THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Exploring the effect of iron-ligand interactions on iron uptake pathways and inflammatory response in human cell lines

Agata Tarczykowska

Food and Nutrition Science

Department of Life Sciences

Chalmers University of Technology

Gothenburg, Sweden 2025

Agata Tarczykowska
ISBN 978-91-8103-264-2

Acknowledgements and personal statements in this thesis reflect the author's own views.

Doktorsavhandlingar vid Chalmers tekniska högskola
Ny serie nr 5722
ISSN 0346-718X

Department of Life Sciences
Chalmers University of Technology
SE-412 96 Gothenburg
Sweden

Cover:
[The cover image was created with the help of AI-assisted image generation (OpenAI, ChatGPT5]

Printed by:
Chalmers Digitaltryck
Gothenburg, Sweden 2025

''It always seems impossible until it's done.''

Nelson Mandela

Exploring the effect of iron-ligand interactions on iron uptake pathways and inflammatory response in human cell lines

AGATA TARCZYKOWSKA

Department of Life Sciences,
Chalmers University of Technology

ABSTRACT

Iron supplementation is essential for treatment of iron deficiency anemia, the most common nutritional disorder worldwide (WHO, 2021). While oral iron supplementation remains the first-line therapy, its use is not without risk, particularly for individuals with pre-existing gastrointestinal conditions such as inflammatory bowel disease (IBD) or Crohn's disease, where pro-inflammatory responses to certain iron formulations could exacerbate the condition. Understanding how the iron-ligand interactions of oral iron compounds influence their inflammatory potential is critical for optimizing treatment safety and efficacy.

Investigating six iron chelates and six iron salts demonstrated that all iron chelates significantly induced the MAPK signaling pathway, as evidenced by elevated amphiregulin levels (Paper I). The two most chemically stable polydentate chelates, ferric EDTA and ferric pyrophosphate, upregulated amphiregulin and IGFr1 at physiologically relevant concentrations ([Fe]=0.05 mM). This effect was not observed with any of the tested iron salts. Ferric pyrophosphate also induced the pro-inflammatory enzyme cyclooxygenase-2 (COX-2) and stimulated production of interleukin-6, indicating the most pronounced pro-inflammatory profile among the tested compounds.

To further investigate these inflammatory responses, we examined COX-2 and lipooxygenase-5 (LOX-5) levels in intestinal cell lines derived from both male and female donors (Paper III). The results demonstrated that solubility and the particle size, formed in cellular medium (pH 7.4) supplemented with ferric pyrophosphate or ferrous fumarate was associated with elevated COX-2 levels in most of the tested cell lines. The fully soluble ferric EDTA did not elicit such response. Importantly, these effects occurred independently of ferritin accumulation, suggesting that inflammatory signaling was driven by the formed particles, rather than by total cellular iron load.

In a mechanistic follow-up study (Paper II), ferrous fumarate was found to utilize both the DMT1 transporter and clathrin-mediated endocytosis for cellular uptake, challenging the common view that the intestinal uptake of iron from iron salts is tightly regulated by means of being transported by the DMT1-mediated pathway. Cellular uptake of iron from ferrous sulfate and ferric EDTA was confirmed to be independent of clathrin-mediated endocytosis. In a gut-liver axis model (Caco-2/HepG2), ferric pyrophosphate treatment (0.2 mM) induced key MAPK/ERK pathway activators, including the IFN-γ receptor, EGF, and CDKN1A, which was further validated in Caco-2 monocultures exposed to commercially available ferric pyrophosphate (0.4 mM), where IL-6 production was observed. This response was not observed in cells treated with a nanoparticulate iron, Iron hydroxide adipate tartrate (IHAT).

In conclusion, this thesis demonstrates that the iron-ligand interactions are critical determinants of both the intestinal uptake route and downstream inflammatory effects in intestinal cell models. These insights highlight the need to carefully consider the choice of iron ligand, when designing an iron supplements in order to minimize adverse effects while ensuring therapeutic efficacy.

Keywords: dietary iron, non-heme iron, iron salts, iron chelates, iron ligand, solubility, precipitation, iron uptake, inflammation, intestinal epithelial cells, Caco-2, Hutu-80

LIST OF PUBLICATIONS

The doctoral thesis is based on the work contained in the following papers:

I Tarczykowska A., Engström N., Dobermann D., Powell J., Scheers N. Differential Effects of Iron Chelates vs. Iron Salts on Induction of Pro-Oncogenic Amphiregulin and Pro-Inflammatory COX-2 in Human Intestinal Adenocarcinoma Cell Lines. Int J Mol Sci. 2023 Mar 14;24(6):5507.

II Tarczykowska A., Malmberg P., Scheers N. Uptake of iron from ferrous fumarate can be mediated by clathrin-dependent endocytosis in Hutu-80 cells. Front Mol Biosci. 2025 Jan 27;12:1460565.

III Tarczykowska A., Mohammadi A.S., Scheers N. COX-2 induction in response to the iron compounds ferric pyrophosphate and ferrous fumarate in human intestinal cell lines (manuscript).

IV Tarczykowska A., Scheers N. Comparative Effects of Ferric Pyrophosphate and IHAT on MAPK/ERK Pathway mediators in single-cultured Caco-2 cells and in the Caco-2/HepG2 co-culture model (manuscript).

CONTRIBUTION REPORT

I Agata Tarczykowska (AT) contributed to the experimental work, was responsible for data management and contributed to the writing, as well as participated in revising the manuscript in collaboration with the co-authors during the editing process.

II AT conducted the experimental work, was responsible for data management, writing the first draft of the manuscript and participated in revising the manuscript in collaboration with the co-authors during the editing process.

III AT was responsible for the design, conducted the experimental work, was responsible for data management and writing the first manuscript draft as well as participating in revising the manuscript in collaboration with the co-authors.

IV AT conducted the experimental work and was responsible for data management, contributed to the writing, as well as participating in revising the manuscript in collaboration with the co-authors.

ABBREVIATIONS

ADAM17	A disintegrin and metalloprotease 17
AI	Anemia of inflammation
Caco-2	Colorectal adenocarcinoma cell line
CCP	Clathrin coated pits
CCV	Clathrin coated vesicles
CDKNA1	Cyclin dependent kinase inhibitor 1A
CME	Clathrin mediated endocytosis
COX-2	Cyclooxygenase 2
CP	Ceruloplasmin
CRC	Colorectal cancer
DCYTB	Duodenal cytochrome b
DMEM	Dulbecco's modified Eagle's medium
DMT1	Divalent metal transporter 1
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FAAS	Flame atomic absorption spectroscopy
FBS	Fetal bovine serum
FDA	Food and drug agency
FPN1	Ferroportin
FTH1	Ferritin heavy chain
FTL	Ferritin light chain
GFAAS	Graphite furnace atomic absorption spectroscopy
HAMP	Hepcidin
HepG2	Hepatocellular carcinoma
HEPH	Hephaestin
HO-1	Heme oxygenase
HRG1	Heme responsive gene 1
Hutu-80	Duodenal adenocarcinoma cells
IHAT	Iron hydroxide adipate tartrate
IL-6	Interleukin 6
IRE	Iron response element
IRP	Iron regulatory proteins
JAK	Janus kinases
STAT	Signal transducer and activator of transcription
LOX-5	Lipoxygenase 5
LRP1	Low density lipoprotein receptor-related protein 1
MAPK	Mitogen-activated protein kinases
NF-κB	Nuclear factor kappa beta
NTBI	Non transferrin bound iron
PEA	Proximity extension assay
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STEAP3	Six-transmembrane epithelial antigen of the prostate 3
SW1417	Colon adenocarcinoma cell line
SW48	Colon adenocarcinoma cell line
TFR1	Transferrin receptor 1

TFR2	Transferrin receptor 2
UTR	Untranslated region
ZIP14	Zinc importing protein

Table of contents

1. INTRODUCTION

Iron is an indispensable micronutrient required by nearly all forms of life, and its biological significance is rooted in the earliest stages of evolution. Life has originated on Earth approximately 3.5 billion years ago in an anoxic environment, where iron was abundant in its soluble form[1]. Presumably, iron-sulfur (Fe-S) minerals provided the catalytic and structural framework, becoming integral components of early enzymatic systems. In particular, they served as cofactors for ancient electron transfer proteins like ferredoxins, which played central roles in early energy metabolism. As cellular complexity increased over evolutionary time, the involvement of iron in biological processes expanded significantly[2]. Living organisms exploit the unique chemical properties of iron, especially its ability to reversibly cycle between ferrous (Fe^{2+}) and ferric (Fe^{3+}) states, for a wide array of functions such as oxygen transport, DNA synthesis or the catalytic activity of numerous enzymes. However, the chemical reactivity of iron is a double-edged sword. While essential for life, free iron can contribute to ROS production damaging proteins, lipids, and nucleic acids[3]. The importance of balanced iron metabolism is underscored by its association with a wide spectrum of clinical conditions.

Iron deficiency leading to anemia remains a major global health concern, affecting approximately 25% of the global population[4]. The regions contributing most significantly to global anemia rates include Central and West Africa as well as South Asia. While the prevalence of anemia in developed countries is estimated at around 9%, it rises sharply to 43% in developing regions, with children and women of reproductive age being especially vulnerable[5], resulting in preterm deliveries and growth retarded infants[6-8].

The gastrointestinal tract is exposed to dietary iron due to its absorptive role[9]. There is evidence suggesting that the source of iron plays a critical role in health outcomes[10,11]. Dietary iron exists in two primary forms: heme iron and non-heme iron. Heme iron, found in animal-based foods such as meat and seafood, contains iron in the ferrous (Fe^{2+}) state bound within hemoglobin and myoglobin. In contrast, non-heme iron, which is present in plant-based foods like nuts, cocoa, beans and cereals, is predominantly in the ferric (Fe^{3+}) form[12]. Although heme iron typically makes up only 10–15% of total dietary iron intake in populations that consume meat, its superior bioavailability, with absorption rates ranging from 15% to 35%, means it can contribute over 40% of the iron actually absorbed by the body[9]. High consumption of red meat in humans has been linked to an increased risk of colorectal cancer, although iron itself has not been definitively established as the causal factor[13-15]. In animal models, dietary iron fortification has been associated with greater tumor burden, while in cell culture studies, different forms of iron have been shown to differentially activate growth-promoting pathways[10,13,16,17].

Oral iron supplementation continues to be the main approach for treating iron deficiency anemia[18], owing to its ease of use, affordability, and accessibility. However, its bioavailability is limited and the remainder accumulates in the gastrointestinal tract, often leading to adverse effects such as mucosal irritation and dysbiosis[19]. The biological effects of iron supplements are highly dependent on their chemical form which determines their physical properties, such as solubility, and which affect cellular uptake routes. The differences in chemical form of iron and uptake pathways raise important questions about how various iron compounds may influence cellular functions and inflammatory responses.

2. AIMS

The aim of this thesis was to investigate differential cellular effects and their mechanisms observed with various iron compounds. Specifically, this work focused on investigating the hypothesis that different iron forms are absorbed through distinct mechanisms, and that certain formulations may bypass normal regulatory pathways, potentially interfering with key processes such as inflammation and apoptosis. Towards the end of this thesis, particular emphasis was placed on the extracellular environment containing iron, related to iron intake, with the aim of exploring its potential link to inflammatory responses.

The specific aims were to:

A. Investigate 12 different iron compounds (6 chelates and 6 salts) and their impact on 96 inflammatory markers, with the main focus on amphiregulin and its targets, as well as COX-2 in male Caco-2 and Hutu-80 cell lines (Paper I)

B. Investigate iron uptake routes, focusing on DMT-1 mediated transport and clathrin-mediated endocytosis, of 3 iron compounds: ferrous fumarate, ferrous sulfate and ferric EDTA in Hutu-80 cells (Paper II)

C. Evaluate whether ferric pyrophosphate induces COX-2 in male-derived (Hutu-80 and Caco-2) and female-derived (SW48 and SW1417) intestinal epithelial cell lines (Paper III)

D. Assess if the extracellular environment containing ferrous fumarate and ferric pyrophosphate, including the formation of iron precipitates, contributes to COX-2 induction (Paper III)

E. Study the effects of two types of iron: ferric pyrophosphate (chelated iron) and IHAT (nanoparticulate iron) on pro-inflammatory mediators and their impact on intracellular signaling associated with inflammation in Caco-2 cells and Caco-2/HepG2 co-culture (Paper IV)

3. BACKGROUND

3.1 Iron as an essential nutrient and its role in the human body

The biological significance of iron stems from its unique ability to exist in multiple oxidation states, ranging from -2 to +6[20]. In physiological systems, it mostly occurs in the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states[21]. This redox activity enables iron to participate effectively in electron transfer reactions and to reversibly bind various ligands. In biological contexts, these ligands typically include oxygen, nitrogen, and sulfur atoms[22]. Iron is involved in numerous physiological functions, including oxygen transport, where it serves as an integral element of hemoglobin in erythrocytes and myoglobin in muscle tissue [23]. In hemoglobin, iron is incorporated into the heme moiety where it binds to oxygen reversibly and mediates the transport of oxygen from lungs to the peripheral tissues[24]. As a component of myoglobin, an oxygen-binding protein found in the cytoplasm of muscle cells, heme iron aids in the diffusion of oxygen from capillaries into the cytosol and mitochondria[25]. It plays a key role in cellular energy metabolism by acting as a cofactor for mitochondrial enzymes (cytochromes) that mediate oxidative phosphorylation and the electron transport chain[26]. Iron is also essential for DNA synthesis[27,28], correct functioning of the immune system, particularly proliferation and activity of lymphocytes and macrophages[20]. Its availability is especially critical during periods of rapid growth and neurodevelopment[29]. Iron must be acquired through dietary intake; insufficient iron supply or malabsorption can result in iron deficiency anemia and various physiological impairments such as perinatal mortality, delayed child mental and physical development, and reduced visual and auditory function[30-32].

Approximately 60-70% of the total iron content of the body is found in hemoglobin within circulating red blood cells[33]. An additional 25% is stored mainly as ferritin and hemosiderin (a readily mobilizable form). The remaining 15% is distributed between myoglobin in muscle tissue and a range of iron-containing enzymes that participate in oxidative metabolism and other essential cellular functions[34].

3.2 Iron compounds and their chemistry

3.2.1 Chemical bonds and iron oxidation states

Although iron can theoretically exhibit oxidation states ranging from -2 to +6, the most common and relevant for human physiology are +2 and +3, since they are central to iron metabolism in humans[35]. Iron has an atomic number of 26, and its ground-state electron configuration in the neutral form is [Ar] $4s^2$ $3d^6$. When iron loses electrons to form cations, the electrons are first removed from the 4s orbital and then from the 3d orbitals. In the ferrous state (Fe^{2+}), iron loses two electrons, usually both from the 4s orbital, resulting in the configuration [Ar] $3d^6$. This configuration is not particularly stable because it does not correspond to a half-filled or fully filled d-subshell, and only one of the d-orbitals contains a pair of electrons. In the ferric state (Fe^{3+}), iron loses one additional electron, this time from the 3d orbital, yielding the configuration [Ar] $3d^5$. A $3d^5$ configuration is considered especially stable because it corresponds to a half-filled d-subshell, which contains five electrons in total, one electron in each of the five d-orbitals with parallel spins. This symmetrical distribution minimizes electron repulsion and provides additional stability through exchange energy [36]. According to Hund's rule, half-

filled orbitals with parallel spins are particularly stable due to maximized exchange interactions[37]. Fe^{2+}, with six d-electrons and being less stable, has more potential for electron donation and is generally more soluble and reactive under physiological conditions. In aerobic environments, where oxygen is abundant and acts as a strong oxidizing agent, Fe^{3+} is favored because its lower electron density and stable half-filled d-orbitals make it less prone to further oxidation than Fe^{2+}. This stability also promotes the formation of stronger coordination complexes with biological ligands, enhancing its role in biological systems such as iron metabolism (e.g., in transferrin, ferritin). However, at neutral to basic pH, Fe^{3+} tends to form insoluble iron oxides or hydroxides, which limit its bioavailability in the intestine[38]. To facilitate iron transport by the divalent metal transporter DMT1, Fe^{3+} must be reduced to Fe^{2+} at the apical membrane of enterocytes by ferrireductases such as Dcytb or by food components such as ascorbic acid, since DMT1 transports iron in the ferrous form.

Iron compounds, used as iron supplements and food fortificants, have different properties such as solubility, stability, and bioavailability[39] depending on the interactions between the iron and its ligand/ligands. In simple iron salts, like ferrous sulfate or ferrous gluconate, the ferrous iron forms ionic bonds with its ligand. These are formed through electrostatic attraction between positively charged ions, cations, Fe^{2+} or Fe^{3+} and negatively charged counterions, anions, sulfate or gluconate, with no electron sharing[40]. In contrast, chelated iron forms, such as iron bisglycinate[41] or ferric EDTA, involve coordinate covalent (dative) bonds where both electrons in the shared pair originate from a donor atom (typically oxygen, nitrogen, or sulfur in the ligand) interacting with the orbitals of the Fe^{2+}/Fe^{3+}. This bonding forms stable ring-like chelate structure (the formation depends on the number of donor atoms the ligand has) that shields the iron from precipitation or oxidation[42]. Heme iron is another example of a chelate, where Fe^{2+} is centrally coordinated in a porphyrin ring through nitrogen donors[43]. Nanoparticulate forms of iron, such as ferric phosphate or iron oxides, often utilize surface coatings stabilized through hydrogen bonds or van der Waals interactions to improve solubility and delivery.

3.2.2 Iron salts

Iron salts, such as ferrous sulfate ($FeSO_4$), ferrous fumarate ($C_4H_2FeO_4$) or ferric chloride ($FeCl_3$), are composed of positively charged metal ions and negatively charged counterions in its solid state. They interact by electrostatic forces, forming ionic bonds[44]. The ionic interactions are responsible for high melting points of iron salts, solubility in water (ferrous fumarate is an exception, because of its strong lattice structure and low hydration energy that make it energetically unfavorable for the solid to dissociate into ions[45]), allowing Fe ions to become available for transport across membranes in living organisms[36]. In aqueous solution, these salts dissociate to release aquated complexes like $[Fe(H_2O)_6]^{2+}$ and $[Fe(H_2O)_6]^{3+}$. As pH rises, Fe^{2+} and Fe^{3+} precipitate as hydroxides ($Fe(OH)_2$ and $Fe(OH)_3$), with ferric hydroxide being especially insoluble at neutral pH[46]. Clinically, ferrous salts such as ferrous sulfate and ferrous fumarate have long been the standard choice for treating iron deficiency (WHO,2021)[47], largely because their bioavailability was believed to be significantly higher than that of ferric iron [48,49]. This view has dominated nutritional and clinical guidelines for decades, based on the understanding that Fe^{2+} is the primary absorbable form of iron through the divalent metal transporter 1 (DMT1) in the intestinal epithelium. However, emerging research challenges this traditional model, showing that iron uptake is not strictly limited to Fe^{2+} and may not rely solely on DMT1-mediated transport. Recent findings suggest that iron in nanoparticulate or oxide forms can be absorbed through endocytic

pathways[50,51], bypassing conventional solubilization and reduction steps. These insights mark the beginning of a paradigm shift in our understanding of iron bioavailability and mean that the role of Fe^{2+}, while still important, may not be as exclusive as previously thought. As this area of research continues to grow, it is becoming increasingly clear that iron absorption is more flexible and dynamic than the classical model implies.

3.2.3 Iron chelates

Iron chelates are coordination complexes in which iron, commonly in the Fe^{2+} (ferrous) or Fe^{3+} (ferric) oxidation state, is bound by ligands through coordinate covalent bonds[52]. A chelate is normally defined as a metal complex having the ligand binding to two or more points on a central metal ion. These ligands can be mono- or polydentate, depending on the number of donor atoms used to bind the metal. In contrast, polydentate ligands (also called chelating agents), such as ethylenediaminetetraacetic acid (EDTA), citrate, pyrophosphate or peptides, possess multiple donor atoms that simultaneously coordinate to the iron ion, forming more stable ring-like structures known as chelates. The stability of a coordination complex generally increases with the denticity of the ligand due to the chelate effect, a thermodynamic phenomenon where polydentate ligands form more stable complexes than equivalent monodentate ligands. The entropy of the system increases when a polydentate ligand binds to a metal ion, displacing multiple monodentate ligand and resulting in higher formation constants, making them less likely to dissociate in solution[52,53]. Most commonly used supplements, in a form of iron chelates, rely on polydentate coordination to ensure solubility and minimize free iron[54-56]. Similarly, biological chelators, like porphyrins in heme or the peptides used in food fortification, bind iron through multiple nitrogen or oxygen atoms, forming highly stable complexes.

3.2.4 Nanoparticulate iron

Iron nanoparticles are gaining attention in the field of iron nutrition, as researchers work to optimize their unique properties that enhance bioavailability while reducing the gastrointestinal side effects commonly associated with conventional iron supplements[57]. The nanoparticles retain iron in either ferric (Fe^{3+}) or ferrous (Fe^{2+}) oxidation states, often in the form of oxides (e.g., Fe_2O_3, Fe_3O_4), phosphates, or pyrophosphates. Their nanoscale dimensions (1–100 nm) significantly increase surface area-to-volume ratio, improving solubility in gastric acid and facilitating more efficient absorption across the intestinal epithelium[58]. Iron nanoparticles are often composed of stabilizing or destabilizing agents or surface coatings, such as polysaccharides[59], peptides[60], or citrate[61], to prevent agglomeration, control reactivity, and enhance biocompatibility[57]. In one study, solid lipid nanoparticles were developed, around 25 nm in size, loaded with ferrous sulphate, which showed extended-release properties and significantly improved iron bioavailability in rabbits, reaching much higher peak plasma concentrations than conventional iron sulphate tablets[62]. Nano-sized ferric phosphate[63] exhibited comparable solubility and relative bioavailability profile to ‘’gold standard’’ iron salt, ferrous sulfate. Another type of iron nanoparticles, including tartrate-modified iron oxo-hydroxide[64] (IHAT), exhibits good absorption (around 80% of ferrous sulfate's efficacy) and the EFSA approved safety profile.

3.2.5 Other forms of iron supplements and fortificants

Carbonyl iron is a unique form of elemental (zero-valent) iron (Fe^0), distinct from iron salts, chelates, or nanoparticles[65,66]. Chemically, it is produced by thermal decomposition of iron pentacarbonyl [$Fe(CO)_5$], yielding highly pure, fine iron particles with a spherical morphology and large surface area[67]. Unlike ionic iron in salts, or complexed iron in chelates, carbonyl iron remains uncharged and insoluble in water, dissolving slowly in gastric acid through surface oxidation and protonation. Its gradual solubilization minimizes free radical formation and gastrointestinal irritation, contributing to its safe profile [68] in oral supplementation[69].

3.2.6 Dietary inhibitors and enhancers of iron absorption

Dietary composition can significantly influence the absorption of iron, whether from food sources or oral supplements[9]. Various dietary components act as either inhibitors or enhancers of iron uptake into enterocytes. In plant-derived foods, phytate (myo-inositol hexakisphosphate) is the principal inhibitor of non-heme iron absorption. It contains six phosphate groups attached to a myo-inositol ring, each capable of donating electron pairs to positively charged metal ions such as iron, zinc, and calcium. Through these interactions, phytate forms coordinate covalent bonds with metals, often acting as a multidentate ligand. This results in the formation of stable, sometimes insoluble, metal-phytate complexes[70]. The presence of phytate has a dose-dependent effect, significantly reducing the absorption at concentrations as low as 7-35 mg oh phytic acid per meal[71]. The phytate-to-iron molar ratio is a key determinant of absorption efficiency; in cereal- or legume-based meals lacking absorption enhancers, a ratio below 0.4:1 is required to support effective iron uptake[72]. There are many ways to reduce the amount of phytates in the food, such as fermentation[73], soaking or germination as well as addition of synthetic phytase, showing improved bioavailability profile[74,75]. Polyphenols are naturally occurring compounds found in a wide range of plant-based foods, including cereals, legumes, tea, coffee, and wine. Their inhibitory effect on non-heme iron absorption has been well documented, particularly in studies involving black and herbal teas[76,77]. In cereals and legumes, polyphenols contribute to the overall inhibitory effect on iron absorption, alongside phytate. Calcium has been shown to inhibit the absorption of both heme and non-heme iron[78]. While this inhibitory effect is consistently observed in short-term and single-meal studies, evidence from multiple-meal studies involving diverse diets suggests that calcium's impact on iron absorption may be attenuated under typical dietary conditions[79]. While animal tissue increases non-heme iron absorption, several isolated animal protein from milk and egg have been shown to inhibit this process. Human studies have demonstrated that the two major protein fractions of bovine milk, casein and whey, as well as egg white, reduce non-heme iron absorption[80,81]. Similarly, proteins derived from soybeans have also been reported to exert an inhibitory effect on non-heme iron absorption.[75]

Dietary ascorbic acid is recognized as the most effective enhancer of non-heme iron absorption[82], particularly important in vegetarian and vegan diets. Its efficacy is partly attributed to its reducing capacity, converting ferric (Fe^{3+}) to ferrous (Fe^{2+}) form, and its ability to form soluble chelates with iron[82,83]. Human radioisotope studies using single meals have demonstrated a clear, dose-dependent enhancement of iron absorption with both native and added ascorbic acid[82]. Ascorbic acid has been shown to counteract the inhibitory effects of several dietary components, including phytate[71],

polyphenols[84], calcium, and proteins found in milk products[85]. Recent findings by Teucher et al. and Pizarro et al. have demonstrated that ascorbyl palmitate, a lipid-soluble ester of ascorbic acid, retains its absorption-enhancing properties even after thermal processing, such as baking into iron-fortified bread[86,87]. Several amino acids have been shown to improve iron absorption by forming stable, soluble complexes with iron, thereby enhancing its bioavailability[88-90]. Studies have demonstrated that the co-ingestion of methionine and threonine with low doses of iron can lead to significantly higher hemoglobin levels compared to iron supplementation alone, even within a short period of four weeks[89]. Enhancing effect on iron absorption was also observed with histidine, cysteine, and lysine speculatively because of the ability to form tridentate chelates with iron, which stabilize iron in solution and may facilitate its transport[90]. Other amino acids such as glutamine, aspartic acid, and methionine have also been identified as capable of forming chelatable complexes with iron[91]. Iron-glycine chelate has shown positive effects on iron absorption across various species, including humans[92].

3.3 The effect of pH on iron bioavailability

The solubility of iron is strongly influenced by the interplay between pH and redox potential of a system (Eh), which together determine both the maximum activity of iron in solution and the relative distribution of ferrous and ferric species. Across most of the pH-Eh spectrum, ferric iron remains only sparingly soluble (forming ferric hydroxo complexes which rapidly precipitate), whereas ferrous iron predominates under more reducing and acidic conditions, highlighting the critical dependence of iron solubility on redox state[93]. Ferrous sulfate, dissolved at very low pH (pH 1), has shown strong positive correlation between solubility and relative bioavailability in rat models, although the pH was lower than the physiological range of gastric acidity and may primarily reflect chemical reactivity rather than physiological digestion[94]. Reductions in gastric acidity, such as those induced by cimetidine (600 mg and 900 mg), have been shown to significantly impair non-heme iron absorption (42% and 65%) in humans[95]. The stomach plays a central role in this process by acidifying food, initiating digestion, and regulating the rate at which nutrients are delivered to the small intestine. Gastric pH rises slightly after food ingestion, but then gradually declines (within approximately two hours) to baseline levels of around pH 2, providing the acidic environment necessary for optimal iron solubilization. Iron compounds are broadly classified by solubility characteristics: water-soluble (e.g., ferrous sulfate, ferric EDTA), poorly water-soluble but acid-soluble (e.g., ferrous fumarate), poorly soluble in both water and acid (e.g., ferric pyrophosphate)[96-98].

3.4 The gastrointestinal tract and iron absorption pathways

The GI tract consists of the oral cavity, pharynx, esophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine, rectum, and anus. The oral cavity is the initial site of digestion, where ingested food is mixed with saliva. After swallowing, the food bolus passes through the esophagus and enters the stomach, where it encounters hydrochloric acid and gastric enzymes, continuing the digestive process[99]. The gastric environment is highly acidic, with a fasting pH around 2[100]. Upon entering the duodenum, the acidic chyme is neutralized by bicarbonate (HCO_3^-) secreted by duodenal epithelial cells and the pancreas[101]. The stomach empties its contents into the duodenum, the primary site for the absorption of iron and other nutrients[102]. Approximately one hour after food intake, the

pH in the upper duodenum rises to around 4 and gradually increases along its length, reaching about 8 in the mid-duodenum due to the influence of bile and pancreatic secretions[100]. Nutrient digestion and absorption continue in the jejunum and ileum, which lead into the large intestine. The upper colon is primarily responsible for the absorption of water, sodium, and potassium, while nutrient absorption is largely complete by this stage[103]. The lower colon serves as a site for fecal storage and transports waste toward the rectum and anus for elimination[104].

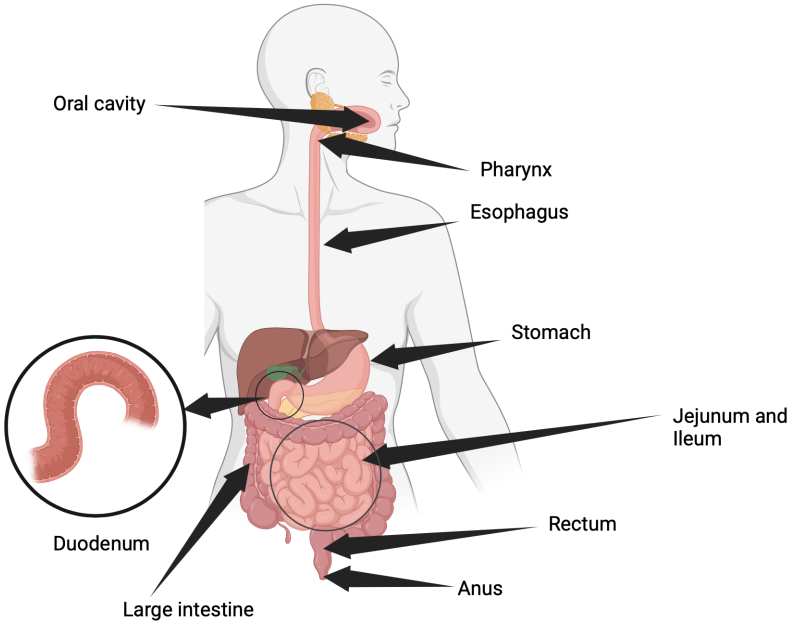

Figure 1. The human gastrointestinal tract (created with Biorender.com).

The GI tract is organized into four main layers: the mucosa, submucosa, muscularis externa, and either serosa or adventitia[105]. The mucosa features a highly folded surface that increases its absorptive capacity. These folds (known as plicae circulares) can extend up to 8 mm into the lumen. Covering these folds are finger-like projections called villi, which are approximately 1 mm in length and composed of a single layer of epithelial cells. Each epithelial cell bears a brush border made up of densely packed microvilli, further amplifying the surface area for nutrient absorption. Villi contain several specialized cell types, including enterocytes (modelled by e.g. differentiated Caco-2 cells), goblet cells that secrete mucus (modelled by e.g. HT29 cells), and L cells, which produce hormones (modeled by e.g. Hutu-80 cells). The epithelium is supported by the lamina propria, connective tissue, that also contains capillaries, lymphatic vessels, and immune cells. Most intestinal epithelial cells originate in the crypts of Lieberkühn, migrate upward along the villus, and are eventually shed at the tip. In the duodenum, the epithelial cell turnover cycle is rapid, taking approximately 3 to 5 days, an essential feature for regulating nutrient absorption and barrier integrity. Beneath the mucosa, the submucosa contains connective tissue, blood and lymphatic vessels, and the submucosal plexus, which contributes to local control of secretion and blood flow. The muscularis externa consists of smooth muscle layers responsible for peristalsis, regulated by the myenteric plexus. The outermost layer is either the serosa, which reduces friction around intraperitoneal organs via secretion of serous

fluid, or the adventitia, which provides structural anchoring for retroperitoneal sections of the GI tract[106].

The structural specialization of the intestinal mucosa, improves absorptive capacity but also ensures efficient regulation of nutrient uptake. Specific membrane transporters are localized to the apical surface of enterocytes to mediate selective uptake. One of them is the divalent metal transporter 1 (DMT1), important for dietary iron acquisition.

3.4.1 DMT1 mediated iron uptake

The divalent Metal Transporter 1 (DMT1/NRAMP2/DCT1/SLC11A2) is a protein with 12 membrane-spanning domains, with N-glycosylation sites located on both its N-terminal and C-terminal tails, responsible for mediating active proton-coupled (H+) transport of e.g. ferrous iron (Fe^{2+}) across the apical membrane of enterocytes in the human intestine. It was first identified through homology with Nramp proteins, and later confirmed as the apical iron transporter essential for non-heme iron absorption via studies in the Belgrade rat and mk mouse models [107,108]. DMT1 is widely expressed across various tissues, although typically at low levels, with particularly high expression in the duodenum where iron absorption occurs[109]. Additionally, iron deficiency enhances the expression and apical localization of DMT1 in enterocytes, thereby increasing iron uptake from the intestinal lumen[110,111]. It also facilitates uptake of other divalent metal ions such as Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}[111,112]. Divalent metal transporter 1 (DMT1) is expressed in four isoforms, generated through the use of two alternative promoters (5') and alternative splicing at the 3' end (IRE)[113,114]. The presence or absence of a 3' iron-responsive element (IRE) in the mRNA's untranslated region regulates isoform stability and translation in response to intracellular iron levels, allowing precise control of DMT1 expression under varying physiological conditions[115]. Promoter 1A primarily drives expression of the 1A isoforms, which are most abundant in the intestinal epithelium, particularly in the duodenum, where the 1A/+IRE isoform is localized to the apical membrane of enterocytes to facilitate dietary iron uptake[111,115]. Promoter 1B gives rise to the 1B isoforms, which are expressed more broadly across tissues such as the liver, kidney, and brain[115,116]. Among these, the 1B isoform is primarily found in endosomal membranes, where it plays a key role in iron transport from transferrin-containing endosomes [117] . Since most dietary iron is present in the ferric (Fe^{3+}) form, it must first be reduced to the ferrous (Fe^{2+}), by an apical ferrireductase duodenal cytochrome B (Dcytb/CYBRD1)[118] before it can be transported into enterocytes via DMT1. Ferric reductase activity is essential for non-heme iron uptake by this transporter, as demonstrated in Caco-2 cell studies[119]. Dcytb is a heme-containing transmembrane protein that facilitates the reduction of Fe3+ to Fe2+ by transferring electrons from intracellular ascorbate, at the brush border membranes[120,121]. Since DMT1 co-transports ferrous ions (Fe^{2+}) along with protons (H^{+}), its activity is pH-dependent, with increased transport efficiency at lower (more acidic) pH levels[122].

3.4.2 Endocytosis-mediated iron uptake

While DMT1-mediated transport of Fe^{2+} is the established pathway for intestinal iron absorption for non-heme iron, recent studies have showed that endocytic mechanisms also contribute to iron uptake, particularly for nanoparticulate Fe(III) compounds[50,51]. Clathrin-mediated endocytosis (CME) is the most thoroughly characterized endocytic pathway and is ubiquitous across mammalian cells[123].

It serves as the primary route for the internalization of essential nutrients, i.e. transferrin[123,124] via clathrin-coated vesicles. Clathrin-mediated endocytosis can be divided into distinct stages: initiation of clathrin-coated pits (CCPs), cargo selection, pit growth and maturation, membrane scission, and the release of clathrin-coated vesicles (CCVs)[125]. Clathrin-mediated endocytosis is tightly regulated at multiple levels and includes selective recruitment of adaptor proteins, the local lipid environment, critical for initiating and stabilizing adaptor and clathrin binding at the plasma membrane as well as post-translational modifications such as phosphorylation and ubiquitination of endocytic proteins[126]. In differentiated Caco-2 cells, luminally hydrolyzed Fe(III) poly oxo-hydroxide nanoparticles are taken up via clathrin- and caveolin-mediated endocytosis, followed by lysosomal dissolution and lysosomal DMT1-mediated transport into the cytosol[50]. Supporting this, Caco-2 cells supplemented with ferric phosphate nanoparticles (NP-$FePO_4$; size 50–400 nm) and treated with endocytosis blockers such as chlorpromazine and sucrose (clathrin-mediated endocytosis) as well as dimethyl amiloride (macropinocytosis), showed a significant decrease in ferritin build-up, indicating a vesicular transport route[51]. Lönnerdal's group demonstrated that Caco-2 cells can absorb ferritin labeled with ^{59}Fe, suggesting that enterocytes are capable of internalizing ferritin via a receptor-mediated mechanism[127], and they reported similar findings for soybean ferritin, showing uptake in both Caco-2 cells and humans using radiolabeled ferritin[128]. However, these conclusions were challenged by Hurrell's group, who studied ferritin digestion and found that iron is efficiently released from the ferritin molecule during cooking and under gastric pH[129], meaning it should be absorbed as efficiently as other non-heme iron present in food. Some findings suggest that at elevated ferritin concentrations, macropinocytosis may function as a secondary pathway for uptake[127].

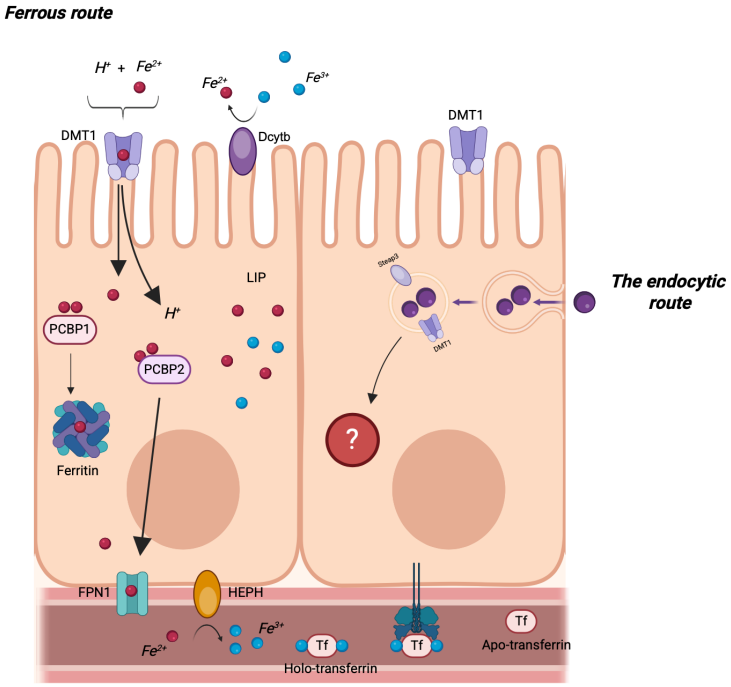

Figure 2. Overview of the established non-heme iron absorption pathways in human epithelial absorptive cells (created with Biorender.com).

3.4.3 Heme iron uptake

Heme is a coordination complex composed of an iron ion bound to a porphyrin ring. When iron is in the ferrous (Fe^{2+}) state, the heme complex is electrically neutral. In the ferric (Fe^{3+}) state, it becomes hemin, and carries a positive charge. Heme is hydrophobic and exists in various natural forms, the most common being heme b, found in hemoproteins such as hemoglobin, myoglobin or cytochromes (e.g., b and P450)[130]. Heme iron enters enterocytes through at least two proposed pathways. The first pathway involves direct transport by heme carriers, such as PCFT/HCP1, which imports intact heme into the cytoplasm[131,132]. A second proposed absorption pathway involves receptor-mediated endocytosis, where heme binds to a yet unidentified apical membrane receptor, is internalized via endocytic vesicles, and subsequently degraded by heme oxygenase (HO-1 or HO-2) to liberate iron[133-135]. Evidence from in vivo studies in rats and dogs demonstrates that heme or hemoglobin administered into closed duodenal loops is internalized by enterocytes via an endocytotic pathway. Electron microscopy, following heme staining, revealed its localization, first on the microvillus membrane, then in apical tubulovesicular structures, and subsequently in secondary lysosomes. The absence of heme in the basal cytoplasm or extracellular space, along with its disappearance from lysosomes within 2-3 hours, supports uptake at the apical membrane by endocytosis. While DMT1 does not transport heme directly, it may contribute by exporting iron from lysosomes into the cytosol following heme degradation[136]. The DMT1 involvement still remains a hypothesis, but if validated, the DMT1 isoform involved would likely be the 1B/+IRE variant, which is known to localize to endosomal membranes, rather than the 1A isoform that is specifically expressed on the apical surface of enterocytes[117]. A third potential mechanism includes basolateral export of heme by Feline Leukemia Virus subgroup C Receptor (FLVCR), although its role in apical uptake remains unclear[137].

3.4.4 Other mechanism for iron uptake

The integrin-mobilferrin-paraferritin pathway has been proposed as a mechanism for ferric iron uptake in intestinal epithelial cell, by Umbreit and Conrad[138,139]. In this model, ferric iron binds to mucin on the luminal surface, which chelates the otherwise insoluble iron, making it bioavailable. This iron-mucin complex interacts with a surface integrin, facilitating iron entry into the enterocyte. Once inside, ferric iron is reduced by a cytoplasmic ferrireductase, referred to as paraferritin[138]. While this pathway offers an alternative to the well-established DMT1-mediated ferrous iron uptake, it remains controversial. There is limited functional evidence supporting its physiological relevance and the findings by Han et al. contradict this model[119].

3.5 Iron storage

Once iron enters enterocytes, it can follow one of two main intracellular pathways: it may be temporarily stored as ferric iron (Fe^{3+}) within ferritin, or it may be exported across the basolateral membrane into the bloodstream. Ferritin is a highly conserved iron-storage protein that plays a central role in maintaining cellular iron homeostasis[140]. It forms a hollow, spherical nanocage composed of 24 subunits of two types: the heavy (FTH1) and light (FTL) chains[141]. Each subunit type serves distinct but complementary functions. The H-chain possesses ferroxidase activity, which catalyzes the oxidation of

Fe^{2+} to Fe^{3+}, facilitating its incorporation into the ferritin core[142]. The L-chain, while lacking enzymatic activity, promotes iron nucleation and stabilizes the mineral core, enhancing long-term iron storage[143]. Together, these subunits allow ferritin to safely sequester up to 4,500 iron atoms, typically as a ferric phosphate complex[144]. In vitro studies, conducted by Glahn and colleagues[145], have shown that cellular iron uptake is directly proportional to intracellular ferritin levels, indicating that ferritin not only reflects iron status but also serves as a predictive marker of iron bioavailability and transport efficiency in cellular systems.

3.6 Cellular regulation of iron metabolism

Many proteins involved in iron metabolism are post-transcriptionally regulated by iron-responsive elements (IREs), conserved RNA stem-loop structures located in the untranslated regions (UTRs) of specific mRNAs[146]. These IREs are recognized and bound by iron regulatory proteins (IRPs), primarily IRP1 and IRP2, which regulate the translation of their target mRNAs in response to intracellular iron levels. For example, ferritin and ferroportin mRNAs contain IREs in their 5′ UTRs, where IRP binding inhibits translation under iron-deficient conditions, thus limiting iron storage and export. In contrast, divalent metal transporter 1 (DMT1) and transferrin receptor 1 (TfR1) mRNAs have IREs in their 3′ UTRs. Here, IRP binding stabilizes the mRNA, preventing degradation and thereby promoting increased iron uptake. TfR1 mRNA contains multiple IRE motifs in tandem, enhancing its sensitivity to IRP-mediated stabilization. This IRE/IRP regulatory system allows for tight coordination of iron absorption, transport, storage, and utilization according to cellular iron availability[146,147].

3.7 Iron export from intestinal cells and systemic regulation

Ferroportin (FPN1/IREG1/SLC40A1) is the only known iron exporter in mammals and is responsible for transporting ferrous iron (Fe^{2+}) out of the enterocyte[148].For efficient loading onto apotransferrin, the Fe^{2+} exported by ferroportin must first be oxidized to ferric iron (Fe^{3+}). This oxidation is catalyzed by the membrane-bound ferroxidase hephaestin (HEPH) which is homologous to ceruloplasmin (CP) and is primarily expressed in enterocytes[149]. Once oxidized, Fe^{3+} binds to apotransferrin in the plasma, forming holo-transferrin, which then circulates to deliver iron to tissues. Ferroportin is tightly regulated by the hormone hepcidin, which binds to ferroportin and triggers its internalization and degradation, leading to iron efflux reduction when body stores are sufficient or during inflammation[150].

There are at least two primary regulatory mechanisms that help prevent iron overload following iron intake. One is the rapid, short-term response at the level of the intestinal epithelium, commonly referred to as the “mucosal block” phenomenon. This occurs within hours after an initial iron dose and reduces the absorption of iron from subsequent doses. Studies using Caco-2 cells[151] and mice[152] , suggest that this response may involve the internalization and downregulation of the divalent metal transporter 1 (DMT1), limiting apical iron uptake into enterocytes. This post-transcriptional modulation of DMT1 helps reduce iron influx when luminal iron levels are high. The second, longer-term regulatory mechanism is systemic, mediated by the iron-regulatory hormone hepcidin, whose mRNA is predominantly expressed in the liver, with lower expression observed in the muscle, intestine, stomach, heart and lungs[153,154]. Hepcidin (HAMP, HEPC, HFE2B, LEAP1) is a peptide hormone

composed of 25 amino acids, and its expression is upregulated in response to elevated systemic iron levels or inflammation. It acts by binding to the basolateral iron exporter ferroportin, leading to its internalization and degradation, which effectively blocks iron efflux from enterocytes into the bloodstream[155]. Through this pathway, hepcidin not only limits dietary iron absorption but also iron release from macrophages and hepatocytes, maintaining systemic iron homeostasis. In iron-replete states or during chronic inflammation, elevated hepcidin levels can significantly reduce plasma iron availability, contributing to iron-restricted erythropoiesis or anemia of inflammation[156]. These two layers of control, an enterocyte-intrinsic response and a systemic feedback loop, both can be mediated by hepcidin, and tightly regulate iron absorption and prevent both deficiency and overload.

3.8 Iron uptake in hepatocytes and macrophages: key regulators of systemic iron homeostasis

Hepatocytes

Iron uptake into hepatocytes occurs through several mechanisms that accommodate the different forms in which iron circulates in the body. The primary pathway involves transferrin receptor 1 (TFR1)-mediated endocytosis of diferric transferrin, which binds to TFR1 with high affinity, is internalized into endosomes, and releases ferric iron upon acidification. This ferric iron is reduced to its ferrous form by STEAP3 and then transported into the cytosol via divalent metal transporter 1 (DMT1)[157]. Hepatocytes also express transferrin receptor 2 (TFR2), which binds diferric transferrin with lower affinity than TFR1 but facilitates iron uptake under physiological transferrin saturation, and also plays a regulatory role affecting hepcidin expression[158]. In states of iron overload, non-transferrin-bound iron (NTBI) becomes a significant source of hepatic iron[159]. NTBI, often complexed with low molecular weight ligands like citrate, is imported into hepatocytes primarily via DMT1 and ZIP14, with the uptake being saturable[160]. Additional iron sources include ferritin, heme-hemeopexin, and hemoglobin-haptoglobin complexes, which are taken up through receptor-mediated endocytosis followed by lysosomal degradation[161,162].

Macrophages

In specialized macrophages such as red pulp macrophages in the spleen and Kupffer cells in the liver, the primary route of iron acquisition is through erythrophagocytosis[163,164]. These cells engulf senescent red blood cells into phagolysosomes, where hemoglobin is degraded and heme is transported across the phagosomal membrane into the cytoplasm by the heme transporter HRG1. There, heme is degraded by heme oxygenase-1 (HO-1) into biliverdin, carbon monoxide, and iron. HO-1 is essential for iron recycling and macrophage viability, with deficiencies causing systemic iron dysregulation and organ damage[165,166]. Additional iron uptake occurs via transferrin receptor 1 (TfR1), particularly in tissue-resident macrophages, where transferrin-bound iron is reduced by STEAP3 and imported via DMT1[167]. Under conditions such as hemolysis, macrophages also internalize hemoglobin-haptoglobin and heme-hemopexin complexes through CD163 and LRP1 receptors, respectively, providing a protective mechanism against the toxicity of free heme[168]. In iron overload states, macrophages actively sequester excess iron through phagocytosis of senescent erythrocytes and uptake of non-transferrin-bound iron, storing it safely in ferritin to prevent oxidative damage[164]. Within the intestinal environment, macrophages infiltrate the lamina propria and localize near the epithelial layer, where they closely interact with enterocytes[169]. These interactions allow macrophages to affect the epithelial responses to luminal iron[170] and inflammation through cytokine signaling[171,172].

3.9 Cell culture models commonly used in iron transport studies in humans

The Caco-2 cell line, derived from human colorectal adenocarcinoma, is the most common in vitro model to study intestinal iron absorption[145,173,174]. When cultured for 14 days (200 000 cells/well in 12 well-plates) on permeable supports (e.g., Transwell inserts), Caco-2 cells spontaneously differentiate into polarized epithelial monolayers, mimicking small intestinal enterocytes. They develop tight junctions, apical microvilli, and express a set of intestinal enzymes and transporters, such as DMT1 (divalent metal transporter 1), ferrireductases (e.g., DcytB), and ferroportin (FPN), which are involved in non-heme iron uptake and transcellular transport[175,176]. Caco-2 monolayers exhibit high transepithelial electrical resistance (TEER > 250 $\Omega \cdot cm^2$), confirming their function as a selective barrier[177]. Uptake studies using radioactive ^{59}Fe have shown preferential absorption of Fe^{2+} over Fe^{3+}, consistent with in vivo findings[145]. Moreover, Caco-2 cells respond to cellular iron status, if the iron levels are low in the culture media, the cells will become iron deficient which leads to upregulation of DMT1 and FPN expression. On the other hand, iron loading suppresses the DMT1 and FPN expression, reflecting post-transcriptional regulation via iron-regulatory proteins (IRPs) binding to iron-responsive elements (IREs) on mRNAs[146].

To incorporate systemic regulation, especially hepcidin-mediated control, coculture system combining Caco-2 with HepG2 (hepatocytes) cells has been developed[178]. In such a model, the two cell types are cultured in bicompartmental setups, allowing signaling between the cell epithelia. HepG2 cells synthesize and secrete hepcidin, the liver-derived peptide hormone that regulates systemic iron homeostasis. In coculture, iron exposure (≥120 µM Fe) increases hepcidin levels in the medium, which downregulates ferroportin and, at concentrations of 10 ng/mL hepcidin, reduces DMT1 expression in Caco-2 cells. This model better reflects the hepcidin–ferroportin axis activity, making it valuable for investigating regulatory responses to iron status and nutritional or pharmacological interventions[178]. Hutu-80 cells are another cell line used for iron transport studies due to their duodenal origin[179], aligning with the primary site of dietary iron absorption in the human body. Hutu-80 cells offer higher transfection efficiency compared to Caco-2 cells, making them particularly suitable for gene silencing and mechanistic studies involving transporters such as DMT1[180]. HT29 cells are a human colorectal adenocarcinoma cell line commonly used as an in vitro model of the intestinal epithelium, most often used as a coculture with Caco-2 cells[181,182]. They are particularly valuable for studying intestinal barrier function and host–microbe interactions due to their ability to differentiate into mucus-producing goblet-like cells under specific culture conditions[183].

Another cell culture model, to be potentially used for iron uptake studies, are intestinal organoids, derived from adult intestinal stem cells or induced pluripotent stem cells (iPSCs). These are 3D cultures that grow on scaffolds and self-organize into mini-gut structures with crypt-villus architecture and multiple cell types (e.g., enterocytes, goblet cells, Paneth cells)[184]. In some cases, compared to traditional 2D cultures, 3D cell culture models may offer superior physiological relevance by preserving tissue-specific architecture and ability of cell-to-cell and matrix interactions. This complexity makes them more predictive of in vivo responses; for example, colon cancer HCT-116 cells grown in 3D exhibit increased resistance to chemotherapeutic agents such as melphalan, fluorouracil, oxaliplatin, and irinotecan, mirroring drug resistance patterns commonly observed in vivo[185].

There have been especially many attempts to develop intestinal organoid models that mimic the intestinal environment in irritable bowel syndrome (IBS)[186]. Studies have begun to explore iron uptake and regulation in organoids[187], but challenges remain, such as access to the apical surface, contained within the interior of the organoid[188]. Some existing models show that compared to Caco-2 cells, differentiated intestinal organoids may more accurately mimic the structural and molecular features of intestinal inflammation, including protein expression patterns and barrier disruption observed in IBS patients[189,190]. Emerging alternative models for studying iron uptake include the use of microfluidic gut-on-a-chip systems. These platforms utilize microchannels lined with i.e. human jejunal enteroids and human umbilical vein endothelial cells (HUVECs), operated under dynamic flow and mechanical strain to mimic physiological conditions such as peristalsis and fluid shear stress [191]. Some versions incorporate endothelial and immune cells or co-cultures with liver cell lines to simulate gut-liver axis interactions. They are promising platforms for studying iron flux, regulation by hepcidin, and iron-drug interactions[192].

3.10 Iron homeostasis

Recent advances in molecular biology have significantly enhanced the understanding of the complex mechanisms governing iron transport and homeostasis, as well as the pathological consequences arising from their disruption. Iron levels require strict regulation to support essential physiological functions such as oxygen transport, cellular metabolism, and redox homeostasis, while preventing overload-associated toxicity mediated by the generation of reactive oxygen species (ROS)[20,21]. Even though it is widely believed that the body lacks major pathways for active iron excretion, there are some studies in both humans and mouse models suggesting otherwise[193,194]. The most important homeostatic mechanism is the intestinal iron absorption: when systemic iron levels are sufficient, dietary iron is not absorbed and is instead eliminated through the gastrointestinal tract[9,152,195]. While iron loss can occur through processes such as epithelial cell shedding, menstrual bleeding, or therapeutic phlebotomy in cases of iron overload[196], there is evidence indicating that excess iron can also be actively secreted into bile and excreted via feces[197,198].

Disruptions in iron homeostasis, whether due to deficiency or overload, are associated with pathological outcomes such as reduced physical performance or impaired cognitive function[199] in iron deficiency; or the development of cirrhosis, cardiomyopathy, and diabetes mellitus[200] (because of toxic accumulation of iron in organs such as the liver, heart, and pancreas), in case of iron overload. Reductions in iron stores through phlebotomy or iron chelation have been shown to improve clinical outcomes in individuals with diabetes, suggesting potential therapeutic strategies for disease management[201,202]. Iron chelation has also been proposed as a potential therapeutic approach in cancer treatment[203]. Neoplastic cells have an elevated demand for iron due to their high proliferation rates therefore they often upregulate transferrin receptor 1 (TFR1), enabling enhanced iron uptake compared to normal cells[204]. Three iron chelators, deferoxamine, deferiprone, and deferasirox, have been approved by the U.S. Food and Drug Administration (FDA) for clinical use[205]. Deferoxamine was the first iron chelator introduced for cancer therapy and showed notable efficacy in reducing bone marrow infiltration by tumor cells in seven out of nine neuroblastoma patients[206]. In studies using murine models of colon adenocarcinoma (C26 cell line and murine tissue) have shown that deferiprone-loaded nanoparticles exhibit strong therapeutic potential in colorectal cancer (CRC), with

targeted nano-platforms significantly reducing tumor cell density and enhancing tumor necrosis [207]. A treatment regimen combining radiotherapy followed by Triapine, a next-generation lipophilic iron chelator, resulted in significant suppression of tumor growth in murine models bearing U251 glioma or PSN1 pancreatic carcinoma, highlighting its potential in combination cancer therapies[208].

The ability of the human body to adapt to insufficient iron intake is linked to enhanced resistance to infection as well as attenuation of inflammation, indicating the interplay between iron metabolism and the immune response. This connection is clearly demonstrated in anemia of inflammation (AI). Lower iron levels in blood (hypoferremia) during inflammation, decreased iron-binding in serum as well as ferritin elevation in cells are hallmarks of AI[209-212] and has been investigated by e.g. Cartwright et al.[213,214]. Anemia of inflammation is regulated by hepcidin (a peptide hormone synthesized primarily in the liver) through binding to ferroportin, the only known iron exporter, causing its internalization and degradation, leading to iron efflux reduction[155]. Inflammatory signaling such as IL-6 production induces hepcidin expression[215,216], in turn leading to reduced systemic iron levels. The regulation of hepcidin involves the integration of several signaling pathways, allowing for control of iron homeostasis during inflammation[217]. This response not only limits iron accessibility to invading pathogens but also contributes to the development of AI. In conclusion, hepcidin is now widely recognized as the central regulator of systemic iron homeostasis.

3.11 Impact of iron on inflammatory signaling

Iron plays an important role in regulating inflammatory pathway by i.e. changes in activation states of immune cells[218] or impacting redox balance[219]. During inflammation, the liver hormone hepcidin is upregulated, limiting iron export and contributing to hypoferremia and anemia of chronic disease[216]. The role of iron in ferroptosis, an iron-involving cell death process linked to lipid peroxidation, also highlights its contribution to chronic inflammatory conditions[26]. In the context of the human diet, iron intake can influence inflammatory signaling by altering systemic and cellular iron availability[155]. Diets high in bioavailable iron (heme iron) have been associated with increased risk of colorectal cancer[11,16,220]. Conversely, inadequate iron intake can impair immune function and reduce the capacity to mount an effective inflammatory response[221].

There are many studies connecting dietary/supplemental iron with a plethora of inflammatory/carcinogenic responses. A randomized supplementation study in healthy adults showed that taking 19 mg/day of ferrous sulfate significantly increased “weakly bound” fecal iron (from 60 to 300 µmol/L) and boosted free radical generation by around 40% in the colon, an effect linked to potential mucosal damage or carcinogenesis promotion in the gut[222]. In a study in the adeno-carcinoma cell lines Caco-2 and SW480, ferrous sulfate concentrations (0.01-0.1 mM) led to increased STAT3 phosphorylation and Wnt signaling, pathways involved in inflammation and tumor growth[223]. Moreover, in lung cancer cells exposure to 100 µM ferrous sulfate significantly enhanced colony formation, implicating a role in tumorigenesis[224]. However, heme iron, a naturally occurring chelate has been also implicated in colorectal cancer development. Epidemiological and animal studies have linked high heme intake with increased colorectal cancer risk and promotion of preneoplastic lesions in rodent models[11,225,226]. Although, the mechanism for pro-oncogenicity and heme-iron intake from red meats have not yet been established, there are few possible causes; 1) heme catalyzes the formation of endogenous N-nitroso compounds, known mutagens, especially in the presence of

dietary nitrites and high cooking temperatures, 2) other iron chelates: ferric citrate and ferric EDTA, have been shown to induce inflammation and tumorigenic signaling in rodent studies[227-229], 3) In vitro studies showed that while ferrous sulfate had no effect, ferric chelates elevated amphiregulin and EGFR levels and activated the MAPK pathway via ERK phosphorylation in Caco-2 and Hutu-80 colorectal cancer cells[10].

3.11.1 The role of amphiregulin in inflammation and tumor progression

Amphiregulin (AREG), a member of the epidermal growth factor (EGF) family and ligand of the EGF receptor (EGFR)[230], plays a critical role in inflammation and tumor progression, particularly in colorectal cancer (CRC)[231]. Amphiregulin is synthesized as a transmembrane precursor that is cleaved by ADAM metalloproteases (particularly ADAM17)[232], potentially by other metalloproteinases as well[233], releasing its active soluble ligand capable of activating EGFR through autocrine or paracrine signaling[234]. Amphiregulin expression is regulated by a diverse set of transcriptional activators, including WT1, HIF-2, p53, β-catenin, CREB, and AP-1[234]. Like other EGFR ligands, amphiregulin is involved in autocrine feedback loops that sustain EGFR and MAPK signaling[235]. Its expression is induced by multiple pro-inflammatory stimuli, including tumor necrosis factor-alpha (TNF-α)[236,237], interleukin-1β (IL-1β)[238], and prostaglandin E2 (PGE2)[239], through the activation of key inflammatory signaling pathways such as MAPK/ERK, JAK/STAT, NF-κB, and AP-1[234]. Amphiregulin has lower affinity for EGFR than EGF or TGF-α but promotes sustained signaling, inducing weaker EGFR downregulation and enabling prolonged receptor signaling leading to enhanced oncogenic potential[234].

In CRC, elevated amphiregulin expression correlates with aggressive tumor behavior, higher tumor burden, liver metastasis, lymphovascular invasion, and poor differentiation[240]. Recent studies demonstrate that amphiregulin expression can be induced by chelated iron compounds, such as ferric citrate and ferric EDTA, but not by ferrous sulfate[10]. These chelated ferric forms induce AREG expression in colon epithelial cells through activation of the ERK1/2 MAPK pathway[10].

3.11.2 Iron-driven inflammatory responses via COX-2 and NF-κB pathways

Iron-driven inflammatory responses are closely tied to the alteration in expression of the COX-2 and NF-κB pathways[241], both of which are involved in human inflammation and tumor progression[242-244]. Cyclooxygenase-2 (COX-2) is a 72 kDa inducible enzyme encoded by the chromosome 1q25.2-q25.3[245]. Structurally, COX-2 operates in the form of a homodimer, with each 581-amino acid subunit containing three key domains: an N-terminal epidermal growth factor domain, a membrane-binding domain comprising amphipathic α-helices for lipid bilayer interaction, and a C-terminal catalytic domain that houses the peroxidase active site[246]. This active site converts arachidonic acid (AA), released from membrane phospholipids by phospholipase A2 (PLA2), into prostaglandin H2, the precursor of various bioactive prostanoids including prostaglandin E2 (PGE2)[247,248]. COX-2 expression is normally low in most tissues but becomes upregulated under pathological conditions such as inflammation, cancer, or stress[249]. It is constitutively expressed in the brain, kidney, and female reproductive organs, but dramatically overexpressed in a variety of tumors including colorectal cancer, where it facilitates angiogenesis and tumor invasion through PGE2 production[250]. Supporting the hypothesis that iron may impact COX-2 expression or directly oxidize arachidonic acid, studies have shown that ferric nitrilotriacetate (FeNTA) elevates PGE2 production in rabbits[251] ,while oral administration of

Deferiprone (Fe(III) chelator) reduces PGE2 levels in rats[207]. Importantly, the COX-2 pathway does not function in isolation; it is closely interconnected with other molecular signaling networks, including NF-κB[252,253], which further influence inflammatory processes and cellular stress responses[254]. Nuclear factor-κB (NF-κB) is a family of transcription factors that regulate genes involved in immune and inflammatory responses. It includes five members: p50, p52, RelA (p65), RelB, and c-Rel, which form active dimers that bind DNA. In resting cells, NF-κB is sequestered in the cytoplasm by inhibitory proteins called IκBs. Upon stimulation, by i.e. cytokines (TNF-α, IL-1β), oxidative stress, or iron-induced ROS, the IκB kinase (IKK) complex becomes activated and phosphorylates IκB proteins, targeting them for ubiquitination and proteasomal degradation. This releases NF-κB, allowing it to translocate into the nucleus, where it binds κB motifs in promoter regions of target genes, including COX-2, TNF-α, IL-6, and various adhesion molecules[255,256]. NF-κB itself also cooperates with the MAPK and PI3K/AKT pathways in promoting survival and resistance to apoptosis[257]. Perhaps, excessive amounts of some forms of iron could affect the balance and push toward a pro-inflammatory induction of NF-κB and COX-2, leading to inflammation-driven cancer progression, particularly in tissues like the colon where iron exposure from the diet or supplementation is high.

3.11.3 MAPK pathway activation by iron chelates-induced inflammation

The MAPK (Mitogen-Activated Protein Kinase) pathway is a signaling cascade regulating essential cellular processes such as growth, differentiation, and survival. It involves a series of kinases that use phosphorylation as an on/off switch, activating or deactivating proteins and thereby controlling downstream signaling events. Conversely, dephosphorylation can reverse these effects, turning the signal off or affecting its intensity[258]. The MAPK pathway is based on three main tiers: MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K), and MAP kinase (MAPK), with ERK1/2 (extracellular signal-regulated kinase), p38 MAPK, and JNK (c-Jun N-terminal kinase) being the principal MAPKs activated in response to extracellular stimuli[259]. Extracellular stimuli such as growth factors or cytokines, act as secondary messengers that can stimulate MAP3K (such as MEKK1, ASK1 or TAK1). MAP3Ks phosphorylate and activate MAP2Ks such as MEK1/2 for ERK, MKK3/6 for p38, and MKK4/7 for JNK, which then phosphorylate their respective MAPKs. Once activated, ERK1/2 primarily mediates cell proliferation and survival, whereas p38 and JNK are involved in inflammatory and stress responses[260]. MAPK signaling contributes to the upregulation of pro-inflammatory genes, including cyclooxygenase-2 (COX-2) and cytokines like IL-1β and TNF-α, through the activation of transcription factors such as AP-1 (composed of c-Fos and c-Jun), CREB, and ATF-2[261]. ERK1/2 phosphorylation has been observed following exposure to iron chelates (ferric citrate and ferric EDTA), but not with ferrous sulfate, in Caco-2 and Hutu-80cells, suggesting that certain forms of iron (chelates) affect MAPK pathway activation[10]. Muñoz et al. observed that in neuronal PC12 cells treated with iron chelate-ferric nitrilotriacetate (Fe-NTA), the MAPK/ERK pathway was activated[262].

3.11.4 IL-6 signaling and activation of the JAK/STAT pathway by specific forms of iron

Iron has been demonstrated to upregulate interleukin-6 (IL-6) expression and production across multiple cancer types, including breast and colorectal cancers[17,263]. In breast cancer cell lines, iron overload (iron chelate- ferric ammonium citrate) induces IL-6 production, which in turn activates the JAK2/STAT3 signaling cascade, facilitating epithelial-mesenchymal transition (EMT) and tumor cell migration[263]. Similarly, in murine model of colitis, excessive dietary iron (carbonyl iron) exacerbates

colonic inflammation, synergistically enhancing IL-6 and IL-11 secretion and promoting STAT3 activation in colonic tissue, thereby accelerating tumorigenesis[17]. In contrast, in a DSS-induced colitis model in rats, a diet rich in ferrous sulfate did not significantly elevate IL-6 levels, suggesting that the form of iron may influence inflammatory responses[264]. IL-6 is a 26-kDa secreted cytokine composed of 184 amino acids, containing two N-glycosylation sites and four cysteine residues[265,266]. It functions as a pleiotropic cytokine involved in diverse biological systems and organ functions[267]. Mechanistically, IL-6 exerts its effects by binding to its membrane-bound receptor (IL-6R) and the signal-transducing co-receptor gp130, resulting in gp130 dimerization[268]. The metalloproteases ADAM10 and ADAM17 mediate proteolytic cleavage of membrane-bound IL-6R, generating soluble IL-6R (sIL-6R). The IL-6/sIL-6R complex can activate gp130 on cells lacking membrane-bound IL-6R, a mechanism termed IL-6 trans-signaling. This expands the spectrum of responsive cell types, accounting for the cytokine's wide-ranging effects[269].

IL-6 signaling initiates downstream activation of the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway[270], as well as the JAK-mitogen-activated protein kinase (MAPK) cascade[271]. JAK associates constitutively with the cytoplasmic domain of gp130 and becomes activated upon IL-6 engagement, leading to STAT3 phosphorylation, dimerization, and nuclear translocation[272]. Activated STAT3 drives transcription of target genes regulating proliferation (e.g., cyclin D1, c-Myc), survival, and inflammation[273]. Notably, IL-6/STAT3 signaling enhances expression of the divalent metal transporter 1 (DMT1), promoting cellular iron uptake and accumulation[274]. This establishes a positive feedback loop reinforcing inflammation and tumor promotion.

4. METHODOLOGICAL CONSIDERATIONS

4.1 Selection of iron compounds

In paper I, twelve iron compounds, six iron salts and six iron chelates, were selected for investigation. All compounds were approved by the European Food Safety Authority (EFSA) and are commercially available for human consumption as dietary iron supplements. The following compounds were used: ferric pyrophosphate (soluble crystals; $Fe_4(P_2O_7)_3$), ferric sodium EDTA hydrate ($C_{10}H_{12}FeN_2NaO_8 \cdot xH_2O$), ferric ammonium citrate ($C_6H_{11}FeNO_7$), ferric citrate hydrate ($C_6H_5FeO_7$), ferrous gluconate hydrate ($(C_6H_{11}O_7)_2Fe \cdot H_2O$), carbonyl iron (Fe), ferrous lactate hydrate ($CH_3CH(OH)COO]_2Fe \cdot H_2O$), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous fumarate ($C_4H_2FeO_4$), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), ferric sulfate hydrate ($Fe_2(SO_4)_3 \cdot H_2O$), and ferrous bisglycinate ($C_4H_8FeN_2O_4$). Except for ferrous bisglycinate, which was obtained as a consumer-grade dietary supplement in capsule form from the online retailer Supersmart (Luxembourg), all other compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of each compound were freshly prepared on the day of the experiment at a final iron concentration of 5 mM. Ferrous fumarate, ferric pyrophosphate, carbonyl iron and ferrous bisglycinate were solubilized in 0.05 M hydrochloric acid (HCl), while all other compounds were dissolved in Milli-Q® ultrapure water. In paper II, three representative iron compounds, ferrous fumarate, ferrous sulfate (considered the clinical "gold standard"), and ferric sodium EDTA were used to assess differences in cellular uptake between iron salts and iron chelates. Paper III utilized ferrous fumarate, ferric sodium EDTA, and ferric pyrophosphate to further investigate the previously observed cyclooxygenase-2 (COX-2) induction in response to ferric pyrophosphate treatment. This set of experiments aimed to verify whether the observed inflammatory response was consistent across intestinal epithelial cell lines of both male and female origin. In paper IV, ferric pyrophosphate and iron hydroxide adipate tartrate (IHAT), a nanoparticulate iron (Nemysis Ltd). Ferric pyrophosphate was selected based on prior results indicating its strong pro-inflammatory potential. In all experiments (papers I-IV), iron compound solutions were prepared fresh approximately one hour before application to cells to ensure chemical stability and reproducibility of biological responses.

4.2 Cell lines and experimental models

Several human cell lines were used across four experimental projects: Hutu-80, Caco-2, SW48, SW1417, and HepG2. In papers I-III, Hutu-80, Caco-2, SW48, and SW1417 cells were cultured in Corning® CellBIND plates. In paper IV, a coculture model with differentiated Caco-2 cell epithelia on permeable Transwell® filters (Corning®) and differentiated HepG2 epithelia in the basal wells, was used to mimic the intestinal-liver axis. Hutu-80, Caco-2, and HepG2 cells were maintained in Minimum Essential Medium (MEM; Gibco) supplemented with fetal bovine serum (10%) and Normocin (0.2%, InvivoGen). SW48 and SW1417 cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium and Ham's F-12 (DMEM/F-12; Gibco), also supplemented with FBS (10%) and Normocin (0.2%). In paper II, Opti-MEM™ Reduced Serum Medium (Gibco) was initially used as the recommended medium for transfection. However, it was later replaced with Minimum Essential Medium (MEM) supplemented with FBS (5%) to minimize the influence of undefined components on the experiments. For all experimental treatments, the culture media were supplemented with

FBS (5%), unless otherwise stated. In the Caco-2/HepG2 co-culture system (paper IV), the medium was supplemented with less FBS (1%) to make cells iron deficient, and increase the iron absorption, while still providing transferrin to support iron exchange between the two cell lines. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. For protein analysis, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher) supplemented with a protease inhibitor cocktail (Roche), to prevent unwanted degradation of proteins by proteases released during the lysis. In experiments involving detection of phosphorylated NF-κB (paper III), phosphatase inhibitors (Roche) were additionally included in the lysis buffer, to preserve protein phosphorylation.

4.3 Iron content measurements

4.3.1 AAS

Atomic Absorption Spectroscopy (AAS) was employed to quantify the iron content in stock solutions and to verify the accuracy and reproducibility of iron concentrations between different preparation batches. In most experiments, iron quantification was performed using Flame Atomic Absorption Spectroscopy (Flame AAS) due to its suitability for measuring relatively high concentrations in standard solutions (1-100 μg/L). However, in the second project involving the analysis of iron content in cell lysates, where greater sensitivity was required, Graphite Furnace Atomic Absorption Spectroscopy (GFAAS) was employed. GFAAS offers significantly enhanced sensitivity and lower detection limits (0.01-1 μg/L), making it more appropriate for detecting trace levels of iron in complex biological matrices such as cell lysates.

4.3.2 ToF-SIMS

Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS; ION-TOF GmbH, Münster, Germany) was employed to visualize and quantify membrane-associated iron at the single-cell level using high-resolution mass spectrometry imaging. The instrument was equipped with a 30 keV Bi3 + cluster ion gun as the primary ion source and a 20 keV Argon gas cluster ion source for sputtering. ToF-SIMS 3D depth profiling was applied (the Ar source at 10 keV using Ar1500 + ions at 5 nA) to selectively remove the outermost ~100 nm of the cell membrane, enabling precise analysis of iron content localized to the plasma membrane region. This technique allowed spatially resolved detection of iron ions (Fe^+) and provided insights into the topographical distribution of iron at the cell surface. All spectra, images and depth profiles were acquired and processed with the Surface Lab software (version 7.3, ION-TOF GmbH, Münster, Germany).

4.4 Biomarker analysis

4.4.1 SDS-PAGE Western blot

Western blot protocols were adapted slightly across the four projects, depending on specific experimental requirements. Variations included loading different amounts of total protein, particularly in cases where the target protein was abundantly expressed, and omitting the boiling step prior to electrophoresis. The latter modification was applied selectively, especially when analyzing

membrane-associated proteins such as DMT1, for which boiling may negatively impact detection, as previously reported[275]. In all projects, following SDS-PAGE, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo™ system (Bio-Rad) with the 3-minute rapid transfer protocol. For all experiments involving protein quantification by western blotting, expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which served as the housekeeping protein control.

4.4.2 ELISA

In selecting ELISA as the primary immunoassay, both the epitope specificity of the antibodies and the availability of validated commercial kits were considered. Particular attention was paid to where on the protein the capture and detection antibodies bind, especially for targets with functionally important domains or post-translational modifications such as phosphorylation. This ensured that the assay would detect the relevant form of the protein and not cross-react with related isoforms or cleavage products. When available, commercial ELISA kits were the first choice, rather than developing custom (home-made) assays. This choice was based on factors such as consistency, rigorous validation, and the availability of standardized controls and calibrators. Commercial kits also come with optimized protocols, saving time in assay development.

4.4.3 Olink proteomics (PEA™)

In projects 1 and 4, we utilized Olink® Target 96 and Olink® Explore 384 panels to assess protein levels in our samples. The provided NPX values, which are log2-transformed and normalized, were first linearized using $\mathbf{2^{NPX}}$. The linearization enabled biological interpretation, particularly of relative fold changes, and supported integration with other linear data types. NPX values below the limit of detection (LOD) were excluded from analyses to avoid artefacts arising from low signal-to-noise ratios. Although the Olink Proximity Extension Assay (PEA™) is designed to minimize issues such as the hook effect, we screened for potential signal suppression at high analyte concentrations and excluded any unusually high NPX values suggestive of such artifacts. Despite these precautions, we encountered discrepancies in Project 4 when attempting to validate selected Olink findings using alternative methods. Specifically, Olink data indicated elevated levels of CD40, CSF-1, and IL-1RN in response to the ferric pyrophosphate treatment. However, follow-up analyses using ELISA and Western blotting failed to replicate these results. Given the expected specificity of the PEA platform and its low cross-reactivity, this discrepancy was unexpected.

4.5 Pharmacological and genetic inhibition of iron uptake pathways

4.5.1 Pharmacological inhibition of endocytosis

Chlorpromazine was used to inhibit clathrin-mediated endocytosis in HuTu-80 and Caco-2 cells, as it is a widely established pharmacological inhibitor of this pathway. Chlorpromazine acts by disrupting the assembly of clathrin-coated pits at the plasma membrane, thereby preventing vesicle formation and internalization of ligands via the clathrin-dependent route.

4.5.2 Gene knockdown (RNAi)

For siRNA-mediated gene silencing, cells were seeded at a density of 50,000 cells per well in 24-well plates (Corning Life Sciences, Lowell, MA, USA), following the manufacturer's protocol provided by

Thermo Fisher Scientific. Transfections were initially performed in Opti-MEM™ Reduced Serum Medium (Thermo Fisher Scientific), a commonly used medium for siRNA delivery. However, Opti-MEM contains iron from proprietary and undefined sources, which significantly influenced ferritin level, which was used as a proxy of iron uptake. Specifically, ferritin levels were observed to be 9.97-fold higher in cells cultured in Opti-MEM compared to those maintained in MEM supplemented fetal bovine serum (5%). To minimize this confounding effect on iron-related readouts, we replaced Opti-MEM with MEM-FBS (5%) for subsequent experiments. Serum-free conditions (MEM alone) were also tested but resulted in poor cell viability in both Hutu-80 and Caco-2 cell lines (data not shown), further supporting the use of MEM-FBS (5%) as the optimal medium for our experimental conditions. Validated Silencer® Select siRNAs (catalog no. 9708, 9709 and 9710; Thermo Fisher Scientific) were used to target gene expression, with final concentrations ranging from 5 to 750 pmol, based on estimated cellular protein levels. To optimize transfection efficiency, various volumes of Lipofectamine™ 3000 Transfection Reagent (0.75–3 µL; Invitrogen™) were tested. All siRNA knockdown experiments were validated by Western blot analysis to confirm the effective reduction of the target protein expression.

4.6 Statistical analyses

In papers 1, 2 and 4, statistical comparisons between treatment groups were conducted using the unpaired two-tailed Student's t-test, implemented in Microsoft® Excel (Redmond, WA, USA). Depending on data distribution and variance homogeneity, tests were performed assuming either equal (2,2) or unequal (2,3) variances. P-values ≤ 0.05 were considered statistically significant. Results were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM), based on a minimum of three biological replicates ($n \geq 3$). Some experiments are reported individually as $n = 1$ (with 3-4 technical replicates), as they originated from independent trials; however, all experiments were independently repeated at least once ($n = 2$). In the paper 3, Spearman's rank correlation coefficient (ρ) was used to evaluate associations between variables in datasets where assumptions of normality were met but sample sizes were small (3 biological replicates × triplicates). Correlation analysis was performed using Python (version 3.10.12) with the scipy.stats module (version 1.14.0). A ρ value of 0 indicates no monotonic relationship, while positive values (up to 1) indicate associations, and negative values (down to -1) indicate decreasing monotonic associations. The Mann-Whitney U test was also applied to confirm the robustness of the t-test results; it yielded comparable outcomes and did not alter the interpretation of significance.

5. RESULTS AND DISCUSSION

5.1 Iron chelates vs. salts: impact on amphiregulin, MAPK, and JAK/STAT pathways

In our initial investigation (Paper I) , we found out all tested iron chelates significantly induced the MAP kinase signaling, as evidenced by elevated amphiregulin ((Figure 1a and 1b), taken from already published work [276]) and IGFr1 levels (Figure 1c and 1d). Certain iron salts at high concentration ([Fe]= 0.5 mM) also increased amphiregulin levels (ferrous fumarate did not), albeit to a much lower degree and importantly, this was the only biomarker for which iron salts had a significant effect. All the cellular responses were independent of total iron content (as measured by ferritin), indicating that the induction of signaling was not simply due to iron accumulation. Among the chelates, ferric pyrophosphate and ferric EDTA induced the strongest responses. The selected dosing was designed to reflect physiologically relevant iron exposure in the human intestine. Iron concentrations of [Fe]= 0.5 mM and [Fe]= 0.05 mM (48 h) were selected, corresponding to estimated equivalent human elemental iron doses of 150 mg and 15mg, respectively. For comparison, a standard 60 mg elemental iron supplement has been reported to elevate postprandial duodenal iron concentrations to approximately 200 µM after accounting for dilution in the stomach and duodenal lumen. Based on these estimates, [Fe]= 0.5 mM was selected to represent a high yet relevant exposure level, while [Fe]= 0.05 mM was included to assess whether any observed effects were attributable to iron itself rather than being dose-dependent.

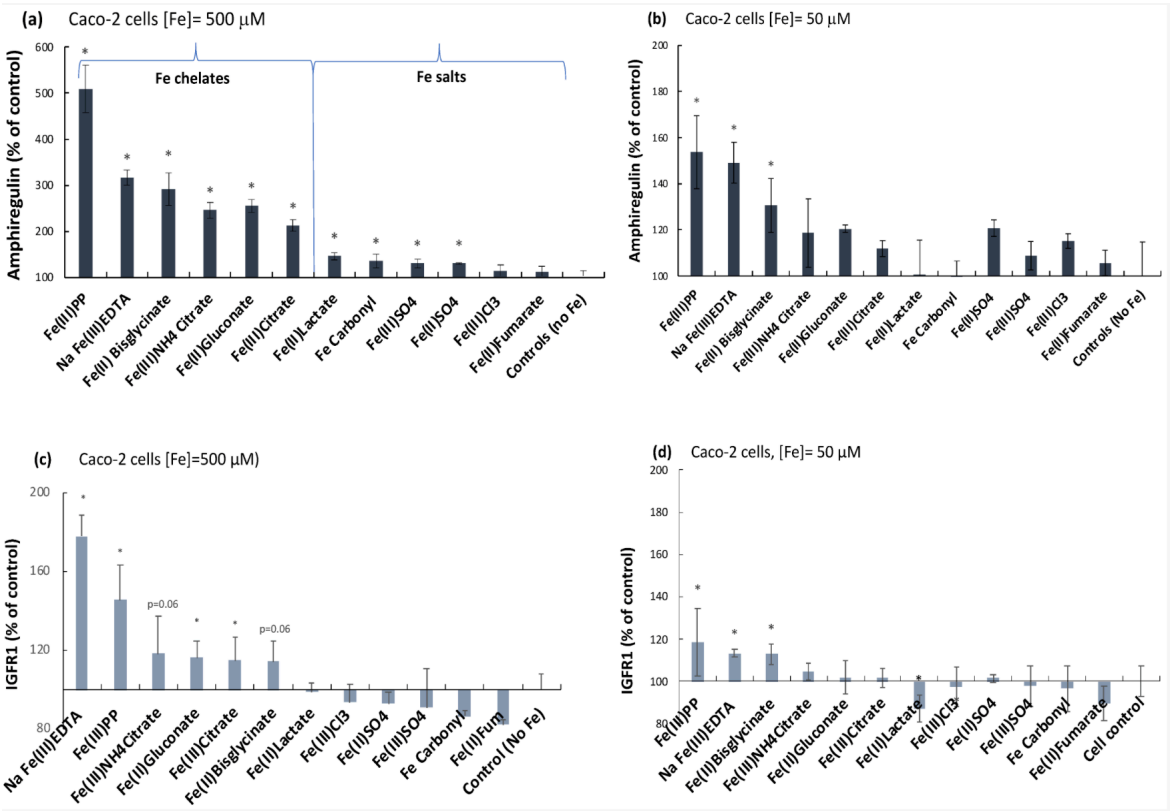

Figure 1. Amphiregulin (a,b) and IGFr1 (c,d) levels (% of untreated controls) in intestinal Caco-2 cells treated with iron compounds (0.5 mM and 0.05 mM, 48 h. A significant difference from untreated control cells is indicated with an asterisk (*). The graph taken from the published paper 1 under a CC BY 4.0 license.

In addition to MAP kinase induction, ferric pyrophosphate and ferric EDTA also upregulated JAK/STAT pathway components, via IFN-γ r1 and its downstream target, CDKN1A, at high ([Fe]= 0.5 mM) concentrations. Ferric pyrophosphate ([Fe]= 0.5 mM) uniquely stimulated IL-6 production and induced COX-2 (Figure 2, taken from already published work[276]), resulting in elevated PGE_2 levels in Caco-2 cells. COX-inhibition studies were performed using the COX-2 inhibitor sulindac sulfone, to explore whether COX-2 mediates amphiregulin upregulation, but the inhibition of COX-2 did not significantly alter the iron-induced expression of amphiregulin, IFNγR nor IL-6, suggesting that their upregulation occurs independently of COX-2 activity.

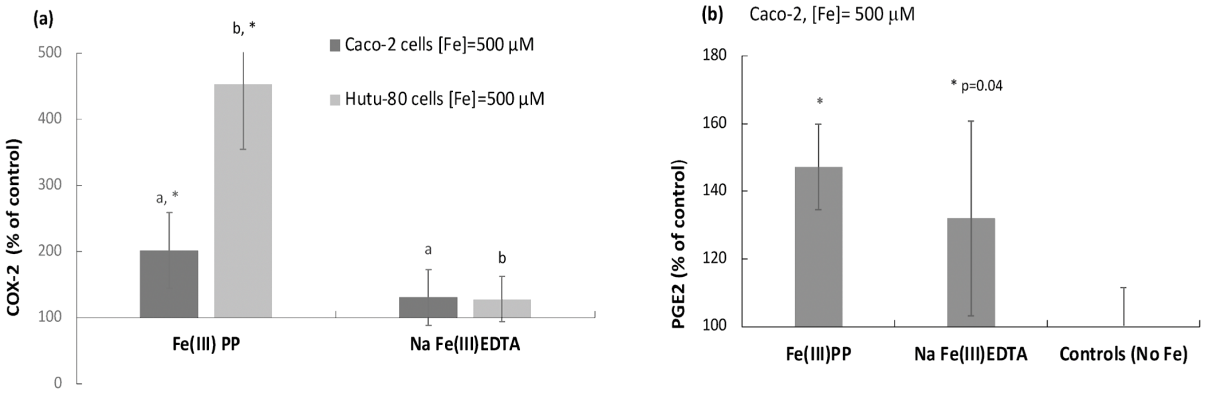

Figure 2. COX-2 levels (% of control) in Caco-2 and Hutu-80 cells. Data are presented as means of 7 cell replicates ‡ Sdev from 2 separate occasions. A copy of the same letter indicates significant differences between the iron treatments (a or b). (b) The COX-2 product PGE2 intracellular levels (% of control) in Caco-2 cells. Data are presented as means of 6 cell replicates ± Sdev from 2 separate occasions. Significant differences from the control (no Fe) are indicated with asterisks (*). The graph taken from the published paper 1 under a CC BY 4.0 license.

To sum up, we observed a dose-dependent relationship between iron compound (ligand-specific) exposure and biomarker response, particularly for chelates, however, increased ferritin levels (representing cellular iron load) did not correlate with increased biomarker expression. While the association between increased inflammation and iron exposure is well established[16,220,277], our findings strongly indicate that the biological effects of iron are highly dependent on its ligand. This suggests that it is not simply the total amount of iron taken up, but the specific chemical form in which it is delivered, that drives the activation of pathways associated with tumorigenesis and inflammation. This is further supported by epidemiological evidence that dietary heme iron intake, rather than total body iron stores, is associated with an increased risk of colon cancer in humans [278,279].

5.2 DMT-1 and clathrin-mediated uptake of iron from ferrous fumarate

Given the differential inflammatory responses observed between iron salts and chelates, we hypothesized that these compounds might be internalized via distinct cellular uptake pathways. To explore this, we investigated the uptake mechanisms of two iron salts ferrous fumarate and ferrous sulfate, as well as iron chelate ferric EDTA in Hutu-80 and Caco-2 cell lines. We selectively targeted two iron absorption mechanisms by silencing DMT1 and pharmacologically inhibiting clathrin-mediated endocytosis. Silencing DMT1 significantly decreased ferritin (L) levels in Hutu-80 cells treated with ferrous fumarate ([Fe]= 0.5mM) and ferrous sulfate ([Fe]= 0.5mM), in comparison to control indicating

reduced iron uptake (Figure 3). We included both ferritin L and H in our analysis in the Hutu-80 cells to assess whether their contributions to iron storage are equivalent, considering that ferritin H facilitates iron oxidation and ferritin L is responsible for long-term storage[143,280]. Silencing DMT1 in Caco-2 cells did not significantly reduce iron uptake following ferrous fumarate treatment, as measured by ferritin production (data not shown). This suggests that DMT1-independent mechanisms, supposedly endocytosis, play a substantial role in the cellular uptake of this iron compound.

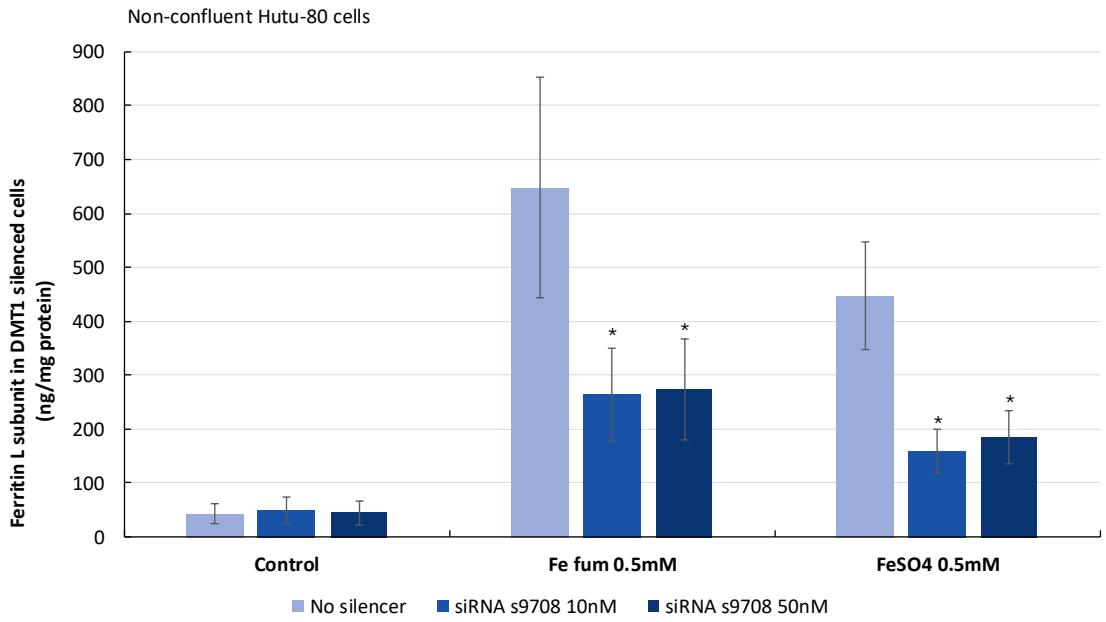

Figure 3. Indirect iron load (ferritin L) in DMT1-silenced Hutu-80 cells incubated with iron compounds for 24 h, presented as ng/mg total cell protein. Data are means ± Sdev, n = 3 separate experiments. An asterisk indicates a significant difference from the iron control ($p < 0.05$). The graph taken from the published paper 2 under a CC BY 4.0 license.

The study confirmed that iron from ferrous fumarate is also absorbed via clathrin-mediated endocytosis in both post-confluent (14 days) and non-confluent (48 h) Hutu-80 cells. In post-confluent cells, inhibition of clathrin-mediated endocytosis with chlorpromazine (100 µM) significantly reduced ferritin L production by 61%, and ferritin H levels by 39.5%. A pilot study using GFAAS to measure total iron content showed that inhibition of clathrin-mediated endocytosis reduced iron uptake from ferrous fumarate by 24%, indicating that endocytic pathways contribute to its absorption. In contrast, no significant change was observed in iron uptake from ferric EDTA under the same conditions, indicating that ferric EDTA is not absorbed by endocytosis. This is supported by the fact that ferric EDTA is fully soluble at physiological pH (manuscript 3) and thus iron can be transported by DMT1 after reduction.

Interestingly, western blot analysis during the development of the DMT1 silencing protocol revealed distinct molecular weights for DMT1 in Caco-2 (~85 kDa) and Hutu-80 (~65 kDa) cells (Figure 4), suggesting isoform-specific expression. Prior studies report that Caco-2 cells predominantly express the 1A isoform, while the lower molecular weight observed in Hutu-80 cells aligns with the 1B isoform (~61-73 kDa). Given the lower DMT1 protein levels in Hutu-80 and their non-polarized phenotype, it is likely that this cell line primarily expresses the 1B variant. Functionally, this difference is relevant:

1A isoforms localize apically for luminal iron uptake, while 1B isoforms are associated with endosomes. Consistent with this, TOF-SIMS analysis showed an 87.4% reduction in membrane-associated iron in Hutu-80 cells after clathrin inhibition, supporting a reliance on endocytic iron uptake pathways.

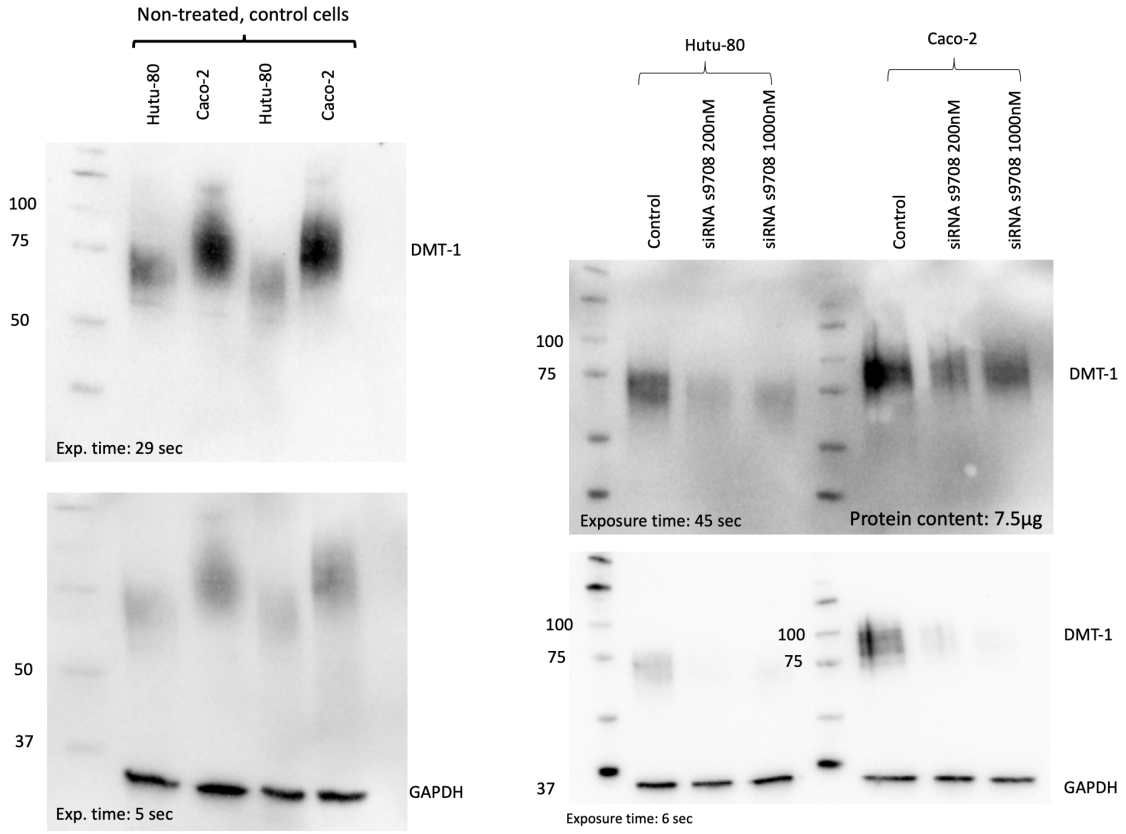

Figure 4. Western blot (left) in confluent non-treated Hutu-80 and Caco-2 cells, (right) Wb of DMT1 protein expression in confluent Hutu-80 and Caco-2 cells treated with 200 and 1000nM of Silencer Select siRNA s9708 (invitrogen) for 48h. The graph taken from the published paper 2 under a CC BY 4.0 license.

Across all three experiments, ferritin levels in Caco-2 and Hutu-80 cells were comparable following treatments with ferrous fumarate and ferric pyrophosphate, indicating similar iron uptake efficiency. In contrast, ferric EDTA induced a threefold higher ferritin response in Caco-2 cells compared to Hutu-80. This difference may be linked to the reliance of ferric EDTA on DMT1-mediated transport, particularly involving the 1A isoform, which is more abundantly expressed and functionally active in Caco-2 cells. Our pilot study further suggest that ferric EDTA uptake does not depend on clathrin-, caveolae-, or macropinocytosis-mediated pathways (Figure 5, our own unpublished data). On the other hand, elevated amphiregulin levels and signaling via both the JAK/STAT and MAPK pathways were observed in Caco-2 and Hutu-80 cells treated with ferric EDTA, whereas none of these effects were seen in cells exposed to ferrous sulfate, a compound known to rely on DMT1-mediated transport. Alternatively, ferric EDTA uptake may involve both DMT-1 as well as different pathway, such as a non-classical endocytic mechanism.

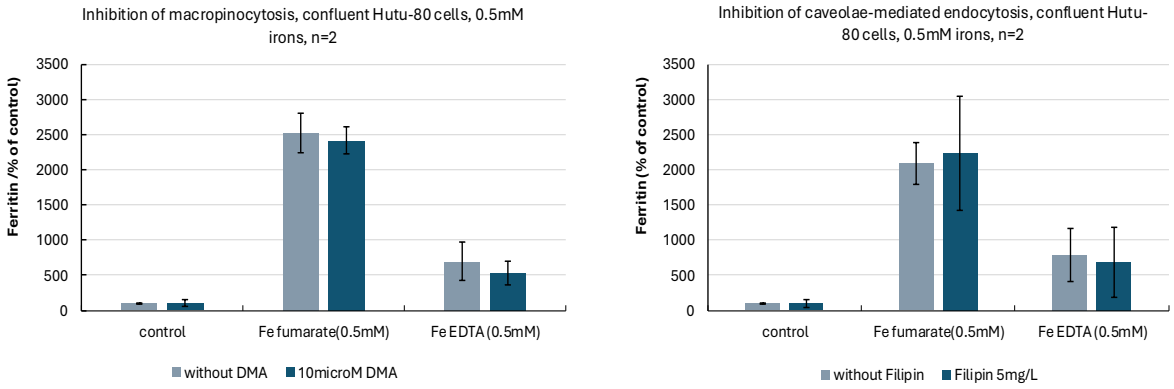

Figure 5. Ferritin levels in confluent Hutu-80 cells, normalized to the untreated control (our own unpublished data).

In summary, our results suggest that iron uptake is likely mediated by multiple, concurrent pathways that are specific to iron compounds. The uptake route appears to be determined by the solubility and physical state of the iron species, soluble iron favoring DMT1-mediated transport (such as ferric EDTA and ferrous sulfate), while poorly soluble or particulate forms (ferrous fumarate) rely on endocytic mechanisms.These findings also raise the possibility that iron formulation and uptake route influence downstream biological responses, with important implications for the safety and selection of oral iron compounds.

5.3 COX-2 induction in response to two iron compounds in human intestinal cells

Prompted by earlier findings showing COX-2 induction in Caco-2 and Hutu-80 cells, we sought to determine whether this response was consistent across other commonly used intestinal cell lines of female origin, at a dosage of 0.5 mM (48 h), equivalent to 150 mg human dose, since a typical 60 mg supplement can transiently raise duodenal luminal iron to ~200 µM [281,282]. A concentration of 0.5 mM was previously shown to be sufficient to induce COX-2 in intestinal epithelial cell lines, Caco-2 and Hutu-80, as well as was used in parallel uptake studies, allowing for direct comparison and integration of COX-2 signaling and transport data across experiments. However, the use of only one iron concentration represents a big limitation since it provides only a single point of reference, and it restricts the ability to draw general conclusions. In this project we selected four widely studied cell lines that vary by chromosomal sex and epithelial origin: male-derived Caco-2 (X) and Hutu-80 (XY), and female-derived SW1417 (XX) and SW48 (XX).

The study revealed differential cyclooxygenase-2 (COX-2) and lipoxygenase-5 (LOX-5) expression, in response to three different iron compounds ferrous fumarate, ferric EDTA and ferric pyrophosphate (at the 0.5 mM iron concentration), commonly used in iron fortified foods and iron supplements [55,96,283]. The exposure to ferric pyrophosphate ([Fe]= 0.5 mM, 48h) significantly induced COX-2 protein expression in male-derived intestinal epithelial cell lines, Hutu-80 and Caco-2, while no such effect was observed in the female-derived SW48 and SW1417 cells (Figure 6 and Figure 7). In contrast, supplementing the medium with ferrous fumarate at the same concentration and exposure time

modestly increased COX-2 levels in Hutu-80 and SW48 cells, with a less pronounced effect than ferric pyrophosphate. Interestingly, ferritin levels were generally lower following ferrous fumarate treatment compared to ferric pyrophosphate (Figure 8). In SW48 cells, COX-2 was induced only by ferrous fumarate, despite ferric pyrophosphate leading to nearly twice the ferritin accumulation. This discrepancy suggests that COX-2 induction is not a consequence of ferritin-mediated storage. Supporting this, previous research has shown that high levels of systemic biomarkers of iron storage are associated with a reduced risk of cancer[278]. An earlier analysis of 26 studies, similarly differentiated between iron exposure and body iron stores, highlighting that not all dietary iron ends up stored in the body[279]. Ferric EDTA (0.5 mM, 48 h) did not increase COX-2 levels in any of the tested cells. Since LOX-5 expression was not evaluated in SW48 cells cultured in MEM, it remains unclear whether the small effect observed (20% increase of LOX-5) was influenced by DMEM/F-12 containing ferric nitrate and ferrous sulfate or by the FeEDTA treatment EDTA (0.5 mM, 48 h) itself. It is possible that higher doses of certain iron compounds, such ferric EDTA, might trigger inflammatory signaling in other cell lines or under different conditions. Future studies should address this by employing a range of iron concentrations to define dose-response relationships more precisely and to better distinguish compound-specific effects across diverse cellular models.

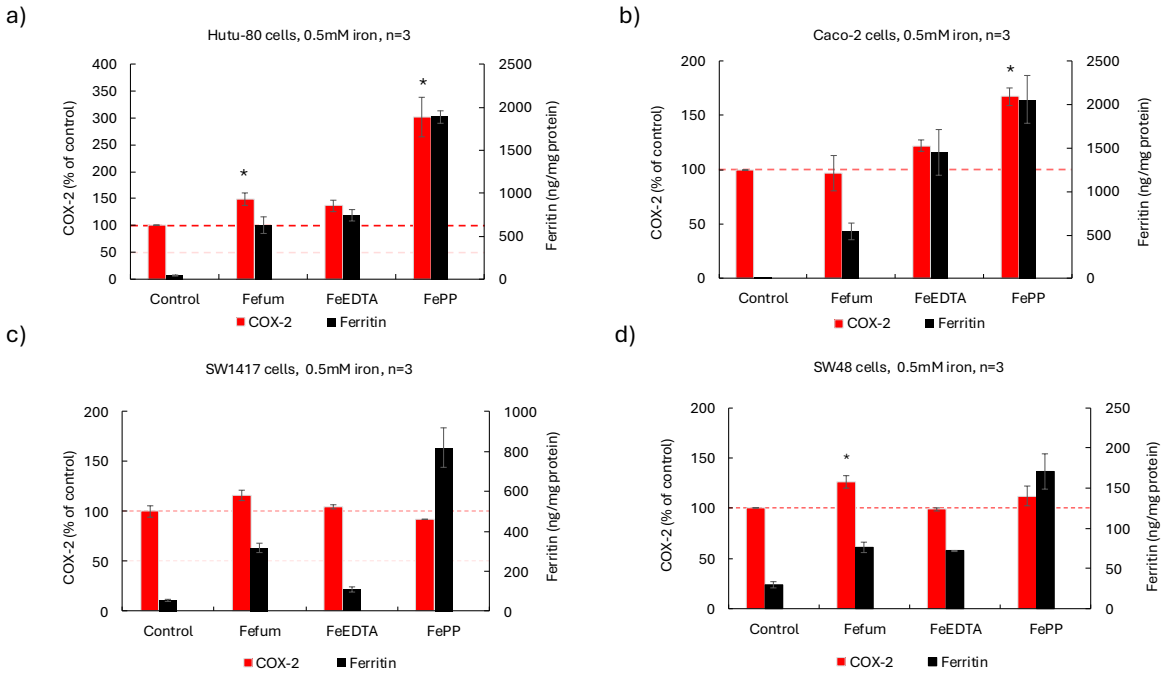

Figure 6. COX-2 (% of control) and ferritin levels (ng/mg total protein) in a) Hutu-80, b) Caco-2, c) SW1417 and d) SW48 cells measured with ELISA. Data are presented as means of 9 cell replicates ± SEM, n=3 separate occasions. Significant differences from control cells (no added Fe) ($p \leq 0.05$) are indicated with asterisks (*). All iron treatments caused a significant increase in ferritin levels compared to control; significance not indicated. The graph taken from the paper 3 (our own unpublished data).

The analysis of intracellular ferritin, a marker of iron uptake[145], showed no correlation with COX-2 in Hutu-80, SW48 and SW1471 cells, further indicating that inflammatory responses are not driven by total iron load. In Caco-2 cells there was a significant inverse correlation between ferritin and COX-2 following ferric pyrophosphate and ferrous fumarate exposure ([Fe]= 0.5 mM, 48h). No overall

correlation was found between LOX-5 and COX-2 levels, although a strong negative correlation was observed in ferric pyrophosphate-treated Hutu-80 cells.

The effect of the iron incubations on cell survival

Total protein content, as a proxy of cell survival, demonstrated that none of the iron compounds stimulated proliferation in any of the four cell lines tested. In contrast, ferric pyrophosphate (Fe=[0.5mM], 48h) exhibited a significant growth-inhibitory effect on Hutu-80 cells (50%) and SW48 cells (51%), Caco2 (21%) and SW1417 (12%). Ferrous fumarate (Fe=[0.5mM], 48h) also significantly reduced proliferation, though to a lesser extent: in Hutu-80 cells (13%) and in SW48 cells (33%) and SW1417 (10%). The observed COX-2 and 5-LOX upregulation occurs independently of proliferative status in Caco-2 cells.

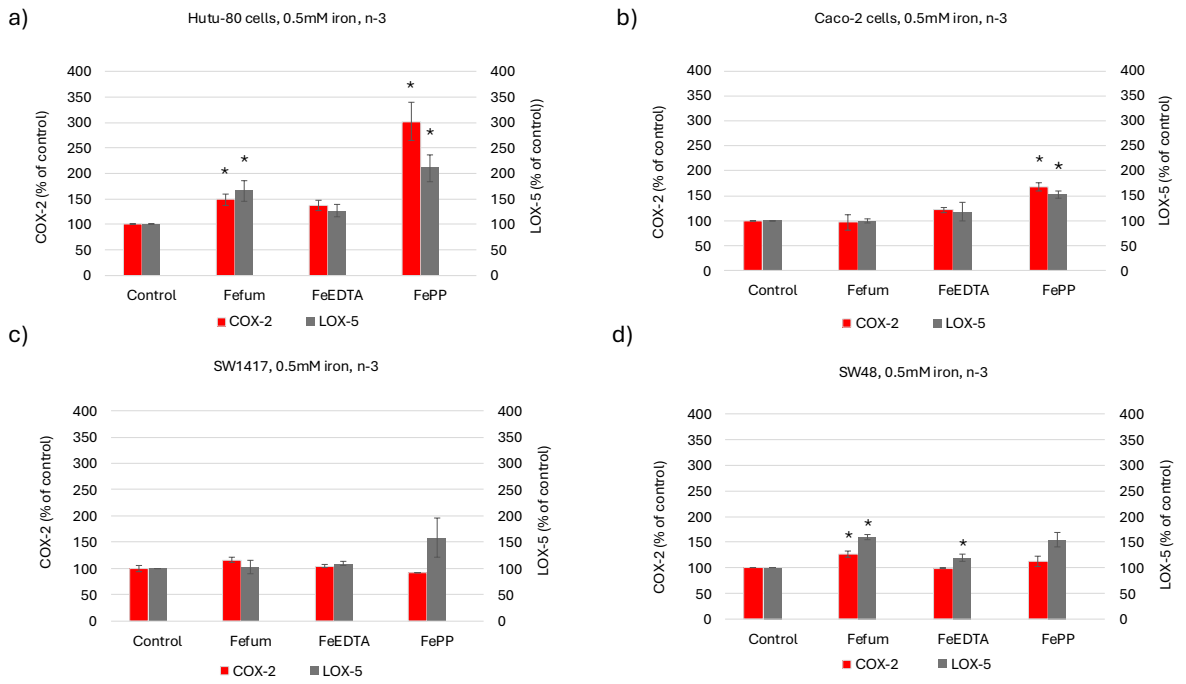

Figure 7. COX-2 and LOX-5 (% of control) in a) Hutu-80, b) Caco-2, c) SW1417 and d) SW48 cells measured with ELISA. Data are presented as means of 9 cell replicates ± SEM, n=3 separate occasions. Significant differences from control cells (no added Fe) ($p \leq 0.05$) are indicated with asterisks (*). All iron treatments caused a significant increase in ferritin levels compared to control; significance not indicated (our own unpublished data).

Interestingly, the susceptibility of the cell lines to iron compound-induced COX-2 upregulation seemed to increase with cellular proliferation rates (Cellosaurus data) : Hutu-80 cells, which have the fastest doubling time (26h), exhibited the most pronounced COX-2 reponse, followed by SW48 (35h), Caco-2 (51h), and SW1417 (68h) cells with progressively slower doubling times. This potential association of rapidly proliferating cells being more susceptible to iron compound-induced inflammatory signalling, could be due to higher metabolic needs[284] and potentially, subsequent elevated iron requirements. However, in cells with ongoing proliferation, most incoming iron bypasses storage (ferritin) and is channeled immediately into functional pools utilizing iron to support biosynthetic processes-production of iron-dependent enzymes, mitochondria, and nuclear replication. This may explain why

ferritin levels do not reflect total iron uptake in these conditions. For instance, following ferrous fumarate treatment (Fe=[0.5mM], 48h), ferritin levels in the rapidly dividing SW48 cells were found to be seven times lower than in the more slowly proliferating SW1417 cells, despite likely higher iron uptake in the former.

Effects on NF-κB Signalling in Hutu-80 Cells

Given that the differential effects of ferric pyrophosphate, ferrous fumarate, and ferric EDTA were most pronounced in Hutu-80 cells, where COX-2 induction inversely correlated with cell viability, we sought to determine whether NF-κB, a known regulator of COX-2 expression, played a role in this response. In a pilot study, Western blot analysis of activated NF-κB (phosphorylated at Ser536) revealed a significant reduction following treatment with ferric pyrophosphate ([Fe]= 0.5mM, 48h), with levels decreasing to 33.75% of control. In contrast, neither ferrous fumarate nor ferric EDTA ([Fe]= 0.5mM, 48h) induced a similar reduction in phosphorylated NF-κB. Importantly, while phosphorylation at Ser536 is traditionally linked to NF-κB transactivation, it also plays a key role in regulating NF-κB turnover and termination. Reduced phosphorylation at this site has been shown to stabilize the p65 subunit, extend its nuclear retention, and sustain its transcriptional activity. Paradoxically, this decrease in Ser536 phosphorylation can enhance NF-κB's pro-inflammatory role by promoting prolonged expression of target genes such as COX-2 and IL-6. This, in turn, reinforces the inflammatory feedback loop driven by MAPK and JAK/STAT signaling pathways. Additonally, we did not assess phosphorylation at other regulatory serine residues, Ser276, Ser311, and Ser468, which are known to influence NF-κB activity through different mechanisms, including co-activator recruitment and modulation of gene-specific transcriptional responses[285]. These unexamined phosphorylation sites could potentially yield different outcomes and may help explain nuances in NF-κB-mediated gene regulation that were not captured in the current study.

Solubility Characteristics of Iron Compounds Tested

A solubility study showed that ferric pyrophosphate remains partially soluble (62.5%) in the stock solution ([Fe]= 5mM Fe) but rapidly precipitates in cell culture medium at 0.5 mM Fe, with only 14% remaining soluble. Ferrous fumarate was fully soluble as a stock solution ([Fe]= 5mM) but became completely insoluble upon dilution into the cell medium ([Fe]= 0.5mM). In contrast, ferric EDTA remained highly soluble (97%) in both stock ([Fe]= 5mM) and medium ([Fe]= 0.5mM), supporting its availability for uptake via the DMT1 transporter. We also noted the presence of particle aggregates in cell culture media (after 48 h) in wells with ferric pyrophosphate and ferrous fumarate, but not in controls, as observed in the microscope with 200 x magnification (Figure 8). The differing size of these particles between ferric pyrophosphate and ferrous fumarate were further supported by DLS measurements. However, they were not yet confirmed to be iron particles as such. To rule out pH as a confounding factor in COX-2 induction, we considered that both ferric pyrophosphate and ferrous fumarate were dissolved in HCl (50 mM), while ferric EDTA was not. However, no COX-2 increase was observed in Caco-2 cells treated with ferrous fumarate, suggesting that the acidic environment of stock solution does not drive the inflammatory response. Moreover, pH measurements taken after the addition of ferric pyrophosphate or ferrous fumarate ([Fe]= 0.5mM) showed that the medium never dropped below pH 6.8, compared to pH 7.3 in untreated controls. Supporting this, a small pilot study showed that when ferric pyrophosphate stock solution was left to sediment before use, COX-2 levels

in Hutu-80 cells dropped, despite unchanged pH (our own unpublished data, not shown). These findings suggest that the visible precipitation/aggregation, rather than acidity of stock solution, plays a central role in COX-2 induction.

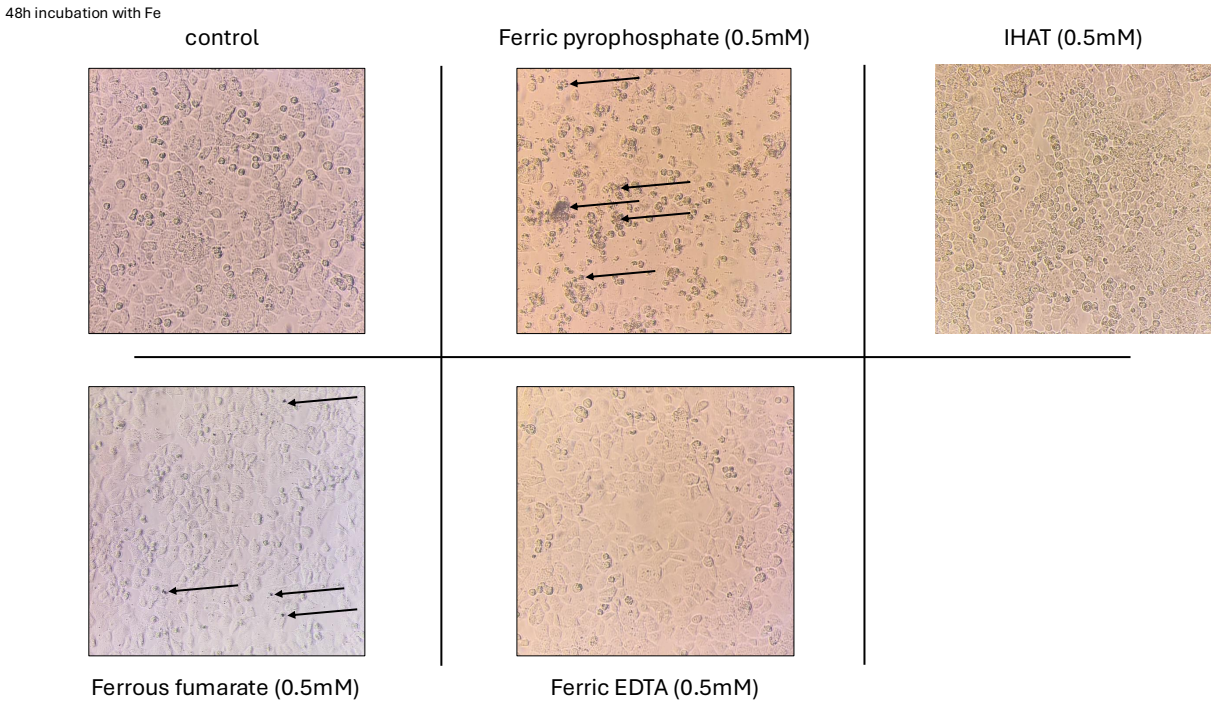

Figure 8. Light microscope pictures of Hutu-80 cells incubated with different iron types (48h) in MEM, 5% FBS (our own unpublished data).

Particles and their diameter in iron-supplemented cell culture medium, MEM-FBS (5%)

Dynamic light scattering (DLS) confirmed particles in ferric pyrophosphate (0.5 mM Fe, 48 h) and ferrous fumarate (0.5 mM Fe, 48 h) treated media (Figure 9). Control medium (MEM + 5% FBS; Figure 9a) showed peaks at 23 nm and 146 nm, consistent with serum nanoparticles/protein aggregates. Ferrous fumarate (Figure 9b) medium showed a 25 nm peak plus peaks at 237 nm and 866 nm, and since ferrous fumarate increased COX-2 expression in Hutu-80 and SW-48, particles bigger than 237 nm may be linked to COX-2 response. Ferric pyrophosphate medium (Figure 9c) showed peaks at 620 nm and 3.8 µm, matching large aggregates seen by microscopy. Whether the particles detected represent the compounds themselves or secondary precipitates will be clarified in future work using SEM.

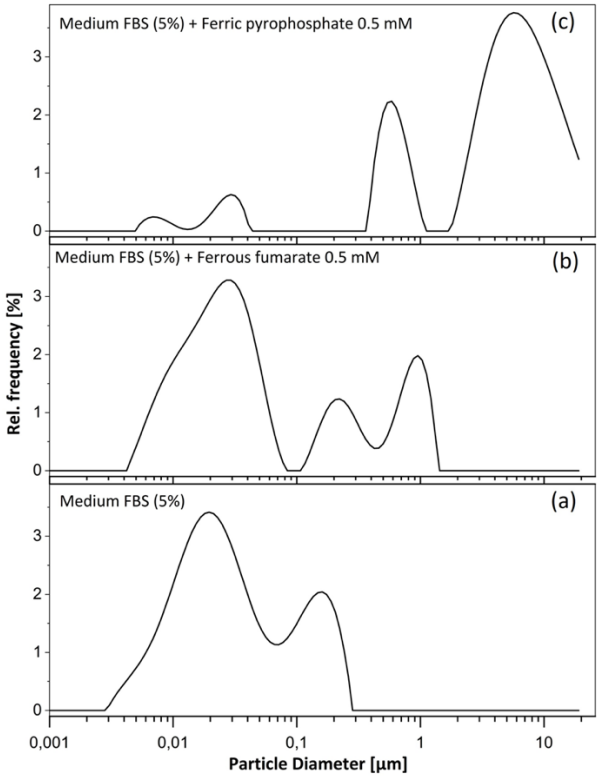

Figure 9. a) Intensity-weighted DLS size distributions of particulate matter in control medium, MEM FBS, 5%). b) MEM FBS, 5%, with ferrous fumarate (0.5 mM Fe), and c) MEM FBS, 5%, with ferric pyrophosphate (0.5 mM Fe). The y-axis shows the relative frequency (%) of scattered light intensity for particles at a given hydrodynamic diameter (µm). Large micro-scale particles were detected only in sample C. The graph taken from the paper 3 (our own unpublished data).

To further explore the potential role of particle size, we performed a pilot experiment with the nanoparticulate iron compound iron hydroxide adipate tartrate (IHAT; [Fe] = 0.5 mM, 48 h, size=2–5 nm) which resulted in no COX-2 induction (unpublished data). To conclude, our findings suggest that particle size could be an important determinant, with larger particles acting as extracellular stimuli capable of triggering COX-2 induction in intestinal cells of both female and male origin.

5.4 Comparison of effects of ferric pyrophosphate and IHAT on MAPK/ERK pathway signaling.

Two key upstream receptors of MAP/ERK signaling IFNγR1 and IGF1R, were assessed, as these were included in both proteomic panels used. IFNγR1 expression was significantly elevated in Caco-2 cells following exposure to Sup-ferric pyrophosphate (0.4 mM Fe, 48 h), with a 169% increase relative to control. (In this study, we refer to Sup-ferric pyrophosphate as a commercially available iron supplement formulation, while Sol-ferric pyrophosphate denotes a soluble crystalline form of ferric pyrophosphate sourced from Sigma-Aldrich). Similar but less pronounced increases were observed in co-cultured Caco-2/HepG2 cells treated with Sol-ferric pyrophosphate at lower concentrations (0.1mM and 0.2mM) and shorter incubation times (36h), indicating a consistent upregulation of IFNγR1 across different experimental conditions. IHAT also induced an increase in IFNγR1, but unlike ferric pyrophosphate, did not lead to downstream IL-6 production, suggesting limited pathway activation (Figure 10). The increase in IGF1R levels with the commercial supplemental form of ferric

pyrophosphate (0.4 mM Fe, 48 h) in monocultured Caco-2 cells, was not significant. Earlier data have shown a significant up-regulation of IGF1r with a non-supplemental form of ferric pyrophosphate marketed as soluble crystals (0.05 mM and 0.5 mM Fe, 48 h). Interestingly, the downstream MAP/ERK effector CDKNA was significantly increased by Sup-ferric pyrophosphate but not by IHAT, reinforcing the notion of differential pathway activation depending on the iron formulation.

Ferritin levels following IHAT treatment were approximately 25% lower than those observed after Sup-ferric pyrophosphate exposure, reflecting differences in intracellular iron accumulation. However, findings from the paper I, in which Sol-ferric pyrophosphate was used (same iron concentration and incubation time), demonstrated that ferritin levels did not correlate with inflammatory responses, specifically MAPK pathway activation, across six tested iron salts and chelates. These results suggest that factors beyond total cellular iron load, such as the nature of the iron ligand or the extracellular environment of the iron compound, may play a more critical role in driving inflammatory signaling.

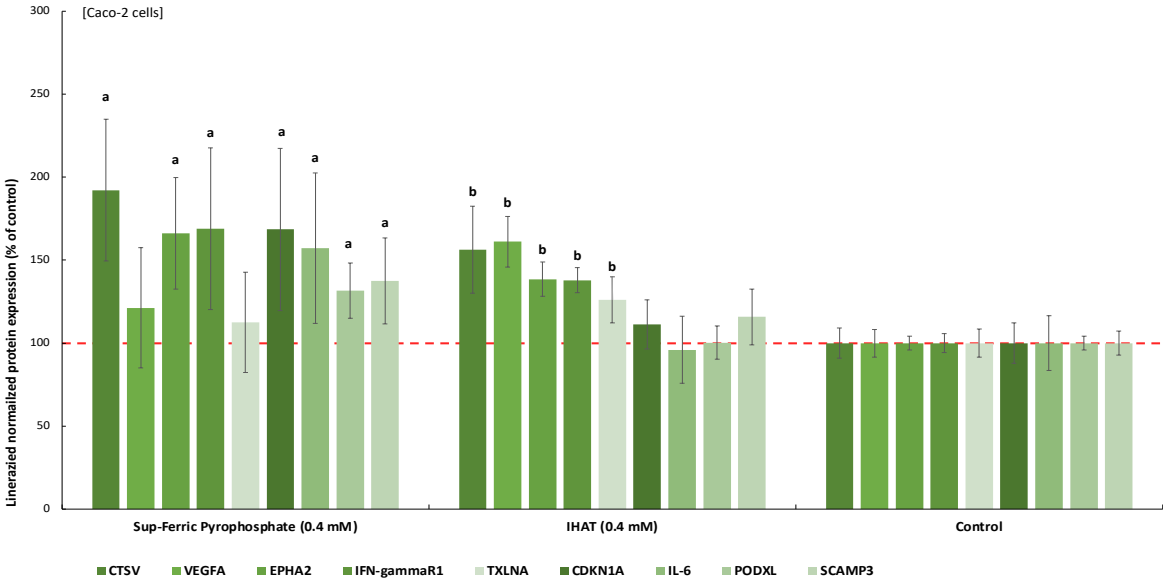

Figure 10. Protein expression in Single cultured Caco-2 cells treated with iron compounds at Fe=0.4 mM, 48 h (n=2, triplicates). Data are presented as linearized normalized protein expression, percentage of the no added iron controls ± Sdevs. Significant differences ($p<0.05$) were expressed as; a) significant difference for sol-Ferric pyrophosphate vs control, b) significant difference for IHAT vs control and c) significant difference between sol-Ferric pyrophosphate and IHAT. The graph taken from the paper 4 (our own unpublished data).

The findings above suggest that ferric pyrophosphate, more strongly activates MAP/ERK signaling than IHAT, which may underline its distinct biological and inflammatory effects in intestinal epithelial cells.

5.5 Iron ligands: biological relevance

The chemical form of iron, defined by the iron and ligand interactions, plays a critical role in determining its cellular uptake mechanism and inflammatory potential. Our studies demonstrate that iron ligands distinctly influence inflammatory and signaling responses, independent of total cellular iron load in intestinal epithelial cell lines. Chelated iron forms, such as ferric pyrophosphate and ferric

EDTA, stimulate pro-oncogenic and inflammatory signaling pathways at iron concentrations relevant for iron supplementation in humans. This occurs even when intracellular iron accumulation, as observed in Caco-2 cells, is comparable to that induced by ferrous sulfate, which does not elicit such effects. The iron ligand seems to be the primary determinant of downstream effects, not intracellular load.

Ferric EDTA (at 0.5 mM Fe, 48 h) failed to induce COX-2 response in any of the four tested epithelial cell lines (Paper III). In contrast, ferrous fumarate at the same concentration (0.5 mM) and exposure time (48h) induced COX-2, but only in Hutu-80 and SW48 cells, with no effect in Caco-2 or SW1417 cells. This selective response raises the possibility of a dose-dependent effect. It is possible that higher concentrations, such as 1 mM, might elicit a broader response across all cell lines. Dose-response studies would therefore be necessary to determine whether the lack of COX-2 induction in certain cell types is due to insufficient exposure. There was no correlation between ferritin levels and COX-2 response, implying that the induction was not driven by iron stored in ferritin. The COX-2 pathway may be affected by the iron ligand as well as the solubility (whether it precipitates and how big the particles are), rather than ferritin-bound iron.

5.6 Possible influence of iron compound solubility on inflammatory mediators

The solubility and in particular particle size of iron compounds in cell culture medium appears to significantly influence their inflammatory potential in intestinal cells. Our findings show that poorly soluble compounds such as ferric pyrophosphate and ferrous fumarate cause rapid precipitation in cell culture medium ([Fe]=0.5mM), where visible aggregates are formed, while ferric EDTA remains highly soluble (97%) in both stock solution ([Fe]= 5mM) and medium ([Fe]=0.5 mM).

At higher concentrations than the tested one ([Fe] = 0.5 mM, 48 h), ferric pyrophosphate and ferrous fumarate, both associated with COX-2 induction in Caco-2 and Hutu-80 cells, may elicit a similar response in SW1417 cells. The COX-2 increase occurred despite similar or even lower ferritin iron levels, suggesting that the extracellular environment (precipitates or protein-bound aggregates) may play an important role in triggering this inflammatory mediator. In contrast, soluble ferric EDTA, effective at activating MAPK pathway (Paper I), did not induce COX-2 production. Meanwhile, ferrous fumarate did not induce amphiregulin in Caco-2 nor Hutu-80 cells (Hutu-80 cells: pilot study, unpublished data), or any other tested markers of MAPK/ERK or JAK/STAT signaling (Paper I). This pattern suggests that COX-2 induction may be driven by extracellular factors such as particle size and membrane interactions, while MAPK and JAK/STAT signaling may depend more on intracellular effects of iron compounds and potentially the specific uptake route. These observations highlight the need to consider both non-solubility/particle size, and transport mechanisms when evaluating the biological effects of different iron compounds. This raises concern about formulations like Sucrosomial® iron, which consists of ferric pyrophosphate encapsulated in large phospholipid-sucrose ester matrices (substantial particle size).

5.7 Future perspective: tentative implications for iron supplementation strategies

While in vitro models offer valuable insight into how intestinal epithelial cells respond to different iron compounds, translating these findings to real-life supplementation strategies must be done with caution. Our data show that the chemical form (salt, chelate, particulate) and particle size of insoluble iron formulas strongly influence not just the uptake, but also the induction of pro-inflammatory and pro-oncogenic pathways, particularly COX-2 and MAPK/ERK signaling. Compounds like ferric pyrophosphate and ferrous fumarate, which cause formation of visible aggregates in cellular media, triggered stronger COX-2 responses despite lower intracellular iron load (Hutu-80 cells treated with ferric EDTA had higher ferritin levels than those treated with ferrous fumarate, despite the latter inducing a COX-2 response), suggesting that particle size of insoluble iron species may provoke local inflammation in the gut. In contrast, ferric EDTA activated intracellular MAPK/ERK and JAK/STAT signaling without inducing COX-2, highlighting the complexity of the effects of iron depending on both its formulation and uptake route. These findings raise important considerations for iron supplementation in individuals with gut inflammation, increased colorectal cancer risk, or impaired intestinal barrier function. Choosing iron compounds based not only on bioavailability but also on their inflammatory potential could improve the safety of oral iron therapies.

6. CONCLUSIONS

Overall, the aims outlined in the aims section have been achieved, and the main findings are as follows:

I Unlike iron salts, iron chelates, particularly ferric pyrophosphate and ferric EDTA, strongly induce amphiregulin and another MAPK pathway mediator IGF-R1, promoting MAPK/ERK signaling in Caco-2 cells. Additionally, ferric pyrophosphate, uniquely among the tested compounds, also induces COX-2 and IL-6 production in male-derived Caco-2 and Hutu-80 cells at the investigated concentration and incubation time (Paper I).

II Ferrous fumarate (iron salt) is taken up via both DMT1 and clathrin-mediated endocytosis (in Hutu-80 cells), but it does not rely on caveolae-mediated, or macropinocytosis pathways for iron uptake (Paper II).

III DMT1-mediated transport, not clathrin-mediated endocytosis, is responsible for the uptake of ferrous sulfate in Hutu-80 cells (Paper II).

IV Ferric EDTA, which is fully soluble at neutral pH, does not induce COX-2 in any of the four cell lines tested; however, MAPK/ERK signaling has been observed (through elevated levels of MAPK mediators) in Caco-2 cells treated with ferric EDTA. Additionally, its uptake is not dependent on clathrin-mediated endocytosis, macropinocytosis or caveolae-mediated endocytosis (Paper I and Paper II).

V Ferric pyrophosphate and ferrous fumarate cause precipitation in cell culture medium (neutral pH), forming visible aggregates. These aggregates may contribute to COX-2 induction, hypothetically via extracellular interactions (Paper III).

VI Ferric pyrophosphate did not induce COX-2 in the tested cells of female origin (Paper III).

VII MAPK/ERK signalling was not affected by ferrous fumarate (iron salt) treatment in Caco-2 cells; however, COX-2 induction was observed in male Hutu-80 and female SW48 cells (Paper I and Paper III).

VIII In differentiated Caco-2/HepG2 co-cultures (mimicking the small intestine-liver axis), ferric pyrophosphate continues to upregulate the MAPK/ERK pathway, reinforcing its effect on growth-related pathways (Paper IV), leading to IL-6 production. In contrast, nanoparticulate IHAT did not elicit the same response and no IL-6 production was observed under the tested conditions.

This work demonstrates that the form of oral iron, whether salt, chelate, or particulate and, in the case of insoluble compounds, particle size, influence not only iron uptake but also the induction of pro-inflammatory pathways. The findings further indicate that inflammatory responses are not driven by the total intracellular iron load (measured as ferritin) and rather by factors such as the iron ligand and the extracellular environment surrounding the compound in the intestinal cells. Our results challenge the prevailing view that intestinal uptake of non-heme iron is tightly regulated and mediated mainly by the DMT1 transporter. This thesis demonstrates that iron salt ferrous fumarate can also be absorbed in its insoluble form via endocytosis, a mechanism previously considered relevant only for nanoparticulate iron supplements. These new insights highlight the importance of carefully evaluating iron-ligand interactions in the design of supplemental iron, and considering lower dosing strategies to reduce the risk of pro-inflammatory and pro-oncogenic effects.

7. STUDY LIMITATIONS

One important limitation of this study is that the majority of experiments (project 1 and partially 4) were performed using non-confluent Caco-2 cells. In this state, Caco-2 cells do not fully differentiate into enterocyte-like cells and are more representative of colonic epithelium rather than the small intestine. As such, the data primarily reflect how colonic cells may respond to iron compounds that bypass absorption in the duodenum and reach the colon. While this provides insight into potential downstream or inflammatory effects of unabsorbed iron, it does not model the primary site of iron uptake. In humans, the vast majority of iron absorption occurs in the duodenum, with the colon contributing minimally (~14%)[286]. The findings should not be directly interpreted in the context of iron absorption efficiency in vivo but rather as a model for post-absorptive or colonic exposure effects.

Another limitation of this study (project 3) is the use of a single iron concentration (0.5 mM) and a fixed incubation time of 48 hours. While this approach allows for controlled comparisons between different iron compounds, it restricts the broader interpretability of the findings. The observed cellular responses, such as changes in cell survival and inflammatory markers, are specific to this concentration and time point, and cannot be assumed to reflect outcomes under different exposure conditions. Therefore, conclusions drawn from these results should be regarded as valid only within the context of this specific experimental setup. To support more generalizable interpretations, future studies should incorporate dose-response and time-course experiments.

The solubility of iron compounds at neutral pH in culture medium represents a limitation, as precipitation can generate additional cellular stress by allowing insoluble particles to settle directly on intestinal cells, potentially leading to inflammatory effects not caused by iron itself but by the physical contact and mechanical tension. However, this also serves as a strength of our research, as it closely reflects physiological conditions in vivo, where oral iron supplements are first exposed to the low gastric pH (~2, similar to the pH of our iron stock solution) of the stomach and subsequently encounter a neutral to slightly alkaline environment (pH 7 in the duodenum, rising to pH 8 in the proximal jejunum) following bicarbonate buffering.

8. ACKNOWLEDGMENTS

I acknowledge the use of ChatGPT5 for text editing during the preparation of this thesis and take full responsibility for the accuracy and integrity of its content.

So, it's really the end of this adventure and time to write the acknowledgements. I can't believe I've reached this point. It has been a challenging and sometimes incredibly hard experience, but also a lot fun (thanks to the FNS people), and overall it was absolutely worth it. I'm deeply grateful I had the opportunity to pursue a PhD. I come out of it more experienced, stronger, more certain of what I want in life, and ready to explore an industrial setting, if I get the chance. I am really grateful for all the help and support I've received along the way.

First and foremost, I'd like to thank Nathalie Scheers, my main supervisor. Thank you for always being there for me, for answering all my questions, guiding me whenever I needed it, and offering your constant support and willingness to help. Writing manuscripts and this thesis would have been a lot more challenging without your insightful feedback. I also really appreciated all the fun we had climbing and our conversations about science and life ☺.

Per Malmberg, thank you for being my co-supervisor, your support with TOF-SIMS and eagerness to help.

Rikard Landberg, thank you for being my examiner, for your support, and for offering thoughtful advice during our discussions about career choices.

Ann-Sofie Sandberg thank you for your feedback on my thesis and for being such an inspiration to all female researchers. Bo Ekstrand and Ulf Jäglid, thanks for your help and invaluable feedback.

A big thank you to all my colleagues in the FNS division, past and present, whom I've had the pleasure of working and socializing with. Special thanks to my former office mates: Mursalin, Helena, Semhar, Helga and Marina, and my current ones: Elahe and Cecilia! I feel very lucky that the people who ended up in our office were always so friendly and fun! Thank you for all the great talks and literally everything. I really hope we'll stay in touch and continue our friendship! ☺

Many thanks to other former and present FNS colleagues such as Hatairad, Jonna (it is great to have a lab buddy to discuss the iron issues ☺), Mar, Mia, Elise, Jakub, Rike, Rebecca, Jon, Nuria, Hui, Gael, Isabelle, Therese, Jose, Suriya (my cat buddy ☺), Gabrielle, Yan, Isabel, Klara, Sebastian, Louka, Rufa, Claudia, Anna, Annalisa, Jumana, Feijian, Busra, João, Viktor, Sudesh, Olle, Vanessa, and everyone else whose names I may not have listed here. I'm so happy to have met you all and you made this PhD journey truly memorable. A big thank you to the research engineers: Karin and Rikard, you are/were fantastic! Thank you for always managing to get me the chemicals I needed, even at odd hours, so I could keep my experiments on track. Thank you Mia G. for your help, support and the nice conversations!

Now it’s time to thank my family. Dziękuję Mamusiu i Tatusiu za wasze wsparcie, za przyjazdy i marnowanie urlopu, żeby pomóc mi skończyć mój doktorat. Częściowo należy on do Was, szczególnie do Ciebie, Mamusiu, bo to Ty spędziłaś z nami najwięcej czasu. Dziękuję z całego serca! Dziękuję Babciu Jadziu, za Twoją wiarę we mnie i kibicowanie mi, bym dotrwała do końca ☺ Podziękowania dla mojej siostrzyczki Marfi, za Twoje ciepłe słowa i wsparcie w chwilach załamania. Jesteś najlepsza!

Many thanks to my parents-in-law, for all your help with the kids and taking care of them so I could attend conferences and focus when I needed to ☺

And finally, I want to thank my husband, Benjamin, you’ve been really supportive and I would have never made it without you. This PhD is 50% yours, for all the help with Python, for letting me work late nights and weekends, and for understanding that sometimes my cells felt like my extra kids ;) I’m so lucky to have you! Ian i Sam: jesteście moim wszystkim, I to Wam dedykuję ten doktorat. Mam nadzieję, że to pokaże Wam, że nie ma rzeczy niemożliwych i że możecie osiągnąć to o czym marzycie.

9. REFERENCES

1 Rodriguez, L. E. *et al.* Chapter 4: A Geological and Chemical Context for the Origins of Life on Early Earth. *Astrobiology* 24, S-76-S-106 (2024). https://doi.org/10.1089/ast.2021.0139

2 K.(Editor), P. Iron Metabolism in Human Health and Disease. *Advances in Experimental Medicine and Biology. Springer.* (2023). https://doi.org/https://doi.org/10.1007/978-3-031-92033-2

3 Winterbourn, C. C. Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol Lett* 82-83, 969-974 (1995). https://doi.org/10.1016/0378-4274(95)03532-x

4 Collaborators, G. B. D. A. Prevalence, years lived with disability, and trends in anaemia burden by severity and cause, 1990-2021: findings from the Global Burden of Disease Study 2021. *Lancet Haematol* 10, e713-e734 (2023). https://doi.org/10.1016/S2352-3026(23)00160-6

5 Alem, A. Z. *et al.* Prevalence and factors associated with anemia in women of reproductive age across low- and middle-income countries based on national data. *Sci Rep* 13, 20335 (2023). https://doi.org/10.1038/s41598-023-46739-z

6 King, J. C. The risk of maternal nutritional depletion and poor outcomes increases in early or closely spaced pregnancies. *J Nutr* 133, 1732S-1736S (2003). https://doi.org/10.1093/jn/133.5.1732S

7 Hurtado, E. K., Claussen, A. H. & Scott, K. G. Early childhood anemia and mild or moderate mental retardation. *Am J Clin Nutr* 69, 115-119 (1999). https://doi.org/10.1093/ajcn/69.1.115

8 Owolabi, A. J. *et al.* Growth and micronutrient status parameters of Nigerian preterm infants consuming preterm formula or breastmilk. *Pediatr Res* 96, 380-387 (2024). https://doi.org/10.1038/s41390-023-02976-6

9 Piskin, E., Cianciosi, D., Gulec, S., Tomas, M. & Capanoglu, E. Iron Absorption: Factors, Limitations, and Improvement Methods. *ACS Omega* 7, 20441-20456 (2022). https://doi.org/10.1021/acsomega.2c01833

10 Scheers, N. M., Pereira, D. I. A., Faria, N. & Powell, J. J. Ferric citrate and ferric EDTA but not ferrous sulfate drive amphiregulin-mediated activation of the MAP kinase ERK in gut epithelial cancer cells. *Oncotarget* 9, 17066-17077 (2018). https://doi.org/10.18632/oncotarget.24899

11 Bastide, N. M. *et al.* A central role for heme iron in colon carcinogenesis associated with red meat intake. *Cancer Res* 75, 870-879 (2015). https://doi.org/10.1158/0008-5472.CAN-14-2554

12 Milman, N. T. A Review of Nutrients and Compounds, Which Promote or Inhibit Intestinal Iron Absorption: Making a Platform for Dietary Measures That Can Reduce Iron Uptake in Patients with Genetic Haemochromatosis. *J Nutr Metab* 2020, 7373498 (2020). https://doi.org/10.1155/2020/7373498

13 Farvid, M. S. *et al.* Consumption of red meat and processed meat and cancer incidence: a systematic review and meta-analysis of prospective studies. *Eur J Epidemiol* 36, 937-951 (2021). https://doi.org/10.1007/s10654-021-00741-9

14 Armstrong, B. & Doll, R. Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int J Cancer* 15, 617-631 (1975). https://doi.org/10.1002/ijc.2910150411

15 Larsson, S. C., Rafter, J., Holmberg, L., Bergkvist, L. & Wolk, A. Red meat consumption and risk of cancers of the proximal colon, distal colon and rectum: the Swedish Mammography Cohort. *Int J Cancer* 113, 829-834 (2005). https://doi.org/10.1002/ijc.20658

16 Kabat, G. C., Miller, A. B., Jain, M. & Rohan, T. E. A cohort study of dietary iron and heme iron intake and risk of colorectal cancer in women. *Br J Cancer* 97, 118-122 (2007). https://doi.org/10.1038/sj.bjc.6603837

17 Chua, A. C. *et al.* Dietary iron enhances colonic inflammation and IL-6/IL-11-Stat3 signaling promoting colonic tumor development in mice. *PLoS One* 8, e78850 (2013). https://doi.org/10.1371/journal.pone.0078850

18 Lo, J. O. *et al.* The role of oral iron in the treatment of adults with iron deficiency. *Eur J Haematol* 110, 123-130 (2023). https://doi.org/10.1111/ejh.13892

19 Bloor, S. R. S., R.; Hobson, A.R. . Oral Iron Supplementation—Gastrointestinal Side Effects and the Impact on the Gut Microbiota. *Microbiol. Res.*12, 491-502 (2021). https://doi.org/ https://doi.org/10.3390/microbiolres12020033

20 Cronin, S. J. F., Woolf, C. J., Weiss, G. & Penninger, J. M. The Role of Iron Regulation in Immunometabolism and Immune-Related Disease. *Front Mol Biosci* 6, 116 (2019). https://doi.org/10.3389/fmolb.2019.00116

21 Roemhild, K. *et al.* Iron metabolism: pathophysiology and pharmacology. *Trends Pharmacol Sci* 42, 640-656 (2021). https://doi.org/10.1016/j.tips.2021.05.001
22 Outten, F. W. & Theil, E. C. Iron-based redox switches in biology. *Antioxid Redox Signal* 11, 1029-1046 (2009). https://doi.org/10.1089/ars.2008.2296
23 Crielaard, B. J., Lammers, T. & Rivella, S. Targeting iron metabolism in drug discovery and delivery. *Nat Rev Drug Discov* 16, 400-423 (2017). https://doi.org/10.1038/nrd.2016.248
24 Baskurt, O. K. & Meiselman, H. J. Blood Rheology and Hemodynamics. *Semin Thromb Hemost* 50, 902-915 (2024). https://doi.org/10.1055/s-0043-1777802
25 Kamga, C., Krishnamurthy, S. & Shiva, S. Myoglobin and mitochondria: a relationship bound by oxygen and nitric oxide. *Nitric Oxide* 26, 251-258 (2012). https://doi.org/10.1016/j.niox.2012.03.005
26 Ru, Q. *et al.* Iron homeostasis and ferroptosis in human diseases: mechanisms and therapeutic prospects. *Signal Transduct Target Ther* 9, 271 (2024). https://doi.org/10.1038/s41392-024-01969-z
27 Kilkenny, M. L., Longo, M. A., Perera, R. L. & Pellegrini, L. Structures of human primase reveal design of nucleotide elongation site and mode of Pol alpha tethering. *Proc Natl Acad Sci U S A* 110, 15961-15966 (2013). https://doi.org/10.1073/pnas.1311185110
28 Prakash, S. & Prakash, L. Translesion DNA synthesis in eukaryotes: a one- or two-polymerase affair. *Genes Dev* 16, 1872-1883 (2002). https://doi.org/10.1101/gad.1009802
29 McCann, S., Perapoch Amado, M. & Moore, S. E. The Role of Iron in Brain Development: A Systematic Review. *Nutrients* 12 (2020). https://doi.org/10.3390/nu12072001
30 Organization., U. N. W. H. Iron Deficiency Anaemia: Assesment, Prevention and Control: a Guide for Programme Managers. *(World Health Organization, 2001)* (2001).
31 Organization., W. H. The Global Prevalence of Anaemia in 2011 *(WHO, 2015)* (2015).
32 Sundararajan, S. & Rabe, H. Prevention of iron deficiency anemia in infants and toddlers. *Pediatr Res* 89, 63-73 (2021). https://doi.org/10.1038/s41390-020-0907-5
33 Ponka P., T. M., Eaton J.W. CHAPTER 30 - Iron. *Handbook on the Toxicology of Metals (Third Edition)*, 577-598 (2007).
34 Medicine), P. o. M. I. o. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. *National Academies Press (US)* (2001).
35 Silva, B. & Faustino, P. An overview of molecular basis of iron metabolism regulation and the associated pathologies. *Biochim Biophys Acta* 1852, 1347-1359 (2015). https://doi.org/10.1016/j.bbadis.2015.03.011
36 Zumdahl S., D. D. J. Chemical Principles *Houghton Mifflin Company* Third Edition ed.R.Stratton (1998).
37 P., F. Applying Electron Exchange Symmetry Properties to Better Understand Hund's Rule. *The Chemical Educator* 13, 141-147 (2008).
38 Gulcin I., A. S. H. Fe3+ Reducing Power as the Most Common Assay for Understanding the Biological Functions of Antioxidants. *Processes* 13 (2025).
39 Lee K., C. F. M. QUANTITATIVE DETERMINATION OF THE ELEMENTAL, FERROUS, FERRIC, SOLUBLE, AND COMPLEXED IRON IN FOODS. *Journal of Food Science* (1979). https://doi.org/ https://doi.org/10.1111/j.1365-2621.1979.tb03833.x
40 Ouellette R.J., R. J. D. Principles of Organic Chemistry. *Elsevier* (2015).
41 Ashmead, H. D. The absorption and metabolism 01 iron amino acid chelate. *Archivos Latinoamericanos de Nutrición- SciELO* (2001).
42 Worsfold P., T. A., Poole C., Miro M. (Editors in Chief). Encyclopedia of Analytical Science. Third Edition. *Elsevier* (2019).
43 Kontoghiorghes, G. J. & Kontoghiorghe, C. N. Iron and Chelation in Biochemistry and Medicine: New Approaches to Controlling Iron Metabolism and Treating Related Diseases. *Cells* 9 (2020). https://doi.org/10.3390/cells9061456
44 Brown, T. L., LeMay, H. E., Bursten, B. E., & Murphy, C. . Chemistry: The Central Science (14th ed.). *Pearson Education.* ((2017)).
45 Skuban S., D. T., Kapor A., Cvejic Z., Rakic S. Dielectric and structural properties of iron- and sodium-fumarates. *Journal of Research in Physics* 36, 21-29 (2012). https://doi.org/doi:10.2478/v10242-012-0010-2
46 Greenwood, N. N., & Earnshaw, A. . Chemistry of the Elements (2nd ed.). *Butterworth-Heinemann.* ((2012)).
47 Organization), W. W. H. Iron deficiency anaemia: assessment, prevention and control. A guide for programme managers. (2001).

48 Brittenham, G. M. New advances in iron metabolism, iron deficiency, and iron overload. *Curr Opin Hematol* 1, 101-106 (1994).
49 World Health Organization; Allen, L. H. D. B., B.; Dary, O.; Hurrell, R. Guidelines on Food Fortification with Micronutrients; World Health Organization. *Geneva, Switzerland* (2006).
50 Pereira, D. I. *et al.* Caco-2 cell acquisition of dietary iron(III) invokes a nanoparticulate endocytic pathway. *PLoS One* 8, e81250 (2013). https://doi.org/10.1371/journal.pone.0081250
51 Perfecto, A. *et al.* Mechanisms of Iron Uptake from Ferric Phosphate Nanoparticles in Human Intestinal Caco-2 Cells. *Nutrients* 9 (2017). https://doi.org/10.3390/nu9040359
52 Housecroft, C. E., & Sharpe, A. G. . Inorganic Chemistry (4th ed.). *Pearson Education.* (2012).
53 Miessler, G. L., Fischer, P. J., & Tarr, D. A. . Inorganic Chemistry (5th ed.). *Pearson Education* (2014).
54 Layrisse, M. & Martinez-Torres, C. Fe(III)-EDTA complex as iron fortification. *Am J Clin Nutr* 30, 1166-1174 (1977). https://doi.org/10.1093/ajcn/30.7.1166
55 Martinez-Torres, C., Romano, E. L., Renzi, M. & Layrisse, M. Fe(III)-EDTA complex as iron fortification. Further studies. *Am J Clin Nutr* 32, 809-816 (1979). https://doi.org/10.1093/ajcn/32.4.809
56 Aiswarya V.V., A. V. V., Devika Krishna S., Premkumar N., Ahmad I. Comparing the Effectiveness of Ferric Pyrophosphate and Ferrous Bis-Glycinate in Non-Dialysis Chronic Kidney Disease Patients. *Asian Journal of Pharmaceutical and Health Sciences* 10, 2220-2223 (2020).
57 Goyal, M., Sawhney, A., & Singh, N. . Iron nanoparticles: a novel approach for iron supplementation in iron deficiency anemia. . *Current Nanoscience* 14, 118–127 (2018). https://doi.org/https://doi.org/10.2174/1573413713666171120220037
58 Ashraf, N. *et al.* Iron/iron oxide nanoparticles: advances in microbial fabrication, mechanism study, biomedical, and environmental applications. *Crit Rev Microbiol* 45, 278-300 (2019). https://doi.org/10.1080/1040841X.2019.1593101
59 Kianpour, S. *et al.* Enterobacter sp. Mediated Synthesis of Biocompatible Nanostructured Iron-Polysaccharide Complexes: a Nutritional Supplement for Iron-Deficiency Anemia. *Biol Trace Elem Res* 198, 744-755 (2020). https://doi.org/10.1007/s12011-020-02101-2
60 Athira, S., Mann, B., Sharma, R., Pothuraju, R. & Bajaj, R. K. Preparation and characterization of iron-chelating peptides from whey protein: An alternative approach for chemical iron fortification. *Food Res Int* 141, 110133 (2021). https://doi.org/10.1016/j.foodres.2021.110133
61 Singh, K., Sethi Chopra, D., Singh, D. & Singh, N. Nano-formulations in treatment of iron deficiency anaemia: An overview. *Clin Nutr ESPEN* 52, 12-19 (2022). https://doi.org/10.1016/j.clnesp.2022.08.032
62 Hosny, K. M., Banjar, Z. M., Hariri, A. H. & Hassan, A. H. Solid lipid nanoparticles loaded with iron to overcome barriers for treatment of iron deficiency anemia. *Drug Des Devel Ther* 9, 313-320 (2015). https://doi.org/10.2147/DDDT.S77702
63 Rohner, F. *et al.* Synthesis, characterization, and bioavailability in rats of ferric phosphate nanoparticles. *J Nutr* 137, 614-619 (2007). https://doi.org/10.1093/jn/137.3.614
64 Pereira, D. I. *et al.* Nanoparticulate iron(III) oxo-hydroxide delivers safe iron that is well absorbed and utilised in humans. *Nanomedicine* 10, 1877-1886 (2014). https://doi.org/10.1016/j.nano.2014.06.012
65 Bothwell, T. H., Charlton, R. W., Cook, J. D., & Finch, C. A. . Iron Metabolism in Man. *Oxford: Blackwell Scientific Publications.* (1979).
66 Gordeuk, V. R., Brittenham, G. M., McLaren, C. E., Hughes, M. A. & Keating, L. J. Carbonyl iron therapy for iron deficiency anemia. *Blood* 67, 745-752 (1986).
67 Schroeer, W., & Becker, P. . Iron, Carbonyl in Ullmann's Encyclopedia of Industrial Chemistry (Vol. 18). *Wiley-VCH* (2000).
68 Whittaker, P., Ali, S. F., Imam, S. Z. & Dunkel, V. C. Acute toxicity of carbonyl iron and sodium iron EDTA compared with ferrous sulfate in young rats. *Regul Toxicol Pharmacol* 36, 280-286 (2002). https://doi.org/10.1006/rtph.2002.1577
69 FDA., U. S. GRAS Notice No. GRN 000129: Carbonyl Iron. (2003).
70 Bohn, L., Meyer, A. S. & Rasmussen, S. K. Phytate: impact on environment and human nutrition. A challenge for molecular breeding. *J Zhejiang Univ Sci B* 9, 165-191 (2008). https://doi.org/10.1631/jzus.B0710640
71 Hallberg, L., Brune, M. & Rossander, L. Iron absorption in man: ascorbic acid and dose-dependent inhibition by phytate. *Am J Clin Nutr* 49, 140-144 (1989). https://doi.org/10.1093/ajcn/49.1.140
72 Hurrell, R. F. Phytic acid degradation as a means of improving iron absorption. *Int J Vitam Nutr Res* 74, 445-452 (2004). https://doi.org/10.1024/0300-9831.74.6.445

73 Brune, M., Rossander-Hulten, L., Hallberg, L., Gleerup, A. & Sandberg, A. S. Iron absorption from bread in humans: inhibiting effects of cereal fiber, phytate and inositol phosphates with different numbers of phosphate groups. *J Nutr* 122, 442-449 (1992). https://doi.org/10.1093/jn/122.3.442
74 Sandberg, A. S. & Andersson, H. Effect of dietary phytase on the digestion of phytate in the stomach and small intestine of humans. *J Nutr* 118, 469-473 (1988). https://doi.org/10.1093/jn/118.4.469
75 Hurrell, R. F. *et al.* Soy protein, phytate, and iron absorption in humans. *Am J Clin Nutr* 56, 573-578 (1992). https://doi.org/10.1093/ajcn/56.3.573
76 Hurrell, R. F., Reddy, M. & Cook, J. D. Inhibition of non-haem iron absorption in man by polyphenolic-containing beverages. *Br J Nutr* 81, 289-295 (1999).
77 Hallberg, L. & Rossander, L. Effect of different drinks on the absorption of non-heme iron from composite meals. *Hum Nutr Appl Nutr* 36, 116-123 (1982).
78 Hallberg, L., Brune, M., Erlandsson, M., Sandberg, A. S. & Rossander-Hulten, L. Calcium: effect of different amounts on nonheme- and heme-iron absorption in humans. *Am J Clin Nutr* 53, 112-119 (1991). https://doi.org/10.1093/ajcn/53.1.112
79 Lynch, S. R. The effect of calcium on iron absorption. *Nutr Res Rev* 13, 141-158 (2000). https://doi.org/10.1079/095442200108729043
80 Hurrell, R. F., Lynch, S. R., Trinidad, T. P., Dassenko, S. A. & Cook, J. D. Iron absorption in humans as influenced by bovine milk proteins. *Am J Clin Nutr* 49, 546-552 (1989). https://doi.org/10.1093/ajcn/49.3.546
81 Hurrell, R. F., Lynch, S. R., Trinidad, T. P., Dassenko, S. A. & Cook, J. D. Iron absorption in humans: bovine serum albumin compared with beef muscle and egg white. *Am J Clin Nutr* 47, 102-107 (1988). https://doi.org/10.1093/ajcn/47.1.102
82 Lynch, S. R. & Cook, J. D. Interaction of vitamin C and iron. *Ann N Y Acad Sci* 355, 32-44 (1980). https://doi.org/10.1111/j.1749-6632.1980.tb21325.x
83 Conrad, M. E. & Schade, S. G. Ascorbic acid chelates in iron absorption: a role for hydrochloric acid and bile. *Gastroenterology* 55, 35-45 (1968).
84 Siegenberg, D. *et al.* Ascorbic acid prevents the dose-dependent inhibitory effects of polyphenols and phytates on nonheme-iron absorption. *Am J Clin Nutr* 53, 537-541 (1991). https://doi.org/10.1093/ajcn/53.2.537
85 Stekel, A. *et al.* Absorption of fortification iron from milk formulas in infants. *Am J Clin Nutr* 43, 917-922 (1986). https://doi.org/10.1093/ajcn/43.6.917
86 Teucher, B., Olivares, M. & Cori, H. Enhancers of iron absorption: ascorbic acid and other organic acids. *Int J Vitam Nutr Res* 74, 403-419 (2004). https://doi.org/10.1024/0300-9831.74.6.403
87 Pizarro, F. *et al.* Ascorbyl palmitate enhances iron bioavailability in iron-fortified bread. *Am J Clin Nutr* 84, 830-834 (2006). https://doi.org/10.1093/ajcn/84.4.830
88 Martinez-Torres, C., Romano, E. & Layrisse, M. Effect of cysteine on iron absorption in man. *Am J Clin Nutr* 34, 322-327 (1981). https://doi.org/10.1093/ajcn/34.3.322
89 Tateishi, Y. *et al.* A short-term intervention of ingesting iron along with methionine and threonine leads to a higher hemoglobin level than that with iron alone in young healthy women: a randomized, double-blind, parallel-group, comparative study. *Eur J Nutr* 62, 3009-3019 (2023). https://doi.org/10.1007/s00394-023-03213-w
90 Li, Y., Jiang, H. & Huang, G. Protein Hydrolysates as Promoters of Non-Haem Iron Absorption. *Nutrients* 9 (2017). https://doi.org/10.3390/nu9060609
91 Van Campen, D. Enhancement of iron absorption from ligated segments of rat intestine by histidine, cysteine, and lysine: effects of removing ionizing groups and of stereoisomerism. *J Nutr* 103, 139-142 (1973). https://doi.org/10.1093/jn/103.1.139
92 Christensen, J. M., Ghannam, M. & Ayres, J. W. Effects of divalent amino acids on iron absorption. *J Pharm Sci* 73, 1245-1248 (1984). https://doi.org/10.1002/jps.2600730913
93 J.D., H. CHEMISTRY OF IRON IN NATURAL WATER. *GEOLOGICAL SURVEY WATER-SUPPLY* (1962).
94 Swain, J. H., Newman, S. M. & Hunt, J. R. Bioavailability of elemental iron powders to rats is less than bakery-grade ferrous sulfate and predicted by iron solubility and particle surface area. *J Nutr* 133, 3546-3552 (2003). https://doi.org/10.1093/jn/133.11.3546
95 Skikne, B. S., Lynch, S. R. & Cook, J. D. Role of gastric acid in food iron absorption. *Gastroenterology* 81, 1068-1071 (1981).
96 Hurrell, R. F. Preventing iron deficiency through food fortification. *Nutr Rev* 55, 210-222 (1997). https://doi.org/10.1111/j.1753-4887.1997.tb01608.x

97 Hurrell, R. F. Fortification: overcoming technical and practical barriers. *J Nutr* 132, 806S-812S (2002). https://doi.org/10.1093/jn/132.4.806S
98 Uauy, R., Hertrampf, E. & Reddy, M. Iron fortification of foods: overcoming technical and practical barriers. *J Nutr* 132, 849S-852S (2002). https://doi.org/10.1093/jn/132.4.849S
99 Hunt, J. N. A possible relation between the regulation of gastric emptying and food intake. *Am J Physiol* 239, G1-4 (1980). https://doi.org/10.1152/ajpgi.1980.239.1.G1
100 Fallingborg, J. Intraluminal pH of the human gastrointestinal tract. *Dan Med Bull* 46, 183-196 (1999).
101 Turney, B. W. Anatomy in a modern medical curriculum. *Ann R Coll Surg Engl* 89, 104-107 (2007). https://doi.org/10.1308/003588407X168244
102 Anderson, G. J. & Frazer, D. M. Current understanding of iron homeostasis. *Am J Clin Nutr* 106, 1559S-1566S (2017). https://doi.org/10.3945/ajcn.117.155804
103 Ferraris, R. P. & Carey, H. V. Intestinal transport during fasting and malnutrition. *Annu Rev Nutr* 20, 195-219 (2000). https://doi.org/10.1146/annurev.nutr.20.1.195
104 Kim, Y. S. & Ho, S. B. Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Curr Gastroenterol Rep* 12, 319-330 (2010). https://doi.org/10.1007/s11894-010-0131-2
105 Berne, R. M., Levy, M. N., Koeppen, B. M., & Stanton, B. A. . Berne & Levy Physiology (6th ed.). . *Mosby Elsevier.* (2008.).
106 Young, B., O'Dowd, G., & Woodford, P. . Wheater's Functional Histology: A Text and Colour Atlas (6th ed.). *Elsevier* (2014).
107 Fleming MD, T. C. r., Su MA, Foernzler D, Beier DR, Dietrich WF, Andrews NC. Microcyticanaemia mice have a mutation in Nramp2, a candidate iron transporter gen. *Nat Gene* 16, 383-386 (1997). https://doi.org/10.1038/ng0897-383
108 Fleming, M. D. *et al.* Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport. *Proc Natl Acad Sci U S A* 95, 1148-1153 (1998). https://doi.org/10.1073/pnas.95.3.1148
109 Shawki, A., Knight, P. B., Maliken, B. D., Niespodzany, E. J. & Mackenzie, B. H(+)-coupled divalent metal-ion transporter-1: functional properties, physiological roles and therapeutics. *Curr Top Membr* 70, 169-214 (2012). https://doi.org/10.1016/B978-0-12-394316-3.00005-3
110 Shah, Y. M., Matsubara, T., Ito, S., Yim, S. H. & Gonzalez, F. J. Intestinal hypoxia-inducible transcription factors are essential for iron absorption following iron deficiency. *Cell Metab* 9, 152-164 (2009). https://doi.org/10.1016/j.cmet.2008.12.012
111 Gunshin, H. *et al.* Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388, 482-488 (1997). https://doi.org/10.1038/41343
112 Illing, A. C., Shawki, A., Cunningham, C. L. & Mackenzie, B. Substrate profile and metal-ion selectivity of human divalent metal-ion transporter-1. *J Biol Chem* 287, 30485-30496 (2012). https://doi.org/10.1074/jbc.M112.364208
113 Lee, P. L., Gelbart, T., West, C., Halloran, C. & Beutler, E. The human Nramp2 gene: characterization of the gene structure, alternative splicing, promoter region and polymorphisms. *Blood Cells Mol Dis* 24, 199-215 (1998). https://doi.org/10.1006/bcmd.1998.0186
114 Mackenzie, B., Takanaga, H., Hubert, N., Rolfs, A. & Hediger, M. A. Functional properties of multiple isoforms of human divalent metal-ion transporter 1 (DMT1). *Biochem J* 403, 59-69 (2007). https://doi.org/10.1042/BJ20061290
115 Hubert, N. & Hentze, M. W. Previously uncharacterized isoforms of divalent metal transporter (DMT)-1: implications for regulation and cellular function. *Proc Natl Acad Sci U S A* 99, 12345-12350 (2002). https://doi.org/10.1073/pnas.192423399
116 Lis, A., Barone, T. A., Paradkar, P. N., Plunkett, R. J. & Roth, J. A. Expression and localization of different forms of DMT1 in normal and tumor astroglial cells. *Brain Res Mol Brain Res* 122, 62-70 (2004). https://doi.org/10.1016/j.molbrainres.2003.11.023
117 Tabuchi, M., Tanaka, N., Nishida-Kitayama, J., Ohno, H. & Kishi, F. Alternative splicing regulates the subcellular localization of divalent metal transporter 1 isoforms. *Mol Biol Cell* 13, 4371-4387 (2002). https://doi.org/10.1091/mbc.e02-03-0165
118 McKie, A. T. *et al.* An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* 291, 1755-1759 (2001). https://doi.org/10.1126/science.1057206
119 Han, O., Failla, M. L., Hill, A. D., Morris, E. R. & Smith, J. C., Jr. Reduction of Fe(III) is required for uptake of nonheme iron by Caco-2 cells. *J Nutr* 125, 1291-1299 (1995). https://doi.org/10.1093/jn/125.5.1291
120 McKie, A. T. The role of Dcytb in iron metabolism: an update. *Biochem Soc Trans* 36, 1239-1241 (2008). https://doi.org/10.1042/BST0361239

121 Asard, H., Barbaro, R., Trost, P. & Berczi, A. Cytochromes b561: ascorbate-mediated trans-membrane electron transport. *Antioxid Redox Signal* 19, 1026-1035 (2013). https://doi.org/10.1089/ars.2012.5065
122 Mackenzie, B., Ujwal, M. L., Chang, M. H., Romero, M. F. & Hediger, M. A. Divalent metal-ion transporter DMT1 mediates both H+ -coupled Fe2+ transport and uncoupled fluxes. *Pflugers Arch* 451, 544-558 (2006). https://doi.org/10.1007/s00424-005-1494-3
123 Conner, S. D. & Schmid, S. L. Regulated portals of entry into the cell. *Nature* 422, 37-44 (2003). https://doi.org/10.1038/nature01451
124 Brodsky, F. M., Chen, C. Y., Knuehl, C., Towler, M. C. & Wakeham, D. E. Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu Rev Cell Dev Biol* 17, 517-568 (2001). https://doi.org/10.1146/annurev.cellbio.17.1.517
125 Conner, S. D. & Schmid, S. L. Identification of an adaptor-associated kinase, AAK1, as a regulator of clathrin-mediated endocytosis. *J Cell Biol* 156, 921-929 (2002). https://doi.org/10.1083/jcb.200108123
126 Mettlen, M., Chen, P. H., Srinivasan, S., Danuser, G. & Schmid, S. L. Regulation of Clathrin-Mediated Endocytosis. *Annu Rev Biochem* 87, 871-896 (2018). https://doi.org/10.1146/annurev-biochem-062917-012644
127 Kalgaonkar, S. & Lonnerdal, B. Receptor-mediated uptake of ferritin-bound iron by human intestinal Caco-2 cells. *J Nutr Biochem* 20, 304-311 (2009). https://doi.org/10.1016/j.jnutbio.2008.04.003
128 Lonnerdal, B. Soybean ferritin: implications for iron status of vegetarians. *Am J Clin Nutr* 89, 1680S-1685S (2009). https://doi.org/10.3945/ajcn.2009.26736W
129 Hoppler, M., Schonbachler, A., Meile, L., Hurrell, R. F. & Walczyk, T. Ferritin-iron is released during boiling and in vitro gastric digestion. *J Nutr* 138, 878-884 (2008). https://doi.org/10.1093/jn/138.5.878
130 (Editor-in-Chief), E. H. E. Comprehensive Biophysics. *Academic Press* (2012).
131 Shayeghi, M. *et al.* Identification of an intestinal heme transporter. *Cell* 122, 789-801 (2005). https://doi.org/10.1016/j.cell.2005.06.025
132 Qiu, A. *et al.* Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. *Cell* 127, 917-928 (2006). https://doi.org/10.1016/j.cell.2006.09.041
133 West, A. R. & Oates, P. S. Subcellular location of heme oxygenase 1 and 2 and divalent metal transporter 1 in relation to endocytotic markers during heme iron absorption. *J Gastroenterol Hepatol* 23, 150-158 (2008). https://doi.org/10.1111/j.1440-1746.2007.05047.x
134 Weintraub, L. R., Weinstein, M. B., Huser, H. J. & Rafal, S. Absorption of hemoglobin iron: the role of a heme-splitting substance in the intestinal mucosa. *J Clin Invest* 47, 531-539 (1968). https://doi.org/10.1172/JCI105749
135 Grasbeck, R., Kouvonen, I., Lundberg, M. & Tenhunen, R. An intestinal receptor for heme. *Scand J Haematol* 23, 5-9 (1979). https://doi.org/10.1111/j.1600-0609.1979.tb02845.x
136 West, A. R. & Oates, P. S. Mechanisms of heme iron absorption: current questions and controversies. *World J Gastroenterol* 14, 4101-4110 (2008). https://doi.org/10.3748/wjg.14.4101
137 Uc, A., Stokes, J. B. & Britigan, B. E. Heme transport exhibits polarity in Caco-2 cells: evidence for an active and membrane protein-mediated process. *Am J Physiol Gastrointest Liver Physiol* 287, G1150-1157 (2004). https://doi.org/10.1152/ajpgi.00157.2004
138 Simovich, M., Hainsworth, L. N., Fields, P. A., Umbreit, J. N. & Conrad, M. E. Localization of the iron transport proteins Mobilferrin and DMT-1 in the duodenum: the surprising role of mucin. *Am J Hematol* 74, 32-45 (2003). https://doi.org/10.1002/ajh.10383
139 Umbreit, J. N., Conrad, M. E., Moore, E. G. & Latour, L. F. Iron absorption and cellular transport: the mobilferrin/paraferritin paradigm. *Semin Hematol* 35, 13-26 (1998).
140 Munro, H. N. *et al.* The ferritin genes: structure, expression, and regulation. *Ann N Y Acad Sci* 526, 113-123 (1988). https://doi.org/10.1111/j.1749-6632.1988.tb55497.x
141 Dorner, M. H. *et al.* Structure of human ferritin light subunit messenger RNA: comparison with heavy subunit message and functional implications. *Proc Natl Acad Sci U S A* 82, 3139-3143 (1985). https://doi.org/10.1073/pnas.82.10.3139
142 Levi S, L. A., Cesareni G, Cozzi A, Franceschinelli F, Albertini A, Arosio P. . Mechanism of ferritin iron uptake: activity of the H-chain and deletion mapping of the ferro-oxidase site. A study of iron uptake and ferro-oxidase activity of human liver, recombinant H-chain ferritins, and of two H-chain deletion mutants. . *J Biol Chem* 263. , 18086-18092 (1988).
143 Levi, S. *et al.* The role of the L-chain in ferritin iron incorporation. Studies of homo and heteropolymers. *J Mol Biol* 238, 649-654 (1994). https://doi.org/10.1006/jmbi.1994.1325

144 Harrison, P. M. & Arosio, P. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta* 1275, 161-203 (1996). https://doi.org/10.1016/0005-2728(96)00022-9

145 Glahn, R. P., Lee, O. A., Yeung, A., Goldman, M. I. & Miller, D. D. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *J Nutr* 128, 1555-1561 (1998). https://doi.org/10.1093/jn/128.9.1555

146 Anderson, C. P., Shen, M., Eisenstein, R. S. & Leibold, E. A. Mammalian iron metabolism and its control by iron regulatory proteins. *Biochim Biophys Acta* 1823, 1468-1483 (2012). https://doi.org/10.1016/j.bbamcr.2012.05.010

147 Eisenstein, R. S. Iron regulatory proteins and the molecular control of mammalian iron metabolism. *Annu Rev Nutr* 20, 627-662 (2000). https://doi.org/10.1146/annurev.nutr.20.1.627

148 McKie, A. T. *et al.* A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Mol Cell* 5, 299-309 (2000). https://doi.org/10.1016/s1097-2765(00)80425-6

149 Chen, H. *et al.* Hephaestin is a ferroxidase that maintains partial activity in sex-linked anemia mice. *Blood* 103, 3933-3939 (2004). https://doi.org/10.1182/blood-2003-09-3139

150 Nemeth, E. & Ganz, T. Hepcidin-Ferroportin Interaction Controls Systemic Iron Homeostasis. *Int J Mol Sci* 22 (2021). https://doi.org/10.3390/ijms22126493

151 Ma, Y., Yeh, M., Yeh, K. Y. & Glass, J. Iron Imports. V. Transport of iron through the intestinal epithelium. *Am J Physiol Gastrointest Liver Physiol* 290, G417-422 (2006). https://doi.org/10.1152/ajpgi.00489.2005

152 Galy, B. *et al.* Iron regulatory proteins control a mucosal block to intestinal iron absorption. *Cell Rep* 3, 844-857 (2013). https://doi.org/10.1016/j.celrep.2013.02.026

153 Pigeon, C. *et al.* A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem* 276, 7811-7819 (2001). https://doi.org/10.1074/jbc.M008923200

154 Park, C. H., Valore, E. V., Waring, A. J. & Ganz, T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem* 276, 7806-7810 (2001). https://doi.org/10.1074/jbc.M008922200

155 Nemeth, E. *et al.* Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 306, 2090-2093 (2004). https://doi.org/10.1126/science.1104742

156 Camaschella, C., Nai, A. & Silvestri, L. Iron metabolism and iron disorders revisited in the hepcidin era. *Haematologica* 105, 260-272 (2020). https://doi.org/10.3324/haematol.2019.232124

157 Hentze, M. W., Muckenthaler, M. U., Galy, B. & Camaschella, C. Two to tango: regulation of Mammalian iron metabolism. *Cell* 142, 24-38 (2010). https://doi.org/10.1016/j.cell.2010.06.028

158 Girelli, D., Nemeth, E. & Swinkels, D. W. Hepcidin in the diagnosis of iron disorders. *Blood* 127, 2809-2813 (2016). https://doi.org/10.1182/blood-2015-12-639112

159 Brissot, P., Ropert, M., Le Lan, C. & Loreal, O. Non-transferrin bound iron: a key role in iron overload and iron toxicity. *Biochim Biophys Acta* 1820, 403-410 (2012). https://doi.org/10.1016/j.bbagen.2011.07.014

160 Wang, C. Y. & Babitt, J. L. Liver iron sensing and body iron homeostasis. *Blood* 133, 18-29 (2019). https://doi.org/10.1182/blood-2018-06-815894

161 Hvidberg, V. *et al.* Identification of the receptor scavenging hemopexin-heme complexes. *Blood* 106, 2572-2579 (2005). https://doi.org/10.1182/blood-2005-03-1185

162 Li, J. Y. *et al.* Scara5 is a ferritin receptor mediating non-transferrin iron delivery. *Dev Cell* 16, 35-46 (2009). https://doi.org/10.1016/j.devcel.2008.12.002

163 Theurl, I. *et al.* On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. *Nat Med* 22, 945-951 (2016). https://doi.org/10.1038/nm.4146

164 Ganz, T. Macrophages and systemic iron homeostasis. *J Innate Immun* 4, 446-453 (2012). https://doi.org/10.1159/000336423

165 Pek, R. H. *et al.* Correction: Hemozoin produced by mammals confers heme tolerance. *Elife* 12 (2023). https://doi.org/10.7554/eLife.93253

166 Gozzelino, R., Jeney, V. & Soares, M. P. Mechanisms of cell protection by heme oxygenase-1. *Annu Rev Pharmacol Toxicol* 50, 323-354 (2010). https://doi.org/10.1146/annurev.pharmtox.010909.105600

167 Muckenthaler, M. U., Rivella, S., Hentze, M. W. & Galy, B. A Red Carpet for Iron Metabolism. *Cell* 168, 344-361 (2017). https://doi.org/10.1016/j.cell.2016.12.034

168 Kristiansen, M. *et al.* Identification of the haemoglobin scavenger receptor. *Nature* 409, 198-201 (2001). https://doi.org/10.1038/35051594

169 Hume, D. A., Perry, V. H. & Gordon, S. The mononuclear phagocyte system of the mouse defined by immunohistochemical localisation of antigen F4/80: macrophages associated with epithelia. *Anat Rec* 210, 503-512 (1984). https://doi.org/10.1002/ar.1092100311

170 Sukhbaatar, N. *et al.* Duodenal macrophages control dietary iron absorption via local degradation of transferrin. *Blood* 141, 2878-2890 (2023). https://doi.org/10.1182/blood.2022016632

171 Bain, C. C. *et al.* Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol* 6, 498-510 (2013). https://doi.org/10.1038/mi.2012.89

172 Li, B., Alli, R., Vogel, P. & Geiger, T. L. IL-10 modulates DSS-induced colitis through a macrophage-ROS-NO axis. *Mucosal Immunol* 7, 869-878 (2014). https://doi.org/10.1038/mi.2013.103

173 Oliveira A.S., F. C., Pereira J.O.,Silva S., Costa E.M., Pereira, A.M. Faustino M., Durão J., Pintado M.E., Carvalho A.P. ron-peptide complexes from spent yeast: Evaluation of iron absorption using a Caco-2 monolayer. *Food Bioscience* 56 (2023). https://doi.org/https://doi.org/10.1016/j.fbio.2023.103106.

174 Fanzaga, M. *et al.* Bioavailability Assessment of an Iron Formulation Using Differentiated Human Intestinal Caco-2 Cells. *Foods* 12 (2023). https://doi.org/10.3390/foods12163016

175 Glahn, R. P., Wortley, G. M., South, P. K., & Miller, D. D. . Inhibition of iron uptake by phytic acid, tannic acid, and Zn is poorly predicted by in vitro digestion/Caco-2 cell model. . *The Journal of Agricultural and Food Chemistry* 50, 398–403 (2002). https://doi.org/https://doi.org/10.1021/jf010897x

176 Pfeiffer, C., & Hurrell, R. F. . Influence of different food components on iron absorption: comparison of Caco-2 cell and human studies. . *The American Journal of Clinical Nutrition* 69, 1289S–1294S. (1999).

177 Hubatsch, I., Ragnarsson, E. G. & Artursson, P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat Protoc* 2, 2111-2119 (2007). https://doi.org/10.1038/nprot.2007.303

178 Scheers, N. M., Almgren, A. B. & Sandberg, A. S. Proposing a Caco-2/HepG2 cell model for in vitro iron absorption studies. *J Nutr Biochem* 25, 710-715 (2014). https://doi.org/10.1016/j.jnutbio.2014.02.013

179 Solomou S., P. K., Hoque R., Sharp P. Duodenal cytochrome b plays a role in copper uptake. *American journal of hematology* 88 (2013).

180 Latunde-Dada, G. O. *et al.* A nanoparticulate ferritin-core mimetic is well taken up by HuTu 80 duodenal cells and its absorption in mice is regulated by body iron. *J Nutr* 144, 1896-1902 (2014). https://doi.org/10.3945/jn.114.201715

181 Wikman-Larhed A., A. P. Co-cultures of human intestinal goblet (HT29-H) and absorptive (Caco-2) cells for studies of drug and peptide absorption. *European Journal of Pharmaceutical Sciences* 3, 171-183 (1995). https://doi.org/
https://doi.org/10.1016/0928-0987(95)00007-Z.

182 Hoffmann, P. *et al.* Caco-2/HT29-MTX co-cultured cells as a model for studying physiological properties and toxin-induced effects on intestinal cells. *PLoS One* 16, e0257824 (2021). https://doi.org/10.1371/journal.pone.0257824

183 Astre, G. *et al.* Protocol for the preclinical evaluation of gut barrier function and immune interaction in an HT-29/PBMC co-culture model. *STAR Protoc* 5, 103416 (2024). https://doi.org/10.1016/j.xpro.2024.103416

184 Fedi, A. *et al.* In vitro models replicating the human intestinal epithelium for absorption and metabolism studies: A systematic review. *J Control Release* 335, 247-268 (2021). https://doi.org/10.1016/j.jconrel.2021.05.028

185 Karlsson, H., Fryknas, M., Larsson, R. & Nygren, P. Loss of cancer drug activity in colon cancer HCT-116 cells during spheroid formation in a new 3-D spheroid cell culture system. *Exp Cell Res* 318, 1577-1585 (2012). https://doi.org/10.1016/j.yexcr.2012.03.026

186 Zhao, Q., Shao, M., Ma, L. & Zhou, R. Insights into Modeling Inflammatory Bowel Disease from Stem Cell Derived Intestinal Organoids. *Stem Cell Rev Rep* (2025). https://doi.org/10.1007/s12015-025-10887-8

187 Kakni, P., Lopez-Iglesias, C., Truckenmuller, R., Habibovic, P. & Giselbrecht, S. PSC-derived intestinal organoids with apical-out orientation as a tool to study nutrient uptake, drug absorption and metabolism. *Front Mol Biosci* 10, 1102209 (2023). https://doi.org/10.3389/fmolb.2023.1102209

188 Co, J. Y., Margalef-Catala, M., Monack, D. M. & Amieva, M. R. Controlling the polarity of human gastrointestinal organoids to investigate epithelial biology and infectious diseases. *Nat Protoc* 16, 5171-5192 (2021). https://doi.org/10.1038/s41596-021-00607-0

189 Kollmann, C. *et al.* Human organoids are superior to cell culture models for intestinal barrier research. *Front Cell Dev Biol* 11, 1223032 (2023). https://doi.org/10.3389/fcell.2023.1223032

190 Takahashi, Y. Human intestinal organoids as a valuable tool to investigate human intestinal biology. *Biosci Biotechnol Biochem* (2025). https://doi.org/10.1093/bbb/zbaf099
191 Yin, J. *et al.* Fluid shear stress enhances differentiation of jejunal human enteroids in Intestine-Chip. *Am J Physiol Gastrointest Liver Physiol* 320, G258-G271 (2021). https://doi.org/10.1152/ajpgi.00282.2020
192 Bhatia, S. N. & Ingber, D. E. Microfluidic organs-on-chips. *Nat Biotechnol* 32, 760-772 (2014). https://doi.org/10.1038/nbt.2989
193 Mercadante, C. J. *et al.* Gastrointestinal iron excretion and reversal of iron excess in a mouse model of inherited iron excess. *Haematologica* 104, 678-689 (2019). https://doi.org/10.3324/haematol.2018.198382
194 Green, R. *et al.* Body iron excretion in man: a collaborative study. *Am J Med* 45, 336-353 (1968). https://doi.org/10.1016/0002-9343(68)90069-7
195 Gulec, S., Anderson, G. J. & Collins, J. F. Mechanistic and regulatory aspects of intestinal iron absorption. *Am J Physiol Gastrointest Liver Physiol* 307, G397-409 (2014). https://doi.org/10.1152/ajpgi.00348.2013
196 Wallace, D. F. The Regulation of Iron Absorption and Homeostasis. *Clin Biochem Rev* 37, 51-62 (2016).
197 Prajapati, M. *et al.* Biliary excretion of excess iron in mice requires hepatocyte iron import by Slc39a14. *J Biol Chem* 297, 100835 (2021). https://doi.org/10.1016/j.jbc.2021.100835
198 Hultcrantz, R., Angelin, B., Bjorn-Rasmussen, E., Ewerth, S. & Einarsson, K. Biliary excretion of iron and ferritin in idiopathic hemochromatosis. *Gastroenterology* 96, 1539-1545 (1989). https://doi.org/10.1016/0016-5085(89)90524-6
199 Agrawal, S. *et al.* Does anemia affects cognitive functions in neurologically intact adult patients: Two year cross sectional study at rural tertiary care hospital. *J Family Med Prim Care* 8, 3005-3008 (2019). https://doi.org/10.4103/jfmpc.jfmpc_599_19
200 Cheng, A. N. & Al-Samkari, H. Impact of iron overload on incidence of diabetes mellitus, cardiac disease, and death in congenital hemolytic anemias. *Blood Adv* 8, 5451-5457 (2024). https://doi.org/10.1182/bloodadvances.2024013666
201 Bofill, C. *et al.* Response to repeated phlebotomies in patients with non-insulin-dependent diabetes mellitus. *Metabolism* 43, 614-620 (1994). https://doi.org/10.1016/0026-0495(94)90204-6
202 Fernandez-Real, J. M. *et al.* Blood letting in high-ferritin type 2 diabetes: effects on insulin sensitivity and beta-cell function. *Diabetes* 51, 1000-1004 (2002). https://doi.org/10.2337/diabetes.51.4.1000
203 Richardson, D. R., Kalinowski, D. S., Lau, S., Jansson, P. J. & Lovejoy, D. B. Cancer cell iron metabolism and the development of potent iron chelators as anti-tumour agents. *Biochim Biophys Acta* 1790, 702-717 (2009). https://doi.org/10.1016/j.bbagen.2008.04.003
204 Le, N. T. & Richardson, D. R. The role of iron in cell cycle progression and the proliferation of neoplastic cells. *Biochim Biophys Acta* 1603, 31-46 (2002). https://doi.org/10.1016/s0304-419x(02)00068-9
205 Entezari, S. *et al.* Iron Chelators in Treatment of Iron Overload. *J Toxicol* 2022, 4911205 (2022). https://doi.org/10.1155/2022/4911205
206 Donfrancesco, A. *et al.* Effects of a single course of deferoxamine in neuroblastoma patients. *Cancer Res* 50, 4929-4930 (1990).
207 Ablin, J., Shalev, O., Okon, E., Karmeli, F. & Rachmilewitz, D. Deferiprone, an oral iron chelator, ameliorates experimental colitis and gastric ulceration in rats. *Inflamm Bowel Dis* 5, 253-261 (1999). https://doi.org/10.1097/00054725-199911000-00003
208 Barker, C. A. *et al.* In vitro and in vivo radiosensitization induced by the ribonucleotide reductase inhibitor Triapine (3-aminopyridine-2-carboxaldehyde-thiosemicarbazone). *Clin Cancer Res* 12, 2912-2918 (2006). https://doi.org/10.1158/1078-0432.CCR-05-2860
209 Wessling-Resnick, M. Iron homeostasis and the inflammatory response. *Annu Rev Nutr* 30, 105-122 (2010). https://doi.org/10.1146/annurev.nutr.012809.104804
210 Ganz, T. Anemia of Inflammation. *N Engl J Med* 381, 1148-1157 (2019). https://doi.org/10.1056/NEJMra1804281
211 Weiss, G., Ganz, T. & Goodnough, L. T. Anemia of inflammation. *Blood* 133, 40-50 (2019). https://doi.org/10.1182/blood-2018-06-856500
212 Nemeth, E. & Ganz, T. Anemia of inflammation. *Hematol Oncol Clin North Am* 28, 671-681, vi (2014). https://doi.org/10.1016/j.hoc.2014.04.005
213 Cartwright, G. E. *et al.* The Anemia Associated With Chronic Infection. *Science* 103, 72-73 (1946). https://doi.org/10.1126/science.103.2664.72
214 Cartwright, G. E., Lauritsen, M. A. & et al. The anemia of infection; hypoferremia, hypercupremia, and alterations in porphyrin metabolism in patients. *J Clin Invest* 25, 65-80 (1946).

215 Dzikaite, V. *et al.* Regulatory effects of tumor necrosis factor-alpha and interleukin-6 on HAMP expression in iron loaded rat hepatocytes. *J Hepatol* 44, 544-551 (2006). https://doi.org/10.1016/j.jhep.2005.07.028
216 Nemeth, E. *et al.* IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 113, 1271-1276 (2004). https://doi.org/10.1172/JCI20945
217 Nemeth, E. & Ganz, T. Regulation of iron metabolism by hepcidin. *Annu Rev Nutr* 26, 323-342 (2006). https://doi.org/10.1146/annurev.nutr.26.061505.111303
218 Pfeifhofer-Obermair, C. *et al.* Regulation of Th1 T Cell Differentiation by Iron via Upregulation of T Cell Immunoglobulin and Mucin Containing Protein-3 (TIM-3). *Front Immunol* 12, 637809 (2021). https://doi.org/10.3389/fimmu.2021.637809
219 Kalinowski, D. S. & Richardson, D. R. Future of toxicology--iron chelators and differing modes of action and toxicity: the changing face of iron chelation therapy. *Chem Res Toxicol* 20, 715-720 (2007). https://doi.org/10.1021/tx700039c
220 Bouvard, V. *et al.* Carcinogenicity of consumption of red and processed meat. *Lancet Oncol* 16, 1599-1600 (2015). https://doi.org/10.1016/S1470-2045(15)00444-1
221 Hassan, T. H. *et al.* Impact of iron deficiency anemia on the function of the immune system in children. *Medicine (Baltimore)* 95, e5395 (2016). https://doi.org/10.1097/MD.0000000000005395
222 Lund, E. K., Wharf, S. G., Fairweather-Tait, S. J. & Johnson, I. T. Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. *Am J Clin Nutr* 69, 250-255 (1999). https://doi.org/10.1093/ajcn/69.2.250
223 Radulescu, S. *et al.* Luminal Iron Levels Govern Intestinal Tumorigenesis after Apc Loss In Vivo. *Cell Rep* 17, 2805-2807 (2016). https://doi.org/10.1016/j.celrep.2016.10.028
224 Xue, X. *et al.* Iron Uptake via DMT1 Integrates Cell Cycle with JAK-STAT3 Signaling to Promote Colorectal Tumorigenesis. *Cell Metab* 24, 447-461 (2016). https://doi.org/10.1016/j.cmet.2016.07.015
225 Sesink, A. L., Termont, D. S., Kleibeuker, J. H. & Van der Meer, R. Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Res* 59, 5704-5709 (1999).
226 Cross, A. J. & Sinha, R. Meat-related mutagens/carcinogens in the etiology of colorectal cancer. *Environ Mol Mutagen* 44, 44-55 (2004). https://doi.org/10.1002/em.20030
227 Liao, J., Seril, D. N., Yang, A. L., Lu, G. G. & Yang, G. Y. Inhibition of chronic ulcerative colitis associated adenocarcinoma development in mice by inositol compounds. *Carcinogenesis* 28, 446-454 (2007). https://doi.org/10.1093/carcin/bgl154
228 Radulescu, S. *et al.* Luminal iron levels govern intestinal tumorigenesis after Apc loss in vivo. *Cell Rep* 2, 270-282 (2012). https://doi.org/10.1016/j.celrep.2012.07.003
229 Seril, D. N., Liao, J., Yang, C. S. & Yang, G. Y. Systemic iron supplementation replenishes iron stores without enhancing colon carcinogenesis in murine models of ulcerative colitis: comparison with iron-enriched diet. *Dig Dis Sci* 50, 696-707 (2005). https://doi.org/10.1007/s10620-005-2560-6
230 Shoyab, M., Plowman, G. D., McDonald, V. L., Bradley, J. G. & Todaro, G. J. Structure and function of human amphiregulin: a member of the epidermal growth factor family. *Science* 243, 1074-1076 (1989). https://doi.org/10.1126/science.2466334
231 Ciardiello, F. *et al.* Differential expression of epidermal growth factor-related proteins in human colorectal tumors. *Proc Natl Acad Sci U S A* 88, 7792-7796 (1991). https://doi.org/10.1073/pnas.88.17.7792
232 Brown, C. L., Meise, K. S., Plowman, G. D., Coffey, R. J. & Dempsey, P. J. Cell surface ectodomain cleavage of human amphiregulin precursor is sensitive to a metalloprotease inhibitor. Release of a predominant N-glycosylated 43-kDa soluble form. *J Biol Chem* 273, 17258-17268 (1998). https://doi.org/10.1074/jbc.273.27.17258
233 Higashiyama, S., Nanba, D., Nakayama, H., Inoue, H. & Fukuda, S. Ectodomain shedding and remnant peptide signalling of EGFRs and their ligands. *J Biochem* 150, 15-22 (2011). https://doi.org/10.1093/jb/mvr068
234 Guernsey-Biddle, C. H., P.; Carmon, K.S. . Exploring the Potential of Epiregulin and Amphiregulin as Prognostic, Predictive, and Therapeutic Targets in Colorectal Cancer. . *Onco* 4, 257-274 (2024). https://doi.org/https://doi.org/10.3390/onco4040019
235 Wen, Y. *et al.* MAPK1E322K mutation increases head and neck squamous cell carcinoma sensitivity to erlotinib through enhanced secretion of amphiregulin. *Oncotarget* 7, 23300-23311 (2016). https://doi.org/10.18632/oncotarget.8188

236 Sisto, M. *et al.* RETRACTED: Blockade of TNF-alpha signaling suppresses the AREG-mediated IL-6 and IL-8 cytokines secretion induced by anti-Ro/SSA autoantibodies. *Lab Invest* (2010). https://doi.org/10.1038/labinvest.2010.168
237 Chokki, M., Mitsuhashi, H. & Kamimura, T. Metalloprotease-dependent amphiregulin release mediates tumor necrosis factor-alpha-induced IL-8 secretion in the human airway epithelial cell line NCI-H292. *Life Sci* 78, 3051-3057 (2006). https://doi.org/10.1016/j.lfs.2005.12.023
238 Liu, F. L., Wu, C. C. & Chang, D. M. TACE-dependent amphiregulin release is induced by IL-1beta and promotes cell invasion in fibroblast-like synoviocytes in rheumatoid arthritis. *Rheumatology (Oxford)* 53, 260-269 (2014). https://doi.org/10.1093/rheumatology/ket350
239 Shao, J., Lee, S. B., Guo, H., Evers, B. M. & Sheng, H. Prostaglandin E2 stimulates the growth of colon cancer cells via induction of amphiregulin. *Cancer Res* 63, 5218-5223 (2003).
240 Chayangsu, C., Khunsri, S., Sriuranpong, V. & Tanasanvimon, S. The correlations between serum amphiregulin and other clinicopathological factors in colorectal cancer. *J Gastrointest Oncol* 8, 980-984 (2017). https://doi.org/10.21037/jgo.2017.08.15
241 Xia, Y. *et al.* Investigation the mechanism of iron overload-induced colonic inflammation following ferric citrate exposure. *Ecotoxicol Environ Saf* 275, 116241 (2024). https://doi.org/10.1016/j.ecoenv.2024.116241
242 Fan, Y., Mao, R. & Yang, J. NF-kappaB and STAT3 signaling pathways collaboratively link inflammation to cancer. *Protein Cell* 4, 176-185 (2013). https://doi.org/10.1007/s13238-013-2084-3
243 Mao, H., Zhao, X. & Sun, S. C. NF-kappaB in inflammation and cancer. *Cell Mol Immunol* (2025). https://doi.org/10.1038/s41423-025-01310-w
244 Sheng, J. *et al.* The Role of Cyclooxygenase-2 in Colorectal Cancer. *Int J Med Sci* 17, 1095-1101 (2020). https://doi.org/10.7150/ijms.44439
245 Turini, M. E. & DuBois, R. N. Cyclooxygenase-2: a therapeutic target. *Annu Rev Med* 53, 35-57 (2002). https://doi.org/10.1146/annurev.med.53.082901.103952
246 Alexanian, A. & Sorokin, A. Cyclooxygenase 2: protein-protein interactions and posttranslational modifications. *Physiol Genomics* 49, 667-681 (2017). https://doi.org/10.1152/physiolgenomics.00086.2017
247 Luo, C., He, M. L. & Bohlin, L. Is COX-2 a perpetrator or a protector? Selective COX-2 inhibitors remain controversial. *Acta Pharmacol Sin* 26, 926-933 (2005). https://doi.org/10.1111/j.1745-7254.2005.00150.x
248 Rouzer, C. A. & Marnett, L. J. Cyclooxygenases: structural and functional insights. *J Lipid Res* 50 Suppl, S29-34 (2009). https://doi.org/10.1194/jlr.R800042-JLR200
249 Moon, H., White, A. C. & Borowsky, A. D. New insights into the functions of Cox-2 in skin and esophageal malignancies. *Exp Mol Med* 52, 538-547 (2020). https://doi.org/10.1038/s12276-020-0412-2
250 Tuncer, S. & Banerjee, S. Eicosanoid pathway in colorectal cancer: Recent updates. *World J Gastroenterol* 21, 11748-11766 (2015). https://doi.org/10.3748/wjg.v21.i41.11748
251 Okazaki, I. *et al.* Iron increases collagenase production by rabbit synovial fibroblasts. *J Lab Clin Med* 97, 396-402 (1981).
252 Poligone, B. & Baldwin, A. S. Positive and negative regulation of NF-kappaB by COX-2: roles of different prostaglandins. *J Biol Chem* 276, 38658-38664 (2001). https://doi.org/10.1074/jbc.M106599200
253 Carothers, A. M., Davids, J. S., Damas, B. C. & Bertagnolli, M. M. Persistent cyclooxygenase-2 inhibition downregulates NF-kappaB, resulting in chronic intestinal inflammation in the min/+ mouse model of colon tumorigenesis. *Cancer Res* 70, 4433-4442 (2010). https://doi.org/10.1158/0008-5472.CAN-09-4289
254 Gasparini, C. & Feldmann, M. NF-kappaB as a target for modulating inflammatory responses. *Curr Pharm Des* 18, 5735-5745 (2012). https://doi.org/10.2174/138161212803530763
255 Beinke, S. & Ley, S. C. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochem J* 382, 393-409 (2004). https://doi.org/10.1042/BJ20040544
256 Liu, T., Zhang, L., Joo, D. & Sun, S. C. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther* 2, 17023- (2017). https://doi.org/10.1038/sigtrans.2017.23
257 Guo, Q. *et al.* NF-kappaB in biology and targeted therapy: new insights and translational implications. *Signal Transduct Target Ther* 9, 53 (2024). https://doi.org/10.1038/s41392-024-01757-9
258 Zhang, W. & Liu, H. T. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res* 12, 9-18 (2002). https://doi.org/10.1038/sj.cr.7290105
259 Kim, E. K. & Choi, E. J. Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta* 1802, 396-405 (2010). https://doi.org/10.1016/j.bbadis.2009.12.009

260 Roux, P. P. & Blenis, J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 68, 320-344 (2004). https://doi.org/10.1128/MMBR.68.2.320-344.2004
261 Zarubin, T. & Han, J. Activation and signaling of the p38 MAP kinase pathway. *Cell Res* 15, 11-18 (2005). https://doi.org/10.1038/sj.cr.7290257
262 Munoz, P. *et al.* Effect of iron on the activation of the MAPK/ERK pathway in PC12 neuroblastoma cells. *Biol Res* 39, 189-190 (2006). https://doi.org/10.4067/s0716-97602006000100021
263 Cheng, M., Liu, P. & Xu, L. X. Iron promotes breast cancer cell migration via IL-6/JAK2/STAT3 signaling pathways in a paracrine or autocrine IL-6-rich inflammatory environment. *J Inorg Biochem* 210, 111159 (2020). https://doi.org/10.1016/j.jinorgbio.2020.111159
264 Carrier, J. C., Aghdassi, E., Jeejeebhoy, K. & Allard, J. P. Exacerbation of dextran sulfate sodium-induced colitis by dietary iron supplementation: role of NF-kappaB. *Int J Colorectal Dis* 21, 381-387 (2006). https://doi.org/10.1007/s00384-005-0011-7
265 Kishimoto, T. The biology of interleukin-6. *Blood* 74, 1-10 (1989).
266 Kishimoto, T. *et al.* The molecular biology of interleukin 6 and its receptor. *Ciba Found Symp* 167, 5-16; discussion 16-23 (1992). https://doi.org/10.1002/9780470514269.ch2
267 Kishimoto, T. Interleukin-6: discovery of a pleiotropic cytokine. *Arthritis Res Ther* 8 Suppl 2, S2 (2006). https://doi.org/10.1186/ar1916
268 Baran, P. *et al.* The balance of interleukin (IL)-6, IL-6.soluble IL-6 receptor (sIL-6R), and IL-6.sIL-6R.sgp130 complexes allows simultaneous classic and trans-signaling. *J Biol Chem* 293, 6762-6775 (2018). https://doi.org/10.1074/jbc.RA117.001163
269 Schumacher, N. *et al.* Shedding of Endogenous Interleukin-6 Receptor (IL-6R) Is Governed by A Disintegrin and Metalloproteinase (ADAM) Proteases while a Full-length IL-6R Isoform Localizes to Circulating Microvesicles. *J Biol Chem* 290, 26059-26071 (2015). https://doi.org/10.1074/jbc.M115.649509
270 Huang, B., Lang, X. & Li, X. The role of IL-6/JAK2/STAT3 signaling pathway in cancers. *Front Oncol* 12, 1023177 (2022). https://doi.org/10.3389/fonc.2022.1023177
271 Fiebelkow, J. *et al.* The tyrosine phosphatase SHP2 increases robustness and information transfer within IL-6-induced JAK/STAT signalling. *Cell Commun Signal* 19, 94 (2021). https://doi.org/10.1186/s12964-021-00770-7
272 Wang, Y. & Fuller, G. M. Phosphorylation and internalization of gp130 occur after IL-6 activation of Jak2 kinase in hepatocytes. *Mol Biol Cell* 5, 819-828 (1994). https://doi.org/10.1091/mbc.5.7.819
273 Yuan, J., Zhang, F. & Niu, R. Multiple regulation pathways and pivotal biological functions of STAT3 in cancer. *Sci Rep* 5, 17663 (2015). https://doi.org/10.1038/srep17663
274 Xie, Q. *et al.* IL-6 signaling accelerates iron overload by upregulating DMT1 in endothelial cells to promote aortic dissection. *Int J Biol Sci* 20, 4222-4237 (2024). https://doi.org/10.7150/ijbs.99511
275 Tsuji, Y. Transmembrane protein western blotting: Impact of sample preparation on detection of SLC11A2 (DMT1) and SLC40A1 (ferroportin). *PLoS One* 15, e0235563 (2020). https://doi.org/10.1371/journal.pone.0235563
276 Tarczykowska, A., Engstrom, N., Dobermann, D., Powell, J. & Scheers, N. Differential Effects of Iron Chelates vs. Iron Salts on Induction of Pro-Oncogenic Amphiregulin and Pro-Inflammatory COX-2 in Human Intestinal Adenocarcinoma Cell Lines. *Int J Mol Sci* 24 (2023). https://doi.org/10.3390/ijms24065507
277 Xue, X. & Shah, Y. M. Intestinal iron homeostasis and colon tumorigenesis. *Nutrients* 5, 2333-2351 (2013). https://doi.org/10.3390/nu5072333
278 Fonseca-Nunes, A., Jakszyn, P. & Agudo, A. Iron and cancer risk--a systematic review and meta-analysis of the epidemiological evidence. *Cancer Epidemiol Biomarkers Prev* 23, 12-31 (2014). https://doi.org/10.1158/1055-9965.EPI-13-0733
279 Nelson, R. L. Iron and colorectal cancer risk: human studies. *Nutr Rev* 59, 140-148 (2001). https://doi.org/10.1111/j.1753-4887.2001.tb07002.x
280 Lawson, D. M. *et al.* Identification of the ferroxidase centre in ferritin. *FEBS Lett* 254, 207-210 (1989). https://doi.org/10.1016/0014-5793(89)81040-3
281 Mergler B., R. E., Bruggraber S., Powell J.J., Pereira D. Development of the Caco-2 Model for Assessment of Iron Absorption and Utilisation at Supplemental Levels. *Journal of Pharmacy and Nutrition Sciences* 2, 27-34 (2012).
282 Dressman, J. B. Comparison of canine and human gastrointestinal physiology. *Pharm Res* 3, 123-131 (1986). https://doi.org/10.1023/A:1016353705970

283 Jyothi, G. S. *et al.* A clinical study evaluating low dose ferrous fumarate vs. standard iron supplements in iron-deficient non-anemic to mild anemic adults. *Sci Rep* 14, 15674 (2024). https://doi.org/10.1038/s41598-024-65878-5

284 Badran, O., Cohen, I. & Bar-Sela, G. The Impact of Iron on Cancer-Related Immune Functions in Oncology: Molecular Mechanisms and Clinical Evidence. *Cancers (Basel)* 16 (2024). https://doi.org/10.3390/cancers16244156

285 Hochrainer, K., Racchumi, G. & Anrather, J. Site-specific phosphorylation of the p65 protein subunit mediates selective gene expression by differential NF-kappaB and RNA polymerase II promoter recruitment. *J Biol Chem* 288, 285-293 (2013). https://doi.org/10.1074/jbc.M112.385625

286 Yilmaz, B. & Li, H. Gut Microbiota and Iron: The Crucial Actors in Health and Disease. *Pharmaceuticals (Basel)* 11 (2018). https://doi.org/10.3390/ph11040098