the Valenti study in Italy), and the Adams study did not obtain liver biopsies, and therefore may have missed subtle improvements in hepatosteatosis and liver inflammation.

It is difficult to draw firm conclusions about the role of iron in NAFLD for several reasons: much of the evidence about increased iron being associated with NAFLD comes

from epidemiological studies, and from studies using ferritin as an iron status marker – and elevated ferritin may reflect inflammation rather than actual iron overload. Intervention trials using iron reduction as therapy have been few, and are hampered by the need for invasive liver biopsies to categorically demonstrate that disease modifying results are secondary to decreased liver iron (rather than some other effect related to phlebotomy); even in the Valenti et al study only some 50 % of participants agreed to post treatment follow up liver biopsy. Animal and in vitro studies that have shown increased liver oxidative stress with iron treatments have typically used non-physiologically high iron doses. For example, a study using mice to look at visceral fat and insulin resistance fed the animals 3750 times more iron (!) compared with controls (Dongiovanni, Ruscica et al. 2013). Although there is evidence that NAFLD is associated with mild liver iron overload, whether iron is causal in disease or simply associated with it remains unanswered.

1.5.3.2: Iron and hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a common cancer worldwide and accounts for significant cancer-related mortality; the incidence has increased in the developed world in the past two decades (El-Serag and Rudolph 2007). HCC typically develops within a diseased, cirrhotic liver, and the most common predisposing factors worldwide are chronic viral infections such as Hepatitis B and C (El-Serag and Rudolph 2007).

HCC can also occur in untreated HH patients, with or without underlying liver cirrhosis, although HCC in HH without cirrhosis is relatively rare; surveys carried out before screening and increased awareness of HH (pre 1992) document that HCC occurred in approximately 8-10 % of patients, and accounted for up to 45 % of deaths in untreated HH (Fargion, Valenti et al. 2010). A more recent study, however, found the incidence of HCC to be only 1.7 % suggesting that the previously documented high rates of HCC related to the very high liver iron levels that can occur in untreated HH (Elmberg, Hultcrantz et al. 2003). Evidence suggests that treatment of even mild iron overload in HH improves mortality (Elmberg, Hultcrantz et al. 2003). In summary, there is good evidence that the very high iron levels in untreated HH increase the risk of liver disease and HCC; conversely treatment of iron overload decreases mortality and morbidity.

The question remains, however, whether mildly elevated liver iron load in patients without frank iron overload disorders (such as HH) contributes to risk of HCC in patients with NAFLD/ NASH (Pietrangelo 2009, Sorrentino, D'Angelo et al. 2009).

The elevated hepatic iron levels in NAFLD are typically mild in comparison with those seen in HH-associated HCC. Furthermore, it is not clear if elevated liver iron in either condition is a primary or secondary event. In other words, is there an underlying factor that leads both to increased risk for HCC and increased liver iron in people with NAFLD. A study by Sorrentino et al attempted to address this question (Sorrentino, D'Angelo et al. 2009). The investigators retrospectively analysed liver iron deposits in 153 people with

NASH-related cirrhosis, of whom 50 had HCC versus 103 HCC-free matched controls; they found that liver iron stores were higher in patients with HCC-NASH versus the matched controls. Interestingly, excess iron was detected in sinusoidal cells (Kupffer cells of the RES) rather than in hepatocytes. HCC is a hepatocyte-derived malignancy, thus these results suggest that cells of the RES may participate in hepatocyte malignant transformation although the study did not explore mechanisms. Nonetheless, as this was a retrospective case-control study neither causality nor directionality of effect can be confirmed.

HH associated liver disease is a result of iron-induced oxidative stress damaging, among other intracellular components, DNA; consequent DNA mutations in genes involved in HCC-related signalling or tumour suppression pathways may then lead to HCC. Whether mildly elevated liver iron, as seen in DIOS or NAFLD, is causative in malignant transformation of liver cells remains to be established.

1.5.3.3: Iron and colorectal cancer

A strong body of epidemiological evidence supports an association between both dietary iron intake and iron status, and colorectal cancer (CRC) development and progression (for example, (Nelson 1992, Knekt, Reunanen et al. 1994, Wurzelmann, Silver et al. 1996, Nelson 2001). Migration studies have demonstrated that adoption of a high red meat "Western" diet is associated with increased risk of CRC (Parkin 1992). However, although the majority of epidemiological studies demonstrate an association between iron and CRC, there are some studies that have found no association (Benito, Stiggelbout et al. 1991, Herrinton, Friedman et al. 1995, Kabat, Miller et al. 2007).

Differences in studies may be as of a result of multiple factors: whether use of iron supplements was recorded; study participant characteristics such as ethnicity and age; accuracy of iron status markers used to measure body iron stores; accuracy of methods used to assess iron intake such as food frequency questionnaires versus 24 hour recall; use of other other multi-nutrient supplements that affect cancer risk; and differentiation between iron and meat intake and whether other components in meat such as saturated fats were measured. Lastly, as with all epidemiological evidence, unaccounted for confounders may complicate the interpretation of findings, and limit the ability to truly confirm causation.

Animal, in vitro and human tissue studies, have suggested molecular mechanisms to support the hypothesis that excess iron may be carcinogenic and/or promote CRC progression (reviewed in (Chua, Klopcic et al. 2010). CRC cells are metabolically active, and like other malignant cells have high iron requirements; adequate iron is thus needed as a nutrient to support growth and replication. Tumour cells generally express high levels of iron acquisition proteins, especially transferrin receptor one (TfR1)(Brookes, Hughes et al. 2006, Daniels, Bernabeu et al. 2012); furthermore, in vitro studies have

demonstrated that cancer cells may alter expression of iron-homeostasis related proteins such that intracellular iron levels are increased (Brookes, Hughes et al. 2006).

A recent study in mice demonstrated that loss of the tumour suppressor gene adenomatous polyposis coli (Apc) led to increased expression of the iron acquisition proteins DMT1 and TfR1, even in the setting of increased cellular iron levels – elevated intracellular iron would be predicted to decrease these proteins; conversely, in the same study, it was found that depletion of gut luminal iron levels suppressed intestinal tumourigenesis (Radulescu, Brookes et al. 2012). Previous work from this same group, using colonic tissue resected from patients diagnosed with CRC, found overexpression of the iron transport proteins DMT1, DcytB and TfR1, and associated increased iron in CRC cells compared with normal tissue (Brookes, Hughes et al. 2006). The colon is potentially exposed to sustained, high levels of luminal iron as only approximately 10-15 % of ingested iron is absorbed, thus the other 85-90 % passes through the colon. Therefore, this portion of the gastro-intestinal tract would be predicted to be especially vulnerable to any iron-associated tumourogenesis effects.

There have been no intervention trials, to the author's knowledge, specifically evaluating whether phlebotomy-induced iron depletion decreases either occurrence or progression of CRC. A phlebotomy intervention trial aimed at decreasing peripheral vascular disease through iron reduction found a significant reduction in all gastro-intestinal malignancies. However, this occurred within the first 6 months of the trial, thus it is not clear that it directly relates to decreased iron status as CRC normally takes decades to develop (Zacharski, Chow et al. 2008).

Although iron reduction intervention trials are lacking in CRC prevention and management, there have been studies evaluating the use of iron chelators in the treatment of several cancers. Results have been mixed and potential benefits have also been limited by treatment generated side effects, but iron chelators for cancer treatment remains a very active area of research (reviewed in (Yu, Gutierrez et al. 2012).

Suggested mechanisms by which iron may promote CRC include ROS induced mutations in the APC and Wnt/beta-catenin signalling pathway (Brookes, Boult et al. 2008), and improved nutrient availability through alteration in iron transport proteins (Boult, Roberts et al. 2008).

In conclusion, there is a significant body of epidemiological evidence that suggests an association between iron and CRC, and in vitro and in vivo work provides plausible pathogenic mechanisms. However, definitive evidence is still lacking in this field.

1.5.3.4: Iron and fructose in NAFLD and HCC

Several carbohydrates have been linked to the development and progression of NAFLD, although the greatest amount of work has focused on fructose as a causative agent. Fructose is a monosaccharide naturally abundantly present in fruits and honey, but also

commercially produced as a sweetener; throughout the developed world it is now most commonly consumed in the form of high fructose corn syrup (HFCS) used as an added sweetener in foods and beverages. The other major source of fructose intake is through sucrose consumption; sucrose is a disaccharide composed of glucose and fructose that is metabolised in the gut by the enzyme sucrase to the individual monosaccharides glucose and fructose (Hunziker, Spiess et al. 1986) that are then absorbed through sodium-dependent glucose transporter 1 (SGLT1) (Hediger and Rhoads 1994) and fructose transporter GLUT5 (Davidson, Hausman et al. 1992, Rand, Depaoli et al. 1993), respectively.

Epidemiological evidence links fructose and development of NAFLD , and there are both animal studies and human intervention trials demonstrating increased hepatic steatosis with high fructose feeding (reviewed in (Nomura and Yamanouchi 2012, Moore, Gunn et al. 2014).

Several mechanisms have been proposed for this effect including: increased hepatic de novo lipogenesis (reviewed in (Moore, Gunn et al. 2014, Softic, Cohen et al. 2016); increased direct activation of liver inflammatory pathways (reviewed in (Nomura and Yamanouchi 2012); fructose induced liver copper depletion leading to increased liver iron, and decreased cellular anti-oxidant enzymes (Song, Zhou et al. 2011, Song, Schuschke et al. 2012); and fructose-induced alterations to the gut microbiome leading to systemic and liver inflammation (Bergheim, Weber et al. 2008, Spruss, Kanuri et al. 2009).

As previously stated patients with NAFLD have an increased risk for HCC, and thus fructose may indirectly increase the risk for HCC through the above discussed related metabolic effects. There is less evidence about whether fructose directly increases the risk of HCC. A recent large cohort study consisting of some 477,000 individuals in Europe found a positive association between HCC and fructose (Fedirko, Lukanova et al. 2013), however another large cohort study found no association between intake of any sugars and cancer once confounding factors such as obesity and type 2 diabetes mellitus (T2DM) were adjusted for in the analysis (Tasevska, Jiao et al. 2012)

A recent study in rats found that fructose enhanced precancerous cell transformation initiated by diethylnitrosamine (a known hepatocarcinogen) suggesting that fructose may support oncogenesis rather than initiate it (Kumamoto, Uto et al. 2013). However, as in many studies involving fructose, intake of the sugar grossly exceeded what would occur in a natural diet – fructose accounted for 60 % of energy intake in experimental animals, far above the 5-15 % that would occur naturally.

Whether fructose and iron together interact within the liver and increase the risk of NAFLD and HCC has been explored in a limited number of studies. Work from the lab of PJ O'Brien conducted in in vitro hepatocyte cell lines suggests that fructose causes liver cell damage by increasing cellular oxidative stress and protein carbonylation facilitated by iron (for example, (Shangari and O'Brien 2004, Feng, Wong et al. 2009, Dong, Yang

et al. 2010). In their model, oxidation of fructose facilitated by iron leads to production of the toxic metabolites glyoxal and methylglyoxal, and these metabolites then go onto to damage the cell leading to increased inflammation, cellular organelle damage, and disease progression.

Studies examining whether fructose and iron together synergistically specifically increase the risk of HCC have not, to the author's knowledge, been conducted.

1.6: Iron nutrition

The main dietary sources of iron vary between and within populations. The diet, food source, and form of iron eaten by the population is important because iron bioavailability (discussed in depth in sections 1.7.- 1.8 of this chapter) differs significantly depending on these factors. Non-haem iron has low bioavailability, ranging from 1 – 10 % (Hallberg 1981) haem iron, in contrast, is highly bioavailable (20-30 %) and although it contributes a relatively low absolute amount of dietary iron may be responsible for up to 50 % of absorbed iron in the developed world where it is more frequently consumed (Carpenter and Mahoney 1992).The bioavailability of ferritin is an area of ongoing research; recent studies both in vitro and in vivo suggest that plant-derived ferritin may have relatively high iron bioavailability (Lv, Zhao et al. 2015).

In the developing world plant-based foods are the primary sources of iron, whereas in the developed world fortified cereal products and animal derived tissue are the main sources. Different diets lead to different iron bioavailability because of the plethora of food factors that can decrease or increase non-haem iron availability in the gut. Total iron bioavailability from mixed, Western diets in industrialised countries is usually estimated at 12-15 % although a recent systematic review found that it may vary from 0.7–22.9 % (Collings, Harvey et al. 2013); in primarily plant based diets bioavailability is estimated at 1-12 % because of the large amounts of iron uptake inhibitors, such as phytates and polyphenols, in plants (Hurrell and Egli 2010).

Population level recommendations for iron intake are based upon a factorial method taking into account population variances (such as age, pregnancy, lactation, and gender) combined with measurement of iron status indices (to indicate what percentage of the population is deficient or at risk for deficiency); along with the iron bioavailability of the general diet of that population. The goal of public health body guidelines is to provide iron to meet the requirements of 97.5 % of the population. Recommendations vary between countries because of differing evidence bases and selection criteria of evidence used to determine needs (Doets, de Wit et al. 2008). The WHO has developed the most extensive recommendations taking into account not only life stage, but also dietary bioavailability of different diets for different life stages and populations (Harvey, Berti et al. 2013).

1.6.1: Iron status

As stated previously approximately two billion people throughout the world suffer from anaemia, however, the WHO estimates that only 50 % of this is secondary to ID (World Health Organization 2008); giving iron to iron replete people with anaemia does not correct the anaemia, and there is evidence that it may actually be harmful in the setting of populations suffering from chronic infections with malaria and gut pathogens (Sazawal, Black et al. 2006, Zimmermann, Chassard et al. 2010). Conversely, iron deficiency may be masked in the setting of inflammation, because the interrelationship between iron homeostasis and immunity/inflammation makes interpretation of iron status blood tests complex. This may either lead to delayed treatment or inappropriate forms of treatment. It is therefore essential to be able to accurately determine iron status – ie the amount of iron present in the body relative to need. Unfortunately, it is also challenging to correctly assess iron status.

There is no single good marker of iron status in deficiency, adequacy or overload except in extreme states; an array of direct and indirect biochemical measurements exist but each has limitations. Iron status marker levels may fluctuate secondary to illness, diurnal variation, and in women, phase of menstrual cycle (Laine, Angeli et al. 2015). Assessment of iron status depends on an integrated interpretation of several tests within the context of the environment in which the tested subjects live (for example, areas of chronic infection and malnutrition). Key iron status markers currently in use are discussed below.

1.6.1.1: Haemoglobin

Anaemia is defined as a condition in which the blood does not have enough RBCs, haemoglobin or total combined. Haemoglobin, which refers to both the oxygen transport iron-containing protein, and in blood tests to the concentration of that protein contained within the blood (usually expressed as grams/decilitre), will be low if iron deficiency is severe enough - the definition of IDA. However, anaemia is a non-specific finding that can be due to a multitude of conditions and occurs late in the development of iron deficiency. Furthermore, iron excess does not lead to elevated levels of haemoglobin.

1.6.1.2: Serum iron

Blood iron levels change little in response to physiological dietary iron intakes, are subject to host iron status and transferrin levels, and may be increased or decreased non-specifically because of disease. Furthermore, blood iron levels normally fluctuate and demonstrate diurnal variation (Sinniah, Doggart et al. 1969) and therefore measurement time should be recorded (but often isn't).

1.6.1.3: Transferrin saturation

In low iron states transferrin saturation falls, whilst in iron overload transferrin saturation increases (transferrin saturation of > 50 % does not typically occur in individuals without an underlying iron overload disorder such as HFE regardless of dietary iron intake) (Beutler, Felitti et al. 2002).

However, transferrin levels fluctuate under normal conditions, and may fall in chronic illness especially in diseases of the liver since that organ synthesizes transferrin. If both serum iron and transferrin levels fall similar amounts related to underlying disease, saturation will appear normal even in iron deficiency, and if transferrin concentration decreases and iron is unchanged the saturation will appear erroneously elevated.

1.6.1.4: Total iron binding capacity (TIBC)

This is a measure of blood iron binding capacity; it may be calculated directly through the addition of iron to blood samples, or inferred based on transferrin saturation; in IDA, TIBC will be low, however it suffers from similar limitations to transferrin saturation (Beutler, Felitti et al. 2002).

1.6.1.5: Serum ferritin

In healthy individuals with no underlying inflammatory conditions, serum ferritin is proportional to liver (storage) ferritin and a good marker of iron status. Serum ferritin, however, is also an acute phase reactant (APR) and may be elevated secondary to inflammation even when iron stores are depleted (Mburu, Thurnham et al. 2008, Thurnham, McCabe et al. 2010). Conversely, elevated blood ferritin levels in inflammatory conditions such as infection or autoimmune disorders, may suggest iron overload when iron status is actually normal (Thurnham, McCabe et al. 2010). As discussed previously, other disorders that we are only now beginning to understand as inflammatory conditions, for instance obesity and T2DM, may cause elevated ferritin levels unrelated to iron overload. A surprisingly large number of studies, especially older studies, assess iron status based on ferritin and haemoglobin alone without adjustments for inflammation (for example (Tuomainen, Nyyssonen et al. 1997, Hua, Stoohs et al. 2001); results from these studies need to be interpreted cautiously in settings or conditions in which there is a high prevalence of inflammation. Iron deficiency often occurs in settings where chronic inflammatory disorders are rife – studies in these environments should measure multiple markers for inflammation such as erythrocyte sedimentation rate (ESR) and chronic reactive phase protein (CRP) to adjust for inflammation in the interpretation of ferritin results to rule out "masked" iron deficiency. For the same reason, serum ferritin alone should not be used to decide if iron stores are high in chronic inflammatory conditions such as NAFLD. Low ferritin, however, is very sensitive and specific for iron deficiency (Lopez, Cacoub et al. 2016).

1.6.1.6: Soluble/Serum transferrin receptor (sTfR)

Transferrin receptor levels increase with iron deficiency, and a percent of these receptors are shed into the serum (sTfR). There is evidence that sTfR is a good iron status marker especially when used in combination with ferritin (Beguin 2003). Hemolysis and ineffective erythropoises also increase sTfR levels; in conditions of increased red blood cell turnover and production, such as malaria, this needs to be taken into account to avoid erroneous diagnosis of ID (Beguin 2003). Furthermore, at the moment, there is a degree of laboratory variability in sTfR blood test results because of different assays used (Infusino, Braga et al. 2012), thus it remains important to interpret this test within the context of other iron status markers.

In conclusion, in otherwise healthy individuals suffering from iron deficiency anaemia the diagnosis is relatively straightforward: low haemoglobin in combination with low serum ferritin and transferrin saturation is diagnostic of iron deficiency anaemia. Very high transferrin saturation combined with very high ferritin levels is also essentially diagnostic of iron overload disorders. The underlying cause of anaemia in populations exposed to chronic infections, such as malaria or helminthic gut infections, is much more challenging to diagnose. Inflammation-related elevated ferritin levels may conceal an underlying iron deficiency, and conversely, individuals with chronic inflammation secondary to non-communicable diseases such as T2DM and NAFLD, may be misdiagnosed with iron overload.

Table 1.3 shows the major iron status markers in clinical use, normal range values, expected range change (i.e. direction of change) in IDA compared with IDA with inflammation, and expected range change in iron overload.

Table 1.3 Iron status markers in adults
(Adapted from Zimmermann 2008, Camaschella 2015).

Status indicator	Normal range	IDA	IDA with inflammation	Comments
Serum ferritin (μg/litre; typically 1 μg/litre = 10mg tissue stored ferritin)	30 - 300	Most laboratories < 20	Most laboratories 20 - 200	Acute phase reactant therefore may be elevated despite low iron stores
Serum iron (μmol/litre)	10 - 30	Most laboratories < 10	Most laboratories < 10	Diurnal variation, may also be increased secondary to cell death and release of iron into the circulation
Transferrin saturation (%)	16 - 45	Most laboratories < 15	Most laboratories 10 -30	May be normal even with iron deficiency if synthesis of transferrin decreased secondary to illness
Erythrocyte zinc protoporphyrin μmol/mol haem	Varies but typically < 80	> 80	> 80	Increased by haemolysis and increased erythropoietic cell turnover
Serum transferrin receptor (sTfR) (mg/litre)	2.8 - 8.5 but varies across laboratories	> 8.5	2.8 - > 8.5	Increased by conditions of increased red cell turnover as may occur in thalassemias and malaria ; values still not standardised across different laboratories
sTfR/Log ferritin	Varies	>2	>2	Values used to calculate ratio not standardized across labs More evidence regarding diagnosing iron deficiency versus iron overload Will also be affected by conditions with increased red cell turnover
Haemoglobin (g/dl)	Men 13 - 18 Women 12 - 18	Men < 13 Women < 12	Men < 13 Women < 12	Non-specific, late finding in the course of iron deficiency
Mean corpuscular volume (femtolitres)	80 - 95	< 80	Variable	Red blood cell size may be a mixture of small and normal sized cells

1.6.2: Iron bioavailability

Bioavailability, the amount of a nutrient that is absorbed and used by the body in relation to the amount ingested, depends on multiple factors that impact to a greater or lesser degree on the specific nutrient. These factors include: absolute amounts of ingested nutrient; the food matrix within which the nutrient is found or co-ingested; food storage and preparation; digestion; absorption; enhancers and inhibitors of absorption; bio-conversion; body distribution and body utilisation including homeostatic controls (Wienk, Marx et al. 1999, Hurrell and Egli 2010, Degerud, Manger et al. 2015).

Iron bioavailability is influenced by all of the above. Systemic, dietary and gut luminal factors interact and have strong effects on iron absorption; the amount of iron absorbed and used by the body cannot be estimated simply by knowing how much iron is in eaten foods.

Systemic control of iron absorption is very tight, and will be the primary determinant of iron absorption under normal physiological states with an iron adequate diet (Hunt and Roughead 2000). Age, gender, genetic variation – both normal and pathological –, disease, inflammation and body iron status in turn determine systemic regulation. Bioavailability becomes important for individuals with increased iron needs; even if systemic regulation is set to "maximal absorption" if there is not enough available iron to absorb deficiency will develop. Bioavailability also matters in conditions of inappropriately elevated iron absorption; iron overload may only manifest if the diet provides the available iron with which to overload (Cade, Moreton et al. 2005, Greenwood, Cade et al. 2005, Moretti, van Doorn et al. 2013) In summary, in situations of dietary iron insufficiency (absolute or relative to need), or disordered iron regulation, bioavailability can influence iron status.

Dietary factors affect non-haem iron bioavailability by altering: metal oxidation state; solubility; chelation; intracellular sequestration; and iron-related protein expression. Haem iron, because it is contained within the porphyrin ring, is shielded from the effects of the majority of dietary factors that affect non-haem iron bioavailability – only factors that hinder digestion and release of haem from food, or change iron egress from the enterocyte, would be predicted to alter haem iron bioavailability. The specific effects of dietary factors on iron bioavailability are discussed below.

Dietary iron intake depends on how much iron is present in the diet eaten, but absorption, outside of systemic controls, depends on bioavailability. Foods high in iron but in which the iron is poorly bioavailable may not provide adequate intakes to prevent deficiency, a situation seen in the largely plant based and monotonous diet of the developing world (World Health Organization 2008).

The two major enhancers of iron absorption are meat and ascorbic acid; other organic acids may also enhance iron absorption under laboratory conditions but have unknown significance in mixed diets (Salovaara, Sandberg et al. 2002).

The major inhibitors of iron absorption are phytates, fibre, polyphenols (such as caffeine and tannins), zinc, calcium, and certain proteins in milk, eggs and soy. These enhancers and inhibitors will now be discussed individually.

1.6.2.1: Dietary factors and iron bioavailability
1.6.2.1.1: Enhancers
1.6.2.1.1.1: "Meat factor"

There is very strong evidence from in vitro models, animal, and human studies that meat, poultry and fish (MPF) increase non-haem iron absorption (Bjorn-Rasmussen and Hallberg 1979, Gangloff, Glahn et al. 1996, Huh, Hotchkiss et al. 2004). The exact factor(s) in MFP that enhance absorption have not been elucidated although various components of MFP such as sulphur rich amino acids (Taylor, Martinez-Torres et al. 1986), glycosaminoglycans (Laparra, Tako et al. 2008), and L-α–Glycerophosphocholine have been proposed (Armah, Sharp et al. 2008). This remains an area of active research.

1.6.2.1.1.2: Ascorbic acid

Ascorbic acid increases iron absorption even at concentrations as low as 8 μmol/L (Hallberg, Brune et al. 1989), and appears to be "the most important luminal accessory for iron" absorption (Miret, Simpson et al. 2003). Ascorbic acid, in a dose dependent manner, increases iron bioavailability both by reducing iron from Fe^{3+} to Fe^{2+}, and by chelating low molecular weight iron and thus increasing its solubility (Crichton 2009). Ascorbic acid may also increase iron uptake by acting as an electron donor to the membrane bound ferri-reductase Dcytb and thus increasing its activity (Wyman, Simpson et al. 2008).

Ascorbic acid overcomes, to varying degrees, the inhibitory effects of phytates, polyphenols and calcium on non-haem iron absorption, although the molar ratio of ascorbic acid to inhibitor, and the precise biochemical form of inhibitors, modulates this effect (Hallberg, Brune et al. 1989, Hallberg, Brune et al. 1989, Walczyk, Muthayya et al. 2014) In order for ascorbic acid to promote absorption in the presence of high levels of inhibitors it needs to be at least at an AA:iron molar ratio ≥ 4:1 (Teucher, Olivares et al. 2004), and in study conducted with a farina based meal increased ferrous sulphate iron uptake was only seen with an AA:iron molar ratio of approximately 5:1 (Forbes, Arnaud et al. 1989). Furthermore, there is evidence that the enhancing effect of ascorbic acid plateaus in the presence of inhibitors, and that it is not additive with the increased uptake seen from MFP (Cook and Monsen 1977, Gillooly, Bothwell et al. 1984, Gillooly, Torrance et al. 1984).

Some studies have failed to show that chronic high dietary vitamin C intakes increase iron status in free-living subjects eating mixed meals (Cook and Reddy 2001). This may be secondary to a threshold effect - once a certain amount of ascorbic acid is present in the diet going above this level has no added benefit (Fleming, Jacques et al. 1998).

Alternatively, it may relate to when the vitamin is ingested; in order to enhance dietary iron absorption ascorbic acid must be eaten with or within one hour of a meal (Cook and Monsen 1977). In the "Framingham Heart Study: dietary determinants of iron stores", total intake of vitamin C was not correlated to iron absorption but there was a positive correlation with mealtime vitamin C intake and improved iron stores as determined by ferritin levels (Fleming, Jacques et al. 1998).

1.6.2.1.1.3: Vitamin A and carotenoids

The effect of vitamin A on iron bioavailability is uncertain; there is more consistent evidence that carotenoids improve iron bioavailability. Vitamin A deficiency (VAD) causes anaemia although how it does so is unclear. Studies have found that VAD impairs iron status (for example, (Bloem, Wedel et al. 1989); this has lead to research on interactions between the two nutrients. VAD is postulated to directly cause anaemia by impairing erythropoiesis (West Jr 2007), but the question is whether vitamin A also affects iron bioavailability and thus deficiency indirectly causes anaemia – the evidence for this is inconclusive. This is an important question as deficiencies of both nutrients are major public health problems, there is overlap between risk groups, and ideally the two micronutrients would be supplied together (Black 2003)

Two Venezuelan human studies found that vitamin A added to baked goods fortified with iron increased iron absorption (Garcia-Casal, Layrisse et al. 1998, Layrisse, Garcia-Casal et al. 2000) however a study by Walczyk et al found no such effect (Walczyk, Davidsson et al. 2003). The food matrix and uptake measurement methods used were similar in the studies, however subjects in the studies by Layrisse and Garcia-Casal et al were more likely to be both iron and vitamin A deficient, whereas the subjects in the later, negative study, were healthy young adults without evidence of any nutrient deficiency. An experiment looking at vitamin A and iron interactions, using both mice and the in vitro Caco-2 cell line, suggested that VAD leads to iron sequestration in the liver and spleen – either directly through increased hepcidin production, or indirectly because of oxidative stress/inflammation induced hepcidin expression and iron sequestration (Citelli, Bittencourt et al. 2012); treatment of Caco-2 cells with retinoic acid (RA, the active form of vitamin A) increased ferroportin expression. Increased ferroportin did not lead to increased ferritin, which was interpreted as RA having no effect on gut iron bioavailability (Citelli, Bittencourt et al. 2012) but in vivo increased ferroportin would allow for improved iron egress out of the gut thus potentially improving iron absorption without changing gut cell ferritin levels. An alternative explanation of the results of the study by Citelli et al suggests that release of sequestered iron, and increased transport from the gut, may explain how vitamin A repletion in VAD improves iron status, and this effect might only be seen in vitamin A deficient subjects.

The evidence regarding whether carotenoids improve iron bioavailability is more consistent compared with the effects of vitamin A.

Both in vitro work with the Caco-2 cell digestion model, and in vivo studies, suggest that carotenoids increase non-haem iron absorption (Garcia-Casal, Layrisse et al. 1998, Layrisse, Garcia-Casal et al. 2000, García-Casal 2006). Furthermore, a study that developed an algorithm to predict bioavailability of iron from vegetarian diets found that β-carotene was a positive modulator of iron bioavailability in the model (Chiplonkar and Agte 2006).

The mechanism(s) by which β-carotene improves iron bioavailability is unknown, but it is postulated that carotenoids improve absorption by chelating non-haem iron and improving its solubility; the specific isomer form of β-carotene may therefore be important in relation to its effects on iron bioavailability.

1.6.2.1.2: Inhibitors

1.6.2.1.2.1: Calcium

In single meal human studies, and in multiple in vitro studies, calcium decreases the bioavailability of haem and non-haem iron – it is the only well established inhibitor of both dietary iron forms (Lonnerdal 2010). The inhibitory mechanism(s) are unclear; inhibition is only seen when the nutrients are co-ingested within 1-2 hours and is also dose dependent – 40 mg or less of calcium in a meal does not inhibit iron uptake, and once levels are higher than approximately 300 mg no further inhibition is seen (Hallberg, Brune et al. 1991, Walczyk, Muthayya et al. 2014). Earlier work suggested that calcium forms complexes with iron in the gut that prevent it being taken up by the enterocyte, however, this proposed mechanism does not explain how calcium would decrease bioavailability of both haem and non-haem iron - they are structurally different complexes and taken up by different pathways. Furthermore, the iron in haem is imbedded within the molecule and not readily available for calcium binding. More recent research suggests that calcium may decrease membrane expression of the iron transporter DMT1 (Thompson, Sharp et al. 2010), but it is not clear how this would explain the mechanism by which haem iron absorption is decreased by calcium since haem is not taken up by DMT1. It is possible that calcium alters iron absorption through multiple mechanisms; the fact that both haem and non-haem iron absorption is decreased suggests that at least one mechanism may involve the trapping of iron intracellularly.

The interaction between iron and calcium is of particular concern because risk of deficiency of both nutrients occurs in the same populations (Ramakrishnan 2002); excluding calcium from iron containing meals/supplements/fortified products is not a practical solution and efforts to treat deficiency of either nutrient would be simpler if they could be given together. Interestingly, a recent study found that ascorbic acid almost entirely negated the inhibitory effects of calcium on non-haem iron absorption indicating that in supplements addition of ascorbic acid could potentially overcome calcium-related inhibition (Walczyk et al., 2014).

Despite the negative effect of calcium on iron bioavailability in multiple studies as discussed above, the majority of both epidemiological and long term calcium supplementation intervention studies find that over the long term calcium intake does not negatively affect iron status (Lonnerdal, 2010) suggesting that homeostatic mechanisms adjust to the short term effects of calcium on iron absorption.

1.6.2.1.2.2: Zinc

Analogously with calcium, zinc and iron deficiency often co-exist within the same vulnerable populations and in the same parts of the world (Black 2003), therefore the same supplementation/fortification/food issues arise; there has been much research on whether zinc decreases iron absorption. Results of both in vitro and in vivo studies have been inconsistent – this is primarily because iron-zinc interactions depend on relative concentrations of the two minerals, their chemical forms, and the food matrix in which they are ingested and digested, and all these parameters can vary between studies (Arredondo, Martinez et al. 2006, Olivares, Pizarro et al. 2007, Olivares, Wiedeman et al. 2012). When iron and zinc are given together in an aqueous solution to fasted subjects, as might happen if they were taken together as a multi-mineral supplement with water and without food, zinc does inhibit iron absorption; the degree of inhibition increases with increasing amounts of zinc relative to iron (Olivares, Wiedeman et al. 2012). Zinc in a milk based food matrix, such as supplemented infant formula, did not decrease iron absorption at a 1:4 Fe:Zn (wt/wt) ratio (Friel, Serfass et al. 1998). These results suggest that within the appropriate food matrix, and with appropriate relative amounts of the two nutrients, iron and zinc can be given together. How zinc inhibits iron absorption, as occurs in aqueous solutions of the nutrients, is unclear. Earlier work suggested that zinc competes with iron for uptake by DMT1, but recent studies do not support this hypothesis (Kordas and Stoltzfus 2004).

1.6.2.1.2.3: Phytates

Phytates, a storage form of energy for plants, are the salts of inosital hexaphosphates (IP_6); they are abundant in cereals, grains, and some vegetables, and although not the most potent inhibitors of non-haem iron absorption in an absolute sense are probably the most important because of their ubiquitous presence in the diet. Inhibition of iron absorption is significant at phytate:iron molar ratios >1:1 in single food studies, and >6:1 in mixed meals that contain AA or meat enhancers (Hurrell and Egli 2010). Processing, storing, cooking and digestion may lead to hydrolysis of IP_6 and generation of lower phosphorylated derivatives such as IP_5,IP_4,$1P_3$; the fewer phosphate groups the less effect on iron bioavailability (Hallberg, Brune et al. 1989, Brune, Rossander-Hulten et al. 1992).

Phytates inhibit iron absorption by forming insoluble chelates such as diferric and tetraferric phytate complexes (Crichton 2009). The inhibitory effects of phytates are partially counteracted by ascorbic acid (Hallberg, Brune et al. 1989), although the extent of improved iron availability is a matter of debate with some investigators arguing that

this effect may only be seen in experimental conditions. Cook and Reddy studied whether ascorbic acid could ameliorate the negative effects of phytates on iron absorption (Cook and Reddy 2001). They used iron-labeled bread rolls eaten as part of a free living diet to assess iron absorption within complete meals, with instructions to participants to either eat high or low ascorbic acid foods, and they found that the high ascorbic acid diet only increased iron absorption by approximately 35 % ; significantly less than in single meal studies. Iron status was determined for the study, and low iron status predicted increased iron absorption. However, dietary ascorbic acid levels were estimated based on subjects' reported intakes, therefore it is possible that the observed lack of effect in this study occurred because actual dietary ascorbic acid was lower than reported.

1.6.2.1.2.4: Polyphenols

Polyphenols are a diverse group of compounds found in vegetables, fruits and commonly consumed beverages such as wine, tea and coffee – examples include quercetin, epicatechin and tannins (Bravo 1998). Polyphenols consist of large organic molecules characterized by multiple phenyl groups with varying numbers of attached hydroxyl groups; iron binds to the hydroxyl groups and may also be oxidized by them. (Bravo 1998). When polyphenols are evaluated either in single meal studies or in in vitro cell lines they powerfully inhibit non-haem iron absorption; ascorbic acid only partly reverses this effect even at high doses (Gillooly, Bothwell et al. 1983, Siegenberg, Baynes et al. 1991, Jin, Frohman et al. 2009, Petry, Egli et al. 2010).Polyphenols chelate iron and render it unavailable for absorption; recent research suggests that certain polyphenols, such as quercetin, may in addition decrease iron absorption by decreasing ferroportin levels and thus trapping the iron intracellularly to be lost with sloughing of the enterocyte (Lesjak, Hoque et al. 2014). This phenomenon may also explain the finding of another recent study that polyphenols decreased haem iron absorption in an in vitro model system – there is no evidence that polyphenols bind haem iron in the gut lumen, but if the iron released from haem were trapped intracellularly the net effect would be decreased absorption (Ma, Kim et al. 2010, Ma, Kim et al. 2011). However, the studies by Ma et al and Lesjak et al were carried out in in vitro cell lines and rodent models, and their findings need to be confirmed in vivo in human beings.

Hallberg speculates that much worldwide iron deficiency relates to the common practice of drinking tea with all meals, but the impact of polyphenols on iron absorption when eaten as part of a mixed diet, is not clear. The inhibitory effect varies with chemical structure (Brune, Rossander et al. 1989), and part of the problem of assessing the effects of polyphenols on iron bioavailability in a mixed, free living diet probably relates to the grouping together of all polyphenols into one category without discriminating between stronger and weaker inhibitory types. However, in populations, or in vulnerable groups within populations, with marginal iron intakes and/or increased needs, even small inhibitory effects on iron absorption secondary to polyphenols may have a clinical impact (Mennen, Walker et al. 2005).

1.6.2.1.2.5: Egg, soy and milk based proteins

In vitro and in vivo studies of the effect milk and milk-based products have indicated that milk proteins (specifically the proteins casein and whey) decrease iron absorption (Hurrell, Lynch et al. 1989). Interestingly, dephosphorylation and hydrolysis of casein decreases this effect (Bernos, Girardet et al. 1997), and an in vitro study using enzymatically hydrolysed casein added to fruit juices actually found improved iron bioavailability (Garcia-Nebot, Alegria et al. 2010). Digestion itself, at least in the mature gut, may therefore lessen the in vivo impact of milk proteins on iron bioavailability. Interestingly, a recent short term study conducted in rats found that high intake of casein protein (40 % of total energy) increased gene expression of the iron-absorption related proteins DMT1, Dcytb and TfR1, and this was correlated with increased iron absorption in the animals as measured by fecal iron balance measurement (Thomas, Gaffney-Stomberg et al. 2013). The study did not document increased protein expression of DMT1, Dcytb or TfR1, nor did animal iron status markers change though this may have related to the short study duration.

Numerous studies have shown that egg proteins decrease non-haem iron absorption (for example, (Ishikawa, Tamaki et al. 2007); it is suggested that the protein phosphovitin is responsible for binding iron and decreasing its bioavailability.

Soybeans have been shown to decrease non-haem iron bioavailability in multiple studies (Hurrell and Egli 2010). Legumes, including soybeans have high phytate levels, but the additional inhibitory effect of soybeans appears to be due to the soy-derived protein conglycine (Hurrell, Juillerat et al. 1992); fermentation may decrease the inhibitory effect of soy products on iron uptake (Baynes, Macfarlane et al. 1990).

1.6.2.1.3: Possible enhancers
1.6.2.1.3.1: Carbohydrates
1.6.2.1.3.1.1: Sugars

The effect of mono and disaccharides (simple sugars) on iron bioavailability is undetermined. Charley et al initially investigated this question in the 1960's (Charley, Sarkar et al. 1963). This group demonstrated that in the test tube simple sugars were able to chelate iron and form stable, low molecular weight complexes, which remained soluble even at a pH of 9 (where normally ferrous iron would form ferrohydroxy precipitants) (Charley, Sarkar et al. 1963). They also found that these complexes were more easily absorbed across the rat intestinal mucosa compared with iron alone, and that their ingestion was associated with increased liver iron deposits (Stitt, Charley et al. 1962). In their seminal paper "Chelation of Iron by Sugars" (1963) Charley et al observed that an excess of sugar over iron favored the formation of stable complexes, and that the "relative sequestering ability of … sugars" was fructose>glucose>sucrose". Work by others suggested that iron to carbohydrate molar ratios of at least 1:50 were required for optimal complex formation (Davis and Deller 1967).

However, further experiments in rodents produced conflicting findings. Several studies found that fructose increased iron absorption (Pollack, Kaufman et al. 1964, Bates, Boyer et al. 1972), but in other studies sugars either had no effect (Beynen, Brouwer et al. 1992)or actually decreased iron absorption (Brouwer, Lemmens et al. 1993). The methods used to assess iron bioavailability varied between these studies, as did iron status of the study animals, and the form in which the iron and sugars were administered– experiments in which the iron and sugars had been premixed and then fed to fasted animals gave the most consistently positive results. One study with negative results induced diarrhea (related to carbohydrate load) and decreased food intake in the study rats (Brouwer, Lemmens et al. 1993) - both of which would have led to decreased iron absorption not actually related to bioavailability.

Human studies to date have been small and results also inconclusive. Davis and Deller, using radioiron absorption measurement, gave a prepared iron-sugar complex and showed that fructose increased iron uptake; another study using the foecal balance method also found that fructose increased iron absorption (Davis and Deller 1967, Holbrook, Smith et al. 1989). But work by two other labs found either no effect of sugars on iron uptake (Heinrich, Gabbe et al. 1974) or a decrease – although similarly to the animal studies in the study with decreased iron uptake test subjects developed diarrhea (Ivaturi and Kies 1992). Subject iron status and dietary back ground varied in these studies, and there was no screening for genetic iron disorders.

Review of the evidence suggests that fructose may increase non-haem iron bioavailability, although this has not been definitively demonstrated, nor a mechanism clearly identified. O'Dell hypothesizes that fructose, a reducing sugar, reduces ferric iron to the bioavailable ferrous form; as discussed previously, earlier work suggests that fructose increases iron bioavailability by forming iron-sugar chelates that keep the metal soluble (O'Dell 1993). Recent research with fructo-oligosaccharides demonstrated that these non-digestible fructose polymers increased the expression of colonic iron transport proteins in a porcine model (Tako, Glahn et al. 2008); although a follow up study did not find an associated increase in colonic iron absorption (Patterson, Rutzke et al. 2009). Studies on the effects of fructose or other simple sugars on iron transport protein expression have not, to the author's knowledge, been carried out.

Recent epidemiological studies looking at fruit and iron interactions found differing results with two showing that fruit consumption predicted higher iron stores, and another two that increased fruit intake was protective against iron overload (Fleming, Jacques et al. 1998, Fleming, Tucker et al. 2002, McCune, Ravine et al. 2006, Milward, Baines et al. 2008) McCune, Ravine et al. 2006, Milward, Baines et al. 2008). But although fruits are a source of fructose they clearly also contain many other compounds that could alter iron bioavailability.

1.6.2.1.3.1.2: Prebiotoics

Prebiotics are defined as non-digestible (by the host animal) dietary factors that are reported to confer health benefits to the host (Gibson and Roberfroid 1995, Gibson, Probert et al. 2004); all identified prebiotics thus far are carbohydrates such as fructo-and galacto-oligosaccharides (FOS and GOS, respectively) (Slavin 2013). There has been extensive research on whether prebiotics improve iron bioavailability and absorption, however results remain inconclusive. For example, work in chickens, pigs, rats, and infants found that prebiotics increased iron absorption or improved iron status (Lopez, Coudray et al. 2000, Yasuda, Roneker et al. 2006, Sazawal, Dhingra et al. 2010, Tako and Glahn 2012); whereas other studies showed no change in iron absorption after addition of the prebiotic inulin (a heterogenous blend of fructo-oligosaccharides) in young iron deficient women (Petry, Egli et al. 2012), or in a porcine model (Patterson, Rutzke et al. 2009). Proposed mechanisms by which prebiotics might alter iron bioavailability include up regulation of iron transporter proteins (Lobo, Gaievski et al. 2014), and increased gut luminal acidity through prebiotic fermentation products (such as short chain fatty acids) thereby favoring the reduction of Fe^{3+} to the more bioavailable Fe^{2+} (Laparra, Tako et al. 2008, Yasuda, Dawson et al. 2009). Both of these mechanisms assume that significant amounts of iron are absorbed in the colon – a question that has not been answered for human beings. In addition, recent work indicates that prebiotics may reduce gut inflammation, and thus reduce systemic hepcidin levels, which would be predicted to improve iron absorption (Yasuda, Dawson et al. 2009). Laparra et al suggest that the conflicting results seen for different studies evaluating the effects of prebiotics relate to the specific prebiotics being evaluated, and their differing abilities to reduce inflammation; they fed rats an iron supplement with the addition of two novel prebiotics not commonly used in other studies (kojibiose and lactulose derived GOS) and showed improved iron absorption that correlated with decreased levels of both inflammatory markers and hepcidin (Laparra, Diez-Municio et al. 2014). If the effect of prebiotics on iron absorption is through alteration of systemic iron homeostatic mechanisms, this may also explain why research looking at prebiotics and iron carried out in vitro cell lines have been negative, as typically systemic effects of iron absorption are not a part of these models (this issue will be discussed later in this thesis).

1.6.2.2: Methods to assess iron bioavailability

There is no simple causal relationship between dietary iron content, iron absorption and subsequent host iron status. As iron deficiency is prevalent worldwide many methods have been developed to assess iron bioavailability, with the goal being to improve iron nutriture by identifying good dietary or supplemental sources of bioavailable iron. These methods can be broadly categorized as follows:

In Vivo studies

In vivo absorption studies – single meal or short term

- Chemical balance
- Isotope tracers
- Plasma iron
- Depletion-repletion studies

- Long term population studies

- Algorithms
- Animal models

In Vitro Studies

- Solubility studies
- Dialysability studies
- Mechanical gastro-intestinal models
- In vitro cell culture studies

The advantages and limitations of these methods are discussed below.

1.6.2.2.1: In vivo absorption studies

In vivo absorption studies are typically conducted over a short time period; duration can vary from one single meal (assessing a supplement, single nutrient or a composite meal) to a period of several weeks.

1.6.2.2.1.1: Chemical balance

Chemical balance studies were the earliest type of absorption study used to measure iron bioavailability. Subjects receive a known amount of iron, and then apparent iron output is measured in the stool; typically urine and sweat losses are presumed to be negligible and not measured (Wienk, Marx et al. 1999). Iron chemical balance studies are accurate if performed correctly, and in theory are straightforward. The method depends on accurate measurement of intake and can be affected by miss/under/over reporting and also by inaccurate measurement of iron in the ingested substance caused by soil iron contamination. Assumptions must be made about iron losses through mucosal sloughing into the gastrointestinal tract, and are typically also made about the amount of iron lost in sweat and urine. Finally, subjects' iron status needs to be taken into account. Accuracy is greatest in studies in which a supplement or prepared meal is given under supervision and stool collected in a controlled environment, and probably worst in assessing free living usual diets and intake (Wienk, Marx et al.1999).

1.6.2.2.1.2: Isotope studies

Iron is primarily used for haemoglobin synthesis, therefore human iron absorption can be evaluated by measuring the incorporation of labeled iron (using radioactive or stable iron isotopes) into RBCs. Uptake of radioactive or stable iron isotopes is a commonly used method to assess bioavailability; radioisotopes are considerably less expensive but can't be used in groups where exposure to radioactivity is a concern such as pregnant women and children – groups in whom iron deficiency is common. For both types of isotope the labeled iron must be incorporated into the nutrient of interest, and one of the limitations of the method relates to incomplete extrinsic isotope-intrinsic food iron equilibration/exchange (Jin, Cheng et al. 2008, Glahn 2015, Glahn, Cheng et al. 2015). After ingestion of the isotope, whole body counting in the case of radioisotopes, or blood measurement for stable isotopes, is used to measure uptake (Wienk, Marx et al.1999).

1.6.2.2.1.3: Depletion-repletion studies

Depletion-repletion studies may be carried out in animals or people. Animals are made ID, fed the food/nutrient/supplement of interest, and subsequent change in haemoglobin measured against a known "gold standard" iron replacement typically ferrous sulphate (Wienk 1999). Animal depletion-repletion studies show good correlation with radio-iron uptake in the same animal models, but extrapolation and applicability to humans depends on the animal.

In humans, a form of depletion-repletion study can be conducted using an iron intervention (food, supplement or fortified product), followed by either measurement of short-term plasma iron levels, or in longer-term studies subsequent changes in iron status in the tested subjects.

Food iron levels are not typically high enough to change iron levels after a meal, however plasma iron levels may change after ingestion of pharmacological doses of iron (such as those found in a supplement). Plasma iron concentration is measured over selected time periods after ingestion, and then the area under the curve calculated or the peak concentration used to assess bioavailability (Barrett, Whittaker et al. 1994).

Blood iron levels can be used to compare differing bioavailability from iron supplements given at pharmacological doses, but assumptions about plasma volume, compartment distribution and underlying iron status are necessary (Wienk, Marx et al.1999).

1.6.2.2.1.4: Single meal studies

Single meal studies may be used to assess iron bioavailability from foods, and identification of iron absorption enhancers and inhibitors. Evidence consistently shows that single meal (whether in vitro or in vivo), or brief (weeks) intervention trials, overestimate dietary components' effects on iron bioavailability in comparison to when that nutrient or food factor is eaten as part of a free-living diet (Collings, Harvey et al.

2013). All of the above discussed balance methods, when used within the context of a single meal or short duration (1-2 weeks), will be prone to this specific limitation. Research suggests that there are homeostatic mechanisms that affect iron absorption over the long term, related both to host iron status, and overall iron bioavailability of the typically consumed diet. This may be one reason underlying discrepancies between short and long term studies.

1.6.2.2.2: Long term population studies

Perhaps the most valid assessment of the effect of a dietary factor/food on iron bioavailability and iron status is to monitor a population eating that factor over the long term. This is time consuming, expensive and still prone to errors because both measurement of iron intake and iron status is challenging. For example, contamination of foods by soil iron may be an unrecorded source of iron intake, and different soil conditions, climate and food processing, in genetically variable populations, mean that even the "same" diet may have different levels of bioavailable iron in foods grown in different geographical conditions. In order to accurately interpret the effects of dietary factors on iron bioavailability in long term population studies there need to be adequate absolute amounts of iron in the whole diet, correction of other nutrient deficiencies, interpretation of results within the context of inflammation and blood loss, and the intervention must be of sufficient duration.

1.6.2.2.3: Algorithms/Predictive models

Whole diet iron bioavailability may be calculated by setting up computer algorithms with information about usual diet along with demographics such as ethnicity, average age, gender. Older algorithms used iron uptake enhancer data only, but newer studies have increased the complexity of factors analysed with improvement in accuracy; the addition of iron status data appears to be especially important for accuracy (Lynch 2005).

These models provide information at a population level, and rely on certain assumptions: this is the "average" diet and it has been accurately measured; the population is well described with similar underlying iron status; information about iron content and bioavailability, enhancers and inhibitors is accurate for the local geographical conditions; cooking methods will be similar and not alter levels of enhancers, for example, ascorbic acid and haem, or inhibitors; diet analysis software is accurate; and dietary factors effects on bioavailability are simply additive. Each of these assumptions is inaccurate in specific circumstances; for example, the enhancing effect of ascorbic acid is reduced in meals with meat (Lynch 2005), and phytate inhibition of iron bioavailability is decreased by phytase (an enzyme that degrades phytates) (Drakakaki, Marcel et al. 2005). The more these sources of error are taken into account the better the predictive value of the algorithm.

1.6.2.2.4: Animal models

Various animal models have been used to study iron bioavailability; the majority of studies use rats, pigs/piglets or chickens, and of these rats are the most frequently used animal – probably because of availability and cost.

Qualitatively there is agreement between rats and human studies for ascorbic acid, soy and fibre; iron deficient rats appropriately increase iron absorption, although the efficiency of this is higher compared with human beings (Reddy and Cook 1991). There are limitations, however, with the rat model (Reddy and Cook 1991). Rats, unlike humans, can synthesize ascorbic acid; this makes it difficult to assess ascorbic acid-related improvements in iron bioavailability for humans when using the rat model. It also probably explains why in rats, compared with humans, ferric iron is well absorbed and phytates only minimally decrease iron bioavailability. All rodent models have the limitation of a different gastrointestinal tract anatomy, gut microbiome and nutritional requirements compared with human beings.

Pigs and piglets are a relatively good model for studying iron bioavailability in human beings (Patterson, Lei et al. 2008). Diet and nutritional requirements are similar between the two species and they share the same iron transporter proteins (DMT1 and ferroportin). The pig and human GIT are structurally similar, although the absolute sizes and anatomical arrangement are different. The effect of iron deficiency, iron form, and dietary factors on iron bioavailability is the same in pigs as in humans with the exception of ascorbic acid – like rats, pigs can synthesize ascorbate and thus the effects of added ascorbic acid may be masked in this model (Patterson, Lei et al. 2008). Limitations of using pigs to study iron uptake include the possibility that the pig colon may be a site of iron absorption in deficiency states (Blachier, Vaugelade et al. 2007), something not demonstrated in humans. Finally, direct validation of results from the porcine model compared with human studies is limited.

The Glahn group have recently used domestic chickens to study iron bioavailability (Tako, Rutzke et al. 2010). The chicken gastrointestinal tract is different compared with humans, but the small intestinal layout is similar and the duodenum, as in humans, is the major site of iron absorption. Expression of gut iron transporters is the same as in people, and transporter expression changes in response to dietary iron manipulations in a predictable manner based on human studies (Tako, Rutzke et al. 2010). The model agrees with Caco-2 cell in vitro digestion results (Tako and Glahn 2010, Tako, Blair et al. 2011). Limitations of the poultry method include limited data on the effects of dietary factors on poultry iron absorption, and lack of direct human validation studies to date.

Iron uptake in animal models can be evaluated with absorption methods as used in human subjects (previously discussed). In addition, animal models allow for study of organ specific iron deposition from supplemental or dietary iron.

1.6.2.2.5: Solubility and dialysability studies

Solubility and dialyzability studies give information on iron bioaccessibility – the amount of iron released from food and available for absorption – but not on how much iron is taken up or used in the body.

The basic assumption behind solubility studies is that iron must be soluble in order to be absorbed, and that all soluble iron will be absorbed. Only non-haem iron bioavailability can be measured with this method.

Typically, the food and/or dietary factors of interest are subjected to an in vitro digestion process – at its simplest, the sequential addition of digestive enzymes in pH appropriate solutions to mimic digestive conditions in the stomach and small intestine – and the end products are called digestates (Wienk, Marx et al.1999). Mechanical gut models have also been developed to simulate the digestive process. For example, the TIM model is an artificial gut model (consisting of stomach, small intestine and colon compartments) that uses a computer to manage transit times through the model and the addition of enzymes, emulsifiers, and other gut secretions (Larsson, Minekus et al. 1997). Digestates from either simple or mechanical systems are centrifuged, and the iron in the supernatant measured and compared with total iron in the original food to provide relative levels of availability. Iron may be measured using colorimetric assays, atomic mass spectroscopy or inductively coupled plasma spectroscopy (Wienk, Marx et al.1999).

Comparison of solubility results with human studies shows that for selected nutrients solubility predicts bioavailability, but there are significant exceptions. The model does not account for small molecular weight iron-food complexes that are soluble but still not absorbed because of iron chelation or inappropriately changed oxidation state (for example ferric citrate is soluble but has poor bioavailability (Heinrich 1987), nor is it accurate in predicting absorption of large MW iron complexes, which though soluble aren't absorbed because they can't cross through the protective mucus layer that lines the gut – size matters. Ferric-phytate complexes are an example of large compounds that are almost completely non-available for absorption despite being soluble (Brune, Rossander et al. 1989).

In order to address size specific solubility study limitations, dialysis membranes with molecular weight cut offs (MWCO) that mimic the gut mucus layer are now routinely used in conjunction with solubility studies; only the soluble iron that can dialyse through the membrane is considered available. Iron dialysability studies correlate fairly well with human studies re relative bioaccessibilty of iron from different foods, and the effects of enhancers and inhibitors; they do not always provide accurate information about the strength of the effect (Wienk, Marx et al. 1999), and markedly underestimate iron available for absorption from large intact iron-protein complexes such as lacto-ferrin and ferritin, for which there is strong evidence that physiological absorption does occur. Furthermore, iron solubility and dialysability studies are usually carried out with isolated

dietary factors/foods or iron supplement forms, thus the impact of the food matrix in which the iron would normally be co-ingested is not investigated (Fairweather-Tait, Lynch et al. 2005).

Dietary factors that alter iron bioavailability either by chelating intracellular iron and thus preventing its release from the cell, or by changing the expression of iron related transporters, can not be assessed by dialysability studies at all. The method has execution issues as well with even very small variations in pH adjustments at various steps altering results between labs (Luten 1996). The impact of systemic control of iron homeostasis on absorption is also clearly lacking in both solubility and dialysability studies.

1.6.2.2.6: In vitro cell lines

In vitro cell line models have been used for decades for analysis of iron absorption mechanisms, kinetics and bioavailability; although originally derived from cancer cells these models have been extensively studied and results validated in comparison with human results.

1.6.2.2.6.1: Caco-2 cells

Caco-2 cells are amongst the original in vitro cell lines used to measure iron bioavailability; work using this cell line in the context of iron bioavailability began in the 1990s (Sharp 2005). Because the cells absorb iron they do assess bioavailability rather than just bioaccesibility (the fraction of the total amount of a substance that is potentially available for absorption) – but only partly since egress from the cells is not typically evaluated, nor are the effects of systemic iron homeostatic controls included in the normally used in vitro digestion method.

Typically, Caco-2 cells are coupled with an in vitro digestion system in which foods are treated with a series of intestinal digestive enzymes to mimic the human digestive process, and then the digestion products (digestates) are "fed" to Caco-2 cells (Figure 1.10, Caco-2 cell in vitro digestion model as developed by R Glahn); iron uptake is then measured either through direct measurement of radio-labelled iron within cells, or through the measurement of ferritin an iron storage molecule as a proxy measure of iron uptake (Gangloff, Glahn et al. 1996, Glahn, Lee et al. 1998).

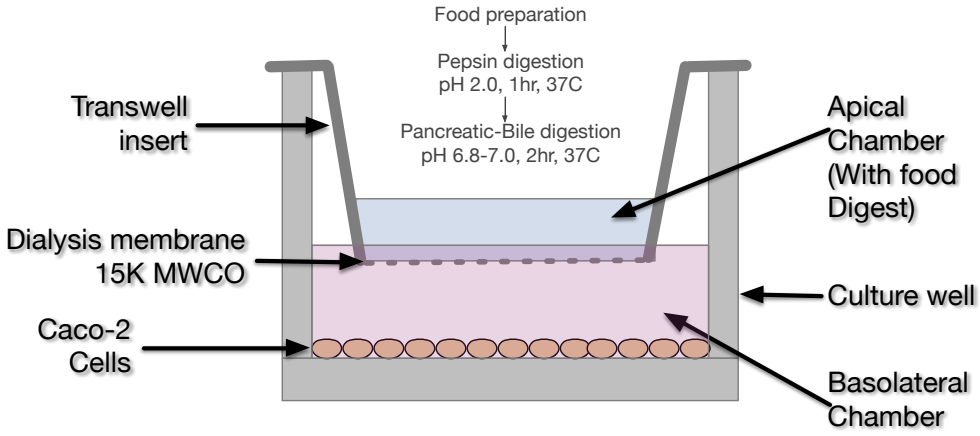

Figure 1.10 Caco-2 cell in vitro digestion model as developed by R Glahn.
The dietary factor or supplement of interest, either with intrinsic iron or added extrinsic iron, is subjected to a simulated in vitro digestion process with gastric and small bowel digestive enzymes. The processed food or supplement now known as the digestate is then placed in the upper chamber of the model formed by the placement of a dialysis membrane over a Transwell insert minus its inherent membrane; the added dialysis membrane both protects the cells from the enzymes, and also mimics the size barrier of the small intestinal mucosal surface. After a total of 24 hours the cells growing on the bottom of the wells are harvested for ferritin analysis (adapted from (Glahn, Lee et al. 1998).

Caco-2 cells are derived from a human adenocarcinoma of the colon (Fogh, Fogh et al. 1977), but when grown in appropriate conditions they differentiate and develop many phenotypic features of small bowel enterocytes; they become polarized, develop microvilli and glands, and express many enterocyte nutrient-related membrane receptors, transporters and enzymes including but not limited to: Sodium Linked Glucose Transporter 1(SLGT1)(Khoursandi, Scharlau et al. 2004); DMT1 (Tandy, Williams et al. 2000); DcytB (McKie, Latunde-Dada et al. 2002); ferroportin (McKie, Marciani et al. 2000); sucrase (Chantret, Rodolosse et al. 1994); and GLUT 5 and GLUT 2 (Mahraoui, Rousset et al. 1992).

Caco-2 cells have been used to study both iron transport kinetics and absorption, and iron bioavailability (Jumarie and Malo 1991, Halleux and Schneider 1994) with validation of these results in several human studies (Garcia, Flowers et al. 1996, Au and Reddy 2000). Au and Reddy conducted a comparison study of iron bioavailability results from published human trials with results obtained in their lab using a modified version of the Caco-2 cell in vitro model with digestates made up of the same foods as those used in the human trials. They demonstrated excellent correlation of results between the two methods (r=0.97), with the exception of ascorbic acid and bovine serum albumin (BSA) – both known enhancers of iron uptake. As the reducing effect of ascorbic acid is pH dependent the authors investigated whether the higher small intestinal phase pH at which Caco-2 in vitro digestions are typically carried (6.9-7 versus the expected pH of the duodenum of 6.4) may have explained this discrepancy, and indeed found that decreasing the pH

improved the effects of ascorbic acid on iron bioavailability in the Caco-2 cell model (Au & Reddy, 2000). In addition, Au and Reddy treated the samples for an additional 30 minutes in a 37°C water bath, placed them on ice, and then only used the supernatant of test meal digestates to apply onto Caco-2 cells; these additional steps may also have affected results compared with the method as developed by Glahn et al in which the entire digestate is applied to a dialysis membrane suspended over the Caco-2 cells. Another study used replicate meals, as prepared in a human trial of iron absorption, in the Caco-2 cell in vitro digestion model, and found good correlation between the two maize-based meals, but lack of correlation with results that compared red-skin bean and white-skin bean iron bioavailability (Beiseigel, Hunt et al. 2007). In the human trial iron absorption from the two bean varieties was equivalent, whereas red-skinned beans were not a bioavailable source of iron in the Caco-2 model. The authors' suggested that polyphenols present in the colored-bean skins may have been inhibitory to iron uptake in the in vitro model but not in human beings, but although they measured total polyphenol levels (gallic acid equivalents) they did not actually identify specific polyphenols present in the tested foods. In a later study conducted by the Glahn lab, evidence suggested that the extrinsic radio-iron isotope used in the human trial of the Beisengel et al study did not equilibrate with the red-bean intrinsic iron, and thus gave an inaccurate assessment of iron uptake in the human trial (Jin, Cheng et al. 2008).

Review of the above evidence suggests that testing parameters in the Caco-2 cell model including pH, exactly how the digestates are prepared and used, and duration of incubation times, may affect results. The cause of the difference in results with red versus white beans remains unclear, although problems with equilibration between extrinsic and intrinsic food iron has been demonstrated in another recent study (Glahn, Cheng et al. 2015), and may underlie the apparent difference in results in the Beiseigel study.

Evaluation of iron uptake with the Caco-2 model depends on the expression of iron-transport related proteins including DMT1 and Dcytb. Caco-2 cells experience mutagenic drift with documented changes in protein expression and enzyme activity related both to cell passage number, and also days in culture (Briske-Anderson, Finley et al. 1997, Sambuy, De Angelis et al. 2005). Therefore, bioavailability studies that use the model need to be conducted with defined parameters including initial cell passage number, number of times cells are passaged and used, and number of days in culture. The Glahn lab, who have worked extensively with this model, conduct experiments with early-mid passage cells, and at 14 days post seeding (Gangloff, Glahn et al. 1996); and Sharp et al have documented that this coincides with maximal and stable DMT1 expression (Sharp, Tandy et al. 2002). Regardless of these measures, Caco-2 cells have relatively low innate expression of Dcytb (Latunde-Dada, Simpson et al. 2008) – thus for meals/foods with low levels of ascorbic acid, or high levels if inhibitors, absorption will be underestimated compared to human studies.

Some researchers have also questioned the gastric phase pH at which experiments are conducted, suggesting that the actual pH of the stomach during digestion is 4 rather than

2 (Dokoumetzidis, Kalantzi et al. 2007). However, stomach pH will vary depending on the age of the person, and the meal composition and therefore buffering capacity of the food. Furthermore, certain substances are recommended to be ingested without food, for example iron supplements, in which case the gastric pH would be expected to be 2. Lastly, validation of the model has been conducted at gastric pH of 2, therefore if the pH were to be changed in experiments re-validation should also be conducted.

The Caco-2 cell model uses fixed digestive phase times, in contrast with the gut in which transit times will vary depending on meal composition, and this might be predicted to introduce a confounding variable if meal types being tested with the model are very different from one another. Generally, however, similar type foods or supplements are tested in the model as it is often used to compare similar foods but with altered levels of iron, or decreased levels of inhibitors (as in many of the biofortification studies, for example (Glahn, Cheng et al. 2002, Wortley, Leusner et al. 2005). Iron bioavailability in these cases is expressed in relative terms, thus the fixed digestion phase times would impact equally on all the foods being tested, and should not alter relative iron bioavailability results.

The preparation and storage conditions of test meals may also artificially alter results; a recent study found that in foods with native iron, levels of soluble, available iron increased after food freezing and thawing compared with fresh food samples (Thompson, Sharp et al. 2010). This is particularly significant as many Caco-2 cell in vitro digestion studies use frozen/freeze dried food samples for analysis; primarily related to convenience and lab operating hours (Ray Glahn, personal communication). Optimal evaluation of iron bioavailability of foods will occur if meals, or foods, being tested are prepared as they would actually be eaten, thus not introducing preparation and storage artifacts; and indeed research demonstrating good correlation between human studies and the Caco-2 cell model has used replicate meals, for example (Au and Reddy 2000, Yun, Habicht et al. 2004)

As stated previously, iron uptake by Caco-2 cells was initially measured using radioactive iron isotopes, however Glahn et al developed a method using ferritin formation as a proxy measure for iron uptake. This method has been validated in human studies and used extensively (Glahn, Lee et al. 1998, Yun, Habicht et al. 2004), however, there is the possibility that cells may make ferritin in response to oxidative stress or inflammation and one laboratory demonstrated that ascorbic acid alone may also induce ferritin formation (Scheers and Sandberg 2008, Scheers and Sandberg 2011). Thus, when using the in vitro digestion system, where possible, an iron free treatment of the Caco-2 cells with the substance of interest – for example, ascorbic acid alone, or prebiotics alone, should be conducted to eliminate non-iron related ferritin formation. Recent work carried out with the Caco-2 cell model demonstrated a good correlation between Caco-2 cell ferritin levels and cellular iron levels as measured by inductively coupled plasma atomic emission spectroscopy- mass spectroscopy (Glahn 2009), a highly sensitive and specific method for analysis of mineral concentrations. Other work with Caco-2 cells has shown that

ferritin increases with increasing iron dose and use of iron-uptake enhancers in test digestates, and decreases with inhibitors (Jin, Frohman et al. 2009).

Body iron status affects dietary iron absorption, but this variable is not assessed in the combined Caco-2 cell in vitro digestion system, as cellular egress of iron isn't measured. Exit of iron from Caco-2 cell monolayers can be measured by growing the cells on inserts suspended over culture wells; this creates two chambers representing the apical and basolateral surfaces of the enterocyte (Figure 1.11). Iron is added to the apical/luminal chamber and the basolateral/blood side may be treated with hepcidin, for example, mimicking circulating signals involved in iron homestasis. Iron levels in media collected from the basolateral chamber represents iron that has egressed from the Caco-2 cells

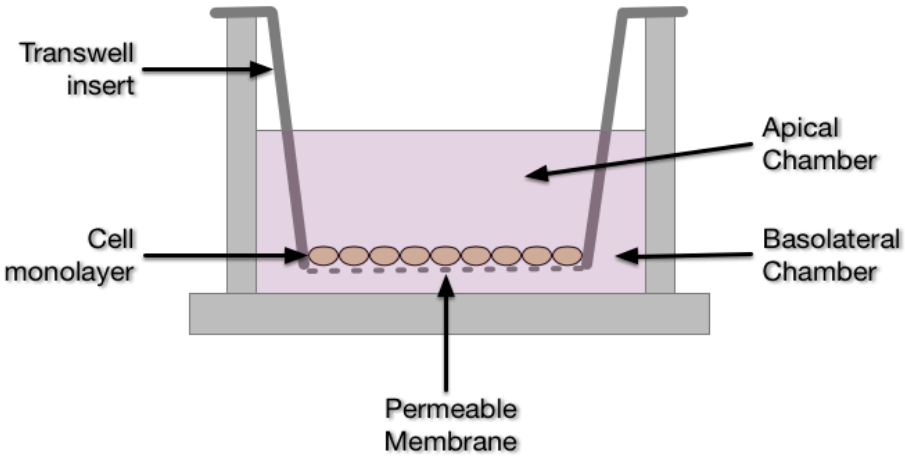

Figure 1.11 Caco-2 cell in vitro system with Transwell insert to assess iron absorption in combination with a model of systemic regulation.
Cells are grown until differentiated on permeable membrane supports creating a bicameral system. The iron and/or dietary factor of interest are placed in the apical chamber representing the gut lumen; systemic controls on iron absorption, such as hepcidin, are placed in the basolateral chamber. Media is collected from both chambers and iron measured in both to calculate the percentage of iron absorbed relative to that applied to the system. The formation of tight cell junctions between cells is essential so that materials pass through cells rather than between them; studies using this method should measure transepithelial electrical resistance(TEER) to demonstrate suitable permeability (diagram produced by T. Christides).

This method would differentiate dietary factors that increase ferritin but actually decrease iron absorption -for example, a study of the polyphenol quercetin found that it increased apical iron uptake and ferritin formation, but inhibited basolateral iron egress, thus decreasing iron bioavailability in the setting of increased enterocyte ferritin (Lesjak, Hoque et al. 2014).

The in vitro digestion system developed by Glahn et al doesn't work with Caco-2 cells grown on inserts, because the digestive enzymes used are toxic if placed directly on the cells (in the Glahn system the cells are grown in the bottom of the well and separated by the dialysis membrane.

Some research groups have circumvented this problem by preparing food digestates, using various methods to inactivate the digestive enzymes such as ice, or high temperature heating and/or centrifuging the samples, and then applying the "inactivated" digestates directly onto the cells growing on the inserts for 1-4 hours (for an example see(Au and Reddy 2000). This introduces unknown effects into the system, as there is the possibility that the inactivating process itself may artificially change iron bioavailability depending on the dietary factor being tested. In addition, if the Caco-2 cell transepithelial electrical resistance (TEER; a measure of the integrity of cell-cell adhesion)) is low this may allow paracellular transport of nutrients that in normal physiological conditions can only traverse the gut through the enterocyte rather than paracellularly; thus if nutrient absorption is being assessed with cells grown on inserts TEER must be checked to ensure integrity of the cell layer. This is not an issue with the in vitro digestion method as developed by Glahn et al in which the cells are grown as a monolayer, and the intracellular protein ferritin is used to measure iron uptake.

Perhaps the most important drawback to the use of Caco-2 cells is one shared with single food or single meal human studies – measuring in isolation the impact of a specific dietary factor or food on iron bioavailability may not accurately reflect the effects that would be seen if that food or factor were eaten within a general mixed diet over a long time period.

In summary, the Caco-2 cell in vitro digestion model is a useful tool for initial screening and ranking of foods and iron supplements in terms of relative iron bioavailability. It is relatively inexpensive compared with animal and human trials (Glahn 2009), and large numbers of samples can be analysed simultaneously. Use of this model may also minimise unnecessary testing in vulnerable groups such as pregnant women and young children in whom iron deficiency is a major public health problem. However, the method requires experienced workers and good cell culture facilities, and quality control of many variables that can affect results including: the pH at which experiments are conducted; cell passage number and days in culture; food preparation and storage; and ensuring that non-iron related increases in ferritin are ruled out.

1.6.2.2.6.2: HepG2 cells

HepG2 cells have also been used to study iron bioavailability in the broadest definition of the word – the metabolism and use of iron within the body. Studies looking at dietary factors and their iron uptake in this cell line, to the author's knowledge, have not been done; HepG2s are used to measure liver iron uptake from different (typically intravenous) iron preparations. Ferritin formation in HepG2 cells changes in response to different iron

formulations and correlates with iron uptake in a dose dependent manner; levels also decrease in response to iron chelators as would be predicted (Scheiber-Mojdehkar, Sturm et al. 2003, Sturm, Goldenberg et al. 2003, Popovic and Templeton 2004). Validation compared with human studies, however, is scarce, and typically inferred (by haemoglobin response, for example) because of the ethical and health issues associated with liver biopsy.

1.6.2.2.6.3: Combined Caco-2 and HepG2 cell culture model for evaluating iron absorption.

Recently, Scheers et al have proposed a modified in vitro model to measure iron absorption that uses Caco-2 and HepG2 cells grown together (Scheers, Almgren et al. 2014). Liver derived HepG2 cells produce hepcidin, and thus in this model use of both cell lines is proposed to add one of the elements of systemic control of iron absorption. The model uses ferritin as a marker for iron uptake. The modified model was responsive to increasing levels of iron, Caco-2 cell ferroportin levels decreased as HepG2 hepcidin levels increased in response to iron loading, and two different breads tested for iron bioavailability gave the same results as with the original Caco-2 cell model. These results suggest that this modified model may be a more physiological method to assess iron absorption in selected circumstances. The model remains to be validated against human studies, and questions about timing may also be important since the evidence suggests that in vivo liver hepcidin release in response to dietary iron load may occur several hours after meal ingestion or may fluctuate (Chung, Chaston et al. 2009, Hwang, Lee et al. 2011).

1.7: Aims

The aims of this thesis were to study the effects of carbohydrates on non-haem iron bioavailability, and iron and fructose effects on colon and liver cell gene and protein expression in relation to pathways implicated in CRC, HCC and NAFLD. The principal carbohydrates to be investigated were the monosaccharide fructose and the related sweetener high fructose corn syrup (HFCS), and the prebiotics FOS and GOS.

The specific objectives of this thesis are outlined below:

1. To determine the effects of sugars and sugar-derived compounds, on iron bioavailability in Caco-2 and HepG2 in vitro cells as models of the human gut and liver, respectively.
2. To determine if fructose as an additive in iron supplements was associated with improved iron bioavailability.
3. To determine if fructose as a naturally occurring dietary factor in a staple crop food matrix, as exemplified by sweet potatoes, improved iron bioavailability.
4. To determine if FOS and GOS improved iron bioavailability in a milk-based food matrix.
5. To examine biological mechanism(s) by which sugars might affect iron bioavailability in Caco-2 and HepG2 cells by analysing iron-homeostasis related protein and gene expression.
6. To determine the effects of iron and fructose treatments on Caco-2 and HepG2 cell gene and protein expression in relation to CRC, NAFLD and HCC.

1.8: Hypothesis

It was hypothesised that fructose, HFCS, and FOS and GOS would increase non-haem iron bioavailability in Caco-2 cells, and that fructose would similarly increase non-haem iron uptake by HepG2 cells. It was further hypothesized that iron and fructose treatments in both cell lines would be associated with changes in CRC-, HCC- and NAFLD-associated gene and protein expression.

CHAPTER TWO: CELL CHARACTERIZATION OF CACO-2 AND HEPG2 CELLS.

2.1: Introduction

In vitro cell line characteristics may change with cell passage number. This appears to be especially true in Caco-2 cells; growth rate, transporter expression and enzyme activity vary between different passages and also different clones (Briske-Anderson, Finley et al. 1997, Sambuy, De Angelis et al. 2005). For this reason the cell lines used in this report, the original Caco-2 cell line (HTB 37), the Caco-2 TC7 clone, and HepG2 cells, were characterised for properties felt to be relevant to the planned studies prior to initial experiments. In particular, Caco-2 iron homeostasis proteins DMT1, ferroportin, and DctyB were assessed to ensure that expression levels were optimal for the planned days that in vitro digestion experiments were to be conducted. The in vitro digestion method as developed and refined by the Glahn lab uses Caco-2 cells 13-15 days (over the entire span of the protocol) status post seeding (Gangloff, Glahn et al. 1996) therefore one of the aims of these studies was to ensure that iron transport proteins were present and stable over the proposed time period of experiments. In addition, as the proposed work involved investigations with sugars, expression of sucrase, the enzyme that metabolises sucrose, were also conducted in the two Caco-2 cell lines.

2.2: Materials and methods

2.2.1: Reagents

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, UK or Fisher Scientific. Glassware used in the sample preparation and analyses was treated with 10 % (v/v) of concentrated Nitric acid (65 %) for 24 h and then rinsed with 18 MΩ purity water before use. All water used in experiments was18 MΩ purity.

2.2.2: Cell culture

The Caco-2 cell original clone (herein referred to as HTB 37) was obtained from ATCC at cell passage 18; experiments were carried out with cell passages 25-35. The TC7 Caco-2 cell clone (developed in the labs of Rousset and others (Caro I 1995) was obtained from the lab of Dr. Paul Sharp and used from passages 35-47. HepG2 cells were obtained from ATCC at cell passage 28 and passages 30-40 were tested. All cells were tested over three separate passages.

Caco-2 cells were maintained in cell culture treated T75 flasks (Corning Inc., Costar) and sub-cultured every 5-7 days. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, 41965) supplemented with 10 % (v/v) foetal bovine serum (LCG Standards, 30-2020**),** 1 % penicillin-streptomycin, 4 mM L-glutamine, 1 % non-essential

amino acids, and Plasmocin 5 μg/ml (Source Bioscience). For experiments, Caco-2 cells were seeded at $1x10^4$ cells/cm^2 in 96, 12 or six-well plates (Corning Inc., Costar).

HepG2 cells were cells were maintained in cell culture treated T75 Tissue Culture Flasks, and seeded at a density of $1x10^5$ cells/cm^2. Cells were sub-cultured every 48-72 hours. Cells were grown in DMEM supplemented with heat inactivated 10 % (v/v) foetal bovine serum, 1 % penicillin-streptomycin, 2 mm glutamine, 1 % non-essential amino acids. Experiments with HepG2 cells were carried out in cell culture treated 12 or six-well plates seeded at $1x10^5$ cells/cm^2.

2.2.3: Cell growth

Cell growth of both Caco-2 cell clones and the HepG2 cell line was assessed using two different methods – cell counts by haemocytometer, and cell viability using the MTT assay.

2.2.3.1: MTT assay

HTB37 and TC7 Caco-2 cell viability was determined by MTT assay ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) (Mosmann 1983). MTT is a tetrazolium salt that is metabolized to visible blue formazan crystals by mitochondrial dehydrogenase enzymes – only live cells can process MTT and formazan production is directly proportional to live cell number and viability.

Cells were seeded in 96-well culture plates; after seeding selected cells were treated with 20 μl MTT solution (5 mg/ml MTT in PBS, sterile filtered) and incubated for 4 hours at 37°C. The MTT and media were then carefully aspirated and 100 μl DMSO (dimethyl sulfoxide) added to dissolve cell membranes and ensure formazon crystals were fully dissolved. Absorbance was read at 540 nm on a Thermo Multiskan Ascent spectrophotometer. Cells were grown for 21 days and assayed on days 3, 5, 7, 10, 14, and 21 (n=8 for each time point).

2.2.3.2: Cell counts

Cell growth of both Caco-2 cell clones, and HepG2 cells, was assessed by cell counts. Cells were grown in either 6 well (Caco) or 12 well (HepG2) plates at the previously stated seeding densities. After seeding cells were released from selected wells by trypsin, diluted with Trypan Blue 1:1 to ensure viability (viability was always ≥ 90 %) and counted using a haemocytometer. HepG2 cells were counted from days 1-5 (n=3 for each time point); Caco-2 cells on days 3, 5, 7, 10, 14, and 21 (n=2 for each time point).

2.2.4: Sucrase activity

Sucrase is an enterocytic membrane-bound enzyme that breaks sucrose down into its constituent monosaccharides glucose and fructose. HTB 37 and TC7 Caco-2 cell sucrase

activity was analyzed using a modified version of the glucose oxidase (GO) method developed by Dahlqvist (Messer and Dahlqvist 1966). GO oxidizes glucose to hydrogen peroxide (H_2O_2) and gluconic acid; in the presence of peroxidase and o-dianiside (a chromogenic reagent) a pink colored solution results whose intensity of color is directly proportional to the amount of glucose produced. The underlying assumption of the assay is that one molecule of produced glucose represents the breakdown of one sucrose molecule. Sucrase activity is expressed as micro-units/µg cell protein (one micro-unit = the enzyme activity that hydrolyzes one µmole sucrose/minute).

Cells were grown in 6 well plates and used for experiments from days 5-21 (days 5,7,10,14,21; n=3 for each time point). On the day of the experiment selected wells were rinsed with sterile PBS and then treated with 1.5 ml 28 mM pH 6.5 sucrose solution (made up in PBS, sterile filtered) for 20 minutes. The applied solution was collected, and 40 µl aliquots in duplicate placed in a 96-well plate alongside with aliquots of known glucose concentration standards. 80 µl of glucose oxidase/peroxidase/o-dianisidine reagents (Sigma-Aldrich Glucose (GO) Assay Kit GAGO-20) were added to each well using a multi-channel pipettor as the assay is very time sensitive, and the plate was incubated in a 37°C water bath for 30 minutes. The reaction was stopped with 80 µl 12N H_2SO_4 (sulphuric acid stops the reaction and stabilises the colored product), and the absorbance read at 540 nm in a Thermo Multiskan Ascent spectrophotometer. Cell layers were harvested for protein analysis with ice cold CelLytic (Sigma) with added 1% protease inhibitor and protein levels were measured using the Pierce Protein bicinchoninic acid (BCA) assay (Fisher 23227). Sucrase activity is expressed as a function of total cellular protein.

2.2.5: Western blot analysis

Cells were grown in 12 or 6-well plates for 1-5 days (HepG2 cells) and 5-21 days (Caco-2 cells) and analyzed for baseline protein expression of several iron-related proteins. On the appropriate day selected cells were twice washed in ice cold phosphate buffered saline (PBS); ice cold CelLytic with 1 % protease inhibitor was added to each tissue culture well. Cell monolayers were removed with a cell scraper and placed in 1.8 ml Eppendorf tubes that were shaken for 15 minutes on a Stuart microtitre plate shaker at 1250 RPM and then spun at 6,000 g for 6 minutes in a 5804R Eppendorf centrifuge. The supernatant was aspirated and stored at -80°C until Western Blot analysis. Protein concentration was measured by Pierce Protein BCA Assay. 2x Laemmli buffer [62.5 mm Tris-HCl (pH 6.8), 25 % glycerol, 2 % SDS, 0.01 % bromophenol blue with 10 % beta-mercaptoethanol (BME) was added in equal volume to a test sample containing approximately 40 µg of the relevant protein, mixed and then loaded onto a precast 10 % SDS-polyacrylamide gel. The gel was run for 1 hour and 30 minutes, at 40 mA per gel.

Separated proteins were electro-transferred to a nitrocellulose membrane (Amersham bioscience) at 40 volts for 90 minutes, and then the membrane was pre-incubated with blocking solution (5 % fat-free milk in PBS containing 0.1 % Tween 20 - PBST) for one

hour at 37ºC followed by overnight incubation at 4^{o}C with the primary antibody of interest in 1 % fat-free milk in PBST. Primary antibodies used were: anti-DMT1 (Santa Cruz Biotechnology) at 1/5000 dilution; anti-DMT1 Abnova (1:2000); anti-Dcytb Santa Cruz (1:500); anti-ferroportin Genetex (1:1000), anti – TfR1 and TfR2 1:1000 (Santa Cruz); anti-villin Genetex (1:2500), and anti-β-actin (Abcam) at 1/2500 dilution. After incubation, the membranes were washed and incubated with the appropriate Horseradish Peroxidase (HRP)-linked secondary antibody (1/2000) for 1 hour at room temperature and blots were detected by ECL chemiluminescence (ECL, Amersham Place, Buckinghamshire, England).

The TC7 Caco-2 cell clone was analysed for DMT1, DcytB, ferroportin and villin protein expression.

The HepG2 cell line was analysed for TfR1 and TfR2 protein expression.

Image J software was used to semi quantify the band corresponding to each antibody and all were normalized to β-actin.

2.2.6: Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism (v.6.0 GraphPad Software, San Diego, CA). Statistical analysis was conducted according to the methods of Motulsky (Motulsky 2010). Data are presented as means ± S.E.M. Data were analysed by one way ANOVA followed by Tukeys multiple comparisons tests post-hoc analysis other than sucrase expression and growth curve analysis which used False Discovery Rates (FDR). The desired false discovery rate (Q) value was set to 1.000 %. Where results were compared on various days, each day was analyzed individually, without assuming a consistent standard deviation. A p-value less than Q was deemed to be a discovery (and therefore statistically significant) if, and only if, the p-value was less than Q divided by the number of comparisons multiplied by the rank of the p value.

2.3: Results

2.3.1: Caco-2 cells: comparison of Caco-2 cell characteristics between different cell passages.

Growth and sucrase activity of three different cell passages (assessed both by MTT assay and cell counts) did not significantly differ between passage numbers for either HTB37, or TC7 Caco-2 cells (Figure 2.1).

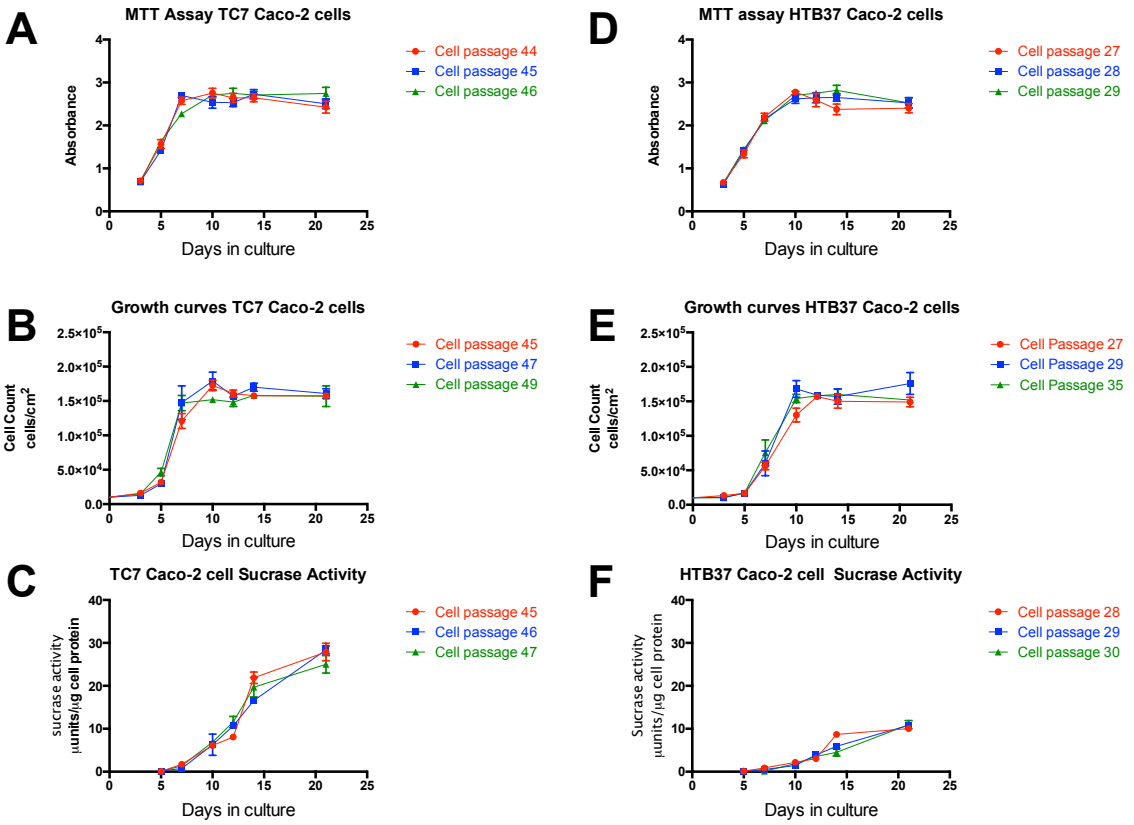

Figure 2.1 Comparison of growth and sucrase activity between three different cell passages for Caco-2 HTB37 and Caco-2 TC7 cells.

Figure 2.1(A): TC7 MTT Assay of cell passages (CP) 44, 45, and 46; **Figure 2.1(B)**: TC7 Growth Curves of CP's 45, 47, and 49; **Figure 2.1(C)**: TC7 sucrase activity of CP's 45, 46, and 47; **Figure 2.1(D)**: HTB37 MTT Assay of CP's 27, 28, and 29; **Figure 2.1(E)**: HTB37 Growth Curves of CP's 27, 29, and 35, **Figure 2.1(F)**: HTB37 sucrase activity of CP's 28, 29 and 30. Cells were tested for growth on days 3,5,7,10,12,14 and 21 (n=2 for cell counts, n=8 for MTT assay; sucrase activity was measured on days 5, 7, 10, 12, 14, 21 (n=3). There were no statistically significant differences between the tested cell passages, for either HTB37 Caco-2 cells or TC7 Caco-2 cells, for growth measured both by MTT assay and cell counts, or sucrase activity (based on FDR rate with Q=1.000 %).

2.3.2: Caco-2 cells: comparison of Caco-2 cell characteristics between HTB37 and TC7 Caco-2 cell clones.

Growth: TC7 Caco-2 cell growth rate was more rapid compared with the HTB37 parental cell line as measured by both MTT assay and cell counts; the doubling time of TC7 cells was approximately 48 hours versus 60 hours for HTB37 cells (Figure 2.2). Growth started to stabilize for both cell lines at approximately 13-15 days post seeding and cells numbers of both cell lines were essentially the same by day 21 (Figure 2.2).

Enzyme activity: Sucrase activity differed significantly between HTB 37 and TC7 Caco-2 cells. HTB 37 sucrase activity became detectable late (after day 10) and was approximately 60 % less compared with sucrase activity of the TC7 clone at day 21

(Figure 2.2(C)). TC7 sucrase activity was detected from day 7 and continued to increase until day 21.

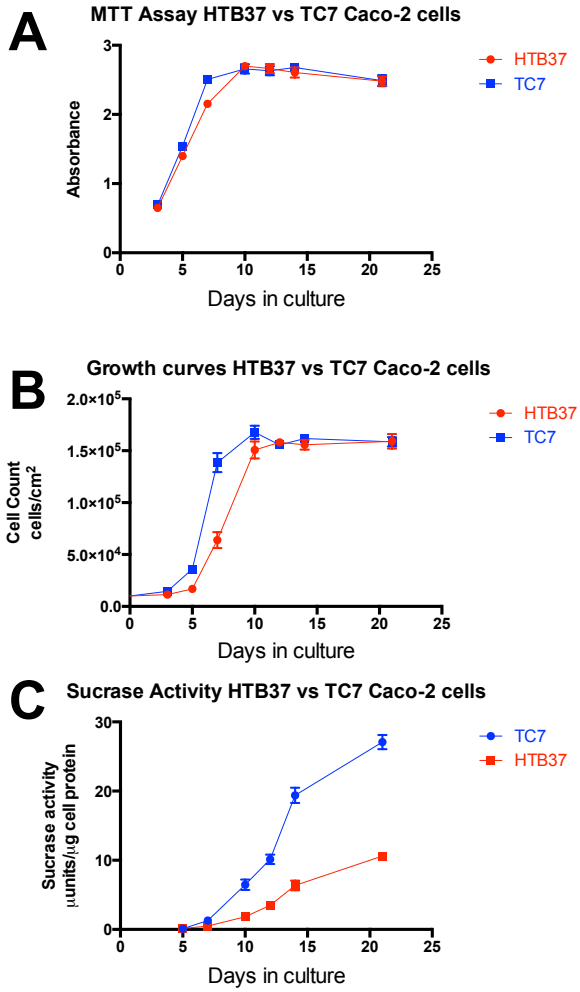

Figure 2.2 Comparison of HTB37 Caco-2 and TC7 Caco-2 cell characteristics.
Figure 2.2(A): Growth assessed by MTT assay; **Figure 2.2(B)**: Growth assessed by cell counts; **Figure 2.2(C)**: Sucrase activity: HTB37 results shown in red, TC7 data in blue. Results are the average of three different cell passages within the cell passage number of cells used for experiments. Cell growth was measured for days 3, 5, 7, 10, 12, 14, 21 (n=2 for cell counts, n=8 for MTT assay); sucrase activity was measured on days 5, 7, 10, 12, 14, 21 (n=3). TC7 cells grew more rapidly compared with HTB37 Caco-2 cells as measured both by MTT assay and cell counts (growth rates differed significantly at day 7 and absorbance differed significantly at days 5 and 7, both based on false discovery rate with Q=1.000 %); by day 14 growth and cell density had equalized between the two cell lines (there was no statistical difference beyond day 10, in either the absorbance or the cell count based on false discovery rate with Q=1.000 %). HTB37 Sucrase activity was not present until day 10 and remained low relative to TC7 Caco-2 cells until day 21. From day 10 onwards sucrase activity between the two cell lines differed significantly based on the false discovery rate with Q=1.000 %.

2.3.3: Caco-2 cells: expression of selected proteins

DMT1 expression was assessed by two different antibodies – Santa Cruz and Abnova (Figure 2.3). Western blot analysis of results from both antibodies demonstrated that the TC7 Caco-2 cell line was expressing DMTI protein by day 5, and that expression continued to increase until day 14. Levels significantly decreased as measured by the Abnova antibody after day 14, but remained stable as assessed by the Santa Cruz antibody.

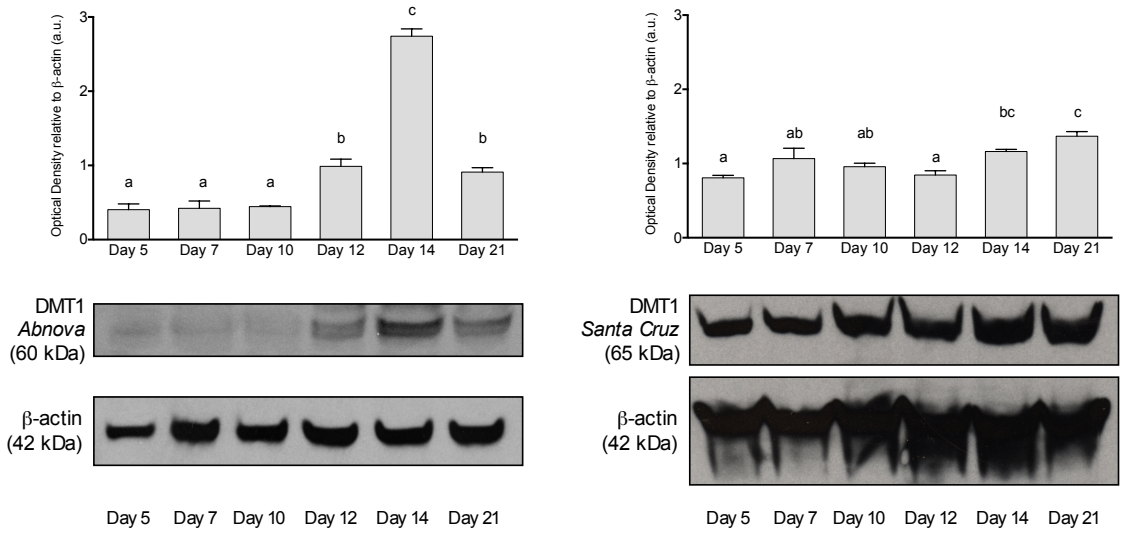

Figure 2.3 Western blot analysis of DMT1 expression as a measure of differentiation relative to days in culture.

TC7 Caco-2 cells were grown on 6 or 12 well tissue culture plates; cells were harvested for analysis days 5, 7, 10, 12, 14, 21 and DMT1 expression measured using two different antibodies (Santa Cruz, Abnova). β actin was used as the loading control protein. DMT1 expression, normalized to β actin and semi-quantified using Image J software, peaked at days 14 as assessed by both antibodies; DMT1 expression remained elevated up to day 21 when measured by the Santa Cruz antibody, however as assessed using the Abnova anti-DMT1 assay levels had dropped by day 21. Representative Western blot result for a 21 day cycle for each antibody is shown. Experiments were repeated three times (n=3) for each antibody. All results are expressed as relative expression levels following normalisation and are in arbitrary units (a.u.). Based on an ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis, bar values with no letters in common are significantly different ($p \leq 0.05$).

The TC7 Caco-2 cell line started expressing DcytB protein on day 5, expression continued to increase until day 21(Figure 2.4).

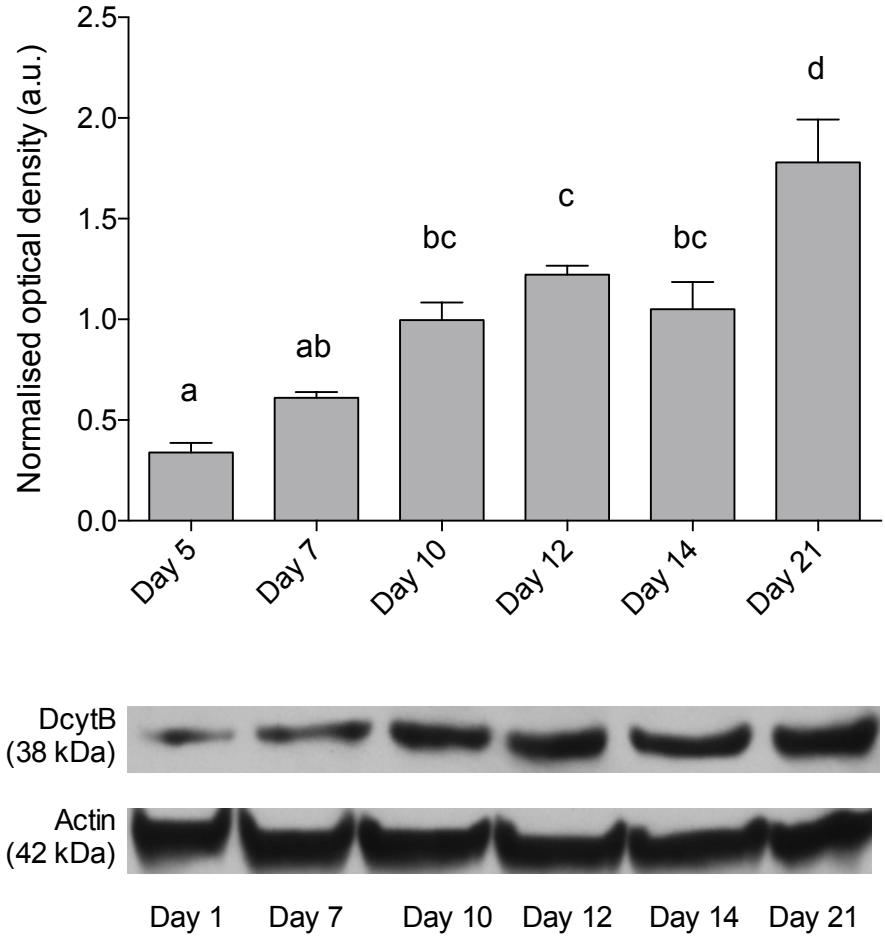

Figure 2.4 Western blot analysis of DcytB expression as a measure of differentiation relative to days in culture.

TC7 Caco-2 cells were grown on 6 or 12 well tissue culture plates; cells were harvested for analysis days 5, 7, 10, 12, 14, 21. β actin was used as the loading control protein. DcytB expression, normalized to β actin and semi-quantified using Image J software, continued to rise through to day 21. Representative Western blot result for a 21 day cycle is shown. The experiment was repeated three times (n=3). All results are expressed as relative expression levels following normalisation and are in arbitrary units (a.u.). Based on an ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all- pairwise basis, bar values with no letters in common are significantly different ($p \leq 0.05$).

Ferroportin expression was minimal at day 5 and continually increased until day 21 (Figure 2.5).

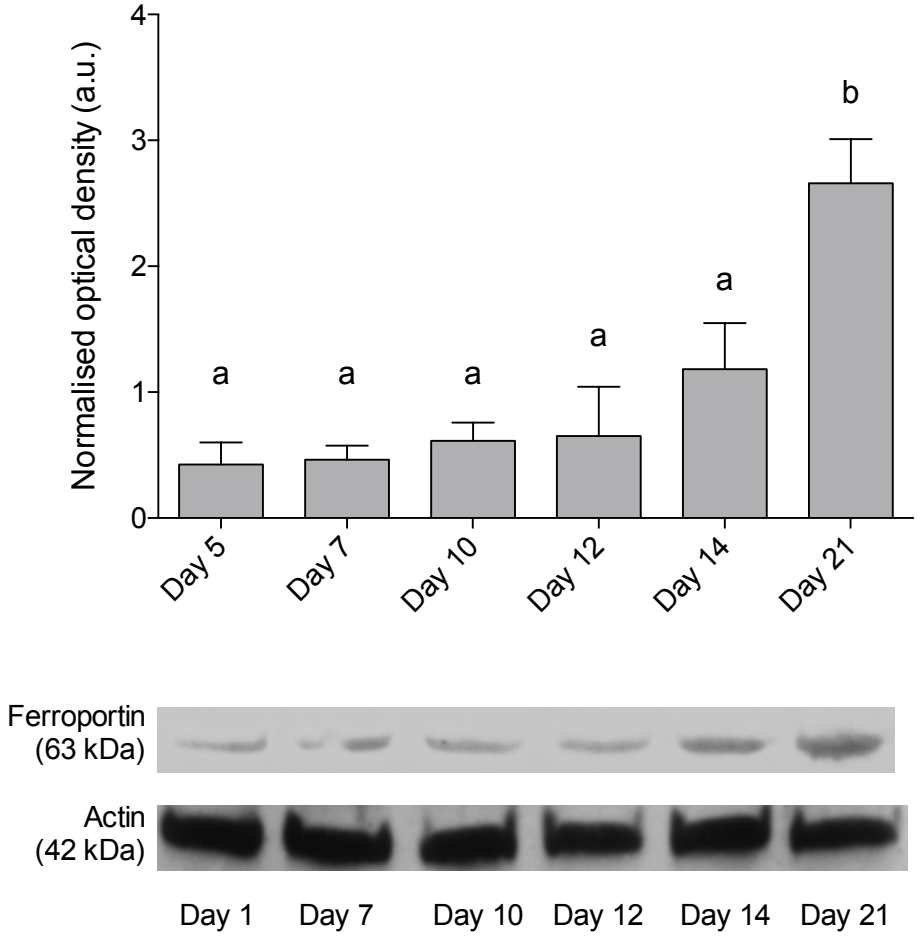

Figure 2.5 Western blot analysis of ferroportin expression as a measure of differentiation relative to days in culture.

TC7 Caco-2 cells were grown on 6 or 12 well tissue culture plates; cells were harvested for analysis days 5, 7, 10, 12, 14, 21. Cells were tested for one three week growth cycle. β actin was used as the loading control protein. Ferroportin expression, normalized to Actin and semi-quantified using Image J software, continued to rise through day 21. Representative Western blot result for a 21 day cycle is shown. The experiment was repeated three times (n=3). All results are expressed as relative expression levels following normalisation and are in arbitrary units (a.u.). Based on an ANOVA ($p<0.0008$) with Tukey's multiple comparisons test post-hoc analysis done on an all- pairwise basis, bar values with no letters in common are significantly different ($p\leq0.05$).

The expression of villin, a structural enterocyte protein, increased until day 14 and then stabilized (Figure 2.6).

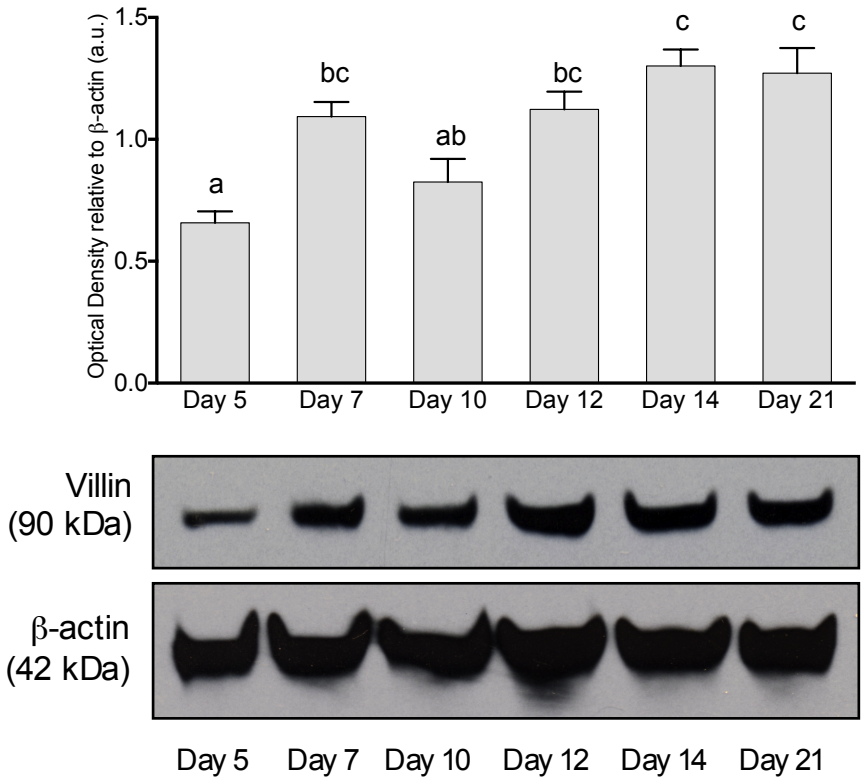

Figure 2.6 Western blot analysis of Villin expression as a measure of differentiation relative to days in culture.

TC7 Caco-2 cells were grown on 6 or 12 well tissue culture plates; cells were harvested for analysis days 5, 7, 10, 12, 14, 21 and villin expression measured using Gene Tex anti-Villin antibody. β actin was the loading control protein. Cells were tested for a three week growth cycle. Villin expression, normalized to β actin and semi-quantified using Image J software, peaked at day 14 and remained stable through day 21. Representative Western blot result for a 21 day cycle is shown. Experiment was repeated three times (n=3). All results are expressed as relative expression levels following normalisation and are in arbitrary units (a.u.). Based on an ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis, bar values with no letters in common are significantly different ($p \leq 0.05$).

2.3.4: HepG2 cell growth and comparison of growth between different cell passages

HepG2 cells grew rapidly with a doubling time of 24 hours; growth started to level out at approximately 4 days. Growth did not significantly vary between tested cell passages (Figure 2.5).

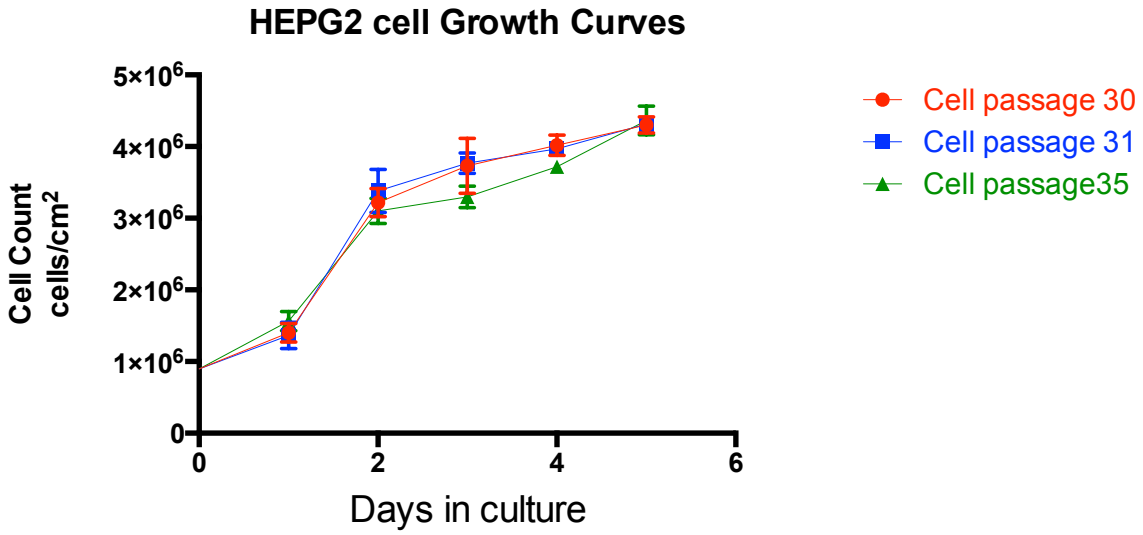

Figure 2.7 Comparison of growth between three different cell passages for HepG2 Cells.
HepG2 cell growth using cell counts was measured on culture days 1, 2, 3, 4, 5. Cell growth was rapid with a doubling time of approximately 24 hours. Cell numbers had started to stabilize by day 4. Growth characteristics did not differ significantly between tested cell passages (based on FDR rate with Q=1.000 %).

2.3.5: HepG2 cells: expression of selected proteins

HepG2 cells expressed TfR1 and TfR2 from day 1 status post seeding; levels peaked at day 4 for both receptors but remained present until day 5 (Figure 2.8).

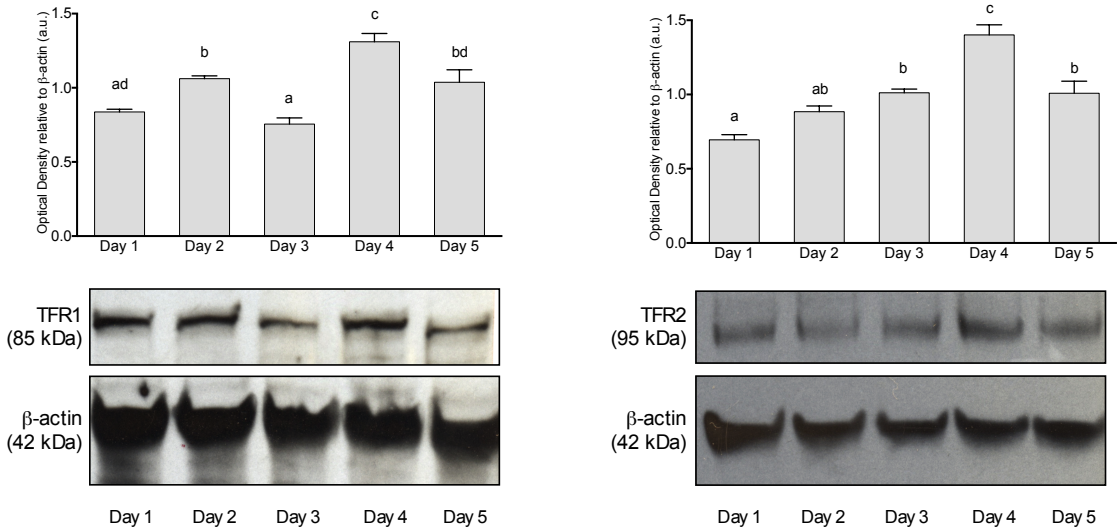

Figure 2.8 Western blot analysis of HepG2 transferrin receptors 1 and 2 (TfR1, TfR2) expression as a measure of differentiation relative to days in culture.
HepG2 cells were grown in 12 well plates and harvested for WB analysis on days 1-5. β actin was the loading control. TfR1 expression was present from day one and peaked at day 4 after which levels fell; TfR2 expression was also present from day one and similarly peaked at day 4 and then decreased. Protein expression was normalized to β actin and semi-quantified using Image J software. Representative Western blot result for a 5 day cycle is shown. The experiment was repeated three times (n=3). All results are expressed as relative expression levels following normalisation and are in arbitrary units (a.u.). Based on an ANOVA ($p \leq 0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis, bar values with no letters in common are significantly different ($p \leq 0.05$).

2.4: Discussion

2.4.1: Caco-2 cells

Consistent with previous reports in the literature the early passage HTB37 Caco-2 cell line displayed initial slow growth rates, and minimal sucrase activity even at 21 days (Briske-Anderson, Finley et al. 1997, Sambuy, De Angelis et al. 2005). In contrast, the TC7 clone had a brisk growth rate and sucrase activity comparable with human enterocytes as was originally described by the Rousset lab in which the clone was developed (Chantret, Rodolosse et al. 1994). Given that the research for this PhD involved the effects of sugars on iron bioavailability, a cell line that metabolises carbohydrates in the most physiological way was assessed to be the most suitable cell line in which to conduct experiments. All further analysis was therefore carried out on the Caco-2 TC7 Caco-2 cell clone, which was the clone used for experiments conducted in

this thesis. DMT1 expression profile over time was consistent with reports in the literature with levels peaking at days 14 (Sharp, Tandy et al. 2002), although the difference in findings between the two anti-DMT1 antibodies post 14 days suggests that either different transcripts, or different post-translational changes, may have occurred with cell maturation that were only recognized by the Santa Cruz antibody. The more rapid growth rate in the TC7 clone in comparison with the HTB37 clone would also be expected to facilitate work with this cell line, as a longer growth time prior to experiments increases contamination risk (although by day 14 these differences were gone and therefore probably would not have an impact on contamination risk at later experimental stages).

The protein expression of TC7 Caco-2 cell DcytB, ferroportin, and villin were also at stable and maximal levels by day 14 and thus suitable for the planned timeframe of experiments as proposed in the established in vitro digestion protocol.

2.4.2: HepG2 Cells

HepG2 cells reached confluence between 48-72 hours after seeding at which point growth significantly slowed; this is consistent with previous studies results that HepG2 growth is rapid and density dependent (Kelly and Darlington 1989). As previously reported in the literature HepG2 expression of transferrin receptors 1 and 2 was seen within 24 hours after seeding (Johnson and Enns 2004) and continued at similar levels through all days tested.

The above results support the use of HepG2 cells 24-72 hours after seeding (depending on confluence) as at that point growth stabilizes and expression of both transferrin receptors is present.

2.5: Conclusion

Characterization of cell lines indicated that the TC7 Caco-2 cell clone was the most appropriate model for use in experiments on the effects of sugars on iron bioavailability; DMT1, DcytB, and ferroportin results also supported using cells for experiments from days 13-15 when expression is maximal and stable. Similarly, the proposed time period of HepG2 cell experiments would be compatible with transferrin receptor expression.

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CHAPTER THREE: Sugars Increase Non-Haem Iron Bioavailability in Human Epithelial Intestinal and Liver Cells

This chapter presents work and evidence related to the effects of sugars and sugar compounds on iron bioavailability in Caco-2 and HepG2 in vitro cells as models of the human gut and liver, respectively. The chapter is based on the published manuscript: "Sugars Increase Non-Heme Iron Bioavailability in Human Epithelial Intestinal and Liver Cells"; Christides and Sharp (2013), *PLoS ONE* 8(12): e83031 (Doi: 10.1371/journal.pone.0083031).

3.1: Introduction

Evidence that simple sugars such as glucose and fructose affect iron bioavailability first arose in the 1960s from work showing that sugars were able to chelate inorganic iron and form stable, low molecular weight soluble complexes (Charley, Sarkar et al. 1963). These sugar-iron complexes were readily absorbed across the intestinal mucosa of rodent models (Charley, Stitt et al. 1963, Pollack, Kaufman et al. 1964). Given that intake of fructose and sucrose has increased worldwide in the past 40 years, especially in the Western world, while at the same time iron deficiency and iron excess remain significant public health concerns (McLean, Cogswell et al. 2009, Wood, Skoien et al. 2009, Fleming and Ponka 2012), understanding the nutritional implications of iron-sugar interactions is particularly relevant.

Excess sugar is blamed for a myriad of modern health problems, but whether sugars might actually be protective against iron deficiency, or contribute to either total body or cellular iron overload is unknown. Insufficient body iron levels are associated with significant health consequences, and affect some one billion people (McLean, Cogswell et al. 2009). Furthermore, iron overload related to either primary (e.g. hereditary haemochromatosis, HH) or secondary (e.g. beta-thalassemia) abnormalities in iron metabolism is prevalent in many populations (Brissot, Ropert et al. 2012, Fleming and Ponka 2012). There is also interest in the role that disordered regulation of intracellular iron levels plays in the pathogenesis of several non-communicable diseases, for example, non-alcoholic fatty liver disease (NAFLD) (Fujita and Takei 2011, O'Brien and Powell 2012).

Non-haem iron is the main source of iron in the diet (Carpenter and Mahoney 1992), and its bioavailability is influenced by a range of factors including solubility, oxidation state and chelation by dietary factors that may either enhance or inhibit iron uptake. Studies investigating the influence of sugars on iron bioavailability have yielded conflicting results; although a number of studies demonstrated improved iron bioavailability (Pollack, Kaufman et al. 1964, Brodan, Brodanova et al. 1967, Davis and Deller 1967,

Bates, Boyer et al. 1972, Roza, VanCampen et al. 1986, Holbrook, Smith et al. 1989), others found either no effect (Heinrich, Gabbe et al. 1974, Beynen, Brouwer et al. 1992), or decreased absorption (Ivaturi and Kies 1992, Brouwer, Lemmens et al. 1993). Human studies have been few, small (number of subjects ranged from 8–25 in the above cited studies), of short duration, and with limited information on iron status and hereditary iron metabolism defects.

The most consistent finding regarding mono- and disaccharides and iron is that fructose increases dietary non-haem iron absorption, possibly by chelating and/or reducing iron to the bioavailable ferrous form (O'Dell 1993). Whilst the dietary burden of fructose alone is low, consumption of sucrose (a glucose-fructose disaccharide cleaved into its constituent sugars prior to absorption), and high fructose corn syrup (HFCS, a widely used liquid sweetener), is high and accounts for approximately 10-15 % of total daily energy intake in the United Kingdom (UK) and United States (US), respectively (Marriott, Olsho et al. 2010, Whitton, Nicholson et al. 2011, Scientific Advisory Committee on Nutrition 2015); representing a significant amount of sugar intake and thus consequently fructose levels in both the gut and portal vein may be elevated. It is thus vital to clarify the effect of sugars and sweeteners on iron bioavailability in the gut and liver, as this could have an impact on iron status, particularly in population groups at risk of iron overload.

The objective of the current study was to investigate the effects of the sugars fructose, glucose and sucrose, as well as high fructose corn syrup 55 (HFCS-55, a mixture of fructose and glucose monomers in a 55:45 ratio), on non-haem iron bioavailability using the Caco-2 cell in vitro digestion model. Furthermore, as the modern diet delivers a loaded cocktail of sugars and iron to the liver, and fructose may be used in studies to induce hepatic metabolic changes, the effect of sugars on liver iron absorption was evaluated using the liver-derived HepG2 cell line. The in vitro digestion Caco-2 cell model is an established tool for assessing gut iron bioavailability and has been validated by comparison with data from human studies (Yun, Habicht et al. 2004), and HepG2 cells have been used to measure liver iron uptake (Scheiber-Mojdehkar, Sturm et al. 2003). Ferritin formation in both cell lines correlates with increasing concentration and duration of iron treatments (Hubert, Lescoat et al. 1993, Gangloff, Glahn et al. 1996).

3.2: Materials and methods

3.2.1: Reagents

Unless otherwise stated, reagents were purchased from Sigma- Aldrich, UK. Glassware used in sample preparation and analyses was treated with 10 % (v/v) concentrated Nitric acid (68 %) for 24 h and rinsed with 18 MΩ purity water. All water used in experiments was 18 MΩ purity.

3.2.2: Cell culture

The Caco-2 TC7 cell clone, developed by Monique Rousset and colleagues (Chantret, Rodolosse et al. 1994, Caro 1995), was kindly gifted to the Sharp lab and was used in experiments at passages 44 – 49. Cells were maintained in cell culture treated T75 flasks (Corning Inc., Costar) and subcultured every 5–7 days. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, 41965) supplemented with 10 % (v/v) foetal bovine serum (LCG Standards, 30-2020), 1 % penicillin-streptomycin, 4 mmol/L L-glutamine, 1 % non-essential amino acids, and Plasmocin 5 μg/ml (Source Bioscience). For experiments, Caco-2 cells were seeded at 1 x 10^4 cells/cm^2 in six- well plates (Corning Inc., Costar) and used 13-15 days post seeding as per the protocol used in the Glahn lab (Glahn, Lee et al. 1998).

HepG2 cells were obtained at passage 28 from American Type Culture Collection and used in experiments at passages 30–40; cells were maintained in cell culture treated T75 Tissue Culture Flasks seeded at a density of 1 x 10^5 cells/cm^2 and sub-cultured every 48 – 72 hours. Cells were grown in DMEM supplemented with heat inactivated 10 % (v/v) foetal bovine serum, 1 % penicillin- streptomycin, 2 mmol/L glutamine, 1 % non-essential amino acids. Experiments with HepG2 cells were carried out in cell culture treated six-well plates seeded at 1 x 10^5 cells/cm^2 and used 24- 48h post seeding.

24 hours prior to all experiments (Caco-2 & HepG2) DMEM medium was removed and the cell culture wells washed with 2.0 ml Minimal Essential Medium (MEM, Gibco, 31095); growth medium was then changed to MEM supplemented with 10 mmol/ L PIPES (piperazine-N, N'-bis- [2-ethanesulfonic acid]), 1 % antibiotic/antimycotic solution, 11 μmol/L hydrocortisone, 0.87 μmol/L insulin, 0.02 μmol/L sodium selenite (Na_2SeO_3), 0.05 μmol/L triiodothyronine and 20 μg/L epidermal growth factor. Foetal bovine serum (FBS) free media was used because different batches of FBS have differing levels of iron and other factors that could add confounding variables; MEM was supplemented to ensure optimal Caco-2 cell growth and differentiation in the absence of FBS while maintaining iron levels < 8 μg Fe/L (Jumarie and Malo 1991, Glahn, Lee et al. 1998).

3.2.3: Caco-2 cell - in vitro digestion studies

Sugar solutions: all solutions were freshly made on the day of the experiment. Stock solutions of 1 mol/L fructose, glucose or sucrose were prepared in 140 mmol/L NaCl, 5 mmol/L KCl, pH 2 solutions. In addition, HFCS-55 (a kind gift from Hanseland, Groningen, Holland) was diluted with water to produce a 1 mol/L fructose stock solution and then shaken with 4 g Chelex 100 resin (Bio-Rad Laboratories, 142-2832) for half an hour to remove possible metal contaminants, followed by elution through a 1.6 cm diameter filtration column (VWR). Iron levels in the Chelex treated HFCS stock solutions were checked by Inductivity Coupled Plasma-Optical Emission Spectrometer and were < 5 μmol/L Fe; iron levels in blank, no food digests were also < 5 μmol/L Fe. Sugar

concentrations were selected to be within the range that might occur in the gut after a meal (after dilution through the in vitro digestion). All solutions were filter sterilized prior to cell culture application.

The in vitro digestion followed a modified version of the protocol developed by Glahn et al for fruit juices (Boato, Wortley et al. 2002). All digestion solutions were prepared fresh for each experiment. On the first day of the experiment the cells were washed with 2.0 ml MEM, and 1.0 ml supplemented MEM was added to each individual plate well.

Food samples were prepared as follows: 25 μg of Fe (added as Fe solubilized in 1% HCl, High-Purity Standards, 100026-2) and 1.0 mL of stock sugar solutions were added to 10 ml 140 mmol/L NaCl, 5 mmol/L KCl pH 2 solutions in sterile 50 ml polypropylene centrifuge tubes. The iron:sugar ratio of ≈ 1:2000 was based on expected relative values of the two nutrients in the gut. Reference control samples of 25 μg Fe added to 11 ml 140 mmol/L NaCl, 5 mmol/L KCl, pH 2 solutions alone, as well as positive controls consisting of 25 μg Fe added to 11 ml 140 mmol/L NaCl, 5 mmol/L KCl, pH 2 solutions containing 265 μmol/L ascorbic acid were performed with each replication. The positive controls' iron:ascorbic ratio was chosen to reflect typical relative levels that might occur in a meal. To ensure no iron contamination of the system ''no food digest'' samples consisting simply of 140 mmol/L NaCl, 5 mmol/L KCl, pH 2 solutions were included with each experiment. Finally, solutions of 140 mmol/L NaCl, 5 mmol/L KCl, pH 2 containing 1 ml of stock sugar solutions without extraneous iron were tested to ensure that sugars alone did not increase ferritin formation. In some experiments, known inhibitors of iron bioavailability, phytic acid and tannic acid, were added to the food digests.

The peptic phase of digestion was initiated by the addition of 0.5 ml pepsin solution (Chelex purified) to each food sample (herein referred to as digests or food digests). The pH was readjusted to pH 2.0 with 1 mol/L HCl and the samples were shaken in a New Brunswick Orbital shaker at 37°C, 200 RPM, for 75 minutes. This phase was terminated with the addition of 1 mol/L $NaHCO_3$ and subsequent pH increase to ≈ pH 5.5. The intestinal digestion phase was initiated with the addition of 2.5 ml Chelex-purified bile/pancreatin solution with subsequent adjustment of the pH to pH 6.9 – 7.0 with 1 mol/L $NaHCO_3$. All food digests were then brought to a final volume of 15 ml by the addition of 140 mmol/L NaCl, 5 mmol/L KCl solution, pH 6.9.

1.5 ml aliquots of digests were gently pipetted into the upper chamber of each cell culture plate well; the upper chamber was created by the fitting of a 15,000 Da molecular weight cutoff dialysis membrane (Tubing Spectra/Por 7 dialysis membrane, Fisher Scientific) to a Transwell tissue culture treated insert ring (Fisher Scientific; the necks of the rings were shortened by 0.1 mm to remove the original filter and prevent excessive pressure on the cell monolayer) held in place with a silicone ring (Parker 2-023 S0613, WebSeal Inc.). Plates were then covered and placed on a platform fitted Multi-function

3D rotator (Fisher Scientific PS- M3D) set at 6 oscillations per minute in a 37°C incubator with a 5 % CO_2/95 % air atmosphere at constant humidity for 120 minutes.

After the 120 min incubation the inserts were removed and an additional 1.0 ml of supplemented MEM was added to each cell culture plate well. Plates were returned to the incubator for a further 22 hours; after this period cells were harvested for analysis of cell ferritin content.

Six replicates of each sugar were tested per experiment, and each experiment was repeated at 3 separate times.

Fructose Analysis: levels of fructose in prepared fructose solutions and HFCS-55, before and after Chelex treatment, were determined using high performance liquid chromatography (HPLC) with refractive index detection using water/methylated spirit extraction method with modifications for high salt levels. Premier Analytical Services, accredited by the United Kingdom Accreditation Service (UKAS), Buckinghamshire, UK, carried out the analysis.

3.2.4: Measurement of iron in blank no-food-digests and HFCS-55 by ICP-OES

Aliquots of the HFCS-55 Chelex treated stock solutions and blank digests were subjected to microwave digestion using an accelerated reaction system (CEM MARS 5H with XP-1500 vessels). 0.5 ml of the solutions (in triplicate) was added to 5.0 ml concentrated 68 % trace analysis grade nitric acid (Fisher). The samples were heated for 20 minutes at 400-psi pressure and 1200- W power. Iron levels were quantitatively analyzed by Inductivity Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Perkin Elmer Optima 4300 DV).

3.2.5: HepG2 cell studies

HepG2 cells were treated for 24 h with MEM containing 1 μmol/L ferric ammonium citrate (FAC) and either 15 mmol/L glucose (inclusive of the 5 mmol/L glucose contained in MEM) or 1 – 15 mmol/L fructose. Cells treated with MEM with 1 μmol/L FAC alone served as a reference in each experiment; in addition, 1 μmol/L FAC + 100 μmol/L ascorbic acid, and MEM alone treated cells, were positive and negative controls, respectively. HepG2 cells were also treated with fructose in the absence of any added iron to ensure that fructose alone did not increase ferritin formation unrelated to iron uptake. After 24 hours cells were harvested for analysis of cell ferritin content.

3.2.6: Ferritin analysis

At the end of each experiment, medium was removed from the wells and cells were rinsed twice with ice cold Phosphate Buffered Saline (PBS). 200 μl ice cold CelLytic™ with 1 % protease inhibitor were added to each well, and cell monolayers were removed

with a cell scraper and placed in 1.8 ml Eppendorf tubes. Tubes were shaken for 15 minutes on a Stuart microtitre plate shaker at 1250 RPM and then spun at 6,000 g for 6 minutes in a 5804R Eppendorf centrifuge. The supernatant was aspirated and stored at -80°C until analysis.

Ferritin analysis using SpectroFerritin MT Enzyme Linked Immunoassay (ELISA; RAMCO, Texas, USA) was carried out on cell extraction supernatants. Absorption readings were performed at 492 nm with subtraction for background at 620 nm in a Thermo Multiscan Ascent Spectrophotometer.

Protein concentration in each sample was measured using the Pierce Protein BCA Assay (Fisher Scientific, 23227). Using this method protein concentrations were consistently 5.0–6.5 mg/ml or 1.0–1.3 mg/well; these are the levels typically found in this lab on day 14 with an initial Caco-2 cell seeding density of 1 x 10^4 cells/cm^2 (the seeding density as recommended by ATCC).

3.2.7: Ferrozine assay

The ferrozine assay is generally used to measure ferrous iron levels in biological samples, and has specifically been used to measure iron in cell extracts (Riemer, Hoepken et al. 2004). It may also, in adapted form, be used to measure ferrous iron in non-biological samples such as intravenous iron sucrose solutions (Venefor) (Sturm, Laggner et al. 2005). For these studies the ferrozine assay was adapted to measure ferrous iron formation in solution after incubation of ferric chloride with fructose, or glucose, or sucrose. The lower limit of detection of the assay is 10 mmol/L; to ensure that production of Fe^{2+} was within the detection limits of the assay levels of iron and sugar were increased in the test tube by a factor of 10 (i.e. 100 μmol/L $FeCl_3$; 500 mmol/ L sugar) while maintaining the same molar ratio (1:50). Carbohydrate solutions containing 500 mmol/L glucose, fructose, or sucrose were prepared in PBS; ferric chloride was added to give a final concentration of 100 μmol/L Fe^{3+}. 150 ml ferrozine reagents (containing 32 mg ferrozine, 32 mg neocuprine and 3.8 g ammonium acetate dissolved in 10 ml water) were mixed with 400 μl carbohydrate solutions. The samples were incubated for two hours at 37°C degrees in a 96 well culture plate. The standard calibration curve was made up with freshly prepared ferrous ammonium sulfate. Absorption readings were performed at 550 nm on a Bio-TekSynergy HT Spectrophotometer.

3.3: Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism (v.6.0 GraphPad Software, San Diego, CA). Statistical analysis was conducted according to the methods of Motulsky (Motulsky 2010). Where noted data from separate experiments were normalized to the relevant reference control. To compensate for unequal variance, GraphPad Prism was used to log transform data. Data are presented as means ± S.E.M.

Except as otherwise noted, data were analyzed by one-way ANOVA followed by Tukey's post-hoc test for pairwise comparisons of experimental groups. Differences between means were considered significant at $p \leq 0.01$.

3.4: Results

3.4.1: Effect of sugars on cell ferritin formation

The effect of sugars on iron bioavailability was assessed by incubating Caco-2 cells with digests of sugar solutions containing iron. Fructose increased iron-induced ferritin formation in Caco-2 cells by approximately 40 % (Figure 3.1). In contrast, ferritin levels were not altered in cells exposed to iron plus either glucose or sucrose. In addition, incubation with digests of HFCS-55 increased iron-induced ferritin levels by approximately the same amount as fructose (Figure 3.2). Incubation with sugars alone (i.e. in the absence of iron) did not increase ferritin formation (data for glucose and sucrose not shown). Fructose levels in stock fructose solutions, and in stock HFCS-55 solutions after Chelex treatment, were on average 1 mol/L ± 0.01 (SEM) and 0.99 mol/L ± 0.04 (SEM), respectively.

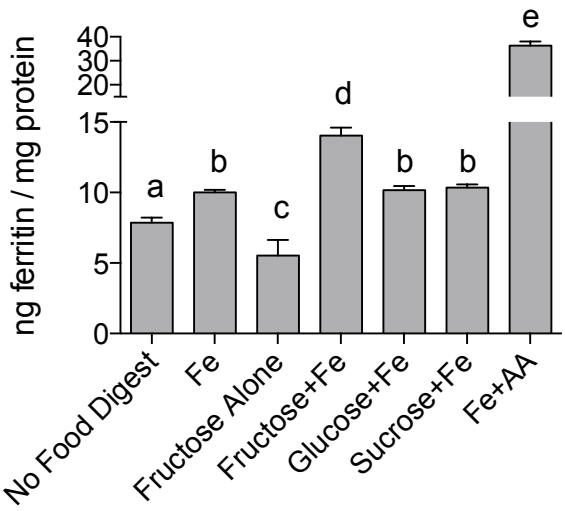

Figure 3.1 Sugar digests effects on iron-induced ferritin.
Measurement of Caco-2 cell ferritin formation from digests of Fe and solutions of sucrose (Sucrose+Fe) or glucose (Glucose+Fe) or fructose (Fructose+Fe) at an iron:sugar ratio of ≈ 1:2000. Equal amounts of iron (25 μg) were combined with sugar solutions (1.0 mL) and subjected to the Caco-2 in vitro digestion process. Digests with fructose alone and no added Fe (No Food Digest) were used as negative controls; digests with Fe alone (Fe) and Fe plus ascorbic acid (Fe + AA) were used as reference controls and positive controls, respectively. Values are means of data normalized to 10 ng of ferritin/mg protein in the reference control (Fe) ± SEM, n=15. Based on an ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis, bar values with no letters in common are significantly different ($p \leq 0.01$).

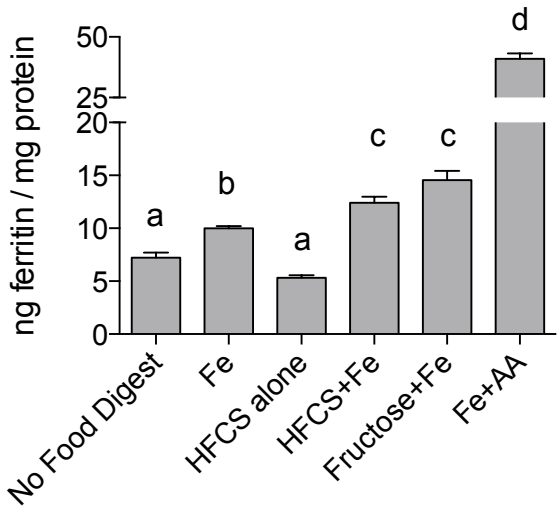

Figure 3.2 Effect of high-fructose corn syrup (HFCS) digests on iron-induced ferritin.
Measurement of Caco-2 cell ferritin formation from digests of Fe and fructose (Fructose+Fe), or Fe and HFCS-55 (HFCS+Fe) at an iron:fructose ratio of ≈ 1:2000. Equal amounts of iron (25 μg) were combined with fructose solutions (1.0 mL) and subjected to the Caco-2 in vitro digestion process. Digests with HFCS alone and no added Fe (No Food Digest) were used as negative controls; digests with Fe alone (Fe) and Fe plus ascorbic acid (Fe + AA) were used as reference controls and positive controls, respectively. Values are means of data normalized to 10 ng of ferritin/mg protein in the reference control (Fe) ± SEM, n≥18. Based on an ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all- pairwise basis, bar values with no letters in common are significantly different ($p \leq 0.01$).

3.4.2: Effect of inhibitors of iron bioavailability on sugar- induced ferritin formation

To determine whether known inhibitors of iron bioavailability could influence the enhancing effect of fructose and HFCS-55 on iron-induced ferritin formation, cells were incubated with either tannic acid (TA) or phytic acid (PA). Incubation with TA or PA alone did not alter basal cell ferritin levels (Figures 3.3 & 3.4). TA (1Fe:1TA molar ratio; Figure 3.3) and PA (1Fe:10PA molar ratio; Figure 3.4) both decreased iron bioavailability. Furthermore, addition of TA (Figure 3.3) or PA (Figure 3.4) to fructose- and HFCS-55-iron digests significantly decreased the sugar-iron- induced increase in ferritin formation to the level of ''no food digests".

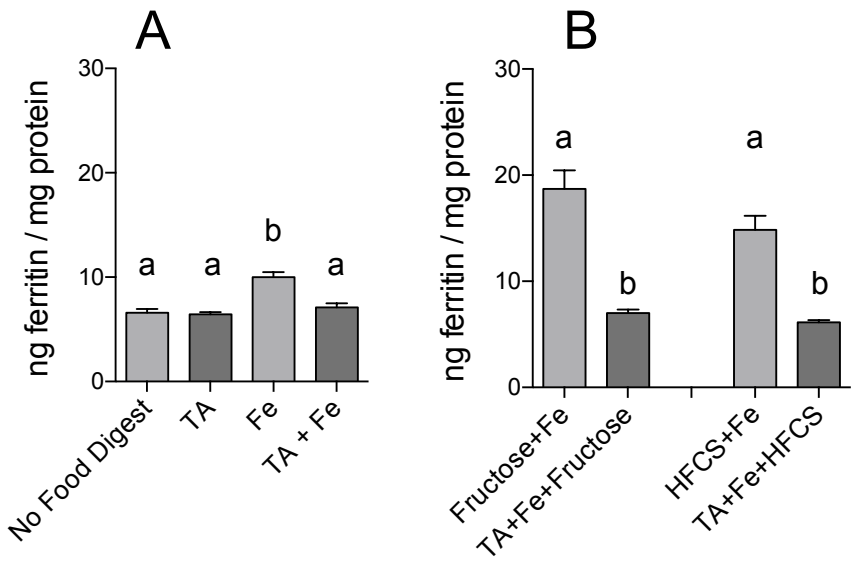

Figure 3.3 Effect of tannic acid (TA) and fructose, or TA and high-fructose corn syrup (HFCS), on iron-induced ferritin formation.

Measurement of Caco-2 cell ferritin formation from digests of Fe and fructose (Fructose+Fe), or HFCS-55 (HFCS+Fe), at an iron:fructose ratio of ≈ 1:2000, plus tannic acid at a 1:1 molar ratio of Fe:TA. Equal amounts of iron (25 μg) were combined with sugar solutions (1.0 mL) and TA and subjected to the Caco-2 in vitro digestion process. Digests without TA are shown with lighter shading and digests with TA added are shown with darker shading. Values are means of data normalized to 10 ng of ferritin/mg protein in the reference control (Fe) ± SEM, n(Fe+AA+TA) = 4, n(TA alone) = 6, all other n = 18. Analysis of **Figure 3.3(A)** was based on a two-factor ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis, bar values with no letters in common are significantly different ($p \leq 0.010$). Analysis of **Figure 3.3(B)** was based on a one-factor ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis, bar values with no letters in common are significantly different ($p \leq 0.01$).

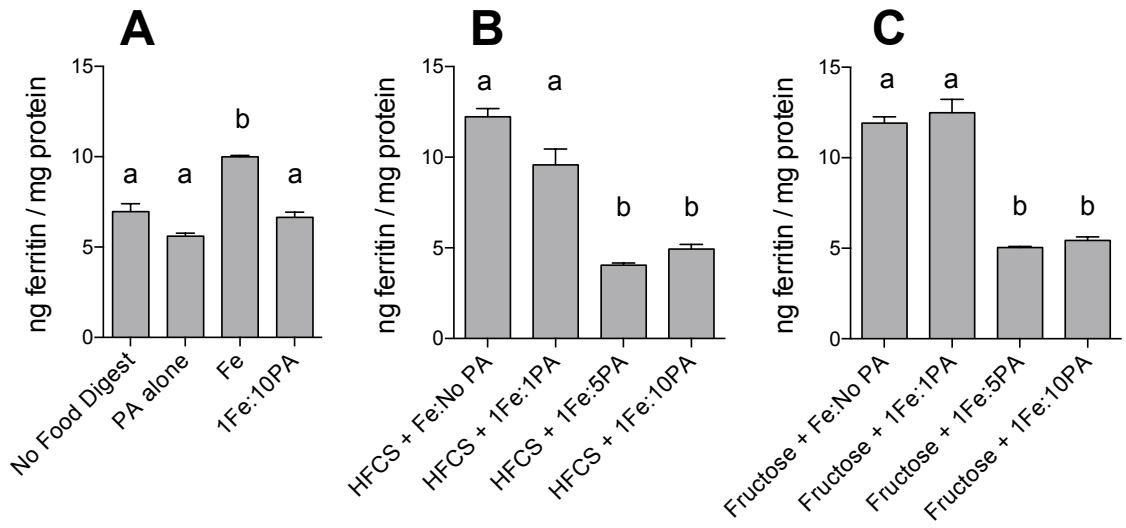

Figure 3.4 Effect of phytic acid (PA) and fructose, or high-fructose corn syrup (HFCS), on iron-induced ferritin formation.

Measurement of Caco-2 cell ferritin formation from digests of Fe and fructose (Fructose+Fe), or HFCS-55 (HFCS+Fe), at an iron:fructose ratio of ≈ 1:2000, plus phytic acid at 1:1, 1:5 or 1:10 Fe:PA molar ratios. Equal amounts of iron (25 μg) were combined with sugar solutions (1.0 mL) and PA and subjected to the Caco-2 in vitro digestion process. Digests of the above without the addition of PA are provided for reference. Digests with Fe alone (Fe), PA alone (PA Alone), and Fe plus PA (1Fe:10PA) were used as controls. Values are means of data normalized to 10 ng of ferritin/mg protein in the reference control (Fe) ± SEM, n(Fe+PA1:10) = 3, all other n ≥ 6. Analysis of **Figure 3.4(A)** was based on a two-factor ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis, bar values with no letters in common are significantly different ($p<0.01$). Analysis of **Figures 3.4(B)** and **3.4(C)** was based on a one-factor ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis, bar values with no letters in common within **Figure 3.4(B)** are significantly different ($p \leq 0.01$) and similarly bar values with no letters in common within **Figure 3.4(C)** are significantly different ($p \leq 0.01$).

3.4.3: Effects of sugars on ferrous iron formation in vitro

It has been reported previously that sugars may have weak iron reducing and chelating activity (Charley, Sarkar et al. 1963, O'Dell 1993). Therefore, to determine whether sugar solutions increased iron bioavailability via reduction of Fe^{3+} to Fe^{2+}, the ferrozine assay was used to measure Fe^{2+} as it selectively detects ferrous iron. Fructose significantly increased ferrozine-chelatable ferrous iron levels by approximately 300 % (Figure 3.5). There was no effect of glucose, sucrose or mannitol on Fe^{2+} formation. $FeCl_3$ alone in MEM gave rise to the lowest levels of ferrozine-chelatable ferrous iron, with levels only 22 % of those formed in the presence of fructose.

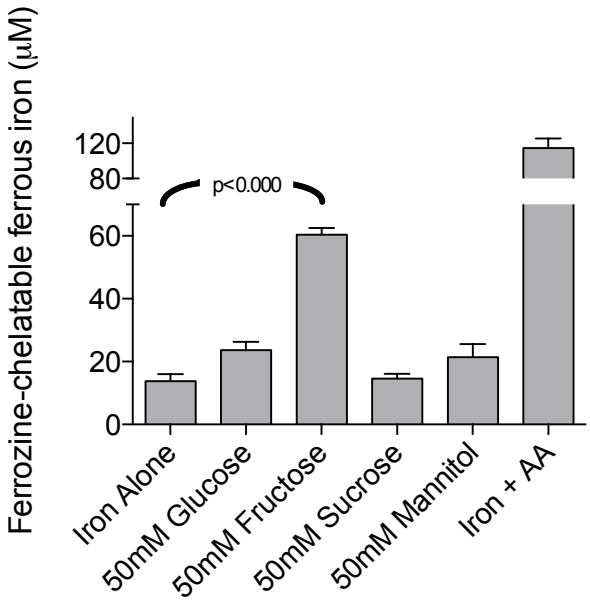

Figure 3.5 Carbohydrate effect on release of ferrozine- chelatable ferrous iron (Fe^{2+}) in vitro.
50 mmol/L solutions of glucose, fructose, sucrose or mannitol were prepared with the addition of $FeCl_3$ at a final concentration of 0.1 mmol/L. Solutions with iron alone and iron plus ascorbic acid were used as controls. Analysis for ferrozine-chelatable ferrous iron was performed after 2 hours incubation. Data in each column are presented as the mean ± SEM, n = 12 per group. Analysis was based on a one-factor ANOVA ($p = 0.0001$). Post-hoc analysis was done versus control. Fructose at a concentration of 50 mmol/L significantly increased ferrous iron levels in comparison to all other tested carbohydrate solutions; compared with 0.1 mmol/L $FeCl_3$ alone ($p \leq 0.0001$).

3.4.4: Effect of fructose on HepG2 cell ferritin formation

To determine whether fructose or glucose might also influence hepatic iron-induced ferritin formation HepG2 cells were exposed to increasing concentrations of fructose, or glucose, and iron. In the presence of iron, liver ferritin levels were unaffected by co-addition of glucose and fructose; however, fructose increased iron-induced HepG2 cell ferritin by approximately 35 % - maximal ferritin formation was observed with 15 mmol/L fructose (Figures 3.6 & 3.7). There was no effect of fructose alone (i.e. without added iron) on ferritin formation (Figure 3.6).

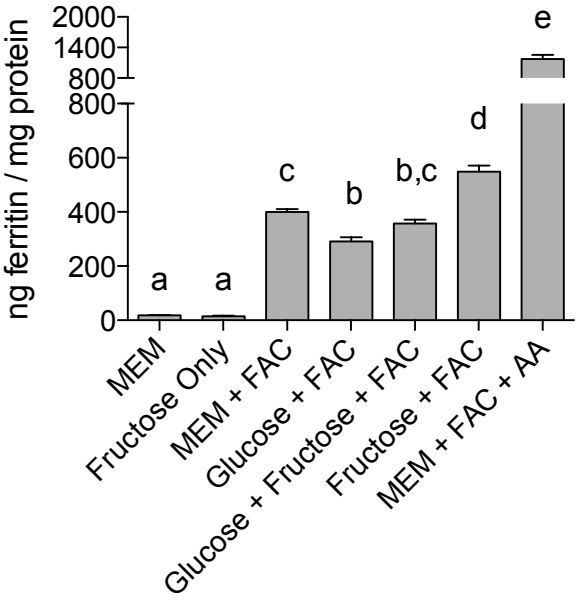

Figure 3.6 HepG2 iron-induced ferritin in response to carbohydrate treatments.
Measurement of HepG2 cell ferritin formation following treatment for 24 hours with 1 μmol/L ferric ammonium citrate (FAC) and one of the following: 15 mmol/L glucose (Glucose+ FAC); 15 mmol/L glucose and 15 mmol/L fructose (Fructose+Glucose+FAC); 15 mmol/L fructose (Fructose+FAC). Cells treated with MEM alone (MEM), or fructose alone (Fructose Only), without the addition of FAC, were used as negative controls. Cells treated with 0.1 μmol/L ascorbic acid and 1 μmol/L FAC were used as positive controls. Values are means of data normalized to 400 ng of ferritin/mg protein in the reference control (MEM+FAC) ± SEM, n(MEM) = 4, (Fructose Only) n= 6, all other n ≥ 12. Based on an ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all- pairwise basis, bar values with no letters in common are significantly different ($p \leq 0.01$).

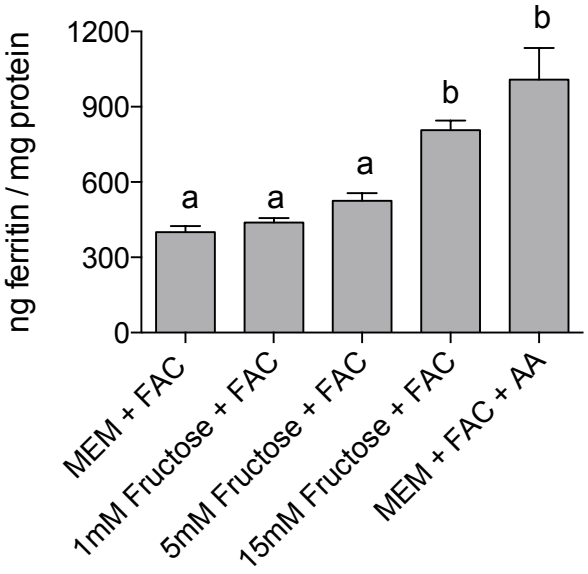

Figure 3.7 Fructose dose response on HepG2 cell iron-induced ferritin.
Measurement of HepG2 cell ferritin formation following treatment for 24 hours with 1 μmol/L FAC and one of the following: 1 mmol/L fructose (1 mM Fruc+FAC); 5 mmol/L fructose (5 mM Fruc +FAC); 15 mmol/L fructose (15 mM Fruc + FAC), to determine dose response of HepG2 cell ferritin relative to fructose concentration. Values are means of data normalized to 400 ng of ferritin/mg protein in the reference control (MEM+FAC) ± SEM, $n \geq 6$. Based on ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis, bar values with no letters in common are significantly different ($p \leq 0.01$).

3.5: Discussion

Non-haem iron bioavailability is influenced by many dietary factors. This in vitro study suggests that fructose increases iron bioavailability in the cell line models of the gut and liver that were used. These results are consistent with previous work in rodent models in which iron- fructose solutions increased both gut iron absorption (Pollack, Kaufman et al. 1964) and liver iron deposition (Stitt, Charley et al. 1962).

Recent human intervention trials looking at the effects of fructose on iron uptake are lacking, but there have been several epidemiological studies that analyzed fruit intake and iron status. Fruit is a dietary source of fructose; observational studies of fruit as a modifier of iron status have yielded conflicting results. Milward et al carried out a study in subjects with hereditary haemochromatosis (HH), which found that non-citrus fruit intake was protective against iron overload (Milward, Baines et al. 2008). In contrast, Fleming et al found that fruit intake was associated with an increased risk for elevated iron stores in the Elderly Framingham Heart Study cohort (Fleming, Tucker et al. 2002). The different results are most likely secondary to several factors. The study by Milward et al differentiated between citrus fruits (which are rich sources of citric and ascorbic acid, known enhancers of iron uptake), and non-citrus fruit; Fleming et al did not differentiate between fruit types. In addition, Milward et al only studied subjects with

HH, whereas HH was one of the exclusion criteria for the Framingham study. Another possible confounding factor is that fruits have varying levels of phytates and polyphenols. The results of this current in vitro study suggest that fructose in non-citrus fruit would not alter iron bioavailability, as fruits contain both phytates and polyphenols, substances that were found to inhibit the enhancing effects of fructose on iron bioavailability.

In Western diets and increasingly worldwide, however, the major source of fructose in the diet is not fruit, but sucrose and high-fructose corn syrup (particularly HFCS-55) (Marriott, Olsho et al. 2010, Whitton, Nicholson et al. 2011, Scientific Advisory Committee on Nutrition 2015). In the USA HFCS represents 15–20 % of total energy intake, the majority coming from sugar sweetened beverages (SSB) (Heckman, Sherry et al. 2010). Here, the effect of HFCS-55 on iron bioavailability was investigated and demonstrated increased ferritin formation in the Caco-2 cell system. Few studies have investigated the effects of sugars from SSB on iron bioavailability. A small study by Hallberg et al found that a low pH cola drink increased iron absorption; however, this was attributed to the low pH of the beverage and data on the carbohydrate composition of the beverage is unavailable (Hallberg and Rossander 1982). A more recent study, which specifically looked at the effect of beverage carbohydrate on iron bioavailability by comparing regular cola with artificially sweetened diet cola, found no effect on iron absorption from either source (Collings, Fairweather-Tait et al. 2011). However, cola drinks contain between 0.5 – 0.7 mmol/L caffeine (Coca-Cola.co.uk), a compound that some studies suggest may decrease non-haem iron bioavailability, and thus a possible confounding factor when assessing SSB sugar effects on iron bioavailability in this study. In this study HFCS-55, used at a concentration comparable to that found in sweetened beverages, significantly increased iron bioavailability, and the effect was comparable to that observed with fructose; however, the effect was negated at polyphenol levels equivalent to those present in cola.

The other sugars tested in this study did not influence iron bioavailability and this is consistent with previous work showing no effects of sucrose or glucose on iron absorption (Pollack, Kaufman et al. 1964). It might have been predicted that the fructose released from digestion of sucrose would increase iron uptake. However, there are suggestions in the literature that sucrose-derived fructose is taken up by the enterocyte immediately upon hydrolysis; it would therefore not be available to interact with iron, in comparison with HFCS which upon ingestion yields free fructose monomers in the intestinal lumen (Riby, Fujisawa et al. 1993).

It has been proposed that fructose increases iron bioavailability by increasing ferrous iron formation (O'Dell 1993). The findings of this study that fructose significantly increased ferrozine-chelatable ferrous iron levels is consistent with this mechanism. Fructose is a reducing sugar giving positive tests for both Benedicts and Fehlings reagents; in solution it exists primarily in the furanose form but is in equilibrium with the straight chain and pyranose forms (Timson 2012). Interestingly, in basic solution fructose is a stronger reducing agent than aldoses such as glucose (Verstraeten 1967, Timson 2012). The

greater part of digestion in the in vitro digestion model occurs at a higher pH (pH 7) thus it could be expected that this aspect of fructose biochemistry would be displayed in the model; furthermore, the majority of digestion in vivo also occurs at a higher pH suggesting that in the gut fructose may have the same effect on iron oxidation state. Sucrose is not a reducing sugar because neither of its carbonyl groups are available to participate in redox reactions (Moreira 2012). In summary, the findings of this study that fructose produced the greatest increase in ferrozine-chelatable ferrous iron is consistent with reports in the literature of its reducing ability in a relatively basic environment in comparison with other tested sugars.

Work by Stitt et al looking at sugar effects on iron bioavailability analyzed iron levels in the liver, as well as gut iron uptake; iron co- administered with fructose resulted in both increased iron absorption and liver iron deposits in a rodent model (Stitt, Charley et al. 1962). In addition, a recent study using mice found that a high-fructose/high-fat diet increased iron liver levels (Tsuchiya, Ebata et al. 2013). These observations are consistent with the findings of this study that HepG2 ferritin levels increased in cells treated with iron and fructose. Data on human blood fructose levels are limited, and there is even less information on human portal vein fructose concentrations to which the liver would be exposed. However, a recent study using an enzyme-based assay, validated by gas chromatography-mass spectroscopy, reported circulating serum post-prandial fructose levels up to 16 mmol/L (Hui, Huang et al. 2009); if these values are correct then portal vein fructose levels would be predicted to be in the order of 27 - 53 mmol/L as the liver extracts 40–70 % of portal vein fructose (Topping and Mayes 1971, Mayes 1993). Older studies in animals, and several small studies in humans, have reported portal vein fructose levels varying from 1 – 2.2 mmol/L (Holdsworth and Dawson 1965, Topping and Mayes 1971, Dencker, Lunderquist et al. 1972, Dencker, Meeuwisse et al. 1973, Niewoehner 1986). The fructose concentrations used in our studies lie somewhere in the middle of these two extremes of possible post-prandial portal circulation levels.

A number of studies have used either high fructose or HFCS diets to augment the effects of a high fat diet on liver metabolism, in order to study the development of fatty liver. Given that liver iron loading is sometimes found in human fatty liver disease and may contribute to disease progression (O'Brien and Powell 2012), the observations in this current study that fructose and HFCS increased iron uptake in in vitro models of both gut and liver suggests that these carbohydrates may have an important pathological role.

Under normal conditions the majority of circulating iron is bound to the plasma iron transport protein transferrin (Gkouvatsos, Papanikolaou et al. 2012), however blood levels of 0 – 1 μmol/L non-transferrin bound iron (NTBI) may occur post-prandially (Graham, Chua et al. 2012). Furthermore, NTBI levels may reach 1 – 20 μmol/L in iron overload disorders as well as in other chronic diseases such as liver cirrhosis (Deugnier and Turlin 2011). NTBI is a heterogeneous mix of compounds; studies suggest that the main form of plasma NTBI is iron(III)citrate (Hider 2002), for this reason FAC is an appropriate model for plasma NTBI. Interestingly, hepatocyte uptake of NTBI, as

opposed to transferrin iron uptake, does not appear to be inhibited by increasing levels of liver iron (Wright, Brissot et al. 1986, Baker, Baker et al. 1998); uptake of fructose mediated liver NTBI may thus escape regulation.

3.6: Conclusion

In conclusion, this study has shown that fructose and HFCS-55 increase iron bioavailability in human intestinal epithelial cells and, furthermore, that fructose increases iron-induced hepatocyte ferritin. Given that substantial amounts of these carbohydrates are present in the modern diet, and also their use in experimental models, these effects may be important in the context of iron homeostasis. Further studies are warranted to examine if these in vitro effects translate into (patho)physiologically relevant changes in animal and human iron status.

Chapter 4: Iron bioavailability from commercially available iron supplements

This chapter presents work and evidence related to whether fructose as an additive in iron supplements was associated with improved iron bioavailability; it is based on the published manuscript: "Iron bioavailability from commercially available iron supplements"; Christides, Wray, McBride, Fairweather, and Sharp (2014), *European Journal of Nutrition (*DOI 10.1007/s00394-014-0815-8).

4.1: Introduction

Iron deficiency is a global health problem; 2 billion people suffer from anaemia, and the World Health Organization (WHO) estimates that iron deficiency accounts for 50 % of these cases (McLean, Cogswell et al. 2009). Although the prevalence of iron deficiency anaemia (IDA) is highest in developing countries, inadequate iron status is also a significant public health problem in developed countries in high-risk groups including older adults, post-bariatric surgery patients, toddlers, and pregnant women and women of child-bearing age (Shankar, Boylan et al. 2010, Miller 2013, Fairweather-Tait, Wawer et al. 2014).

Three ferrous iron salts, sulphate, fumarate and gluconate, are the standard recommended supplements for IDA (Andrews 1999, Pavord, Myers et al. 2012). Clinical trials with pregnant women document that these iron supplements do improve IDA (Zhou, Gibson et al. 2009); however, non-compliance secondary to supplement-associated gastrointestinal (GI) problems, such as constipation, black stools, nausea, reflux and vomiting, means that real-life efficacy may be low (Ekstrom, Hyder et al. 2002, Hyder, Persson et al. 2002, Seck and Jackson 2008, Habib, Alabdin et al. 2009). Pregnancy itself induces gastrointestinal symptoms, related to the mechanical effects of the growing foetus, changes in water homoeostasis, and hormonal effects on GI motility— all of which contribute to increased incidence of reflux, constipation and bloating (Bonapace and Fisher 1998, Keller, Frederking et al. 2008). Higher doses of iron increase GI symptoms in pregnant women (Beard 2000), and studies document that lower doses are associated with fewer GI symptoms (Ekstrom, Kavishe et al. 1996, Zhou, Gibson et al. 2009), thus it is possible that the use of iron preparations with higher bioavailability, enabling lower absolute doses of iron to be ingested, may cause less GI upset.

IDA is associated with increased health risks to both mother and foetus, reviewed in (Stoltzfus 2011), and it is therefore vital that effective supplement forms are available to pregnant women. A human volunteer study demonstrated that iron absorption from a naturally iron-rich mineral water (Spatone Iron-Plus) was higher compared with ferrous sulphate tablets ($FeSO_4$) (Worwood, Evans et al. 1996). Furthermore, a subsequent study

looking specifically at pregnant women with IDA found that the mean absorption from 25 mg of Spatone-derived Fe was 28 % — comparable or greater to that reported from a higher dose of $FeSO_4$ (Halksworth, Moseley et al. 2003).

The physical form of iron administered in supplements could have a bearing on bioavailability. A recent in vitro study found that dissolution rates varied significantly amongst different iron tablet formulations; $FeSO_4$ conventional-release tablets dissolved most quickly at 48–64 min, and this was associated with the highest iron uptake in the in vitro Caco-2 cell model used in this study (Zariwala, Somavarapu et al. 2013). Therefore, it was hypothesised that liquid iron formulations, whether naturally occurring or synthetic, might provide alternative supplement forms that have higher bioavailability compared with iron delivered in tablet form. In addition, previous work found that fructose improved iron bioavailability in a water-based media (Christides and Sharp 2013); therefore it was hypothesised that liquid iron supplements with added fructose would have the highest iron bioavailability.

In this study, the Caco-2 cell in vitro digestion model was employed to assess iron bioavailability, using cell ferritin as a surrogate marker for iron absorption (Glahn, Lee et al. 1998), from five commercially available iron formulations including three liquid iron supplements. Two naturally mineral-rich waters, Spatone® and Spatone Apple® (Spatone with added apple concentrate, fructose, and ascorbic acid), and Iron Vital F® (a synthetic liquid iron supplement with added ascorbic acid, fructose and other micronutrients), were compared with immediate release $FeSO_4$ tablets, and Vitabiotics Pregnacare® tablets (multivitamin and mineral tablets marketed to pregnant women).

4.2: Materials and methods

4.2.1: Reagents

Chemicals, hormones and enzymes were purchased from Sigma-Aldrich, UK, unless otherwise noted. Cell culture media, flasks, tissue culture plates and culture reagents were obtained from Thermo Fisher Scientific. Acids used in Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) analysis, in digestions, and for glassware cleaning, were purchased from VWR, UK. Glassware and plastic ware used in experiments were soaked in 10 % trace metal grade 68 % nitric acid for 24 h prior to use and then rinsed with 18 mΩ pure water.

Commercial iron preparations were purchased from Boots Pharmacy, UK. These consisted of: Spatone® and Spatone Apple® (A Nelson and Co Ltd, UK); Iron Vital F® (Anton Hübner GmbH and Co, Germany); Pregnacare Original® (Vitabiotics Ltd, UK) and Almus Ferrous Sulphate tablets (Almus Pharmaceuticals, UK). Table 1 provides reported iron concentration for the different formulations.

4.2.2: Cell culture

Caco-2 cells were used from cell passages 46–49, and grown as previously described (Christides and Sharp 2013). Briefly, for experiments, cells were grown in six-well tissue cultures plates seeded at a density of 1×10^4 cells/cm^2 and used on days 13–15 post-seeding in the method developed by the Glahn laboratory (Glahn, Lee et al. 1998). Cells were grown in supplemented Dulbecco's Modified Eagle Medium as previously described (Christides and Sharp 2013).

Twenty-four hours prior to the initiation of in vitro digestion experiments, medium was changed to supplemented MEM as previously described (Christides and Sharp 2013).

4.2.3: Iron supplements and in vitro digestions

Iron-containing tablets were sealed inside a disposable plastic sleeve and crushed with a mortar and pestle; the particles were then solubilised in 250 mL 0.2 mol/L hydrochloric acid (HCl) (Glahn, Rassier et al. 2000). Liquid formulations were used directly from the sachets (Spatone® and Spatone Apple®) or from the bottle (Iron Vital F®). The supplements all had different iron concentrations; therefore, the volumes of prepared solutions, or liquid supplements, were adjusted (based on the manufacturers reported iron concentrations) to achieve a notional final digest iron concentration of 50 μmol/L.

Fresh tablet solutions were prepared, and new sachets were used for every experiment. In addition, all experiments had a set of controls consisting of: a digest with no added iron to ensure no iron contamination of our system; a reference digest of 50 μmol/L Fe added as Fe solubilized in 1 % HCl (High-Purity Standards, 100026-2); and a positive control digest of 50 μmol/L Fe and 500 μmol/L ascorbic acid.

Iron levels of digests were quantitatively analysed by ICP-OES. All iron-containing digests, and undiluted samples of Spatone® and Spatone Apple®, were subjected to microwave digestion using an accelerated reaction system (CEM MARS 5H with XP-1500 vessels). 0.5 mL of the solutions (in triplicate) was added to 5.0 mL concentrated 68 % trace analysis grade nitric acid. Samples were processed for 20 min at 400 psi pressure and 1200 W power. After digestion, samples were reconstituted with 50 mL 18 mΩ water and aliquots used to measure iron levels on a Perkin–Elmer Optima 4300 DV Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES).

Fructose levels in Iron Vital F® and Spatone Apple®, the only supplements containing fructose, were measured by high performance liquid chromatography (HPLC) with refractive index detection using water/methylated spirit extraction method with modifications for high salt levels. Premier Analytical Services, accredited by the United Kingdom Accreditation Service (UKAS), Buckinghamshire, UK, carried out the analysis.

Digests were prepared as previously described (Christides and Sharp 2013). Briefly, iron solutions or liquid iron supplements were added to 10 mL of 140 mmol/L NaCl and 5 mmol/L KCl followed by the sequential addition of pepsin, and then bile and pancreatin digestive enzymes to mimic the digestive process. 1.5 mL of the above digests was then placed in a chamber suspended over a layer of Caco-2 cells grown on the bottom of the tissue culture wells of a six-well plate. Plates were placed on a platform-fitted multi-function 3D rotator in a 37°C incubator with a 5 % CO_2/95 % air atmosphere at constant humidity for 60 min. Inserts were then removed, and an additional 1 mL of supplemented MEM was added to the cells, which were returned to the incubator for a further 22 h and then harvested for ferritin. All supplements were tested on three separate occasions, with n = 6 for each treatment.

Digest experiments were also carried out with digests containing 50 μmol/L Fe and 500 μmol/L ascorbic acid (equivalent to the regular positive controls used in the in vitro digestions) with added fructose (66 mmol/L, the same concentration used in experiments in chapter three of this thesis) in order to evaluate if fructose had an added positive effect with ascorbic acid on iron bioavailability, as two of the liquid supplements contained both fructose and ascorbic acid. The combination of iron, ascorbic acid and fructose solution digestions were tested on four separate occasions, $n \geq 3$.

Direct application of iron supplements onto Caco-2 cells was performed to assess dose and treatment duration responses. The experiments described in this paragraph were carried out by R. Fairweather in the lab of Dr Paul Sharp, King's College London. Caco-2 cells were seeded and maintained as described for in vitro digestion experiments. On the day of the experiment, fresh solutions of Spatone®, Spatone Apple® or ferric ammonium citrate (FAC) were mixed with unsupplemented MEM (herein referred to as MEM) and placed directly onto the cell monolayers at iron concentrations of 10, 30 and 100 μmol/L. In order to assess ferritin formation as a function of treatment duration/time, one set of cells was treated continuously for 24 h, while another set was treated for 4 h (begun 20 h after medium was changed to MEM). At the end of both time periods, cells were harvested for ferritin. Experiments were repeated on three separate occasions with n = 6 for each treatment.

4.2.4: Cell harvest and ferritin analysis

Cells were harvested and lysed as previously described, then spun at 6,000 × g for 6 min and the supernatant stored in a −80 °C freezer until analysed (Christides and Sharp 2013). For analysis, samples were thawed on ice and ferritin was measured with SpectroFerritin MT Enzyme Linked Immunoassay (ELISA; RAMCO, Texas,USA). Final ferritin levels were adjusted for cell protein using Pierce Protein BCA assay (Fisher Scientific, 23227).

4.3: Statistical analysis

Statistical analysis of the data was performed using Graph- Pad Prism (version 6.0c GraphPad Software, San Diego, CA, USA). Digest experiments were analysed using the

statistical methods of Motulsky (Motulsky 2010). Data are presented as mean ± SEM and were analysed by one-way ANOVA followed by Tukey's multiple comparisons test (all-pairwise across experimental groups); where the comparison was made to a single control, Dunnett's post hoc test was used. Except as noted, differences between means were considered significant at $p \leq 0.05$. Ordered increases in data were tested using the non-parametric Jonckheere-Terpstra test. Ordered increases were considered significant at $p \leq 0.05$. The Jonckheer-Terpstra test was computed in Microsoft Excel.

4.4: Results

Iron supplements contained different stated iron concentration (Table 4.1). Each digest was prepared to give a notional iron concentration of 50 µmol/L based on the manufacturers' stated iron concentrations. However, ICP-OES measurements revealed that the iron content of digests varied between different iron sources (Table 4.2). To adjust Caco-2 cell ferritin levels for the variable iron content of digests, a dose–response study was carried out to measure ferritin formation following exposure to different concentrations of iron (Figure 4.1). The dose–response study demonstrated that ferritin levels in digests were proportional to the log of iron concentration, and thus measured ferritin levels were adjusted using the equation: Fadj = F × ln(50)/ln(Feicp), where Fadj equals adjusted ferritin, and Feicp equals ICP measured digest iron levels. Using *unadjusted* ferritin results Spatone Apple® had the highest levels of ferritin, followed by Iron Vital F®; there was no statistically significant difference in ferritin formation between the other three tested formulations (Table 4.2).

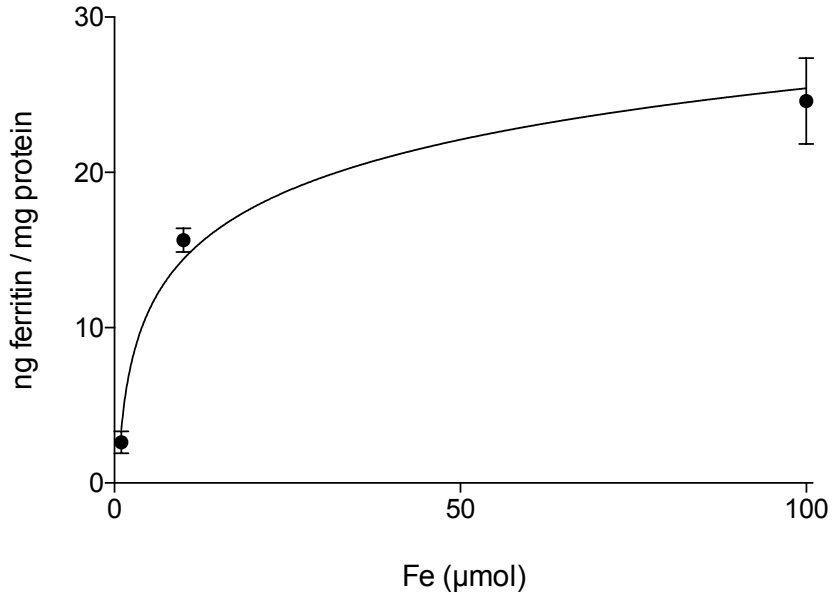

Figure 4.1: Caco-2 cell ferritin levels as a function of iron dose.
Caco-2 cells were treated for 24 hours with increasing concentrations of iron ranging from 1-100 µmol/L. Ferritin levels increased as a function of the log of iron concentration.

Table 4.1 Manufacturers' information including reported iron content for tested iron preparations

Product name	Reported iron content	Manufacturer
Spatone®	0.25 mg $FeSO_4$/ml	A Nelson & Co Ltd (UK)
Spatone Apple®	0.20 mg $FeSO_4$/ ml	A Nelson & Co Ltd (UK)
Iron Vital F® (IVF)	1 mg iron-II-gluconate/ml	Anton Hubner Gmbh & Co (Germany)
Pregnacare Original® (PG)	51.4 mg ferrous fumarate equivalent to 17 mg ferrous iron/tablet	Vitabiotics Ltd. (UK)
Almus Ferrous Sulphate tablets	200 mg $FeSO_4$ equivalent to 65 mg ferrous iron/tablet	Almus Pharmaceuticals (UK)

Table 4.2 Iron digest levels and Caco-2 cell ferritin after treatment with iron supplements—unadjusted for iron digest levels

Product name	Digest iron concentrations (mg/L)	ng ferritin/mg protein (unadjusted)
Almus Ferrous Sulphate tablets	2.80 ± 0.10 (0 %)	11.82 ± 0.67 (0 %)
Iron Vital F® (IVF)	2.90 ± 0.37 (4 %)	25.76 ± 2.07^{a} (118 %)
Pregnacare Original® (PG)	2.83 ± 0.68 (1 %)	10.35 ± 0.82 (−12 %)
Spatone®	3.40 ± 0.62 (18 %)	12.24 ± 0.91 (4 %)
Spatone Apple®	4.00 ± 0.75* (30 %)	83.94 ± 4.46^{b} (610 %)

Ferritin levels of Caco-2 cells unadjusted for differing digest iron levels as measured by ICP-OES. Data are expressed as mean ± SEM. Means with different superscript letters or asterisk in a column are statistically different. Percentage figures in parentheses by digest iron levels are the percent by which the mean digest iron levels of other formulations compared with mean $FeSO_4$ iron digest levels; percentage figures in parentheses by ferritin values are the percent by which the mean ferritin levels of cells treated with the other formulations compared with ferritin of $FeSO_4$ treated cells. Digest iron concentrations for IVF, PG and Spatone®, were not statistically different from that of $FeSO_4$; however, digest iron concentrations in Spatone Apple® were significantly different based on one-way ANOVA followed by Tukey's multiple comparisons test ($p \leq 0.05$). Unadjusted ferritin levels were highest in Spatone Apple®-treated cells followed by IVF; levels of the three other treatments were not statistically significantly different based on one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$)

Caco-2 cell *adjusted* ferritin levels were still the highest in cells exposed to Spatone Apple® (Figure 4.2). Levels were approximately 600 % higher than both Spatone® original and $FeSO_4$ tablets. Iron Vital F® (IVF)-induced adjusted ferritin formation was approximately 120 % higher compared with $FeSO_4$, Spatone® and Pregnacare Original® (PG), but was only about a third that of Spatone Apple® (Figure 4.2). Adjusted ferritin formation following exposure to Pregnacare Original® was equivalent to that of cells treated with Spatone® and $FeSO_4$.

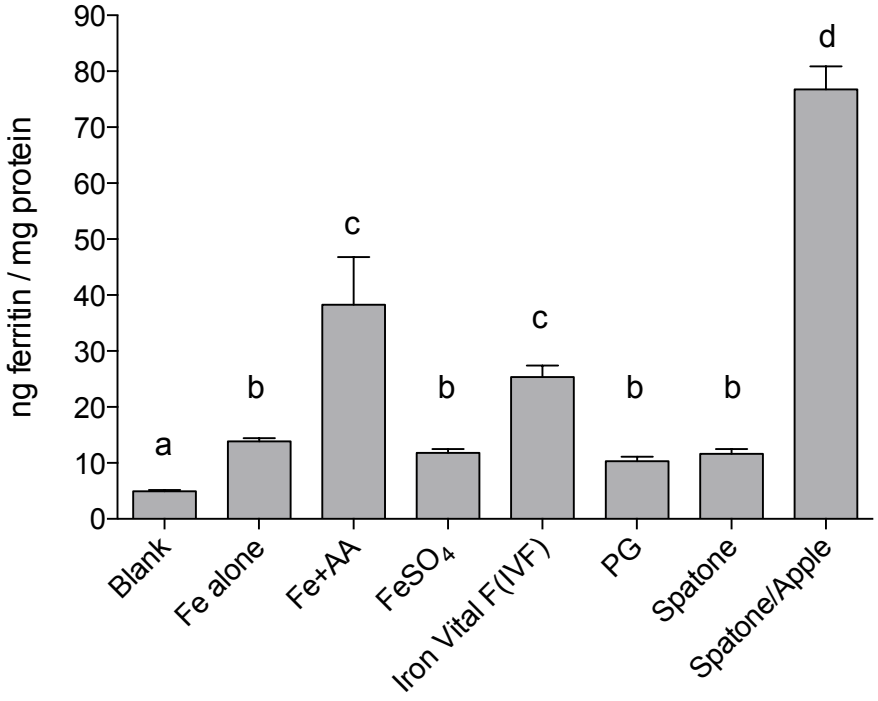

Figure 4.2 Comparison of ferritin formation from digests with different commercial iron formulations adjusted for measured iron levels in digests.

Measurement of Caco-2 cell ferritin formation from digests of $FeSO_4$ tablets, Iron Vital F® (IVF, a synthetic liquid iron supplement), Vitabiotics Pregnacare® tablets (PG, a mineral and multi-vitamin supplement marketed to pregnant women), Spatone® and Spatone Apple® (naturally iron-rich mineral waters, the latter with added ascorbic acid and fructose). Digests with no added Fe (Blank) were used to rule out iron contamination; digests with Fe alone and Fe plus ascorbic acid (Fe + AA) were used as reference controls and positive controls, respectively (not adjusted for digest iron levels). Treatment with Spatone Apple® yielded the highest ferritin levels followed by IVF treatment; ferritin levels from the three other supplements were equivalent. Values are means of data adjusted for the iron content of the digests (as determined by microwave analysis) ± SEM, n = 18. Based on ANOVA ($p < 0.0001$) with Tukey's multiple comparisons test post hoc analysis done on an all-pairwise basis after log adjustment, bar values with no letters in common are significantly different ($p \leq 0.01$)

Fructose levels in Iron Vital F® averaged 11.2 grams/100 ml supplement (n=3, standard deviation = 0.1); the final fructose concentration in digests was 1734 µmol/L (based on the addition of 41.88 µL of Iron Vital F® solution to each digest), giving a fructose to iron molar ratio of ≈ 33:1 (using the measured iron digest concentrations rather than reported). Spatone Apple® fructose concentration averaged 5.8 grams/100 ml supplement (n=3, standard deviation = 0.15); final fructose concentration in digests was 4494 µmol/L (based on the addition of 209.5 µL supplement to each digest, giving a fructose to iron molar ratio of ≈ 62:1 (using the measured iron digest concentrations rather than reported). Thus, the fructose:iron molar ratio was greater in Spatone Apple® compared with Iron Vital ®, and only in Spatone Apple® digests did the fructose:iron molar ratio exceed 50.

The addition of fructose to iron and ascorbic acid in MEM (in the same concentrations as used for the positive controls in previous experiments) increased Caco-2 cell ferritin levels two-fold compared with the positive control of iron with added ascorbic acid alone, suggesting an additive effect of fructose and ascorbic acid on iron bioavailability (Figure 4.3).

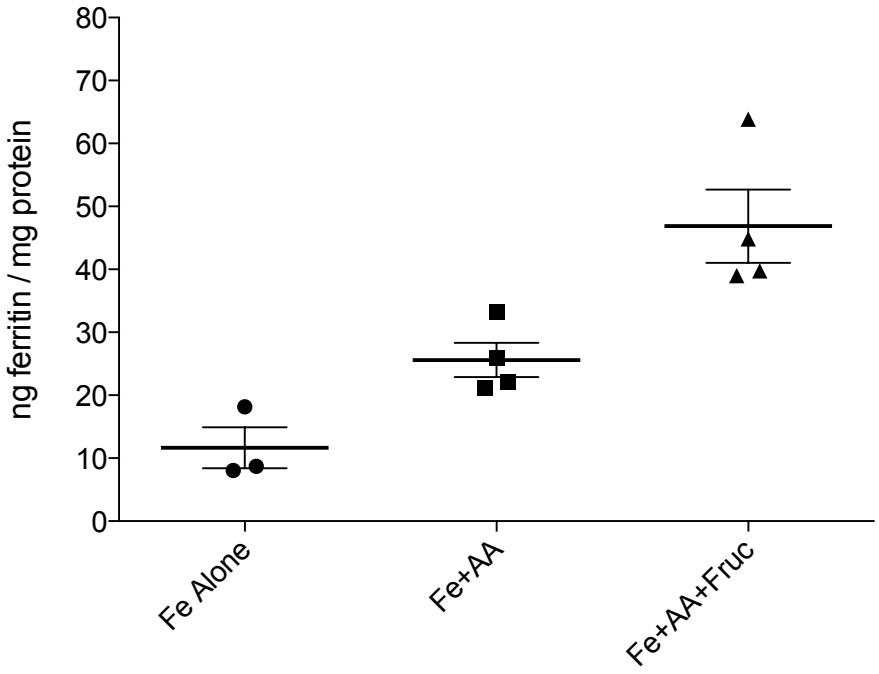

Figure 4.3 Ferritin levels of Caco-2 cells treated with digests of Fe alone, Fe with added ascorbic acid (AA), and Fe with added AA and fructose.
Caco-2 cell ferritin was measured after cells were exposed to digests of iron (Fe Alone), iron and AA (Fe+AA, 1:10 molar ratio), and iron, AA and fructose solutions (Fe+AA+Fruc, Fruc:Fe molar ratio > 50:1). Each point represents the resultant mean value from each of 4 individual experiments having an $n \geq 3$. A bold horizontal line shows the mean of these values with the SEM shown as error bars. Increase in ferritin formation was tested using the non-parametric Jonckheere-Terpstra test, and is significant in the order Fe alone, Fe + AA, and Fe+AA+Fructose ($p \leq 0.01$).

The effects of Spatone® and Spatone Apple® on ferritin levels in Caco-2 cells were further investigated over a range of iron concentrations and following different exposure times. Exposure to Spatone Apple® either for 4 h (Figure 4.4A) or 24 h (Figure 4.4B) resulted in significantly enhanced ferritin levels at all concentrations compared with the untreated controls. Spatone Apple®-induced ferritin levels were significantly higher than those produced following exposure to iron alone (all concentrations at both 4 h and 24 h time points) and Spatone® original (all concentrations at 24 h; 30 and 100 μmol/L at 4 h).

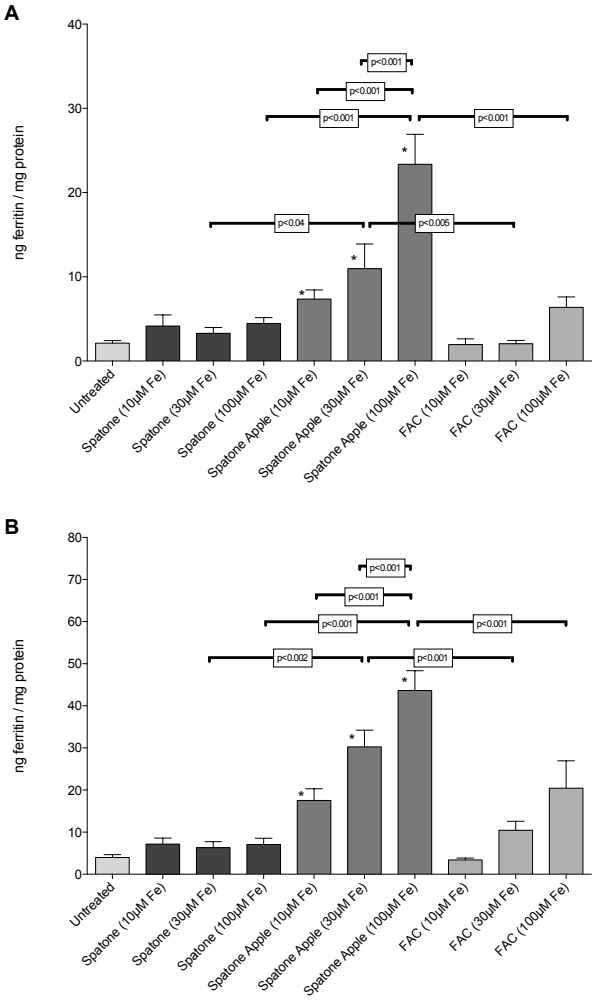

Figure 4.4(A) Caco-2 cell ferritin levels as a function of iron dose and time—4 h exposure. Caco-2 cells were treated for 4 h with three different concentrations of iron. Ferritin levels increased as a function of concentration with treatments with Spatone® and Spatone Apple®; this only reached statistical significance for Spatone Apple®. Ferritin levels were highest after treatment with Spatone Apple® at all three iron doses tested. Asterisks represent treatments with mean values of ferritin that are significantly greater than the blank control value (labelled untreated) based on Dunnett's multiple comparisons test. Columns connected by lines have significantly different means from one another based on ANOVA followed by Tukey's multiple comparisons test where the Tukey's adjusted p value is shown within the line ($p \leq 0.05$ is considered significant). **Figure 4.4(B) Caco-2 cell ferritin levels as a function of iron dose and time—24 h exposure.** Caco-2 cells were treated for 24 h with three different concentrations of iron. Ferritin levels increased as a function of concentration with treatments with Spatone® and Spatone Apple®; this only reached statistical significance for Spatone Apple®. Ferritin levels were highest after treatment with Spatone Apple® at 30 and 100 µmol/L iron. Asterisks represent treatments for which mean values of ferritin are significantly greater than the blank control value (labelled untreated) based on Dunnett's multiple comparisons test. Columns connected by lines have significantly different means from one another based on ANOVA followed by Tukey's multiple comparisons test where the Tukey's adjusted p value is shown within the line ($p \leq 0.05$ is considered significant). **NB:** The above experiments were carried out by R. Fairweather in the laboratory of Dr Paul Sharp at King's College London; statistical analysis as presented was carried out on the data by R. Fairweather, P.Sharp and T. Christides.

4.5: Discussion

Current strategies for the treatment of IDA are based on oral ferrous iron supplements; however, these may be poorly tolerated by patients due to a range of GI side effects (Hyder, Persson et al. 2002, Seck and Jackson 2008, Habib, Alabdin et al. 2009, Zhou, Gibson et al. 2009). This in turn may lead to poor compliance with therapy, which has consequences for the efficacy of treatment. Iron preparations with higher bioavailability, thus enabling lower absolute doses of iron to be ingested, may cause less GI upset and be better tolerated by patients. This notion is supported by a study undertaken in pregnant women who had been non-compliant with taking $FeSO_4$ tablets and were then switched to Spatone iron-rich water; 57 % of subjects were compliant with the new iron supplement compared with 67 % in the controls given placebo (plain water), and dyspepsia scores did not differ between the two groups (McKenna, Spence et al. 2003).

The bioavailability of a range of commercially available iron supplements were compared in this study using a well-characterized in vitro digestion model. Spatone® and ferrous sulphate tablets had equivalent bioavailability in the in vitro model, which is consistent with previous absorption studies in human volunteers (Halksworth, Moseley et al. 2003). The iron in Spatone® is $FeSO_4$ (i.e. the same as in ferrous sulphate tablets); this suggests that being in liquid form per se does not increase iron bioavailability. Spatone Apple® demonstrated the highest iron bioavailability in the in vitro model, followed by the synthetic liquid iron formulation Iron Vital F®; this result remained highly significant even after adjustment for the increased iron in Spatone Apple® digests. Both Spatone Apple® and Iron Vital F® contain ascorbic acid, a known enhancer of iron uptake. In addition, both of these formulations have added fructose, and the results of this study and previously published research (Christides and Sharp 2013) suggests that fructose increases non-haem iron bioavailability. While Spatone Apple® has an ascorbic acid:iron molar ratio of 16:1 (manufacturer's information), and a fructose:iron molar ratio of 62:1, the ascorbic acid:iron molar ratio in Iron Vital F is 6:1 (manufacturer's information), and the fructose:iron molar ratio 33:1. The enhancing effects of ascorbic acid and fructose on iron bioavailability are dose dependent, and fructose-enhanced iron bioavailability is optimal at fructose:iron molar ratios $\geq$ 50:1 (Charley, Sarkar et al. 1963, Gillooly, Torrance et al. 1984, Teucher, Olivares et al. 2004); therefore, the higher ascorbic acid:iron and fructose:iron molar ratios in Spatone Apple® may explain the difference in iron uptake between the two supplements. Furthermore, Iron Vital F®, according to the manufacturer's ingredients list, also contains plant extracts, pectin and thickening agents. These dietary factors are sources of polyphenols and phytates, both of which inhibit iron absorption (Gillooly, Bothwell et al. 1983, Petry, Egli et al. 2010). The effects of these inhibitors are only partly counteracted by ascorbic acid, and the effects of fructose on iron bioavailability are negated, in a dose dependent manner, by both polyphenols and phytates (Teucher, Olivares et al. 2004, Christides and Sharp 2013). Interestingly, Vitabiotics Pregnacare® had the same bioavailability as $FeSO_4$, despite having added ascorbic acid (70 mg/tablet). The ascorbic acid:iron molar ratio, however, was 4:1 and

thus lower compared with both Iron Vital F® and Spatone Apple®. In addition, the presence of other minerals contained in Pregnacare®, such as zinc, may partially inhibit iron uptake especially in a water-based solute matrix such as that used in these studies (Olivares, Pizarro et al. 2012).

Pregnant women are at high risk of IDA (Scholl 2005); however, iron supplement recommendations during pregnancy differ between countries. The WHO and the USA recommend that all pregnant women receive prophylactic prenatal iron (CDC 1998, WHO 2012). Currently, the UK does not suggest routine prenatal iron supplementation (NICE 2008); historically, pregnant women in the UK were advised to take iron but this was found either to be ineffective (Fenton, Cavill et al. 1977), or to have no demonstrated benefit on maternal or foetal outcomes (Cuervo and Mahomed 2001, Pena-Rosas and Viteri 2009). Recent research may challenge the current UK guidelines (Krafft 2013); two large systematic reviews with meta-analyses found that daily prenatal iron reduced maternal anaemia, IDA and risk of foetal low birth weight (Pena-Rosas, De-Regil et al. 2012, Haider, Olofin et al. 2013). However, it has been noted that dose and regimen recommendations for routine iron supplementation need refining and updating (Pena-Rosas, De-Regil et al. 2012), and within this context, the results of this study suggest that further research with the tested formulations used is warranted.

Another group that might benefit from both Spatone Apple® and Iron Vital F® are post-bariatric surgery patients who are at high risk of IDA (Jauregui-Lobera 2013), and in whom treatment with standard iron tablets appears to often be ineffective (Gasteyger, Suter et al. 2008, Sawaya, Jaffe et al. 2012). In one study, post-operative gastric bypass patients given 100 mg $FeSO_4$ tablets as an oral challenge absorbed inadequate amounts of iron as measured by change in serum iron concentration (Gesquiere, Lannoo et al. 2014); altered gut physiology after bariatric surgery may not allow for efficient iron absorption from $FeSO_4$ tablets. Furthermore, several studies have also documented low plasma ascorbic acid levels in patients after bypass procedures (Clements, Katasani et al. 2006, Netto, Moreira et al. 2012); therefore, liquid iron preparations with added ascorbic acid — such as Spatone Apple® and Iron Vital F® (which also has other micronutrients) — may be helpful in this population.

The data presented here for Spatone Apple® suggest that this, or similar products, are highly bioavailable as they gave rise to a rapid increase in cell ferritin in the in vitro model used in this study. Furthermore, 10 µmol/L iron given as Spatone Apple® gave a comparable ferritin response to 100 µmol/L FAC, and a higher ferritin level than 100 µmol/L $FeSO_4$ (from Spatone®), suggesting the possibility of obtaining better iron absorption with a lower dose of iron. An important caveat is that only in vitro studies of Spatone Apple® and Iron Vital F® have been performed to date. The bioavailability of these formulations has not been assessed in human volunteers, and the data presented here suggest that these studies are warranted. The Caco-2 in vitro digestion model provides a validated method for screening iron bioavailability of a range of compounds

and test meals. Furthermore, it has been shown to predict the direction, although not necessarily the magnitude, of iron bioavailability in humans (Fairweather-Tait, Lynch et al. 2005). If the in vitro results of this study are confirmed *in vivo*, it would suggest that lower doses of a more bioavailable iron from supplements such as those tested could be effective in combating iron deficiency. This would be particularly advantageous for groups who are both at increased risk of IDA and less tolerant to high doses of iron, such as pregnant women.

4.6: Conclusion

In conclusion, the results of this in vitro study demonstrate that naturally iron-rich mineral waters, or synthetic liquid iron formulations, are equivalent to the standard of care $FeSO_4$ recommended for IDA, and those with added ascorbic acid, and possibly fructose, have better bioavailability compared with $FeSO_4$ alone. If these results are confirmed in randomized control studies in human volunteers at risk of IDA, such subjects could then be offered a greater choice of more bioavailable and potentially better tolerated iron preparations.

4.7: Author Contributions

Conceived and designed the experiments: TC and PS. Performed the experiments: TC, DW (supervision ICP analysis), RM, and RF. Analysed the data: TC, RM, RF and PS. Contributed reagents/ materials/analysis tools: TC and PS. Wrote the paper: TC and PS.

Chapter 5: Iron Bioavailability and Provitamin A from Sweet Potato- and Cereal-Based Complementary Foods

This chapter presents work and evidence related to whether fructose, as a naturally occurring dietary factor in a food product made from the staple crop sweet potato, affected iron bioavailability. The chapter is based on the published manuscript: "Iron Bioavailability and Provitamin A from Sweet Potato- and Cereal-Based Complementary Foods"; Christides, Amagloh and Coad (2015), *Foods*, 4: 463-476 (doi: 10.3390/foods4030463). This article belongs to the Special Issue Infant and Child Nutrition and Foods.

5.1: Introduction

Iron deficiency (ID) and Vitamin A deficiency (VAD) are prevalent in the developing world, especially in vulnerable groups such as infants and young children (World Health Organization 2015). These micronutrient deficiencies are particularly problematic in young children; both ID and VAD during infancy can cause long-term health problems that may not be able to be reversed even with adequate intake later in life (Grantham-McGregor and Ani 2001, Grantham-McGregor 2003, Nyaradi, Li et al. 2013). Infants and toddlers are at increased risk for ID and VAD when complementary foods, which are usually cereal-based, are introduced (Khan and Bhutta 2010). These cereal-based foods are often poor dietary sources of both vitamin A (Amagloh, Hardacre et al. 2012, Amagloh and Coad 2014), and bioavailable iron as they contain high levels of iron absorption inhibitors such as phytates (Gibson, Bailey et al. 2010, Roos, Sorensen et al. 2013) or polyphenols (Roos, Sorensen et al. 2013). The ideal complementary food should have adequate levels of nutrients and high mineral bioavailability. VAD and ID often co-exist in low-income countries due to long-term poverty and plant-based monotonous diets (World Health Organization 2015), thus sustainable strategies, particularly food-based approaches, which will lead to high intake of dietary vitamin A and bioavailable iron are desirable.

Sweet potato-based complementary food formulations, prepared from cream- or orange-fleshed sweet potato (CFSP or OFSP, respectively) cultivars, contain significantly more β-carotene, the precursor of vitamin A, than cereal-based products (Amagloh, Hardacre et al. 2012, Amagloh and Coad 2014); have acceptable sensory attributes; and, desirable physical properties, such as viscosity, for complementary feeding (Amagloh, Mutukumira et al. 2013, Mahmoud and El Anany 2014). Additionally, sweet potato is relatively low in phytate compared to cereals (Phillippy 2003, Phillippy, Bland et al. 2003), thus it would be expected that the sweet potato food matrix would have a less inhibitory effect on iron absorption in human infants. Furthermore, compositional analysis of sweet potato-based complementary foods found high concentrations of ascorbic acid and fructose (Amagloh and Coad 2014)[6]; ascorbic acid is a known enhancer of iron uptake (Hurrell and Egli 2010), and research indicates that both ß-carotene and fructose may also improve iron

bioavailability (García-Casal 2006, Christides and Sharp 2013). Therefore, it would be predicted that sweet potato-based complementary foods, with comparable amounts of iron as in cereal-based infant foods, would have better iron bioavailability. There is support for this in the research literature. In an in vitro study conducted by Lung'aho & Glahn (Lung'aho and Glahn 2009), the trend of data suggested that the addition of sweet potato (cultivar not specified) to food mixes potentiated iron bioavailability from household-level recipes, and a review suggested that sweet potato, particularly the OFSP food matrix, would be expected to promote iron absorption (Glahn 2009).

Sweet potato, however, is also a rich source of polyphenols (Teow, Truong et al. 2007, Ojong, Njiti et al. 2008). Polyphenols are potent inhibitors of non-haem iron (inorganic iron and the most prevalent form in the diet) uptake, and may even inhibit absorption of haem-iron (Brune, Rossander et al. 1989, Ma, Kim et al. 2010, Ma, Kim et al. 2011, Tako, Reed et al. 2015). Studies have demonstrated that tannic acid (a commonly occurring dietary polyphenol) at a 0.1:1 tannin to iron molar ratio decreased iron uptake by almost 100 % in the Caco-2 cell model, and a single meal study conducted in humans found that a tannic acid:iron 1:1 molar ratio led to almost 90 % decreased iron absorption (Brune, Rossander et al. 1989, Glahn, Wortley et al. 2002). In addition, different sweet potato cultivars may contain different types of polyphenols (Teow, Truong et al. 2007, Ojong, Njiti et al. 2008); the iron inhibitory effects of polyphenols vary with structure, thus knowing only total levels of food polyphenols may limit the ability to predict their effects on iron absorption (Brune, Rossander et al. 1989, Tuntipopipat, Judprasong et al. 2006).

Dietary iron bioavailability (outside of host factors) represents the sum of interactions between total iron levels, and iron uptake inhibitors and enhancers; compositional proximate analysis of inhibitors and enhancers can be used to predict iron absorption, but experiments directly measuring iron uptake are needed to more fully evaluate the net effects of complex food matrices on iron bioavailability.

The in vitro digestion/Caco-2 cell model, using cell ferritin formation as a surrogate marker for iron uptake, is a validated method for initial screening of food iron availability (Glahn, Lee et al. 1998, Yun, Habicht et al. 2004). Furthermore, it has specifically been applied to evaluate iron bioavailability from complementary foods (Lung'aho and Glahn 2009, Lung'aho and Glahn 2009). Therefore, this model is suitable for the assessment of iron bioavailability of sweet potato-based household-level food formulations as alternatives to cereal-based complementary foods.

The objective of this study was to assess the iron bioavailability from sweet potato-based complementary food formulations (OFSP ComFa and CFSP ComFa) in comparison with a non-commercial cereal-based infant food composed of maize-soyabean-groundnut (Weanimix: a food blend developed by the Ghanaian Ministry of Health Nutrition Division and the United Nations Children's Fund in 1987), and a commercial infant cereal (Cerelac®), using the Caco-2 cell in vitro digestion model. In addition, a further

objective of this study was the evaluation of iron bioavailability in the tested food formulations in relation to concentrations of iron absorption modifiers; specifically, we evaluated the effects of the inhibitors phytates, total polyphenols, and total dietary fibre, and the enhancers ascorbic acid, ß-carotene and fructose.

5.2: Materials and methods

5.2.1: Complementary Food Samples

The food samples, as previously described (Amagloh and Coad 2014), were packaged in air- and light-safe containers, and couriered from Massey University, New Zealand (NZ) to the University of Greenwich, United Kingdom (UK), for testing in the in vitro digestion/Caco-2 cell model to assess their iron bioavailability. The samples were stored at 4°C in air and light tight containers prior to the in vitro digestion for use in the Caco-2 cell studies. Samples were coded, and the investigator conducting the in vitro digestion/Caco-2 cell studies was blinded to the composition (i.e., which formulation) of tested samples.

Infant formulations were prepared from a blend of OFSP or CFSP, soya bean, fish powder and soya bean oil (Amagloh and Coad 2014). Weanimix is a blend of maize (usually the white cultivar), soya bean or cowpea, and groundnut; in this study, soya bean was used, and the blend was further enriched with fish powder. Cerelac® is a commercial infant cereal commonly used in Africa; the sample evaluated in this study was Cerelac® Infant Cereal Wheat & Ikan Bilis that also contained dried anchovies, and was donated by Nestlé Malaysia.

5.2.2: In vitro digestion/Caco-2 model to measure iron bioavailability

Two experiments were carried out. In Experiment I, iron bioavailability was investigated from all the formulations as prepared in NZ and received in the UK, herein referred to as "as provided". In Experiment II, β-carotene alone, and β-carotene plus fructose, were used to fortify CFSP ComFa to levels equivalent with OFSP ComFa (13,353 µg β-carotene/100 g; and 13,353 µg β-carotene/100 g plus 7.15 g fructose/100 g, respectively) to investigate their effect on iron bioavailability. Analytical standard fructose and β-carotene were obtained from Sigma, solubilized, and added to the CFSP ComFa digest immediately prior to the beginning of the digestive process.

Iron uptake was assessed using the TC7 Caco-2 cell clone (INSERM U505) as used in previous studies (Christides and Sharp 2013, Christides, Wray et al. 2014).

In both experiments, 1 g of the food formulation was weighed, taking into account an adjustment for respective moisture content of different foods. The method used for the in vitro digestion procedure and iron bioavailability estimations has been published elsewhere (Christides and Sharp 2013, Christides, Wray et al. 2014).

Briefly, cells were grown in six-well tissue culture plates and maintained in supplemented DMEM. On day 13, cell media was changed to supplemented MEM, and on day 14, foods were subjected to in vitro digestion with sequential addition of digestive enzymes to mimic exposure to the stomach and small intestine (pepsin at pH 2, followed by bile/pancreatin at pH 7). Digested foods (digestates), and controls were then applied to Caco-2 cells as previously described; cells were treated for two hours, digestates removed, and the cells returned to the incubator. Twenty-four hours after the initiation of the digestive process cells were harvested for ferritin. Ferritin was measured using a commercial enzyme-linked immunosorbent assay (Spectro ferritin, RAMCO Laboratories Inc., Stafford, TX, USA), and corrected for differing cell numbers/wells by measurement of cell protein; cell protein was measured using the Pierce protein bicinchoninic acid assay (BCA). Ferritin values are expressed as ng ferritin/mg cell protein.

5.2.3: Compositional Analysis: β-Carotene, Vitamin C, Fructose, Iron, Total Polyphenols and Phytate

The methods used for determination of β-carotene, ascorbic acid (vitamin C), fructose, iron, total polyphenols and phytate have been described in depth in previous publications (Amagloh, Brough et al. 2012, Amagloh and Coad 2014).

Briefly, levels of inhibitors were measured as follows. Phytates were measured using the K-PHYT05/07 assay kit (Megazyme International, Bray, Co. Wicklow, Ireland); a quality control sample of oat flour was analysed alongside with samples and values were within the 10 % variation as specified in the protocol. Sample polyphenol levels were measured using the redox Folin–Ciocalteu reagent, and quantified by UV/visible-light spectrophotometry; values are expressed as Gallic acid equivalents. Soluble and insoluble dietary fibres were determined by the enzymatic-gravimetric method (AOAC 991.43).

Levels of food components enhancing iron absorption were determined as follows. β-carotene was measured by the Carr–Price method (AOAC 974.29) using high-performance liquid chromatography (Shimadzu HPLC, Tokyo, Japan), ascorbic acid was measured by reversed-phase, high-performance liquid chromatography (HPLC), and fructose was determined by gas chromatography.

Assays for polyphenols, sugars, and ascorbic acid were carried out by a commercial laboratory (Nutrition Laboratory, Massey University, New Zealand, accredited by the IANZ and a NZ Ministry of Health recommended agency).

Sample iron levels were measured by quadrupole inductively coupled mass spectrometry (Agilent 7500ce; Agilent Technologies Inc., Santa Clara, CA, USA) after high temperature digestion in 68 % HNO3; analysis was conducted by the Campbell Microanalytical Laboratory, Department of Chemistry, University of Otago, New Zealand. Use of certified reference materials demonstrated a mean recovery of 103 % for iron, which is within acceptable experimental standards.

5.3: Statistical Analysis

The data generated from the in vitro digestion/Caco-2 cell studies were analysed using general linear models. For the compositional data, One-Way ANOVA was used, and the results are presented as the mean ± SD. Tukey's studentised range test was used to compare differences between means when the ANOVA result was significant ($p < 0.05$). Minitab 16.2.2 (Minitab Inc., State College, PA, USA) statistical package was employed for the statistical analysis.

5.4: Results

5.4.1: Iron Bioavailability of Tested Complementary Foods as Provided

Data on iron bioavailability from the in vitro digestion/Caco-2 cell model for Experiment I are presented in Figure 5.1.

The commercial blend, Cerelac®, caused the greatest uptake of iron as measured by the ferritin assay; approximately 100 % more than the average of that of the sweet potato- and maize-based blends investigated in this study. Iron uptake promoted by OFSP ComFa and Weanimix was equivalent, and approximately half that of Cerelac® treated cells. CFSP ComFa treated cells had the lowest iron uptake; approximately 65 % less than Cerelac®, and 25 % less than both OFSP ComFa and Weanimix.

5.4.2: Iron Bioavailability of OFSP ComFa Compared with CFSP ComFa Fortified with Fructose and β-Carotene

In Experiment I, with the complementary foods as provided, ferritin formation from OFSP ComFa was approximately 30 % higher compared with CFSP ComFa. In Experiment II, the fortification of CFSP ComFa with the addition of β-carotene, and β-carotene and fructose, to levels equivalent to those in OFSP ComFa, did not increase ferritin levels in cells treated with fortified CFSP ComFa to the levels seen in cells treated with OFSP ComFa (Figure 5.2), and ferritin levels were comparable to those of unfortified CFSP ComFa (Figure 5.1). The difference in ferritin between fortified CFSP- and OFSP ComFa remained approximately 30 % — i.e., the same difference as between unfortified CFSP- and OFSP ComFa.

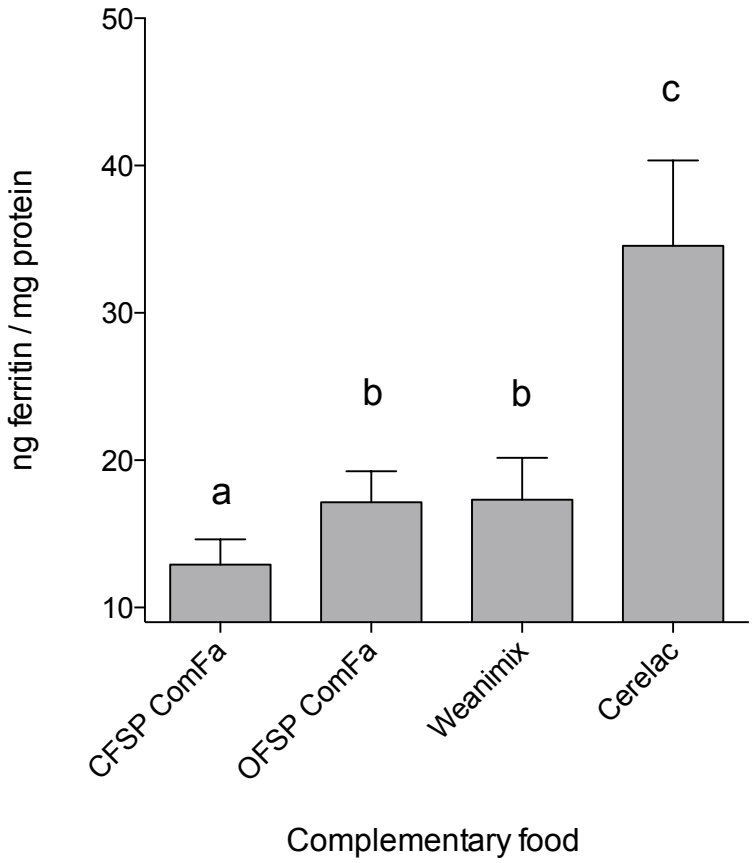

Figure 5.1 Ferritin formation per gram (adjusted for moisture content) of infant complementary weaning foods from: Cerelac®; CFSP ComFa; OFSP ComFa and Weanimix.
Caco-2 cell ferritin formation was highest after exposure to the commercial infant cereal Cerelac®. OFSP ComFa and Weanimix ferritin levels were equivalent and approximately 50 % less than Cerelac® induced ferritin formation. CFSP ComFa treated cells formed the lowest amount of ferritin: 25 % less than the levels formed in OFSP ComFa and Weanimix. Values shown are the mean ±SEM (n=18). Values have been normalised to the average blank digest (i.e., no added iron) ferritin level. Means per group with different letters are significantly different ($p \leq 0.05$).

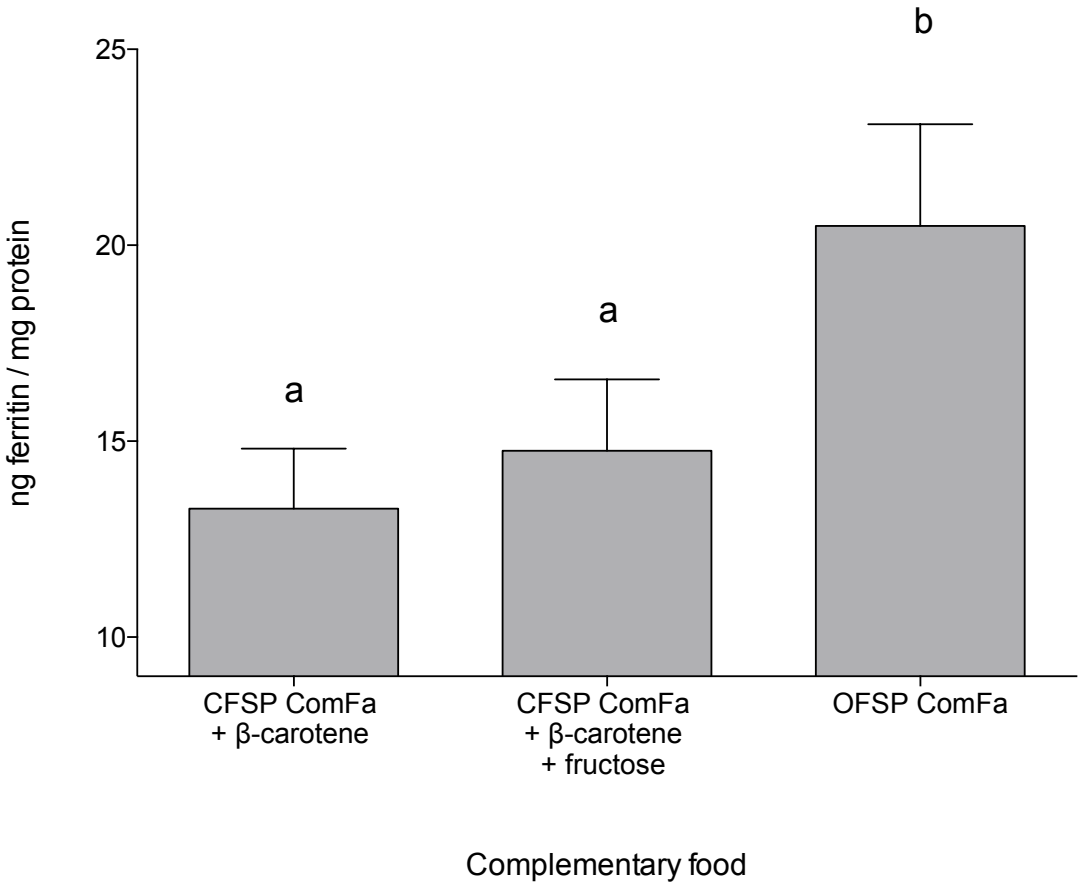

Figure 5.2 Ferritin formation per gram (adjusted for moisture content) of cream-fleshed sweet potato-based complementary food (CFSP ComFa) fortified with β-carotene, and β-carotene plus fructose, in comparison with orange-fleshed sweet potato-based food (OFSP ComFa).

Ferritin formation was highest for Caco-2 cells exposed to OFSP ComFa; CFSP ComFa ferritin levels remained approximately 25 % less than OFSP ComFa levels whether fortified with β-carotene alone, or β-carotene and fructose, and comparable to the CFSP ComFa ferritin concentration seen in Figure 5.1 (approximately 14 ng ferritin/mg protein). OFSP ComFa mean ferritin formation (labeled b) was significantly higher than both other treatments (labeled a). Values shown are the mean ±SEM (n=6). Values have been normalised to the average blank digest (i.e., no added iron) ferritin level. Means per group with different letters are significantly different ($p \leq 0.05$).

5.4.3: Concentrations of Total Iron and Iron Absorption Inhibitors in Tested Foods

Table 5.1 shows the data for total iron and iron absorption inhibitors.

All tested foods had similar levels of total iron. According to the manufacturer's label, Cerelac® was fortified with ferrous fumarate; data on the specific biochemical iron form of the other foods are not available.

Levels of inhibitors varied for the tested foods. Cerelac® had the lowest levels of all measured inhibitors; however, this did not reach statistical significance for Cerelac® polyphenol levels compared with Weanimix, nor Celerac® phytate levels compared with CFSP ComFa. OFSP ComFa had the highest concentration of total dietary fibre (TDF), followed by CFSP ComFa (12.28 g/100 g and 10.05 g/100 g respectively). Weanimix TDF was approximately 50 % of that in each of the sweet potato-based foods. Cerelac® TDF was the lowest of all tested foods at 1.48 g/100 g.

Weanimix had the highest phytate levels: approximately twice that of OFSP ComFa, and approximately 5 times that of CFSP ComFa and Cerelac®, which had equivalent phytate concentrations.

The sweet potato-based foods had the highest levels of polyphenols, the most potent inhibitors of iron absorption; concentrations were nearly identical in OFSP- and CFSP ComFa, and approximately 80 % and 100 % higher than Weanimix and Cerelac®, respectively.

Table 5.1 Amount of inhibitors and enhancers of iron absorption, and the concentration of iron in sweet potato- and cereal-based complementary foods.

	Inhibitor (/100g)			Enhancer(/100g)			
Complementary Food	**Phytate (mg)**	**Total Polyphenols (mg GA equ)**	**Total Dietary Fibre (g)**	**Ascorbic Acid (mg)**	**β-Carotine (µg)**	**Fructose (g)**	**Iron (mg/100 g)**
OFSP ComFa	229.85 ±19.36^{b}	466.28 ±9.68^{a}	12.28 ±1.04^{a}	32.48 ±0.48^{b}	13353 ±1792.60^{a}	7.15 ±0.17^{a}	7.76 ±1.22^{a}
CFSP ComFa	78.62 ±3.50^{c}	466.42 ±34.98^{a}	10.05 ±0.18^{b}	37.40 ±0.61^{a,b}	1263 ±22.30^{b}	3.09 ±0.07^{b}	7.26 ±0.08^{a}
Weanimix	438.10 ±8.58^{a}	263.68 ±17.82^{b}	6.90 ±0.64^{c}	BDL	34 ±11.77^{b}	BDL	6.53 ±1.55^{a}
Cerelac	66.92 ±0.96^{c}	213.45 ±29.92^{b}	1.48 ±0.50^{d}	53.11 ±12.07^{a}	66 ±15.83^{b}	BDL	8.85 ±0.17^{a}
p-value	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*0.10*

BDL, below detection limit. Values are the mean ± SD of triplicate determinations on dry matter basis; values within a column with unlike superscript letters are significantly different. Ascorbic acid, phytate, total polyphenols and fructose have previously been published (Amagloh and Coad 2014); copyright 2014, The Nevin Scrimshaw International Nutrition Foundation (used with permission).

5.4.4: Concentrations of Iron Absorption Enhancers in Tested Foods

Data on iron absorption enhancers are shown in Table 5.1.

Cerelac® had the highest levels of ascorbic acid, an established enhancer of iron absorption; levels were 50 % higher than in either of the sweet potato-based foods. OFSP- and CFSP ComFa ascorbic acid levels were similar, and 500 % higher than Weanimix, for which levels were below the detection limits (BDL) of the assay.

OFSP ComFa had the highest concentration of β-carotene (13353 µg/100 g), followed by CFSP ComFa (1263 µg/100 g); levels were very low in Cerelac® and Weanimix and not statistically different from one another.

Fructose concentrations were below the level of detection in both Cerelac® and Weanimix. OFSP ComFa had the highest amount of fructose at 7.15 g/100 compared with 3.09 g/100 g in CFSP ComFa.

5.5: Discussion

As expected, Cerelac® had the highest iron bioavailability as estimated by ferritin formation (Figure 5.1). Cerelac® infant cereal is fortified with ferrous fumarate, the current recommended form of inorganic iron for fortification of complementary foods because of its relatively high bioavailability (Hurrell 2010). In contrast, the household formulations contained endogenous iron, which is typically in the less bioavailable ferric iron form (Miret, Simpson et al. 2003). Furthermore, levels of inhibitors were lower in Cerelac®; total dietary fibre (TDF) was statistically significantly lower compared with all other tested food formulations. Although there is some debate about the role of TDF itself (versus the phytates it contains) in decreasing iron absorption in humans (Gibson 2007), the results of this study are consistent with other reports in the literature that would predict lower TDF would be associated with better iron bioavailability (Lestienne, Besancon et al. 2005). Concentrations of both polyphenols and phytates in Cerelac® were also lower than the other formulations. In addition to lower concentrations of iron uptake inhibitors, Cerelac® had approximately 50 % higher levels of ascorbic acid. Cerelac®'s high iron bioavailability is thus explained by the combination of low levels of iron absorption inhibitors, high levels of ascorbic acid, and fortification with a relatively bioavailable form of inorganic iron.

Despite higher levels of iron absorption enhancers in OFSP ComFa compared with Weanimix, the results of this study indicate that the presence of inhibitors in the OF sweet potato-based formulation led to OFSP ComFa and Weanimix having similar levels of bioavailable iron as measured by ferritin formation (Figure 5.1)—approximately half the amount seen with Cerelac®. Proximate analysis of the ComFa formulations, and review of the literature, suggested that the OFSP food matrix would promote iron absorption, and that OFSP ComFa would have more bioavailable iron compared with Weanimix (Amagloh, Brough et al. 2012), but this was not found in this study. Although OFSP ComFa contained 32-, 393-, and 7-times higher levels of ascorbic acid, β-carotene and fructose, respectively, than found in Weanimix, apparently these enhancers did not counteract the inhibitory effects of the approximately 1.8 times higher levels of OFSP ComFa polyphenols (Table 5.1). Ascorbic acid, β-carotene and fructose have been shown to improve the bioavailability of iron from foods, however, for ascorbic acid and fructose, this effect is dose dependent, affected by the food matrix, and diminished or eliminated by polyphenols (Siegenberg, Baynes et al. 1991, Hurrell, Reddy et al. 1999, Christides and Sharp 2013). As previously stated, sweet potato has high concentrations of a range of

different polyphenols, and although knowledge about the inhibitory effects of all the specific polyphenolic compounds in sweet potato is lacking, the results of this study suggest they are strong inhibitors of iron uptake. This is consistent with in vitro and in vivo evidence demonstrating that polyphenols are potent inhibitors of inorganic iron absorption, and limit food iron bioavailability (Hurrell, Reddy et al. 1999, Tako, Beebe et al. 2014, Tako, Reed et al. 2015). Indeed, the results of our study suggest that the presence of polyphenols is critical to the overall iron bioavailability of foods, even when large amounts of known iron absorption enhancers are present. One caveat to this is that there is evidence that the food matrix itself also impacts on the relative importance of inhibitors and enhancers. A human study looking at a cowpea based food found that low iron bioavailability was associated with higher phytate/iron molar ratios and total phytate but not the amount of polyphenols (Abizari, Moretti et al. 2012), in contrast with the findings of our study.

It is noteworthy that the iron content (Table 5.1) in each of the tested formulations was similar, underscoring the fact that inorganic iron bioavailability is not primarily determined by absolute iron levels, but rather by the biochemical form of dietary iron, in combination with the balance of inhibitors and enhancers of iron absorption in the food matrix.

Interestingly, ferritin formation with OFSP ComFa was higher than with CFSP ComFa (Figure 5.1) despite similar levels of polyphenols and ascorbate, and lower TDF and phytate levels in CFSP ComFa (Table 5.1); this suggests that other factors accounted for the difference in iron bioavailability between these two sweet potato-based foods. Fructose and β-carotene levels were higher in OFSP ComFa compared with CFSP ComFa; therefore, whether these enhancers were responsible for the observed difference in ferritin levels between OFSP- and CFSP ComFa was investigated. Experiment II tested whether exogenous fortification of CFSP ComFa with fructose and β-carotene at the concentrations found in OFSP ComFa would result in equivalent iron uptake from the two different types of sweet potato-based foods. However, neither the addition of β-carotene alone, nor β-carotene and fructose together, improved the amount of bioavailable iron from CFSP- compared with OFSP ComFa (Figure 5.2).

The negative findings of Experiment II may be the result of the specific single type of β-carotene used (versus the variety of forms naturally occurring in foods), or because, in order for β-carotene to improve iron bioavailability, it needs to be present together with iron in the original food matrix. Alternatively, in this food matrix β-carotene may not act as an iron uptake enhancer.

The mechanism by which fructose increases iron bioavailability is uncertain, although a recent in vitro study suggested that fructose reduces non-haem ferric iron to the more bioavailable divalent ferrous form (Christides and Sharp 2013). However, the enhancing effects of fructose on iron bioavailability demonstrated in that study were negated by both phytates and polyphenols at levels lower than those found in either OFSP- or CFSP

ComFa. Thus, the negative findings in this current study are consistent with previous work that found the effects of fructose on iron uptake are negated by phytate and polyphenols at levels commonly found in consumed foods, and indicate that higher fructose levels do not explain the different iron bioavailability of OFSP- compared with CFSP ComFa. In summary, the exact reason for the difference in iron uptake between the two sweet potato-based formulations remains undetermined.

It is known that sweet potato colour may indicate the presence of various polyphenolic compounds; research analysing sweet potato cultivars has demonstrated differences both in type, and amount, of polyphenols between cultivars with different colours (Teow, Truong et al. 2007). The results of the present study suggest that different polyphenolic compounds present in cream-fleshed versus orange-fleshed sweet potato might account for their differing iron bioavailability. A recent in vitro study by Hart et al. using different coloured beans found that polyphenol type varied with bean colour, and specific polyphenols had differing effects on iron bioavailability at equivalent concentrations (Hart, Tako et al. 2015); these findings are consistent with the hypothesis proposed above, that although the total polyphenol concentrations of CFSP- and OFSP ComFa were the same, differences in types of polyphenols led to differing iron uptake. A limitation of this study is that only total polyphenol levels were measured; the findings of this research suggest a need for specific analysis of polyphenols found in sweet potato-based foods, and the sweet potato cultivars used to make them, for their effects on iron bioavailability.

The amount of iron available from CFSP ComFa was significantly lower (1.3 times) than that of Weanimix (Figure Table 5.1) although CFSP ComFa contained higher amounts of ascorbic acid (37-fold), β-carotene (37-fold) and fructose (3-fold) than Weanimix (Table 5.1). Furthermore, Weanimix had 5.5 times the amount of phytate compared with CFSP ComFa, and the iron:phytate molar ratio of CFSP ComFa was 1:0.92; this molar ratio would be expected to have minimal inhibitory effects on iron uptake (Hallberg, Rossander et al. 1987, Hallberg, Brune et al. 1989). TDF levels were higher in CFSP ComFa compared with Weanimix, which might, in part, explain the lower bioavailability. Perhaps more significantly, CFSP ComFa, similarly to OFSP ComFa, contained approximately twice the amount of total polyphenols compared with the cereal-based complementary foods (Table 5.1), which would be predicated to decrease iron absorption. Significantly, the non-haem iron inhibitory effects of polyphenols are only partially counteracted by ascorbic acid at the levels found in the ComFa foods (Hallberg, Brune et al. 1986), as opposed to phytates for which ascorbic acid has been shown to more completely reverse the inhibitory effects at lower concentrations (Hallberg, Brune et al. 1986, Hallberg, Brune et al. 1989). Polyphenols also decreased the enhancing effects of fructose on iron bioavailability at a 1:1 molar ratio, whereas a 5:1 phytate:iron molar ratio was required for phytate inhibition in a recent in vitro study (Christides and Sharp 2013). Furthermore, two in vitro studies suggested that polyphenols decrease haem iron bioavailability (Ma, Kim et al. 2010, Ma, Kim et al. 2011); therefore, uptake of the haem-

iron in CFSP ComFa (from the added anchovy powder) might also be decreased. There are no data (to the authors' knowledge) about the effects of β-carotene on polyphenol-related inhibition of iron absorption. In summary, the research conducted in this study indicates that the decreased iron uptake seen with CFSP ComFa compared with Weanimix is most likely secondary to inhibition by polyphenols.

Overall, the findings of this study support the well-documented inhibitory effects of polyphenols on iron bioavailability, and also that this inhibition is only partially reduced by iron absorption enhancers. OFSP ComFa had higher concentrations of phytate and TDF than CFSP ComFa, but more bioavailable iron, and polyphenol levels were almost identical. Thus, the better iron bioavailability of OFSP ComFa is not explained by differences in absolute polyphenol levels, suggesting that CFSP ComFa-specific phenolic compounds were the most potent inhibitors, or that there are other unidentified iron uptake inhibitors in cream-fleshed sweet potato. The results of this in vitro study, demonstrating low iron bioavailability from Weanimix enriched with fish powder, confirm earlier findings of a randomised, community-based feeding trial, which found that infants fed enriched Weanimix for six months had decreased plasma ferritin and haemoglobin levels at the end of the intervention (Lartey, Manu et al. 1999).

Although the findings of this study did not confirm the earlier suggestion that sweet potato-based foods would be less inhibitory on iron bioavailability compared with cereal-based formulations, these foods could be a good dietary source of other essential nutrients such as ascorbic acid and provitamin A (β-carotene) when used as a home produced complementary food. Furthermore, whilst the Caco-2 cell model overall accurately predicts whether substances enhance or inhibit iron uptake, it may not always precisely predict the magnitude of the effect (Fairweather-Tait, Lynch et al. 2005), therefore in vivo studies to confirm these results are warranted.

5.6: Conclusions

CFSP- or OFSP ComFa are not better sources of bioavailable iron compared with non-commercial cereal-based weaning foods as measured using the Caco-2 cell in vitro digestion model. However, they may be a good source of provitamin A. Furthermore, sample preparation may affect iron bioavailability, and thus it is possible that sweet potato-based complementary foods prepared in a different manner might have more bioavailable iron. Studies to identify and analyse polyphenols from different sweet potato cultivars, and their specific effects on iron absorption, and investigation of the interaction between sweet potato polyphenols, phytates and fibre in the presence of ascorbic acid, β-carotene and fructose, could help to identify optimal sweet potato cultivars, and sample preparation methods, to be used as a basis for complementary foods. Follow up in vivo studies to confirm results are also warranted.

5.7: Acknowledgments

We acknowledge the Nutricia Research Foundation, the Netherlands (Project number: 2012-E6), for funding for the development of the sweet potato-based complementary foods, and the compositional analyses. We are indebted to Delta Produce Co-op Ltd, Dargaville, New Zealand for providing the sweet potato and Nestlé Malaysia, for donating the Nestlé Cerelac® Infant Cereal Wheat & Ikan Bilis. We thank the University of Greenwich at Medway for funding the Caco-2 studies.

5.8: Author Contributions

FKA and JC conceived the ComFa formulations for complementary feeding, particularly for infants in low-income countries, and arranged for analysis of ascorbic acid, polyphenols, phytates, iron levels and sugars of the formulations. TC designed and carried out the in vitro digestion/Caco-2 studies. TC and FKA analysed the data and wrote the first draft; TC and JC oversaw and edited all other draft versions. All authors read and approved the final version submitted for peer-review and publication.

Chapter 6: In vitro assessment of iron availability from commercial Young Child Formulae supplemented with prebiotics

This chapter presents work and evidence related to whether galacto- and fructo-oligosaccharides improve iron bioavailability in a milk-based food matrix. The chapter is based on the manuscript accepted for publication in the *European Journal of Nutrition* entitled "In vitro assessment of iron bioavailability from commercial Young Child Formulae supplemented with prebiotics"; Christides, Ganis and Sharp (2016).

6.1: Introduction

Iron is essential for growth and neurodevelopment in infants, and young children (children between the ages of one-three). Humans from birth until three years of age undergo rapid brain growth and development; iron is required for multiple processes underlying this developmental surge including myelination, neurotransmitter function, brain energy homeostasis, and neuronal- and synaptogenesis (Nyaradi, Li et al. 2013). Inadequate iron during this period of childhood is associated with impaired cognitive and psychomotor development that may not reverse even if children become iron-replete later in life (Lozoff, Jimenez et al. 2000). Full term infants are protected from iron deficiency and iron deficiency anaemia (ID/IDA) for the first four-six months after birth as a result of gestationally-derived iron stores (Domellof, Braegger et al. 2014). Unfortunately, for the rest of the critical time period up to three years of age, children are at high risk for ID/IDA because of rapid overall growth and associated increased iron needs, and often, inadequate intake of bioavailable dietary iron (Domellof, Braegger et al. 2014, Paoletti, Bogen et al. 2014).

Infant formula is fortified with iron to prevent deficiency (American Academy of Pediatrics Committee on Nutrition 1989), and the evidence suggests that this mandatory fortification is effective - in the developed world less than 5 % of infants under 12 months have IDA (Domellof, Braegger et al. 2014, Scientific Advisory Committee on Nutrition 2010). However, the prevalence of ID/IDA increases between one–three years of age, ranging from 6-15 % in the developed world (Brotanek, Gosz et al. 2008, Bates, Lennox et al. 2014), due to accelerated growth at this developmental stage, and also possibly the switch from formula to cow's milk that has less iron compared with formula (Ziegler 2011, Ghisolfi, Fantino et al. 2013, McCarthy, Ni Chaoimh et al. 2016). In response, the food industry has developed "Young Child Formulae" (YCF), milk-derived products fortified with multiple micronutrients and other dietary factors, aimed at one-three year old children. YCF contain more iron compared with cow's milk, and are also fortified with ascorbic acid, a known enhancer of non-haem iron absorption.

There are numerous commercially available YCF throughout the developed world. In the United Kingdom (UK), according to the Diet and Nutrition Survey of Infants and Young children, 18 % of children between 12-18 months received YCF as part of their diet, representing a significant number of children consuming these products (Lennox, Ong et al. 2011). Furthermore, a cross-sectional study of one-two year old children conducted in France found that use of YCF was associated with improved dietary iron intake compared with children exclusively receiving cow's milk (Ghisolfi, Fantino et al. 2013); however, the specific nutrient composition of the YCF being consumed with reference to modifiers of iron absorption was not specified, nor were iron status markers measured in study subjects to assess whether the observed increase in iron intake correlated with good iron status.

In addition to added iron and ascorbic acid, several YCF products are supplemented with prebiotics. Prebiotics are non-digestible dietary substances, typically oligosaccharides such as fructo- and galacto- oligosaccharides (FOS and GOS, respectively) that encourage the growth of beneficial microorganisms in the large intestine (Gibson, Roberfroid 1995, Gibson, Probert et al. 2004). Prebiotics have also been shown to improve the bioavailability of minerals, although results with regard to iron are not consistent (Yeung, Glahn et al. 2005). The type and amount of added prebiotic varies between YCF, and some YCF do not have any added prebiotics (source of information: manufacturers' nutrition labels and websites); of note, The European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) Committee on Nutrition in a recent review found that there was no evidence of adverse effects from the addition of prebiotics to formula, however they also concluded that evidence was insufficient to recommend routine supplementation and that further research was needed (Braegger, Chmielewska et al. 2011). The European Commission regulates infant and follow-on formulae composition in the European Union and stipulates a maximum amount of prebiotics that may be added, but does not make more specific reference to recommended amounts (Commission Directive 2006/141/EC, reviewed and updated July 2016).

Although young children are at increased risk for ID/IDA, there is also concern that excess dietary iron may affect their gut microbiome with negative local and systemic health effects (Krebs, Domellof 2015). A study conducted in Kenya found that intake of iron-fortified foods led to a higher number of gut pathogens, increased intestinal inflammation, and more frequent diarrhoea episodes requiring treatment (Jaeggi, Kortman et al. 2015). In another study, consumption of iron-fortified biscuits was associated with an unfavorably altered gut microbiome (Zimmermann, Chassard et al. 2010. Unabsorbed iron reaching the colon is suggested to mediate these effects (Quinn 2014). Of note, however, a study in children from 9-18 months living in the UK, who continued to receive iron supplemented formula, found no evidence of adverse effects over the nine months of the study even in iron replete children (Singhal, Morley et al. 2000), thus the effects of iron on the young gut may vary depending on underlying risk for gut inflammation and infection. None the less, foods fortified with highly bioavailable iron

are desirable because of their efficacy in improving iron nutriture as a result of increased absorption. They may also, as a consequence of improved small bowel uptake, deliver less iron to the large intestine that may have long term health implications related to the gut microbiome.

The objective of this study was to compare the iron bioavailability of four YCF commonly consumed in the UK containing approximately equal amounts of iron, but with different ascorbic acid concentrations, and varying amounts and types of FOS and GOS. The aim of our research was to evaluate YCF iron bioavailability relative to supplemental prebiotic and ascorbic acid concentrations. We utilised the Caco-2 cell/in vitro digestion model with the formation of Caco-2 cell ferritin as a surrogate marker for cellular iron uptake. This model has been used in multiple studies to assess iron bioavailability from foodstuffs including milks, milk-derived products, and infant formulae, for examples see (Etcheverry, Wallingford et al. 2004, Viadel, Perales et al. 2007, Laparra, Tako et al. 2008). We hypothesised that the YCF with the highest concentrations of prebiotics and ascorbic acid would have the highest levels of bioavailable iron, while the YCF with no added prebiotics and lower levels of ascorbic acid would have the lowest iron availability.

6.2: Materials and methods

6.2.1: Reagents

All chemicals and enzymes, unless otherwise stated, were purchased from Sigma-Aldrich (UK). Acids required for glassware cleaning and in vitro digestions were acquired from VWR (UK). Thermo Fisher Scientific was the provider of cell culture media, flasks, tissue culture plates and cell culture reagents. Twenty-four hours prior to each experiment glass- and plastic ware needed for experiments were soaked in 10 % (v/v) trace metal grade 68 % nitric acid and then subsequently rinsed with 18 mΩ pure water.

GOS powders (100 % purity) used in experiments were either purchased from Megazyme International (Ireland), or kindly provided by King-Prebiotics. FOS powder (100 % purity) was purchased from Health Plus (Seaford, East Sussex).

6.2.2: Samples

Four YCF were obtained from three different leading UK supermarkets on three separate occasions, herein identified as YCF A, B, C and D. Products were tested before the use by date, and within one month of purchase. YCF were stored unopened at room temperature, similar to their distribution and market environment. YCF A and B were fortified with equivalent amounts of prebiotics in the forms of GOS and FOS by the manufacturer; YCF A specified the ratio of GOS to FOS, but neither YCF A or B provided nutrient label information on the specific forms of GOS and FOS used. YCF C was fortified with GOS alone by the manufacturer, and at a lower concentration compared with YCF A and B, and YCF D had no added prebiotics. YCF A, B and D had 1.2 mg iron/100 ml product; YCF C had 1.1 mg/100 ml. Ascorbic acid levels also

differed between YCF; YCF A and B had the highest reported levels, followed by YCF D. YCF C had the lowest reported concentration of vitamin C. The nutrient composition of the various YCF in relation to total iron, ascorbic acid and prebiotics, is provided below (Table 6.1)

Table 6.1: Nutrient composition of YCFs A-D according to the manufacturers' labels and websites.

Nutrient (mg/100 ml unless otherwise noted)	YCF Sample			
	A	B	C	D
Ascorbic Acid	15	15	10	12
Iron	1.2 ($FeSO_4$)	1.2 (Fe_2lactate)	1.1 ($FeSO_4$)	1.2 ($FeSO_4$)
FOS	120	Not stated	0	0
GOS	1080	Not stated	500	0
Total FOS & GOS	1200	1200	N/a	0
Fibre (g/100 ml)	0.8	0.8	0.5	0

6.2.3: Cell culture

The TC7 Caco-2 cell line was used for all experiments (kindly endowed to the Sharp laboratory by Monique Rousset and Edith Brot-Laroche, INSERM U505) (Caro, Rousset et al. 1995); work with this cell line investigating iron uptake and bioavailability has been published in numerous studies, for example (Christides, Sharp 2013, Christides, Wray et al. 2014, Johnson, Yamaji et al. 2005).

Details of the in vitro digestion method have been previously published (Christides, Sharp 2013, Christides, Wray et al. 2014, Glahn, Lee et al. 1998). Briefly, cells were grown in T75 flasks, and seeded into six-well tissue culture plates for experiments. According to the method developed by the Glahn laboratory (Glahn, Lee et al. 1998) the in vitro digestions were carried out 13–15 days post-seeding. The media used to feed cells was

Dulbecco's Modified Eagle Medium supplemented with 10 % v/v foetal bovine serum, 1 % penicillin–streptomycin, 4 mmol/L L-glutamine, and 1 % non-essential amino acids.

Media was changed to supplemented MEM (10 mmol/L PIPES [piperazine-N, N′-bis- (2-ethanesulfonic acid)], 1 % antibiotic/antimycotic solution, 11 μmol/L hydrocortisone, 0.87 μmol/L insulin, 0.02 μmol/L sodium selenite (Na_2SeO_3), 0.05 μmol/L triiodothyronine and 20 μg/L epidermal growth factor), without foetal bovine serum, to ensure satisfactory cell growth but with low basal media iron levels (Glahn, Lee et al. 1998, Jumarie, Malo 1991).

6.2.4: YCF and in vitro digestions

All experiments were carried out with freshly prepared reagents and freshly opened cartons of YCF; only liquid forms of YCF were tested in experiments. 1.8 - 2 ml of YCF sample were used per digestate depending on sample iron concentration.

All experiments contained a set of controls: a digest with no added iron to ensure no iron contamination of our system (Blank food digest); a reference digest of 30 μmol/L $FeCl_3$ (Fe alone) equivalent to the iron concentration in YCF digestates; and a positive control digest of 30 μmol/L $FeCl_3$ and 300 μmol/L ascorbic acid (Fe+AA at a 1:10 molar ratio).

The digests were prepared as previously described (Christides, Sharp 2013, Christides, Wray et al. 2014). Briefly, pepsin was added to the controls or YCF samples to initiate the gastric phase of the in vitro digestion (tested controls or samples herein referred to as digestates), and the pH adjusted to pH 2. As the literature suggests the stomach pH of infants and young children may be higher compared with adults (reaching a pH of 4) (Bowles, Keane et al. 2010), we also conducted a subset of experiments, with a limited number of YCF, in which digestions were initiated with a gastric phase pH of 4. During the gastric phase (at both pHs) digestates were placed in a shaking incubator for 75 minutes. Subsequently, the pH was raised to 5.0-6.0, bile salts and pancreatin digestive enzymes were added, and the pH was readjusted to 6.9-7.0 followed by another 75 minutes in the shaking incubator to mimic the small intestinal phase of digestion.

A chamber was created over each individual cell culture well containing the Caco-2 cell monolayer using a 15 kD molecular weight cut-off dialysis membrane fitted over a Transwell insert and held in place with a silicon ring. 1.5 mL of the digestate were then placed in the chamber that was in contact with the cell culture media of the well. The tissue culture plates were placed in a 37 °C incubator for 60 minutes. While in the incubator they were rotated using a platform-fitted multi-function 3D rotator set at 6 oscillations/minute. The inserts were then removed, and an additional 1 mL of supplemented MEM added to each well. The cells were returned to the incubator for the following 22 hours, and then harvested for ferritin.

All YCF samples were tested on a minimum of three separate occasions at gastric phase pH 2, with n = 6 for each treatment. Further in vitro digestions were carried out on one or more occasions with total n ≥ 6 (specified on figures and tables) for: gastric phase digestion at pH 4; YCF with exogenously added GOS and FOS so that total levels of prebiotics were equivalent across all YCF samples; YCF with exogenously added ascorbic acid so that levels of ascorbic acid were equivalent across all YCF samples; YCF with exogenously added GOS, FOS and ascorbic acid so that levels were equivalent across all YCF; and, heat-treated digestates.

6.2.5: Cell harvest and ferritin analysis

Cells were harvested as previously described (Christides, Sharp 2013, Christides, Wray et al. 2014). In brief, the monolayers of cells were rinsed, and then detached with cell lysis buffer (CelLyticTM – Sigma-Aldrich), and subsequently centrifuged at 6,000×g for 6 min; supernatant was separated and stored at −80 °C. The samples were analysed for ferritin using the SpectroFerritin MT Enzyme Linked Immunoassay (ELISA; RAMCO, TX, USA). Ferritin levels were adjusted for varying cell numbers/well by measurement of cell protein/well (Pierce Protein BCA assay) and expressed as ng ferritin/mg of cell protein.

6.2.6: Ascorbic acid analysis

All samples, and GOS and FOS used to fortify Samples C and D to levels equivalent with Samples A and B, were analysed for reduced ascorbic acid (herein referred to rAA) concentrations using the 2,6-Dichloroindophenol Titrimetric method (AOAC 985.33). Briefly, 5 ml of the YCF being analysed, or 5 grams of the GOS and FOS powders being tested (prebiotic powders were initially ground in a mortar and pestle with fine sand, then strained), were added to a mixture of glacial acetic acid and metaphosphoric acid, and titrated with a freshly prepared Indophenol standard solution. A rAA standard solution was freshly prepared and used to standardise the Indophenol titration solution. rAA levels were calculated based on the ascorbic acid equivalent mass titrated to 1 ml of Indophenol indicator solution (in relation to the rAA standard solution), adjusted for dilutions factors and the test solution blank titration level.

6.2.7: Heat treatment

To evaluate the contribution of ascorbic acid to iron uptake, selected YCF digestates, and the iron plus ascorbic acid positive control, were treated for 7.5 minutes in a 100 °C water bath, and then plunged into ice, in order to degrade the ascorbic acid (Davey, Inze et al. 2000).

6.3: Statistical analysis

Statistical analysis of the data was performed using Graph-Pad Prism (version 6.0c GraphPad Software, San Diego, CA, USA). In vitro digestion experiments were analysed using the statistical methods of Motulsky (Motulsky 2010). Data are presented as means ± SEM and were analysed by one-way ANOVA followed by either Tukey's multiple comparisons test (all-pairwise across experimental groups) or, where the comparison being made was only between select pairs, Sidak's multiple comparisons test. Differences between means were considered significant at $p \leq 0.05$. Ordered increases in data were tested using the non-parametric Jonckheere-Terpstra test. Ordered increases were considered significant at $p \leq 0.05$. The Jonckheer-Terpstra test was computed in Microsoft Excel. Unless stated otherwise, in the results section the word "significantly" is used to denote statistical significance as indicated by the relevant p shown in parenthesis at the end of the statement.

6.4: Results

6.4.1: Iron Bioavailability of Tested YCF at gastric phase pH 2

Data on iron bioavailability of YCF A-D from the in vitro digestion/Caco-2 cell model are presented in Figure 6.1.

YCF A and B, which had the highest levels of prebiotics and ascorbic acid, had the highest iron bioavailability as measured by ferritin formation. YCF A ferritin levels were 43 %, and 62 % greater than YCF C and D, respectively ($p<0.0001$). YCF B ferritin levels were 25 %, greater than YCF C, although this was not statistically significant ($p=0.1007$), and 41 % greater than YCF D ($p=0.0113$). YCF C and D did not have statistically different ferritin concentrations

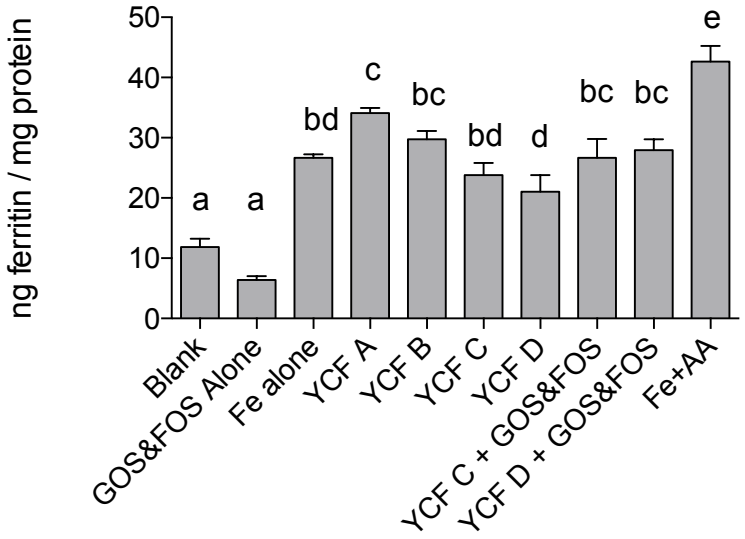

Figure 6.1 Caco-2 ferritin levels from treatment of cells with digestates of: controls; YCF A-D; and YCF C and D supplemented with GOS & FOS to levels equivalent to those found in YCF A and B.

Values presented are means of data normalized to the Fe reference control (Fe alone) ± SEM ($n\geq18$ for YCF alone, all others $n\geq6$). Treatment with YCF A yielded the highest ferritin compared with all other YCF although not statistically so when compared to YCF B. The addition of GOS & FOS to YCF C and D increased ferritin to levels statistically equivalent to YCF A and B. Based on an ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis; means with different superscript letters in a column are statistically different ($p\leq0.05$).

6.4.2: Iron Bioavailability of Tested YCF at gastric pH 4

YCF A, B and D (representing the YCF with the highest and lowest bioavailability, respectively) were tested using a gastric phase pH of 4 (Table 6.2). Changing the pH to 4 did not alter iron bioavailability for any of the tested YCF.

Table 6.2 Ferritin values of Caco-2 cells treated with YCF A, B and D at gastric phase digestions of pH 2 compared with pH4.

YCF Sample	pH2 ng ferritin/mg protein	pH4 ng ferritin/mg protein	p-value
YCF A	34.1 ± 0.9	29.9 ± 1.1	0.4239
YCF B	29.7 ± 1.4	30.1 ± 2.5	0.9990
YCF D	21.1 ± 2.8	17.1 ± 3.2	0.5407

Values presented are the mean +/- SEM. There was no statistical difference between pH2 and pH4 gastric phase digestions for any of the three samples using Sidak's multiple comparisons test ($n \geq 6$).

6.4.3: Iron Bioavailability of YCF to which exogenous GOS & FOS were added

Data on iron bioavailability for these experiments presented in Figure 6.1.

Caco-2 cells ferritin concentration as a marker for iron uptake (herein referred to as "Ferritin") in YCF C and D were significantly lower than those in YCF A. Ferritin levels of YCF C were 10.25 ng ferritin/mg protein lower than YCF A (30 % lower; $p<0.0001$), and ferritin levels of YCF D were 13.01 ng ferritin/mg protein lower than YCF A (38 % lower; $p<0.0001$). Whilst not statistically significant, ferritin levels of YCF C were also 20 % lower than YCF B ferritin levels ($p=0.1007$). YCF D was significantly lower than YCF B by 29 % ($p=0.0113$). YCF C originally contained 0.5 grams GOS/100ml milk, whereas YCF D originally contained no supplemental prebiotics. After the addition of a mixture of GOS and FOS to both YCF C and D, so that total prebiotic levels were equivalent to those found in YCF A and B, there was no longer a statistically significant difference between the ferritin levels in any of the four tested YCF; although YCF C and D ferritin levels were still 7.42 and 6.15 ng ferritin/mg protein lower, (by 22 % and 18 %), respectively, compared with YCF A.

6.4.4: Iron Bioavailability of YCF relative to ascorbic acid with and without heat treatment

YCF A and B had equal amounts ascorbic acid according to stated information on the manufacturer's label (15 mg ascorbic acid/100 ml milk); measured levels were 14.2 and 16.3 mg rAA/100 ml milk, respectively. Ascorbic acid levels in YCF C were reported as

10 mg/100 ml milk (the lowest of all tested YCF) and measured as 10.2 mg/100ml; YCF D rAA levels were reported as 12 mg/100 ml and measured as 10.1 mg/100ml.

The reported ascorbic acid:iron molar ratios were 4:1 for YCF A and B, and 3:1 for YCF C and D, respectively. Ascorbic acid:iron molar ratios based on measured rAA were: 3.75:1; 4.3:1; 3:1; and 2.7:1; for YCF A, B, C and D, respectively.

In order to ensure that our heat treatment inactivated the ascorbic acid present in YCF samples, we tested whether heat-treating our positive control (Fe+AA) affected ferritin formation. Heat treatment of the positive control significantly decreased ferritin levels compared with the non-heat treated positive control ($p=0.0002$); levels were equivalent to those seen in our iron alone controls (i.e. iron added to digestates without any added ascorbic acid; $p=0.2227$; Figure 6.2A). Heat treatment of YCF A significantly decreased ferritin levels by approximately 24 % (from 53.42 to 40.83 ng ferritin/mg protein; $p=0.0074$), however YCF A ferritin concentration, even after heat treatment, was still significantly higher by 13.52 ng ferritin/mg protein compared with YCF C levels (the sample with the lowest reported and measured AA levels; $p=0.0082$). Lastly, we added exogenous ascorbic acid to YCF C to levels equivalent to those found in YCF A; ferritin levels increased by 5.90 ng ferritin/mg protein, or approximately 22 %, but were still 20.20 ng ferritin/mg protein less (38 %) than those measured in cells treated with YCF A ($p=0.0002$; Figure 6.2B).

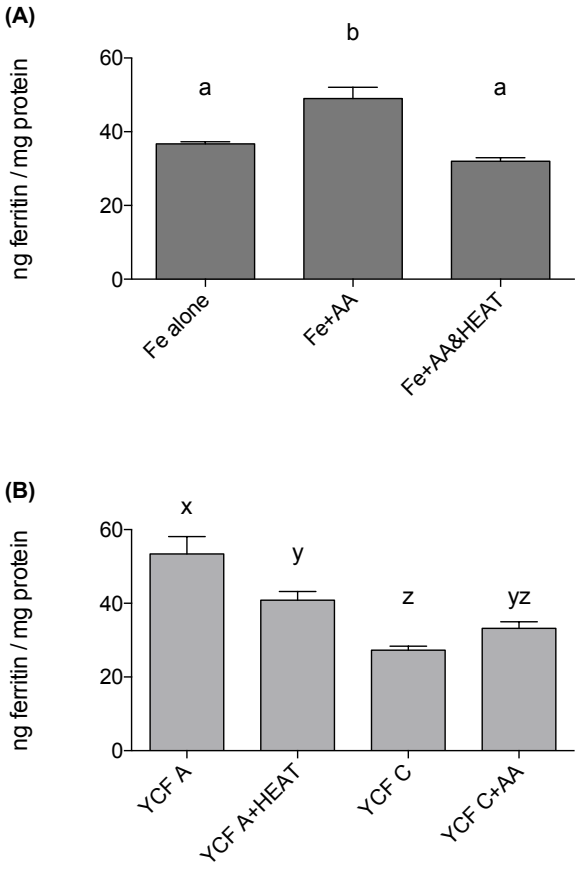

Figure 6.2 Ferritin levels of Caco-2 cells exposed to control digests, both with, and without, heat treatment to degrade ascorbic acid (AA).

Figure 6.2(A) Ferritin levels of Caco-2 cells exposed to control digests, both with, and without, heat treatment to degrade ascorbic acid (AA). Heat treatment of the positive control (Fe+AA at a 1:10 Fe:AA molar ratio) decreased the enhancing effect of AA on iron uptake; ferritin formation was equivalent between the Fe alone and Fe+AA controls. Based on an ANOVA ($p=0.0003$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis; means with different superscript letters in a column are statistically different ($p\leq0.0015$).

Figure 6.2(B) Caco-2 ferritin levels of cells treated with YCF A digestates, with, and without, heat treatment, and YCF C digestates, with, and without, added AA (≈1:4 Fe:AA molar ratio). Ferritin levels of YCF A decreased after heat-treatment; ferritin levels of YCF C increased after the addition of AA to levels equivalent with YCF A, however ferritin was still less than non-heat treated YCF A levels. All presented values are means normalized to the Fe reference control (Fe alone) ± SEM. Based on an ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis; means with different superscript letters in a column are statistically different ($p\leq0.0074$).

6.4.5: Iron bioavailability of YCF C and D with the addition of exogenous GOS&FOS, with, or without, added ascorbic acid

In order to test whether prebiotics and ascorbic acid had an additive effect on iron bioavailability, we measured ferritin levels of digestates of YCF C and D with added GOS&FOS alone, compared with digestates of YCF C and D with added GOS&FOS

and ascorbic acid. There was an ordered increase in ferritin formation in the order of digestates of YCF C alone, YCF C and GOS&FOS, and YCF C, GOS&FOS and ascorbic acid (p=0.0048; Figure 3A); similarly, there was an ordered increase in ferritin formation in the order of digestates of YCF D alone, YCF D and GOS&FOS, and YCF D, GOS&FOS and ascorbic acid (p=0.0001; Figure 6.3B).

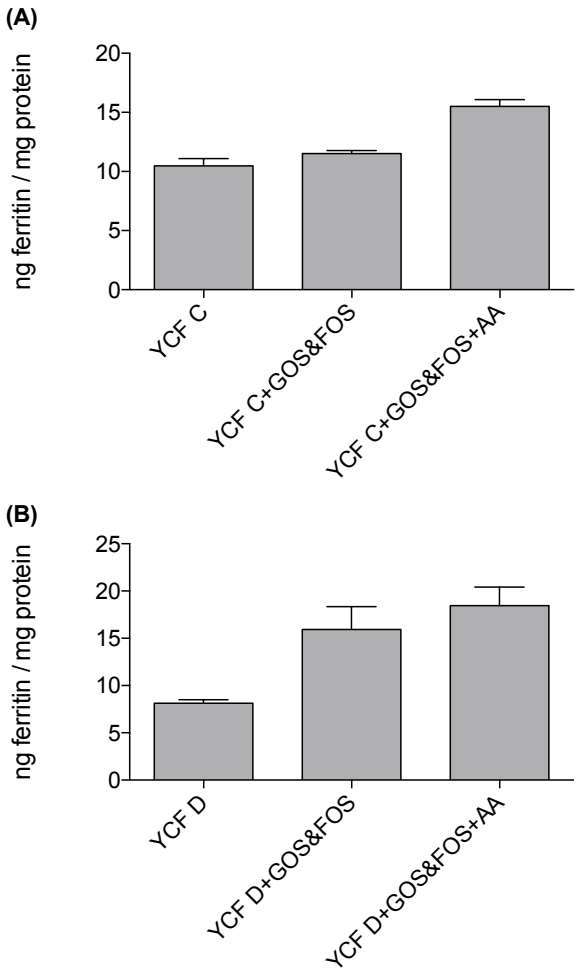

Figure 6.3(A) Ferritin levels of Caco-2 cells treated with digests of: YCF C alone; YCF C + GOS&FOS; and YCF C, GOS & FOS, + ascorbic acid (AA, 114 µmol/L; ≈ 1:4 Fe:AA molar ratio). Increase in ferritin formation was tested using the Jonckheere-Terpstra test and is significant in the order YCF C alone, YCF C + GOS & FOS, and YCF C, GOS & FOS, + AA (p=0.0048).

Figure 6.3(B) Ferritin levels of Caco-2 cells treated with digests of: YCF D alone; YCF D + GOS&FOS; and YCF D, GOS & FOS, + AA (114 µmol/L; ≈ 1:4 Fe:AA molar ratio). Increase in ferritin formation was tested using the Jonckheere-Terpstra test and is significant in the order YCF D alone, YCF D + GOS & FOS, and YCF D, GOS & FOS, & AA (p=0.0001).

6.5: Discussion

YCF A and B, with the highest levels of prebiotics and ascorbic acid, had the highest iron bioavailability as measured by Caco-2 cell ferritin formation. YCF A ferritin levels were approximately 1.5 times greater than those of YCF C and D, while YCF B ferritin levels were approximately 1.3 times greater than those of YCF C and D. The addition of prebiotics (a mixture of GOS and FOS) and ascorbic acid, to YCF C and D increased iron uptake by approximately 1.5 and 2.3 times, respectively, but neither exogenously added ascorbic acid alone, nor prebiotics alone, elevated ferritin levels to those seen in YCF A, suggesting both factors contributed to the greater iron bioavailability of YCF A.

Previous reports in the literature found that an ascorbic acid to iron molar ration of 4:1 is associated with higher iron bioavailability in high phytate food products (Allen, de Benoist et al. 2006); consistent with this in our study YCF A and B, which had the most bioavailable iron, also had both higher absolute amounts of ascorbic acid, and higher ascorbic acid to iron molar ratios compared with the other tested YCF.

Relative to prebiotics, the results of our study are consistent with numerous studies demonstrating prebiotics enhance iron absorption in rodents and pigs (Lobo, Gaievski, et al. 2014, Freitas, Amancio et al. 2012, Wang, Zeng et al. 2010, Pérez-Conesa, López et al. 2007, Yasuda, Roneker et al. 2006). Potential mechanisms driving this increase included: enhanced solubility of iron (Yasuda, Roneker et al. 2006); a decrease in colonic pH (Yasuda, Dawson et al. 2009, Laparra, Glahn et al. 2009); changes in the profile of the gut microbiota (Patterson, Yasuda et al. 2010); a direct effect on iron transporter expression either in duodenum, or colon (Lobo, Gaievski et al. 2014, Tako, Glahn et al. 2008, Marciano, Santamarina et al. 2015); or a systemic decrease in inflammation leading to decreased production of hepcidin, a peptide secreted by the liver in response to inflammation that decreases iron absorption (Yasuda, Dawson et al. 2009). However, it is not clear whether any of these mechanisms would be relevant in our short-term in vitro studies.

Results from studies evaluating the effects of prebiotics on iron availability carried out in human beings, and also in vitro with the Caco-2 cell line, have been mixed. A recent study in women with low iron status who were given approximately 20 g/d of the FOS inulin, found that although inulin did modify the large bowel microbiome and also lowered colonic pH, there was no influence on iron absorption as measured by the stable isotope technique; iron absorption in the inulin group was 14 % higher but this was not statistically significant (Petry, Egli et al. 2012). Another study carried out in young men using the same method to assess iron absorption also found no statistically significant effect of inulin, FOS, or GOS on iron absorption, though absorption was some 20 % higher in the FOS group compared with control (van den Heuvel, Schaafsma et al. 1998). Both of these studies were relatively small (n=32, n=15, respectively), and therefore may have been underpowered to detect small, but physiologically relevant, increases in iron

uptake. Absorption of iron is primarily determined by host iron status, and differences in iron absorption from foods containing less potent enhancers of absorption (e.g. prebiotics) may not be revealed unless some degree of iron deficiency is present, or absolute amounts of bioavailable dietary iron are too low to meet needs. Our results are consistent with a study carried out in infants and young children that demonstrated improved iron status amongst children receiving a mixture of prebiotic oligo-saccharides (type not specified) and probiotics (Sazawal, Dhingra et al. 2010). A possible reason for these different study results is that inulin may not be the optimal FOS for increasing iron absorption. Shorter chain FOS more closely resemble fructose (Schaafsma, Slavin 2015), which we have shown increases non-haem iron bioavailability in the Caco-2 cell model (Christides, Sharp 2013). Our previous work demonstrated that fructose added to a ferric iron solution increased levels of ferrous iron by approximately 300 % (Christides, Sharp 2013), suggesting that the observed increase in iron bioavailability was caused by fructose reducing ferric iron to the more bioavailable ferrous iron. Shorter chain FOS may be better at improving iron bioavailability compared with longer chain FOS-inulin if they share some degree of this reducing effect, or secondary to other structural-related differences. In support of this, recent work using GOS suggests that specific structural differences in prebiotics alter their influence on iron absorption (Laparra, Diez-Municio et al. 2014). Lastly, human studies evaluating the effects of GOS and FOS given together on iron bioavailability have not, to the authors' knowledge, been conducted; the two prebiotics together may act synergistically.

Several in vitro studies evaluating the effect of prebiotics on iron uptake have been carried out in the past decade. Two Caco-2 cell-based studies evaluating iron bioavailability from milk- or soy-based yogurts, and common beans, both found that inulin did not improve iron bioavailability from the test food matrix (Laparra, Tako et al. 2008, Laparra, Glahn et al. 2009). In contrast, and in agreement with our findings, another in vitro study using iron-fortified cereal biscuits, to which inulin had been added, demonstrated improved iron bioavailability that correlated with increased iron solubility (Vitali, Cetina-Čižmek et al. 2011). Both the yogurt and bean based studies used a higher dose of inulin compared with our study (approximately 17 mg vs. 2.4 mg per digestate), and did not add any GOS; the amount of prebiotics applied to Caco-2 cells in the biscuit study was not specified. In conclusion, the positive results in our present study may relate to several factors: different dose of FOS; different forms of FOS and GOS; and the addition of GOS and FOS together to the tested food matrix.

Young children have the highest risk of ID/IDA amongst pre-adolescent children. The ESPGHAN Committee on Nutrition recommends young children receive a diet rich with iron-containing foods and that excess intake of iron-poor cow's milk be avoided (Domellof, Braegger et al. 2014). However, ID/IDA persists in young children, and thus YCF fortified with iron may be beneficial, as part of a varied diet, in the prevention of ID/IDA. In addition, fortified milk products with the most bioavailable iron would also be predicted to deliver less unabsorbed iron to the young colon. The findings of this study

suggest that both prebiotics and ascorbic acid contribute to improved iron bioavailability in YCF. However, ascorbic acid is a relatively unstable micronutrient, and levels may fluctuate depending on storage conditions and timing of use from purchase date (Davey, Inzé et al. 2000), whereas prebiotics are relatively stable in foods (Klewicki 2007, Vega, Zuniga-Hansen 2015). Thus the use of both dietary factors to improve iron bioavailability in fortified milks may be complementary.

Several observational studies in Europe (Ghisolfi, Fantino et al. 2013, Walton, Flynn 2015) found that intake of YCF was associated with increased dietary intake of iron in young children. A recent cross-sectional study carried out in Western Europe amongst healthy young children found that 11.9 % and 3.9 % of tested children had ID, and IDA respectively, and use of cow's milk as the main milk source was associated with an increased risk of ID (Akkermans, van der Horst-Graat et al. 2016). The prevalence of ID in children primarily receiving cow's milk was 19.7 % in contrast with children whose main source of milk was YCF (ID prevalence 5.4%); this difference was highly significant ($p<0.001$)(Akkermans, van der Horst-Graat et al. 2016). The specific YCF used in this study were not specified, nor were study participants' intakes of ascorbic acid or prebiotics.

There are several limitations to our study. Although the Caco-2 cell system is a validated model for assessing iron bioavailability including in the food matrixes we tested, the magnitude of effects may not always be accurate (Fairweather-Tait, Lynch et al. 2005). It thus remains for our results to be confirmed in vivo. In addition, we did not analyse the specific forms of GOS and FOS used in this study, and there is evidence that there is structural specificity in terms of the effects of prebiotics on iron bioavailability. Studies looking at specific prebiotic structure and its relation to iron availability would be helpful. Furthermore, although the European Commission regulates the absolute amounts both of prebiotics (and the ratio of GOS to FOS) that may be added to YCF, dose-response analysis of prebiotic effects on iron bioavailability would enable producers to optimise formulation.

6.6: Conclusion

In conclusion, use of YCF may contribute significantly to iron intake in young children and thus potentially reduce ID/IDA; however, in the case of iron, the absolute amount ingested does not necessarily reflect the fraction absorbed. Therefore, ensuring that iron in YCF is delivered in a bioavailable form, whilst minimising the delivery of excess iron to the young gut, would improve the nutritional benefits of YCF. Our results suggest that ascorbic acid and prebiotics in YCF improve iron bioavailability. Further in vivo work to confirm our results, and also to explore whether absolute amounts of iron fortification in YCF could be decreased through optimisation of concentration and forms of prebiotics and ascorbic acid warrants investigation.

6.7: Author contributions

TC and JCG designed and carried out the in vitro digestion/Caco-2 studies. TC and PAS analysed the data. TC and JCG wrote the first draft of the manuscript; TC and PAS oversaw and edited all other draft versions. All authors read and approved the final version submitted for peer-review and publication.

Chapter Seven: Iron-fructose effects on gene and protein expression in Caco-2 and HepG2 cells

This chapter presents work and evidence exploring biological mechanisms underlying the observed effects of fructose on iron bioavailability in Caco-2 and HepG2 cells, and evaluation of the effects of iron and fructose treatments on Caco-2 and HepG2 cell gene and protein expression related to the pathogenesis of colorectal cancer (CRC), hepatocellular carcinoma (HCC) and non-alcoholic fatty liver disease (NAFLD).

7.1: Introduction

Iron is essential for multiple physiological functions, but excess iron is toxic to cells. In the two in vitro cell line models used in this thesis it was found that fructose increases non-haem iron bioavailability as measured by ferritin formation (chapter three, see also (Christides and Sharp 2013). The mechanism underlying this increase is not definitively known, nor if there are pathophysiological consequences to the iron and fructose treatments, and observed increased ferritin levels in these cell lines, and therefore potentially in vivo.

Biochemical evaluation of the effect of fructose on inorganic iron in the aforementioned experiments suggested the observed increase in iron uptake related to fructose-mediated reduction of ferric to ferrous iron; ferrous iron is more bioavailable compared with ferric iron (Miret, Simpson et al. 2003). However, work by others in in vitro and animal models, examining mechanisms by which different nutrients affect iron bioavailability, has found that dietary factors may also alter iron bioavailability by increasing expression of intestinal iron transporter and homeostasis proteins (Tako, Glahn et al. 2008, Thomas, Gaffney-Stomberg et al. 2013). Indeed, iron uptake and absorption would be expected to be improved by increased expression of the iron uptake protein Divalent Metal Transporter 1 (DMT1), the ferri-reductase DcytB, and in the gut the iron export protein ferroportin. Intracellular iron levels, and therefore ferritin, would also be expected to increase with increased expression of transferrin receptor 1 (TfR1).

The objectives of this current study were to measure the expression of selected iron-related molecules in response to iron and fructose treatments, in order to evaluate whether fructose improves iron bioavailability by altering levels of iron-transport and homeostasis proteins. Specifically, TfR1 mRNA and protein expression were measured, and the protein expression of DMT1, DcytB, ferroportin, and Iron Responsive Protein 2 (IRP2) was measured. At this time HepG2 DMT1, DcytB, ferroportin and IRP2 mRNA expression levels were not evaluated.

High intake of iron and fructose have been associated with development and progression of colorectal cancer (CRC) (Michaud, Fuchs et al. 2005, Chua, Klopcic et al. 2010)

hepatocellular carcinoma (HCC) (Pietrangelo 2009, Kew 2014), and non-alcoholic fatty liver disease (NAFLD) (Mouzaki and Allard 2012). Therefore, the effects of iron, fructose, and combined iron and fructose, on the expression of selected genes and proteins central to disease pathways of these disorders was also evaluated. Caco-2 cells, an in vitro cell line derived from a colonic adenocarcinoma, were used as a model of CRC, and HepG2 cells, an in vitro cell line derived from a hepatoma, as a model of HCC.

Initial screening for iron and fructose effects on HepG2 gene expression was carried out using Affymetrix microarray-based gene expression profiling, followed by quantitative real-time reverse transcriptase-Polymerase Chain Reaction (rt-PCR) of selected genes of interest.

Literature review was also used to identify important pathways and proteins in the development and progression of CRC, HCC and NAFLD; the effect of fructose and iron on expression of selected genes and proteins in these pathways was then explored.

The P13K-Akt-mTOR and Wnt-β-catenin pathways play a pivotal role in disease pathogenesis of both HCC and CRC (Lee, Kim et al. 2006, Schneikert and Behrens 2007, Villanueva, Chiang et al. 2008, Ekstrand, Jonsson et al. 2010, Wang and Zhang 2014). Furthermore, expression levels of the tumour suppressor proteins Adenomatous Polyposis Coli (APC) and Tumour Protein p53 (p53) in turn modulate these pathways. In addition, Heat Shock Protein 90 (HSP90) noted as the "Cancer Chaperone" (Neckers 2007) also interacts with several key proteins in the above disease pathways, and can therefore affect disease development and progression. For example, HSP90 transcriptionally decreases expression of p53 in HCC, and alterations in this tumour suppressor gene have been found in iron-associated HCC (reviewed in (Tirnitz-Parker, Glanfield et al. 2013) Akt (also known as Protein Kinase B), a protein kinase over-activated in many many cancers including HCC (Whittaker, Marais et al. 2010), is also a HSP90 client protein. Therefore, the effects of fructose and iron on expression levels of APC, beta-catenin, and p53, were measured by quantitative real-time rt-PCR because of the centrality of these genes to development of CRC and HCC as identified in the research literature. In addition, assessment of expression levels at the gene and protein level of HSP90 was also carried out.

NAFLD is a metabolic disorder characterized by increased triglycerides in hepatocytes with associated insulin resistance. It covers a spectrum of liver findings, ranging from simple hepatosteatosis, to nonalcoholic inflammatory steatohepatitis (NASH), and in approximately 5 % of cases cirrhosis (Adams, Lymp et al. 2005). Research suggests that multiple factors act synergistically in the development and progression of NAFLD in genetically predisposed individuals; fructose, and iron are both postulated to be involved in NAFLD pathogenesis (Lim, Mietus-Snyder et al. 2010, Dongiovanni, Fracanzani et al. 2011) Specifically, the Transforming Growth Factor Beta (TGF-β) and Nuclear Factor- κ Beta (NF-κβ) pathways have been implicated in NAFLD disease pathogenesis, therefore expression of selected genes in these pathways was evaluated.

It was hypothesised that the increase in ferritin formation seen in both Caco-2 and HepG2 cells treated with iron and fructose together, compared with iron alone, would not be as a result of increased iron-uptake proteins. It was also hypothesised that fructose, iron, and fructose and iron together, would increase expression of genes and proteins implicated in disease pathogenesis in CRC, HCC and NAFLD.

7.2: Materials and methods

Chemicals and reagents: all products were purchased from Thermo Fisher Scientific UK and Sigma-Aldrich UK unless otherwise noted. Prior to use, glassware was soaked for 24 hours in 65 % trace metal grade nitric acid, and rinsed with 18 mΩ water. RNAse free products were used for all cell culture RNA work.

7.2.1: Cell Culture

HepG2 and Caco-2 cells were grown and cultured as previously described (Christides and Sharp 2013).

For experiments, HepG2 cells were grown in either 6-well or 12-well plates at 1 x 10^5 cells/cm^2 and used 24- 48 hours after seeding unless noted otherwise. Caco-2 cells were grown in 6-well plates at 1 x 10^4 cells/cm^2; experiments investigating expression of iron uptake proteins in response to iron and fructose were conducted 14-16 days after seeding to correlate with the days in which in vitro digestions had been conducted in previously cited studies. For experiments looking at gene expression of proteins related to CRC, experiments were conducted on Caco-2 cells at 19-21 days to ensure complete differentiation and full protein expression.

7.2.2: Cell Treatments and RNA Extraction

7.2.2.1: HepG2 cells

HepG2 cell media was changed to Foetal Bovine Serum (FBS)-free Minimal Essential Medium (MEM) supplemented with 1 % antibiotic/antimycotic solution, 11 μmol/L hydrocortisone, 0.87 μmol/L insulin, 0.02 μmol/L sodium selenate (Na_2SeO_3), 0.05 μmol/L triiodothyronine and 20 μg/L epidermal growth factor (supplemented MEM as in previous experiments but without PIPES buffer) 24 hours prior to initiation of treatments with iron and fructose for microarray gene expression profiling studies, and initial quantitative real-time rt-PCR experiments (these treatments are labeled as "with wash-out"). MEM was used because it has low levels of glucose (5 mmol/L) and no other carbohydrates, and therefore the effects of fructose would not be obscured by the presence of high levels of other sugars. After 24 hours of treatment cells were harvested for RNA.

On the day of the experiment cells were treated with one of the following (made up in supplemented MEM): 1 μmol/L ferric ammonium citrate (**FAC**; low dose iron, labelled as **FAC** or **Fe1** depending on the exact protocal); 15 mmol/L fructose (**Fruc**); 1 μmol/L

FAC plus 15 mmol/L fructose (labelled as **FF** or **FF1** depending on the exact protocal); 10 μmol/L FAC (**Fe10**; high dose iron); or 10 μmol/L FAC plus 15 mmol/L fructose (**FF10**).

HepG2 cell treatments for second phase quantitative real-time rt-PCR experiments were as described above, with the adaptation that cells were treated without the 24 hour pre-experimental change to FBS-free and low glucose MEM (these treatments are labeled as "no wash-out").

Caco-2 cells were treated with the following solutions: 30 μmol/L iron ($FeCl_3$, **Fe30**; low dose iron) or 30 μmol/L $FeCl_3$ in combination with 66 mmol/L fructose (as used in digest experiments; **FF30**), or 100 μmol/L $FeCl_3$ (**Fe100**; high dose iron), or 100 μmol/L $FeCl_3$ in combination with 66 mmol/L fructose (**FF100**). Caco-2 cell media was changed to supplemented MEM (minus PIPES buffer) 24 hours prior to the start of experiments as in the established in vitro digestion protocol.

RNA was extracted using the PureLink™ RNA Mini Kit in combination with Trizol (Ambion/Life Technologies). Media was removed from wells and 1 ml Trizol was added to each well. Cells were homogenized in Trizol; the homogenate was then separated into an aqueous phase with the addition of 0.2 ml chloroform followed by centrifugation. The aqueous phase containing the RNA was aspirated and washed as per the kit instructions, and then the extracted RNA was treated with On-Column PureLink DNAse (Ambion/Life Technologies) to ensure DNA-free RNA for microarray analysis and quantitative real-time PCR. RNA was stored in RNAse free microcentrifuge tubes at -80°C until needed for analysis.

RNA concentration and quality was checked by NanoDrop spectrophotometer ND-1000 (NanoDrop) followed by Agilent Technologies (AT) Bioanalyzer. All RNA samples used for quantitative real-time rt-PCR had 260/280 and 260/230 absorbance ratios greater than 2.0 indicating high purity RNA without evidence of protein or phenol contamination. The RNA integrity number as checked by AT Bioanalyzer for all samples was ≥ 8.5.

For microarray analysis HepG2 treatments (with wash-out) were carried out over 4 different cell passages; samples were then pooled together so that one microarray chip was treated with a sample that represented a mix of the same treatment, but carried out over four different times. Initial quantitative real-time rt-PCR was carried out with the same pooled RNA samples used for microarray analysis. Second phase PCR analysis did not use pooled samples; rather individual samples from independent experiments were analysed for mRNA expression (n=3).

The King's College Genomics Centre performed the preparation of cDNA from RNA for microarray application, and carried out the microarray expression study. The Affymetrix GeneChip® probe array HumanGenome U133A 2.0 was used for gene expression analysis; this is a single array genechip representing 14,500 well-characterized human genes.

7.2.3: Microarray Gene Data Analysis

Gene expression was analysed using bioinformatics analysis software. Data was initially transformed from intensity to expression level by Genechip® Operating software (GCOS; Affymetrix, USA), and normalized against the median expression level of each microarray chip. Expression values were then compared between two arrays whose values had been normalized. Genes were analysed both for degree of expression change and actual levels of expression (related to signal strength). Genes that showed statistically significant changes of ≥ 1.5 fold increased or decreased expression from one treatment compared with another, were imported into Genego Metacore Software bioinformatics (GO) for network and pathway analysis. The treatments analysed by microarray chip results were fructose (15 mmol/L) treatment versus MEM (Fruc), 1 μmol/L ferric ammonia citrate (FAC) versus MEM (FAC), and fructose (15 mmol/L) and FAC (1 μmol/L) together (FF) versus MEM. In addition, the genes that changed uniquely to Fruc, FAC, and FF, were entered into GO.

Microarray analysis with Caco-2 cells was not conducted.

7.2.4: Initial quantitative real-time rt-PCR analysis of HepG2 gene expression

Genes were selected for verification of altered expression by quantitative real-time rt-PCR based on significant GO enrichment analysis, and involvement in relevant process networks related to CRC, HCC and NAFLD. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Ribosomal Protein 27S were chosen as reference genes after analysis for least changed microarray gene expression conducted by King's College Genomic Centre. Initial quantitative real-time rt-PCR was performed at King's College Genomics Centre; the centre provided details of the process.

Forward and reverse primers (Table 7.1) and the appropriate probes were selected using the Roche PCR Assay Design Center, and purchased from Roche, UK.

Single strand complementary DNA (cDNA) was synthesized from RNA aliquots. The cDNA was mixed with the appropriate primers and probes, and amplification carried out on the ABI Prism 7000 sequence detection system thermo cycler (Applied Biosystems). The linearity of dissociation curves was analyzed using ABI7000 SDS software of standard dilution curves for each analysed gene. Samples were run in quadruplicate. Assay efficacy was checked by running standard curves, with a minimum of four 10-fold dilution points for each gene. Standard curve efficiencies were 96 –100 %, and the correlation for all standard curves was > 0.980 indicating high amplification efficiency and comparability between assays.

The threshold cycle (Ct, the reading at which signal exceeds back ground noise) was measured for each analysed gene. The comparative Ct method -- $2^{-(\Delta\Delta CT)}$ -- was used to quantify gene expression relative to the two selected reference genes (Schmittgen and Livak 2008). In the comparative Ct method the gene of interest is normalized to one or

more reference gene(s) (in this case to GADPH and Ribosomal Protein 27S) signals, and then the gene signal of the control samples (i.e. no treatment) is subtracted from the Ct of the gene of interest. Data was generated using ABI Prism software.

Table 7.1 Forward and reverse primer sequences used for initial quantitative real-time rt-PCR

Gene symbol	Forward Primer	Reverse Primer
ACTB	5′ ATT GGC AAT GAG CGG TTC 3′	5′ CGT GGA TGC CAC AGG ACT 3′
GAPDH	5′ AGC CAC ATC GCT CAG ACA C 3′	5′ AAT ACG ACC AAA TCC GTT GAC T 3′
HNF4A	5′ AGC AAC GGA CAG ATG TGT GA 3′	5′ TCA GAC CCT GAG CCA CCT 3′
HSP90	5′ GGC TAT CCC ATC ACC CTT TAT T 3′	5′ TCT TCC TCT TCT TTC TCA CCT TTC 3′
NFKB1	5′ CTG GCA GCT CTT CTC AAA GC 3′	5′ TCC AGG TCA TAG AGA GGC TCA 3′
RPS27A	5′ CCT GAT CAG CAG AGA CTG ATC TT 3′	5′ TTT TCT TAG CAC CAC CAC GA 3′
SMAD2	5′ GGC CTT TAC AGC TTC TCT GAA C 3′	5′ ATG TGG CAA TCC TTT TCG AT 3′
SMAD3	5′ CAC CAC GCA GAA CGT CAA 3′	5′ GAT GGG ACA CCT GCA ACC 3′
STAT3	5′ CCC TTG GAT TGA GAG TCA AGA 3′	5′ AAG CGG CTA TAC TGC TGG TC 3′

7.2.4.1: Second phase quantitative real-time rt-PCR

After the initial evaluation of HepG2 cell gene expression, further quantitative real-time rt-PCR experiments were carried out at the University of Greenwich. The 24 hour pre-experimental wash-out period prior to treatment of HepG2 cells, characterized by a total of 48 hours of energy depletion in control samples (MEM alone), was felt to possibly have added a metabolic stress to HepG2 cells and confounded results. HepG2 cells, in comparison with Caco-2 cells, are more rapidly growing cells with high metabolic demands (as discussed in chapter two of this thesis they have a doubling time of approximately 24 hours in comparison with 48 hours in Caco-2 cells), and energy and FBS deprivation are documented to increase expression of reactive oxygen species and apoptosis within 48-72 hours (Zhuge and Cederbaum 2006).

In order to explore whether observed changes in mRNA expression related to experimentally induced energy/FBS deprivation, and thus guide the methods used in further PCR experiments, a limited number of second phase PCR experiments were initially carried out in which the mRNA expression levels of NF-κβ in HepG2 cells both with, and without, the extended 48 hour energy/FBS depletion were measured. Control cells were "treated" with MEM both with (M+), and without a 48 hour wash-out period (M), and levels of NF-κβ mRNA were quantified. Comparison of mRNA levels in M versus M+ cells demonstrated that NF-κβ RNA levels were indeed significantly increased 1.4 fold in controls cells that had had the wash-out period compared with mRNA from cells with no wash-out (Figure 7.12). Therefore, further quantitative real-time rt-PCR

analysis in HepG2 cells was carried out without the 24 hour pre-experimental wash-out period. These experiments were categorised as no wash-out.

Analysis of Caco-2 cells after treatment with iron and fructose, for TfR1 expression, as well as measurement of the expression of selected genes relevant to CRC, was also carried out in the second phase set of PCR experiments.

TaqMan® hydrolysis probes were used for gene expression analysis with FAM (6-carboxyfluorescein) as the fluorescent reporter dye, and a nonfluorescent quencher. The reaction mixture was prepared using ThermoFisher Scientific proprietary Taqman Master Mix (containing AmpliTaq Gold® DNA Polymerase, nucleotide triphosphates, ROX reference dye, and buffer) combined with hydrolysis probes and reverse-transcriptase (MultiScribe™ Reverse Transcriptase) using the one-step method in which the reverse transcription (rt) and cDNA amplification are conducted in the same well. Components used in the second phase PCR analysis are shown in Table 7.2. Hydrolysis probes were selected on the basis of "Best coverage for standard gene expression" as recommended by the manufacturer (ThermoFisher Scientific) based on probe specificity and short amplicon length to maximize PCR efficiency; specific Taqman hydrolysis probes used are shown in Table 7.3. A StepOne Real-Time PCR System™ (Applied Biosystems) was used for quantitative real-time rt-PCR with the following cycling parameters: stage 1a, 48°C for 15 minutes (reverse transcription RNA to cDNA); stage 1b, 95°C for 10 minutes (initial DNA denaturation); stage 2a, 95°C for 15 seconds (melting); and stage 2b (annealing and extending), 60°C for one minute – stages 2a and 2b repeated for 40 cycles.

Samples were run in triplicate, and three biological replicates were carried out for each experiment. Assay efficacy of all hydrolysis probes was checked by running standard curves for each gene of interest, with four 10-fold dilution points for each gene probe used. Standard curves efficiencies for all hydrolysis probes used were between 97 – 105 %, indicating excellent and comparable amplification efficiency between different probe sets. Probe negative control PCR experiments indicated that there was no significant DNA contamination of RNA extractions.

The comparative Ct method -- $2^{-(\Delta\Delta CT)}$ -- was used to quantify gene expression (Schmittgen and Livak 2008); both GAPDH and 18S ribosome expression were used as reference control genes.

Table 7.2 Components for second phase quantitative real-time rt-PCR (per reaction)

Component	Volume	Final Concentration
TaqMan Mastermix (2x)	5.0 µl	1x
Reverse Transcriptase (40x)	0.25 µl	1x
Hydrolysis probe-primer set	0.5 µl	0.5 µmol/L
Template	1.0 µl	≈100-500 ng/reaction
RNAse free water	3.25 µl	----
Total volume	10.0 µl	----

Table 7.3 TaqMan® hydrolysis probes used for quantitative real-time rt-PCR

Protein/gene of interest	Gene Symbol	Hydrolysis Probe
Adenomatous Polyposis Coli	APC	Hs01568269
Beta catenin (cadherin-associated protein, beta 1)	CTNNB1	Hs00355049
Cyclin D2	CCND2	Hs00153380
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Hs02758991
Hepatocyte Nuclear Factor 4, Alpha	HNF4A	Hs00230853
Nuclear factor kappa-light-chain-enhancer of activated B cells	NFKB1	Hs00765730
RNA, 18S ribosomal 5	RNA18S5	Hs03928985
S-phase kinase-associated protein 2, E3 ubiquitin protein ligase	SKP2	Hs01021864
Transferrin receptor 1	TFRC	Hs00951083
Transferrin receptor 2	TFR2	Hs01056398
Tumour protein p53	TP53	Hs01034249

7.2.5: Western Blot

Caco-2 and HepG2 cells were treated with iron alone ($FeCl_3$ for Caco-2 experiments and ferric ammonium citrate – FAC-- for HepG2 experiments), fructose alone, and iron and fructose solutions as detailed above in the cell treatments used in the RNA extraction section; 6- or 12-well plates were prepared in duplicate, where possible, for analysis of both protein and mRNA expression over the same time period. After 24 hours of cell treatments, cells were rinsed with ice cold Phosphate Buffered Saline (PBS), and then harvested for protein with CelLytic™ (propriety lysis buffer, Sigma) with added protease inhibitor. Lysed cells were placed in ice and shaken for 15 minutes, then centrifuged for 6 minutes at 6 x 1000 g. Supernatants were collected and stored at -80°C until needed for

Western Blot analysis. Pierce Protein BCA Assay was used to measure protein concentration prior to Western Blot analysis to ensure approximately even protein loading of samples onto gels.

For gel loading 4x Laemmli buffer [62.5 mm Tris-HCl (pH 6.8), 25 % glycerol, 2 % SDS, 0.01 % bromophenol blue] with 10 % beta-mercaptoethanol (BME) was added in a 1:4 ratio (v/v) to a sample containing approximately 40 μg protein; the mixture was loaded and run on precast 8 or 10 % SDS-polyacrylamide gels (Thermo Scientific™ Precise™ Tris-Glycine Gels).

Separated proteins were electro-transferred to a nitrocellulose membrane (Amersham Bioscience) at 40 volts in ice cold Tris-Glycine transfer buffer for 70-80 minutes. The membrane was then pre-incubated with blocking solution (5 % fat-free milk in PBS containing 0.1 % Tween 20 - PBST) for one hour at 37^{o}C followed by overnight incubation at 4^{o}C with the primary antibody of interest in 1 % fat-free milk in PBST. Primary antibodies used are listed in Table 7.4.

Table 7.4 Western Blot Antibodies

Antibody	Type	Raised In	MW (kD)	Dilution	Source	Catalog No.
Anti-Actin	Primary	Mouse	42	1:2500	Abcam	ab3280
Anti-Actin	Primary	Rabbit	42	1:2500	Abcam	ab1801
Anti-DcytB	Primary	Donkey	35	1:500	Santa Cruz	sc-161025
Anti-DMT1	Primary	Mouse	62	1:500	Abnova	H00004891
Anti-DMT1	Primary	Mouse	62	1:1000	Santa Cruz	sc-166884
Anti-Ferroportin	Primary	Rabbit	63	1:1000	GeneTex	GTX54821
Anti-GAPDH	Abcam	Mouse	37	1:2500	Abcam	ab8245
Anti-GAPDH	Abcam	Rabbit	37	1:2500	Abcam	ab9485
Anti-Heat Shock Protein 90AB (HSP90)	Primary	Mouse	85	1:1000	Abcam	ab13492
Anti-HNF4A	Primary	Rabbit	53	1:500	Cell Signaling	G162
Anti-IRP2	Santa Cruz	Mouse	105	1:250-500	Santa Cruz	sc-33682
Anti-TfR1	Santa Cruz	Mouse	85-95	1:1000	Santa Cruz	sc-65882
Anti-TfR2	Santa Cruz	Mouse	97-105	1:500	Santa Cruz	sc-32271
Anti-Villin	Primary	Mouse	93	1:2500	GeneTex	GTX76074

Initial Western blots used actin as the reference loading protein, and this was unchanged for experiments looking at native expression of proteins related to cell passage or

maturation (chapter two). However, as the microarray PCR analysis, and follow up quantitative real-time rt-PCR identified actin as a changed gene (it was up-regulated by iron, Table 7.5 and Figure 7.11) subsequent Western blots used GAPDH as the loading reference protein.

Membranes were washed after the 24 hour primary antibody incubation, and incubated with the appropriate HRP-linked secondary antibody for 1 hour at room temperature. Signal was detected by ECL chemiluminescence (ECL, Amersham Place, Buckinghamshire, England).

ImageJ software was used to semi-quantify the band corresponding to each antibody and all were normalized to GAPDH as described above.

7.3: Statistical analysis

Statistical analysis varied in accordance with experimental purpose and design. Non-parametric methods were used when n was small, or the distribution of test results was either known, or found, to be non-Gaussian. The specific analysis for each type of experiment is outlined below.

7.3.1: Gene expression

7.3.1.1: Hypothesis generating microarray analysis

HepG2 gene expression was initially evaluated by microarray analysis to identify genes of interest (Discoveries) in relation to NAFLD and HCC and was hence hypothesis generating. For analysis of microarray results (gene expression changes between treatments), the False Discovery Rate (FDR) was used to compensate for multiple comparisons in the method of Motulsky (Motulsky 2010); the desired False Discovery Rate (q) value was set to 5.000 % and a value Q was computed based upon the p-value associated with each gene. Q less than the desired rate q was deemed to be a Discovery.

7.3.1.2: Real-time rt-PCR analysis of $2^{-(\Delta\Delta CT)}$ data

An initial set of quantitative real-time rt-PCR experiments were conducted on biologically relevant Discoveries. This initial PCR analysis was done both in order to verify the results of the microarray analysis, and also to help identify statistically significant Discoveries. Holm-Sidak's multiple comparisons test (herein referred to as Sidak's multiple comparisons test) was used in this case to test for statistical significance between different treatment effects compared with: the control treatment (MEM); and also between iron alone compared with the equivalent concentration iron but with added fructose treatments. Statistical significance for Sidak's multiple comparisons test was set at $p \leq 0.05$.

Second phase PCR experiments were conducted on new RNA extractions in order to repeat and/or further investigate initial results. Sidak's multiple comparisons test was used for analysis of this data ($p \leq 0.05$).

Trend analysis was conducted using "Multiple Comparisons Linear Trend Analysis" in the method of Motulsky (Motulsky 2010) using GraphPad Prism (v.6.0h GraphPad Software, San Diego, CA). Significance for Trend Analysis was defined by the significance of the slope of the resultant line in the predicted direction (i.e. either negative or positive) and set at $p \leq 0.05$.

7.3.2: Western Blots

ImageJ software (v2.0.0-rc-44-1.50e) was used to semi-quantify bands corresponding to each antibody and all were normalized to GAPDH as described above. Semi-quantified Western blot derived data were analysed either by Sidaks' multiple comparisons test; or where an ordered response was hypothesized, either in response to changing iron levels, or the addition of fructose, trend analysis was used to test for statistical significance. Significance for Trend Analysis was defined by the significance of the slope of the resultant line in the predicted direction (i.e. either negative or positive) and set at $p \leq 0.05$. Trend analysis was conducted using "Multiple Comparisons Linear Trend Analysis " in the method of (Motulsky 2010) using GraphPad Prism (v.6.0h GraphPad Software, San Diego, CA).

7.4: Results

7.4.1: Caco-2 cells – iron transport and homeostasis

7.4.1.1: Effect of treatments on Caco-2 cell mRNA expression of transferrin receptor one (TfR1)

Fructose alone (Fruc) did not alter levels of TfR1 mRNA in comparison with control cells. All iron-fructose treatments significantly decreased TfR1 mRNA levels; FF30 decreased levels by 1.6 fold; and FF100 decreased levels by 2.5 fold ($p \leq 0.05$). Both Fe30 and Fe100 decreased TfR1 levels by 1.2 fold but this did not reach statistical significance. The difference in mRNA expression levels of TfR1 between Fe30 and FF30 was not statistically significant; the difference between Fe100 and FF100 was significant ($p \leq 0.05$) (Figure 7.1)

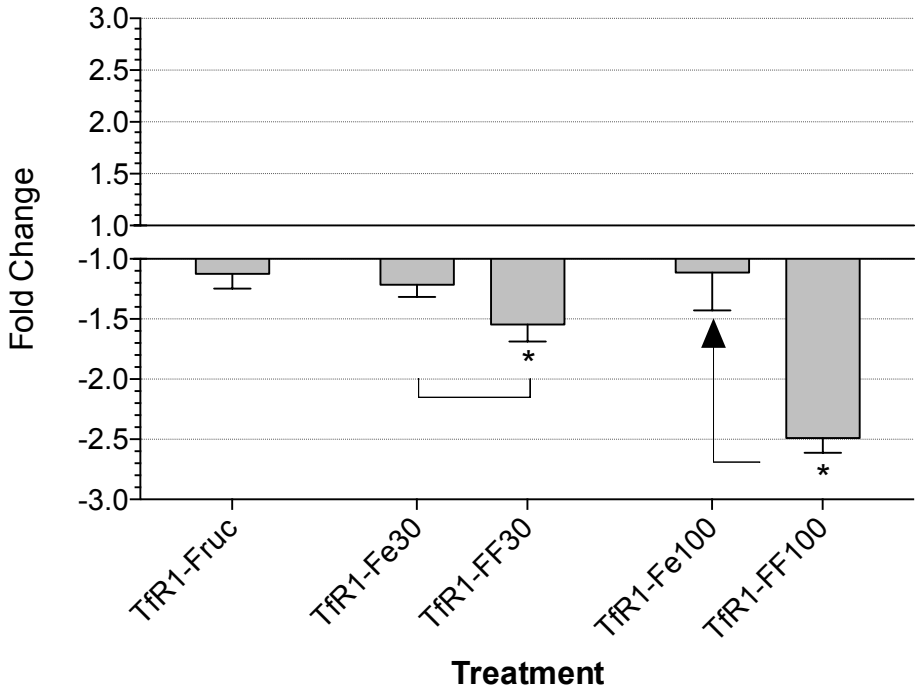

Figure 7.1 Effect of fructose (Fruc), low dose iron (Fe30), low dose iron and fructose together (FF30), high dose iron (Fe100), and high dose iron and fructose together (FF100) on Caco-2 cell Transferrin Receptor 1 (TfR1) mRNA expression.

Caco-2 cells grown as a monolayer were treated at 14 days with one of the following: 66 mmol/L fructose (Fruc); low dose iron, 30 µmol/L $FeCl_3$ (Fe30); 30 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF30); high dose iron, 100 µmol/L $FeCl_3$ (Fe100); or 100 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF100). Changes in mRNA expression were quantified by quantitative real-time rt-PCR, normalised to both 18S and GAPDH. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as fold change following normalisation to the control group (MEM alone; denoted as M). Each treatment was compared as a ratio to the control M; the logs of these ratios were compared and a significant difference is indicated by an * (Sidak's multiple comparisons test; p<=0.05). Similarly, data for linked pairs were analysed using the log of the ratio to the control M using Sidak's multiple comparisons test and considered significant at p<=0.05; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis indicated a significant ordered response (p≤0.05).

7.4.1.2: Effect of treatments on Caco-2 cell protein expression of iron-transport and iron-homeostasis related proteins – DcytB, DMT1, ferroportin, IRP2 and TfR1

Fructose alone (Fruc) did not significantly alter levels of DcytB, DMT1, ferroportin, IRP2 or TfR1 in comparison with controls (MEM) in Caco-2 cells (Figures 7.2-7.6).

Caco-2 cell DcytB protein expression levels were not significantly changed by any treatment; there was a linear trend indicating increased DctyB expression with increasing iron levels, but this did not reach statistical significance (Figure 7.2).

Caco-2 cell DMT1 protein levels decreased with all iron treatments; decreased DMT1 formation was significant in the order Fe30 and FF30, Fe100 and FF100 as tested by GraphPad Multiple Comparisons Linear Trend Analysis herein referred to as "Trend Analysis" ($p \leq 0.0001$). MEM and fructose DMT1 levels were significantly greater than Fe30, FF30, Fe100 and FF100 (Sidak's multiple comparisons test, $p \leq 0.05$); there was no significant difference in DMT1 between Fe30 and FF30, nor between Fe100 and FF100 (Figure 7.3).

All iron treatments decreased Caco-2 cell ferroportin levels in comparison with both MEM and fructose, although the decrease in Fe30 did not reach statistical significance. The decrease was significant in the order Fe30, FF30, Fe100 and FF100 as tested by Trend Analysis ($p \leq 0.05$). FF30 and FF100 Ferroportin levels were 50 % and 10 % less, respectively, compared with Fe30 and Fe100, but this did not reach statistical significance. Ferroportin levels in control cells was significantly greater than FF30, Fe100 and FF100 (Sidak's multiple comparisons test, $p \leq 0.05$) (Figure 7.4).

IRP2 protein levels were highest in Caco-2 control and fructose treated cells, and decreased in all cells treated with iron although no changes in comparison with control cells reached statistical significance. The decrease was significant in the order Fe30 and FF30, and Fe100 and FF100 as tested by Trend Analysis; $p \leq 0.05$). There was no statistically significant difference between Fe30 and FF30, nor between Fe100 and FF100. (Figure 7.5).

TfR1 protein levels were highest in Caco-2 control and fructose treated cells, and in parallel with mRNA changes decreased in all cells treated with iron though this only reached statistical significance for Fe100 and FF100 ($p \leq 0.05$). The decrease was significant in the order Fe30 and FF30, and Fe100 and FF100 as tested by Trend Analysis ($p \leq 0.05$). There was no statistically significant difference between Fe30 and FF30, nor between Fe100 and FF100, for either protein. This is in contrast with PCR results for which there was a statistically significant decrease of TfR1 between Fe100 vs FF100 (Figures 7.1 and 7.6).

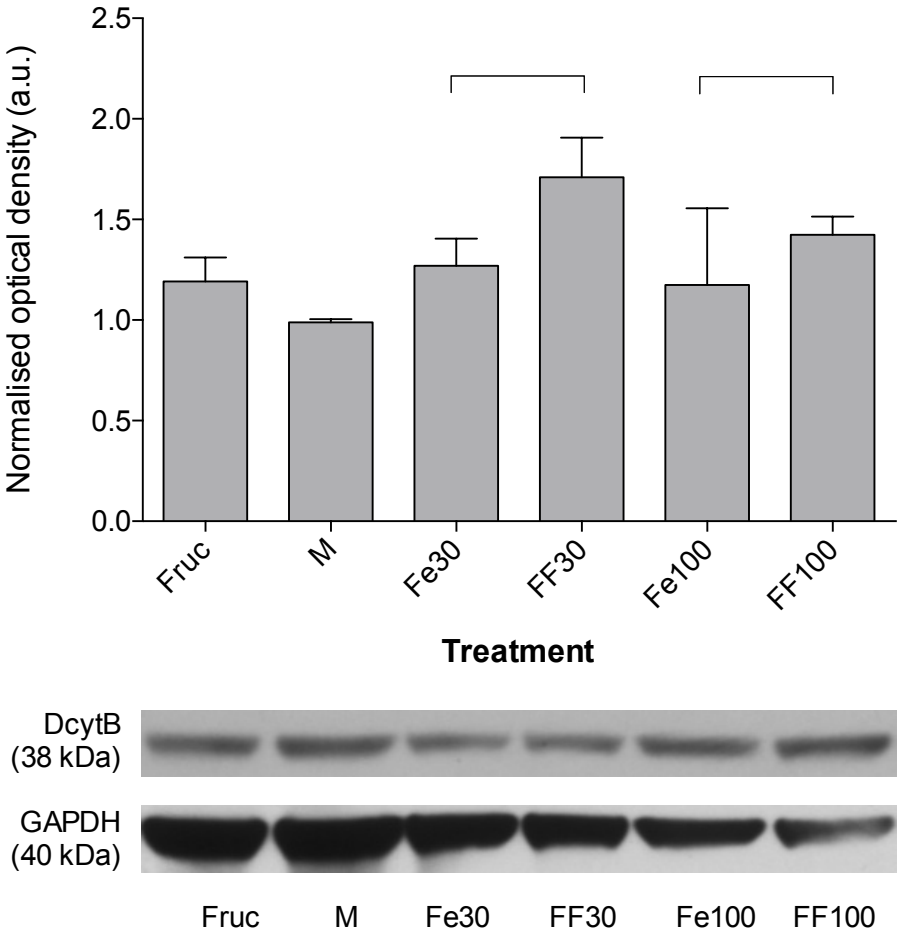

Figure 7.2 Effect of fructose (Fruc), low dose iron (Fe30), high dose iron (Fe100), low dose iron and fructose together (FF30), and high dose iron and fructose together (FF100) on Caco-2 cell DcytB protein expression.

Caco-2 cells grown as a monolayer were treated at 14 days with one of the following: 66 mmol/L fructose (Fruc); low dose iron, 30 µmol/L $FeCl_3$ (Fe30); 30 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF30); high dose iron, 100 µmol/L $FeCl_3$ (Fe100); or 100 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF100). Protein expression changes of DcytB were semi-quantified by Western blot analysis normalised to GAPDH using ImageJ software; representative Western blot results for each treatment are shown above. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as relative expression levels following normalisation to the control group (MEM alone). Each treatment was compared to the control M and a significant difference indicated by an * (Sidak's multiple comparisons test; $p \leq 0.05$). Data for linked pairs were analysed by Sidak's multiple comparisons test and considered significant at $p \leq 0.05$; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis did not indicate a significant ordered response.

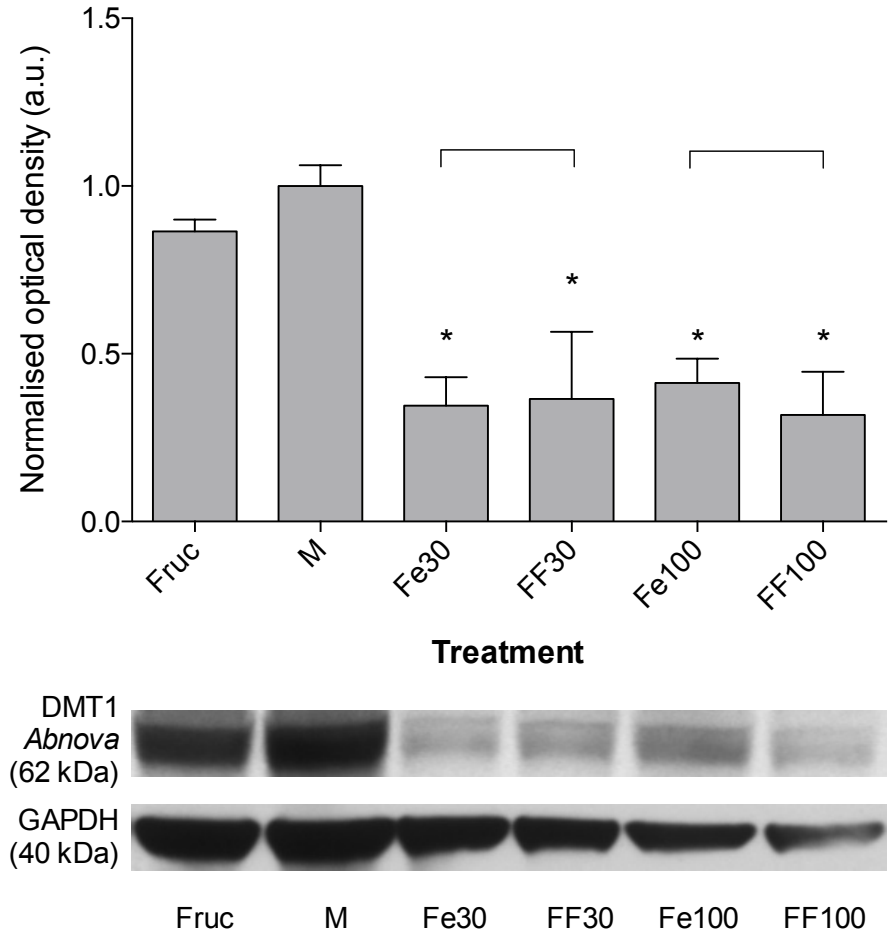

Figure 7.3 Effect of fructose (Fruc), low dose iron (Fe30), high dose iron (Fe100), low dose iron and fructose together (FF30), and high dose iron and fructose together (FF100) on Caco-2 cell DMT1 protein expression.

Caco-2 cells grown as a monolayer were treated at 14 days with one of the following: 66 mmol/L fructose (Fruc); low dose iron, 30 µmol/L $FeCl_3$ (Fe30); 30 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF30); high dose iron, 100 µmol/L $FeCl_3$ (Fe100); or 100 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF100). Protein expression changes of DMT1 were semi-quantified by Western blot analysis normalised to GAPDH using ImageJ software; representative Western blot results for each treatment are shown above. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as relative expression levels following normalisation to the control group (MEM alone). Each treatment was compared to the control M and a significant difference indicated by an * (Sidak's multiple comparisons test; $p \leq 0.05$). Data for linked pairs were analysed by Sidak's multiple comparisons test and considered significant at $p \leq 0.05$; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis indicated a significant ordered response ($p \leq 0.05$).

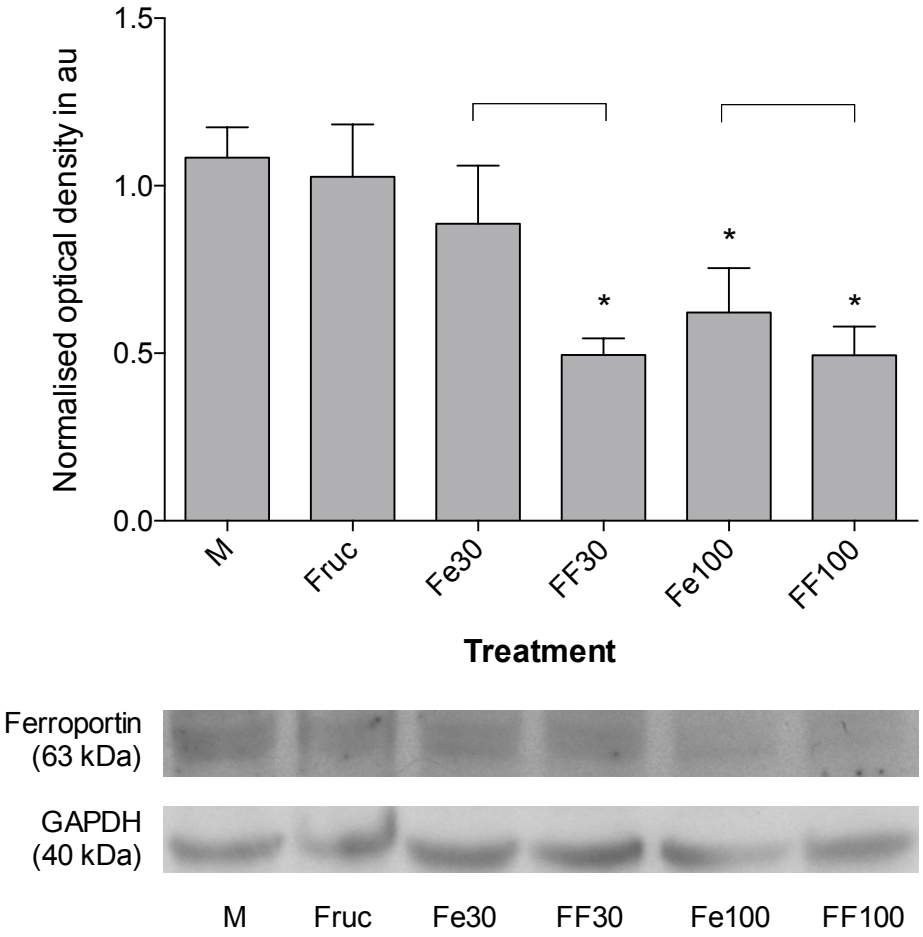

Figure 7.4 Effect of fructose (Fruc), low dose iron (Fe30), high dose iron (Fe100), low dose iron and fructose together (FF30), and high dose iron and fructose together (FF100) on Caco-2 cell ferroportin protein expression.

Caco-2 cells grown as a monolayer were treated at 14 days with one of the following: 66 mmol/L fructose (Fruc); low dose iron, 30 µmol/L $FeCl_3$ (Fe30); 30 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF30); high dose iron, 100 µmol/L $FeCl_3$ (Fe100); or 100 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF100). Protein expression changes of ferroportin were semi-quantified by Western blot analysis normalised to GAPDH using ImageJ software; representative Western blot results for each treatment are shown above. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as relative expression levels following normalisation to the control group (MEM alone). Each treatment was compared to the control M and a significant difference indicated by an * (Sidak's multiple comparisons test; $p \leq 0.05$). Data for linked pairs were analysed by Sidak's multiple comparisons test and considered significant at $p \leq 0.05$; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis indicated a significant ordered response ($p \leq 0.05$).

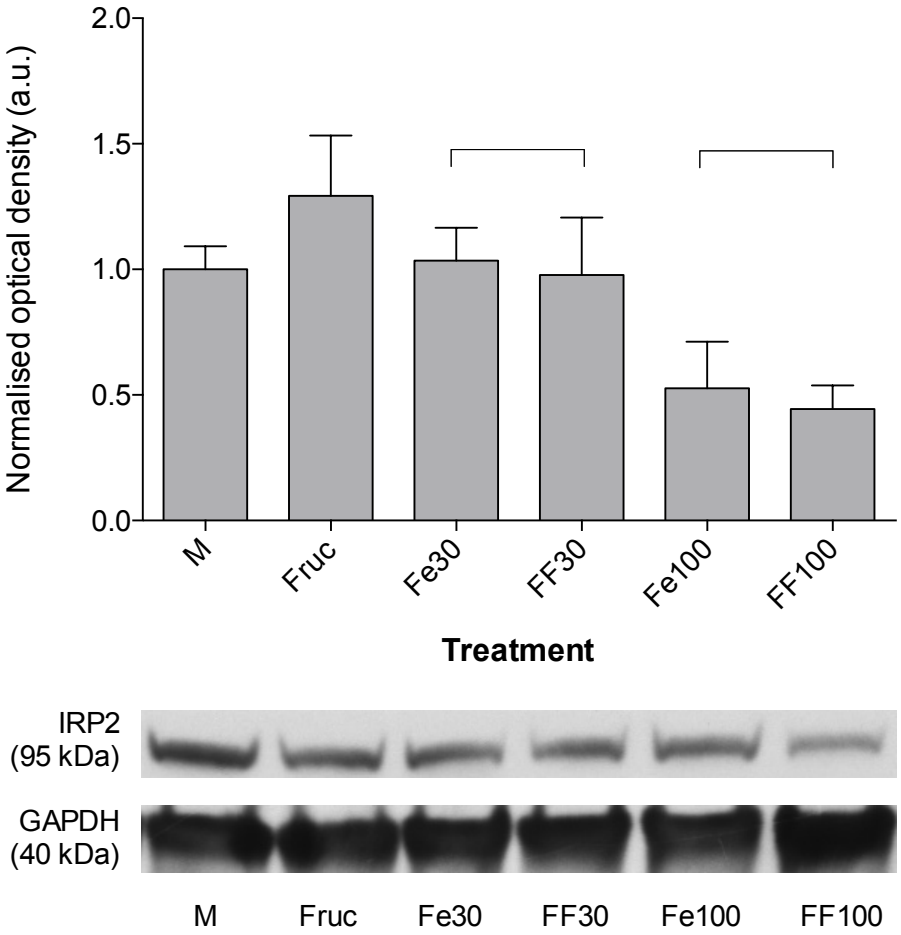

Figure 7.5 Effect of fructose (Fruc), low dose iron (Fe30), high dose iron (Fe100), low dose iron and fructose together (FF30), and high dose iron and fructose together (FF100) on Caco-2 cell IRP2 protein expression.

Caco-2 cells grown as a monolayer were treated at 14 days with one of the following: 66 mmol/L fructose (Fruc); low dose iron, 30 µmol/L $FeCl_3$ (Fe30); 30 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF30); high dose iron, 100 µmol/L $FeCl_3$ (Fe100); or 100 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF100). Protein expression changes of IRP2 were semi-quantified by Western blot analysis normalised to GAPDH using ImageJ software; representative Western blot results for each treatment are shown above. Experiments were repeated ≥ 3 over three separate time points except for Fe30, FF30 and FF100 for which n=2. All results are expressed as relative expression levels following normalisation to the control group (MEM alone). Each treatment was compared to the control M and a significant difference indicated by an * (Sidak's multiple comparisons test; $p \leq 0.05$). Data for linked pairs were analysed by Sidak's multiple comparisons test and considered significant at $p \leq 0.05$; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis indicated a significant ordered response ($p \leq 0.05$).

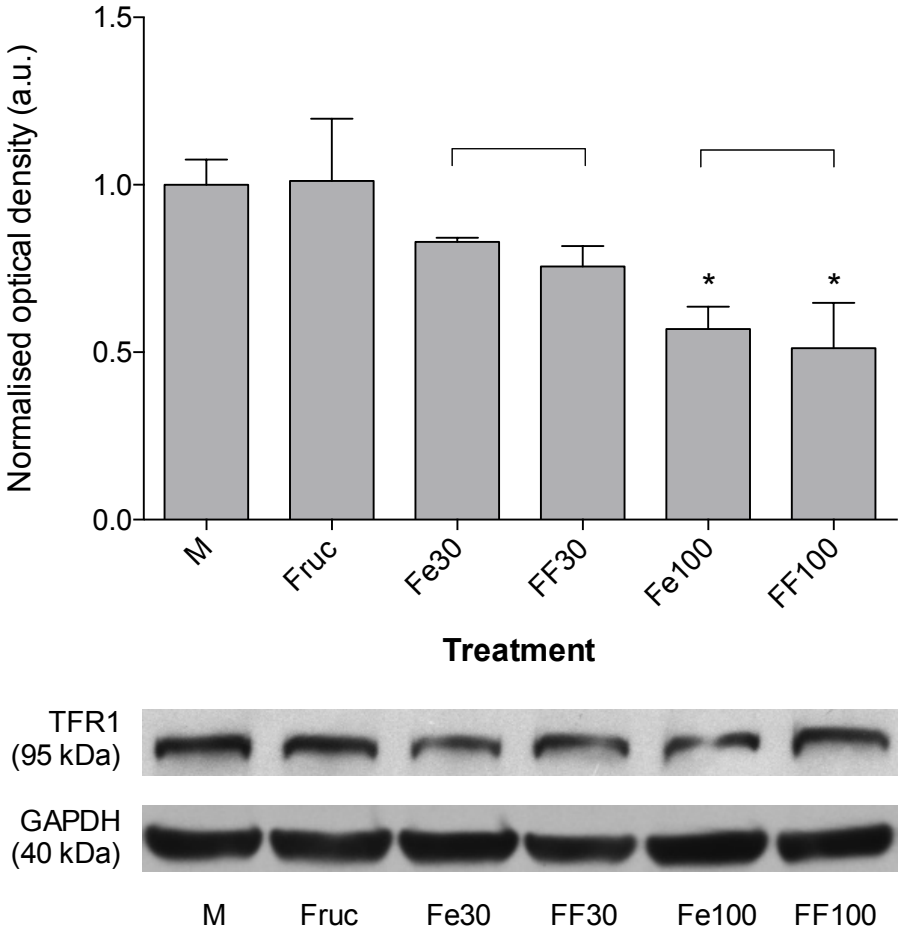

Figure 7.6 Effect of fructose (Fruc), low dose iron (Fe30), high dose iron (Fe100), low dose iron and fructose together (FF30), and high dose iron and fructose together (FF100) on Caco-2 cell Transferrin Receptor 1 (TfR1) expression.

Caco-2 cells grown as a monolayer were treated at 14 days with one of the following: 66 mmol/L fructose (Fruc); low dose iron, 30 µmol/L $FeCl_3$ (Fe30); 30 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF30); high dose iron, 100 µmol/L $FeCl_3$ (Fe100); or 100 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF100). Protein expression changes of TfR1 were semi-quantified by Western blot analysis normalised to GAPDH using ImageJ software; representative Western blot results for each treatment are shown above. Experiments were repeated ≥ 3 over three separate time points except for Fe30, FF30 and Fe100 for which n=2.. All results are expressed as relative expression levels following normalisation to the control group (MEM alone). Each treatment was compared to the control M and a significant difference indicated by an * (Sidak's multiple comparisons test; $p \leq 0.05$). Data for linked pairs were analysed by Sidak's multiple comparisons test and considered significant at $p \leq 0.05$; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis indicated a significant ordered response ($p \leq 0.05$).

7.4.2: HepG2 cells - iron transport and homeostasis

7.4.2.1: Effect of treatments on HepG2 cell mRNA expression of TfR1 and TfR2

Measurement of HepG2 TfR1 and TfR2 gene expression was conducted in no wash-out treated HepG2 cells.

Fructose alone did not alter mRNA levels of either TfR1 or TfR2 in comparison with control cells (data for TfR2 not shown). As expected, levels of TfR1 mRNA went down with iron treatments; TfR1 mRNA expression significantly decreased 1.4 fold compared with controls after treatment with Fe1 ($p \leq 0.05$). Treatment of cells with FF1 resulted in a reduction in TfR1 mRNA expression of 1.9 fold compared with the control ($p \leq 0.05$), and the difference between cells treated with Fe1 compared with FF1 was also significant for Fe1 versus FF1 ($p \leq 0.05$). FF10 decreased TfR1 levels 2.1 fold; the effect of Fe10 treatment alone in HepG2 cells (10 μmol/L FAC) TfR1 RNA expression was not measured. Trend analysis indicated a significant ordered decrease in the order Fe1, FF1, FF1, FF10 ($p \leq 0.05$) (Figure 7.7).

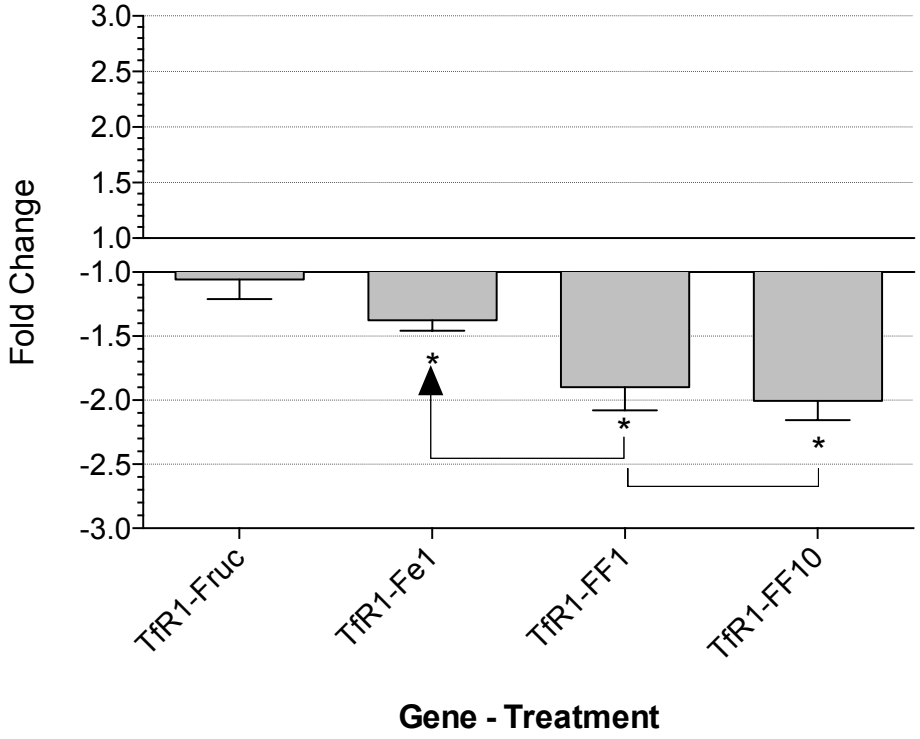

Figure 7.7 Effect of fructose (Fruc), low dose iron (Fe1), low dose iron and fructose together (FF1), and high dose iron and fructose together (FF10) on HepG2 Transferrin Receptor 1 (TfR1) mRNA expression.

HepG2 cells grown as a monolayer were treated at 24-48 hours with one of the following: 15 mmol/L fructose (Fruc); low dose iron, 1 µmol/L FAC (Fe1); 1 µmol/L FAC and 15 mmol/L fructose together (FF1); or 10 µmol/L FAC and 15 mmol/L fructose together (FF10). Changes in mRNA expression were quantified by quantitative real-time rt-PCR, normalised to both 18S and GAPDH. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as fold change following normalisation to the control group (MEM alone; denoted as M). Each treatment was compared as a ratio to the control M; the logs of these ratios were compared and a significant difference is indicated by an * (Sidak's multiple comparisons test; $p<=0.05$). Similarly, data for linked pairs were analysed using the log of the ratio to the control M using Sidak's multiple comparisons test and considered significant at $p<=0.05$; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis indicated a significant ordered response ($p\leq0.05$).

7.4.2.2: Effect of treatments on HepG2 cell protein expression of iron-transport and iron-homeostasis related proteins ferroportin, IRP2 and TfR1

HepG2 cell levels of ferroportin did not alter with fructose alone treatments compared with control cells; all iron treatments significantly increased HepG2 ferroportin expression, in contrast with Caco-2 cell ferroportin protein expression after iron treatments. HepG2 ferroportin protein expression increase was significant in the order Fe1 and FF1, and Fe10 and FF10, as tested by Trend Analysis ($p\leq0.05$) (Figure 7.8). There were no significant differences between Fe1 and FF1, nor between Fe10 and FF10;

FF10 was higher compared with FF1, and both Fe10 and FF10 were higher compared with MEM (Sidak's multiple comparisons test, $p \leq 0.05$) (Figure 7.8).

HepG2 cell IRP2 protein levels were highest in control cells (MEM) and fructose treated cells. IRP2 levels decreased with all iron treatments although this did not reach statistical significance for Fe1; decreased IRP2 formation was significant in the order Fe1 and FF1, and Fe10 and FF10, as tested by Trend Analysis ($p \leq 0.05$). There was no statistically significant difference in protein levels of IRP2 between Fe1 versus FF1, nor between Fe10 versus FF10. MEM and fructose treated cell IRP2 levels were significantly greater compared with FF1, Fe10 and FF10 by approximately 100 % (Sidak's multiple comparisons test, $p \leq 0.05$) (Figure 7.9).

Fructose alone did not alter levels of HepG2 TfR1 protein in comparison with MEM. Fe10 and FF10 decreased TfR1 protein levels; decreased TfR1 protein formation was significant in the order Fe1 and FF1, and Fe10 and FF10, as tested by Trend Analysis ($p \leq 0.05$). There was no statistically significant difference in protein levels of TfR1 between Fe1 versus FF1, nor between Fe10 versus FF10. MEM and fructose TfR1 protein levels were significantly higher compared with Fe10 and FF10, by approximately 100 % (Sidak's multiple comparisons test, $p \leq 0.05$) (Figure 7.10).

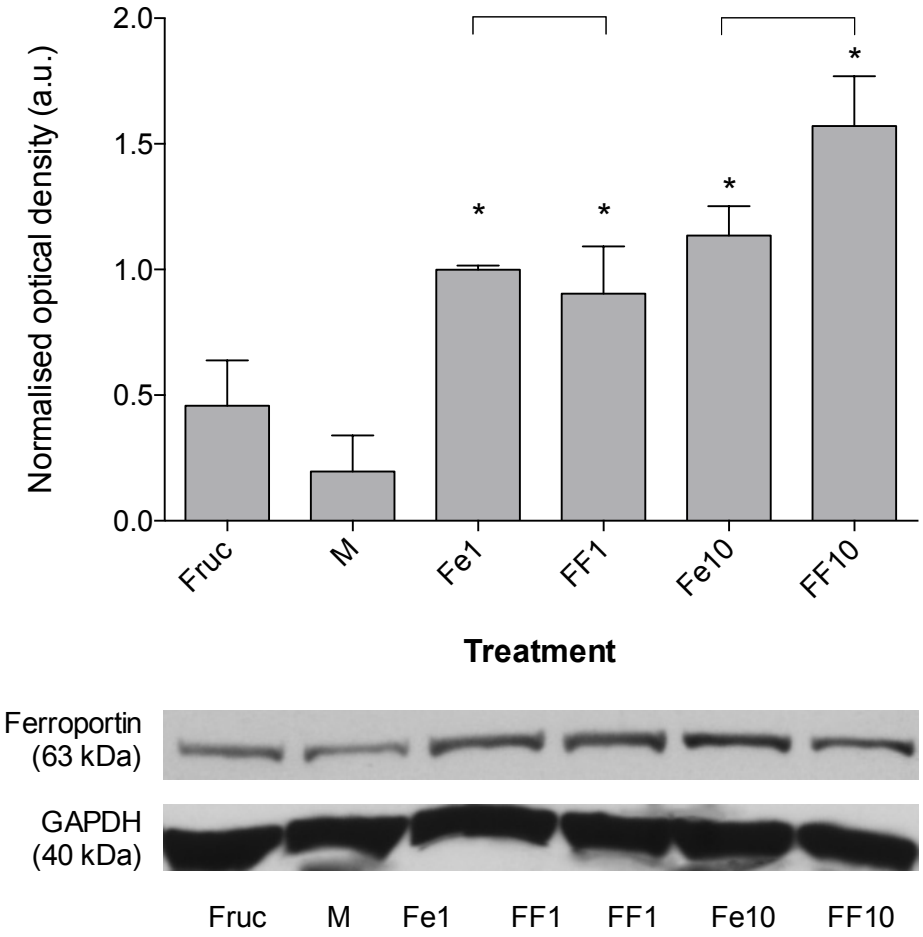

Figure 7.8 Effect of fructose (Fruc), low dose iron (Fe1), low dose iron and fructose together (FF1), high dose iron (Fe10), and high dose iron and fructose together (FF10) on HepG2 ferroportin protein expression.

HepG2 cells grown as a monolayer were treated at 24-48 hours with one of the following: 15 mmol/L fructose (Fruc); low dose iron, 1 µmol/L FAC (Fe1); 1 µmol/L FAC and 15 mmol/L fructose together (FF1); high dose iron, 10 µmol/L FAC (Fe10); or 10 µmol/L FAC and 15 mmol/L fructose together (FF10). Protein expression changes of ferroportin were semi-quantified by Western blot analysis normalised to GAPDH using ImageJ software; representative Western blot results for each treatment are shown above. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as relative expression levels following normalisation to the control group (MEM alone). Each treatment was compared to the control M and a significant difference indicated by an * (Sidak's multiple comparisons test; $p \leq 0.05$). Data for linked pairs were analysed by Sidak's multiple comparisons test and considered significant at $p \leq 0.05$; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis indicated a significant ordered response ($p \leq 0.05$).

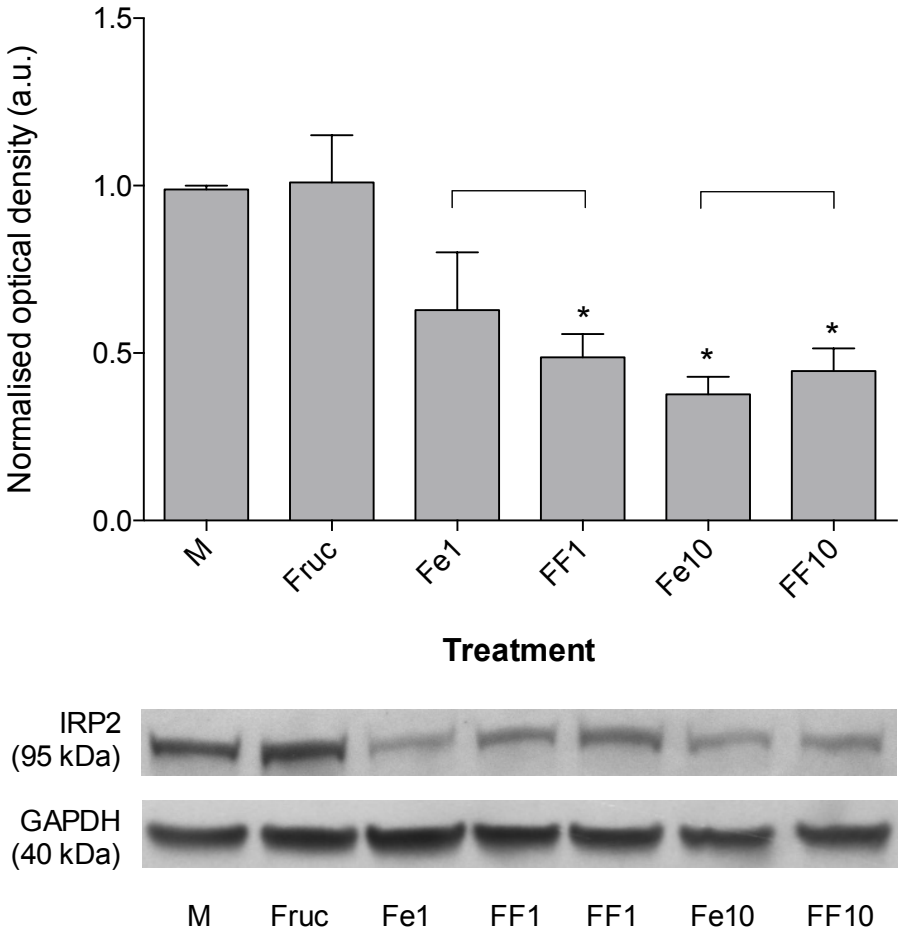

Figure 7.9 Effect of fructose (Fruc), low dose iron (Fe1), low dose iron and fructose together (FF1), high dose iron (Fe10), and high dose iron and fructose together (FF10) on HepG2 IRP2 protein expression.

HepG2 cells grown as a monolayer were treated at 24-48 hours with one of the following: 15 mmol/L fructose (Fruc); low dose iron, 1 µmol/L FAC (Fe1); 1 µmol/L FAC and 15 mmol/L fructose together (FF1); high dose iron, 10 µmol/L FAC (Fe10); or 10 µmol/L FAC and 15 mmol/L fructose together (FF10). Protein expression changes of IRP2 were semi-quantified by Western blot analysis normalised to GAPDH using ImageJ software; representative Western blot results for each treatment are shown above. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as relative expression levels following normalisation to the control group (MEM alone). Each treatment was compared to the control M and a significant difference indicated by an * (Sidak's multiple comparisons test; $p \leq 0.05$). Data for linked pairs were analysed by Sidak's multiple comparisons test and considered significant at $p \leq 0.05$; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis indicated a significant ordered response ($p \leq 0.05$).

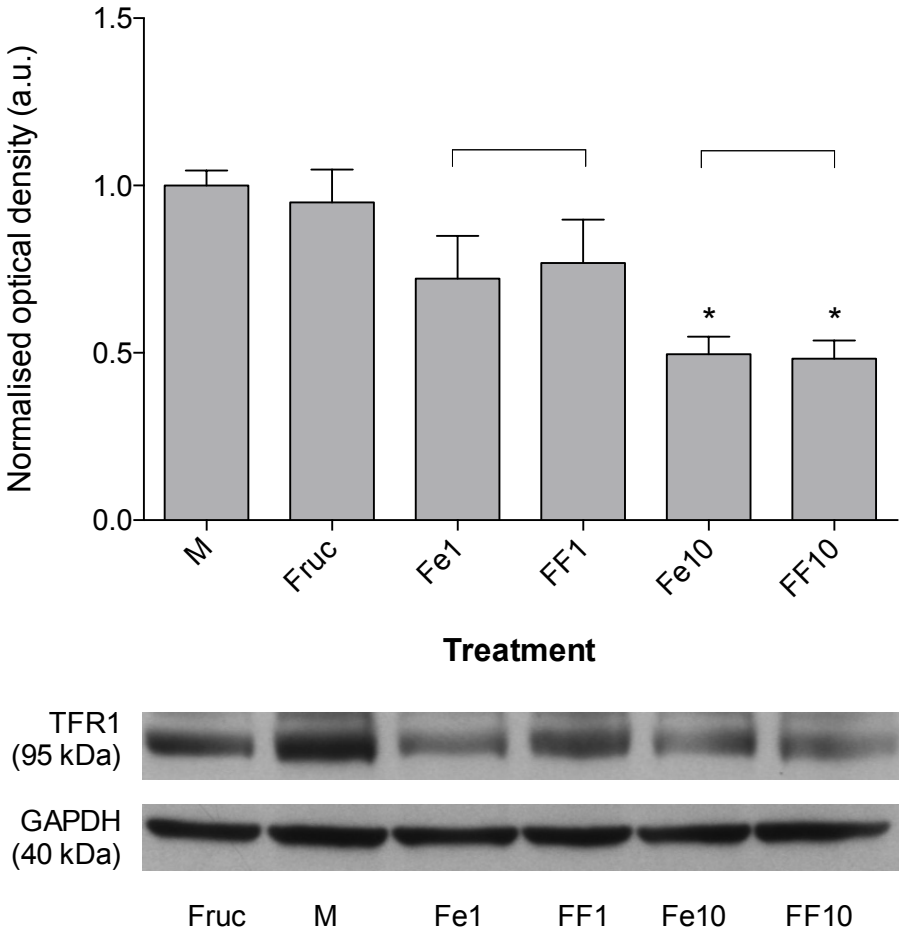

Figure 7.10 Effect of fructose (Fruc), low dose iron (Fe1), low dose iron and fructose together (FF1), high dose iron (Fe10), and high dose iron and fructose together (FF10) on HepG2 Transferrin Receptor 1 (TfR1) protein expression.

HepG2 cells grown as a monolayer were treated at 24-48 hours with one of the following: 15 mmol/L fructose (Fruc); low dose iron, 1 µmol/L FAC (Fe1); 1 µmol/L FAC and 15 mmol/L fructose together (FF1); high dose iron, high dose iron, 10 µmol/L FAC (Fe10); or 10 µmol/L FAC and 15 mmol/L fructose together (FF10). Protein expression changes of TfR1were semi-quantified by Western blot analysis normalised to GAPDH using ImageJ software; representative Western blot results for each treatment are shown above. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as relative expression levels following normalisation to the control group (MEM alone). Each treatment was compared to the control M and a significant difference indicated by an * (Sidak's multiple comparisons test; $p \leq 0.05$). Data for linked pairs were analysed by Sidak's multiple comparisons test and considered significant at $p \leq 0.05$; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis indicated a significant ordered response ($p \leq 0.05$).

7.4.3: Caco-2 and HepG2 - genes and proteins related to colorectal cancer (CRC), hepatocellular carcinoma (HCC), and non-alcoholic fatty liver disease (NAFLD).

7.4.3.1: HepG2 cells

7.4.3.1.1: Microarray Results

Full available microarray enrichment results and Genego maps are in Appendix One.

Expression of 940 genes was uniquely altered by treatment with **fructose and ferric ammonium citrate together (FF)** compared with MEM (M), fructose (15 mmol/L; FRUC) or ferric ammonium citrate (1 µmol/L FAC). The top three canonical pathways (determined by statistical significance) in which these genes were found were: triacylglycerol metabolism; IL-27 signalling pathway; and Activin A signalling regulation. Selected specific genes related to CRC, HCC and/or NAFLD changed within these top three pathways, as well other altered genes related to these diseases from statistically significant lower ranked pathways, follow below and are also shown in Table 7.5.

Altered expression of genes in the top three pathways is indicated by the colour blue. Gene expression of the following were **increased**: Aldehyde dehydrogenase family 1 member B1 (AL1B1); aldo-keto reductase family 7, member A2 (AKR7A2); Cytosolic glycerol-3-phosphate dehydrogenase 1 (GPD1); histone deacetylase 1 (HDAC1); C-terminal binding protein (CTBP1); Cylin D (CCND1); Cyclin D2 (CCND2); and S-Phase Kinase-Associated Protein 2, E3 Ubiquitin Protein Ligase (SKP2).

Gene expression of the following were **decreased**: Nuclear Factor- κ Beta (NFKB1); SMAD2; SMAD3; STAT3; Casein kinase 1 alpha (CSNK1A1); Janus Kinase 1; and c-Jun & c-Fos.

The expression of 574 genes was uniquely changed by treatment with **fructose alone**. The top three canonical pathways (determined by statistical significance) altered by fructose treatment alone were immune response alternative complement pathway, immune response lectin-induced complement pathway, and immune response classical complement pathway. Selected specific genes related to CRC, HCC and/or NAFLD changed within these top three pathways, as well other altered genes related to these diseases from statistically significant lower ranked pathways, follow below and are also shown in Table 7.5.

Altered expression of genes in the top three pathways is indicated by the colour blue. Gene expression of the following were **increased**: complement component three (C3); C5 Convertase (C2); CD59 Molecule (CD59); C8 Gamma subunit (C8G); Membrane Attack Complex (C8A); Clusterin (CLU); and Heat shock protein 90 (HSP90AB1).

Treatment with **FAC alone** uniquely changed expression of 88 genes. The top three canonical pathways (as determined by statistical significance) altered by FAC alone were Carbohydrate Responsive Element-binding protein (ChREBP), Unsaturated fatty acid

biosynthesis, and Prostaglandin E2 Cancer pathways. Selected specific genes related to CRC, HCC and/or NAFLD changed within these top three pathways, as well other altered genes related to these diseases from statistically significant lower ranked pathways, follow below and are also shown in Table 7.5.

Altered expression of genes in the top three pathways is indicated by the colour blue. Gene expression of the following were **increased**: 5' AMP-activated protein kinase gamma subunit (AMPK gamma subunit); Actin/Actin, Beta (ACTB).

Altered expression of genes in the top three pathways is indicated by the colour blue. Gene expression of the following were **decreased**: Acyl CoA synthetase (ACSL); Acyl-CoA synthetase long-chain family member 4; Stearoyl-CoA desaturase-1 (SCD1); Fatty Acid Desaturase 1 (FAD1); Prostaglandin E4 Receptor (PGE2R4); Hepatocyte Nuclear Factor 4A (HNF4A); T-cell factor/lymphoid enhancer factor (TCF/LEF).

Table 7.5 Microarray results showing genes altered ≤ or ≥ 1.5 fold (Discoveries based on a FDR analysis where q=0.050; literature references for comments available in discussion section).

	Fructose and Ferric ammonium citrate (FF) unique		
	Gene	Gene Symbol	Comments
Increased	Aldehyde dehydrogenase family 1 member B1	AL1B1	Metabolizes retinaldehyde to generate retinoic acid -vitamin A derivative necessary for cell growth and development; increased expression associated with CRC
	Aldo-keto reductase family 7, member A2	AKR7A2	Liver enzyme involved in detoxification of aldehydes and ketones including methyl glyoxal – a potential toxic by product of fructose metabolism
	C-terminal binding protein	CTBP1	Transcriptional regulator, mutations associated with CRC &HCC
	Cyclin D	CCND1	Regulator of associated cyclin dependent kinases that function in regulation of cell cycle; mutations associated with CRC & HCC
	Cyclin D2	CCND2	Regulator of associated cyclin dependent kinases that function in regulation of cell cycle; mutations associated with CRC & HCC
	Cytosolic Glycerol-3-phosphate Dehydrogenase	GPD1	Essential enzyme for carbohydrate and lipid metabolism; catalyses the conversion of dihydroxyacetone phosphate and NADH to glycerol-3-phosphate & NAD+. Mutations associated with fatty liver disorders.
	Histone deacytylase 1	HDAC1	Enzyme that participates in control of gene expression; mutations associated with HCC
	S-Phase Kinase-Associated Protein 2, E3 Ubiquitin Protein Ligase (Skp2)	SKP2	Proto-oncogene; mutations associated with multiple cancers including CRC & HCC
Decreased	Casein kinase 1 alpha	CKA2	Kinase involved in multiple signalling pathways including Wnt & Akt-Mtor; mutations associated with increased CRC and HCC risk
	c-Jun & c-Fos	JUN	Proto-oncogenes; together make up Activator Protein 1 (AP-1) – transcription factor involved in cell cycle regulation & inflammatory pathways
	Nuclear Factor- κ Beta (NF-κβ)	NFKB1	Transcription factor involved in inflammation and cancer through its effects on cytokines, apoptosis & angiogenesis
	SMAD2	SMAD2	Signal transducer and transcriptional modulator integral to multiple signalling pathways including iron homeostasis & inflammation/part of TGF-β pathway
	SMAD3	SMAD3	Signal transducer and transcriptional modulator integral to multiple signalling pathways including iron homeostasis & inflammation/ part of TGF-β pathway
	STAT3	STAT3	Component of a pleiotropic signalling cascade involved in iron homeostasis, inflammation & oncogenesis/part of NF-κβ pathway

Table 7.5 Microarray results (cont.)

Fructose Alone			
	Gene	Gene Symbol	Comments
Increased	C3	C3	Component of the complement activation cascade immune system; disturbances associated with metabolic disease.
	C5 convertase	C2	Component of the complement attack cascade immune system; disturbances associated with metabolic disease.
	C8 gamma subunit	C8G	Component of the complement attack cascade immune system; disturbances associated with metabolic disease.
	CD59	CD59	Component of the complement attack cascade immune system; disturbances associated with metabolic disease.
	Clusterin	CLU	Interacts with terminal complement attack cascade; also extracellular chaperone protein involved in cell proliferation, apoptosis, & carcinogenesis
	Heat shock protein 90	HSP90AB1	Intracellular chaperone protein released in response to stress that controls transport, folding/unfolding, assembly of complexes, and correct subcellular location of client proteins; transports many oncogenic client proteins - mutations associated with multiple malignancies
	Membrane Attack Complex	C8A	Component of the complement attack cascade; disturbances associated with metabolic disease.

Table 7.5 Microarray results (cont.)

FAC Alone			
	Gene	Gene Symbol	Comments
Increased	Actin beta subunit	ACTB	Component of the cellular cytoskeleton; involved in apoptosis and malignant cell contraction, deformability and invasion
	Actin complex	---	Component of the cellular cytoskeleton; involved in apoptosis and malignant cell contraction, deformability and invasion
	AMPK γ subunit	PRKAG2	Regulatory subunit of kinase that functions as a cell energy sensor and master modulator of energy pathways; mutations may either promote or suppress malignancy
Decreased	Acyl-CoA synthetase long-chain family member 4	ACSL4	Enzyme that form acyl-CoAs, involved in fatty acid metabolism, membrane modification and lipid signalling; involved in liver lipid distribution & malignant cell proliferation
	Fatty Acid Desaturase 1	FAD1	Enzyme egulates unsaturation of fatty acids; mutations associated with malignancy
	Hepatocyte Nuclear Factor 4A (HNF4A)	HNF4A	Hepatic nuclear receptor involved in regulation of metabolism, differentiation and proliferation and possibly iron; mutations associated with diabetes and HCC
	Prostaglandin E4 Receptor (PGE2R4)	PGE2R4	Receptors regulating tumour cell growth, invasion and migration involved in multiple malignancies including CRC
	Stearoyl-CoA Desaturase-1(SCD)	SCD1	Key regulator of the fatty acid composition of cellular lipids; mutations associated with fatty liver and cancer cell proliferation
	TCF/LEF Transcription Factors	TCF7/ LEF1	Transcription factors that are part of the Wnt signalling pathways; mutations associated with CRC,HCC & Diabetes

7.4.3.1.2: Quantitative real-time rt-PCR – HepG2 cells

Initial phase PCR was carried out with the same HepG2 cell samples used for microarray analysis, and second phase quantitative real-time rt-PCR (unless otherwise specified) was carried out in cells with no wash-out period. All initial phase quantitative real-time rt-PCR results are shown in Figure 7.11; second phase quantitative real-time rt-PCR results are shown in Figures 7.12-7.16.

As suggested by the microarray results, initial phase quantitative real-time rt-PCR experiments verified that 1 μmol/L FAC and 15 mmol/L fructose together (FF1) treatments *decreased* relative mRNA expression of SMAD 3, and STAT 3 and NF-κβ ($p \leq 0.05$). Fructose alone also decreased the expression of these genes, but this only reached statistical significance for NF-κβ. Initial phase quantitative real-time rt-PCR showed no change in relative mRNA expression of SMAD2, in contrast with microarray results (Figure 7.11).

Follow up second phase quantitative real-time rt-PCR experiments demonstrated a 1.2 fold *increased* expression of NF-κβ mRNA after treatment with FF10, but this did not reach statistical significance. Confirming initial phase real-time rt-PCR findings, when HepG2 cells were treated with FF1 and compared to "with-wash-out" control cells mRNA levels of NF-κβ were less, whereas mRNA levels of NF-κβ of FF1 treated cells in comparison with control cells with no wash-out were increased; the difference between FF1 versus M+ and FF1 versus M was statistically significant ($p \leq 0.05$) (Figure 7.12).

As SMADs 2 and 3 are both part of the Transforming Growth Factor Beta 1 (TGF-β1) signalling pathway, gene expression of this key cytokine was evaluated by second phase quantitative real-time rt-PCR; neither FF1 or FF10 altered mRNA expression of TGF-β1 (Figure 7.13). Iron alone, and fructose alone, were not tested.

Alterations in Wnt/β-catenin pathways have been implicated in both HCC and CRC development in multiple studies, therefore levels of β-catenin mRNA were measured although no changes had been noted in the microarray findings. β-catenin mRNA levels measured by second phase quantitative real-time rt-PCR were not changed either by FF1 or FF10, consistent with microarray results (Figure 7.14); iron alone, and fructose alone, were not tested.

Second phase quantitative real-time rt-PCR confirmed the increase in Cyclin D2 by fructose and iron treatments seen in microarray analysis; both FF1 and FF10 increased relative mRNA expression of Cyclin D2 1.5 fold ($p \leq 0.05$) (Figure 7.14).

Consistent with microarray results, FF1 treatment of HepG2 cells significantly increased expression of proto-oncogene Skp2 mRNA 2.3 fold with FF1, and 1.6 fold with FF10 as measured by second phase quantitative real-time rt-PCR ($p \leq 0.05$). Effects of iron alone, and fructose alone on Skp2 RNA levels were not tested (Figure 7.15).

In contrast with microarray results in which fructose alone increased HSP90 expression, follow up initial phase PCR demonstrated that HSP90 mRNA expression was significantly increased with fructose and iron together (1.3 fold; $p \leq 0.05$); interestingly both iron alone, and fructose alone, did minimally increase mRNA relative expression but this did not reach statistical significance (Figure 7.11).

Also in contrast with microarray findings, in which HNF4A had been decreased by iron alone, follow up initial *and* second phase PCR found that HNF4A was significantly decreased only by iron and fructose together (FF); FF1 decreased expression by 1.4 fold, and FF10 by 1.6 fold in second phase PCR studies ($p \leq 0.05$) (Figures 7.11 and 7.16). Of note, however, second phase PCR did demonstrate decreased HNF4A mRNA by 1.6 fold after HepG2 cell treatment with iron alone, but this was not statistically significant.

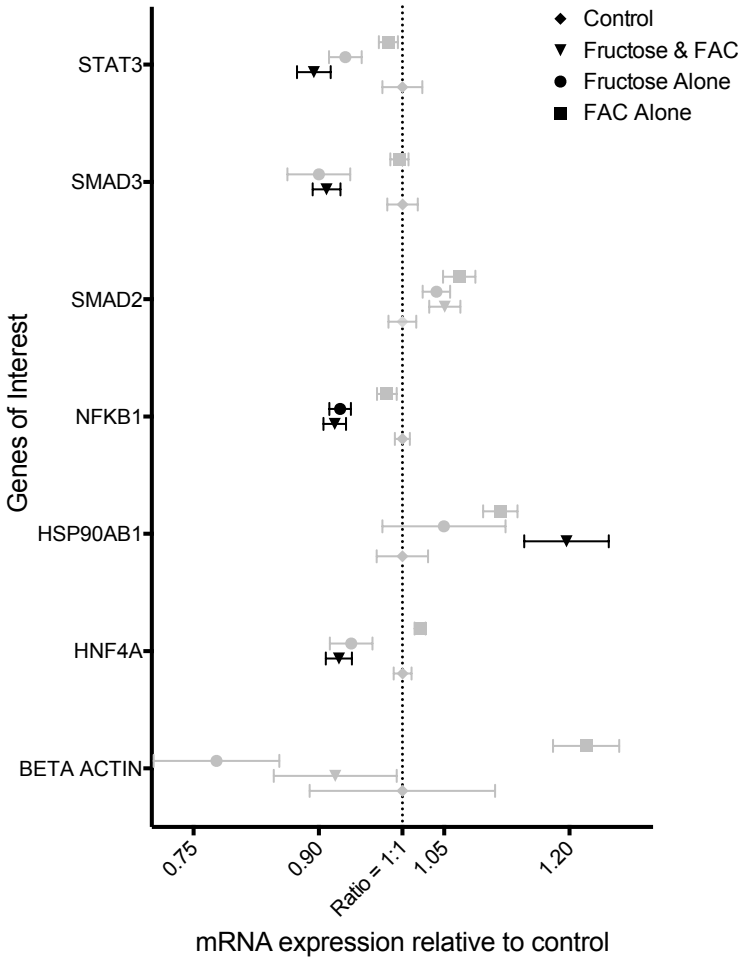

Figure 7.11 Initial phase quantitative real-time rt-PCR effects of fructose (Fruc), iron (FAC), and iron and fructose together (FF) on mRNA expression relative to controls in HepG2 cells.
HepG2 cells grown as a monolayer were treated at 24-48 hours with one of the following: 15 mmol/L fructose (Fruc); 1 µmol/L FAC (FAC); 1 µmol/L FAC and 15 mmol/L fructose together (FF; Fructose and FAC). Changes in RNA expression were quantified by quantitative real-time rt-PCR, normalised to both 27S ribosome and GAPDH. RNA samples were derived from pooled RNA samples used in microarray analysis. All results are expressed as relative expression levels following normalisation to the control group (MEM alone). Data shown in grey were not significantly different from MEM; data shown in black were different compared with MEM (Sidak's multiple comparisons test $p \leq 0.05$).

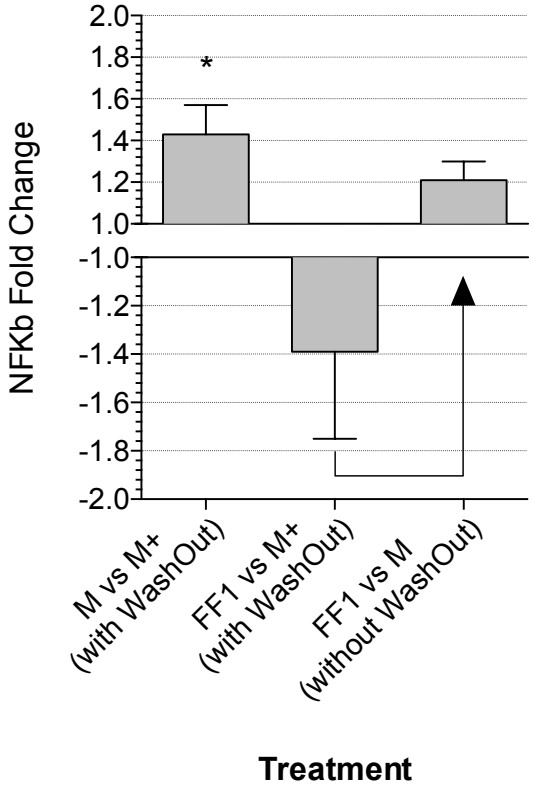

Figure 7.12 Effect of foetal bovine serum (FBS) and energy deprivation on HepG2 NF-κβ mRNA fold changes.

HepG2 control cells that were not FBS and energy deprived (M; no wash-out) were compared both to control cells with a longer period of FBS and energy deprivation (M+; with wash-out) and iron and fructose treated cells (FF1). HepG2 cells grown as a monolayer were treated at 24 or 48 hours with one of the following: MEM alone for 24 hours (M), MEM alone for 48 hours (M+), and 1 µmol/L FAC and 15 mmol/L fructose together (FF1). Changes in RNA fold expression were measured by quantitative real-time rt-PCR, normalised to both 18S and GAPDH reference genes. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as fold change following normalisation to the control group (MEM alone; denoted as M). Each treatment was compared as a ratio to the control M; the logs of these ratios were compared and a significant difference is indicated by an * (Sidak's multiple comparisons test; $p<=0.05$). Similarly, data for linked pairs were analysed using the log of the ratio to the control M using Sidak's multiple comparisons test and considered significant at $p<=0.05$; significance is indicated by an *.

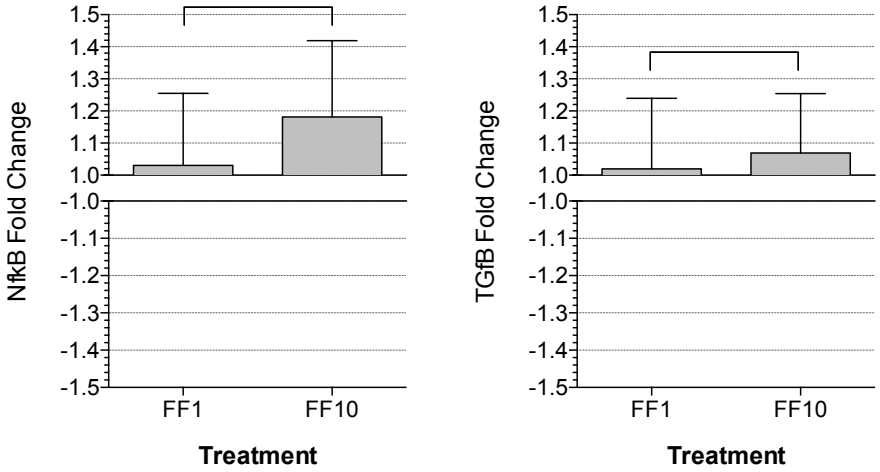

Figure 7.13 HepG2 NF-κβ and TGF-β mRNA fold change expression after treatment with low dose iron and fructose (FF1), and high dose iron and fructose (FF10).

HepG2 cells grown as a monolayer were treated at 24-48 hours with one of the following: 1µmol/L FAC and 15 mmol/L fructose together (FF1); or 10 µmol/L FAC and 15 mmol/L fructose together (FF10). Changes in RNA expression were quantified by quantitative real-time rt-PCR, normalised to both 18S and GAPDH. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as fold change following normalisation to the control group (MEM alone; denoted as M). Each treatment was compared as a ratio to the control M; the logs of these ratios were compared and a significant difference is indicated by an * (Sidak's multiple comparisons test; $p<=0.05$). Similarly, data for linked pairs were analysed using the log of the ratio to the control M using Sidak's multiple comparisons test and considered significant at $p<=0.05$; a significant difference between linked pairs is indicated by a link with an arrow.

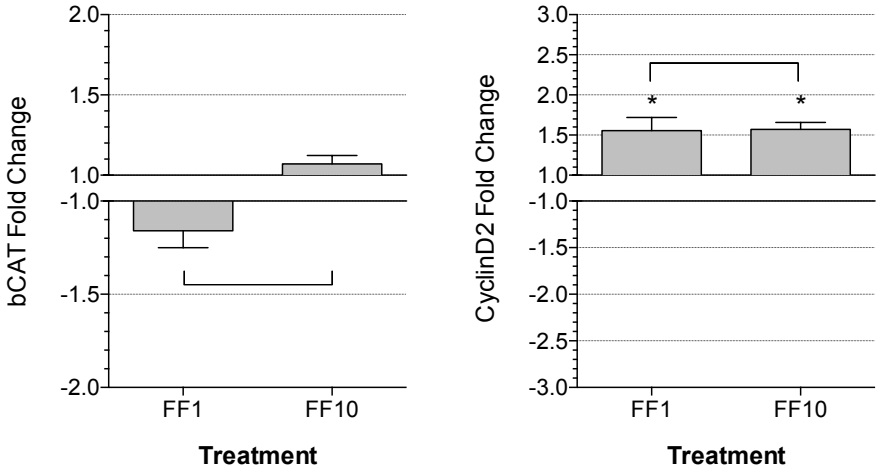

Figure 7.14 HepG2 β-catenin and Cyclin D2 mRNA fold expression change after treatment with low dose iron and fructose, and high dose iron and fructose.

HepG2 cells grown as a monolayer were treated at 24-48 hours with one of the following: 1 µmol/L FAC and 15 mmol/L fructose together (FF1); or 10 µmol/L FAC and 15 mmol/L fructose together (FF10). Changes in RNA expression were measured by quantitative real-time rt-PCR, normalised to both 18S and GAPDH. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as fold change following normalisation to the control group (MEM alone; denoted as M). Each treatment was compared as a ratio to the control M; the logs of these ratios were compared and a significant difference is indicated by an * (Sidak's multiple comparisons test; $p<=0.05$). Similarly, data for linked pairs were analysed using the log of the ratio to the control M using Sidak's multiple comparisons test and considered significant at $p<=0.05$; a significant difference between linked pairs is indicated by a link with an arrow.

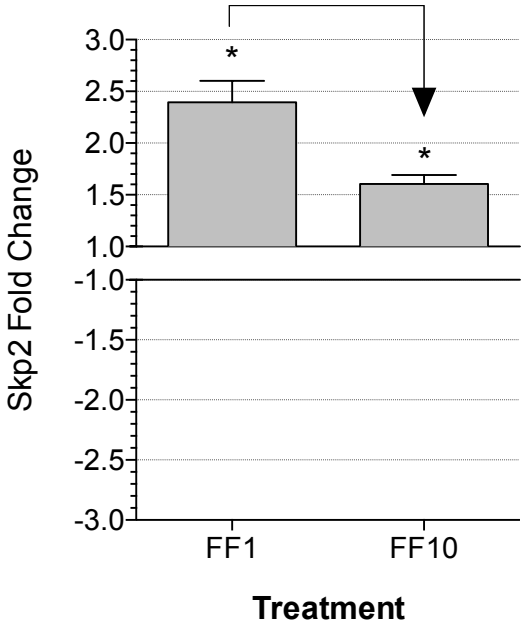

Figure 7.15 HepG2 Skp2 mRNA fold expression change after treatment with low dose iron and fructose, and high dose iron and fructose.

HepG2 cells grown as a monolayer were treated at 24-48 hours with one of the following: 1µmol/L FAC and 15 mmol/L fructose together (FF1); or 10 µmol/L FAC and 15 mmol/L fructose together (FF10). Changes in RNA expression were measured by quantitative real-time rt-PCR, normalised to both 18S and GAPDH. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as fold change following normalisation to the control group (MEM alone; denoted as M). Each treatment was compared as a ratio to the control M; the logs of these ratios were compared and a significant difference is indicated by an * (Sidak's multiple comparisons test; $p<=0.05$). Similarly, data for linked pairs were analysed using the log of the ratio to the control M using Sidak's multiple comparisons test and considered significant at $p<=0.05$; a significant difference between linked pairs is indicated by a link with an arrow.

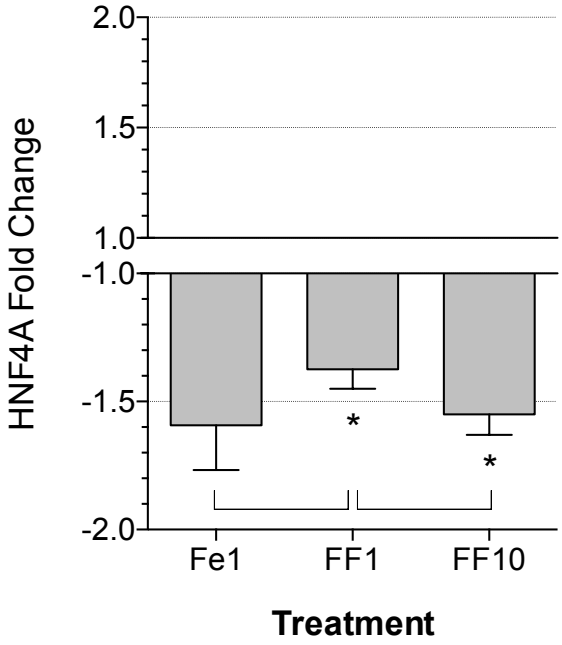

Figure 7.16 HepG2 HNF4a mRNA fold expression change after treatment with low dose iron, low dose iron and fructose, and high dose iron and fructose.

HepG2 cells grown as a monolayer were treated at 24-48 hours with one of the following: 1µmol/L FAC (Fe1); 1µmol/L FAC and 15 mmol/L fructose together (FF1); or 10 µmol/L FAC and 15 mmol/L fructose together (FF10). Changes in RNA expression were measured by quantitative real-time rt-PCR, normalised to both 18S and GAPDH. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as fold change following normalisation to the control group (MEM alone; denoted as M). Each treatment was compared as a ratio to the control M; the logs of these ratios were compared and a significant difference is indicated by an * (Sidak's multiple comparisons test; $p<=0.05$). Similarly, data for linked pairs were analysed using the log of the ratio to the control M using Sidak's multiple comparisons test and considered significant at $p<=0.05$; a significant difference between linked pairs is indicated by a link with an arrow.

7.4.3.1.3: Effect of treatments on HepG2 cell HSP90 protein expression.

HSP90 protein levels did not increase with fructose alone treatments compared with control. Levels increased with treatment with Fe1 and Fe10 by approximately 25 and 10 %, respectively, but this was not statistically significant. Levels increased with treatment with FF1 and FF10 by approximately 45 and 10 % respectively, though this was only significant for FF1 versus MEM ($p \leq 0.05$) (Figure 7.17).

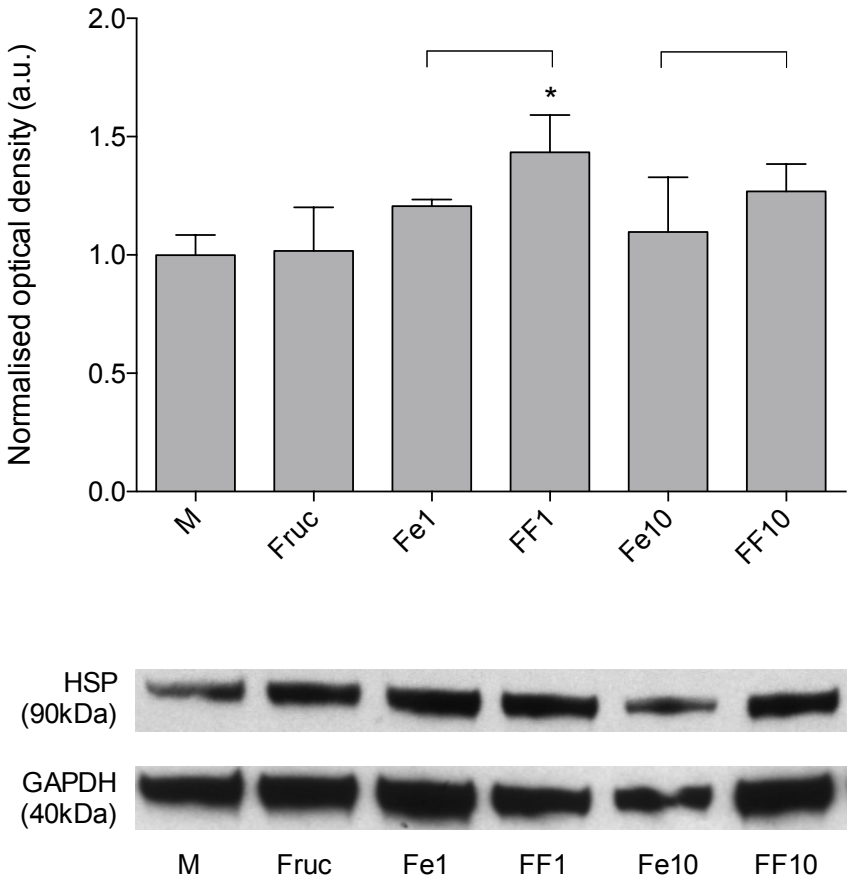

Figure 7.17 Effect of fructose (Fruc), low dose iron (Fe1), high dose iron (Fe10), low dose iron and fructose together (FF1), and high dose iron and fructose together (FF10) on HSP90 protein expression in HepG2 cells.

HepG2 cells grown as a monolayer were treated at 24-48 hours with one of the following: 15 mmol/L fructose (Fruc); low dose iron, 1 µmol/L FAC (Fe1); 1 µmol/L FAC and 15 mmol/L fructose together (FF1); high dose iron, 10 µmol/L FAC (Fe10); or 10 µmol/L FAC and 15 mmol/L fructose together (FF10). Protein expression changes of HSP90 were semi-quantified by Western blot analysis normalised to GAPDH using ImageJ software; representative Western blot results for each treatment are shown above. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as relative expression levels following normalisation to the control group (MEM alone). Each treatment was compared to the control M and a significant difference indicated by an * (Sidak's multiple comparisons test; $p \leq 0.05$). Data for linked pairs were analysed by Sidak's multiple comparisons test and considered significant at $p \leq 0.05$; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis did not indicate a significant ordered response.

7.4.3.1.4: Effect of treatments on HepG2 cell protein expression of HNF4A

There was no significant difference in protein expression of HNF4A in cells treated with fructose alone. Contrary to the results seen in our PCR experiments, protein expression of HNF4A *increased* for cells treated with both Fe1 and Fe10, by approximately 15 and 100 % respectively, and increased for cells treated with FF10 by approximately 40 %.

Individually these differences did not reach statistical significance, but the trend was significant for an increase in the order MEM/Fructose, Fe1, FF1, F10 and FF10 as analysed by Trend analysis ($p \leq 0.05$) (Figure 7.18).

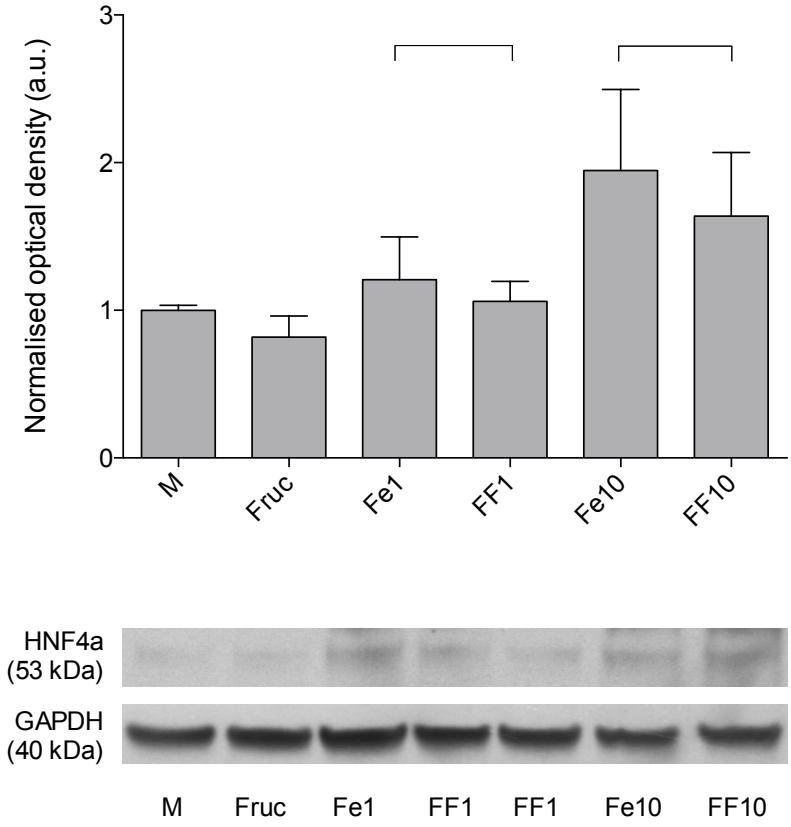

Figure 7.18 Effect of fructose (Fruc), low dose iron (Fe1), high dose iron (Fe10), low dose iron and fructose together (FF1), and high dose iron and fructose together (FF10) on HepG2 HNF4A protein expression.

HepG2 cells grown as a monolayer were treated at 24-48 hours with one of the following: 15 mmol/L fructose (Fruc); low dose iron, 1 µmol/L FAC (Fe1); 1 µmol/L FAC and 15 mmol/L fructose together (FF1); high dose iron, 10 µmol/L FAC (Fe10); or 10 µmol/L FAC and 15 mmol/L fructose together (FF10). Protein expression changes of HNF4A were semi-quantified by Western blot analysis normalised to GAPDH using ImageJ software; representative Western blot results for each treatment are shown above. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as relative expression levels following normalisation to the control group (MEM alone). Each treatment was compared to the control M and a significant difference indicated by an * (Sidak's multiple comparisons test; $p \leq 0.05$). Data for linked pairs were analysed by Sidak's multiple comparisons test and considered significant at $p \leq 0.05$; a significant difference between linked pairs is indicated by a link with an arrow. Trend analysis: trend analysis indicated a significant ordered response ($p \leq 0.05$).

7.4.3.2: Caco-2 cells - second phase quantitative real-time rt-PCR.

7.4.3.2.1: Effect of treatments on Caco-2 cell gene expression of APC and p53.

Fe30 and FF100 did not alter Caco-2 cell APC mRNA levels; Fe100 decreased APC gene expression by approximately 1.3 fold, and FF30 decreased expression of APC by approximately 1.5 fold ($p \leq 0.05$) although this did not reach statistical significance (Figure 7.19). The effect of fructose alone on APC expression has not been tested.

P53 mRNA levels did not change with treatments with Fe30, Fe100, FF30 or FF100 (Figure 7.19). The effect of fructose alone on p53 expression has not been tested.

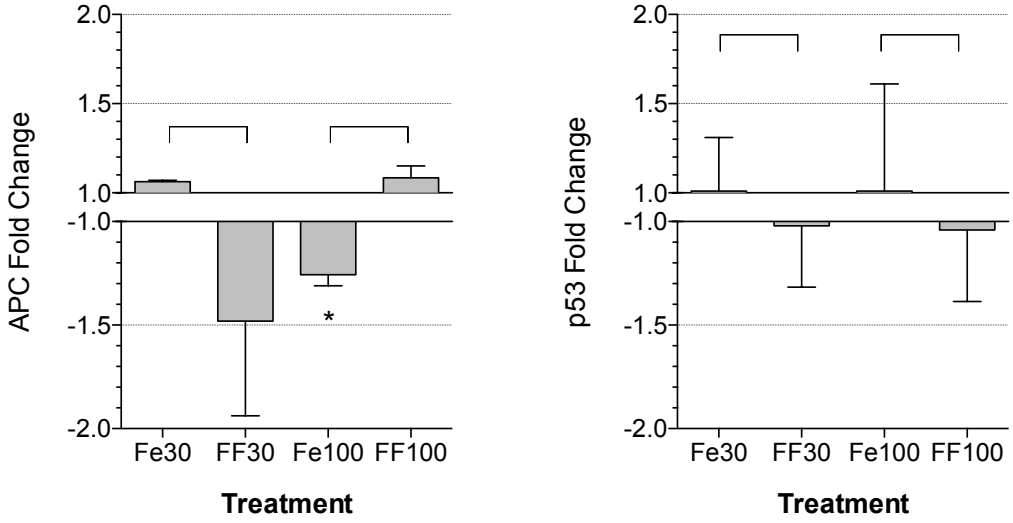

Figure 7.19 Caco-2 cell APC and p53 mRNA fold changes after treatment with iron and fructose.

Caco-2 cells grown as a monolayer were treated at 19-21 days with one of the following: 30 µmol/L $FeCl_3$ alone (Fe30); 30 µmol/L $FeCl_3$ and and 66 mmol/L fructose together (FF30); 100 µmol/L $FeCl_3$ alone; or 100 µmol/L $FeCl_3$ and 66 mmol/L fructose together (FF100). Changes in RNA expression were measured by quantitative real-time rt-PCR, normalised to both 18S and GAPDH. Experiments were repeated ≥ 3 over three separate time points. All results are expressed as fold change following normalisation to the control group (MEM alone). All results are expressed as fold change following normalisation to the control group (MEM alone; denoted as M). Each treatment was compared as a ratio to the control M; the logs of these ratios were compared and a significant difference is indicated by an * (Sidak's multiple comparisons test; $p<=0.05$). Similarly, data for linked pairs were analysed using the log of the ratio to the control M using Sidak's multiple comparisons test and considered significant at $p<=0.05$; a significant difference between linked pairs is indicated by a link with an arrow.

7.5: Discussion

7.5.1: Effects of fructose on iron transporter and homeostasis proteins

7.5.1.1: Caco-2 cells

Enterocyte iron levels are finely adjusted both to meet intracellular iron needs and also whole body iron homeostasis; in iron deficiency gut DMT1 and ferroportin levels increase, which results both in increased iron uptake and also flux through the enterocyte (Zoller, Koch et al. 2001, Taylor, Qu et al. 2011). An increase in these iron transport proteins was not observed in Caco-2 cells after treatment with fructose alone, suggesting that the observed increase in Caco-2 cell ferritin seen with combined fructose and iron treatments was not secondary to increased expression of these transport proteins. However, a limitation of this study was that transporter expression was measured at only a single time point – therefore a transient but physiologically relevant change in transporter expression may have been missed.

Control and fructose treated cells demonstrated the highest levels of DMT1 and ferroportin; this is as expected since these cells would have been cultured in essentially iron free media for 48 hours and thus be iron deficient. Iron treatments alone, and equivalent iron with added fructose treatments, decreased expression of both DMT1 and ferroportin in an iron-dose respondent manner consistent with the expected effects of increased intracellular iron on the expression of these proteins in the gut (Zoller, Koch et al. 2001*)*. However, although fructose and iron together produced higher ferritin levels, compared with iron alone at the same iron concentrations (chapter three and also see reference(Christides and Sharp 2013), the protein expression of DMT1 and ferroportin was not significantly different between these two treatments in this current set of experiments (Figures 7.3 and 7.4), suggesting either that the difference in iron uptake was not high enough to change translational or post-translational levels of these iron transporters, or that the Western blot technique was not sensitive enough to detect subtle differences in protein expression. Ferritin levels in Caco-2 cells treated with iron alone, compared with iron combined with fructose, increased by approximately 40 % (chapter three and also see reference (Christides and Sharp 2013), but in absolute terms ferritin levels only went from ≈ 10 ng ferritin/mg protein to ≈ 14 ng ferritin/mg protein, which may not be enough to significantly alter iron-transport protein expression levels.

As would be predicted, Caco-2 TfR1 mRNA expression decreased with increasing concentrations of iron treatments (Ponka and Lok 1999). Furthermore, there was a significant two-fold difference in TfR1 mRNA expression between Caco-2 cells treated with high dose iron combined with fructose, compared with high dose iron alone, and although not statistically significant, a 30 % difference in mRNA expression between low dose iron combined with fructose, compared with low dose iron alone (Figure 7.1). However, there was no associated difference in TfR1 *protein* expression between iron and fructose versus iron alone treatments, at either high or low dose iron. Similarly, with the protein expression of the two other membrane bound iron transporters discussed above,

this discrepancy may be the result of Western blot lack of sensitivity, an issue that is particularly relevant for membrane associated proteins such as DMT1, ferroportin and TfR1 (Kaur and Bachhawat 2009). Alternatively, the time frame of experiments may have missed an earlier or later occurring alteration in protein expression.

The IRP/IRE system is the main translational control point of cellular iron homeostasis, and the predominant and rapid-response system that maintains intracellular iron levels appropriate to cellular needs (Muckenthaler, Galy et al. 2008). IRP1 is constitutively expressed functioning as a cytoplasmic aconitase in iron replete cellular conditions, and as part of the IRE/IRP system under low iron condition (Walden, Selezneva et al. 2006). In contrast, IRP2 is degraded by the proteasome under iron replete conditions (Vashisht, Zumbrennen et al. 2009) and thus levels are a more direct measure of intracellular iron status. IRP2 levels were highest in control and fructose treated cells indicative of underlying low intracellular iron status and reflecting the 48 hour period of iron depletion prior to and during in vitro digestions; levels decreased with increasing concentration of iron treatments, but there was no difference in IRP2 protein expression between iron alone, and iron and fructose treated cells. Western blot detection conditions suggest that overall Caco-2 cell levels of IRP2 are low as bands were not detected until an extended period of film exposure (20 minutes), and band density was lower compared with other tested proteins; thus detection of small changes in IRP2 protein levels may not have been observable.

In summary, the observed increase in Caco-2 cell ferritin after treating cells with iron and fructose, in comparison with iron alone, does not appear to be related to fructose-induced changes in the iron transporters DyctB, DMT1, ferroportin, TfR1 or the iron-homeostasis protein IRP2, within the experimental time frame of these studies.

7.5.1.2: HepG2 cells

As increased ferritin levels were also observed in HepG2 cells treated with a combination of iron and fructose, compared with iron alone (chapter three, also see reference (Christides and Sharp 2013), evaluation of expression of a number of iron-related genes and proteins was carried out in this cell line. Consistent with the results for Caco-2 cells, no changes in the measured iron-homeostasis proteins in response to fructose alone treatments were found, suggesting that the observed increase in HepG2 cell ferritin after treatment with iron and fructose is not a consequence of increased levels of the measured iron transporters or homeostasis proteins. Once again, however, the important caveat about the single time point nature of these experiments also applies to HepG2 studies.

In contrast with Caco-2 cells, HepG2 cell ferroportin protein levels significantly increased with iron treatments (Figure 7.8). Hepatocyte ferroportin, however, has a 5' IRE that results in ferroportin mRNA degradation in iron-replete conditions, thus this response is consistent with increased iron loading of HepG2 cells (Anderson, Shen et al. 2012). Enterocyte ferroportin is primarily the non-IRE variant (Zhang, Hughes et al.

2009); the increase observed in gut ferroportin levels with iron deficiency is mediated through a hypoxic response element in the gene's promoter area (Taylor, Qu et al. 2011). Similarly, with Caco-2 cell results, no differentiation between cells treated with iron alone, versus same-dose iron combined with fructose, was observed despite documented increases in ferritin for possibly the same reasons outlined above re Caco-2 cell results.

HepG2 TfR1 mRNA expression decreased significantly with iron treatments, and there was a significant difference between iron alone treated cells compared with combined same-dose iron and fructose, suggesting that intracellular iron levels were different enough between iron alone versus iron and fructose treated cells to transcriptionally down regulate TfR1.

Both IRP2 and TfR1 protein levels decreased in HepG2 cells with iron treatments in a dose dependent manner, but in contrast with mRNA changes no difference in protein levels of the transporters between iron and fructose, and iron alone treated cells was observed. This suggests that, as with Caco-2 cells, the Western blot may not be sensitive enough to detect subtle differences in the expression of these proteins, or that intracellular iron differences are not great enough to change protein expression. Lastly, protein changes may have occurred outside the time frame of the conducted experiments and thus transient, or delayed, changes missed.

HepG2 mRNA expression of TfR2, a receptor that is involved in regulatory pathways of iron metabolism, rather than cellular uptake for metabolic needs (Rapisarda, Puppi et al. 2010) was unchanged by fructose treatments suggesting that fructose did not increase iron uptake in HepG2 cells by altering iron homeostatic control mechanisms in the TfR2 pathway within the time frame of these experiments.

7.5.2: Effects of iron and fructose on genes and proteins related to CRC, HCC and NAFLD

Caco-2 and HepG2 cell gene and protein analysis demonstrated significant changes in pathways related to CRC, HCC and NAFLD; there were changes in metabolic pathways, and also in expression of proteins involved in cell signalling underlying inflammation and oncogenesis.

There is epidemiological evidence of an association between iron intake and CRC risk (Nelson 2001, Ashmore, Rogers et al. 2016). Suggested mechanisms by which iron increases CRC risk include reactive oxygen species (ROS) mediated mutations, nutrient support for rapidly proliferating cells, and alterations in cell signalling pathways that underlie malignant cell transformation and promotion (Chua, Klopcic et al. 2010). Aberrations in the canonical Wnt-β-catenin-TCF/LEF signalling pathway underlie the majority of spontaneously occurring cases of CRC (Schneikert and Behrens 2007). Wnt, a secreted protein, binds to one of several membrane receptors; the heterodimer formed then stabilises cytosolic β-catenin allowing it to translocate to the nucleus, bind with TCF/LEF, and function as a transcription complex acting on genes related to

differentiation, proliferation and migration. In low Wnt conditions β-catenin is degraded by a destruction complex consisting of a scaffold with binding domains for β-catenin, Axin, Adenomatous Polyposis Coli (APC), kinases Glycogen synthase kinase 3 beta (GSK3β) and Casein kinase 1α/ε, that then targets β-catenin to the proteasome (Schneikert and Behrens 2007). Mutations in the tumour suppressor APC gene underlie the majority of hereditary CRC cases, and approximately 50 % of spontaneously occurring cases of CRC (Schneikert and Behrens 2007). Decreased expression or loss-of-function APC mutations lead to overexpression of cytosolic β-catenin and its translocation to the nucleus. There β-catenin interacts with the transcription factors TCF/LEF, and increases expression of target genes that promote cancer development (Schneikert and Behrens 2007). Mutations of Casein kinase 1α/ε may have the same effect, as function of the destruction complex is compromised (Schittek and Sinnberg 2014). Microarray analysis in *HepG2* cells revealed decreased levels of Casein kinase 1α (CK1α) and TCF/LEF after treatment with iron alone (Table 7.5); investigation of whether these changes also occur in Caco-2 cells exposed to iron treatments is warranted to evaluate if these changes may play a part in the increased incidence of CRC seen in epidemiological studies in association with iron intake and status. Another interesting microarray finding in HepG2 cells that should be investigated in colon cancer cells was the finding that iron-fructose treatments increased expression of Aldehyde dehydrogenase family 1 member B1 (AL1B1). A recent in vitro and mouse xenograft study demonstrated that suppression of this enzyme, which is responsible for metabolisng the vitamin A derivative retinaldehyde to retinoic acid, was associated with slower growth of colon cancer cells and tumours xenografts (Singh, Arcaroli et al. 2015).

Treatment of Caco-2 cells with high dose iron alone did significantly decrease APC mRNA expression by 1.3 fold, and low dose iron and fructose together also decreased APC expression by 1.5 fold although this did not reach statistical significance (Figure 7.19). Decreased APC levels would be predicted to lead to increased expression of β-catenin and thus Wnt target genes such as Cyclins D1 and D2, as indeed was observed in the HepG2 cell line; dysregulation of Cyclin Ds is associated with malignant transformation of cells because of disordered cell cycle regulation (Musgrove, Caldon et al. 2011*)*. Research using in vitro and mouse models has demonstrated iron induced increased Wnt signalling including increased expression of Cyclin D2, but only in cells with a non-functional APC protein, (Brookes, Boult et al. 2008, Radulescu, Brookes et al. 2012) as is the case in Caco-2 cells, and thus consistent with the results of the studies in this thesis. Further evaluation of the effects of iron and fructose on Caco-2 cell APC gene and protein expression, as well as downstream targets of Wnt signalling, is warranted to explore these findings. Furthermore, exploration of the effects of iron and fructose in other colon cancer derived cells would provide more robust evidence that the findings have broad implications. A significant amount of iron and fructose is consumed in the modern diet, therefore even modest changes in a pathway that is central to CRC disease pathogenesis may have important consequences.

Figure 7.20 presents a simplified version of Wnt signalling including the possible effects of iron and fructose treatments as observed in the experiments in this thesis chapter.

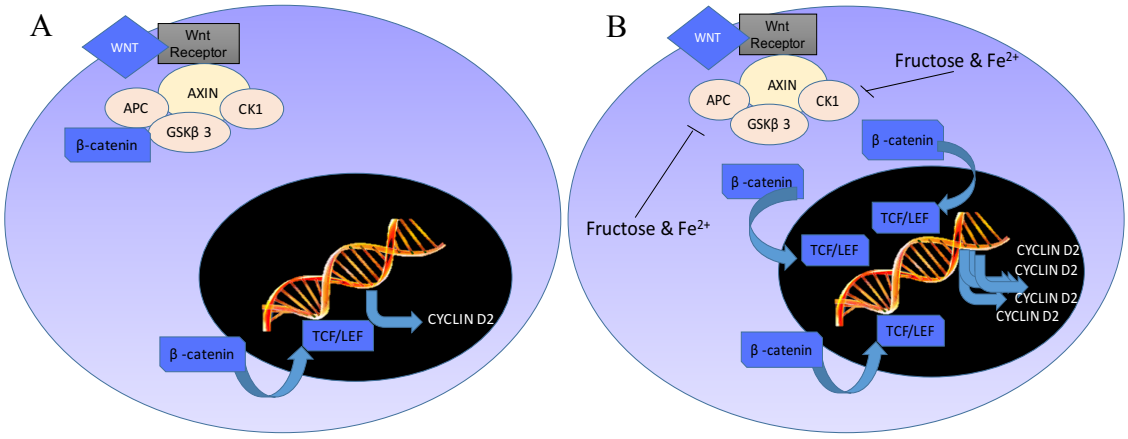

Figure 7.20 Simplified Wnt signalling pathway under: A) Physiological conditions, and B) After iron and fructose exposure.
Figure **7.20(A)**: Under physiological conditions with low levels of Wnt, β-catenin is primarily bound to the destruction complex consisting of APC, Axin, Gskβ 3 kinase (GSKβ3) and Casein kinase 1α (CK1) and degraded by the proteasome (not shown); therefore transcription of Wnt target genes including Cyclin D2 is limited. Figure **7.20(B)**: Iron and fructose decrease expression of APC and Casein kinase 1α leading to decreased binding of β-catenin by the destruction complex, and therefore increased intracellular levels of β-catenin that then translocate to the nucleus, bind with TCF/LEF and increase transcription of Wnt target genes such as Cyclin D2. If this leads to unregulated cell replication and proliferation malignant transformation may occur.

There is also an intriguing possible connection between Wnt signalling and metabolic reprogramming in colon cancer. Cancer cell metabolism is increasingly recognised as part of the oncogenic process itself, rather than simply a by-product of mutagenesis, or an adaption required for rapidly proliferating cells (Seyfried and Shelton 2010, Seyfried 2012). Aerobic glycolysis, in which cancer cells in the presence of oxygen ferment glucose (or fructose) to lactate rather than pyruvate -- and thus effectively uncouple glycolysis from pyruvate oxidation - was originally described by Otto Warburg and is known as the Warburg Effect (Warburg 1956). Current evidence suggests that cancer cells reprogramme glycolysis to produce anabolic substrate precursors and reducing equivalents that support the production of nucleotides, lipids and proteins required for cell proliferation, in addition to providing energy. An in vitro study using colon cancer cells and in vivo tumour xenografts found evidence that Wnt-induced TCF/LEF alterations changed metabolism-related target genes; the changes then created a favourable tumour environment (Pate, Stringari et al. 2014). Both iron and fructose can alter cell metabolism, therefore how the two nutrients together affect colon cell metabolism, and indeed metabolism of liver cells that are also exposed to significant amounts of both of these nutrients, requires further attention as iron and fructose may act synergistically to

support cancer cell metabolism and proliferation. In the proposed model of cancer as a metabolic disorder, it is hypothesised that mitochondrial dysfunction as a result of an insult, such as oxidative stress, both impairs cellular respiration and leads to mutations that promote tumourigenesis (Seyfried 2012). Furthermore, some researchers suggest that disordered intracellular iron metabolism underlies or contributes to this mitochondrial dysfunction (Robert L Elliott 2012). Lastly, fructose is also metabolised through the glycolytic pathway, but critically *after* the main point of homeostatic control in glycolysis by phosphofructokinase (Mayes 1993, Timson 2012), thus fructose effectively will continue through glycolysis without negative feedback and glycolytic products will be a function of fructose concentration . Putting all of the above together, intracellular iron may lead to cancer causing mutations, with associated metabolic changes and cellular requirements that are then supported by the unregulated flux of fructose through glycolysis. The importance of glycolysis to cancer metabolism is underscored by the observation that one of the functions of the tumour suppressor protein p53 is suppression of this metabolic pathway, and loss of p53 may contribute to conversion of cells to aerobic glycolysis (Olovnikov, Kravchenko et al. 2009). In the research for this thesis neither iron alone nor iron and fructose together altered p53 mRNA expression, but analysis of p53 protein expression and localisation after iron and fructose treatments would more definitely rule out fructose-iron induced changes in this tumour suppressor. Figure 7.21 provides a simplified schematic presentation of possible mechanisms by which iron and fructose could promote cancer cell transformation and proliferation.

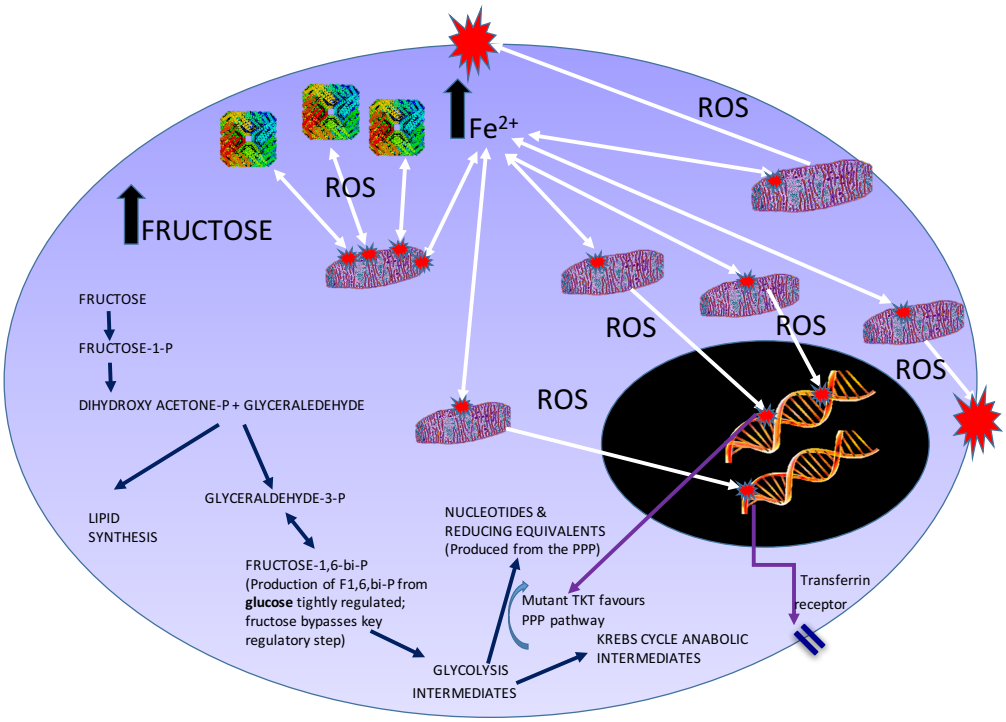

Figure 7.21 Model of disordered iron and carbohydrate metabolism in cells that may synergistically lead to initiation and promotion of cancer.
Excess or dysregulated intracellular iron increases reactive oxygen species (ROS) that then lead to damage of lipid membranes, intracellular proteins, mitochondria and DNA. The result is activation of cellular stress responses, mutations in enzymes that lead to tumour-favourable metabolic products such as changed transketolase isoforms producing more nucleotides (Liu, Huang et al. 2010), mutations in tumour promotion and suppressor genes, and also in iron import proteins such as Transferrin Receptor 1 (Brookes, Hughes et al. 2006, Boult, Roberts et al. 2008) to support increased cellular metabolic and proliferative needs. ROS impaired ferritin damage may also release more iron that then through Fenton chemistry increases ROS thus continuing the cycle and further increasing oxidative stress. Iron induced mitochondrial damage may both impair cellular respiration, and also lead to increased release of ROS, once again continuing the cycle. As the cell begins to undergo malignant transformation it converts to aerobic glycolysis to meet metabolic requirements; fructose facilitates the production of needed anabolic substrates through unregulated entry into glycolysis thus supporting malignantly transformed cells survival and proliferation.

The metabolic changes observed by Warburg are seen in the majority of cancer cells, and thus the above discussion may also apply to the effects of iron and fructose on liver cells, and both iron and fructose intake have been associated with HCC in epidemiological studies (Tasevska, Jiao et al. 2012, Kew 2014). As mentioned, the gene changes in Casein kinase 1α, Cyclins D1 and D2, and TCF/LEF were actually observed in HepG2 cells, although at this time only the increase in Cyclin D2 has been verified by quantitative real-time rt-PCR. Altered Wnt signalling is implicated in HCC pathogenesis (Clevers and Nusse 2012), therefore further verification of microarray results including analysis of Wnt signalling, β-catenin, TCF/LEF, Cyclin D1, and Casein Kinase 1α gene

and protein expression, and cellular localisation, with iron and fructose treatments is warranted. Investigation of HepG2 β-catenin expression by quantitative real-time rt-PCR was carried out and found to be unchanged by any treatment. This is not surprising given that Wnt- β-catenin associated mutations typically act by changing either protein expression or intracellular location (i.e. cytoslic versus nuclear) rather than gene expression of β-catenin.

Expression of several other HCC-related genes was changed by iron and fructose treatments. HSP90 expression increased; the change was confirmed by quantitative real-time rt-PCR, and furthermore, Western blot analysis confirmed a moderate but significant increase in HSP90 protein expression with iron and fructose treatments.

Mutations and over expression of HSP90 are associated with HCC. HSP90 is a constitutively expressed molecular chaperone that co-chaperones some 200 proteins involved in signalling pathways, metabolism, and stress adaptation, and elevated expression of HSP90 is associated with increased risk and progression of HCC (Sun, Xing et al. 2007). Recently, evidence has emerged that suggests cancer cells hijack HSP90 and use it to chaperone and protect abnormal proteins essential for cancer cell survival (Whitesell and Lindquist 2005, Trepel, Mollapour et al. 2010); the centrality of HSP90 to oncogenesis is illustrated by the large number of anti-HSP90 drugs currently being developed as chemotherapeutic agents including for HCC (Trepel, Mollapour et al. 2010). Therefore, further study of the effects of iron, and combined iron and fructose, on expression of HSP90, and in particular key HSP90 client protein expression and trasnport analysis is warranted.

There were expression changes noted in HepG2 cells in several other genes associated with CRC and HCC after iron-fructose treatments. The largest increase in gene expression occurred in the proto-oncogene S phase kinase-associated protein (Skp2); Skp2 mRNA levels increased 2.5 fold after iron-fructose treatments as measured by second phase quantitative real-time rt-PCR. Skp2 over expression is associated with worse prognosis in both CRC and HCC; it is part of a ligase complex that mediates the degradation of cell cycle regulatory proteins and it is believed that the mechanism by which it promotes oncogenesis is through increased/dysregulated destruction of key molecules in cell cycle progression (Hershko 2008, Shin, Kim et al. 2012). Furthermore, an in vitro study found that Akt1, a HSP90 client protein, controls Skp2 protein stability and RNA expression, and it was postulated that this was one of the underlying mechanisms by which Akt promotes tumourigenesis (Gao, Inuzuka et al. 2009).

Expression of Histone Deacytylase 1 and C-terminal binding protein (CtBP1), transcriptional co-repressors that when mutated are associated with HCC, was elevated by iron-fructose treatments. HDAC1 has been proposed to cause HCC by silencing of tumour suppressor genes (Iakova, Timchenko et al. 2011). Lastly, microarray analysis demonstrated decreased expression of c-Jun/c-Fos, and Janus kinase 1, after treatments with iron and fructose together. C-Jun and c-Fos together make up AP-1, a transcription

factor involved in control of the cell cycle amongst many other functions. Overexpression or dysregulation of these proto-oncogenes is associated with multiple malignancies and has also been associated with disease progression in NAFLD (Kodama and Brenner 2009). Decreased expression of these proto-oncogenes would be predicted to be protective against malignant transformation. Further research is needed to determine if the observed alterations in gene expression of HepG2 cells exposed to iron-fructose treatments leads to associated alterations in protein expression and localization, and also downstream targets of these genes. Furthermore, other than for Skp2 in which results were confirmed under non-energy/FBS deprived conditions, all the remaining changes need to be evaluated in no wash-out experiments, and verified by further PCR work.

Research both in vivo and in vitro suggests that both iron and fructose increase the risk for disease progression in NAFLD. One of the major pathways in the development of NASH is the NF-κβ/STAT inflammatory pathway. This pathway is stimulated both by fatty acids and also by reactive oxygen species (ROS), and studies have demonstrated that fructose increases de novo hepatic lipogenesis (DNL) and free fatty acid (FFA) flux; it has been proposed that by increasing DNL and FFAs fructose increases NF-κβ activity and thus promotes an inflammatory response in the fatty liver (Mouzaki and Allard 2012). Iron has also been shown to increase ROS-induced NF-κβ signalling (Bhattacharyya, Ghosh et al. 2012). In addition, the TGF-β/SMAD pathway is the other pathway implicated in NAFLD pathogenesis (Yang, Roh et al. 2014), and once again there is evidence that iron and fructose increase TGF-β/SMAD pathway activity (Lee and Friedman 2011). Thus it was an unexpected finding that gene expression of SMADs 2 and 3, and NF-κβ and STAT 3-- key proteins in the above pathways -- were *decreased* by iron and fructose treatments in liver cells both in the microarray analysis, and then follow up initial quantitative real-time rt-PCR. It was hypothesised that comparatively decreased levels of the above genes was in response to energy provided with fructose, or fructose and iron treatment of cells, compared with iron alone or MEM alone treatment of HepG2 cells. Treatment of cells with iron alone in the microarray analysis resulted in increased expression of 5' AMP-activated protein kinase (AMPK) gamma subunit, the regulatory subunit of this key kinase involved in sensing cellular energy homeostasis. AMPK increases in response to low cellular energy levels leading to increased activity of energy generating pathways, and decreased activity of anabolic pathways (Hardie, Ross et al. 2012). Consistent with a low cellular energy state in iron treated cells, expression levels of stearoyl-CoA desaturase (SCD), fatty acid desaturase 1 (FAS) and acyl-CoA synthetase (ACC)- key anabolic proteins involved in fatty acid synthesis- were decreased in cells treated with FAC alone; AMPK decreases transcription of all of these anabolic related genes (Hardie, Ross et al. 2012). Thus one possible interpretation of the microarray results is that low energy induced stress changes in control cells, though not as significant as in iron alone treated cells, led to comparatively lower levels of SMADs 2 and 3, and NF-κβ and STAT 3 in combination iron and fructose (i.e. energy provided) treated cells. Consistent with this hypothesis, initial phase quantitative real-time PCR

revealed that fructose alone also decreased expression of NF-κβ, STAT3 and SMAD3 (Figure 7.11).

Another possible explanation for the observed changes in the SMAD and NF-κβ pathways is that the increased ferritin formation found in previous experiments is actually protective against inflammation by trapping iron within ferritin, rather than increasing levels of "free" intracellular iron. ROS production and cell stress are related to "free" iron from the labile iron pool, and ferritin is protective against oxidative stress by binding iron. Thus if iron and fructose treatments only increased ferritin rather than "free" iron there might be no deleterious effects observed. Lastly, work by Spasojević et al, and others, suggests that short-term exposure to fructose may be protective against oxidative stress by chelating free intracellular iron, and thus rendering it less reactive (Valeri, Boess et al. 1997, Spasojevic, Bajic et al. 2009).The second phase PCR experiments, conducted in HepG2 cells with no wash-out, suggests that at least the NF-κβ expression changes did relate to energy deprivation as NF-κβ mRNA levels were increased after iron-fructose treatments of control cells with a wash-out in comparison with no wash-out control cells (Figure 7.12).

Expression of several genes involved in lipid and carbohydrate metabolism were altered by iron-fructose HepG2 treatments. Microarray analysis demonstrated increased expression of the enzyme Aldo-keto reductase family 7, member A2 (AKR7A2). A recent in vitro study in HepG2 cells found that methyl glyoxal induced expression of this enzyme as a protective mechanism (Li, Ma et al. 2015), and previous work by several research groups suggests that fructose may be hepatotoxic and mutagenic, in part, through iron Fenton-chemistry associated production of toxic metabolites such as methyl glyoxal (Manini, La Pietra et al. 2006, Lee, Bruce et al. 2009). The observed increase of this enzyme in these experiments is consistent with the above, and it is significant that increased expression was only noted in iron-fructose treated cells rather than fructose alone suggesting that exogenous iron did indeed exacerbate toxin production. Lastly, another enzyme induced by iron-fructose treatments, but not by fructose alone, was cytosolic glycerol-3-phosphate dehydrogenase (GPD1); this enzyme catalyses the reversible conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) and regenerates NAD+ from NADH (simplified role of G6PD in glycolysis is illustrated in Figure 7.22). GPD1 is central to both glucose and fructose metabolism, maintains reducing equivalents for biosynthesis, and also provides the glycerol backbone for lipid biosynthesis. Indeed, one study suggested that increased expression of this enzyme in adipose tissue increased lipogenesis/triacylglycerol synthesis, and mutations in this gene have been associated with fatty liver and fibrosis (Swierczynski, Zabrocka et al. 2003, Basel-Vanagaite, Zevit et al. 2012). It is interesting that the addition of iron would increase its expression over and above fructose alone, suggesting a possible mechanism by which iron and fructose together would synergistically increase hepatosteatosis if this effect also occurred in vivo.

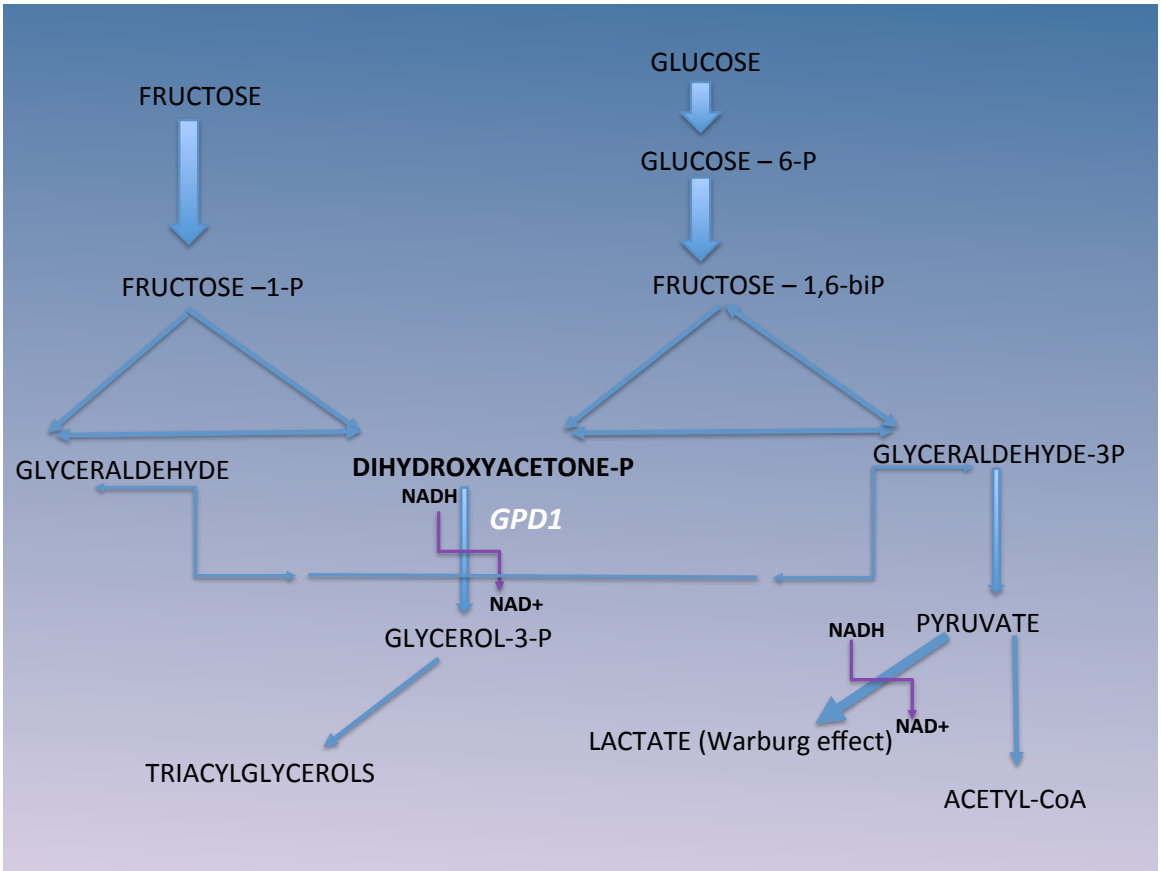

Figure 7.22 Role of cytosolic-glycerol-3-phosphate dehydrogenase (GPD1) in glucose and fructose metabolism.
Fructose or glucose enter the cell and are phosphorylated at the 1 and 6 carbon, respectively (fructose-1-P, glucose-6-P). Glucose-6-P undergoes isomerisation to fructose-6-P, and then undergoes another phosphorylation at the one carbon to form fructose 1,6 biphosphate (fructose-1,6-biP) – this is a one of the rate limiting steps in glycolysis and also a major regulatory point of the cycle. ATP and citrate, indicators of a high energy cellular state inhibit glycolysis at this point, but because fructose does not undergo this enzymatic step it is not inhibited and continues through glycolysis dependent on concentration. Both fructose-1-P and fructose-1,6-biP are then cleaved to **dihydroxyacetone phosphate**; the other three carbons in glucose form glyceraldehyde-3phosphate (glyceraldehyde-3P), whereas glyceraldehyde is formed from the remaining three fructose carbons. However, fructose-derived glyceraldehyde can be freely phosphorylated to glyceraldehyde-3P, and dihydroxyacetone phosphate and glyceraldehyde-3P are also in equilibrium. Glyceraldehyde-3P can then be further metabolised to pyruvate, or lactate (the Warburg effect if this occurs in the presence of oxygen). GPD1 catalyses the reversible conversion of dihydroxyacetone phosphate (DHAP) and reduced nicotine adenine dinucleotide (NADH) to glycerol-3-phosphate (G3P) and NAD+. G3P can then be used as the backbone for triacyglycerol synthesis.

Another potentially significant effect of iron and fructose treatments on HepG2 cells were changed levels of HNF4A mRNA and protein expression. Initial microarray analysis demonstrated that iron alone decreased expression of HNF4A, a member of the nuclear receptor superfamily that is involved in transcriptional regulation of numerous genes

involved in liver glucose and lipid metabolism, cellular development and differentiation, and inflammation. Mutations of the HNF4A gene, and alterations in HNF4A expression have been associated with diabetes, liver and colon inflammation, fatty liver, CRC and HCC (Hayhurst, Lee et al. 2001, Tanaka, Jiang et al. 2006, Saif-Ali, Harun et al. 2012, Babeu and Boudreau 2014, Ning, Ding et al. 2014). Follow up initial quantitative real-time rt-PCR revealed that combined iron and fructose decreased HNF4A mRNA expression by approximately 10 %; iron alone had no statistically significant effect (Fig 7.11). Second phase PCR confirmed the finding that both low, and high dose iron, combined with fructose significantly decreased HNF4A mRNA expression; iron alone also decreased mRNA levels 1.5 fold but this was not statistically significant (Figure 7.16). The relative consistency of these findings over different experimental conditions and different assays suggests that iron and fructose do indeed decrease HNF4A mRNA in a dose dependent manner. However, the opposite effect was noted for protein expression of HNF4A in which increasing iron concentration, with or without fructose, was significantly associated with increased protein expression in a dose dependent manner (Figure 7.18). HNF4A itself is transcriptionally, translationally and post-translationally regulated by multiple factors that may alter its activity through acetylation, phosphorylation and methylation (Schrem, Klempnauer et al. 2002) – levels of these modified forms of HNF4A might be relatively increased despite overall decreased mRNA expression, explaining the discrepancy between mRNA and protein expression levels depending on what variant was recognised by the primary antibody. Alternatively, HNF4A has at least 7 different splice variants. The Western blot antibody used recognises the canonical HNF4A isoform one (Cell Signaling Technology technical support, Dr R Duffy, personal communication March 2016), whereas the RNA hydrolysis probe recognizes the isoform two HNF4A variant (ThermoFisher Scientific website, accessed March 2016) – the two isoforms share some homology but differ overall, therefore it is possible that the iron and fructose differentially alter HNF4A isoform expression at the translational level, decreasing mRNA expression of isoform one (recognized by the PCR probe), but increasing expression of isoform 2 (recognized by the Western blot antibody). Until these different possibilities have been resolved it is difficult to predict what impact the observed iron-fructose effects on HNF4A would have.

The most robust expression change seen in fructose-alone treated HepG2 cells was the activation of multiple genes of all three complement pathways, and the associated increased expression of Clusterin and the Membrane Attack Complex. Complement activation is typically induced by infection, but there were no gene changes identified to suggest bacterial HepG2 cell infection; levels of bacterial ribosomes were not detected (for example Ribosomal 30-, 23-, 20-, 16- or 5S expression), nor on gross inspection of the cells was there evidence of bacterial or fungal infection. A low grade viral infection can not, however, be ruled out although the observed robust growth of HepG2 cells in culture argues against this. On balance this suggests that fructose itself did increase expression of these genes. Recently, complement activation has gained recognition as a contributory factor in metabolic disorders including NAFLD and type 2 two diabetes

(Rensen, Slaats et al. 2009). An in vitro study demonstrated that fructoselysine, a glucose derived amadori product formed by the binding of glucose to protein/peptide lysine residues, caused complement activation (Fortpied, Vertommen et al. 2010). The cell culture media used in the experiments in this thesis contained amino acids, therefore it is possible that added fructose could form amadori products in culture media over the duration of these experiments, and by a similar mechanism activate the HepG2 complement systems. There are no documented reports of this, to the author's knowledge, therefore initial investigations should look at whether the observed findings can be replicated, and if so over what time periods and fructose doses.

Iron-alone treated HepG2 cells displayed the least number of changed genes, and surprisingly there was no expression change of any directly iron-related genes in the top 10 pathways. Microarray analysis is a powerful and broad screening tool for exploring altered gene expression in response to myriad exposures, however it is subject to false

positive results secondary to the vast amount of data generated, and furthermore both the technical processes involved and data analysis are complex with multiple systems available to carry out tests and interpret results. Both low sensitivity of probes and discrepancies in fold-change expression have been described by others (Kothapalli, Yoder et al. 2002), therefore the microarray may have missed subtle changes in iron-related genes. This is corroborated by quantitative real-time rt-PCR HepG2 experiments that clearly showed changes in TfR1 mRNA expression in response to iron treatments at the same iron concentration as used in microarray experiments (Figure 7.7).

An interesting finding in relation to cancer pathogenesis and iron-alone treatments revealed by microarray analysis was the iron-induced decreased expression of prostaglandin E2 Receptor (PGE2R). Increased PGE2R signalling underlies multiple cancer-associated pathways and cancers (Wang and Dubois 2010), therefore iron would be predicted to have a protective or anti-tumour effect if it did indeed decrease expression of this receptor. The mechanism by which iron might decrease expression of this receptor, however, is not clear.

Microarray analysis revealed that Beta actin was increased by iron-alone treatments; quantitative real-time rt-PCR also revealed a 1.2 fold increased expression of actin with iron-alone treatments although this did not reach statistical significance. The actin cytoskeleton is essential for multiple cellular functions, and pertubations in actin secondary to oxidative stress are well described (Dalle-Donne, Rossi et al. 2001). An in vitro study in isolated rat endothelial cells specifically found that iron-induced oxidative stress led to actin remodelling, although gene expression changes were not analysed (Gorbunov, Atkins et al. 2012). The above suggest that HepG2 iron treatments induced increased actin mRNA expression secondary to iron-induced oxidative stress, and it would be valuable to confirm if this change translates to protein expression both as a mechanism of cell injury related to liver fibrosis, but also in view of the ubiquitous use of actin alone as a loading control protein for Western blot analysis.

7.6: Conclusion

In summary, fructose did not alter levels of measured iron transporter and homeostasis proteins within the time frame of these experiments; the increase in both Caco-2 and HepG2 cell ferritin levels associated with combined iron and fructose does not appear to be secondary to changes in iron-related proteins.

Iron and fructose did alter expression of multiple genes and proteins associated with CRC, HCC and NAFLD including genes associated with inflammatory pathways such as NF-κB, several key cell cycle related genes and proteins including Cyclin D2, and a key liver transcription factor HNF4A. If these changes are confirmed in vivo they may have important physiological ramifications in relation to CRC, HCC and NAFLD.

7.7: Limitations

There are multiple limitations in the above described studies. Although no changes in iron-transport and homeostasis molecules was observed that would explain the mechanism(s) by which iron-fructose treatments increased Caco-2 and HepG2 ferritin levels, the time frame of experiments means that such changes may have been missed. Further experiments over several time points would address this question. In addition, a broader range of proteins involved in iron transport and homeostasis, in both cell lines, should be evaluated to more fully address this question. Lastly, investigation of the effects of different doses of fructose and iron would also be useful to see if there is a dose-response effect on any of the above proteins.

Microarray, PCR and protein expression analysis studies revealed many interesting changes in relation to all cell treatments that potentially could have pathophysiological consequences if they also occurred in vivo. However, the microarray studies should be repeated without a wash-out period, in both Caco-2 and HepG2 cells, and ideally rather than using pooled samples the experiments should be repeated over three separate times (i.e. three biological replicates) to improve the statistical robustness of results. There were changes identified in follow up PCR studies, conducted under appropriate conditions, that may help to start to explain molecular mechanisms underlying observed effects of iron and fructose on CRC, HCC and NAFLD pathogenesis; for example, the effects of iron and fructose on HSP90, but deeper exploration of the complex networks associated with these changes is needed before any conclusions can be drawn.

Lastly, repetition of the experiments carried out in this thesis in several different cell lines as models of the gut and liver would increase the probability that the results are broadly applicable.

Chapter Eight: conclusion and future work

8.1: Conclusion

The main hypothesis of this research work was that fructose, the related sweetener high fructose corn syrup (HFCS), and the prebiotics fructo- and galactoligosaccharides (FOS & GOS) would increase non-haem iron bioavailability as measured by ferritin formation in two in vitro cell line models of the gut and liver, respectively.

The findings of this research support the above hypothesis with the important caveat that the presence of polyphenols and phytates, known iron uptake inhibitors, negate this effect. Fructose, and a mixture of FOS & GOS, respectively, added to cell culture media, as a component of liquid iron supplements, or added to milk-based products, increased ferritin formation from non-haem iron in Caco-2 cells. Furthermore, fructose added to ferric iron-ascorbic acid solutions at a neutral pH had an additive effect on iron bioavailability compared with the known enhancing effects of ascorbic acid. Fructose added to ferric iron in solution also increased HepG2 ferritin levels. Fructose present in a sweet potato-based infant food with high levels of phytates and polyphenols, however, did not appear to improve iron bioavailability indicating that in a mixed diet with high levels of these inhibitors the effects of these carbohydrates on iron would be inhibited. The addition of fructose to liquid iron supplements, however, might improve their iron bioavailability, and as only small amounts of supplements are used on a daily basis this would not be expected to lead to excessive fructose intake. Some supplements already add ascorbic acid to improve iron absorption, however, fructose may further improve supplement iron bioavailability as it had an additive effect with ascorbic acid, and may be more stable in solution over time. Its sweet taste could also improve palatability. Lastly, FOS & GOS prebiotics added to milk-based products may improve the iron absorption from these formulations targeted at toddlers, a group who are at high-risk for iron deficiency anaemia.

Biochemical assessment of ferrous iron formation using the ferrozine assay suggested that the basis for the observed improved non-haem iron bioavailability after fructose treatments related to fructose-induced reduction of ferric iron to the more bioavailable ferrous iron form. No evidence for a molecular based improvement in iron uptake related to alterations in iron-transport proteins was demonstrated, although such changes may have been missed within the time frame of experiments.

The secondary hypothesis of this research was that epithelial colon and liver cell exposure to iron and fructose would alter gene and protein expression of molecules involved in colorectal cancer (CRC), hepatocellular carcinoma (HCC) and non-alcoholic fatty liver disease (NAFLD). Such changes were found by microarray, polymerase chain reaction and protein expression analysis studies. In particular, there were changes in expression of genes and proteins: in the the Wnt signalling pathway that underlies the majority of cases

of CRC; the NF-κβ and TGF β pathways implicated in development of NAFLD; several cell cycle related molecules including Cyclin D1 and Cyclin D2, and the proto-oncogene Skp2; and HNF4A, a key liver transcription factor, associated with CRC, HCC and NAFLD. The assay conditions that produced these results may not have been optimal in all studies, but given the significant intake of both iron and fructose in the modern Western diet, and the high exposure levels of the two organ systems represented by the in vitro models used, follow up of these findings is warranted. This could also be an important issue for individuals with high levels of blood non-transferrin bound iron, such as those with hereditary iron overload disorders, or haemoglobinopathies. In addition, these possible effects should be evaluated before any recommendations to add fructose to iron supplements are suggested. Furthermore, there are already supplements containing iron and fructose, thus there is a need to evaluate the effects of such supplements, and indeed the effects of a high iron meal eaten with a large sized fructose or HFCS sweetened beverage.

8.2: Future work

The molecular investigation of iron and fructose effects on expression of key proteins in colon and liver cells needs to be completed, both with respect to iron-homeostasis and transport proteins, and also with respect to changes that may have pathological consequences. Suggested further work on changes to disease-associated molecules after iron and fructose exposure in Caco-2 and HepG2 cells is discussed in chapter 7. In addition, it would be valuable to look at iron-fructose changes induced in other cell types that are associated with colon and liver disease. For example, there is evidence that hepatic stellate cells and macrophages may play roles in both NAFLD and HCC, and also that alterations in the gut immune system cells cause disease. As with colonocytes and hepatocytes, these cells are also exposed to high levels of both fructose and iron.

The effects of fructose on ferritin and transferrin-bound iron remain unknown at this time. It would be valuable to evaluate whether fructose alters their bioavailability, or leads to any change in significant genes or proteins.

Lastly, the above work has been conducted in in vitro models. As George Box stated - "The most that can be expected from any model is that it can supply a useful approximation to reality: All models are wrong; some models are useful". Caco-2 and HepG2 models are useful, but before the findings of this thesis can be disseminated for practical applications the results need to be confirmed in vivo.

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Enrichment analysis report

Enrichment by Pathway Maps

#	Maps	Total	pValue	Min FDR	FAC+Fruc vs control unique to FAC+Fruc changes genego_FAC/Fruc vs control unique fold change (values) p-value	FDR	In Data	Genes from Active Data
1	Triacylglycerol metabolism p.1	28	0.000	1.497	0.000	1.497	10	ACSL6, AKR1C1, AL1B1, ACSL1, GKP3, GLPK, GNPAT, ACSL3, AKR7A2, GPD1
2	Immune response_IL-27 signaling pathway	24	0.001	0.828	0.001	0.828	8	STAT3, gp130, NF-kB, JAK1, ICAM1, Tyk2, IL-27 receptor, IL27RA
3	Signal transduction_Activin A signaling regulation	31	0.001	0.828	0.001	0.828	9	Ubiquitin, FKBP12, N-CoR, HDAC1, SMAD3, Histone H3, SMAD2, Histone H2, Inhibin alpha subunit
4	Immune response_HSP60 and HSP70/ TLR signaling pathway	52	0.001	0.823	0.001	0.823	12	Ubiquitin, MHC class I, AP-1, NF-kB, CD14, NF-kB1 (p105), HSP60, ICAM1, HSP70, MEK3(MAP2K3), MEK6(MAP2K6), c-Jun
5	Vitamin E (alfa-tocopherol) metabolism	17	0.002	0.657	0.002	0.657	6	FACVL1, ACSL6, ACSL1, DHB4, AMACR, ACSL3
6	Immune response_IL-2 activation and signaling pathway	45	0.003	0.657	0.003	0.657	10	HMGI/Y, AP-1, c-Raf-1, Calcineurin A (catalytic), NF-kB, SMAD3, JAK1, PDK (PDPK1), SHP-2, ELF1
7	Immune response_IL-5 signalling	38	0.003	0.657	0.003	0.657	9	STAT3, CrkL, c-Raf-1, JAK1, JunB, ICAM1, PDK (PDPK1), SHP-2, c-Jun
8	Cell cycle_Regulation of G1/S transition (part 1)	38	0.003	0.657	0.003	0.657	9	Cyclin D2, Ubiquitin, Skp2/TrCP/FBXW, p70 S6 kinase1, SMAD3, JunB, SMAD2, TGF-beta receptor type II, Cyclin D
9	Development_Angiotensin activation of Akt	25	0.004	0.657	0.004	0.657	7	AGTR1, Calmodulin, GPX1, PDK (PDPK1), MEK3(MAP2K3), MEK6(MAP2K6), NSGPeroxidase
10	Development_WNT signaling pathway. Part 1. Degradation of beta-catenin in the absence WNT signaling	19	0.004	0.657	0.004	0.657	6	Cullin 1, Ubiquitin, CtBP, Skp2/TrCP/FBXW, HDAC1, Casein kinase I alpha

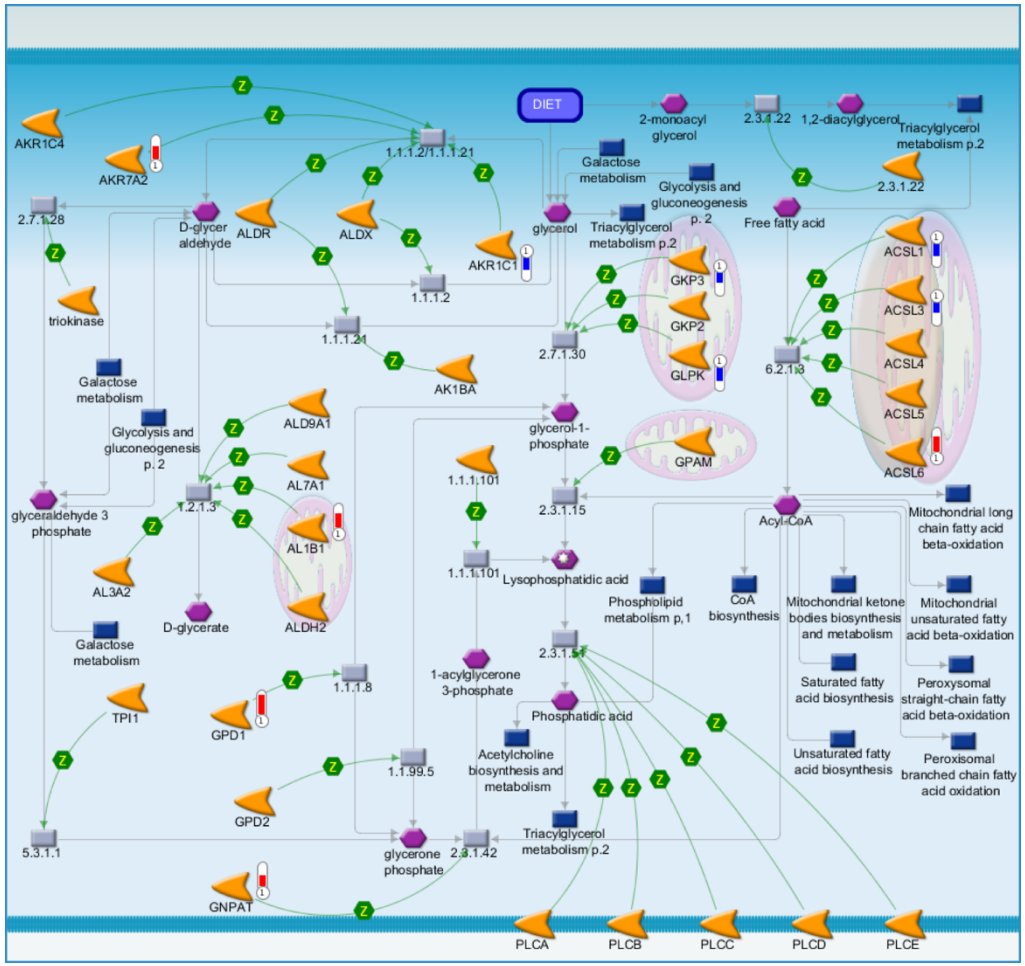

Figure 1. The top scored map (map with the lowest p-value) for iron and fructose (FAC&FRUC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

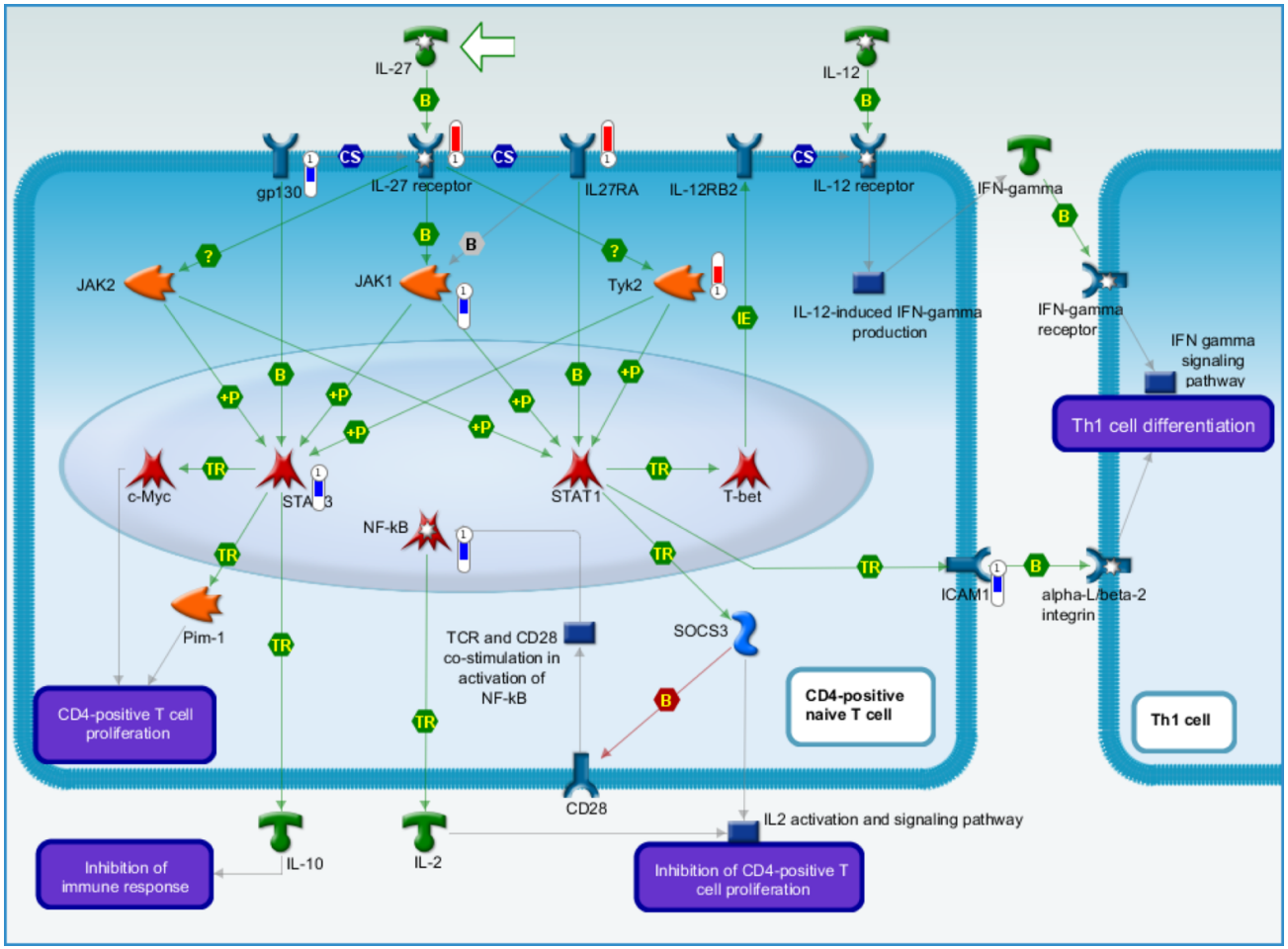

Figure 2. The second scored map (map with the second lowest p-value) for iron and fructose (FAC&FRUC) based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

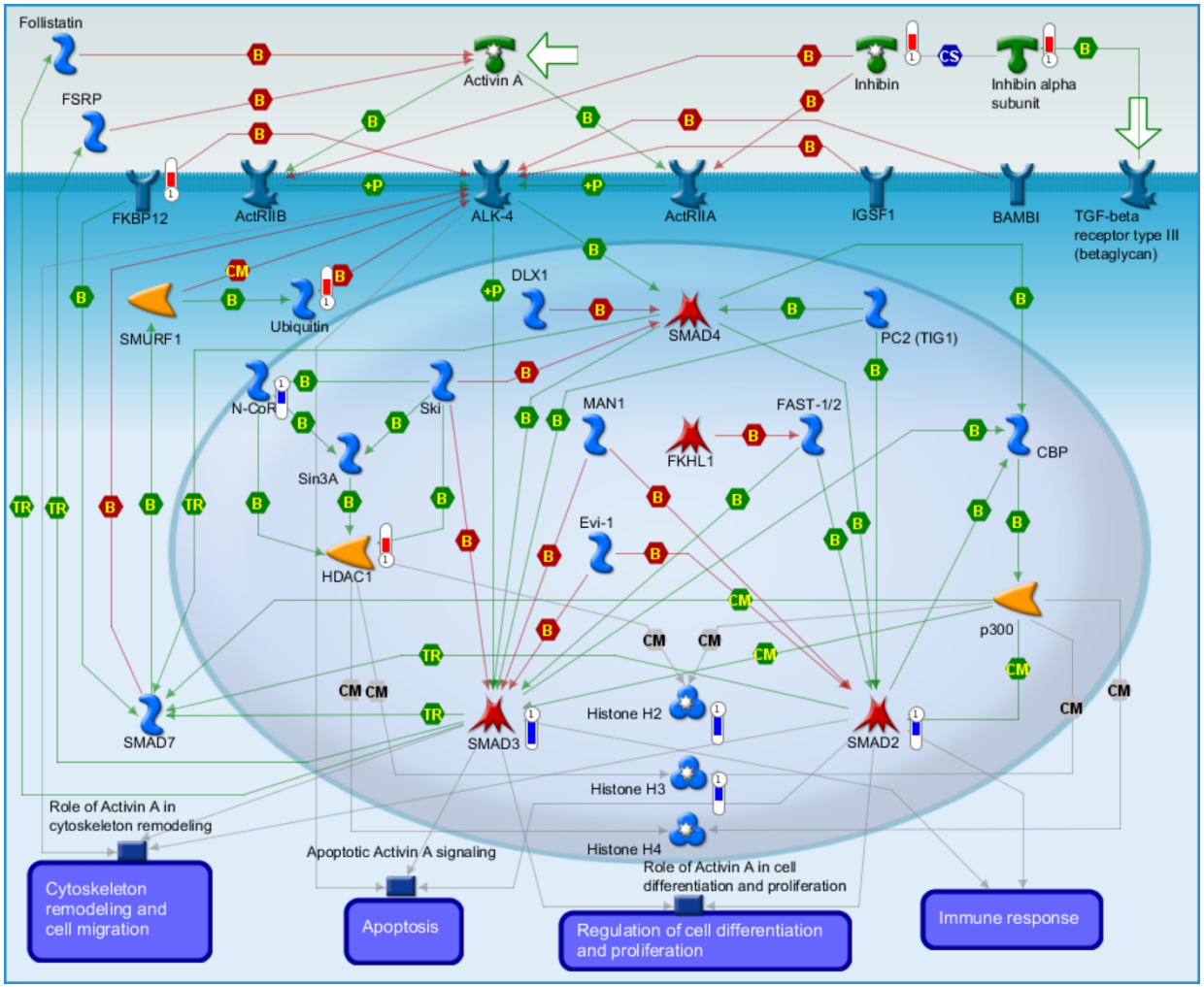

Figure 3. The third scored map (map with the third lowest p-value) based on the enrichment distribution for iron and fructose (FAC&FRUC) treatments unique sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

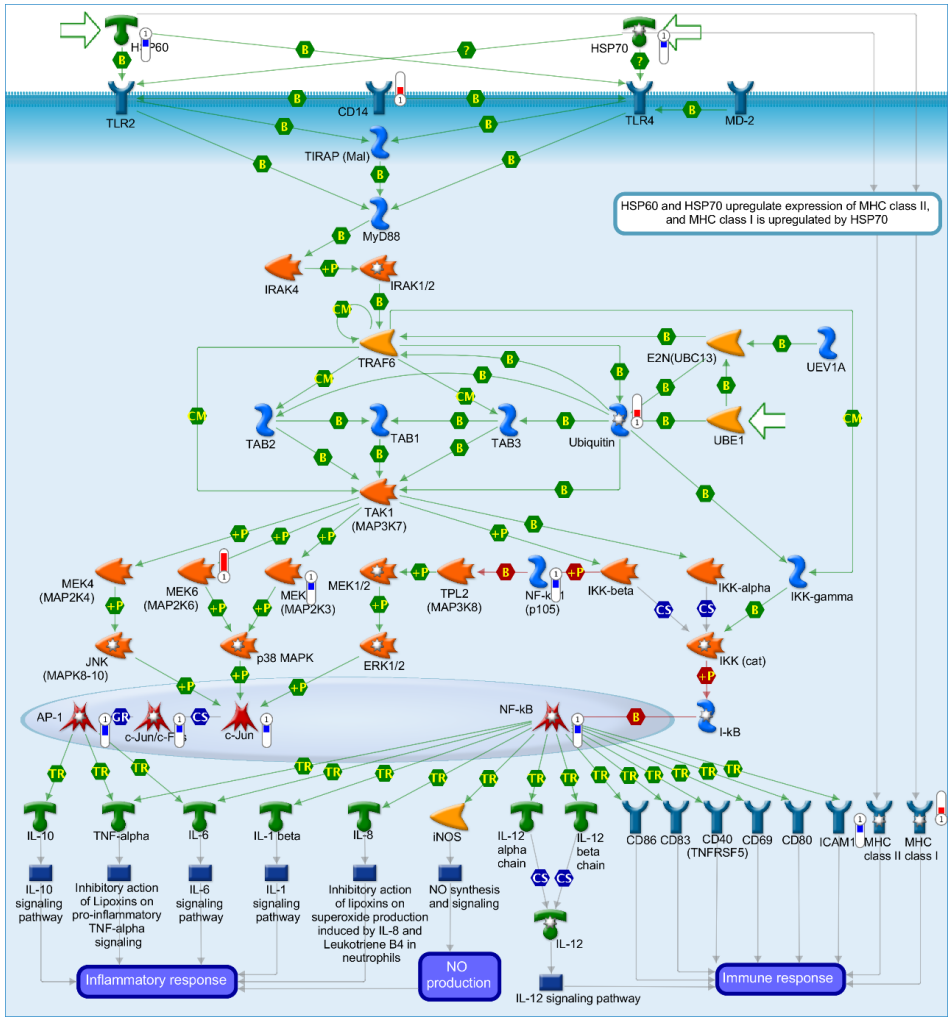

Figure 4. The fourth scored map (map with the fourth lowest p-value) for iron and fructose (FAC&FRUC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

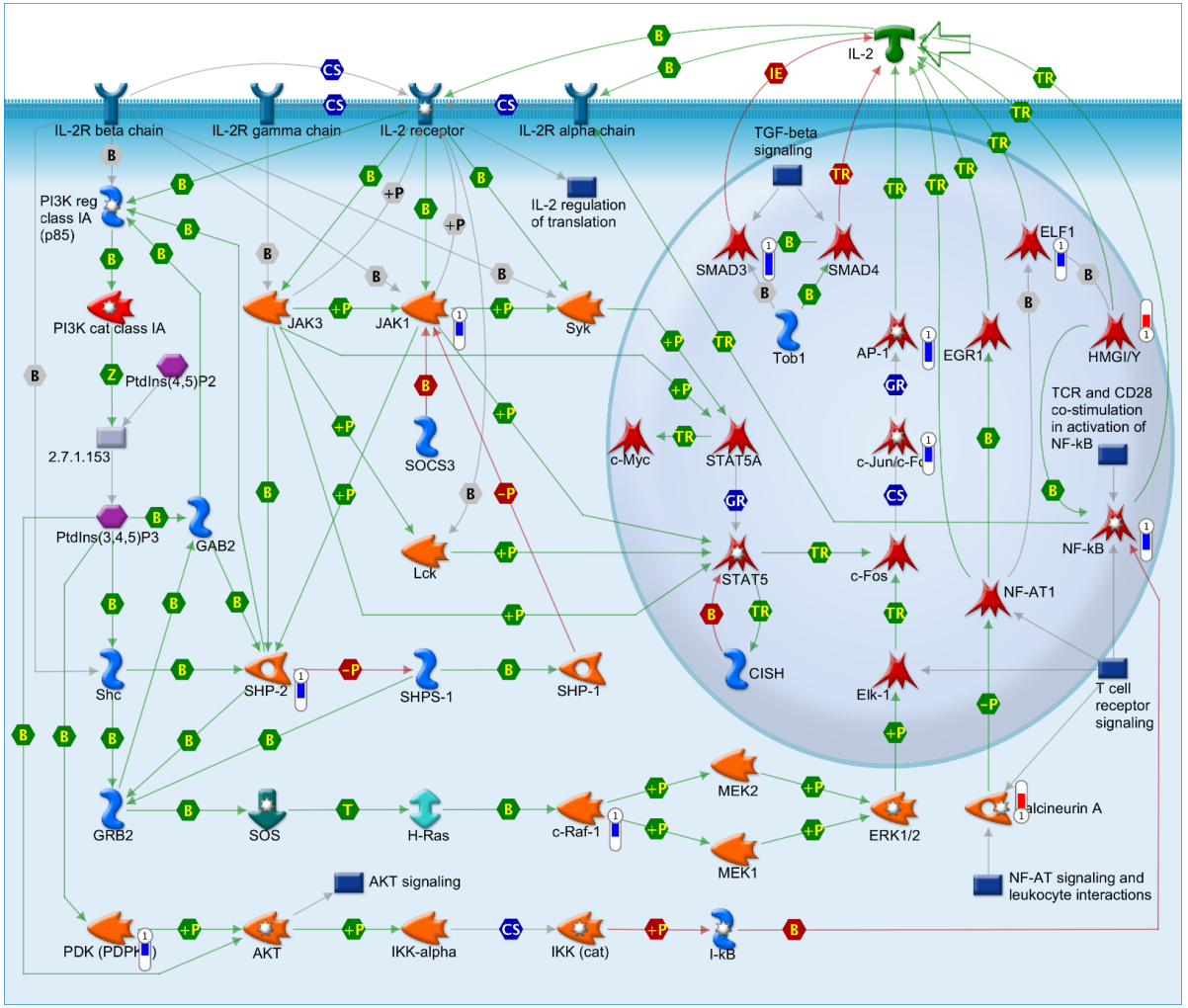

Figure 5. The fifth scored map (map with the fifth lowest p-value) for iron and fructose (FAC&FRUC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

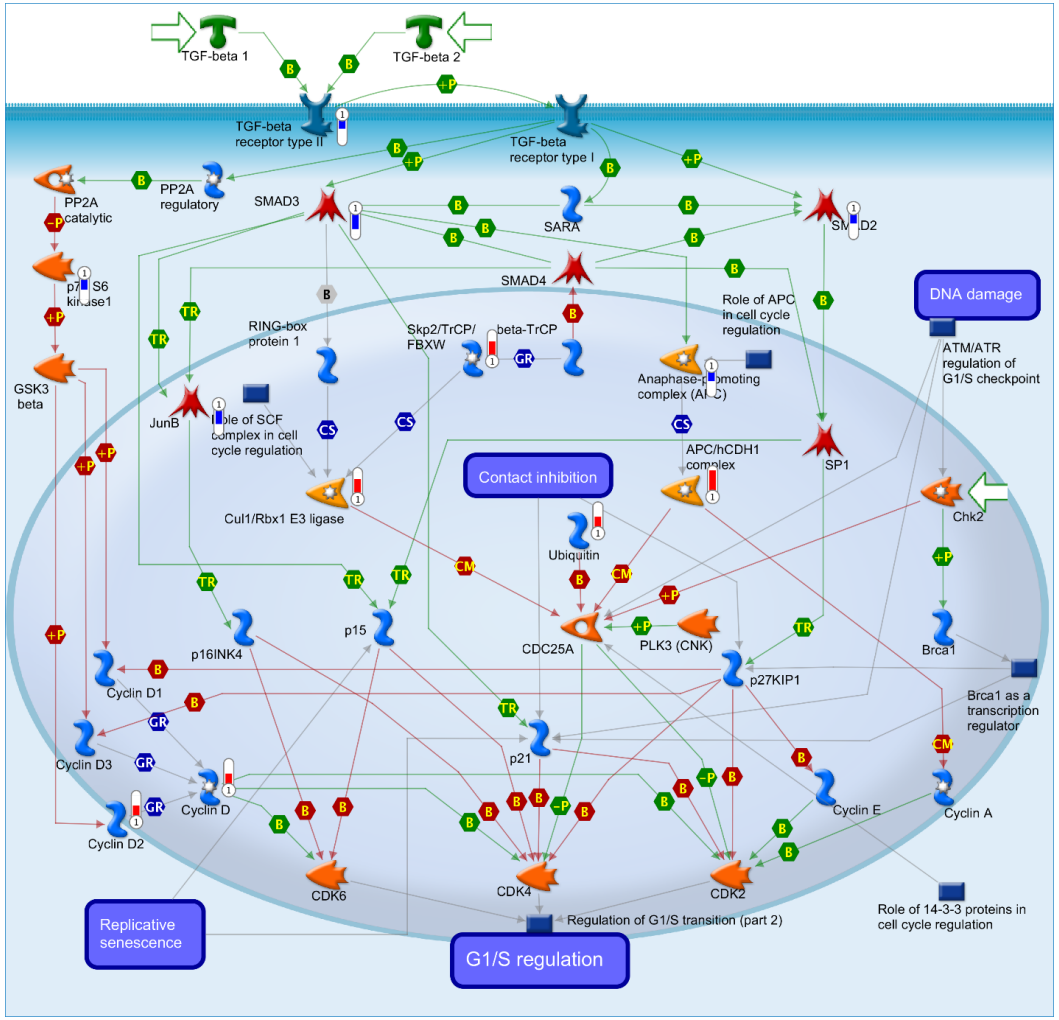

Figure 6. The sixth scored map (map with the sixth lowest p-value) for iron and fructose (FAC&FRUC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

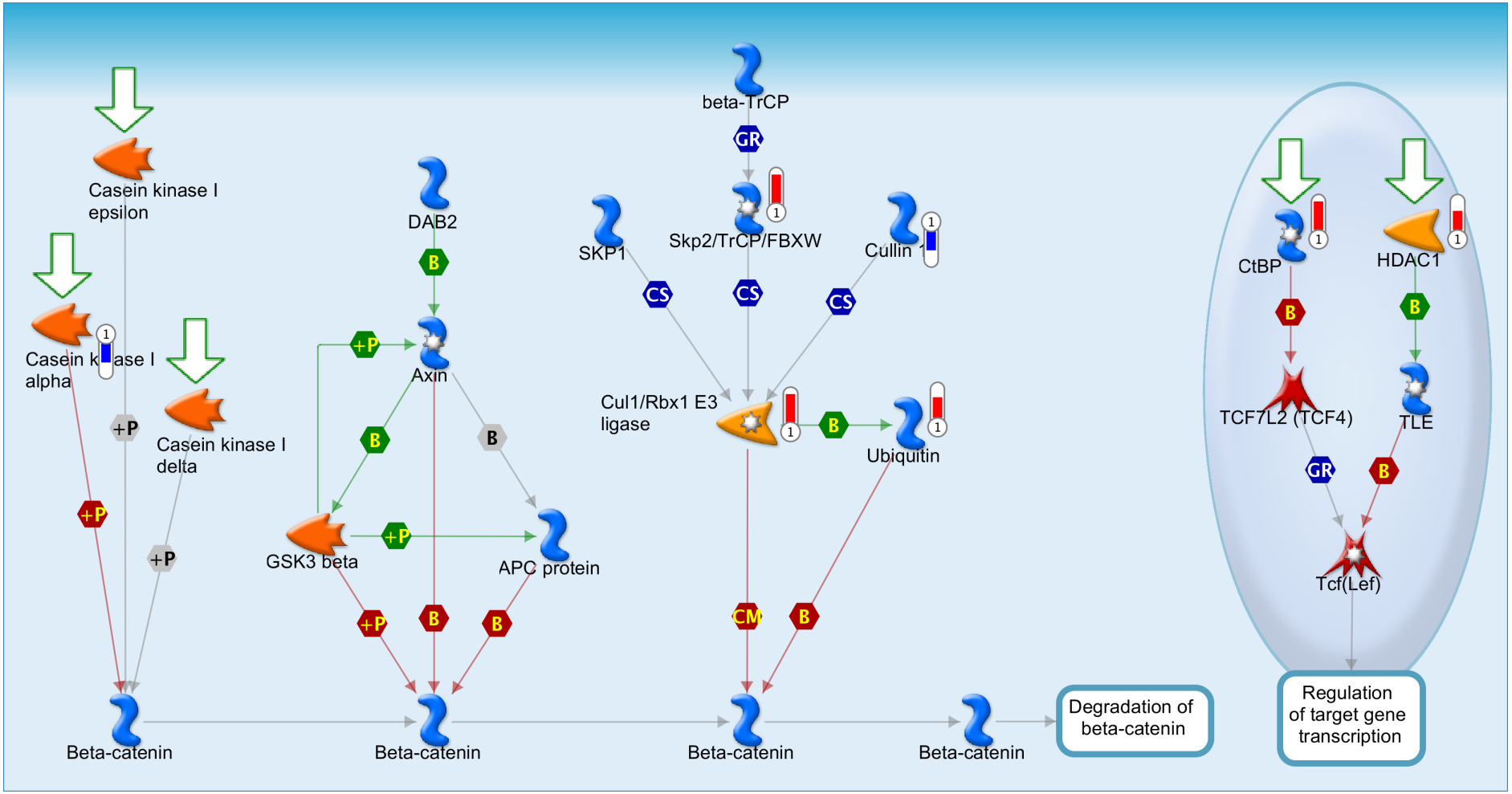

Figure 7. The seventh scored map (map with the seventh lowest p-value) for iron and fructose (FAC&FRUC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

Enrichment analysis report

Enrichment by Pathway Maps

					Fruc vs cont unique changed genes_genelist			
#	Maps	Total	pValue	Min FDR	p-value	FDR	In Data	Genes from Active Data
1	Immune response_Alternative complement pathway	39	0.000	4.244	0.000	4.244	9	C8gamma, C3a, C3, C3b, iC3b, CD59, C3dg, C3c, Clusterin
2	Immune response_Lectin induced complement pathway	49	0.000	3.641	0.000	3.641	9	C8gamma, C3a, C3, C3b, iC3b, CD59, C3dg, C3c, Clusterin
3	Immune response_Classical complement pathway	52	0.000	3.589	0.000	3.589	9	C8gamma, C3a, C3, C3b, iC3b, CD59, C3dg, C3c, Clusterin
4	Immune response_Antigen presentation by MHC class I	28	0.000	2.502	0.000	2.502	6	HSP90, MHC class I, TAP1 (PSF1), PSME3, HSP90 beta, Calreticulin
5	Development_Regulation of telomere length and cellular immortalization	35	0.000	2.020	0.000	2.020	6	HSP90, TRF1, hnRNP C, hRap1, E2F1, Max
6	Regulation of CFTR activity (norm and CF)	58	0.000	1.712	0.000	1.712	7	PP2A regulatory, Filamin B (TABP), PP2A structural, Annexin II, Calcineurin A (catalytic), PP2C, G-protein alpha-s
7	Apoptosis and survival_BAD phosphorylation	42	0.000	1.712	0.000	1.712	6	Bcl-XL, Calcineurin A (catalytic), SOS, PP2C, G-protein alpha-s, CDK1 (p34)
8	Cell cycle_Role of Nek in cell cycle regulation	32	0.001	1.440	0.001	1.440	5	Tubulin beta, NEK7, Aurora-A, Histone H3, CDK1 (p34)
9	Cell cycle_Spindle assembly and chromosome separation	33	0.001	1.428	0.001	1.428	5	Importin (karyopherin)-beta, Aurora-A, NUMA1, Separase, CDK1 (p34)
10	Development_EPO-induced Jak-STAT pathway	35	0.001	1.353	0.001	1.353	5	Bcl-XL, Lyn, SHP-2, XIAP, SOS

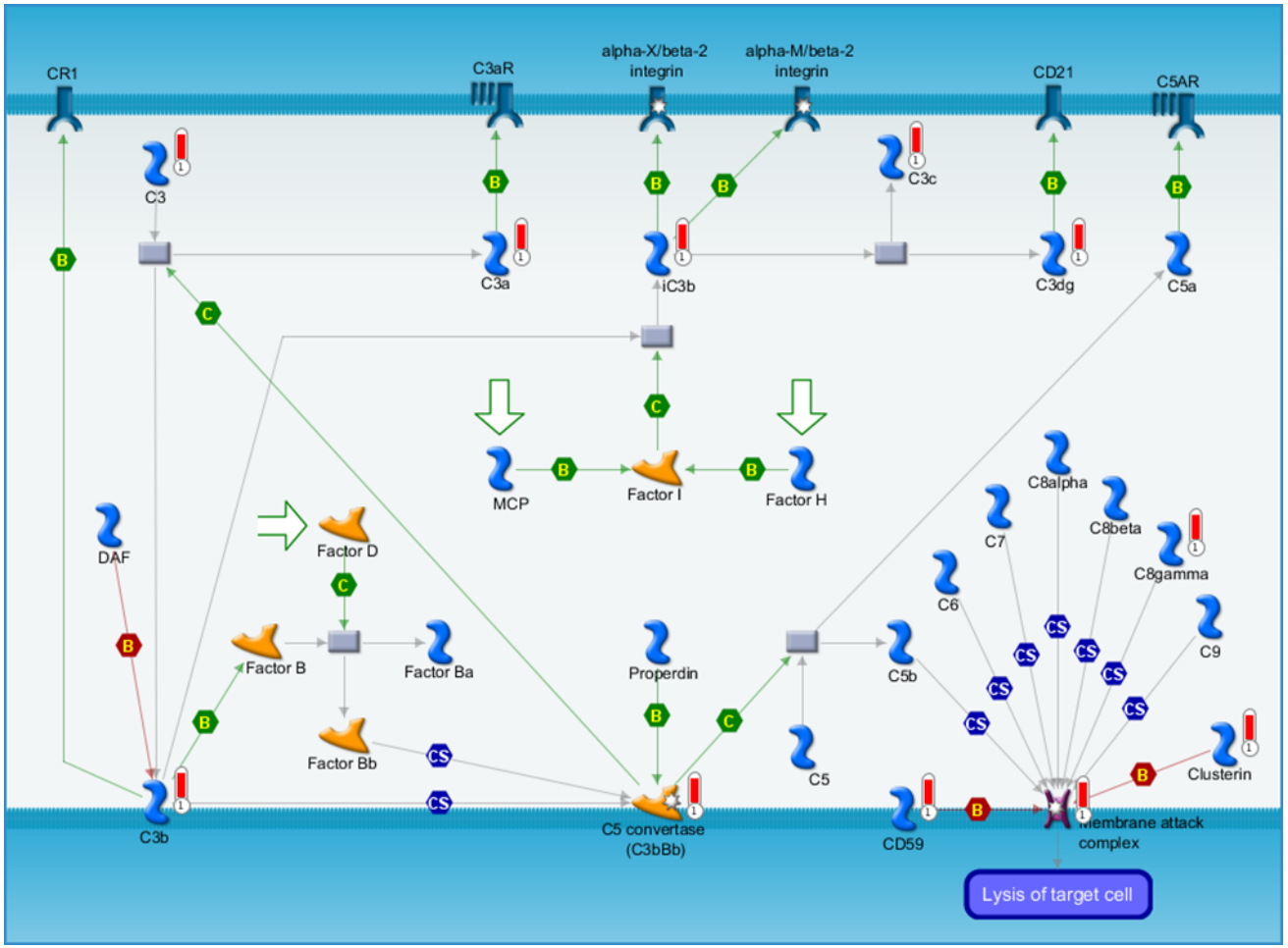

Figure 8. The top scored map (map with the the lowest p-value) for fructose alone (FRUC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

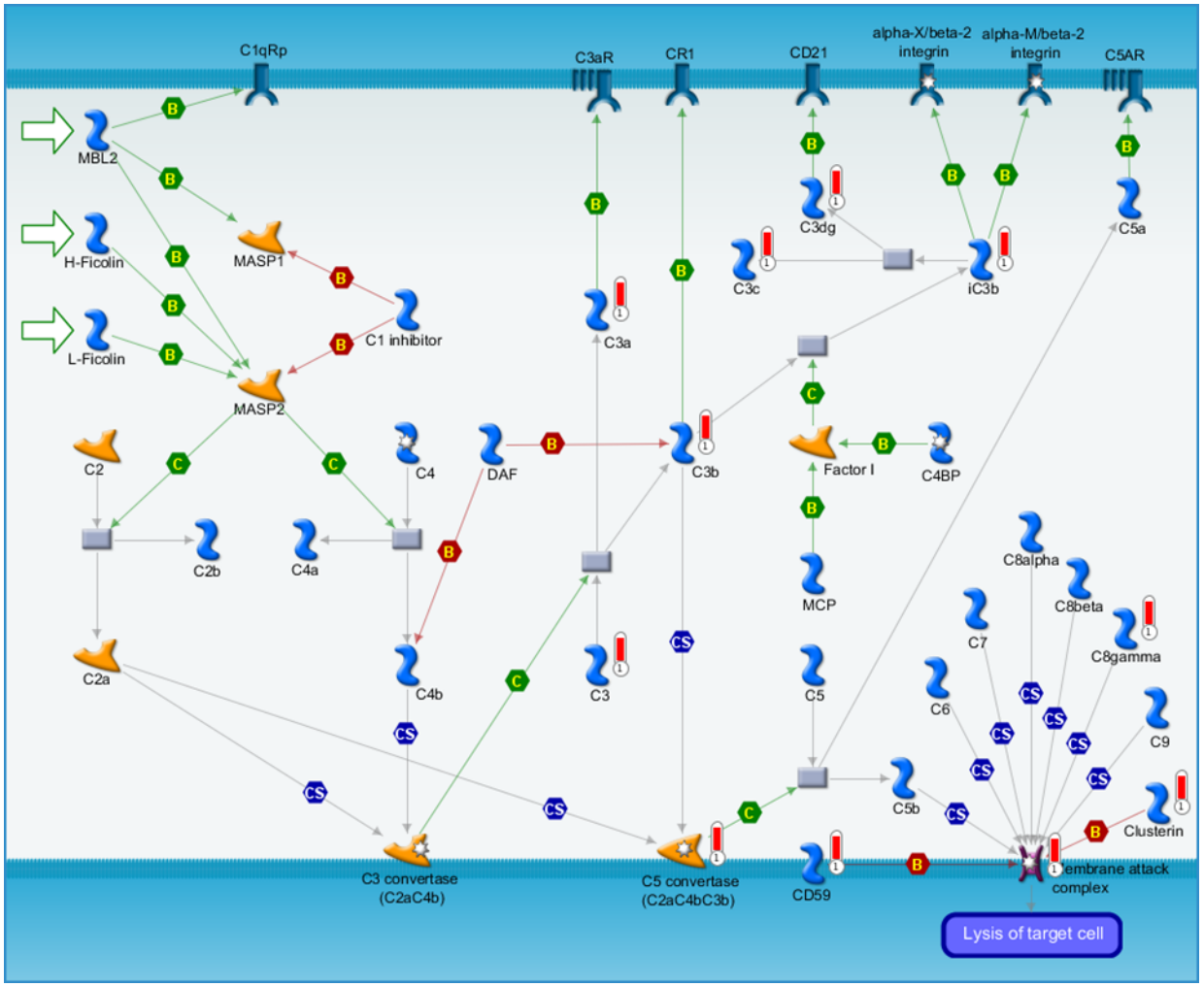

Figure 9. The second scored map (map with the second lowest p-value) for fructose alone (FRUC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

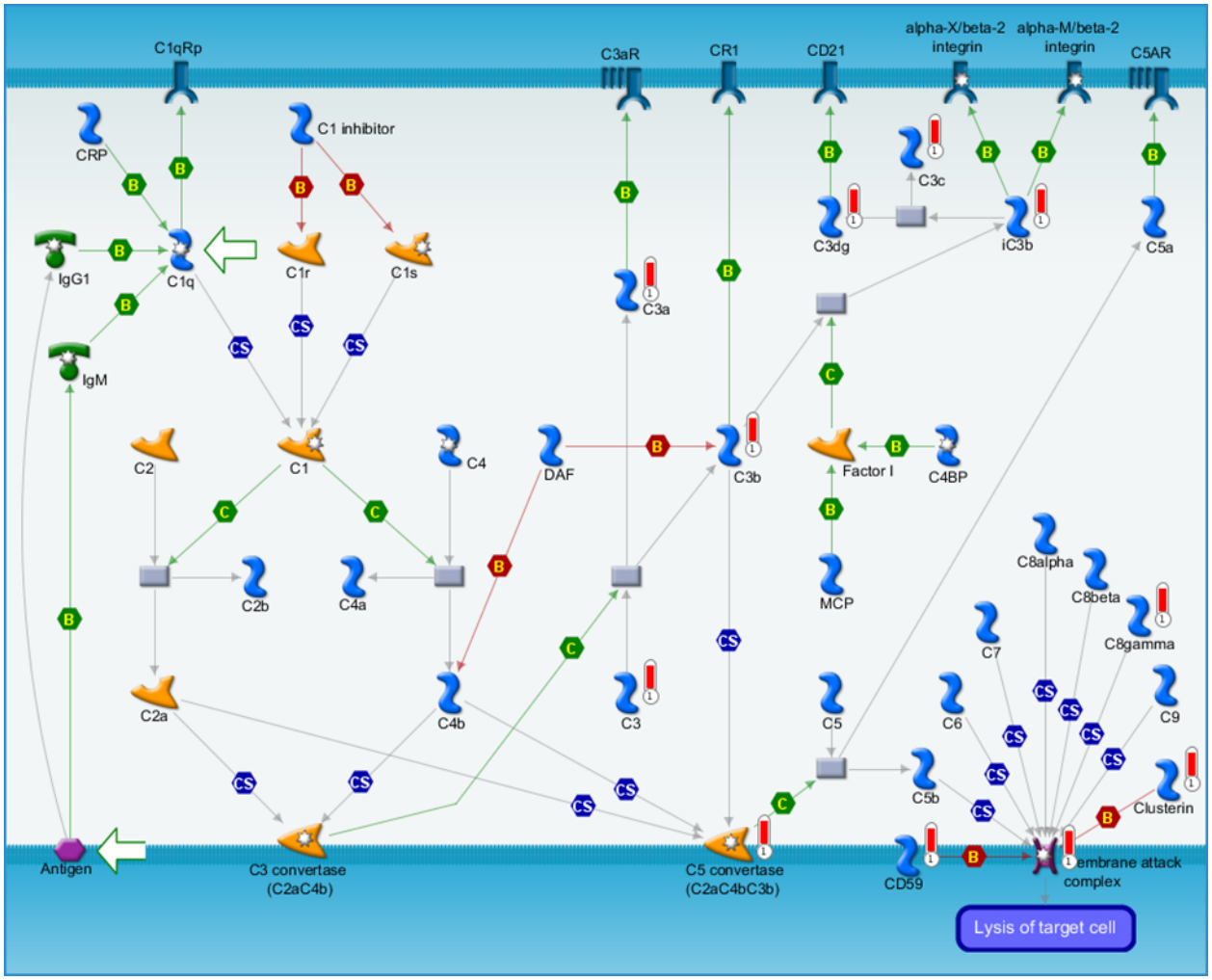

Figure 10. The third scored map (map with the third lowest p-value) for fructose alone (FRUC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

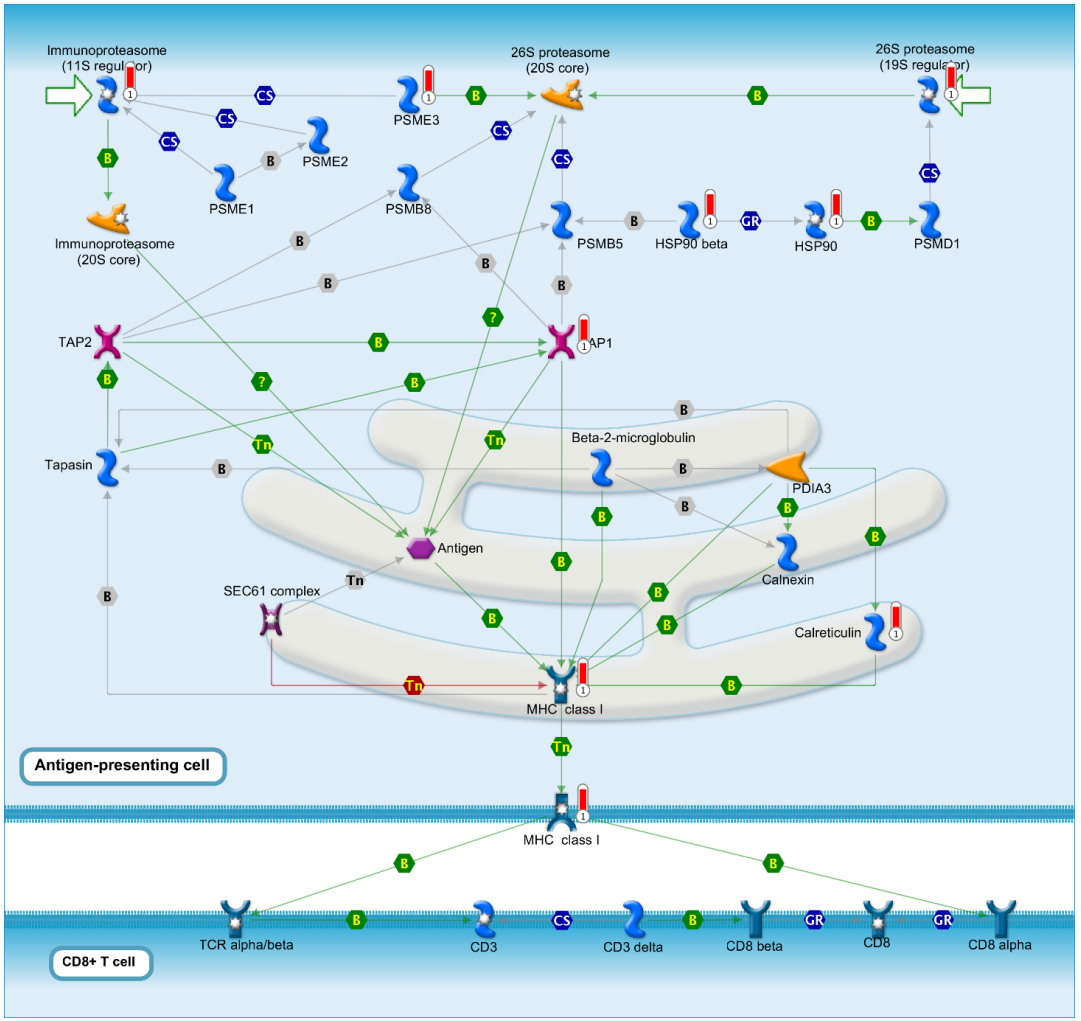

Figure 11. The fourth scored map (map with the fourth lowest p-value) for fructose alone (FRUC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

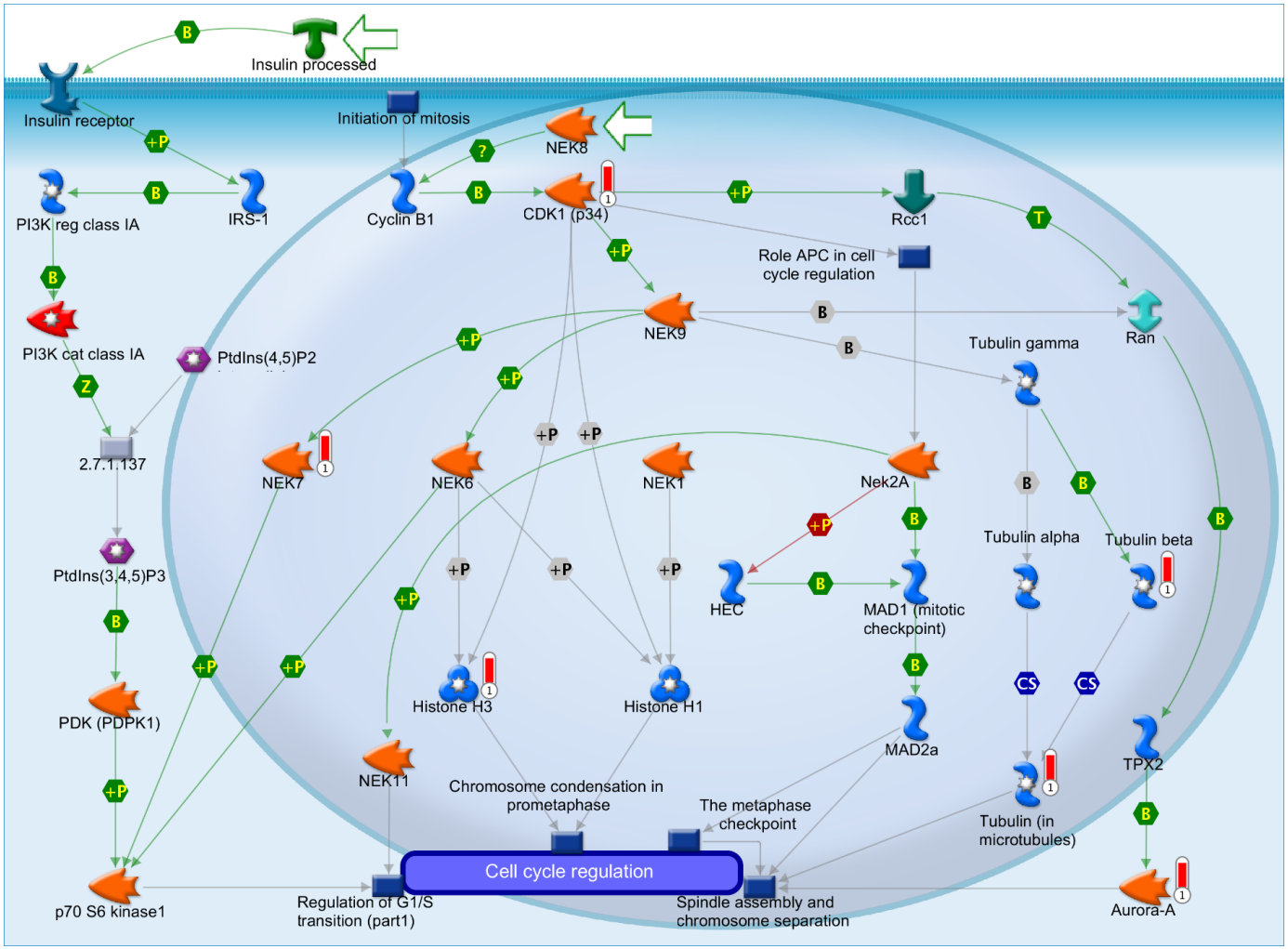

Figure 12. The fifth scored map (map with the fifth lowest p-value) for fructose alone (FRUC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

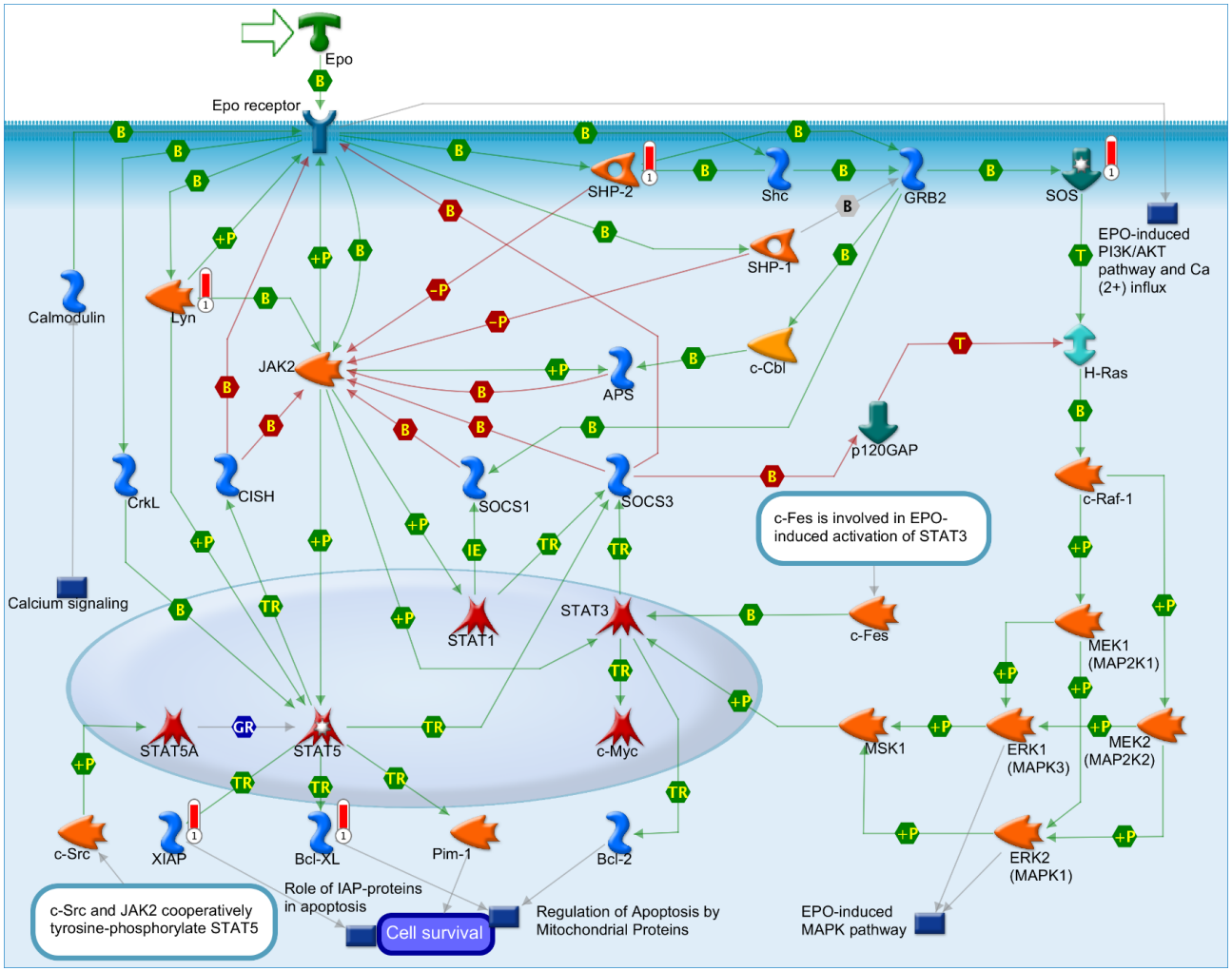

Figure 13. The sixth scored map (map with the sixth lowest p-value) for fructose alone (FRUC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

Enrichment analysis report

Enrichment by Pathway Maps

					FAC vs cont unique changed genes_genelist			
#	Maps	Total	pValue	Min FDR	p-value	FDR	In Data	Genes from Active Data
1	Transcription_ChREBP regulation pathway	13	0.000	1.144	0.000	1.144	3	PKA-cat (cAMP-dependent), AMPK gamma subunit, Acyl-CoA synthetase
2	Unsaturated fatty acid biosynthesis	16	0.001	1.144	0.001	1.144	3	FADS1, SCD, ACSL4
3	PGE2 pathways in cancer	41	0.001	1.144	0.001	1.144	4	TCF7L2 (TCF4), PKA-cat (cAMP-dependent), PGE2R4, Tcf(Lef)
4	Regulation of CFTR gating (normal and CF)	7	0.002	0.853	0.002	0.853	2	PKA-cat (cAMP-dependent), AMPK gamma1
5	Development_Slit-Robo signaling	28	0.003	0.847	0.003	0.847	3	Actin cytoskeletal, ACTB, Actin
6	Regulation of lipid metabolism_Regulation of lipid metabolism via LXR, NF-Y and SREBP	29	0.003	0.847	0.003	0.847	3	CYP51A1, AMPK gamma subunit, SCD
7	LRRK2 in neurons in Parkinson's disease	31	0.004	0.831	0.004	0.831	3	PKA-cat (cAMP-dependent), Actin cytoskeletal, ACTB
8	Immune response_PGE2 signaling in immune response	33	0.005	0.811	0.005	0.811	3	IL-2, PKA-cat (cAMP-dependent), PGE2R4
9	G-protein signaling_Rap2A regulation pathway	14	0.009	0.614	0.009	0.614	2	PKA-cat (cAMP-dependent), cAMP-GEFII
10	Development_TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK.	42	0.010	0.614	0.010	0.614	3	Caldesmon, ACTB, Actin

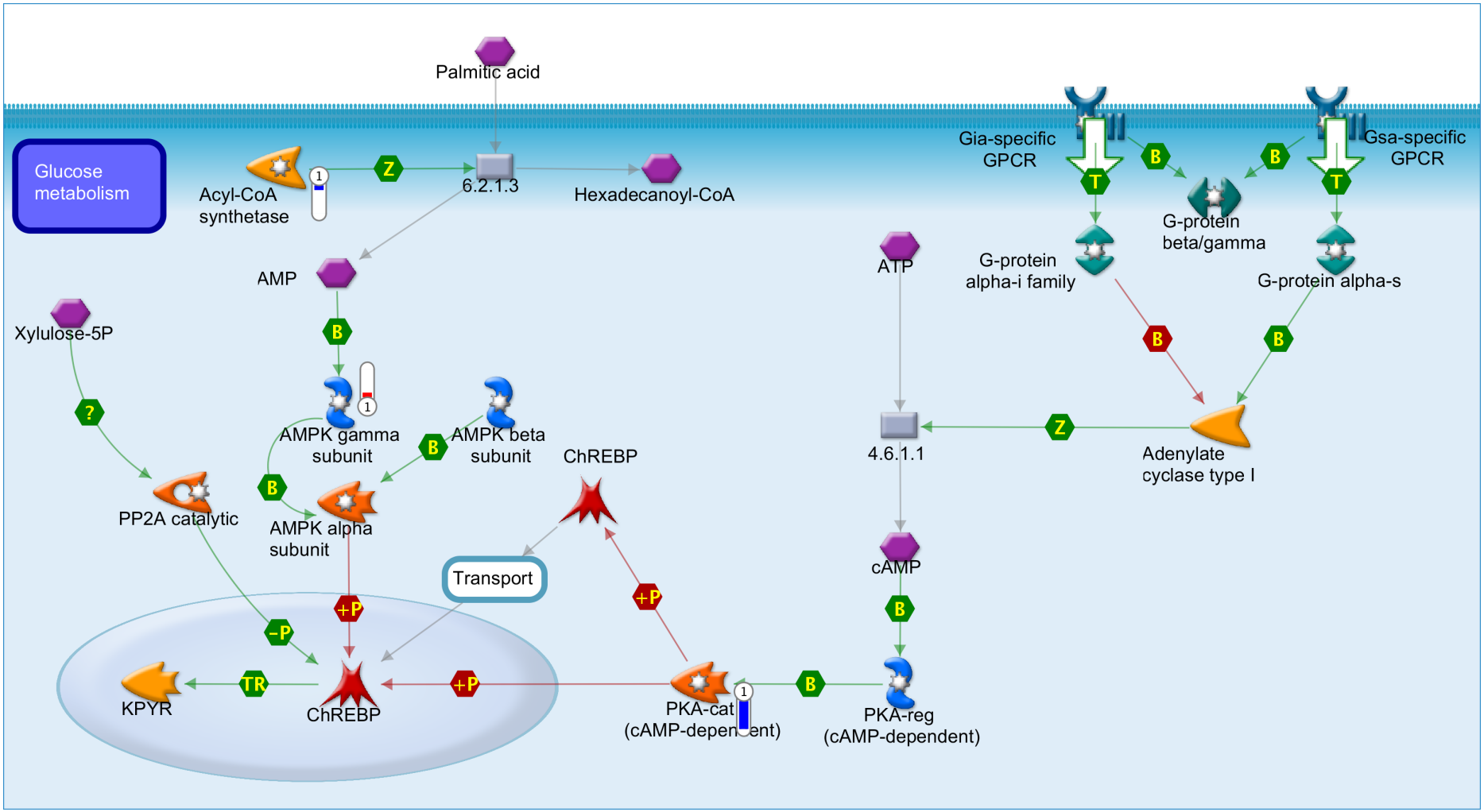

Figure 14. The top scored map (map with the lowest p-value) for iron alone (FAC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

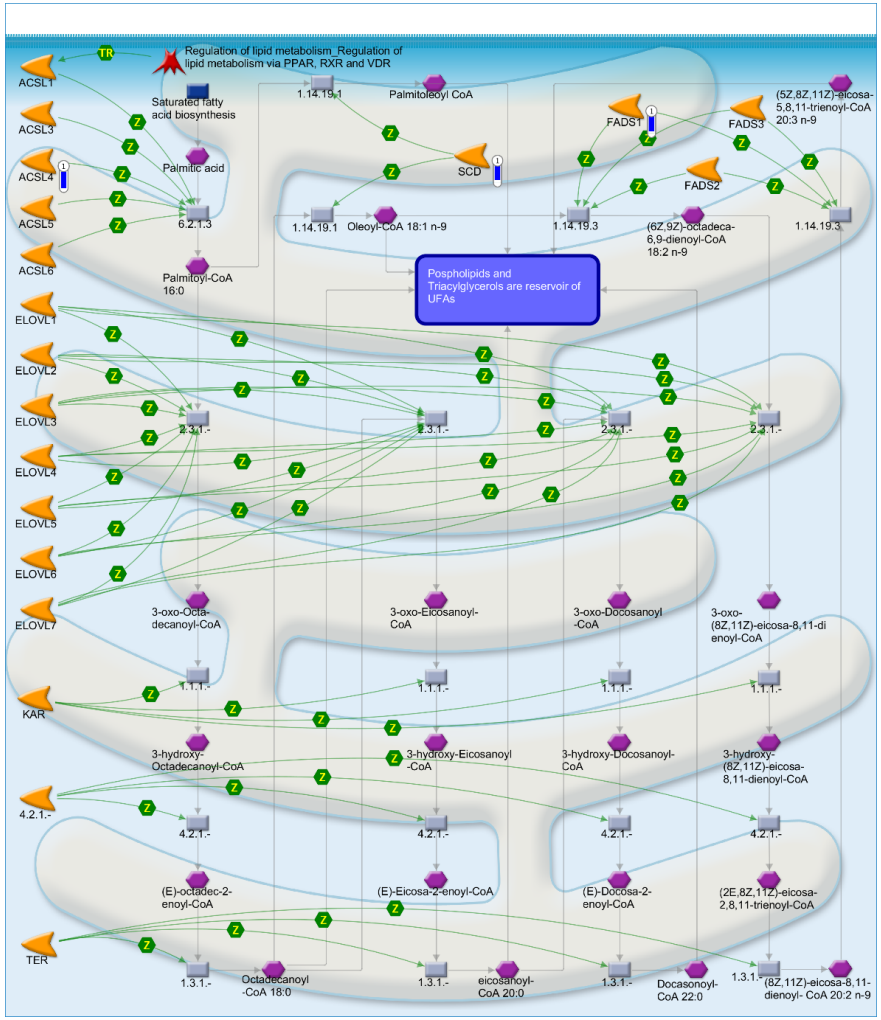

Figure 15. The second scored map (map with the second lowest p-value) for iron alone (FAC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

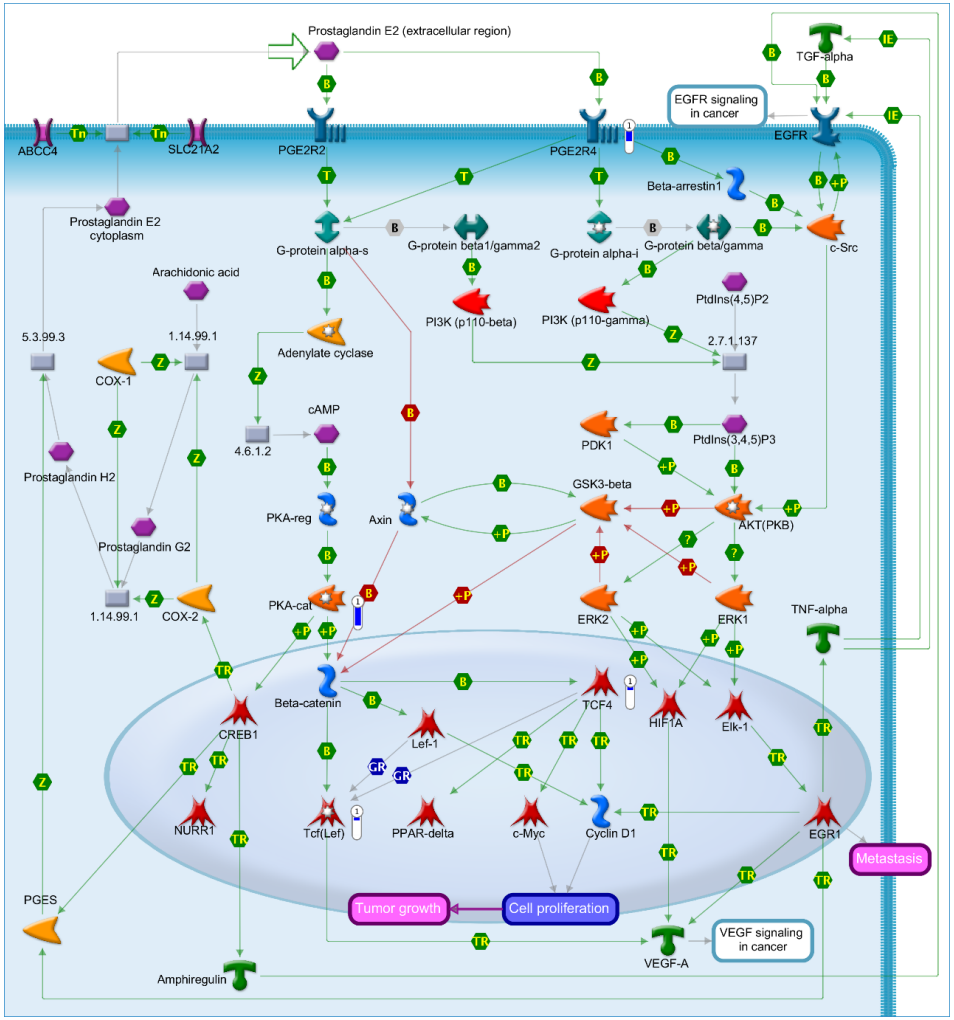

Figure 16. The third scored map (map with the third lowest p-value) for iron alone (FAC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

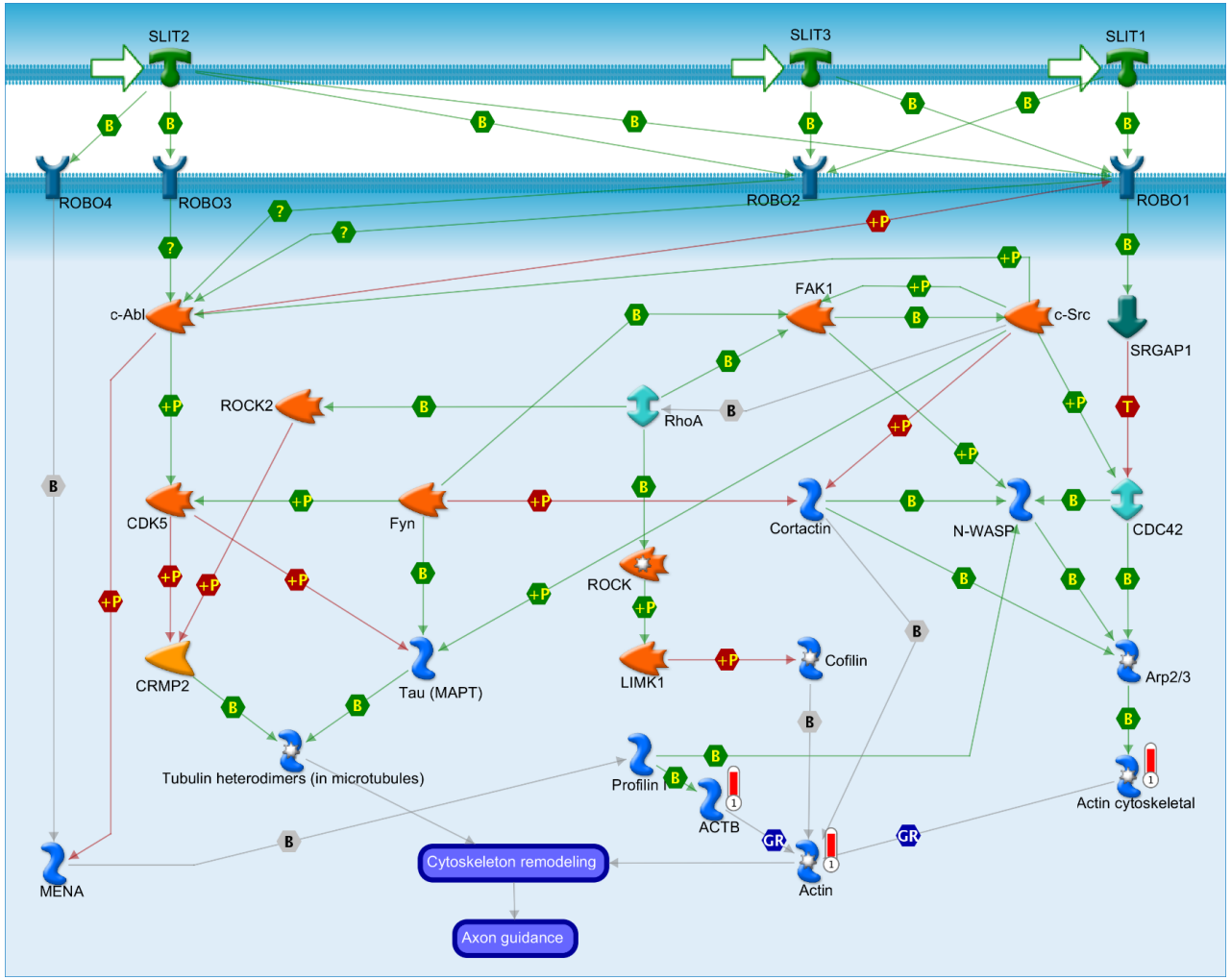

Figure 17. The fourth scored map (map with the fourth lowest p-value) for iron alone (FAC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

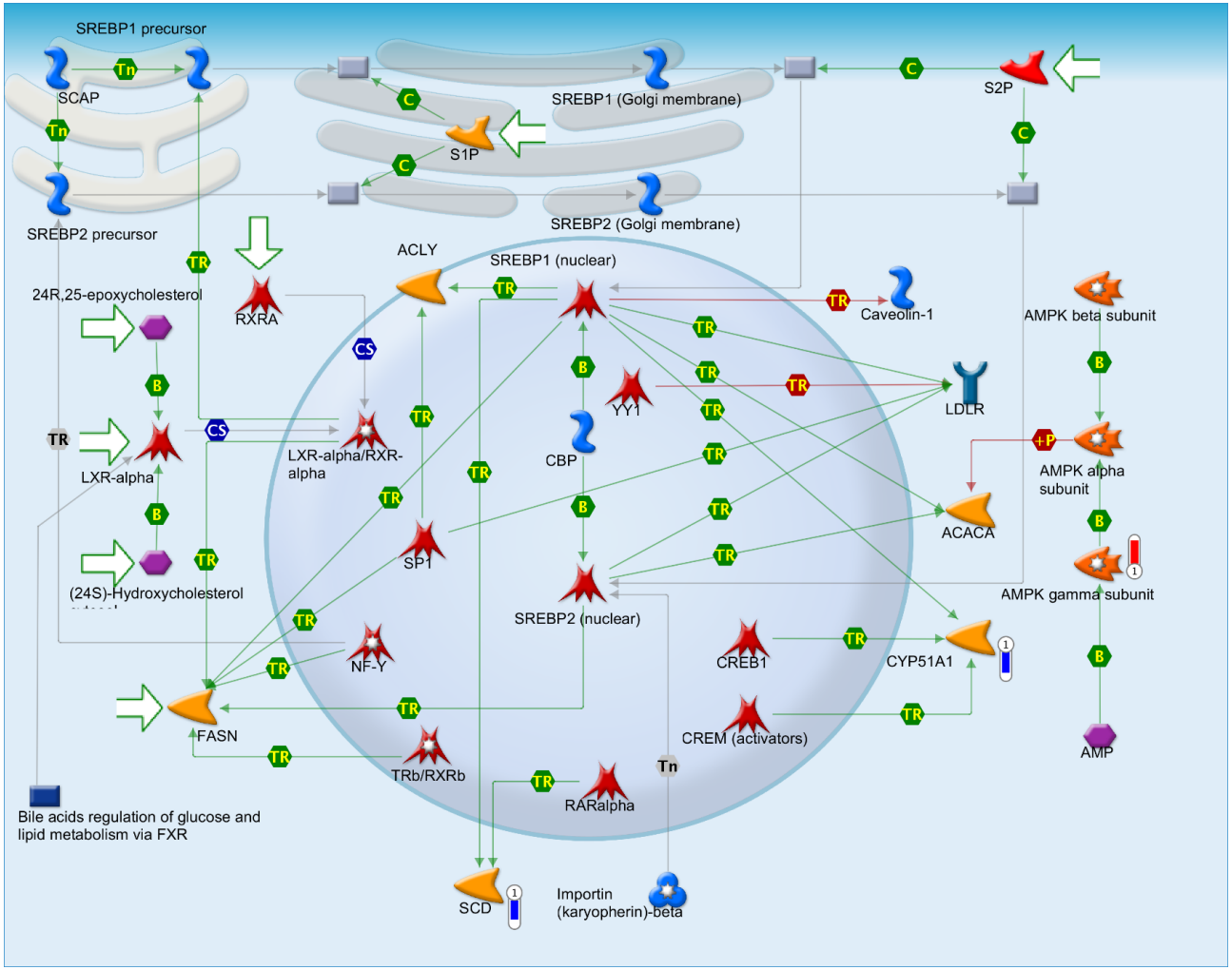

Figure 18. The fifth scored map (map with the fifth lowest p-value) for iron alone (FAC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

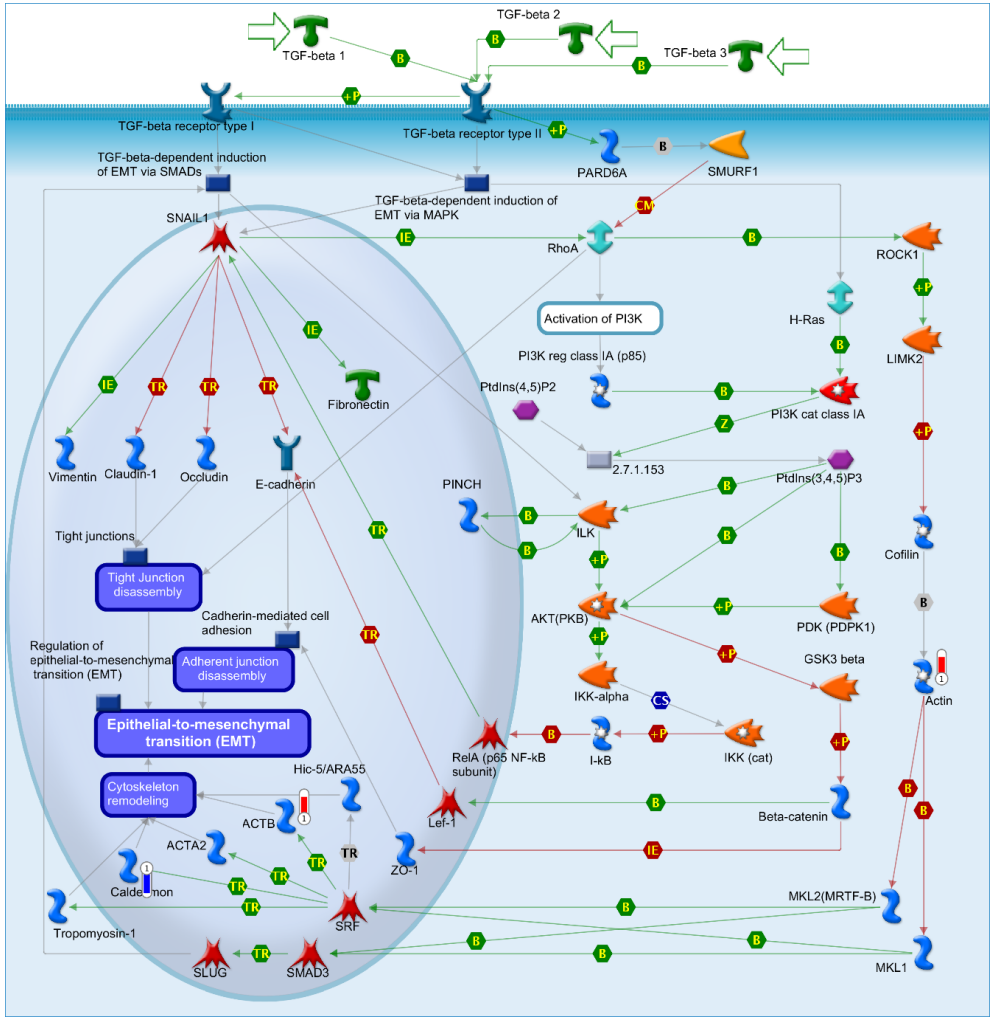

Figure 19. The sixth scored map (map with the sixth lowest p-value) for iron alone (FAC) treatments unique based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.