Role of polyphenols in iron homeostasis

thesis submitted by

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Declaration

"I, **Marija Lesjak**, declare that the all work presented in this thesis is the result of my own work. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. The work herein was carried out while I was a graduate student at University College London, Research Department of Structural and Molecular Biology under the supervision of Professor Kaila Srai"

Abstract

Balancing systemic iron levels within narrow limits is critical for human health, as both iron deficiency and overload lead to serious disorders. There are no known physiologically controlled pathways to eliminate iron from the body and therefore iron homeostasis is maintained by modifying dietary iron absorption. Several dietary factors, such as polyphenols, are known to greatly affect iron absorption. Furthermore, it is proposed that polyphenols can affect iron status by regulating expression and activity of proteins involved in either the systemic regulation of iron metabolism or iron absorption. To reveal how polyphenols affect iron metabolism, experiments which included intraperitoneal (IP) or forced feeding (gavage) treatment of Sprague Dawley rats with quercetin, polyphenol which is plentiful in the diet, were performed. These treatments were followed by the evaluation of iron-related genes and iron content in duodenum, liver, spleen, kidney and serum. Results revealed that quercetin treatment, IP or gavage, provoked iron deficiency. Oral treatment mainly affected iron absorption, mostly by changing the expression of iron transporters. Additionally, with *in vivo* uptake studies it was shown that quercetin reduces duodenal iron uptake by direct chelation of iron consequently increasing apical iron uptake and decreasing basolateral iron release from enterocyte. IP treatment mainly affected systemic iron homeostasis, mainly through up-regulation of hepcidin expression in liver and kidney. Additionally, in *in vitro* studies quercetin metabolites and other polyphenols showed a notable effect on hepcidin expression in human liver HepG2 cells, as well as on inflammatory and iron-related genes in THP1 cells. Results showed that polyphenols have multiple effects on iron homeostasis. Thus, polyphenols may have important consequences for conditions that are low in iron such as anaemia. Alternatively, polyphenols have therapeutic potential for iron overload diseases, potentially as a part of chelato-therapy.

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Abbreviations

ARE	antioxidant responsive element
ACD	anaemia of chronic disease
AI	anaemia of inflammation
apo–Tf	apo-transferrin
BMP	bone morphogenetic protein
cDNA	complementary DNA
COX–2	cyclooxygenase–2
Cp	ceruloplasmin
Ct	cycle threshold
DAHP	2–dehydro–3–deoxyarabinoheptulosonate–7–phosphate
Dcytb	duodenal cytohrome b
DEPC	diethyl pyrocarbonate
DFO	deferoxamine
DFP	deferiprone
DFX	deferasirox
DMSO	dimethyl sulfoxide
DMT1	divalent metal transporter 1
dw	dry weight
Epo	erythropoietin
FBS	foetal bovine serum
Fe–S protein	iron-sulphur protein
FPN	ferroportin
GAPDH	glyceraldehyde 3–phosphate dehydrogenase
GLUT	glucose transporter
GST	glutathione S–transferase
H	heavy
HAMP	gene encoding hepcidin
HCP1	haem carrier protein 1
Heph	hephaestin
HFE	haemochromatosis protein
HH	haemochromatosis
Hif	hypoxia-inducible factor
HJV	hemojuvelin
HO–1	haem oxygenase
holo–Tf	holo-transferrin
HRE	hypoxia-response elements
IDA	iron deficiency anaemia
IL	interleukin
iNOS	inducible nitric oxide synthase
IP	intraperitoneal
IRE	iron-responsive element
IRIDA	iron-refractory iron deficiency anaemia
IRP	iron-regulatory protein
JHH	juvenile haemochromatosis
Keap1	kelch-like ECH-associated protein 1
L	light
LPH	lactase phlorizin hydrolase
LPS	lipopolysaccharide
MDCK	Madin–Darby canine kidney

Nrf2	nuclear factor erythroid 2-related factor
PAL	phenylalanine ammonia–lyase
PBS	phosphate buffered saline
PEP	phosphoenolpyruvate
PHD	prolyl hydroxylase
PMA	phorbol 12–myristate 13–acetate
QR	quinone reductase
RNase	ribonuclease
ROS	reactive oxygen species
SD	Sprague Dawley
SEM	standard error of the mean
SGLT1	sodium-dependent glucose transporter
sla	sex-linked anaemia
Tf	transferrin
TfR	transferrin receptor
TIBC	total iron binding capacity
TMPRSS6	transmembrane protease, serine 6
TNF–α	tumour necrosis factor–α
UIBC	unsaturated iron binding capacity
UTR	untranslated region

1. INTRODUCTION

1.1 Biological importance of iron

Studying chemistry of iron in detail, it is easy to see why iron is essential for life. Namely, under physiological conditions, iron is mainly present in two forms, ferrous (Fe^{2+}) and ferric (Fe^{3+}). The Fe^{2+}/Fe^{3+} system facilitates variety of redox potentials that can be fine adjusted by different ligands (from about -0.5 V to about +0.6 V), which almost entirely corresponds to the redox potential range of utmost importance for biological systems. That is why iron complexes are uniquely suitable for a variety of catalytic processes and reactions which are of great biological significance, such as electron transfer and acid-base reactions (Crichton, 2001; Lieu et al., 2001).

Iron carries out a variety of significant roles in biological systems, mostly as a part of iron-containing proteins. Haemoproteins are a large group of iron-containing proteins where the iron is bound to a porphyrin molecule (haem) which is bound to the different proteins with diverse functions. There are three main categories of haemoproteins: oxygen carriers (haemoglobins, myoglobins and neuroglobins), activators of molecular oxygen (cytochrome oxidase, cytochrome P450s, catalases and peroxidases) and electron transport proteins (cytochromes; Yehuda and Mostofsky, 2010; Ying–Wu and Jiangyun, 2013).

The second group of iron-containing proteins is the iron-sulphur proteins (Fe–S proteins), where iron is bound to sulphur by thiol groups from cysteine or inorganic sulphide. Fe–S proteins are widespread in all living organisms and express numerous actions. Namely, they are included in redox and non-redox reactions as part of

different enzymes, like succinate dehydrogenase and aconitase, and proteins involved in the electron transfer chain (Crichton, 2001; Lill and Muhlenhoff, 2006).

The third class of iron-containing proteins presents a diverse group of proteins that do not contain iron in a haem or Fe–S form. One group is mononuclear non-haem iron enzymes, which include lipoxygenases, aromatic amino-acid hydroxylases, prolyl and lysyl hydroxylases, etc. Additionally, there is the dinuclear non-haem iron protein group, consisting of ribonucleotide reductase and ferritins or proteins involved in iron transport, such as transferrins (Crichton, 2001).

Summing activities of the above mentioned proteins, it is apparent that iron is crucial for many important processes, such as: oxygen transport and storage, cellular respiration and energy production, the electron transport chain of mitochondria, synthesis of DNA, RNA and proteins, regulation of gene expression, cell proliferation and differentiation. In addition, iron is indispensable for normal brain function, psychomotor development and cognitive performance (especially in infants), endurance and physical performance, the inflammatory response (iron deficiency greatly reduces resistance to infection), pregnancy (40% of all maternal prenatal deaths are linked to anaemia), thyroid function, production and metabolism of catecholamines and other neurotransmitters, drug metabolism, etc. Hence it is evident that nearly every cell and organism require iron for life (World Health Organization, 2001; Dunn et al, 2007; Sharp and Srai, 2007; Yehuda and Mostofsky, 2010).

On the other hand the property of iron to easily change its oxidative stage can also be toxic, mainly due to its ability to produce free radicals when it is not bound by

proteins and is free in a labile iron pool. Iron takes part in a reaction, known as the Fenton reaction, where the hydroxyl radical ($HO^{\bullet}$) is the end product. $HO^{\bullet}$ is the most toxic reactive oxygen species (ROS) which can damage all classes of biomolecules. Consequently, unrestrained production of $HO^{\bullet}$ leads to cell injuries and death and gives rise to numerous severe pathological states (Halliwell and Gutteridge, 2007).

The Fenton reaction initiates the chain reaction (equation 1), which is then followed by the reactions (equations 2 and 3) in which more and more $HO^{\bullet}$ is produced (Koppenol, 2001).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-} \qquad \text{equation 1}$$

$$HO^{\bullet} + H_2O_2 \rightarrow H_2O + O_2^{\bullet-} + H^{+} \qquad \text{equation 2}$$

$$O_2^{\bullet-} + H^{+} + H_2O_2 \rightarrow O_2 + HO^{\bullet} + H_2O \qquad \text{equation 3}$$

Thus, balancing systemic iron levels within narrow limits in an organism is crucial, as both iron deficiency and iron overload lead to serious haematological, metabolic and neurodegenerative disorders, which belong to the most frequent disorders worldwide, as well as carcinogenesis (Hentze et al., 2004).

1.2 Distribution and homeostasis of body iron

The total iron content of the adult human organism is estimated around 4 g (~ 35 mg/kg woman, ~ 45 mg/kg for men). About 52% of total body iron is found as part of haemoglobin in circulating erythrocytes or erythrocyte precursors, 40% in complex with ferritin or hemosiderin as intracellular pool (liver and reticulo-endothelial macrophages), 7.5% in muscle as part of myoglobin, 0.5% as part of the catalytic centre of a variety of enzymes (cytochromes, catalase, peroxidases,

flavoproteins, etc.) and 0.1% as transferrin-bound iron in the circulation (see Figure 1–1; Andrews, 1999; Lieu et al., 2001).

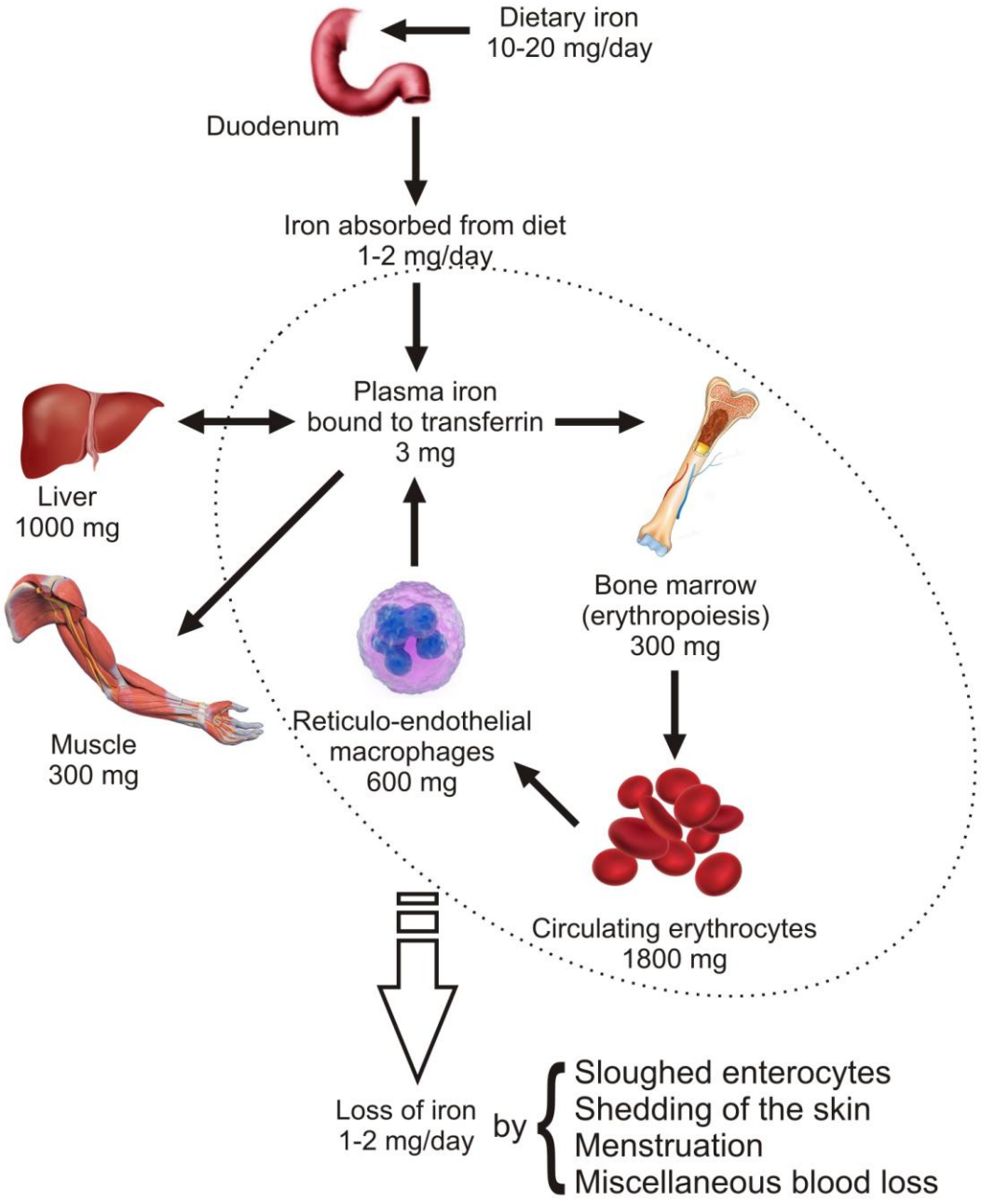


Figure 1–1 Distribution of body iron
The adult human body have approximately 4 g of iron, with more than half (>2 g) incorporated in the haemoglobin of developing erythroid precursors (300 mg) and mature circulating erytrocytes (1800 mg). Remaining body iron is found in a transit pool in reticulo-endothelial macrophages (600 mg) or stored in hepatocytes (1000 mg) complexed with ferritin. A smaller part is present in muscles within myoglobin (300 mg), while only a minor amount is present in plasma bound to transferin (3 mg) or incorporated in other proteins and enzymes that include iron in their structures. Approximately, 10–20 mg of iron is daily consumed by diet, from which only 1–2 mg is absorbed. The same amount is lost every day by blood loss of different etiology, shedding of the skin and sloughed enterocytes.

Body iron homeostasis is maintained by regulating the iron levels in plasma (transferrin-bound iron), which is determined by four coordinated processes: duodenal iron absorption, macrophage iron recycling, hepatic iron storage and erythropoiesis. Erythropoiesis, the production of red blood cells in bone marrow, requires nearly 30 mg iron each day, the main part of which comes from the recycling of iron via reticulo-endothelial macrophages (> 28 mg/day). Macrophages ingest old or damaged erythrocytes, process them and release recycled iron to plasma transferrin. The pool of transferrin-bound iron (~ 3 mg) is very dynamic and undergoes recycling more than 10 times daily. Furthermore, when in balance, each day the body absorbs 1–2 mg of iron by duodenal enterocytes and at the same time loses 1–2 mg of iron by nonspecific iron losses, such as exfoliation of enterocyte, skin and hair loss, menstruation and some gastrointestinal blood loss (see Figure 1–1). Bearing in mind that there is no known physiologic mechanism for controlling iron excretion and that macrophage-mediated iron recycling cannot be sufficient for maintaining erythropoiesis over the long term, absorption of dietary iron in duodenum is of great importance in keeping iron homeostasis in balance (Papanikolaoua and Pantopoulos, 2005; Beaumont and Delaby, 2009).

1.3 Mechanism of dietary iron uptake

Nutritional iron absorption occurs primarily in the duodenum, on the apical (luminal) membrane of the enterocytes, and is tightly regulated by bioavailable iron, iron stores, erythropoietic drive and inflammation. The average diet daily contains about 10–20 mg of iron from which only 1–2 mg is absorbed. There are two types of

dietary iron: non-haem iron, which is present in food from both animal or plant origin, and haem iron, which is present only in food of animal origin.

Absorption of non-haem iron in the intestine comprises the following (Srai et al., 2002; see Figure 1–2):

1. *Reduction of Fe^{3+} and uptake of Fe^{2+} from the diet through the apical membrane of enterocytes.* In the diet iron is mainly present as Fe^{3+}. However, the absorption of Fe^{2+} is more efficient than Fe^{3+}. In order to increase Fe^{3+} bioavailability, Fe^{3+} firstly needs to be reduced. Duodenal cytohrome b (Dcytb) is an iron-regulated ferric reductase, highly expressed on the apical membrane of duodenal enterocytes (described in detail in Section 1.3.1.1; McKie et al., 2001). After being reduced by Dcytb, Fe^{2+} is transported across the apical membrane by the divalent metal transporter 1 (DMT1; described in detail in Section 1.3.1.2; Gunshin et al., 1997).

2. *Intracellular processing of iron and iron transport to the basolateral membrane of enterocytes.* There is not much information about the mechanism of intracellular iron transport. However, it is supposed that it is closely related to vesicular transport. The fate of absorbed iron is closely related to the body's demands for iron. If there is a need for more iron, then iron is exported from the cell via the basolateral membrane of enterocytes which is followed by iron binding to transferrin (Tf; described in detail in Section 1.3.1.6) and transport to peripheral tissues that require iron. If there is no need for additional iron in the body, iron is stored in the cell in the form of ferritin (described in detail in Section

1.3.1.3), the main iron storage protein, and returned to the lumen at a time when the villus enterocytes die (Sharp and Srai, 2007).

3. *Transfer of iron through the basolateral membrane to the circulation.* The mechanism of Fe^{2+} transport through the basolateral membrane includes synchronized activity of two proteins, ferroportin (FPN; described in detail in Section 1.3.1.4; Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000) and transmembrane copper-dependent ferroxidase, hephaestin (Heph; described in detail in Section 1.3.1.5; Vulpe et al., 1999; Chen et al., 2004). Before entering the circulation, Fe^{2+} firstly needs to be oxidized to the Fe^{3+} state, which is catalyzed by hephaestin, the intestinal ferroxidase. Fe^{3+} then binds to the serum glycoprotein Tf (described in detail in Section 1.3.1.6; MacGillivray et al., 1983), the key iron transporting protein in the serum and extracellular fluids.

The uptake mechanism for non-haem and haem iron differs across the apical membrane of the enterocyte, while it follows the same pathway once iron is inside the cell (see Figure 1–2). Even though the mechanism of haem absorption is not fully characterized, haem carrier protein 1 (HCP1) was identified as protein for haem uptake on the apical membrane of duodenal enterocytes (Shayeghi et al., 2005). Once inside the cell, haem is degraded by haem oxygenase (HO–1; Tenhunen et al., 1969) and the released iron enters an intracellular iron pool. After that, absorbed iron from the haem source follows the pathway of absorbed non-haem iron.

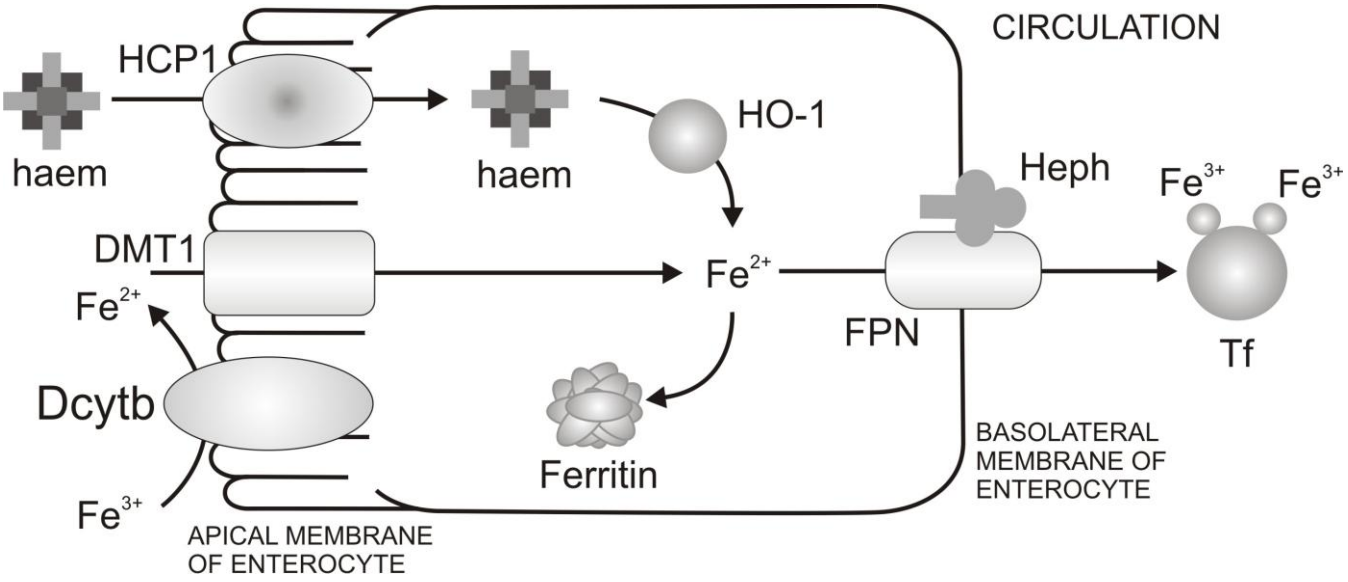


Figure 1–2 Mechanism of non-haem and haem iron absorption in duodenal cells
Non-haem iron from food is firstly reduced by the ferric reductase Dcytb yielding Fe^{2+}, which afterwards enters the enterocytes via DMT1. On the other hand, haem is absorbed via HCP1, subsequently broken down by HO–1, after which free Fe^{2+} from haem joins a common cell iron pool with iron from the non-haem source. If body iron stores are high, iron may be stored in the cell complexed with ferritin as Fe^{3+} and eventually lost when the cell is discarded from the intestinal villus tip. Otherwise, iron efflux into the circulation via FPN, subsequently being re-oxidised through Heph to enable loading into Tf, after which it is transferred to peripheral tissues that require iron.

1.3.1 Proteins involved in iron absorption and transport

1.3.1.1 Duodenal cytochrome B (DcytB)

Dcytb (or Cybrd1) is an iron-regulated protein with ferric reductase activity, highly expressed on the human duodenal apical membrane next to DMT1. The primary role of Dcytb is to reduce non-haem iron (Fe^{3+} to Fe^{2+}) prior to its transport inside the cell via DMT1 (see Figure 1–2; McKie, 2008). The structure of the Dcytb protein consists of 286 amino acids with six transmembrane domains and was revealed using a subtractive cloning strategy by McKie et al. in 2001. Furthermore, it has been

shown *in vitro* that Dcytb expresses dual, ferric and cupric reductase activity (Wyman et al., 2008).

Dcytb mRNA and protein levels are up-regulated by hypoxia (described in detail in Section 1.4.3) and iron deficiency, which strongly supports its role in iron uptake and metabolism (Zoller et al., 2003; Wyman et al., 2008). Recently it has been shown that the transcriptional modulation of Dcytb expression can be regulated by transcriptional factor hypoxia-inducible factor (Hif) –2α (Shah et al., 2009). Specifically, the activity of enzymes that modulate Hif–2α ubiquitination is supported by ascorbate, Fe^{2+} and O_2. Thus, low ascorbic acid levels, iron deficiency and hypoxia, hold back Hif–2α ubiquitination, enhance Hif–2α levels and support Dcytb expression. In addition, ascorbic acid has been recognized as a Dcytb helper by supplying electrons for Fe^{3+} reduction, and thus has a dual role in regulation of Dcytb activity (Oakhill et al., 2008; Luo et al., 2014).

Interestingly, in contrast to other genes important for iron uptake, such as DMT1 and FPN, Dcytb transcript lacks an iron-responsive element (IRE), one of the key players in the control of iron metabolism (described in detail in 1.4.1).

Moreover, it was shown that over-expression of Dcytb in Caco–2 or MDCK (Madin–Darby canine kidney) cells, significantly increased iron absorption, clearly indicating that Dcytb plays an important role in iron transport (Latunde–Dada et al., 2008; Wyman et al., 2008). Also, it was shown that Dcytb is increased in the duodenum of iron-deficient patients, which is associated with a desirable increase of iron absorption. On the other side, it was shown that in iron-overload patients, Dcytb levels in the duodenum are decreased (Zoller et al., 2003). However, the essential

role of Dcytb in iron uptake in humans was the subject of uncertainty. Namely, there are no reports on a direct connection between mutations in human Dcytb and iron metabolism disorders. Additionally, it was shown that there was no significant disturbance of iron homeostasis in Dcytb knockout mice. Still, absence of iron deficiency in humans with mutated Dcytb and Dcytb knockout mice could be explained by the presence of other reductase enzymes in the gut and substrates that could reduce iron, such as ascorbic acid (Gunshin et al., 2005). However, to date Dcytb still remains the only known reductase in the human duodenum that is regulated by increased physiological demands for iron.

1.3.1.2 Divalent metal transporter 1 (DMT1)

DMT1 (Nramp2 or DCT1) is a divalent metal protein transporter located on the apical membrane of enterocytes which, after reduction of iron by Dcytb, transports Fe^{2+} inside the cell (see Figure 1–2). DMT1 was first identified in 1995 (Gruenheid et al., 1995), but its activity as an iron transporter was revealed subsequently when a DMT1 mRNA construct was transfected into *Xenopus* oocytes and iron uptake activity was observed (Gunshin et al., 1997). It is believed that DMT1 acts as proton symporter, transporting one H^+ for each Fe^{2+}, and therefore iron uptake is supported by the mildly acidic environment of the duodenum (Gunshin et al., 1997). Moreover, DMT1 is confirmed to have the ability to transport other divalent metals, such as Mn^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Pb^{2+} (Garrick et al., 2003).

The importance of DMT1 in iron uptake was highlighted by study with DMT1 gene mutations in two animal models, microcytic anaemia mice and Belgrade rats (Fleming et al. 1997; Fleming et al., 1998). Both models showed a severe iron

deficiency phenotype due to impaired iron uptake. Moreover, humans with DMT1 mutations exhibited hypochromic microcytic anemia, which undoubtedly indicates a significant role for DMT1 in iron homeostasis (Mims et al., 2005; Lam–Yuk–Tseung et al., 2006).

Humans have two types of DMT1 proteins, with 561 and 570 amino acids, having 12 transmembrane domains. Two variants of mRNA differ at their C-terminus by the presence or absence of a specific IRE sequence on the 3' untranslated region (UTR). Thus, +IRE and -IRE DMT1 types are controlled by both the intracellular iron pool or an IRE-independent mode of metal regulation, respectively (described in detail in Section 1.4.1; Gunshin et al., 1997; Fleming et al., 1998; Lee et al., 1998; Au et al., 2008). However, it was shown *in vitro* in an intestinal Caco–2 cell line that hepcidin, a main controller of systemic iron homeostasis, is a negative regulator of DMT1 expression of both RNA and protein levels (Yamaji et al., 2004; Mena et al., 2008). Recently it was revealed that duodenal DMT1 is down-regulated by hepcidin via proteasome internalization and degradation, similar to FPN in macrophages (described in detail in Sections 1.3.1.4 and 1.4.2; Brasse–Lagnel et al., 2011). Hepcidin is a small, liver-secreted hormone which plays a central role in iron metabolism by inhibiting iron absorption in the duodenum, liberation of iron from macrophages after recycling of senescent erythrocytes and iron which is stored in hepatocytes (described in detail in Section 1.4.2). Additionally, it was recently shown that Hif–2α, but not Hif–1α, induces DMT1 transcription, in the same manner as Dcytb, which is evidence that DMT1 levels are also controlled by hypoxia (described in detail in Section 1.4.3; Mastrogiannaki et al., 2009).

1.3.1.3 Ferritin

Ferritin is the main intracellular iron storage protein present in almost all living organisms and expressed in numerous cell types. Ferritin was discovered more than 70 years (1937) by the French scientist Laufberger who crystallized ferritin from horse spleen (Laufberger, 1937). Inside the cell (e.g. the enterocyte) surplus iron is stored by ferritin in a redox inactive form, thus preventing formation of new free radicals, which can be highly toxic for the cell. However, under conditions of iron demand in the cell or organism, iron can be released from ferritin. In other words, in humans ferritin acts as a buffer against cell iron deficiency and iron overload.

Ferritin is in the form of a shell with a cavity (a.k.a. nanocage structure) where up to 4500 iron atoms can be loaded in a form of ferric oxide, Fe_2O_3 (Theil, 2011). It comprises 24 subunits of two types: H (heavy; 21 kDa) and L (light; 19 kDa) which combine in different ratios with a tissue-specific distribution. Prior to iron storage, oxidation of Fe^{2+} to Fe^{3+} occurs in the ferritin H subunit (Arosio et al., 2009; Bou–Abdallah, 2010).

The ferritin structure is highly conserved in bacteria, plants and animals and follows the same pattern of expression regulation: iron availability and oxidative stress. In mammals, post-transcriptional regulation is based on the presence of an IRE sequence on both H and L ferritin mRNA 5'UTRs, which facilitate up-regulation of ferritin synthesis when iron is present in excess. In contrast, during iron deficiency ferittin synthesis is blocked in order to increase iron bioavailability (described in detail in Section 1.4.1; Theil, 2007). Oxidative stress regulates levels of ferritin at the transcriptional level by an upstream antioxidant responsive element (ARE) on ferritin

genes. Both nuclear factor erythroid 2-related factor (Nrf2) and JunD transcriptional factors are involved in the transcriptional activation of H and L ferritin via ARE after oxidative stress, therefore protecting cells from oxidative stress induced by higher levels of iron. Moreover, the Nrf2 pathway is enhanced by haem through which haem also up-regulates ferritin levels on transcription level (described in detail in Section 1.4.3; Hintze et al., 2007). Additionally, ferritin levels are up-regulated by the cytokines tumour necrosis factor-α (TNF–α) and interleukin (IL)–6 and inflammation (Torti and Torti, 2002).

1.3.1.4 Ferroportin (FPN)

FPN is the single known mammalian iron exporter which facilitates efflux of iron to the circulation through the basolateral membrane of the enterocyte together with the coordinated action of a ferrioxidase Heph (see Figure 1–2). Besides, being essential for iron absorption from the diet, FPN is the crucial iron exporter in different cell types, such as hepatocytes and macrophages, thus takes part in the distribution of iron between tissues (Ward and Kaplan, 2012). FPN is a protein comprising 571 amino acids with a mass of 62 kD, whose discovery was made at the same time by three independent groups, using three different methods, and thus was given three different names: metal transporter 1, iron-regulated protein 1 and FPN1 (Abboud and Haile 2000, McKie et al., 2000, Donovan et al., 2000, respectively). In this work, the name ferroportin or its abbreviation FPN will be used.

The mechanism of iron transport via FPN is not fully understood. However, it is considered that Fe^{2+} is substrate for FPN, because FPN needs additional ferroxidase activity in order to provide iron for Tf (Ward and Kaplan, 2012).

The pivotal role of FPN in iron homeostasis has been extensively documented showing that the absence of FPN provokes disruption of iron homeostasis. An experiment with mice, where removal of FPN was done *in utero*, resulted in viable but underdeveloped mice with severe iron deficiency. Specifically, selective inactivation of FPN in the duodenum resulted in low iron concentrations right after the birth, demonstrating that FPN is of great importance for iron absorption. Also, general inactivation of FPN in an organism revealed iron overload in the liver, spleen and duodenum, provoking anaemia and pointing out that FPN is a major iron exporter in the body (Donovan et al., 2005). The connection between mutations in FPN and loss of its function and subsequently iron retention in Kupffer cells and macrophages in humans was first identified by Montosi et al. 2001, and was named "FPN disease". Nowadays, this disorder is classified as most common non-haemochromatosis protein (HFE) haemochromatosis (HH), which belongs to the group of hereditary iron loading disorders associated with the reduction in levels of the peptide hormone hepcidin (described in detail in 1.4.2 and 1.4.4; Pietrangelo, 2010; Pietrangelo et al., 2011).

FPN levels are regulated by a number of mechanisms. Like Dcytb and DMT1, FPN transcription is also supported by the presence of Hif–2α induced by hypoxia (described in detail in Section 1.4.3; Mastrogiannaki et al., 2009). Also, like ferritin, the FPN gene contains an ARE promoter and its transcription is controlled by oxidative stress through the Nrf2 pathway. Similarly, transcription of FPN is demonstrated to be up-regulated by iron and haem and it is considered that these paths of regulation include Nrf2 transcriptional factor (described in detail in Section 1.4.3; Marro et al., 2010; Ward and Kaplan, 2012). In addition, transcription of FPN is proven to be up-regulated by other metals (e.g. Zn and Cd; Troadec et al., 2010).

At the transcriptional level FPN is down-regulated by inflammation induced by lipopolysaccharide (LPS; Ludwiczek et al., 2003; Harada et al., 2011).

Post-transcriptionaly, FPN is regulated by the IRE/IRP (iron-regulatory protein) system (described in detail in Section 1.4.1). mRNA of FPN has an IRE sequence at its 5'UTR, by which translation of FPN is inhibited by low levels of iron and increased by high levels of iron in the cell (Hentze et al., 2004).

Post-translationally, FPN can be regulated by two known mechanisms. Firstly, FPN levels can be regulated by the hepatic hormone hepcidin. This mechanism was elucidated by Nemeth et al. 2004b, who demonstrated that hepcidin attached to FPN on the cell membrane induced its internalization (described in detail in Section 1.4.2). Additionally, post-translational internalization of FPN can be driven by a hepcidin-independent mechanism. Specifically, FPN is degraded in the absence of the multicopper oxidase ceruloplasmin (Cp), while it is stable when Cp is present (Jeong and David, 2003).

1.3.1.5 Hephaestin (Heph)

Heph is a membrane-bound copper-dependent ferroxidase that takes part in iron export from the intestinal epithelium to the circulation, where it binds to Tf, in synchronized activity with the FPN transporter (see Figure 1–2). It is believed that Heph facilitates iron trafficking by oxidizing the soluble Fe^{2+} into the Fe^{3+}, prior to its release by FPN. The role of Heph in iron export from enterocytes to the circulation was established by experiments with the sex-linked anaemia (*sla*) mouse, where iron export was inhibited leading to intestinal iron accumulation and systemic iron deficiency. The *sla* mouse contains a mutation that generates Heph with

impaired structure and thus can take up iron normally from the diet into enterocytes, but is unable to facilitate iron export (Vulpe et al., 1999; Chen et al., 2004).

Heph is 50% identical with Cp, the serum multi-copper ferroxidase, which assists in iron export from various tissues. Both ferroxidases contain iron- and copper-binding sites, have similar functions but different locations. Namely, Heph is mainly expressed in the small intestine and anchored to the membrane where it plays an important role in the uptake of iron from the diet. Cp is soluble plasma protein, mainly expressed in liver and has a role in the redistribution of iron from the liver and other internal organs (Petrak and Vyoral, 2005; Chen et al., 2006).

In contrast to most proteins involved in iron homeostasis, Heph activity is neither regulated via the IRE/IRP system nor hepcidin (described in detail in Sections 1.4.1 and 1.4.2). Also, it is recognized that Heph expression is more responsive to systemic iron levels than to iron levels in enterocytes. In iron-deficient conditions, Heph mRNA levels increase, whereas in contrast, high iron concentration causes a decrease in Heph mRNA *in vivo* (Sakakibara and Aoyama, 2002; Chen et al., 2003). Specifically, a down-regulation of Heph expression was proven in the duodenum of patients with hereditary HH (Stuart et al., 2003), while mRNA and protein levels are up-regulated by iron deficiency, which strongly supports its role in iron uptake and metabolism (Zoller et al., 2003). Also, reduced interactions between FPN and Heph in rats after iron ingestion indicate that this is a regulatory mechanism for limiting further iron absorption (Yeh et al., 2011). Still, by now, no human disease has been associated with a Heph mutation (Petrak and Vyoral, 2005).

Also Heph requires copper for its structural stability and enzymatic activity and consequently copper levels affect Heph activity. To be exact, copper deficiency leads to a marked decrease in Heph expression and ferroxidase activity (Nittis and Gitlin, 2004), while it was observed when copper is abundant an increase in Heph mRNA occurs (Han and Wessling–Resnick, 2002).

1.3.1.6 Transferrin (Tf)

Tf is monomeric non-haem iron-binding glycoprotein. Its main function is to transport iron safely around the body from site of iron absorption to places of iron storage and usage. One molecule of plasma Tf can bind reversibly two atoms of Fe^{3+} with high affinity (Aisen et al., 1978). Chelation of iron by Tf has a few functions: it maintains Fe^{3+} in a soluble form under physiologic conditions, it maintains Fe^{3+} in a redox-inert state preventing the generation of toxic free radicals, it facilitates regulated iron transport and cellular uptake and it has an indirect defensive role against infections by making iron unavailable to potential pathogens which require iron for growth (Brock et al., 1987; Gkouvatsos et al., 2012). When not bound to iron, Tf is known as apo-transferrin (apo–Tf), while holo-transferrin (holo–Tf) is the iron-saturated form of Tf. Tf is mainly synthesized in liver (Zakin, 1992), while it is present in different body fluids, such as plasma, bile, lymph, cerebrospinal fluid, breast milk and amniotic fluid (Qian et al., 2002). The concentration of Tf in plasma ranges from 2 g/L to 3 g/L, and the *in vivo* half-life of the Tf is eight days (Gomme et al., 2005).

At any time plasma Tf binds less than 0.1% of total body iron (3 mg; see Figure 1–1). Two millions of new erythrocytes are produced every second by the bone

marrow, which requires a daily supply around 30 mg of iron. In order to meet the needs of erytrocyte production, plasma Tf turns over more than 10 times a day (Cavill, 2002). Because iron absorbed from diet is limited, the main source of plasma iron is the reticulo-endothelial macrophages: senescent erythrocytes are phagocytized by macrophages in the spleen, liver and bone marrow. Macrophages degrade haemoglobin, liberate Fe^{2+} and export it through FPN by synchronized iron re-oxidation to Fe^{3+} by Cp, and load it to Tf, if the body requires extra iron (Wang and Pantopoulos, 2011). Tf saturation reflects body iron stores and the balance between reticulo-endothelial iron release and bone marrow uptake. Around 30% of the Tf iron-binding sites are saturated under normal conditions. This partial saturation of Tf presents a shield for preventing iron toxicity, because it makes it possible to tolerate a sudden change of plasma iron levels. In humans, Tf saturation less than 15% indicates iron deficiency, whereas 45% and more is sign for iron overload (Hentze and Muckenthaler, 2010).

The mechanism of cellular uptake of Tf-bound iron from plasma involves diferric Tf binding to transferrin receptor (TfR) 1 and endocytosis of the complex Tf/TfR1. After releasing Fe^{3+} from Tf into the endosome, iron is reduced and transported to the cytosol via DMT1. Consequently, apo–Tf is exported back to the circulation, while TfR1 is recycled on to the cell surface ready for another iron uptake cycle. TfR1 is expressed in many cell types, such as developing erythroid cells, hepatocytes and placental syncytiotrophoblasts (Hémadi et al., 2004).

Interestingly, holo–Tf expresses a significant regulatory function in the expression of hepcidin, a systemic regulator of iron homeostasis (described in detail in Section

1.4.2). Namely, the increase in Tf saturation (i.e. increase in holo–Tf concentration), but not apo–Tf provokes an increase in hepcidin excretion (Lin et al., 2007).

Similarly to DMT1, TfR1 mRNA has an IRE on its 3'UTR, and thus is post-transcriptionaly regulated by the IRE/IRP system (described in detail in Section 1.4.1). Therefore in iron deficiency, IRE/IRP interactions stabilize TfR1 mRNA and support increased iron uptake and prevention of iron storage (Gkouvatsos et al., 2012). Furthermore, during iron deficiency Tf synthesis is increased significantly in the liver (Idzerda et al., 1986). Also, levels of Tf are increased after hypoxia through Hif–1α (Rolfs et al., 1997).

Additionally, besides TfR1 there is also TfR2, which differs in a couple aspects from the TfR1. While TfR1 is ubiquitously expressed, TfR2 expression is limited to hepatocytes and erythroid precursors. Unlike TfR1, TfR2 is not regulated by IRPs, as its mRNA does not contain IRE elements. Moreover, even though in transfected cells TfR2 binds holo–Tf, *in vivo* it does not rescue the embryo-lethality of *Tfr1*$^{-/-}$ mice, implying that TfR2 has a function that is distinct from TfR1 and unrelated to iron transport. Also, the affinity of TfR2 for holo–Tf is significantly lower than that of TfR1 (Fleming et al., 2000; West et al., 2000; Silvestri et al., 2014). Even though it seems that TfR2 does not have an important role in acquisition of iron, undoubtedly it has an essential role in regulation of iron homeostasis. Namely, TfR2 has an ability to bind HFE and this action represents a key sensor for circulating iron and activating hepcidin synthesis as a response to an elevated transferrin saturation (described in detail in Section 1.4.2). Contrary, TfR1–HFE interaction may serve to withdraw HFE away from participation in a hepcidin up-regulatory pathway, which is greatly different from TfR2–HFE interaction intention.

The importance of Tf in humans is evident with individuals with atransferrinaemia (described in detail in Section 1.4.4). Also, in humans mutation in the TfR2 gene lead to HH (described in detail in Section 1.4.4), while this is not a case with TfR1 (Schmidt et al., 2008; Fleming, 2009).

1.4 Regulation of iron homeostasis

1.4.1 IRE/IRP system – cellular regulation of iron homeostasis

The central role in the cellular regulation of the iron homeostasis is the post-transcriptional IRE/IRP control machinery which is highly dependent on iron availability in the cell. More specifically, inside the cell iron metabolism is controlled by the binding of IRP1 or IRP2 to *cis*-regulatory mRNA sequences IREs. IRE/IRP interactions regulate the expression of the mRNAs which encode proteins important for iron acquisition (TfR1 and DMT1), storage (ferritin H and L subunit) and export (FPN). Additionally, this system controls expression of Hif–2α, which is also responsible for Dcytb, DMT1 and FPN expression (Muckenthaler et al., 2008).

The presence of iron inside the cell is the key regulator of the IRE/IRP system. Namely, the IRE-binding activity of both IRP (IRP1 and IRP2) is increased when iron is low and decreased when iron is high. Additionally, the regulatory outcome depends on the position of the IRE on the mRNA sequence. Namely, if IRPs bound to IREs that are on the 3'UTR end of mRNA this will stimulate its translation by mRNA stabilization and prevention of its degradation by a ribonuclease (RNase). Contrary, if IRE is on the 5'UTR end of mRNA, binding of IRP will lead to inhibition of translation (see Figure 1–3). Specifically, when iron is limited, IRPs

bind to IREs which stabilize the mRNAs that have IREs on their 3'UTR (such as TfR1 and DMT1) and repress the translation of the mRNAs which have IREs on their 5'UTR (such as ferritin and FPN). Together, the sequence of these events leads to an increase of cellular iron uptake (i.e. absorption in duodenum, hepatocyte intake). In contrast, high iron levels diminish the stability of IRE-IRP complex, which supports translation of ferritin and FPN mRNAs, and blocks translation of TfR1 and DMT1 mRNAs, and eventually decreases intracellular iron levels (Leipuviene and Theil, 2007; Recalcati et al., 2010).

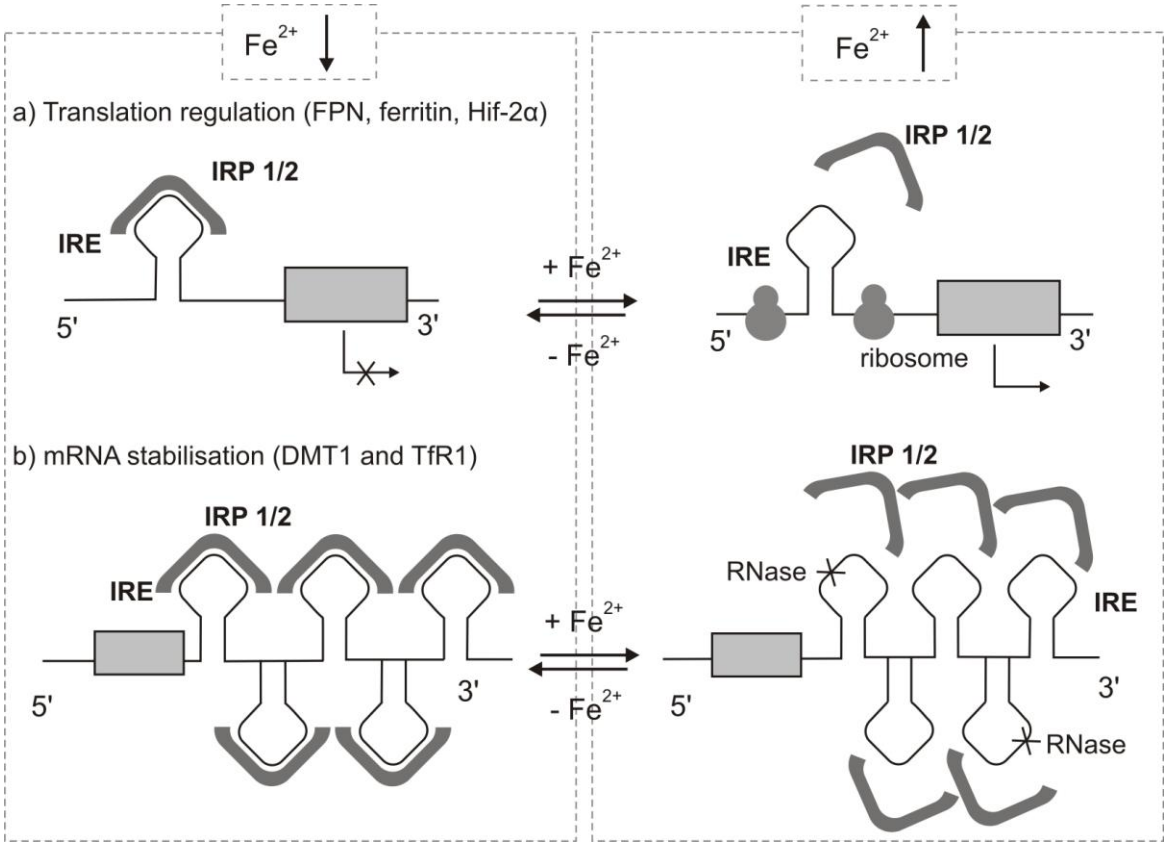


Figure 1–3 Iron-dependent regulation of IRE/IRP system
When iron levels are low, IRPs bind to IREs, and thus repress translation of 5' IRE-containing mRNAs (a) or support translation of 3' IRE containing mRNAs (b). In contrast, when iron levels are high IRPs lose binding affinity to IREs, and increase the translation of 5' IRE containing mRNAs (a) and the degradation of 3' IRE-containing mRNAs (b).

IREs are 26–30 nucleotide-long hairpin-forming sequences on the 3’ or 5’UTR of mRNA of proteins important for iron absorption. The loop sequence, which is highly conserved in all IREs, is presumably required for efficient IRP binding (Piccinelli and Samuelsson, 2007).

IRP1 and IRP2 defer partially in their structure and mechanism of action, but have the same role in iron metabolism. IRP1 is a protein containing an iron-sulphur cluster unit, which besides its role in regulation of iron metabolism has cytosolic aconitase activity. In iron-deficient cells, IRP1 operates as a RNA-binding protein, but in iron-replete cells the [4Fe–4S] cubane cluster is formed inside the IRP1, leading to destabilization of IRP1-IRE complex and expression of aconitase activity. During a change in iron levels, there is no change in IRP1 protein levels, just a change in its binding activity to IRE. However, IRP2 is produced *de novo* during iron deficiency, while it is rapidly degraded during high iron concentrations in the cells. IRP2 is highly homologous to IRP1, however IRP2 does not have a Fe–S cluster unit and does not display aconitase activity (see Figure 1–4). Both IRPs are present in all tissues, but IRP1 is mainly dominant in liver, spleen, kidney, heart, while in duodenum IRP1 and IRP2 are present in similar amounts (Iwai et al., 1998; Cairo and Pietrangelo, 2000; Volz, 2008).

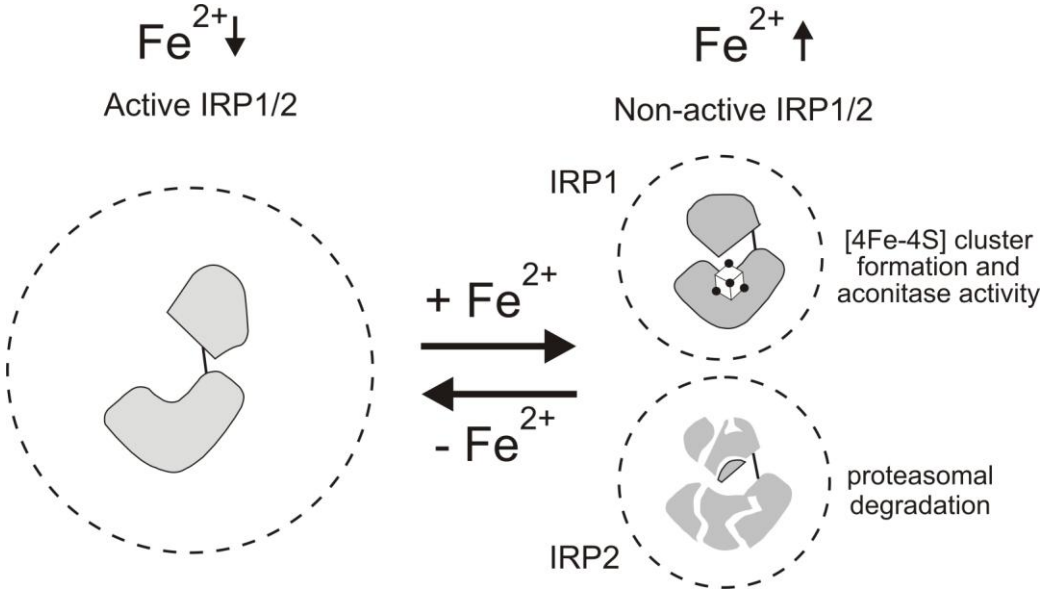


Figure 1–4 Diferences in IRP1 and IRP2
High iron levels favor the formation of the iron-sulphur cluster, which obstruct IRE binding and generate aconitase activity in IRP1, while it leads to IRP2 degradation. In contrast, low iron levels inhibit iron-sulphur cluster formation in IRP1 and support *de novo* synthesis of IRP2, thus facilitating IRP1 and IRP2 binding to IRE.

There are known disorders in humans in which the molecular basis of the disease can be addressed to IRE/IRP system regulatory components. Namely, in the hereditary disease hyperferritinaemia cataract syndrome mutations in the IRE sequence of ferritin L-chain occur, while autosomal dominant iron overload is a consequence of a mutation in the apical loop sequence on the IRE sequence of ferritin H-chain (Girelli et al., 1995; Kato et al., 2001; Roetto et al., 2002). Also, it was shown that mice with no both types of the IRP gene die early during embryonic period, which indicates a significant function of the IRE/IRP system in development. Conversely, analysis of mice lacking either IRP1 or IRP2 reveals that the two IRPs can largely compensate the function of each other (Meyron–Holtz et al., 2004; Galy et al., 2005; Smith et al., 2006; Galy et al., 2008).

1.4.2 Hepcidin – systemic regulation of iron homeostasis

1.4.2.1 Discovery and structure of hepcidin

Exactly thirteen years ago, two serendipitous discoveries occurred where two different groups of scientists isolated a new protein, one from blood (Krause et al., 2000) and one from urine (Park et al., 2001). No one thought that the discovery of this new small protein would have a crucial role in iron metabolism and that it presented the start of a novel era in iron metabolism research. This new protein was named hepcidin.

Hecidin is a 25-amino acid peptide hormone, containing four disulphide bridges, with weak antimicrobial activity and a highly conserved structure. It is primarily synthesized in hepatocytes as an 84-amino acid pre-pro-peptide which undergoes rapid intracellular processing into several smaller peptides, among which the 25 residue protein with eight cysteine residues is the biologically active form of hepcidin (Hunter et al., 2002).

1.4.2.2 Role of hepcidin

Hepcidin is a main regulator of systemic iron homeostasis and its essential role in iron metabolism was confirmed in a number of *in vivo* studies. In 2001, the leading role of hepcidin in iron homeostasis was demonstrated by Nicolas et al. in hepcidin gene-knockout mice which developed severe iron overload with iron accumulation in liver, pancreas and heart. Furthermore, the same group demonstrated that mice over-expressing hepcidin were born with severe iron-deficiency anaemia and died soon after birth (Nicolas et al., 2002). Apart from hepcidin's role in systemic iron

regulation it was shown that hepcidin is in a close relationship with rate of the iron absorption in the duodenum. Namely, an experiment with adult rats that were switched from an iron-replete diet to an iron-deficient diet showed that hepcidin expression inversely correlates with the expression of duodenal iron transporters and iron absorption (Frazer et al., 2002). These findings were followed by numerous confirmations of the essential role of hepcidin in iron homeostasis in humans. Generally, the discovery of hepcidin and its role in iron homeostasis remarkably improved our understanding of the pathogenesis of the most common and often fatal iron disorders, such as HHs (described in detail in1.4.4).

FPN, as it was described in detail in Section 1.3.1.4, is a transporter crucial for iron efflux from different cell types. Nowadays, it is confirmed that FPN is not only an iron exporter, but is also a receptor for hepcidin, which is particularly important during iron overload. As a result of hepcidin binding to FPN endocytosis of FPN occurs, leading to its proteolysis in lysosomes. This action makes hepcidin the main regulator of systemic iron homeostasis. Namely, hepcidin controls iron efflux from cells and keeps circulating iron at the proper level, preventing iron overload and production of toxic ROS (Nemeth et al., 2004b). By binding to FPN, hepcidin controls the main supply routes of iron into the circulation, such as: iron absorbed from diet in duodenal enterocytes, iron from macrophages liberated during recycling of senescent erythrocytes and iron which is stored in hepatocytes. In other words, when hepcidin concentrations are low, FPN is re-synthesized and re-expressed at the cell surface and iron enters into circulation at a high rate. When hepcidin concentrations are high, FPN is internalized and iron is trapped in enterocytes, macrophages and hepatocytes (Ganz, 2011).

1.4.2.3 Regulation of hepcidin expression

The gene encoding hepcidin (*HAMP*) is strongly expressed in liver hepatocytes. However, there is slight, but not insignificant, production of hepcidin by other cell types, such as: macrophages, kidney, adipocytes and pancreatic β-cells. To limit iron toxicity in periods of iron overload, hepcidin expression is physiologically induced to decrease circulating iron levels. Vice versa, during iron deficiency, erythropoesis and hypoxia, hepcidin expression is reduced to allow efficient iron mobilization (described in detail in Section 1.4.3). Interestingly, hepcidin production is induced during the inflammation process by what is believed to be related to its ability to lower the extracellular iron, a nutrient whose reduced availability can limit the proliferation of invading microorganisms (described in detail in Section 1.4.3; Viatte and Vaulont, 2009).

Hepcidin mRNA does not contain an IRE, as other proteins important for iron homeostasis do. This indicates that hepcidin is regulated by iron and signaling pathways other than the IRE/IRP system. The only known routes of regulation of hepcidin expression are at the transcriptional level. On the basis of all the information on the regulation of hepcidin expression this model arises (see Figure 1–5): (1) the membrane bone morphogenetic protein (BMP) receptor and its signaling components are the key regulatory machinery which control the transcription of hepcidin via the SMAD (mainly SMAD4) pathway in hepatocytes; (2) hemojuvelin (HJV) is an iron-specific adaptor ligand of the BMP receptor that boosts its sensitivity to BMPs; (3) neogenin binds to HJV also enhancing BMP signaling; (4) iron levels in the liver are sensed by BMP6, an activating ligand of the BMP receptor, that increases in hepatic iron overload (5) extracellular iron concentration is

sensed through the interaction of holo–Tf with TfR1 and TfR2, in coordination with HFE as a mediator of the holo–Tf uptake by TfRs; (6) TfR2 and HFE interaction increase the sensitivity of the BMP receptor to its ligands in a holo-Tf-dependent manner, perhaps by interactions with HJV; (7) transmembrane protease, serine 6 (TMPRSS6) seems to be stabilized by iron deficiency, leading to cleavage and inactivation of HJV and thus inhibits further hepcidin expression (Ganz, 2011; Pietrangelo, 2011; Ganz and Nemeth, 2012).

When research on hepcidin's role in humans started to emerge, it was firstly discovered that patients with juvenile haemochromatosis (JHH) had negative hepcidin mutations generating severe iron overload (Roetto et al., 2003). Namely, JHH is one of several types of HH, where mutation of HJV is the major cause (Gkouvatsos et al., 2014). Moreover, deficiency in hepcidin is the fundamental issue in every HH, which confirms the essential role of hepcidin as a negative regulator of iron absorption and iron efflux in humans (described in detail in Section 1.4.4).

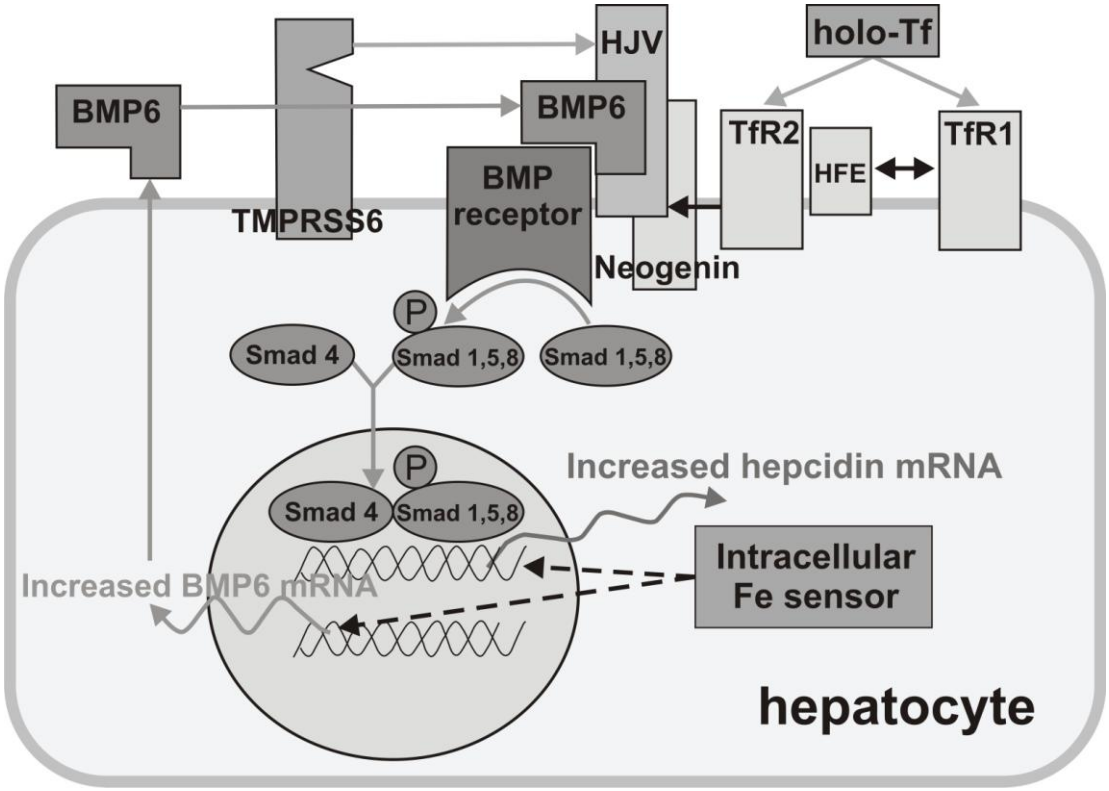


Figure 1–5 Regulation of hepcidin transcription by iron
The BMP receptor and its signaling components are the key regulatory machinery which controls the transcription of hepcidin via the SMAD pathway in hepatocytes. Iron levels in the liver are sensed by BMP6, an activating ligand of the BMP receptor, that increases during hepatic iron overload while extracellular iron concentration is sensed through the interaction of holo–Tf with TfR1 and TfR2, in coordination with HFE as a mediator of the holo–Tf uptake by TfRs. TfR2 and HFE interaction increase the sensitivity of the BMP receptor to its ligands in a holo-Tf-dependent manner, perhaps by interactions with HJV. HJV is an iron-specific adaptor ligand of the BMP receptor, while neogenin binds to HJV, both boosting its sensitivity to BMPs during iron overload. TMPRSS6 seems to be stabilized by iron deficiency, leading to cleavage and inactivation of HJV and thus inhibits further hepcidin expression.

1.4.3 Erythropoiesis, hypoxia, inflammation and oxidative stress – additional regulation mechanisms of iron homeostasis

Increased production of erythrocytes in bone marrow, in order to improve the O_2-carrying capacity of the circulation, presents a major adaptation pathway to anaemia and hypoxia. In order to support erythrocyte production during anaemia or hypoxia, up-regulation of protein erythropoietin (Epo) by kidneys and liver occurs. This event

decreases hepcidin levels which results in an increase absorption of dietary iron and mobilization of iron from macrophages and hepatocytes to maintain erythropoiesis, autonomously of iron stores. Even though it was proven *in vitro* and *in vivo* that Epo inhibits hepcidin production, the exact mechanism of action is still not fully understood (Pinto et al., 2008; Huang et al., 2009).

During hypoxia, O_2-regulated transcription factors Hif–1 and Hif–2 are elevated in order to induce erythropoiesis by activation of renal and hepatic Epo synthesis. Hif–1 and Hif–2 comprise O_2-sensitive α subunits (Hif–1α and Hif–2α) and a constitutively expressed β subunit (Hif–β). While O_2 levels are normal (normoxia), a regulatory α subunit is modified by iron-dependent prolyl hydroxylases (PHDs) and degraded through the ubiquitin/proteasome pathway. Under hypoxia, PHD activity is inhibited and the α subunit is translocated into the nucleus, where it binds to the β subunit. The heterodimeric Hif binds to the hypoxia-response elements (HREs), the regulatory sequence of the target gene, supporting the transcription of corresponding genes. Epo is one of these target genes, while *in vivo* studies showed that Hif–2 is the main regulator of Epo. Interestingly, it was proven *in vivo* that *HAMP* suppression by the Hif pathway occurs indirectly via stimulation of Epo-induced erythropoiesis. However, even though *HAMP* promoter contains several HREs, Hifs binding to them remains controversial (Peyssonnaux et al., 2007; Liu et al., 2012a). Additionally, hypoxia controls expression of genes important for iron absorption in the duodenum through Hif–2α, such as Dcytb, DMT1 and FPN (Mastrogiannaki et al., 2009; Shah et al., 2009). Also, levels of Tf are increased after hypoxia through Hif–1α (Rolfs et al., 1997). Interestingly, expression of Hif–2α is control by IRE/IRP machinery. This provides evidence that different ways of iron homeostasis control are mutually

dependent, include many factors and make complex network of machinery for fine tuning of iron levels in organism.

During infection and inflammation, hepcidin levels are increased as a host defence mechanism in order to reduce iron levels and make it non-available to invading microorganisms. Thus, inflammation has a great influence on iron homeostasis and by up-regulation of hepcidin cause down-regulation of intestinal iron absorption, iron export from macrophages and hepatocytes and thereby decreased serum iron levels. It was proven *in vivo* that IL–6 has a stimulatory effect on hepcidin transcription. Specifically, in urine of human volunteers levels of hepcidin increased 7-fold after infusion with recombinant human IL–6 which was followed with a significant decrease in serum iron and transferrin saturation (Nemeth et al., 2004a). Additionally, IL–6 knock-out mice failed to produce surplus hepcidin mRNA in response to induced inflammation, indicating that IL–6 is necessary for hepcidin induction and hypoferremia during inflammation in mice (Nemeth et al., 2004a). In a separate study, after individuals were injected with LPS, IL–6 was significantly up-regulated within 3 hours after injection, which was followed by an urinary hepcidin increase which peaked within 6 hours and a large decrease in serum iron (Kemna et al., 2005). This study confirmed the previous statement and highlighted the importance of general inflammation in iron homeostasis. It is speculated that cytokines other then IL–6 may also contribute to the up-regulation of hepcidin during inflammation. However, this effect was not proven *in vivo* up to now (Armitage et al., 2011; Nairz et al., 2014).

Oxidative stress presents a disturbance in the equilibrium between the presence of ROS and antioxidant defence mechanisms, in favour of ROS, leading to damage of

cell components and provoking pathogenesis of numerous disorders. However, oxidative stress induces a battery of genes encoding antioxidant and detoxifying enzymes. Nrf2 is a transcription factor which binds to ARE in response to oxidative stress, initiating the synthesis of enzymes that will "fight" against elevated levels of reactive species. The mechanism by which the binding of Nrf2 to ARE is induced is still not fully understood. Though, it is known that when oxidative stress is not present Nrf2 forms an inactive complex with kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. This interaction supports Nrf2 ubiquitination and degradation. In contrast, when increased levels of intracellular ROS occur, destabilization of the Keap1-Nrf2 interaction takes place, allowing Nrf2 to dissociate from Keap–1 and translocate to the nucleus. In the nucleus, Nrf2 will join to an additional transcriptional factor Maf in order to form a complex that binds to ARE on DNA. This will up-regulate transcription of numerous antioxidative and cytoprotective proteins which have ARE region, such as: HO–1, superoxide dismutase 1, quinone reductase (QR), catalase, sulfiredoxin, thioredoxin, peroxiredoxins, glutathione peroxidase, glutathione reductase, glutathione S–transferase (GST) and γ–glutamine cysteine ligase (Halliwell and Gutteridge, 2007; Johnson et al., 2008; Klaassen and Reisman, 2010; Gan and Johnson, 2014). Oxidative stress is strongly associated with high levels of free iron in the cell. With this in mind, it is no surprise that in periods of oxidative stress actions that maintain iron export out of the cell and storage are supported. Thus, in order to avoid additional oxidative stress in the cell, levels of FPN and ferritin H- and L-chain are up-regulated by the Nrf2 pathway. Interestingly, haem acts like a stabilizer of the Nrf2-Maf complex and thus supports its binding to ARE. By bringing together knowledge about haem and its proven role in the Nrf2 pathway, it can be concluded

that phagocytosis of erythrocytes by reticulo-endothelial macrophages and release of haem up regulates: HO–1 to activate haem degradation, ferritin H- and L-chain to accumulate iron released from the haem and FPN to export iron out of the cell for further transport via circulation (Marro et al., 2010).

1.4.4 Disorders of iron metabolism of different etiology

In this chapter iron metabolism disorders, such as: HH, iron-refractory iron deficiency anaemia (IRIDA), atransferrinaemia, β-thalassaemia, iron deficiency anaemia (IDA), anaemia of inflammation (AI) or anaemia of chronic disease (ACD), are described. Additionally, a description of how these disorders are treated is also included.

HHs mainly belongs to disorders of hepcidin and ferroportin functions, and are characterized as a group of genetic disorders described by excessive absorption of dietary iron and its accumulation in the liver and other organs. The prime consequence is liver injury leading to cirrhosis and hepatocellular carcinoma. In addition, iron toxicity can also damage other organs. Early diagnosis and use of recommended therapy to maintain normal body iron stores is crucial and can prevent all known complications of HHs. If untreated, HHs may lead to death from cirrhosis, diabetes, malignant hepatoma or cardiac disease (Niederau et al., 1994). Deficiency of hepcidin is the fundamental issue in every HH (see Figure 1–6). In humans, there are several types of HH: HFE-associated, TfR2-associated, HJV-associated, FPN-associated and *HAMP*-associated, as a consequence of mutation of genes for HFE, TfR2, HJV, FPN and hepcidin, respectively. The most severe types of HH are associated with *HAMP* and HJV. Loss of TfR2 or HFE causes an intermediate level

of hepcidin deficiency, while loss of FPN results in hepcidin-resistance type of HH (Pietrangelo, 2010).

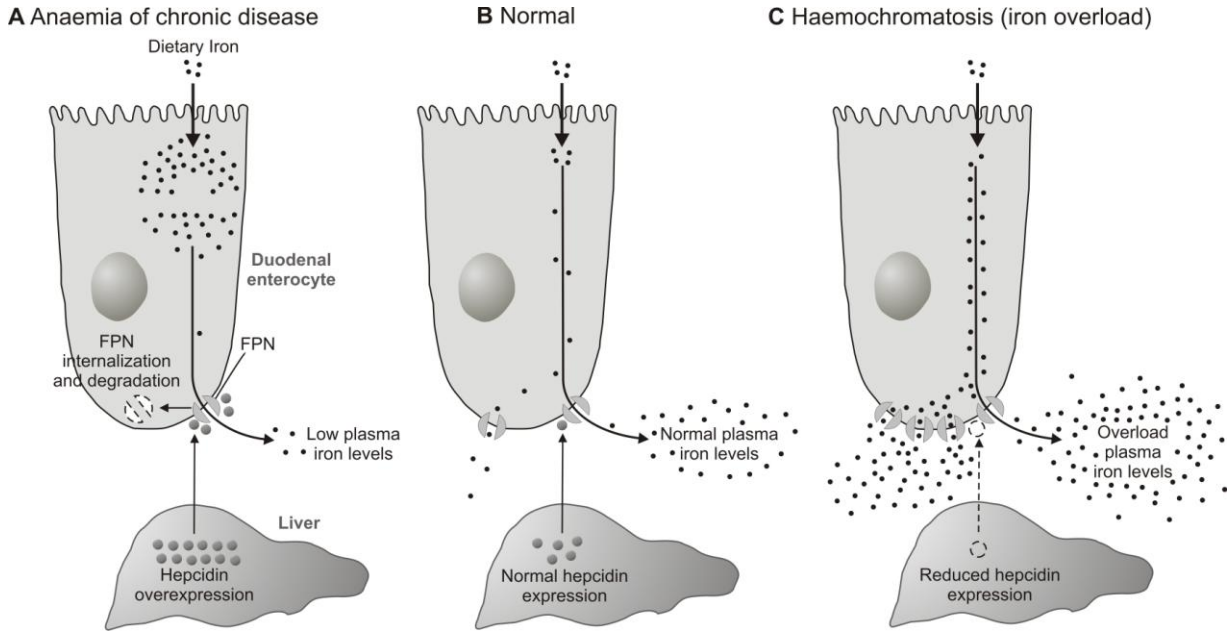


Figure 1–6 Iron absorption during some iron metabolism disorders connected with hepcidin
During high and long-lasting levels of hepcidin expression in liver, caused by inflammatory stimuli, internalization and degradation of FPN in enterocyte occurs leading to impaired iron absorption through the enterocyte, intracellular iron accumulation and low plasma iron levels (A). While iron metabolism is in balance, hepcidin levels are normal and in accordance with iron demands (B). HH is a consequence of impaired hepcidin expression, causing increased iron import from the diet and overload of iron in plasma (C).

Human iron disorder caused by mutations in the gene encoding the TMPRSS6 enzyme is known as IRIDA. IRIDA is characterized by low iron and transferrin saturation and inappropriate elevation of hepcidin levels in serum.

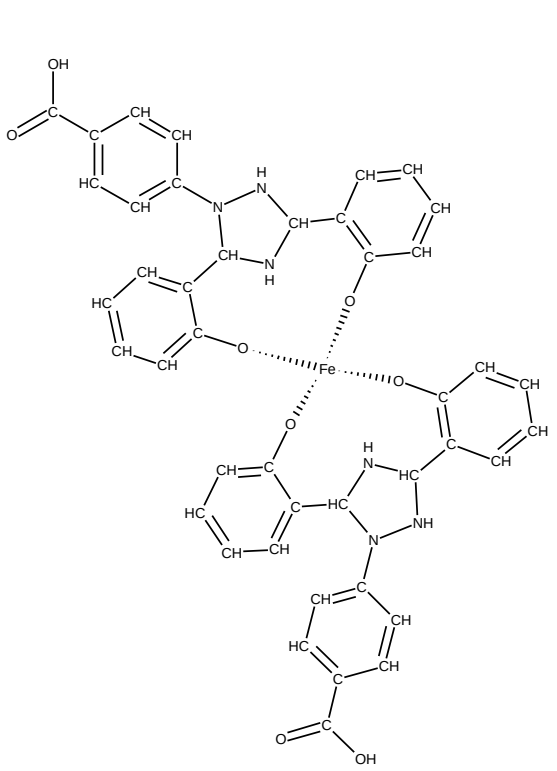
Atransferrinaemia, hereditary deficiency of Tf, is a rare autosomal recessive disorder as a consequence of Tf gene mutations. Without Tf and impaired supply of iron to the bone marrow, individuals develop severe, microcytic, hypochromic, iron-deficiency anaemia. These individuals need erythrocyte transfusions from birth in

order to survive. This state leads to increased intestinal iron absorption. However, absorbed iron is transported inadequately in the plasma because non-Tf bound iron cannot be imported by erythroid precursors. Consequently, iron is taken up by other organs, which leads to a paradoxical iron overload of non-erythropoietic tissues. This disorder also leads to low hepcidin levels (Beutler et al., 2000; Heeney and Andrews, 2004)

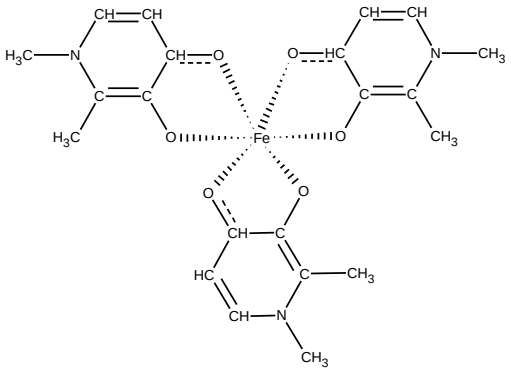
β-thalassaemia, a type of iron-loading anaemia, is a secondary disorder of hepcidin function in which mutation of the gene for β-globin, the subunit which together with α-globin makes up haemoglobin, occurs. Therefore, synthesis of haemoglobin is impaired, facilitating ineffective erythropoiesis which is stimulated by constant high levels of Epo, leading to low levels of hepcidin. Thus, both significant iron overload, with hyper-absorption of dietary iron despite high serum transferrin, and anaemia, due to impairment in erythrocyte synthesis, occur. A transfusion is effective treatment for these patients because it increases hepcidin due to lowering of Epo concentrations and provides normal erythropoiesis, at least for a while (Origa et al., 2007; Ganz, 2011).

Standard therapies for iron overload disorders are phlebotomy and use of chelation therapy. During phlebotomy, around 500 mL of blood is removed once or twice weekly from patients until levels of ferritin falls under 20 mg/mL. This leads to anaemia, which induces iron mobilization from iron stores. Once the patient has reached a satisfactory iron levels, phlebotomy is performed several times per year over the lifetime (Adams and Barton, 2010; Yehuda and Mostofsky, 2010).

When phlebotomy is not an adequate way for iron overload treatment, chelation therapy is often used to remove excess iron stores. Additionally, it is considered by many that phlebotomy is an obsolete technique and that chelation therapy is the gold standard for treating iron overload of different etiologies. The aim of chelation therapy is to scavenge free iron from circulation and tissues by forming complexes which are excreted in the faeces and/or urine. Several iron chelator drugs have been developed, such as deferoxamine (DFO), deferiprone (DFP) and deferasirox (DFX), which are administrated orally or subcutaneously. DFO, DFP and DFX bind iron at a different molar ratio chelator : iron, 1:1, 3:1 and 2:1, respectively (see Figure 1–7). Today, research in the hope of finding new iron chelator drugs which could chelate iron from tissues more effectively is evolving (Poggiali et al., 2012). Interestingly, it has been confirmed that iron is a crucial element for tumour proliferation, thus the potential role of iron chelation therapy in the treatment of cancer should be considered in the near future (Hann et al., 1988; Zacharski et al., 2008).



DFO-Fe complex



DFX_2-Fe complex

DFP_3-Fe complex

Figure 1–7 Complexes of chelation therapy drugs with iron

Contrary to iron overload disorders, there are iron deficiency disorders, generally known as anaemias. Commonly, anaemia is a condition in which there are not enough healthy erythrocytes in the circulation which leads to inadequate oxygen distribution and consequently disturbance in the maintenance of normal physiological function of tissues, such as liver, brain, muscles, etc. (World Health Organisation, 2007).

There are many types of anaemia and these can arise as a result of a wide variety of causes that can be single, but more often coexist. Globally, the most significant contributor to the anaemia is iron deficiency, known as IDA. The main causes for

IDA are low dietary iron intake, poor absorption of iron from diet at a period of life when iron requirements are particularly high (described in detail in Section 1.4.5). Other recognized causes of anaemia, such as heavy blood loss or extensive menstruation are also recognized (World Health Organisation, 2008).

Additionally, anaemia and hypoferraemia that occurs as consequence of chronic infections and inflammatory disorders is known as AI or ACD. AI is a systemic iron disorder characterized with decreased iron, iron binding capacity and intestinal iron absorption, as well as impaired erythropoiesis, while iron is trapped in macrophages and liver, indicating impaired mobilization of iron from stores. AI is a consequence of cytokine (mainly IL–6) mediated induction of hepcidin production as a response to chronic inflammation (see Figure 1–6; Franchini et al., 2010; Yehuda and Mostofsky, 2010).

To be more precise, anaemia is a consequence of both poor nutrition and poor health. Increased risk of maternal and child mortality is one of the main concerns of severe anaemia. Additionally, the negative consequences of IDA on cognitive and physical development of infants and on general performance, particularly work productivity in adults, are also great concern. To be more precise, iron deficiency is the most common and widespread nutritional disorder in the world. The World Health Organization declares iron deficiency as one of the 10 leading risk factors for disease, disability and death in the world today. Iron deficiency affects mostly children and women in practically all countries. It can be estimated that most preschool children in non-developed countries and at least 30–40% in developed countries are iron-deficient, and nearly half of the pregnant women in the world are estimated to be anaemic (World Health Organisation, 2001; World Health

Organization, 2002; World Health Organisation, 2007; World Health Organisation, 2008).

Details on how diet habits affect iron deficiency, as well as routes how iron deficiency can be overcome are described in detail in Sections 1.4.5 and 1.6.

1.4.5 Bioavailability of iron

Mechanisms of keeping iron metabolism in homeostasis greatly differ from those of other metals. Namely, there is no known physiologic mechanism for iron excretion and thus 90% of daily needs for iron are obtained from an endogenous source, such as the breakdown of old or damaged erythrocytes by reticulo-endothelial macrophages. Unavoidably, there are regular daily iron losses, such as shedding of skin cells, sweating, miscellaneous bleeding in intestine, menstrual bleeding, etc. Thus, to keep iron in balance, it is essentially that iron is supplied by diet, especially during growth of infants, children and adolescents and the reproductive period in women, particularly during pregnancy (Hurrell and Egli, 2010; Abbaspour et al., 2014). In

Table 1–**1** it can be clearly seen that daily requirements of absorbed iron differ greatly between individuals of different age, sex and state.

Table 1–1 Daily requirements of absorbed iron in individuals of different age, sex and state

age/state	absorbed iron in duodenum[a] (mg/day)
4–12 months	0.96
13–24 months	0.61
2–5 years	0.70
6–11 years	1.17
12–16 years (girls)	2.02
12–16 years (boys)	1.82
adult males	1.14
women during lactation	1.31
women during menstruating period	2.38
women during postmenopausal period	0.96
pregnant women	*requirement during pregnancy greatly depends on the woman's iron status before pregnancy, these values are given as average*
1st trimester of pregnancy	0.8
2nd & 3rd trimester of pregnancy	6.3

[a]calculations were done on basis on average weight (DeMaeyer et al., 1989; Abbaspour et al., 2014)

As mentioned previously, dietary iron occurs in two forms: haem and non-haem. Haem iron makes 10–15% of total iron from diet in meat-eating populations, but, it is estimated to contribute ≥40% of total absorbed iron. However, non-haem iron absorption is much lower, and it varies between 2%–20%. In contrast to non-haem iron whose bioavailability is highly dependent on the presence of iron absorption promoters or inhibitors in the diet, dietary factors have little effect on haem iron absorption (Hurrell and Egli, 2010).

The low bioavailability of non-haem iron contributes greatly to IDA, which is the most prevalent nutritional deficiency worldwide, estimated to affect two billion people especially in low-income populations where consumption of meat is low (World Health Organisation, 2007). On the other hand, low bioavailability of non-haem iron is a problem in population groups eating only a plant-based diet,

vegetarians and vegans, whose popularity is rising in modern societies. In order to compensate for lost iron and keep iron homeostasis in balance, it is of utmost importance that absorption of iron is sufficient. Thus, it is essential to understand in detail the mechanism of iron absorption in the duodenum as well as to target its promoters or inhibitors. Additionally, for individuals affected with iron deficiency it is important to know what food is rich in highly bioavailable iron and try to consume it as much as possible. In Table 1–2 it can be seen what are the average levels of total iron in common foods.

Table 1–2 Amount of total iron in common foods

food	total iron μg/g
sources of non-haem iron	
spinach	260
tea	250
salad rocket	154
parsley	90
lettuce	65
red cabbage	61
tomato	36
red bean	35
red pepper	31
cucumber	29
broccoli	29
garlic	28
banana	27
cauliflower	25
corn flour	25
onion	23
Nescafe	22
wheat	20
strawberry	19
potato	15
carrot	15
apple	15
eggplant	14
flour	12
kiwi	6
peach juice	1.53 mg/L
orange juice	0.68 mg/L
sources of haem iron	
ostrich fillet	24
lamb chop	22
beef topside	19
pork loin	4
chicken breast	4

(Lombardi–Boccia et al., 2002; Tokalıoğlu and Gürbüz, 2010)

However there are recognized inhibitors of iron absorption whose occurrence in food should be addressed in iron-deficient individuals. Major inhibitors of iron absorption from the diet are phytate, polyphenols, calcium and proteins.

Phytate (inositol hexakisphosphate; see Figure 1–8) is a primary phosphorous storage molecule in plants and cannot be digested by humans.

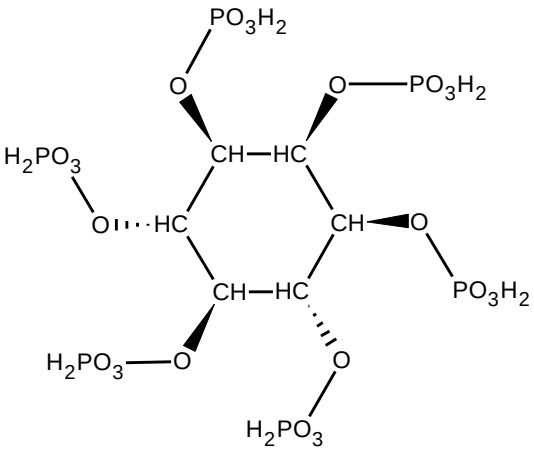


Figure 1–8 Structure of phytate

It is believed that phytate forms a complex with iron through its phosphate ester groups making it nonabsorbable and it is considered as the main inhibitor of non-haem iron absorption. The inhibitory effect of phytate has been proven, but particular food preparation methods, such as milling, heat treatment, soaking, germination, fermentation, addition of ascorbic acid or enzyme phytase, can remove or degrade phytate and thus partially or totally eliminate its negative effect on non-haem iron absorption (Siegenberg et al., 1991; Hurrell et al., 2003; Hurrell, 2004; Hurrell and Egli, 2010). However, low concentrations of phytate (2–10 mg/meal) express a negative effect on non-haem iron absorption. Considering that some foods contain phytate in considerable concentrations (see Table 1–3), even much more than common food containing non-haem iron (see Table 1–2), consumption of phytate-rich plants should be under attention.

Table 1–3 Phytate content in selected foods

food	phytate mg/g
walnut	40
pistachio nuts	28
hazelnut	23
wheat cereal	11
wheat bread	3

(Harland et al., 2004)

Polyphenols are group of natural products greatly distributed in food of plant origin (described in detail in Section 1.5). It has been proven by numerous studies that polyhenols significantly inhibit iron absorption. The connection between polyphenols and iron metabolism is described in detail in1.6.

Calcium has been shown to have an inhibitory effect on both non-haem and haem iron absorption. The mechanism of the inhibitory effect of calcium on iron absorption is not known, but it is speculated that it could block initial iron uptake by the enterocyte (Hallberg et al., 1992). Inhibition of iron absorption has been demonstrated even with a calcium concentration that is common in the daily dietary intake. This fact could represent a general health problem because widespread and recommended use of calcium supplements, manly for the prevention of osteoporosis, can bring about problem with iron absorption (Roughead et al., 2005; Hurrell and Egli, 2010).

Particular proteins are also proven to have an inhibitory effect on iron absorption such as: milk, soybean and egg proteins, albumin, casein and whey (Cook and Monsen, 1976; Hurrell et al., 1988; Hurrell et al., 1989; Lynch et al., 1994).

On the other hand, the main dietary enhancers of iron absorption are ascorbic acid and muscle tissue. It is proven that ascorbic acid improves non-haem iron absorption, mainly due to its ability to reduce Fe^{3+} to Fe^{2+} and thus make it available for

transport by DMT1. The amount of ascorbic acid that expresses a positive effect on non-haem iron absorption is approximately 30–100 mg daily, which corresponds to the recommended dietary intake for ascorbic acid. However, in foods of plant origin, such as, fruits and vegetables, the supporting effect of ascorbic acid might be reduced by the inhibiting effect of polyphenols and phytate (Gillooly et al., 1983; Ballot et al., 1987; Hallberg et al., 1989; Siegenberg et al., 1991; Carr and Frei, 1999; Teucher et al., 2004). In contrast to the positive effect of ascorbic acid on non-haem iron absorption after a single meal, improvement in iron status after chronic supplementation with ascorbic acid was not observed in humans. The reason for this occurrence is not yet fully understood (Cook and Reddy, 2001).

Muscle tissue, known as "meat factor", also showed a positive effect on non-haem iron absorption, the same as ascorbic acid, but it was hard to demonstrate the same activity after a longer consumption. There is evidence that this could be attributed to: cysteine-containing peptides, glycosaminoglycans and L–α–glycerophosphocholine and their ability to reduce and chelate iron (Hurrell et al., 2006; Storcksdieck Bonsmann and Hurrell 2007; Armah et al., 2008).

Nowadays three approaches are recognized as ways to deal with IDA and they can be practices alone or in combination with each other: change in dietary habits by means of diversity and modification of the diet in order to improve nutritional value and iron bioavailability, supplementation (intake of iron in higher doses not with food), and fortification (the addition of iron into food during food processing). A change of dietary habits so that intake of food rich in both haem and non-haem iron, as well as promoters of iron absorption, is increased, while intake of inhibitors of iron uptake should be decreased. Even though it showed significant practical limitations, a

change of dietary habits is the favoured way of treating IDA. Apart from fact that it is hard to change individuals' dietary preference, food rich in highly bioavailable iron, such as meat, is expensive especially in developing countries.

Supplementation is an efficient and cost-effective way of treating IDA over short periods of time, such as pregnancy. However, insufficient coverage of all parts of the world and compliance is a major limitation to the effectiveness of iron supplementation programs (Yip and Ramakrishnan, 2002). Iron supplementation is carried out orally or, very rarely by injection. Frequently used forms of iron in supplements include Fe^{2+} and Fe^{3+} salts, such as SO_4^{2-}, gluconate, fumarate and citrate. High doses of supplemental iron may cause gastrointestinal side effects, such as nausea and constipation. Other forms of supplemental iron, such as haem iron, carbonyl iron, iron amino-acid chelates and polysaccharide-iron complexes, are also available and are believed to manifest fewer gastrointestinal side effects compared with salts (World Health Organization, 2001; Lynch, 2005).

Iron fortification of food is considered as the most cost-effective route for lowering incidence of IDA all over the world. Generally, iron fortification refers to the addition of iron to foods consumed by all or most of the population and it is regulated by the government. Milled cereals are frequently the subject of iron fortification and showed a successful outcome in making populations less iron-deficient. Also, it was estimated that iron fortification is economically more favourable than iron supplementation (Baltussen et al., 2004; Lynch, 2005).

1.5 Plant phenols

Phenols are plant secondary metabolites that include a great number of structurally diverse compounds. Chemically speaking, phenols are compounds which contain one (phenol) or more (polyphenols) aromatic rings, bearing one or more hydroxyl groups, which can be esterified, etherified or glycosylated. Generally, phenols and polyphenols represent all secondary metabolites whose syntheses go through the shikimate/phenylpropanoid or the "polyketide" acetate/malonate pathway, or by combination of two of them, producing monomeric or polymeric phenols. Additionally, phenols are uncommon in bacteria, fungi and algae but are ubiquitously present in the plant kingdom. The phenolic profile of an individual plant strongly depends on plant species and thus can be used as a reliable taxonomic marker (Cheynier et al., 2013).

Throughout evolution, plants have developed adaptive mechanisms which are reflected in their ability to produce a great number of phenolic secondary metabolites. Though, phenols are not compulsory in the processes such as plant growth and development, they have pivotal role for plants' interactions with the environment, reproduction and defence. From an evolutionary point of view it is easy to see why plants produce such a great collection of secondary compounds compared with animals. Namely, they cannot rely on physical mobility to escape predators or perform successful pollination. Thus, they had to developed exuberant chemical systems in order to survive. Plants need phenols for protection against herbivores, microbes, viruses or other plants, as signal compounds to attract pollinating or seed dispersing animals, protection from ultraviolet radiation or oxidants and fluctuation of organic and inorganic nutrients from soil. Phenols are generally soluble in polar

organic solvents, unless being entirely esterified, etherified or glycosylated. Also, most phenol glycosides are water-soluble but the corresponding aglycones are usually less so. Due to the presence of an aromatic ring, all phenols demonstrate intense absorption in the ultraviolet part of the spectrum. Furthermore, phenols that give colour to plants absorb light in the visible region as well.

On the basis of the phenol skeleton, several classes of phenols have been categorized: C_6 (phenols, benzoquinones), C_6–C_1 (phenolic acids), C_6–C_2 (acetophenones, phenylacetic acids), C_6–C_3 (hydroxycinnamic acids, coumarins, phenylpropanes, chromones), C_6–C_4 (naphthoquinones), C_6–C_1–C_6 (xanthones), C_6–C_2–C_6 (stilbenes, anthraquinones), C_6–C_3–C_6 (flavonoids, isoflavonoids), $(C_6$–$C_3)_2$ (lignans, neolignans), $(C_6$–C_3–$C_6)_2$ (biflavonoids), $(C_6$–$C_3)_n$ (lignins), $(C_6)_n$ (catechol melanins) and $(C_6$–C_3–$C_6)_n$ (condensed tannins) (Robards, 2003; Lattanzio et al., 2006; Cheynier et al., 2013).

1.5.1 Flavonoids

Flavonoids are one of the largest groups of plant phenols and, by now, more than 8000 structures of flavonoids have been identified. These secondary metabolites are widely distributed in plants and are classified in a number of subgroups, of which one representative of flavones, flavonols, isoflavones, flavanones, flavanonols, flavanols, anthocyanins, chalcones and aurones subgroup is presented in Figure 1–9.

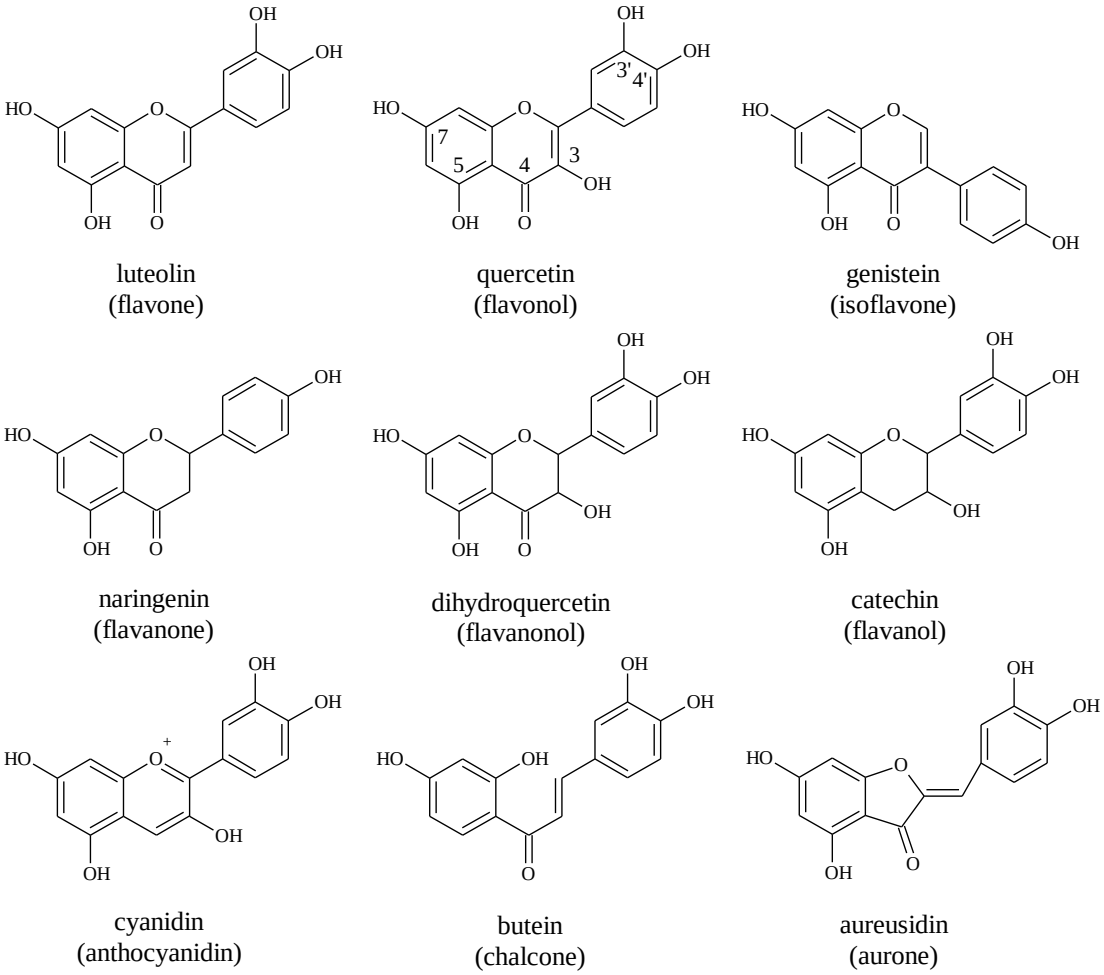


Figure 1–9 Structure of some classes of flavonoids

As with other phenols, flavonoids also have numerous functions in plants, such as: protection against ultraviolet radiation and phytopathogens, a protective response during stress, signaling during development and growth, auxin transport and coloration of flowers for attraction of insects during pollination (Bradshaw and Schemske, 2003; Falcone Ferreyra et al., 2012).

Apart of being valuable for the plant kingdom, flavonoids are also beneficial to human health. Namely, flavonoids have played a key role in the successful traditional medical treatments of ancient times and their use has continued up to the present day. In addition to being highly bioactive, they express low toxicity, which

makes their medicinal use very attractive. Flavonoids express many positive therapeutical properties which have been experimentally confirmed. For medicine, the most valuable property of flavonoids is their ability to effectively scavenge highly toxic free radicals. Free radical species occur in the course of numerous physiological processes and can initiate damage of nucleic acid, lipid and protein structures, resulting in disturbance of vital cellular functions and causing a wide range of disorders. Thus, today it is almost impossible to separate free radical reactions from almost any disorder. Apart from keeping biomolecules safe from free radical attack, flavonoids take part in many biochemical processes in an organism, such as: regulation of expression of cell cycle regulatory proteins, inhibition of signal transduction pathways or enzyme activity. As a consequence, flavonoids express many beneficial health actions, such as: lowering blood pressure and risk of cardiovascular disorders, decreasing the incidence of carcinogenesis and neurodegeneration, inhibiting platelet aggregation and the inflammatory response, as well as lowering levels of bad LDL cholesterol (Sharma, 2014).

To give answer to question as to why flavonoids express numerous physiological properties is not easy, but the most probable answer lies in the fact that they are highly reactive and can enter into almost any type of reaction known to organic chemistry. Namely, they can take part in oxidation-reduction, acid-base and free-radical reactions and hydrophobic interactions, while their substituents can modify electronic induction, resonance and steric hindrance. Additional, flavonoids make stable complexes with metal ions, such as iron, and thus express their antioxidative property, which is all together of particular interest for this thesis (described in detail in Section 1.6).

All flavonoids share a common origin – the amino acid phenylalanine. Initially, phenylalanine is synthesized in the shikimate pathway, while its further processing goes through the phenylpropanoid pathway until a certain flavonoid structure is formed (see Figure 1–10; Cheynier et al., 2013). As one of the main aims of this thesis is to elucidate the effect of the flavonoid quercetin on iron homeostasis, the biosynthesis of flavonoids in plants will be described with quercetin as an example. Biosynthesis of other flavonoids follows a similar route, but due to page limitations other routes will not be described. For the same reason, the following headings will be mainly concentrated on quercetin.

Synthesis of quercetin (see Figure 1–10) starts with the shikimate pathway and its initial reaction of aldol condensation of phosphoenolpyruvate (PEP; glycolytic intermediate) and erythrose–4–phosphate (pentose phosphate pathway intermediate) to give a C7 sugar, 2–dehydro–3–deoxyarabinoheptulosonate–7–phosphate (DAHP), which will convert to shikimate. Shikimate will after phosphorylation and reaction with PEP give chorismate. Chorismate will transform to phenylalanine giving the basic phenylpropanoid skeleton. Phenylpropanoid metabolism is controlled by the enzyme phenylalanine ammonia–lyase (PAL), which catalyzes a non-oxidative deamination of phenylalanine to cinnamic acid. Hydroxylation of cinnamic acid will give 4–coumaric acid whose carboxyl group will be activated in a reaction with CoA–SH, giving 4–coumaroyl–CoA. 4–coumaroyl–CoA combines with three molecules of malonyl–CoA (an intermediate in fatty acid biosynthesis) forming the flavonoid skeleton naringenin chalcone by the enzyme chalcone synthase. Afterwards, enzyme chalcone isomerase catalyzes the cyclization of chalcone to the flavanone naringenin, a basic structure for further synthesis of all classes of flavonoids. Quercetin is formed after hydroxylation and oxidation of naringenin.

Furthermore, quercetin is glycosylated with different sugars to make glycosides, the main forms of quercetin in plants (Cheynier et al., 2013; Sharma, 2014).

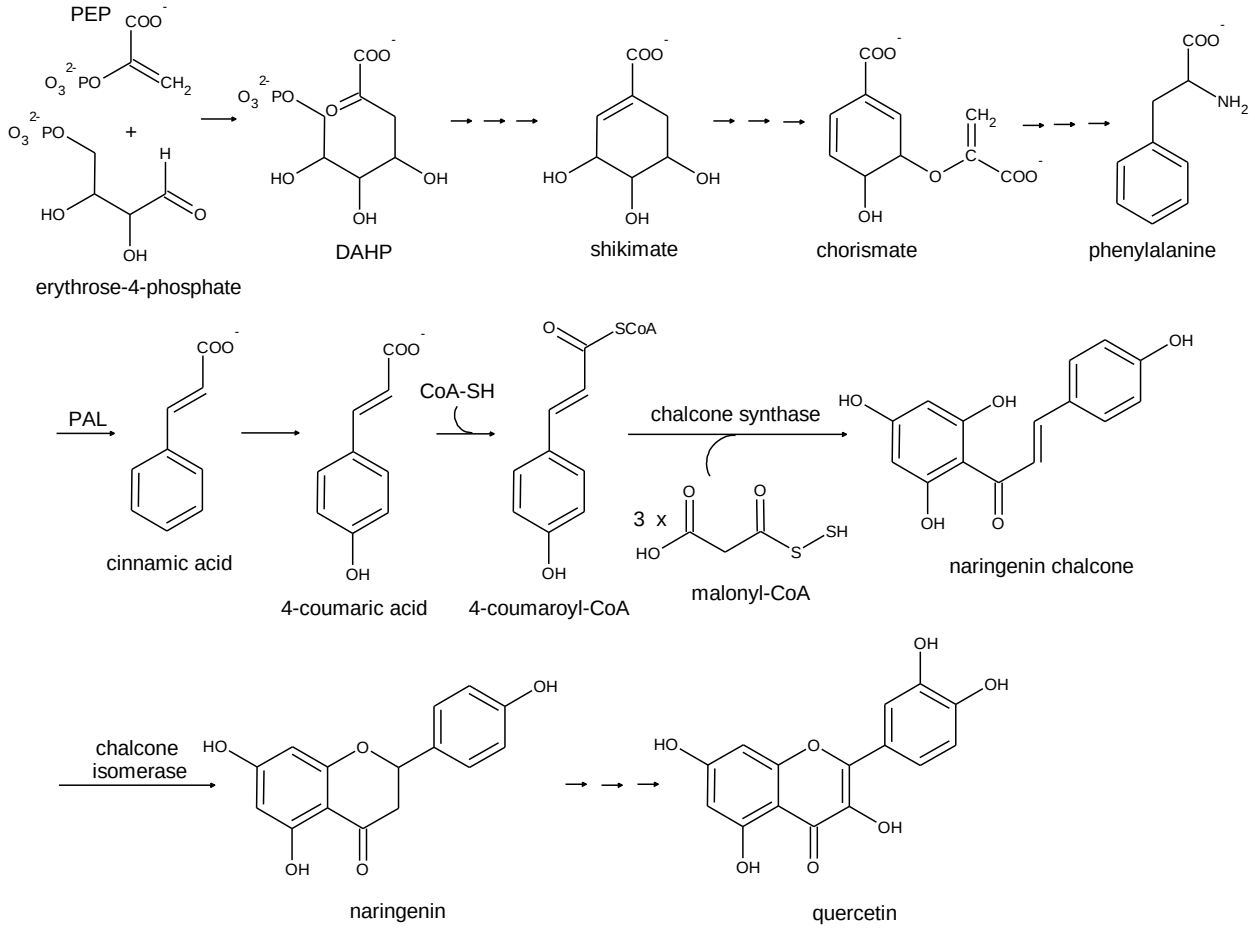


Figure 1–10 Synthesis of quercetin

1.5.2 Absorption and metabolism of flavonoids in humans

Absorption and metabolism of flavonoids will be explained with quercetin as an example due to limited space and the specific aim of this thesis. However, other flavonoids follow the same or similar mechanism of absorption and metabolism described for quercetin.

Quercetin, the most abundant flavonol in plant diet, is mainly present in plants in its highly hydrophilic glycosylated forms, mainly as β-glycosides of various sugars. The dominant types of quercetin glycosides differ in plants. However, the main forms present in plants are quercetin–3–*O*–rutinoside (rutin), quercetin–3–*O*–galactoside (hyperoside), quercetin–3–*O*–glucoside (isoquercitrin), quercetin–3–*O*–rhamnoside (quercitrin) and quercetin–4'–*O*–glucoside (spiraeoside; Lee and Mitchell, 2012).

Prior to absorption in the gut, flavonoids first need to be freed from plant tissue by chewing in the oral cavity and then processed by digestive juices in the intestine or by microorganisms in the colon. Generally, there are two main routes of quercetin glycoside absorption by the enterocyte. Firstly, absorption goes via a transporter followed by deglycosylation within the enterocyte by cytosolic glycosidase. Secondly, deglycosylation can occur firstly by luminal hydrolases followed by transport of the aglycone by passive diffusion or via different transporters. It is demonstrated that quercetin glucosides can be taken up by the enterocyte through the sodium-dependent glucose transporter (SGLT1) with subsequent deglycosylation inside the enterocyte by cytosolic β–glycosidase. Also, quercetin glucosides can firstly undergo luminal hydrolysis by lactase phlorizin hydrolase (LPH) and afterwards are absorbed inside the enterocyte by passive diffusion or a transporter-mediated mechanism (Walle et al., 2000; Wolffram et al., 2002; Day et al., 2003; Ziberna et al., 2014). Specifically, quercetin can use glucose transporter (GLUT)–1, –3 and –4 to enter cells and thus operate as an inhibitor of glucose transport (Strobel et al., 2005). The nature of the sugar moiety greatly influences the route and rate of quercetin absorption in the gut. Namely, it is suggested that 3–*O*–glucosylation of quercetin improves its absorption in the small intestine, even compared with absorption rate of quercetin alone. On the other hand, quercetin glucosides

containing rhamnose (rutin) could not be absorbed in the small intestine, and are believed to be absorbed in the colon after deglycosylation (Hollman et al., 1999; Morand et al., 2000; Day et al., 2003).

Definition of bioavailability states that bioavailability is the portion of an initially administered dose of drug that reaches the systemic circulation unchanged. Considering that, flavonoid bioavailability is very low mostly due to extensive metabolism at the intestinal level. Namely, further biotransformation of quercetin aglycone involves glucuronidation, sulfonation and methylation of hydroxyl groups, which primarily occur in enterocytes and hepatocytes. Specifically, the major quercetin metabolites detected in plasma are quercetin–3'–sulphate and quercetin–3–glucuronide. It is assumed that they are produced in the small intestine, pass into the portal vein and are further converted into other metabolites in the liver, such as isorhamnetin–3–glucuronide, quercetin diglucuronide, quercetin glucuronide sulphate, methylquercetin diglucuronide, etc. After returning to the bloodstream they are excreted in urine via kidneys. Additionally, a portion of quercetin is converted to low molecular weight phenolic acids, such as 3–hydroxyphenylpropionic acid, 3,4–dihydroxyphenylpropionic acid and 3–methoxy–4–hydroxyphenylpropionic acid (Olthof et al., 2003; Mullen et al., 2006).

1.5.3 Occurrence and intake of dietary flavonoids

Nowadays, a growing body of evidence confirms different beneficial health effects of dietary flavonoids. Consequently, the scientific community takes more and more interest in the levels and types of flavonoids that are taken up in the diet. This is particularly interesting in the scope of modern concept of so called "functional food",

food that apart from nutritional value expresses additional functions, such as health-promotion or disease prevention. Namely, flavanols and anthocyanidins have been associated with reduction of risk of cardiovascular disease, while anthocyanidins efficiently protect LDL cholesterol oxidation (Schroeter et al., 2010). It had been shown that flavonoids express organ-specificity for cancer prevention, so intake of a quercetin–rich diet was proven to be in positive correlation with protection against lung and intestinal cancer (Lam et al., 2010; Ekström et al., 2011).

Flavonoids are present in nearly all edible fruits, vegetables and other foods of plant origin. Generally, the human population is consuming notable amounts of flavonoids on a daily basis being more in regions where the diet is mainly based on plant sources. It is estimated that the average daily intake of flavonoids in the United States of America is 20–34 mg, in Finland 24 mg, Japan 63 mg and Netherlands 73 mg (Beecher, 2003). The daily intake of quercetin in the Western diet was estimated to be approximately 15 mg. In Table 1–4 contents of dominant dietary flavonoids and quercetin in selected foods that are regularly consumed in Western diet are listed.

Table 1–4 Flavonoids and quercetin content of selected foods

food	mg of flavonoid aglycone*/ 100 g of fresh weight of edible portion	mg of quercetin aglycone/ 100 g of fresh weight of edible portion
red onion	56.61	39.21
onion	26.02	20.30
cranberry	132.08	14.84
blueberry	180.82	7.67
fig	8.07	5.47
lettuce	4.63	4.16
apple	15.15	4.01
spinach	11.44	3.97
broccoli	11.96	3.26
tomato	5.95	2.76
green tea	137.93**	2.49**
black tea	118.27**	2.19**
red wine	171.88***	2.11***
garlic	3.61	1.74
cauliflower	1.02	0.54
potato	0.49	0.49
strawberry	13.35	0.48
red cabbage	210.67	0.36
parsley	233.16	0.28
red pepper	0.86	0.23
carrot	0.6	0.21
banana	13.69	0.06
cucumber	0.17	0.04
eggplant	85.73	0.04
kiwi	2.18	0.04
white wine	2.22***	0.04***

*total content represents sum of 26 dominant dietary flavonodis (isorhamnetin, kaempferol, myricetin, quercetin, apigenin, luteolin, eriodictyol, hesperetin, naringenin, catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin–3–gallate, epigallocatechin–3–gallate, theaflavin, theaflavin–3–gallate, theaflavin–3'–gallate, theaflavin–3,3'–digallate, thearubigins, cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin)

**values for tea are given as mg/100 g (100 mL) of tea infusions, while tea was prepared with 1 g tea leaves/100 mL boiling water

***values are converted from liquid to weight basis

(Bhagwat et al., 2014)

1.6 The connections between quercetin and iron homeostasis

Flavonoids are known for their numerous health benefits which are mostly attributed to their ability to scavenge highly reactive free radical species. However, flavonoids' antioxidative potential is, at least partially, associated with their ability to chelate

iron. By chelating iron, flavonoids reduce the accessibility of iron to oxygen and consequently diminish oxygen high toxicity, e.g. by inhibiting production of $HO^{\bullet}$ in Fenton reaction (Mladěnka et al., 2011). Like most other flavonoids, it was proven that quercetin possesses a high ability to chelate iron (Leopoldini et al, 2006). As one of the main aims of this thesis is to investigate the effect of quercetin on iron homeostasis, this section will be mainly concentrated on quercetin in the light of iron metabolism. The preferred site for iron chelation by quercetin is its 3–hydroxyl and 4–carbonyl group. Specifically, for complexes containing one iron and one quercetin, the binding strength has an order 3–4 > 4–5 > 3'–4'. Moreover, the 3–4 chelation site is also preferred for complexes which are formed between one iron and two or three quercetin molecules (see Figure 1–11; Ren et al., 2008). In addition, it is estimated that quercetin, like most other flavonoids, forms a complex with Fe^{3+} with a greater stability than Fe^{2+}. Even though when quercetin initially forms a complex with Fe^{2+}, Fe^{2+} will autooxidise to Fe^{3+} (Perron and Brumaghim, 2009).

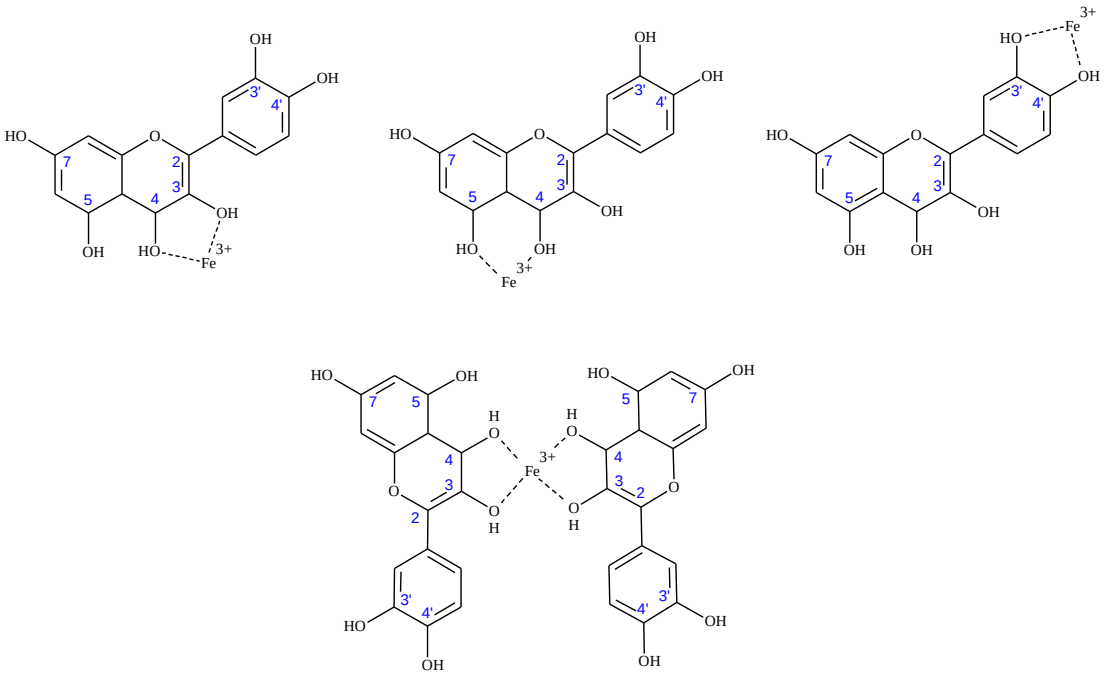


Figure 1–11 Structures of complexes between Fe^{3+} and quercetin

Considering that quercetin is a powerful chelator of iron, a couple of ways of how it can modulate iron homeostasis come to mind. Namely, intracellularly it could lower free iron and thus change the influence of the IRE/IRP regulatory system in iron homeostasis. Bearing in mind that many proteins that have a pivotal role in iron homeostasis, such as FPN, ferritin, DMT1, TfR1 and Hif–2α, are regulated by the IRE/IRP system (described in detail in1.4.1), the quercetin effect becomes even more important. Additionally, quercetin could inhibit iron absorption and redistribution during iron overload by chelating it and thus affecting iron status. Over 30 years ago it was shown that consumption of tea is in accordance with low non-haem iron bioavailability (Disler et al., 1975; Rossander et al., 1979). Consequently, flavonoids, or polyphenols, from the tea were recognized as the main cause for low non-haem iron absorption. Today, flavonoids, among them quercetin, are considered as one of the main dietary inhibitors of iron absorption in the duodenum. Even though the exact mechanism of how flavonoids inhibit non-haem iron absorption is still not fully elucidated, it is strongly believed that its power to chelate iron is mainly responsible for this action (Petry, 2014). In contrast, it was shown that quercetin may operate as a substrate for DcytB by increasing its reduction potential and providing more Fe^{2+} for cellular uptake by DMT1 (Vlachodimitropoulou et al., 2010). Furthermore, it has been proven in cells that quercetin is able to activate Nrf2 pathway by supporting its nuclear translocation and transcriptional activity (Granado–Serrano et al., 2012). In view of the fact that levels of FPN and H and L ferritin are also known to be transcriptionaly up regulated by Nrf2 pathway (described in detail in1.4.3), quercetin could affect iron homeostasis and help cells defending against oxidative stress.

In diseases connected with an imbalance in iron homeostasis (described in detail in Section 1.4.4) organ-specific iron accumulation is present, even under conditions of

anaemia. In order to bring iron levels back into balance, chelato therapeutics are applied. Potent chelato therapeutics should be able to go through iron-overloaded tissues, complex iron by forming stable and redox-inactive iron and transfer it to transferrin in the circulation. Known chelato therapeutic drugs fulfill more or less listed requirements (Poggiali et al., 2012; described in detail Section 1.4.4). However, it has been proven *in vitro* that quercetin is able to decrease intracellular iron and to transfer it to transferrin. These significant findings suggest that quercetin could be a valuable representative of chelato therapeutics for iron-redistribution therapy. Yet, this fact still needs to be proven with *in vivo* studies (Baccan et al., 2012). On the other hand, it is clear that quercetin should be avoided in IDA, especially during oral consumption of iron either as a natural constituent of the diet or as a food supplement.

All of the above mentioned possible impacts of quercetin on iron homeostasis become even more significant in view of the fact that quercetin is consumed regularly in considerable amounts (see Table 1–4) and that nowadays its supplementation is supported due to numerous health benefits. On the other hand, as imbalance in iron homeostasis is connected with many diseases, flavonoids may have important applications in their treatment. Hence it is of great importance to fully understand how dietary flavonoids interact with intake and homeostasis of iron. The main body of this thesis will try, at least partially, to address this issue.

1.7 Aims of study

The main aim of this thesis was to elucidate the mechanisms underlying the effects of polyphenols, mainly quercetin, on iron homeostasis *in vivo*.

The specific aim of each Chapter of this thesis was:

1. To investigate the effect of oral quercetin on iron metabolism, mainly iron absorption in duodenum, in an *in vivo* rat model (Chapter 3).

2. To investigate the chelation effect of polyphenols on non-haem iron absorption in duodenum applying an *in vivo* uptake method in rats (Chapter 4).

3. To investigate the effect of intraperitoneal (IP) quercetin on iron metabolism, mainly systemic iron homeostasis, in an *in vivo* rat model (Chapter 5).

4. To elucidate the effects of different polyphenols on iron-related genes expression in HepG2 cells, an *in vitro* hepatic model (Chapter 6).

5. To elucidate changes in iron-related and inflammation-related gene expression induced by different polyphenols in THP1 cells, a macrophage iron-recycling *in vitro* model (Chapter 7).

2. GENERAL MATERIALS AND METHODS

2.1 Chemicals and standards

chemical or standard	*supplier*	*catalogue number*
3,4'–dimethoxy–5,7,3'–trihydroxyflavone (3,4'–dimethylquercetin)	abcr GmbH, Germany	AB151842
3',4',5,7–tetrahydroxy–3–methoxyflavone (3–*O*–methylquercetin)	Extrasynthese, France	1342
^{55}Fe	PerkinElmer, USA	NEZ 043
^{59}Fe	PerkinElmer	NEZ 037
acetic acid (CH_3COOH)	Sigma–Aldrich, UK	ARK2183
acetone	Sigma–Aldrich	270725
Antibiotic/Antimycotic solution	Sigma–Aldrich	A5955
ascorbic acid	Sigma–Aldrich	95210
bathophenanthrolinedisulfonic acid disodium salt hydrate	Sigma–Aldrich	B1375
carbonyl iron	Sigma–Aldrich	C3518
chloroform	Sigma–Aldrich	C2432
diethyl pyrocarbonate (DEPC) treated water	Life Technologies Ltd., Ambion	AM9916
dimethyl sulfoxide (DMSO)	Sigma–Aldrich	D8418
DNase I	Life Technologies Ltd., Invitrogen	AM2222
DNase I Buffer	Life Technologies Ltd., Ambion	AM8170G
Dulbecco's phosphate–buffered saline (PBS; 1×) without Ca^{2+} and Mg^{2+}	PAA Laboratories Ltd., UK	H15–002
epicatechin	Extrasynthese	0977 S
ethanol	VWR, USA	20821.330
Foetal Bovine Serum (FBS)	Life Technologies Ltd., Gibco, USA	10270–106
FBS, heat inactivated	Life Technologies Ltd., Gibco	10500–064
formic acid (HCOOH)	Sigma–Aldrich	56302–50ML–F
genistein	Sigma–Aldrich	92136
GlutaMAX™–I	Life Technologies Ltd., Gibco	35050–038
HEPES sodium salt (Na–HEPES)	Sigma–Aldrich	H8651
hydrochloric acid (HCl)	Sigma–Aldrich	H1758
iron (III) chloride hexahydrate ($FeCl_3 \times 6H_2O$)	Sigma–Aldrich	F2877

iron-deficient diet	Special Diet Service, UK	
isopropyl alcohol	Sigma–Aldrich	I9516
isorhamnetin	Extrasynthese	1120 S
isorhamnetin–3–*O*–glucoside	Extrasynthese	1228
LPS	Enzo Life Sciences Inc., UK	ALX–581–008–L002
methanol	Sigma–Aldrich	34860
pentobarbitone sodium	Pentoject – obtained from Animalcare Ltd., UK	/
phorbol 12–myristate 13–acetate (PMA)	Sigma–Aldrich	P1585
potassium chloride (KCl)	VWR	BDH9258
quercetin	Sigma–Aldrich	Q4951
quercetin–3,3',4',7–tetramethylether (tetra–methylquercetin)	Extrasynthese	1074
quercetin–3,4'–di–*O*–glucoside	Extrasynthese	1347 S
quercetin–3,5,7,3',4'–pentamethylether (penta–methylquercetin)	Extrasynthese	1285
quercetin–3–*O*–glucuronide	Extrasynthese	1315
resveratrol	Extrasynthese	4963 S
RM1 diet	Special Diet Service, UK	
RPMI–1640 Medium	Sigma–Aldrich	R8758
sodium acetate (CH_3COONa)	Sigma–Aldrich	W302406
sodium chloride (NaCl)	VWR	27810.295
Solvable™	PerkinElmer	6NE9100
tamarixetin (4'–*O*–methylquercetin)	Extrasynthese	1140 S
thioglycolic acid	Sigma–Aldrich	T3758
trichloroacetic acid (CCl_3COOH)	Sigma–Aldrich	T4885
TRIzol® reagent	Life Technologies Ltd., Ambion	10296–028
Trypsin EDTA (1×) 0.05%/0.02% in Dulbecco's phosphate–buffered saline	PAA Laboratories Ltd.	L11–004
William's E Medium, without phenol red, without L–Glutamine	PAA Laboratories Ltd.	E15–073

2.2 Animal care and treatments

Rats were supplied by the Comparative Biology Unit, Royal Free Campus, UCL Medical School, London, UK. All experimental procedures were approved by the University College London local animal ethics committee and were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986. In all

experiments male Sprague Dawley (SD) rats were used. After weaning (three weeks old) SD rats were placed on a low iron (25 ppm iron) diet or a regular (RM1, 156 ppm iron) diet for two weeks and allowed free access to water throughout. Subsequently, animals were subject to different gavage or IP treatments with quercetin or 10% DMSO. Additionally, separate groups of animals had no specified treatments prior to uptake studies (described in detail in Section 2.3). In Figure 2–1 time line of performed treatments and in Table 2–1 and Table 2–2 a detailed description of performed treatments is given. At the end of the experimental procedure animals were killed by administering a terminal dose of pentobarbitone sodium (120 mg/kg body weight, IP) and blood samples were removed via cardiac puncture. Subsequently, serum was separated from clotted blood sample after centrifugation for 10 minutes at 5000*g*, rapidly frozen in liquid nitrogen before being stored at -80ºC and afterwards used for serum iron and transferrin saturation measurements (described in detail in Section 2.4). Additionally, duodenum, liver, spleen and kidney were removed and rapidly frozen in liquid nitrogen before being stored at -80ºC and subsequently used for tissue non-haem iron measurements (described in detail in Section 2.5) and gene expression levels determination (described in detail in Section 2.5). However, animal groups 5 and 6 (see Table 2–1) and 11 and 12 (see Table 2–2) were the subject of uptake studies (described in detail in Section 2.3) and were neither killed by the specified procedure nor blood or tissue samples were collected as detailed above.

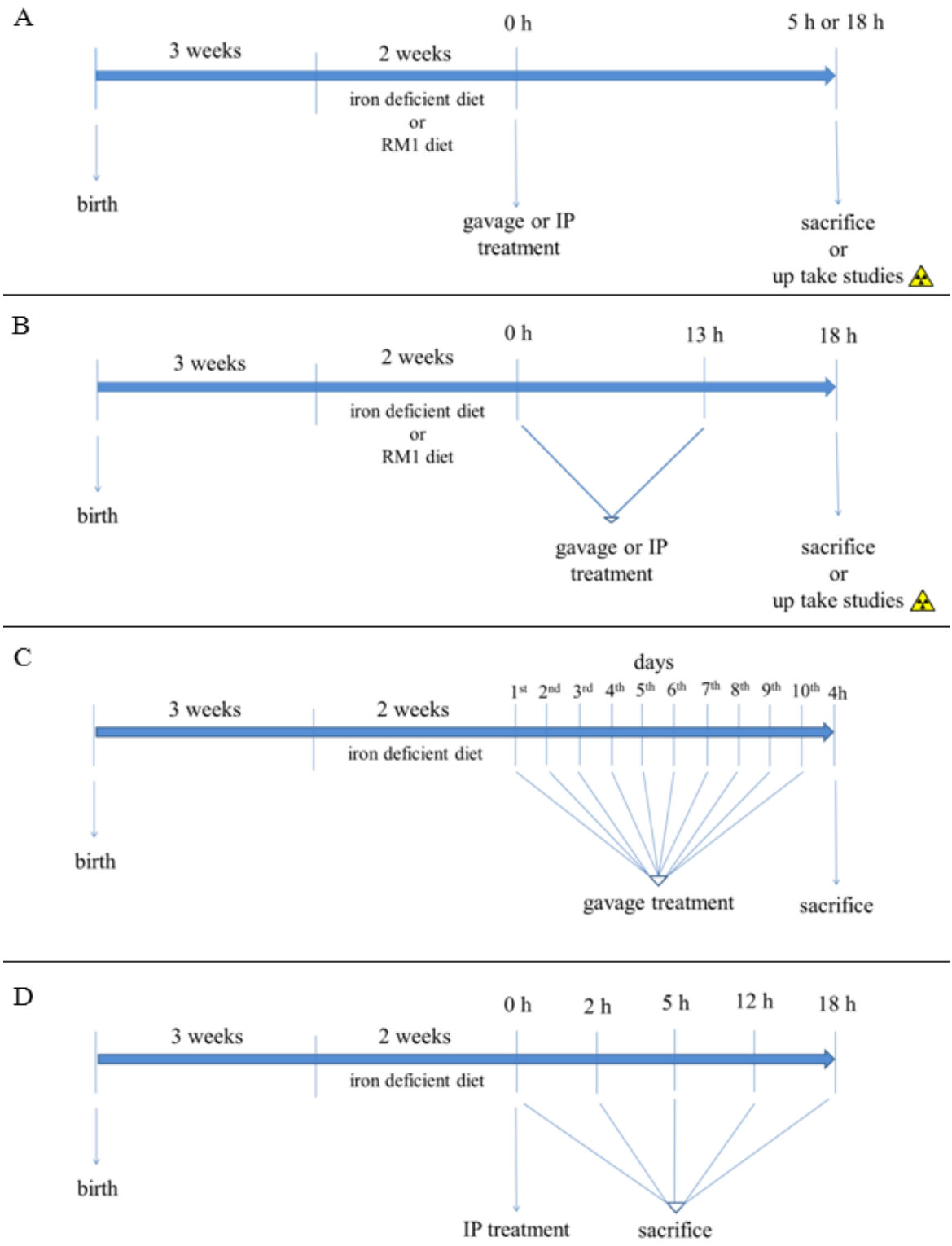


Figure 2–1 Time line of performed treatments

Table 2–1 Detailed description of performed gavage treatments

group		type of treatment	time of treatment	number of animals per group	type of diet	corresponding treatment from Figure 2–1
1	control	10% DMSO	5 hours prior to dissection	5	iron-deficient diet	A
	treated	quercetin 50 mg/kg		5		
2	control	10% DMSO	18 hours prior to dissection	4	iron-deficient diet	A
	treated	quercetin 50 mg/kg		4		
3	control	10% DMSO	18 hours prior to dissection	3	RM1 diet	A
	treated	quercetin 50 mg/kg		3		
4	control	10% DMSO	10 days in the row; the 10th day treatment was performed 4 hours prior to dissection	5	iron-deficient diet	A
	treated	quercetin 50 mg/kg		5		
5☢*	control	10% DMSO	5 hours prior to uptake experiment	5	iron-deficient diet	C
	treated	quercetin 50 mg/kg		5		
6☢*	control	10% DMSO	18 hours and 5 hours prior to uptake experiment	5	iron-deficient diet	B
	treated	quercetin 50 mg/kg		5		

administration by gavage

*animals were subject to uptake studies (described in detail in Section 2.3)

Table 2–2 Detailed description of performed IP treatments

group			type of treatment	time of treatment	number of animals per group	type of diet	corresponding treatment from Figure 2–1
7	treated	administration by IP	quercetin 50 mg/kg	0 hours prior to dissection	4	iron-deficient diet	D
	treated			2 hours prior to dissection	4		
	treated			5 hours prior to dissection	4		
	treated			12 hours prior to dissection	4		
	treated			18 hours prior to dissection	4		
8	control		10% DMSO	18 hours and 5 hours prior to dissection	5	iron-deficient diet	B
	treated		quercetin 50 mg/kg		5		
9	control		10% DMSO	18 hours and 5 hours prior to dissection	4	RM1 diet	B
	treated		quercetin 50 mg/kg		4		
10	control		10% DMSO	5 hours prior to dissection	4	iron-deficient diet	A
	treated		quercetin 2 mg/kg		4		
	treated		quercetin 5 mg/kg		4		
	treated		quercetin 10 mg/kg		4		
	treated		quercetin 20 mg/kg		4		
11☢*	control		10% DMSO	5 hours prior to uptake experiment	5	iron-deficient diet	A
	treated		quercetin 50 mg/kg		5		
12☢*	control		10% DMSO	18 hours and 5 hours prior to uptake experiment	5	iron-deficient diet	B
	treated		quercetin 50 mg/kg		5		

*animals were subject to uptake studies (described in detail in Section 2.3)

2.3 *In vivo* iron uptake

After the animal care procedure and treatments (described in detail in Section 2.2), groups of SD rats were subjected to uptake studies. Namely, SD rats were anesthetized with 60 mg/kg pentobarbitone sodium IP and 10 cm long segments of duodenum (starting 1 cm distal to the pylorus) were cannulated and rinsed free of their contents with warm saline (0.9% w/v of NaCl), followed by air. Uptake buffer (200 µL), containing Na–HEPES (14.6 mmol/L), NaCl (127.4 mmol/L), KCl (3.2 mmol/L), ascorbic acid (4.0 mmol/L) and $^{59}Fe^{2+}$ (^{55}Fe; 0.2 mmol/L) was instilled into the duodenal segment, which was then tied off. However, in experiments with SD rats that were not treated prior to uptake studies, 1 mmol/L of either quercetin or 3–*O*–methylquercetin or 4'–*O*–methylquercetin or 3,4'–dimethylquercetin or penta–methylquercetin or resveratrol (stock solutions prepared in DMSO:ethanol [1:1]) was added to uptake buffer. In the corresponding control groups, DMSO:ethanol (1:1) was added to buffer instead of the polyphenols. The effect of each polyphenol on iron absorption was investigated in a group of five SD rats. In uptake studies with quercetin, 3–*O*–methylquercetin, 4'–*O*–methylquercetin, 3,4'–dimethylquercetin and penta–methylquercetin ^{59}Fe was used. However, due to a change in regulations at the time when the experiment with resveratrol was performed instead of ^{59}Fe, ^{55}Fe was used.

During the experiment rat body temperature was maintained at 37°C using a thermostatically controlled heating blanket. After 30 minutes, blood samples (≤2 mL) were collected via cardiac puncture and put in pre-weighed tubes, and blood weight was determined afterwards. In the experiment where ^{55}Fe was used, after 30 minutes 1 mL blood was also removed via cardiac puncture and but placed in tubes

containing anticoagulant. The segment of duodenum was removed and washed with approximately 40 mL of solution containing 154 mmol/L NaCl, 0.1 mmol/L ascorbic acid, 0.01 mmol/L $FeCl_3$ to displace any ^{59}Fe or ^{55}Fe bound to the mucosal surface. The duodenal segment was cut longitudinally to form a flat sheet, and the mucosa was removed by scraping, placed into a pre-weighed tube, and its weight determined. Appropriate blood and mucosa samples were gamma counted (Wallac 1282 Compugamma Counter Model 1283) for the determination of ^{59}Fe activity. However, in the experiment where ^{55}Fe was used mucosa was digested in 2 mL Solvable™ and a 50 μL sample of the digestant was counted on the liquid scintillation counter (Packard Scintillation Counter Model 2900) in duplicate for the determination of ^{55}Fe activity. In the corresponding animal group blood was centrifuged and a 50 μL aliquot of plasma was counted in duplicate to determine ^{55}Fe activity. Results were expressed as a percentage of absorbed radioactive iron retained in duodenal mucosa or transferred to blood. Namely, it was considered that absorbed radioactive iron is distributed between mucosa of cannulated duodenum and total body blood, and that sum of their counts is 100%. The percentage of ^{59}Fe or ^{55}Fe transferred to the entire blood volume of the animal was calculated using the equation: total blood volume = (body weight*0.06) +0.77 (Lee and Blaufox, 1985). In Figure 2–2 the experimental procedure is presented schematically.

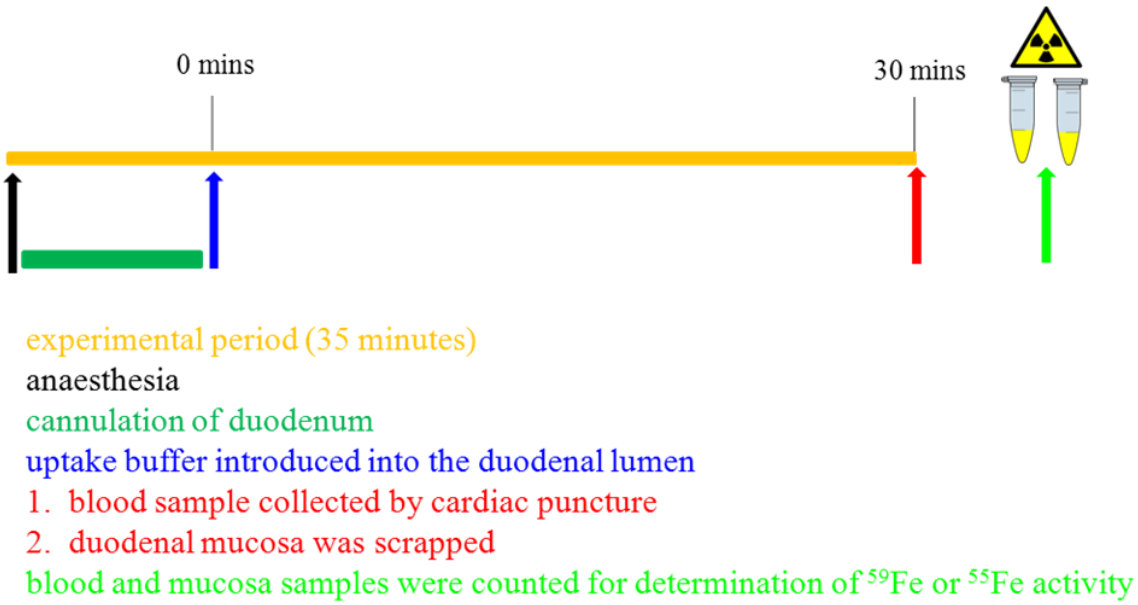


Figure 2–2 Time line of uptake experiment

2.4 Serum iron and transferrin saturation measurements

Serum iron and transferrin saturation were measured by using a commercial kit (Pointe Scientific Inc., USA cat. no. I7504) as instructed by the manufacturer and described below.

2.4.1 Measurement of serum iron

All samples, blank and standard probes were prepared for spectrophotometric measurements in duplicate in 96-well plate. Forty microliters of each standard, serum sample and water was added into standard, sample and blank wells. Iron buffer reagent (200 µL) was added to each well and the absorbance reading (A1) was taken at 560 nm by a plate reader (Thermo/LabSystems 352 Multiskan MS Microplate Reader) after mixing. Iron colour reagent (4 µL) was added to each well and plate was incubated at 37ºC for 10 minutes. Again the absorbance was measured at 560 nm named A2 and the serum iron was calculated with the following equation:

(A2 sample - A1 sample)/(A2 standard - A1 standard) × 500 = serum iron (μg/dL)

2.4.2 Determination of transferrin saturation levels

All samples, blank and standard probes were prepared for spectrophotometric measurements in duplicate in 96-well plates. Forty microliters of each standard and sample was added to standard and sample wells. Eighty microliters of water was added to the blank well and 40 μL of water was added to standard well. 40 μL of iron standard was added to the sample wells. Unsaturated iron binding capacity (UIBC) reagent (160 μL) was added to each well and after mixing the absorbance reading (A1) was measured at 560 nm. Iron colour reagent (4 μL) was added to each well and plate was incubated at 37ºC for 10 minutes. Again the absorbance was measured at 560 nm named A2. UIBC, total iron binding capacity (TIBC), and transferrin saturation were measured by using the following equations:

500 - (A2 sample - A1 sample)/(A2 standard - A1 standard) × 500 = UIBC (μg/dL)

serum iron + UIBC = TIBC (μg/dL)

serum iron/TIBC × 100 = transferrin saturation (%)

2.5 Tissue non-haem iron determination

The tissue non-haem iron content was determined by using a modified method of a method firstly reported by Torrance and Bothwell (1980). Namely, tissues (duodenum, liver, spleen) were oven dried at 50ºC for 72 hours and subsequently weighed. The dried tissues were digested in 1 mL of acid mixture (30% HCl and

10% CCl_3COOH) at 65°C over 20 hours. Blanks were prepared in the same way, but without tissue. After cooling, samples, blank and standard probes were prepared for spectrophotometric measurements in triplicate in plastic cuvettes as presented in Table 2–3 Preparations of samples for tissue non-haem iron determination.

Table 2–3 Preparations of samples for tissue non-haem iron determination

	sample/blank µL	working chromogen reagent (mL)	dH_2O µL	working iron solution (µL)
sample	25 sample	1	225	0
standard	25 blank	1	100	125
blank	25 blank	1	225	0

All samples were incubated at 37°C for 10 minutes, after which measurements of absorbance (A) were made at 535 nm using a spectrophotometer (Cary 100, Agilent Technologies) and non-haem levels in tissues were calculated using the following equation:

[(A sample - A blank)/(A standard - A blank)]×[11.169/dry weigh (dw; g)] × [(2500/150)/(2500/250)] = tissue non-haem iron (µg/g dw)

The working chromogen reagent and working iron solution were freshly prepared just before the assay. The composition of the reagents was as follows:

Chromogen reagent was prepared by mixing 50 mg bathophenanthrolinedisulfonic acid disodium salt hydrate and 500 µL of thioglycolic acid and afterwards made up to 50 mL of dH_2O (stable for 1 month in dark bottle).

Working chromogen reagent was prepared on the day of experiment by mixing one volume of previously described chromogen reagent, five volumes of saturated solution of CH_3COONa and five volumes of dH_2O.

Stock iron standard solution was prepared by mixing 22.3 mg of carbonyl iron powder with 1096 µL HCl. Afterwards, solution was left to stand overnight and made up to 20 mL with dH_2O (stable indefinitely when stored tightly sealed).

Working iron solution was prepared on the day of experiment by mixing 50 µL of stock iron standard solution, 27 µL of HCl and 423 µL dH_2O.

2.6 Cell culture

2.6.1 HepG2 cells

HepG2 cells were cultured in William's E medium without phenol red and L–glutamine. The medium was supplemented with 10% (v/v) FBS, 2 mM L–glutamine (1% v/v GlutaMAX™–I) 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B (1% v/v Antibiotic/Antimycotic solution). All supplements were filtered through a 0.2 µm pore size filter (Merck Millipore, USA, Cat no. SLGS033SB). The cell were seeded onto 75 cm^2 cell plastic culture flasks with vent cap (Corning, USA, cat. no. 430641) and grown in an incubator at 37ºC with 5% CO_2 until they were 70–75% confluent. The culture medium was changed every two days. To subculture the HepG2 cells the culture medium was carefully removed and the adherent cells were washed three times with 10 mL PBS without Ca^{2+} and Mg^{2+}. To release the cells from the flask 10 mL of 0.05% Trypsin–EDTA was added for 10 minutes. Detached cells were transferred into a 50 ml sterile tube (Thermo Fisher Scientific Inc., USA, Cat. no. 352070) containing 10 mL of culture medium in order to deactivate trypsin activity and pelleted by centrifugation (1400*g* for 3 minutes). The supernatant was removed and the cell pellet was re–suspended in 10 mL of fresh

culture medium. To disperse the cells further, the cell suspension was filtered through a 40μm sterile cell strainer (Corning, USA, cat. no. 352340) on top of a 50 mL sterile tube. Cell numbers and percentage of viable cells were determined by using the haemocytometer and the trypan blue exclusion test.

2.6.1.1 Treatment of HepG2 cells

Prior to treatments cells were plated in 6-well plates (Greiner Bio–One, USA, cat no. 657160) with seeding density 5×10^5 (viable cells/well). HepG2 cells were treated with polyphenols at 90% confluence. Polyphenols, epicatechin, 3–*O*–methylquercetin, isorhamnetin, tamarixetin, 3,4'–dimethylquercetin, penta–methylquercetin, quercetin–3–*O*–glucuronide, resveratrol and quercetin were dissolved in serum–free culture medium in concentration of 100 μM, and were added to the cell plates for 5 hours. In control wells for 3–*O*–methylquercetin and resveratrol an equal volume of ethanol was added instead of the compound (final concentration 0.1%); for epicatechin, isorhamnetin, tamarixetin, 3,4'–dimethylquercetin, quercetin–3–*O*–glucuronide, quercetin an equal volume of DMSO was added instead of the compound (final concentration 0.125%); for penta–methylquercetin equal volume of ethanol:DMSO was added instead of compound (final concentration 0.09% ethanol/0.09% DMSO). All probes were done in triplicate. After treatment cells were washed 3 times in PBS and were subject to RNA isolation and gene expression (hepcidin, FPN and HO–1) analysis (described in detail in Section 2.7).

2.6.2 THP1 cells

The THP1 cells were cultured in RPMI–1640 with L–Glutamine and $NaHCO_3$. The medium was supplemented with 10% (v/v) heat inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B. All supplements were filtered through a 0.2 μm pore size filter. The cells were seeded onto 75 cm^2 straight neck vented tissue culture flasks (Thermo Scientific, Nunc, USA, cat. no. 153732) and grown in an incubator at 37ºC with 5% CO_2. The THP1 cells remained in cell suspension and were maintained between 2–9 x 10^5 cells/mL. To subculture the cells, the cell suspension was collected in a 50 mL sterile tube and pelleted by centrifugation (1400*g* for 3 minutes). The supernatant was removed and the pellet was re-suspended in 5 mL of culture medium. Cell numbers and percentage of viable cells were determined by using the haemocytometer and the trypan blue exclusion test.

2.6.2.1 Treatment of THP1 cells

Prior to treatments cells were plated in 6-well plates for 24 hours (Greiner Bio–One, USA, cat no. 657160) with seeding density 1×10^6 (cells/well). 100 nM PMA in DMSO was added for a further 24 hours to promote differentiation of monocytes into macrophages. The PMA-containing medium was removed and replaced with serum–free medium for 24 hours before treatment.

THP1 cells were treated with quercetin, quercetin–3–*O*–glucuronide and tamarixetin (30 μM) for 0, 5 and 18 hours. In control wells an equal volume of DMSO was added instead of the compound (final concentration 0.04%). All probes were done in

triplicate. After treatment, cells were subject to RNA isolation and gene expression (hepcidin and FPN) of analysis (described in detail in Section 2.7).

Other batches of THP1 cells were treated with LPS in the presence or absence of quercetin, isorhamnetin and quercetin–3–*O*–glucuronide (30 μM). Namely, LPS (500 ng/mL, dissolved in medium) was added to cells 2 hours after polyphenols. Cells were treated with LPS for 18 hours. In control wells an equal volume of DMSO was added instead of the compound (final concentration 0.04%). All probes were done in triplicate. After treatment cells were subject to RNA isolation and gene expression (IL–6, IL–1β, TNF–α, inducible nitric oxide synthase (iNOS) and cyclooxygenase–2 (COX–2)) analysis (described in detail in Section 2.7).

2.7 Gene expression levels by Real–Time Polymerase Chain Reaction (RT–PCR)

2.7.1 RNA extraction by TRIzol® reagent

Precautions were taken against contamination of samples with RNAses. The bench working area was cleaned with RNaseZAP (Life Technologies Ltd., Ambion, USA AM9780 and AM9784), disposal gloves were worn at all times, and sterile disposable plasticware and pipettes for RNA work only were used.

RNA extraction was carried out using TRIzol®/chloroform extraction and isopropyl alcohol precipitation according to the manufacturer's instructions (Chomczynski and Sacchi, 1987). TRIzol® reagent was added to the cells or frozen tissue (1 mL was used per well of six well plate or 100 mg tissue) in sterile 1.5 mL micro centrifuge tube. Cell samples were homogenized by repeatedly passing the cell suspension

through a syringe and needle (BD, UK, Cat no. 300600, 25 G (orange)). Tissue samples were homogenized by pellet pestles (Sigma–Aldrich, UK, Cat no. Z359971 and Z359947). Samples were then incubated at room temperature for 5 minutes for complete dissociation of nucleotide/protein complexes. Chloroform (200 µL) was added to the samples and samples were shaken vigorously by hand for 15 seconds. Tubes were incubated at room temperature for 3 minutes and then centrifuged at 12 000*g* for 15 minutes at 4ºC. The mixture separated into a lower red, phenol-chloroform phase, which contained DNA and protein, and a colourless upper aqueous phase containing the RNA. The aqueous phase was carefully removed with a pipette and transferred to a clean 1.5 mL tube and the lower organic phase was discarded.

The RNA was precipitated form the aqueous phase with isopropyl alcohol (500 µL) by inverting the tubes. Samples were incubated at room temperature for 10 minutes and then centrifuged at 12000*g* for 15 minutes at 4ºC. The supernatant was discarded and the RNA pellet was washed with 1.2 mL 70% ethanol by vortexing followed by centrifugation at 7500*g* for 5 minutes at 4ºC. Ethanol was discarded and the final RNA pellet air–dried. RNA pellet was resuspended in DEPC-treated water (approximately 50 µL for cell sample and 500 µL for tissue sample) and kept at 60ºC for 10 minutes. After this, RNA samples were kept on ice at all times.

The concentration of extracted RNA was measured using a NanoDrop 2000c UV–VIS spectrophotometer, Thermo Scientific. RNA quality was assessed by using the A260/280 ratio. RNA with a ratio between 1.8 and 2.00 indicates RNA free of protein contamination. The RNA samples were kept at -80ºC until required.

2.7.2 Complementary DNA (cDNA) synthesis

In order to prevent DNA contamination, firstly RNA samples were incubated in a thermal cycler (MJ Research PTC–200) with DNase I (RNase-free) enzyme for 30 minutes and an additional 10 minutes at 37ºC and 70ºC, respectively. A total reaction mixture consisted of 1 µg of RNA, 1 µL DNase I Buffer, 0.5 µL DNase I and made up by DEPC treated water to 11 µL for each sample. As soon as treatment was finished samples were placed at 4ºC until required.

After DNase I treatment cDNA synthesis was performed using a VersoTM cDNA kit (Thermo Scientific, Cat no. AB–1453/B). The following components were added into each tube: 4 µL 5 × cDNA synthesis buffer, 2 µL dNTP mix, 1 µL anchored Oligo dT, 1 µL RT enhancer and 1 µL Verso enzyme mix. The tubes were places back into the thermal cycler for further 60 and 2 minutes incubation periods at 42ºC and 95ºC, respectively. Samples were kept at 4ºC for immediate use or at -20ºC to be used later.

2.7.3 Real-Time PCR amplification

Real-time quantitative gene analysis was performed using a LightCycler® 480 System (Roche Diagnostics GmbH, Germany) and a LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, Cat no. 04707516001). SYBR green is a fluorescent dye that binds to the minor groove of DNA and fluoresces only when bound to double stranded DNA, but not single stranded DNA. SYBR green is excited at 494 nm and emits light at 521 nm. Monitoring the emission at 521 nm allows indirect quantification of double stranded DNA in the reaction tube. Fluorescence was measured at 521 nm within each reaction tube of a 96-well plate following completion of every PCR extension step. The PCR cycle at which the fluorescence

reaches the threshold value is used as a measure of relative template concentration. The second derivative maximal method was used to determine threshold values of fluorescence. Cycle threshold (Ct) values were obtained for each gene of interest and the glyceraldehyde 3–phosphate dehydrogenase (GAPDH) internal standard. Gene expression was normalized to GAPDH and represented as ΔCt values. For each sample the mean of the ΔCt values was calculated. Relative gene expression was normalized to 1.0 (100%) of controls. Each PCR reaction contained 0.3 μM of each specific primer, forward and reverse, 6 μL of SYBR Green I Master, 1 μL of cDNA made up by DEPC treated water to 11 μL for each sample. Samples without cDNA were included as negative controls. The primers for genes of interest were synthesized by Sigma Aldrich. The primers for the internal standard gene GAPDH was obtained from Primer Design, UK (Cat no. HK–SY–ra–600). For the primers' sequences see

Table 2–4 Primer sequences used in this study. qPCR plates were centrifuged and placed in a LightCycler® 480 System real-time cycler with the following programme:

step	temperature	time	cycles
initial denaturation	95ºC	10 minutes	1
denaturation	95ºC	10 seconds	50
annealing	60ºC	10 seconds	
extension	72ºC	10 seconds	
metltcurve	95ºC	5 seconds	1
cooldown	65ºC	1 minute	1

Table 2–4 Primer sequences used in this study

gene	forward (5'→3')	reverse (5'→3')
Dcytb (rat)	TCCTGAGAGCGATTGTGTTG	TTAATGGGGCATAGCCAGAG
DMT1 (rat)	GCTGAGCGAAGATACCAGCG	TGTGCAACGGCACATACTTG
ferritin (rat)	CACTCTTCCAGGATGTGCAG	ACAGAGGTGAGGGTCTGTGC
FPN (rat)	TTCCGCACTTTTCGAGATGG	TACAGTCGAAGCCCAGGAC
GST (rat)	AGACATCCACCTGCTGGAAC	GGCTGCAGGAACTTCTTCAC
hepcidin (rat)	AGACACCAACTTCCCCATATG	ACAGAGACCACAGGAGGAATTCT
Hif–1α (rat)	TGCTTGGTGCTGATTTGTGA	GGTCAGATGATCAGAGTCCA
Hif–2α (rat)	CCCCAGGGGATGCTATTATT	GGCGAAGAGCTTCTCGATTA
OH–1 (rat)	TGCTCGCATGAACACTCTG	TCCTCTGTCAGCAGTGCC
QR (rat)	GCTTTCAGTTTTCGCCTTTG	GAGGCCCCTAATCTGACCTC
COX–2 (human)	CAGCACTTCACGCATCAGTT	CGCAGTTTACGCTGTCTAGC
FPN (human)	CAGTTAACCAACATCTTAGC	AAGCTCATGGATGTTAGAG
GAPDH (human)	TGGTATCGTGGAAGGACTC	AGTAGAGGCAGGGATGATG
hepcidin (human)	CTGCAACCCCAGGACAGAG	GGAATAAATAAGGAAGGGAGG
IL–1β (human)	TGAGCTCGCCAGTGAAATGA	CATGGCCACAACAACTGACG
IL–6 (human)	AGTACCCCCAGGAGAAGATTCC	TGAAGAGGTGAGTGGCTGTCTGT
iNOS (human)	AAAGACCAGGCTGTCGTTGA	ACGGGACCGGTATTCATTCT
OH–1 (human)	GTTGGCACCATGGAGCGTCCG	AGCCGTCTCGGGTCACCTGG
TNF–α (human)	TGGCGTGGAGCTGAGAGATA	TGGGTGAGGAGTACATGGGT

2.8 LC–MS/MS analysis of quercetin and selected quercetin metabolites in rat serum

2.8.1 Preparation of serum

In order to remove proteins from serum prior to LC–MS/MS analysis, acetone (50 µL) and CH_3COOH (2 µL) were vigorously mixed with serum (50 µL). Additionally, 1 µL genistein (1 µg/mL in methanol) was added to the mixture as an internal standard. The mixture was centrifuged at 7000*g* for 15 minutes; the supernatant was removed and was subject to further LC–MS/MS analysis.

2.8.2 LC–MS/MS analysis

Analysis was performed on an Agilent Technologies 1200 Series high–performance liquid chromatograph coupled with Agilent Technologies 6410A Triple Quad tandem mass spectrometer with electrospray ion source, and controlled by Agilent Technologies MassHunter Workstation software – Data Acquisition (ver. B.03.01). Separation was carried out using a Zorbax Eclipse XDB–C_{18} analytical column (4.6 × 50 mm, 1.8 µm particle size). The column was maintained at 50ºC and a binary gradient separation was performed using a flow rate of 1 mL/min. The mobile phase consisted of 0.05% HCOOH in water (A) and 100% methanol (B). The gradient profile started at 30% B, reaching 70% B in 6.00 minutes, then 100% B at 9.00 minutes, holding 100% B until 12.00 minutes, with a post-time of 3 minutes. The injection volume was 10 µL and the autosampler needle was washed with acetonitrile between injections to eliminate carryover. Eluted components were detected by MS, using the ion source parameters as follows: nebulization gas (N_2) pressure 60 psi,

drying gas (N_2) flow 11 L/min and temperature 350ºC, capillary voltage 4 kV. All compounds were quantified in selected reaction monitoring mode. Compound–specific, optimized MS/MS parameters are given in Table 2–5. For all the compounds, peak areas were determined using the Agilent MassHunter Workstation Software – Qualitative Analysis (ver. B.03.01). Calibration curves were plotted and samples' concentrations calculated using OriginLab's Origin Pro (ver. 8.0) software.

Table 2–5 Optimized LC–MS/MS parameters

compound	retention time (min)	ionisation mode	fragmentor voltage (V)	precursor ion (*m/z*)	product ion (*m/z*)*	collision energy (V)*
quercetin–3,4'–di–*O*–glucoside	1.46	ni	200	625	463, 301	15, 35
quercetin–3–*O*–glucuronide	2.11	ni	145	477	301, 151	20, 20
isorhamnetin–3–*O*–glucoside	2.94	ni	180	477	314, 243	30, 45
quercetin	3.65	ni	130	301	151	20
genistein	4.09	ni	145	269	133	30
tamarixetin/isorhamnetin	4.76	ni	150	315	300	20
penta–methylquercetin	6.17	pi	175	373	312, 357	25, 30

ni – negative ionization
pi – positive ionization
*second number represents qualifier ion parameter

2.9 Statistical analysis

All quantitative data are presented as mean ± standard error of the mean (SEM) of at least three different independant trials. Statistical significant difference between two groups through all study was determined using the Student's two-tailed unpaired t-test. Statistical significance was set at $p \leq 0.05$. Charts were drawn using Microsoft Office Excel 2007. Graphs were drawn using Origin software version 8.0.

3. EFFECT OF ORAL QUERCETIN ON IRON HOMEOSTASIS

3.1 Introduction

In mammals iron excretion is not regulated, therefore regulation of iron homeostasis is tightly controlled by intestinal iron absorption, so it matches daily obligatory loses. This is particularly important throughout periods of high iron demand, such as growth of infants, children and adolescents and the reproductive period for women, particularly in pregnancy, during which poor iron absorption can lead to impairment of health, in both mother and child (Papanikolaoua and Pantopoulos, 2005; Abbaspour et al., 2014). It is important that systemic levels of iron must be balanced within a narrow range, as both iron overload and iron deficiency are detrimental to human health leading to metabolic, neurodegenerative and haematological disorders.

There are two forms of iron, haem and non-haem iron. Non-haem iron availability in duodenum is regulated by a number of dietary factors, which either enhance or inhibit absorption in the duodenum. Non-haem iron is mainly present as the Fe^{3+} form which is very poorly bioavailable. In order for this to be available/absorbed, Fe^{3+} iron must be reduced to Fe^{2+} form. This is achieved by the combined action of Dcytb, a ferrireductase that is present on the apical membrane of the duodenal enterocyte or dietary reducing agent, such as dietary ascorbic acid. Reduced iron in the Fe^{2+} form is then transported across the apical iron symporter, DMT1. Fe^{2+} is then transferred across the basolateral membrane via an iron exporter, FPN, reoxidised by a membrane-attached ferroxidase, Heph, and loaded on to Tf.

In addition to dietary enhancers there are number of components in our diet which have an inhibitory effect on non-haem iron absorption in duodenum, included amongst these are phytic acid and polyphenols. This property of polyphenols is mostly attributed to their ability to chelate iron. The inhibitory effect of polyphenols has been demonstrated in single meal studies in human volunteers and acute *in vitro* studies. The long term effect of consuming elevated levels of polyphenols on iron status and mechanism of action is not clear (Disler et al., 1975; Rossander et al., 1979; Hurrell et al., 1999; Hurrell and Egli, 2010; Petry, 2014).

The flavonol quercetin is the most abundant flavonoid in our diet and is especially enriched in onions, tea and apples.

The aims of this study were to investigate the acute and long term effect of oral quercetin on iron metabolism *in vivo* (in rats) and to determine the mechanism of how quercetin influences iron absorption in rats.

3.2 Results

3.2.1 Acute effect of quercetin on iron metabolism *in vivo*

The acute effect of quercetin on iron methabolism was studied *in vivo* in rats 5 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control). After weaning rats were placed on low iron diet for two weeks. At the end of the experimental procedure animals were whether killed by administering a terminal dose of pentobarbitone sodium and blood samples were removed via cardiac puncture or were subject to uptake studies. Regarding first animal group, serum was separated and used for serum iron and transferrin saturation measurements using stadard spectrophotometric methods. Additionally, duodenum, liver and spleen were removed and subsequently used for tissue non-haem iron spectrophotometric measurements and gene expression levels determination. Uptake studies comprised of instaling $^{59}Fe^{2+}$ inside the cannulated duodenum for 30 minutes while rats were anesthetized. After 30 minutes, blood samples were collected via cardiac puncture and duodenal mucosa was scraped away and subsequently gamma counted for determination of ^{59}Fe activity.

3.2.1.1 Acute effect of quercetin on serum iron and transferrin saturation

Serum iron and transferrin saturation levels were significantly decreased in rats treated with a single dose of quercetin (50 mg/kg) by gavage five hours before being used for experiments (see Figure 3–1).

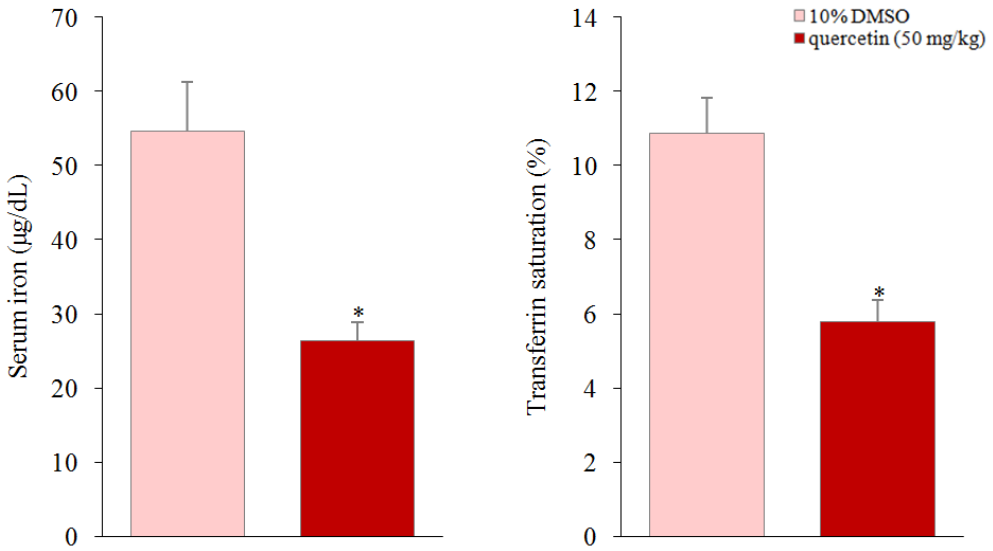


Figure 3–1 Acute effect of orally administered quercetin on serum iron and transferrin saturation in rats.
The acute effect of quercetin on serum iron and transferrin saturation were measured 5 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control) to rats. Data are mean ± SEM; n=5 rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

3.2.1.2 Acute effect of quercetin on duodenal iron transporters and Dcytb gene expression and iron content

Five hours after oral administration of a single dose of quercetin, quantitative RT–PCR analysis of rat duodenal iron transporters and Dcytb revealed a significant up-regulation of DMT1. But no change in expression in any of the other genes was observed (see Figure 3–2). In addition, no significant difference was observed in duodenal iron levels between the treated and control groups (see Figure 3–3).

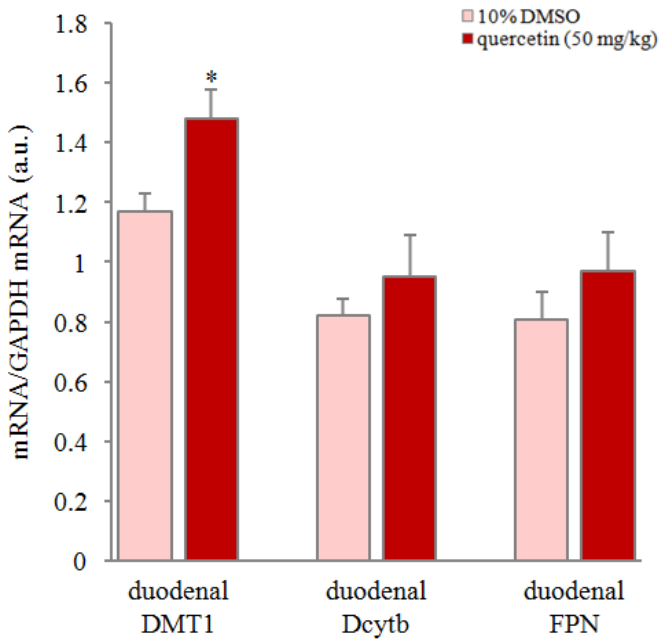


Figure 3–2 Acute effect of orally administered quercetin on duodenal iron transporters and Dcytb gene expression in rats.
Rats were given a single gavage containing quercetin (50 mg/kg body weight) or DMSO 10% (control). After 5 hours, RNA was isolated from duodenal tissue to measure changes in iron transporters and Dcytb mRNA levels. Data are mean ± SEM; n=5 rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

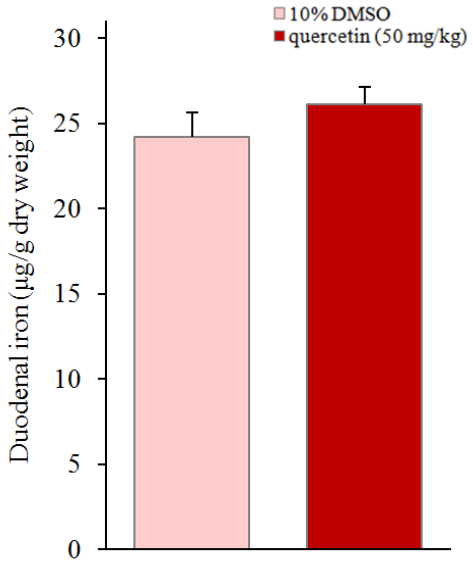


Figure 3–3 Acute effect of orally administered quercetin on duodenal iron content in rats.
The acute effect of quercetin on duodenal iron content was measured 5 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control) to rats. Data are mean ± SEM; n=5 rats per group.

3.2.1.3 Acute effect of quercetin on liver hepcidin, FPN and HO–1 gene expression and iron content

Analysis of hepcidin, FPN and HO–1 gene expression in liver of rats sacrificed five hours after oral treatment with quercetin, showed a significant, more then double, reduction in hepcidin mRNA levels (see Figure 3–4). However, expression of FPN and HO–1 mRNA stayed the same after the same treatment (see Figure 3–4). In addition, no difference was observed in liver iron levels between the treated and control groups (see Figure 3–5).

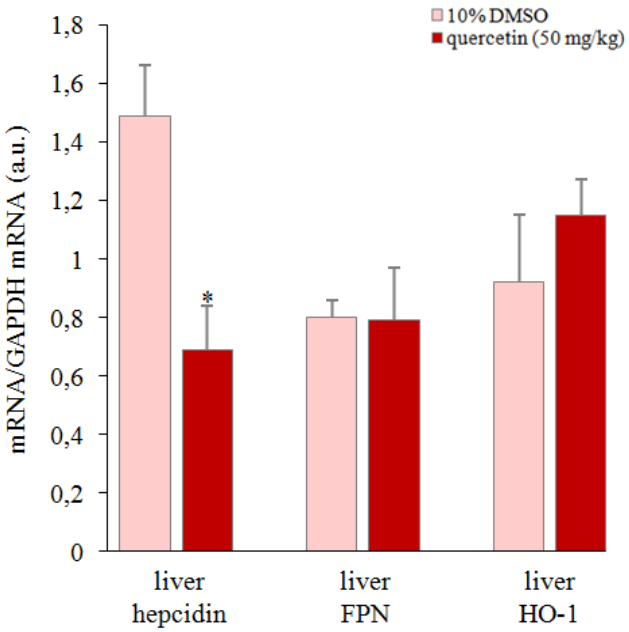


Figure 3–4 Acute effect of orally administered quercetin on liver hepcidin, FPN and HO–1 gene expression in rats.
Rats were given a single gavage containing quercetin (50 mg/kg body weight) or DMSO 10% (control). After 5 hours RNA was isolated from liver tissue to measure changes in corresponding mRNA levels. Data are mean ± SEM; n=5 rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

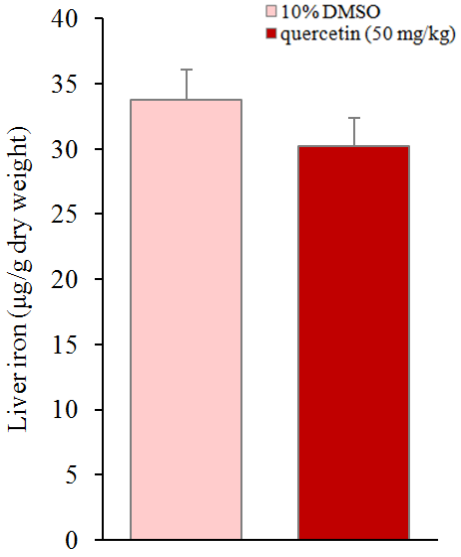


Figure 3–5 Acute effect of orally administered quercetin on liver iron content in rats.
The acute effect of quercetin on liver iron content was measured 5 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control) to rats. Data are mean ± SEM; n=5 rats per group.

3.2.1.4 Acute effect of quercetin on spleen hepcidin and FPN gene expression and iron content

Five hours after oral treatment with quercetin rats were sacrificed and estimation of hepcidin and FPN mRNA levels was done. Analysis showed a significant increase in both hepcidin and mRNA levels (see Figure 3–6). Additionally, the spleen iron level was significantly greater in the treated animal group compared with the control group (see Figure 3–7).

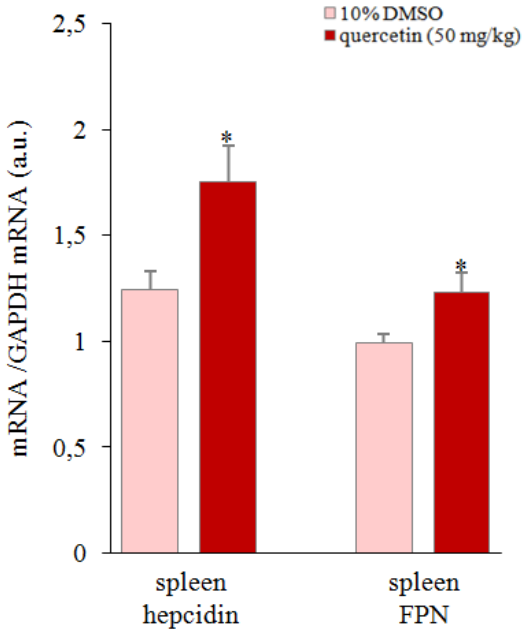


Figure 3–6 Acute effect of orally administered quercetin on spleen hepcidin and FPN gene expression in rats.
Rats were given a single gavage containing quercetin (50 mg/kg body weight) or DMSO 10% (control). After 5 hours RNA was isolated from spleen tissue to measure changes in corresponding mRNA levels. Data are mean ± SEM; n=5 rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

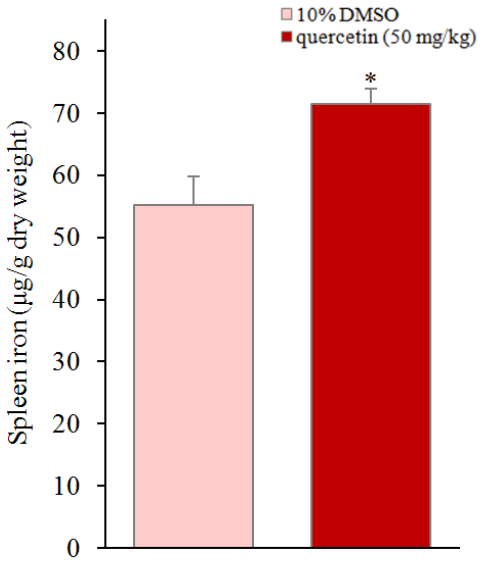


Figure 3–7 Acute effect of orally administered quercetin on spleen iron content in rats.
The acute effect of quercetin on spleen iron content was measured 5 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control) to rats. Data are mean ± SEM; n=5 rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

3.2.1.5 Acute effect of quercetin on iron absorption in duodenum

Mucosal ^{59}Fe uptake in rat duodenum was significantly increased five hours after a single dose of quercetin given orally by gavage (50 mg/kg; see Figure 3–8). In contrast, in the same group of animals, mucosal ^{59}Fe transfer was significantly decreased after the same treatment (see Figure 3–8).

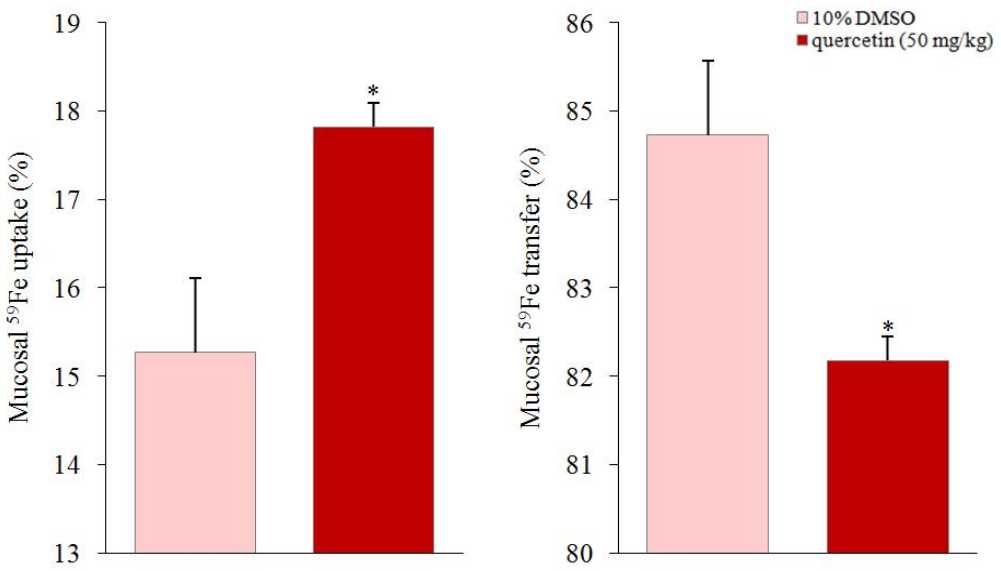


Figure 3–8 Acute effect of orally administered quercetin on iron absorption in duodenum in rats.
Rats were given a single gavage containing quercetin (50 mg/kg body weight) or DMSO 10% (control). After 5 hours mucosal ^{59}Fe uptake and ^{59}Fe transfer were measured. Data are mean ± SEM; n=5 rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

3.2.2 Longer-term effect of quercetin on iron metabolism *in vivo*

The longer-term effect of quercetin on iron methabolism was studied *in vivo* in rats 18 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control). After weaning rats were placed on low iron diet or regular diet for two weeks. At the end of the experimental procedure animals were killed by administering a terminal dose of pentobarbitone sodium and blood samples were removed via cardiac puncture. Subsequentlly, serum was separated and used for serum iron and transferrin saturation measurements using stadard spectrophotometric methods. Additionally, duodenum, liver and spleen were removed and subsequently used for tissue non-haem iron spectrophotometric measurements and gene expression levels determination.

Longer-term effect of quercetin on iron absorption in duodenum was sudied in rats treated by a double gavage containing quercetin (50 mg/kg body weight) or 10% DMSO, for 18 hours and then 5 hours. Afterwards, uptake studies were performed where $^{59}Fe^{2+}$ was put inside the cannulated duodenum for 30 minutes while rats were anesthetized. After 30 minutes, blood samples were collected via cardiac puncture and duodenal mucosa was scraped away and subsequently gamma counted for determination of ^{59}Fe activity.

3.2.2.1 Longer-term effect of quercetin on serum iron and transferrin saturation

Serum iron and transferrin saturation levels were significantly decreased in rats fed on iron-deficient diet and treated with a single dose of quercetin (50 mg/kg) by gavage eighteen hours before being used for experiment (see Figure 3–9; A). However, the oppsite outcome was observed in the same parameters in rats fed on RM1 diet and treated with single dose of quercetin (50 mg/kg) by gavage eighteen hours before being used for experiment. In the treated group serum iron and transferrin saturation levels were significantly increased compared with control (see Figure 3–9; B).

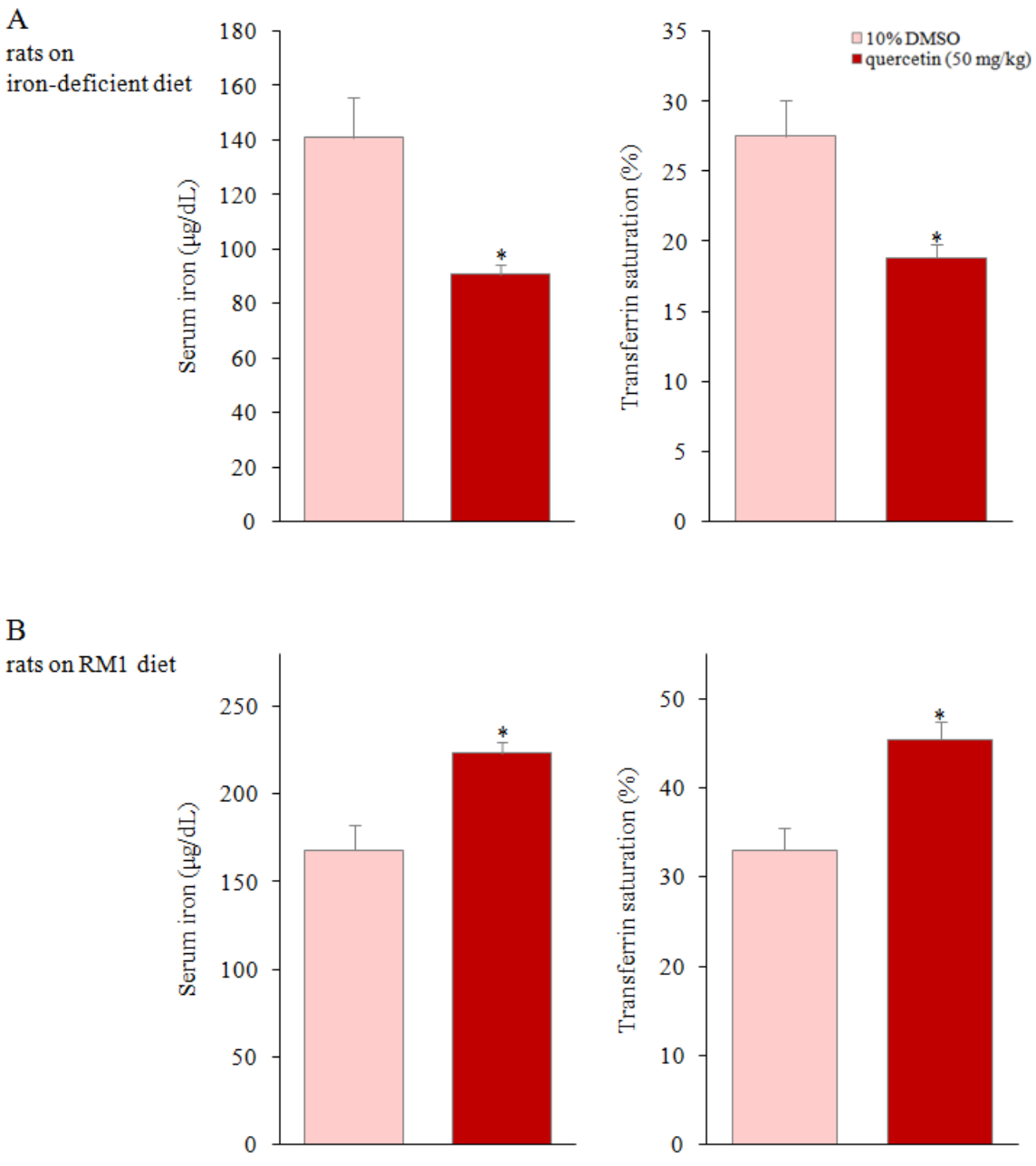


Figure 3–9 Longer-term effect of orally administered quercetin on serum iron and transferrin saturation in rats.

The longer-term effect of quercetin on serum iron and transferrin saturation was measured 18 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control). Data are mean ± SEM; n=4 (A); 3 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

3.2.2.2 Longer-term effect of quercetin on duodenal iron transporters and Dcytb gene expression and iron content

Analysis of the iron transporters (DMT1 and FPN) and Dcytb mRNA expression in duodenum of rats fed an iron-deficient diet and sacrificed eighteen hours after oral treatment with quercetin showed a significant reduction in DMT1 and FPN mRNA levels. It should be noted, that mRNA levels of DMT1 changed nearly 10-fold, while levels of FPN changed nearly 2–fold. Additionally, after the same treatment levels of Dcytb mRNA levels stayed the same (see Figure 3–10; A). Furthermore, in the duodenum of the same group of animals, levels of HIF–1α and HIF–2α mRNA were also examined, but no expression was observed (results not shown). The same treatment, but with rats fed on RM1 diet, brought about a significant increment in Dcytb and FPN mRNA levels between 2- to 3-fold (see Figure 3–10; B). In the same group of animals levels of DMT1 mRNA were also increased but not significant (see Figure 3–10; B). Duodenal iron levels increased significantly after single longer-term oral quercetin treatment in rats fed on an iron-deficient and a normal diet with respect to iron content (Figure 3–11; A and B, respectively).

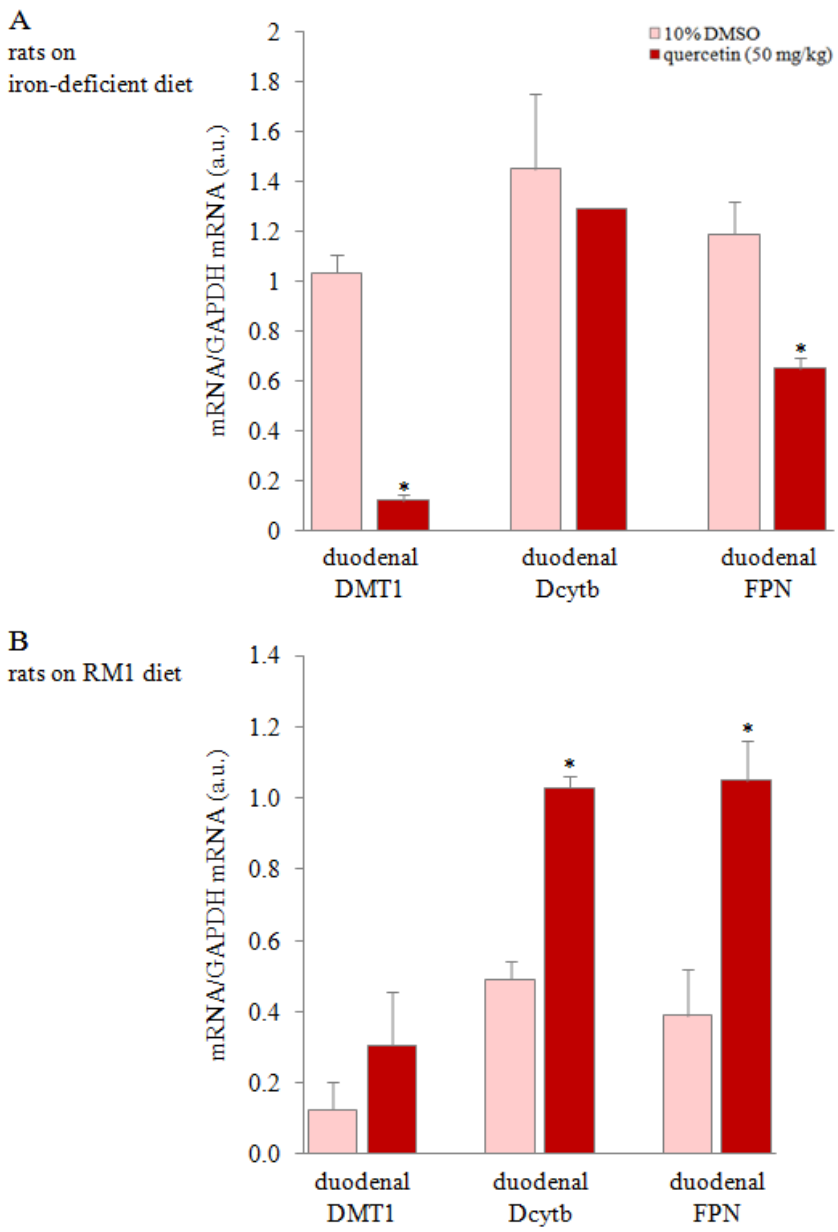


Figure 3–10 Longer-term effect of orally administered quercetin on duodenal iron transporters and Dcytb gene expression in rats.
The longer-term effect of quercetin on duodenal iron transporters and Dcytb gene expression was measured 18 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control). Data are mean ± SEM; n=4 (A); 3 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$)

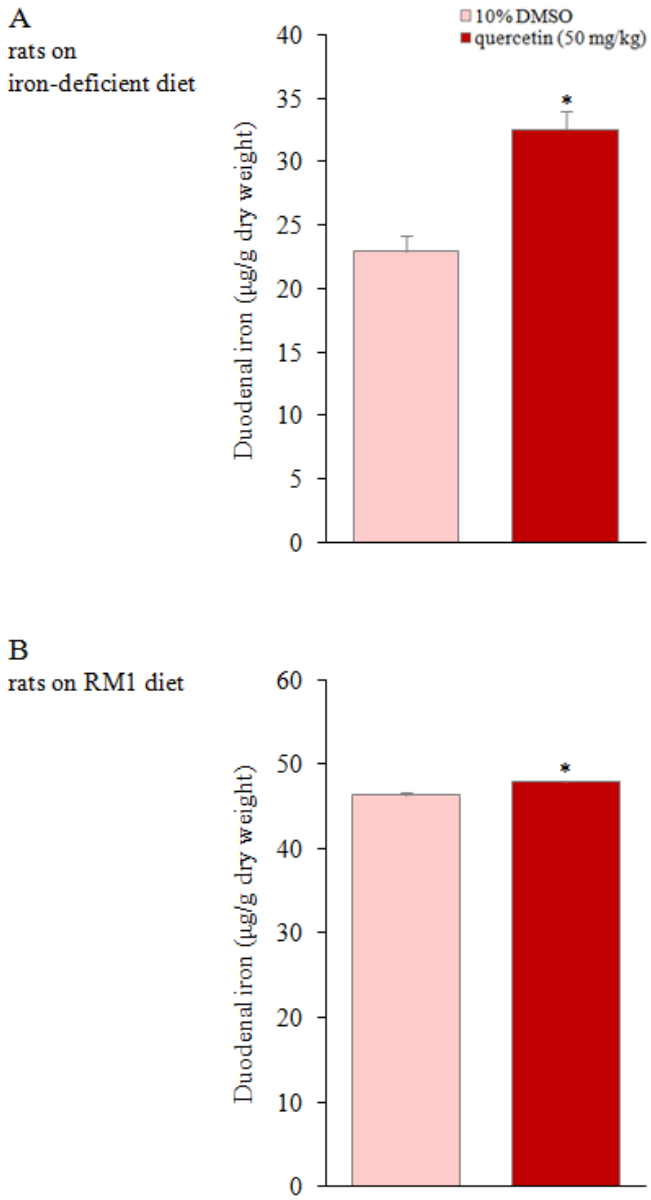


Figure 3–11 Longer-term effect of orally administered quercetin on duodenal iron content in rats.
The longer-term effect of quercetin on duodenal iron content was measured 18 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control). Data are mean ± SEM; n=4 (A); 3 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

3.2.2.3 Longer-term effect of quercetin on liver hepcidin, FPN and related gene expression and iron content

Analysis of hepcidin, FPN and HO–1 mRNA expression in liver of rats fed an iron-deficient diet and sacrificed eighteen hours after oral treatment with quercetin did not show significant changes in their levels compared with controls (see Figure 3–12; A). Furthermore, in the liver of the same group of animals levels of GST and QR mRNA were also followed, but no significant change was evidenced (results not shown). The same treatment, but with rats fed on a RM1 diet, caused a significant increase in FPN mRNA levels of nearly 4-fold, while mRNA levels of hepcidin and HO–1 were not affected with the applied treatment (see Figure 3–12; B). In the same group of animals levels of GST were analyzed, but no significant change occurred (results not shown). Liver iron levels increased in both groups of animals, but a significant change was only evidenced after single longer-term oral quercetin treatment in rats fed on an iron-deficient diet where iron levels increased 1.5-fold (Figure 3–13; A).

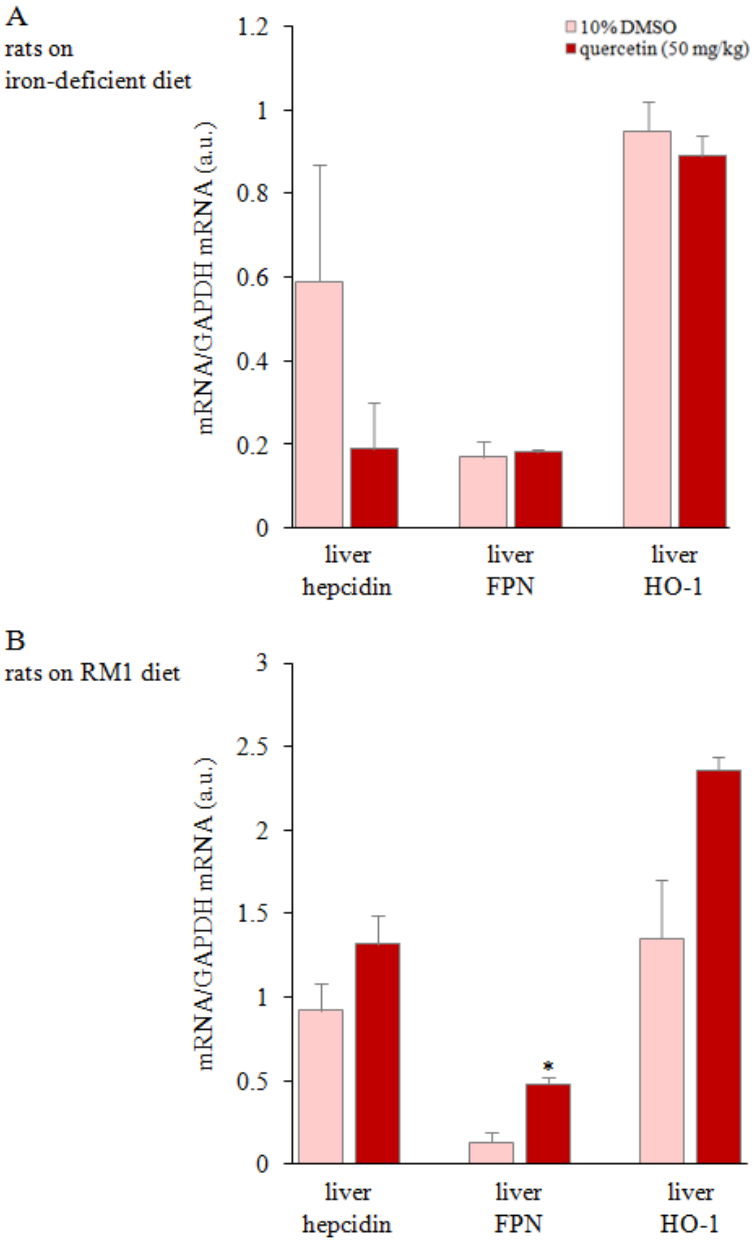


Figure 3–12 Longer-term effect of orally administered quercetin on liver hepcidin, FPN and HO–1 gene expression in rats.
The longer-term effect of quercetin on liver hepcidin, FPN and HO–1 gene expression was measured 18 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control). Data are mean ± SEM; n=4 (A); 3 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

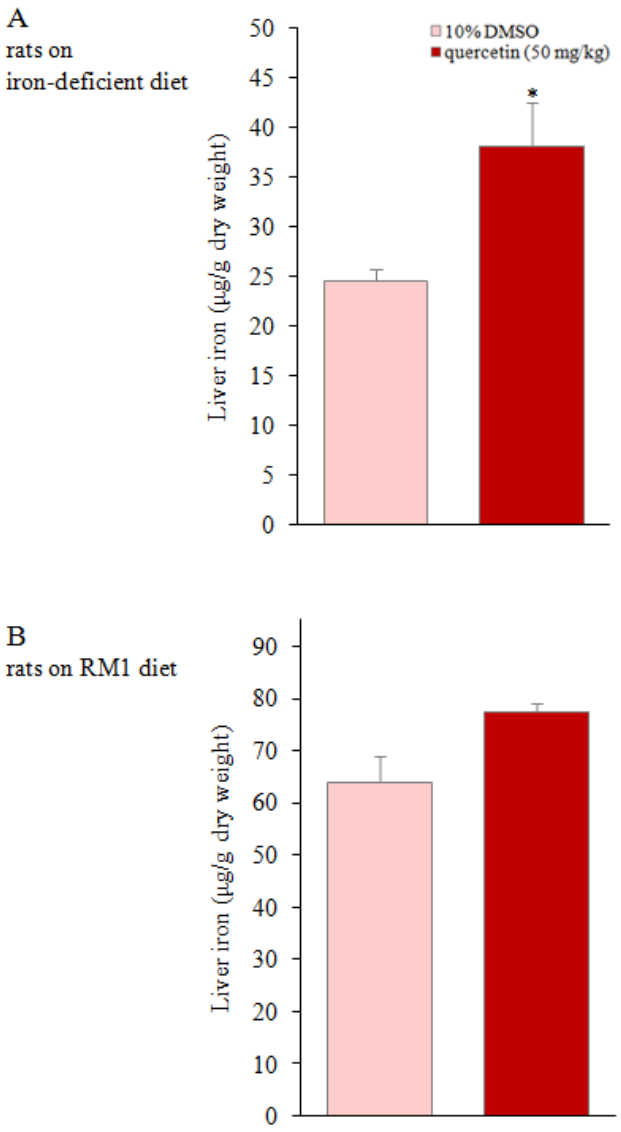


Figure 3–13 Longer-term effect of orally administered quercetin on liver iron content in rats.
The longer-term effect of quercetin on liver iron content was measured 18 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control). Data are mean ± SEM; n=4 (A); 3 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

3.2.2.4 Longer-term effect of quercetin on spleen hepcidin and FPN gene expression and iron content

Analysis of hepcidin and FPN mRNA expression in the spleen of rats fed on an iron-deficient diet and sacrificed eighteen hours after oral treatment with quercetin showed a noteworthy decrease in hepcidin mRNA expression. In addition, FPN mRNA levels in the same group of animals were lower but not significant (see Figure 3–14; A). The same treatment, but in rats fed on a RM1 diet, did not cause a significant change in the two examined mRNA levels (see Figure 3–14; B). Spleen iron levels significantly increased in the first group of animals (see Figure 3–15; A), while in animals where the RM1 diet was applied, no significant change in spleen iron levels was observed (Figure 3–15; B).

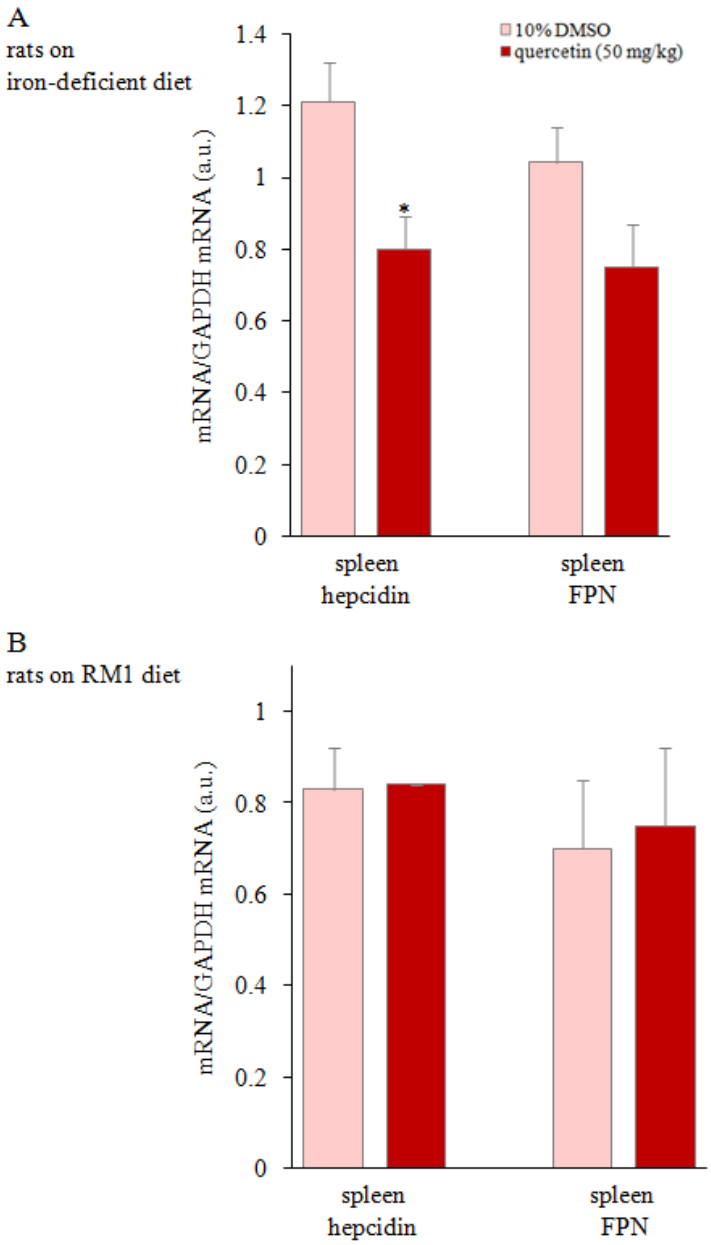


Figure 3–14 Longer-term effect of orally administered quercetin on spleen hepcidin and FPN gene expression in rats.
The longer-term effect of quercetin on splenic hepcidin and FPN gene expression was measured 18 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control). Data are mean ± SEM; n=4 (A); 3 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

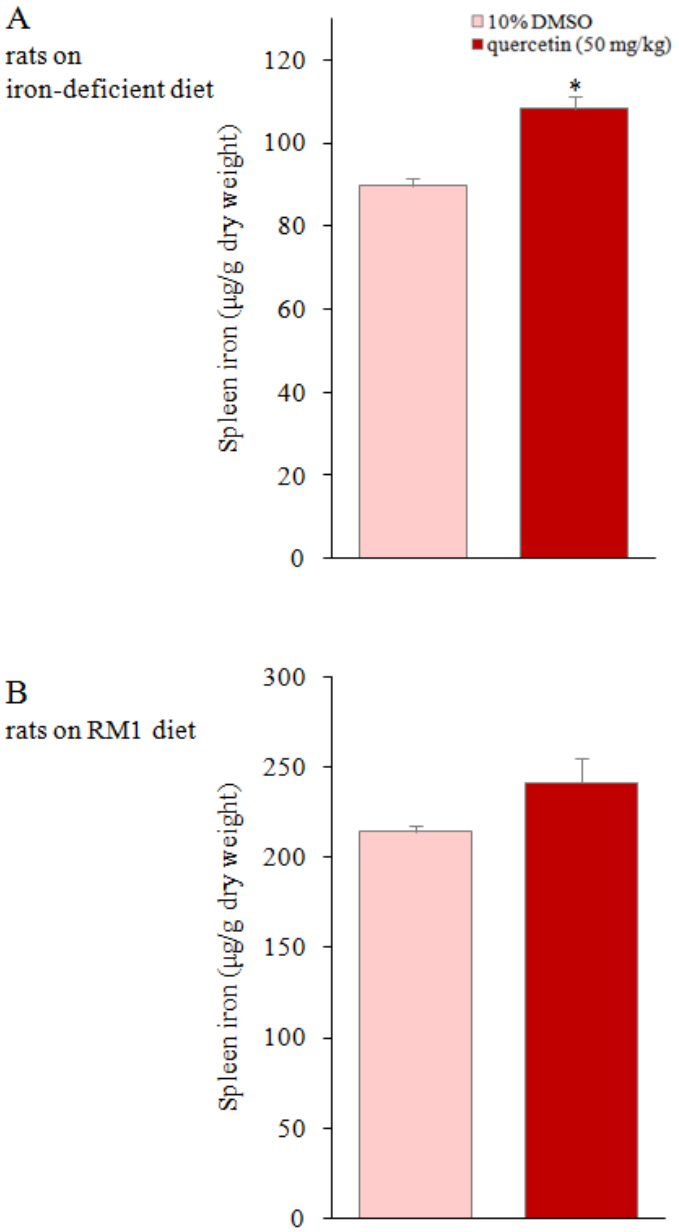


Figure 3–15 Longer-term effect of orally administered quercetin on spleen iron content in rats.

The longer-term effect of quercetin on splenic iron content was measured 18 hours after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control). Data are mean ± SEM; n=4 (A); 3 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

3.2.2.5 Longer-term effect of quercetin on iron absorption in duodenum

The effect on iron absorption in duodenum after longer-term gavage was similar to that following the shorter-term quercetin gavage treatment shown previously (see 3.2.1.5). Namely, mucosal ^{59}Fe uptake was significantly increased after a double dose of quercetin (50 mg/kg) given orally by gavage eighteen hours and then five hours before the experiment (see Figure 3–16). In contrast, in the same group of animals, mucosal ^{59}Fe transfer was significantly decreased after the same treatment (see Figure 3–16).

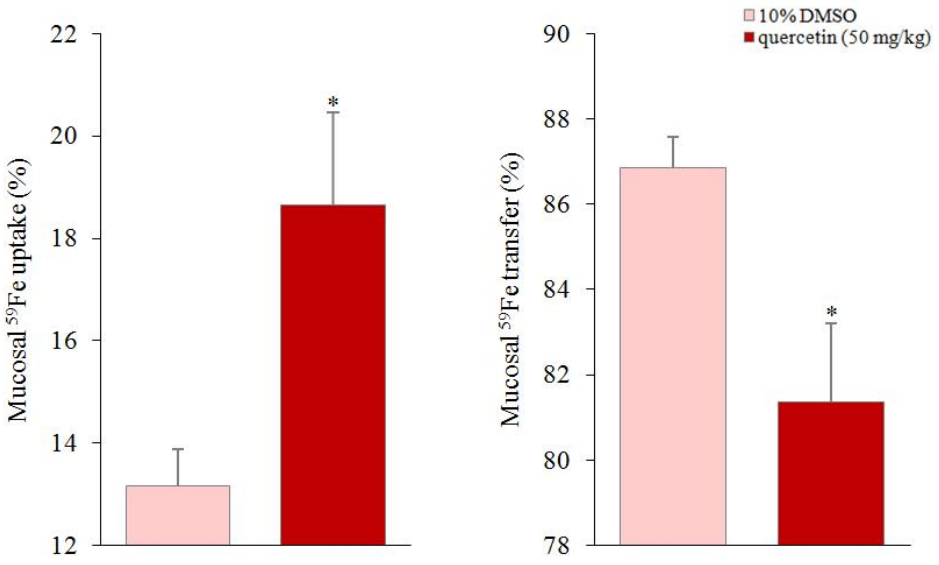


Figure 3–16 Longer-term effect of orally administered quercetin on iron absorption in duodenum in rats.
Rats were given a double gavage containing quercetin (50 mg/kg body weight) or 10% DMSO, for 18 hours and then 5 hours, after which mucosal ^{59}Fe uptake and ^{59}Fe transfer were measured. Data are mean ± SEM; n=5 rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

3.2.3 Chronic effect of quercetin on iron metabolism *in vivo*

The chronic effect of quercetin on iron methabolism was studied *in vivo* in rats after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control), where quercetin tretement was applied once per day during 10 days. Afterwards, animals were killed by administering a terminal dose of pentobarbitone sodium and blood samples were removed via cardiac puncture. Subsequentlly, serum was separated and used for serum iron and transferrin saturation measurements using stadard spectrophotometric methods. Additionally, duodenum, liver and spleen were removed and subsequently used for tissue non-haem iron spectrophotometric measurements and gene expression levels determination.

3.2.3.1 Chronic effect of quercetin on serum iron and transferrin saturation

Serum iron and transferrin saturation levels were unchanged in rats after ten days oral quercetin treatment (50 mg/kg each day) compared with controls (see Figure 3–17).

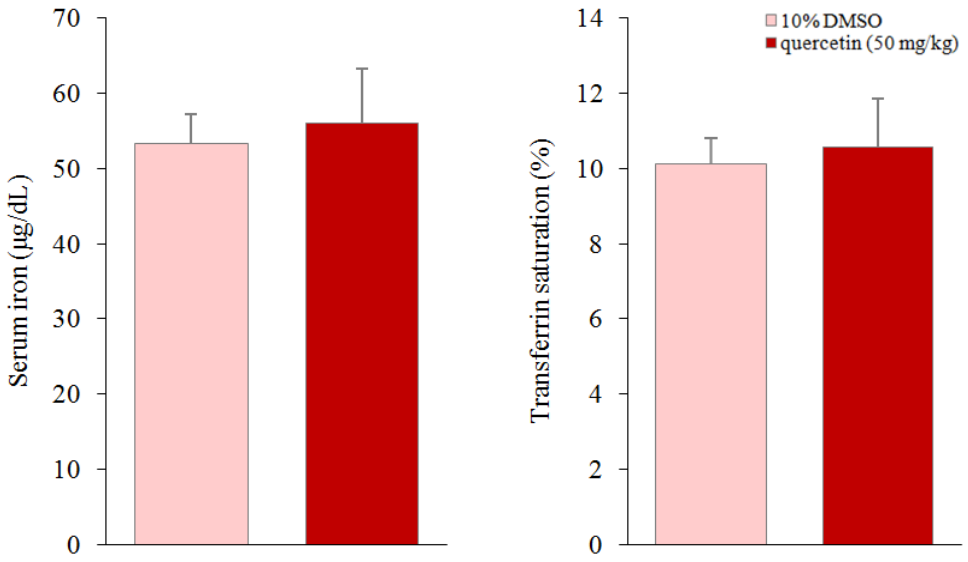


Figure 3–17 Chronic effect of orally administered quercetin on serum iron and transferrin saturation in rats.
The chronic effect of quercetin on serum iron and transferrin saturation were measured after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control), during 10 days, single per day. Data are mean ± SEM; n=5 rats per group.

3.2.3.2 Chronic effect of quercetin on duodenal iron transporters and Dcytb gene expression and iron content

Analysis of iron transporters (DMT1 and FPN) and Dcytb mRNA expression in duodenum of rats day–to–day treated with quercetin during ten days showed a significant decrease in DMT1, Dcytb and FPN mRNA levels (2.2, 1.6 and 1.8 fold, respectively; see Figure 3–18). Furthermore, in the duodenum of the same group of animals levels of HIF–1α and HIF–2α mRNA were also followed. However there was no change in HIF–1α levels, while there was no expression of HIF–2α mRNA levels (results not shown). In addition, no difference was observed in duodenal iron levels between the treated and control groups (see Figure 3–19).

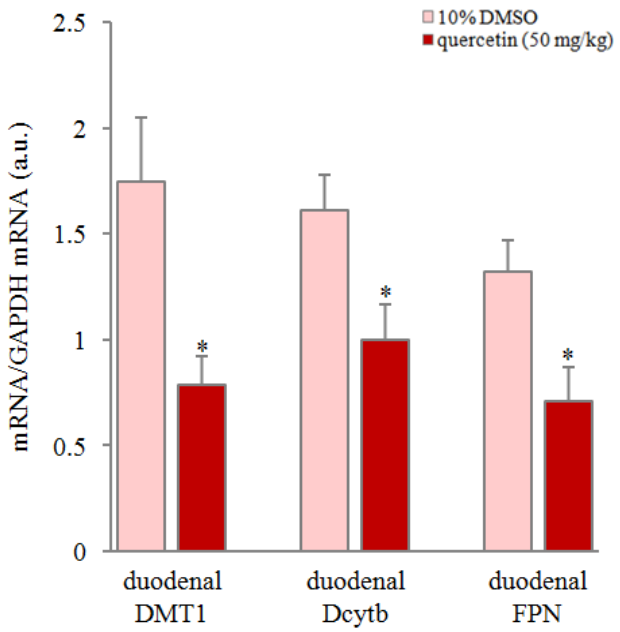


Figure 3–18 Chronic effect orally administered quercetin on duodenal iron transporters and Dcytb gene expression in rats.
The chronic effect of quercetin on duodenal iron transporters and Dcytb gene expression was measured after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control), during 10 days, single per day. Data are mean ± SEM; n=5 rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

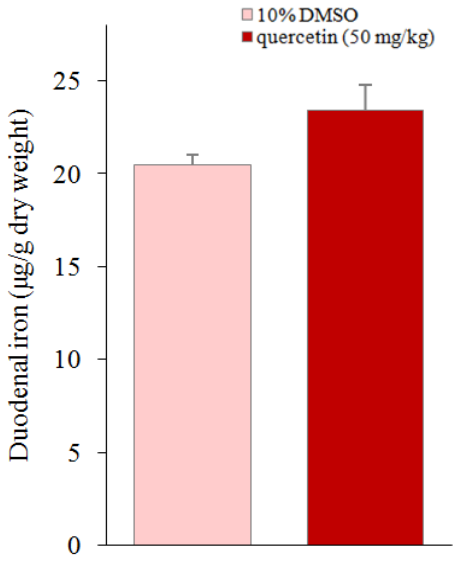


Figure 3–19 Chronic effect of orally administered quercetin on duodenal iron content in rats.
The chronic effect of quercetin on duodenal iron content was measured after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control), during 10 days, single per day. Data are mean ± SEM; n=5 rats per group.

3.2.3.3 Chronic effect of quercetin on hepcidin, FPN and HO–1 gene expression and iron content

Analysis of hepcidin, FPN and HO–1 mRNA expression in liver of rats fed on an iron-deficient diet and treated daily with quercetin for ten days showed a noteworthy increased in HO–1 levels (see Figure 3–20). However, other mRNA levels (hepcidin and FPN) were unchanged after chronic treatment with quercetin compared with control (see Figure 3–20). In addition, liver iron levels decreased significantly after the same treatment compared with the control (see Figure 3–21).

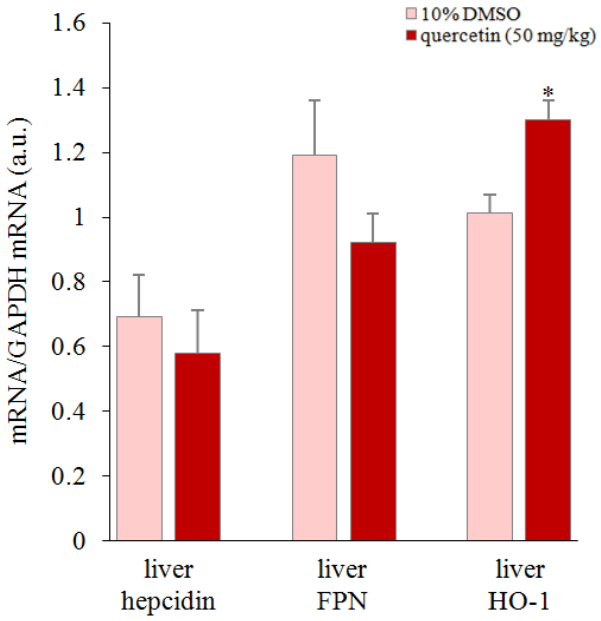


Figure 3–20 Chronic effect of orally administered quercetin on liver hepcidin, FPN and HO–1 gene expression in rats.
The chronic effect of quercetin on liver hepcidin, FPN and HO–1 gene expression was measured after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control), during 10 days, single per day. Data are mean ± SEM; n=5 rats per group. Data are mean ± SEM; n=5 rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

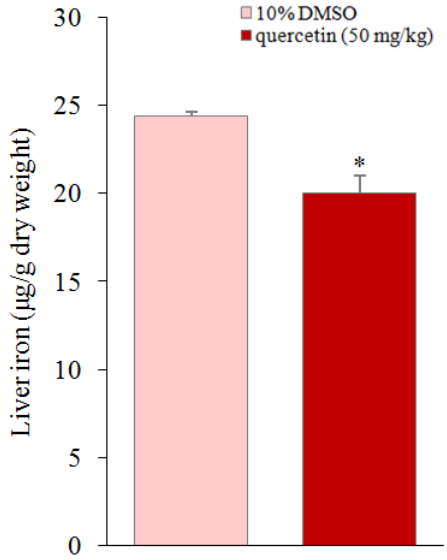


Figure 3–21 Chronic effect of orally administered quercetin on liver iron content in rats.
The chronic effect of quercetin on liver iron content was measured after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control), during 10 days, single per day. Data are mean ± SEM; n=5 rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

3.2.3.4 Chronic effect of quercetin on spleen hepcidin and FPN gene expression and iron content

Analysis of hepcidin and FPN mRNA expression in spleen of rats fed on an iron-deficient diet and treated daily with quercetin for ten days showed no significant change between treated and control animal group (see Figure 3–22). However, spleen iron levels decreased significantly after the same treatment compared with the control group (see Figure 3–23).

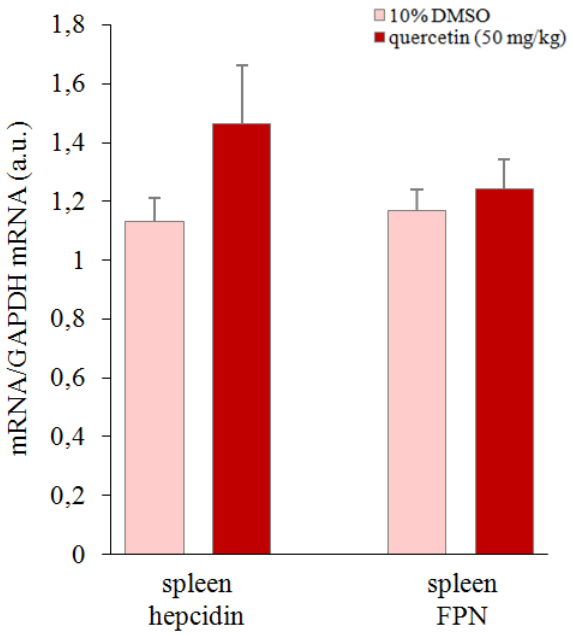


Figure 3–22 Chronic effect of orally administered quercetin on spleen hepcidin and FPN gene expression in rats.
The chronic effect of quercetin on spleen hepcidin and FPN gene expression was measured after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control), during 10 days, single per day. Data are mean ± SEM; n=5 rats per group.

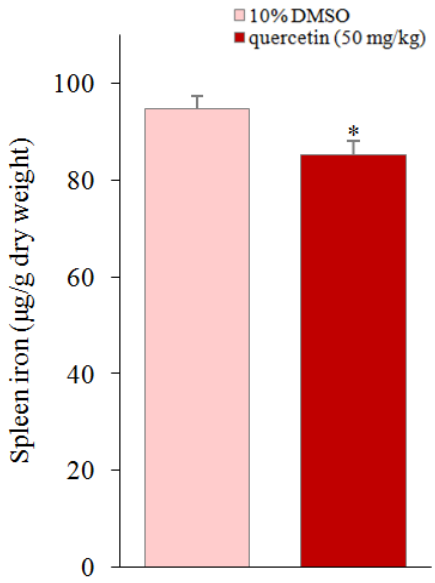


Figure 3–23 Chronic effect of orally administered quercetin on spleen iron content in rats.
The chronic effect of quercetin on splenic iron content was measured after oral administration of quercetin (50 mg/kg body weight) or 10% DMSO (control), during 10 days, single per day. Data are mean ± SEM; n=5 rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

3.2.3.5 Distribution of quercetin metabolites in serum after oral guercetin administration

Quantitative analysis of quercetin and five selected quercetin metabolites in rat serum, after short-term, longer-term and chronic oral quercetin treatment, was performed using the LC–MS/MS technique. The contents of the determined compounds are presented in Table 3–2. Only quercetin–3–*O*–glucuronide was detected in the serum of the rats treated short-term or longer-term with quercetin, while no other examined compounds were identified. After short-term oral quercetin treatment (rats were treated with single dose of quercetin (50 mg/kg) given by gavage five hours before being used for experiment) the concentration of quercetin–

3–*O*–glucuronide was 12.65 ng/mL of serum. The lower concentration of quercetin–3–*O*–glucuronide (0.37 ng/mL of serum) was determined in rats fed a RM1 diet and treated with a single dose of quercetin (50 mg/kg) by gavage eighteen hours before the experiment. After chronic oral quercetin treatment neither quercetin nor the five selected metaboplites were detected.

Table 3–1 Concentrations of quercetin and selected quercetin metabolites in rat serum after acute, longer-term and chronic oral quercetin treatment determined by LC–MS/MS technique

treatment*		content of quercetin and selected quercetin metabolites (ng/mL serum)					
		quercetin–3,4'–di–*O*–glucoside	quercetin–3–*O*–glucuronide	isorhamnetin–3–*O*–glucoside	quercetin	isorhamnetin	quercetin–3,5,7,3',4'–penthamethylether
acute treatment (5 hours before experiment; iron-deficient diet)	10% DMSO (control)	nd	nd	nd	nd	nd	nd
	quercetin (50 mg/kg)	nd	12.65 ± 0.99	nd	nd	nd	nd
longer–term treatment (18 hours before experiment; RM1 diet)	10% DMSO (control)	nd	nd	nd	nd	nd	nd
	quercetin (50 mg/kg)	nd	0.37 ± 0.023	nd	nd	nd	nd
chronic treatment (single day-to-day during ten days; iron-deficient diet)	10% DMSO (control)	nd	nd	nd	nd	nd	nd
	quercetin (50 mg/kg)	nd	nd	nd	nd	nd	nd

nd – not detected

*serum from rats treated with quercetin 18 hours before experiment and fed on an iron-deficient diet are not included in the study due to the lack of serum

3.3 Discussion

In this chapter short–term, longer–term and chronic effects of quercetin administered orally by gavage on iron homeostasis in rats were investigated. All the results are summarised in Table 3–2. The results indicate that the period of gavage with quercetin had significant, but somewhat different, effects on iron homeostasis and gene expression. To be more precise, in the shor-term, longer-term and chronic setting quercetin generally caused iron deficiency in rats that were fed on an iron-deficient diet prior to the experiment. Iron deficiency was evident from the significant reduction in liver and spleen iron pools, as well as the reduction in serum iron and transferrin saturation levels. However, these results came as no surprise because many authors previously have shown that consumption of a diet rich in polyphenols caused iron deficiency *in vivo*. For example, thirty years ago Merhav et al. (1985) recognized that the main cause of IDA in infants in Israel was regular tea drinking, while Hamdaoui et al. (2005) and Marouani et al. (2007) provided evidence for iron deficiency after tea consumption in rats. My results indicate that oral quercetin treatment generally induced iron deficiency in all treated groups. However, there is a great difference in the change of gene expression of the monitored mRNA levels and tissue iron levels in duodenum, liver and spleen, between the different treated groups.

Table 3–2 Summary of the effect of oral quercetin on iron homeostasis

treatment			obtained results
acute treatment (5 hours before experiment; iron-deficient diet)		10% DMSO (control)	*Serum*: Iron↓*, transferrin saturation↓* *Duodenum*: DMT1↑*, Dcytb↑, GLUT1↑, SGLT1=, FPN↑, Iron= *Liver*: Hepcidin↓*, FPN=, HO–1↑, Iron= *Spleen*: Hepcidin↑*, FPN↑*, Iron↑*
		quercetin (50 mg/kg)	
longer-term treatment	(18 hours before experiment; iron-deficient diet)	10% DMSO (control)	*Serum*: Iron↓*, transferrin saturation↓* *Duodenum*: DMT1↓*, Dcytb↓, GLUT1=, SGLT1↑*, FPN↓*, HIF–1α (no expression), HIF–2α (no expression), Iron↑* *Liver*: Hepcidin↓, FPN=, HO–1=,GST↑, QR↓, Iron↑* *Spleen*: Hepcidin ↓*, FPN↓ Iron↑*
		quercetin (50 mg/kg)	
	(18 hours before experiment; RM1 diet)	10% DMSO (control)	*Serum*: Iron↑*, transferrin saturation↑* *Duodenum*: DMT1↑, Dcytb↑*, GLUT1 no expression, SGLT1↑*, FPN↑*, Iron↑* *Liver*: Hepcidin↑, FPN↑*, HO–1↑, GST=, Iron↑ *Spleen*: Hepcidin=, FPN=, Iron=
		quercetin (50 mg/kg)	
chronic treatment (single day-to-day during ten days; iron-deficient diet)		10% DMSO (control)	*Serum*: Iron=, transferrin saturation= *Duodenum*: DMT1↓*, Dcytb↓*, GLUT1↓, SGLT1↓*, FPN↓*, HIF–1α↓, HIF–2α no expression, Iron↑ *Liver*: Hepcidin=, FPN↓, HO–1↑*, Iron↓* *Spleen*: Hepcidin↑, FPN=, Iron↓*
		quercetin (50 mg/kg)	

results in red and marked with * indicate that parameter significantly differ from treated and corresponding control group
↑ indicate that parameter increased, but not significantly, between treated and corresponding control group
↓ indicate that parameter decreased, but not significantly, between treated and corresponding control group
= indicate that parameter did not change in treated group compared with corresponding control group

Specifically, short-term oral quercetin treatment, caused a significant up-regulation of mRNA for the iron transporter DMT1 mRNA in enterocytes. This result seems logical as an organism during iron deficiency makes every effort to boost iron uptake. This outcome was also confirmed previously in duodenal biopsy specimens of iron-deficient patients. Zoller et al., (2001) showed that both mRNA and protein levels of DMT1 and FPN were significantly up-regulated in iron-deficient patients compared with healthy subjects. Similarly, up-regulation of DMT1, Dcytb and FPN mRNA levels was reported in the duodenum of four iron-deficient mice strains (Dupic et al., 2002), while McKie et al. (2001) confirmed an increase in Dcytb mRNA and protein levels in the duodenum of iron-deficient mice. However, in this thesis, Dcytb and FPN mRNA levels were increased but not significantly, regardless of iron deficiency. However, it is not clear whether changes in mRNA levels of proteins that are involved in iron uptake are due to iron deficiency caused by quercetin or directly by quercetin itself. There is no evidence in the literature that polyphenols, or specifically quercetin, affect duodenal gene expression in this manner *in vivo*, apart from a recent study where following polyphenol-rich bean consumption for six weeks by chickens, levels of DMT1, Dcytb and FPN mRNA did not show any significant difference (Tako et al., 2014). An *in vitro* experiment with Caco–2 cells treated with quercetin for 24 hours did not show a significant change in DMT1 or Dcytb, while FPN mRNA levels were decreased (Hoque and Sharp, 2010), whereas treatment with flavonoid-rich berry extract for 16 hours decreased DMT1 mRNA levels in the same cells (Alzaid et al., 2010).

Furthermore, in our study quercetin administered orally short-term to rats caused a decrease in liver hepcidin mRNA expression. This is likely to reflect the low

transferrin saturation and low iron in the serum of rats treated with quercetin for 5 hours. A decreased expression of liver hepcidin has been shown to be associated with iron deficiency and induction of DMT1 mRNA (Frazer et al., 2004; Papanikolaou et al., 2005). In contrast to liver hepcidin, in the same group of rats, spleen hepcidin mRNA levels were significantly up-regulated. One possible explanation for this is that polyphenols increase hepcidin expression in both tissues, but only the liver hepcidin expression is sensitive to serum iron levels and this is the dominant factor in the repression of hepcidin expression. The other possible explanation is that hepcidin expression is regulated in a tissue-specific manner in response to quercetin administration. Independent of the mechanism of increased hepcidin expression in spleen, it is tempting to suggest that the increased hepcidin in the spleen binds to spleen FPN, leading to its internalization which leads to a decreased iron efflux thereby causing an increased spleen iron and decreased serum iron and transferrin saturation as observed in this study. However, this view should be confirmed with an increase in FPN protein levels, not just with mRNA. Furthermore, decreased serum iron will also be contributed by a decreased efflux of iron from the duodenum in quercetin-treated rats as shown in this study with uptake experiments. The effect of quercetin on iron efflux is likely to be independent of hepcidin as liver hepcidin expression levels, the major contributor to serum iron, are significantly decreased after quercetin treatment. However, increased spleen FPN expression in quercetin treated rats is in agreement with previous studies which have shown that polyphenols increase FPN expression by promoting its expression by increasing Nrf2 levels which then bind to an ARE in the promoter of FPN (Marro et al., 2010). Also it could be proposed that the increased spleen FPN expression is a direct consequence of a decrease in hepatic hepcidin levels, which leads to splenic FPN re-expression on

the membrane. Also, these results support the view that internalization of FPN as a response to hepcidin is tissue- and cell-specific. Namely, it was shown that macrophages respond more acutely to a hepcidin challenge, while the duodenum appears to be less sensitive to a rise in hepcidin levels (Chaston et al., 2008; Masaratana et al., 2011). Moreover, FPN levels in spleen might be under post-transcriptional IRE/IRP control, which when iron levels in tissue are high, expression of FPN is up-regulated (Muckenthaler et al., 2008).

However, uptake studies after short–term, as well as after longer-term, quercetin treatment caused a significant increase of mucosal iron uptake, while there was a significant decrease in iron efflux from enterocytes. These results solidly confirm that quercetin plays an important role in the bioavailability of non-haem iron in the duodenum. In particular, decreased iron transfer is probably due to chelation of iron by quercetin which increases apical uptake of iron, but prevents basolateral transport. This explanation can be applied to all polyphenols that have a noticeable capacity to chelate iron, particularly those which are abundant in the diet. These results were only preliminary to a further, more detailed, study of the chelation property of quercetin and its effect on iron absorption in duodenum, which is presented in Chapter 4.

Furthermore, the possibility cannot be discarded that quercetin, or its metabolites, have direct effects on the expression of proteins involved in iron absorption and systemic homeostasis, and that together with chelation this modifies iron metabolism. This fact is even more important bearing in mind that uptake studies after longer-term quercetin treatment showed a decrease in iron uptake. Eighteen hours is a long period for quercetin to remain in lumen or inside the enterocyte, thus,

it is more likely that after 18 hours there is no quercetin in place to form a complex with iron, and the only explanation of its effect is to cause changes in expression of proteins involved in iron metabolism.

It was pointed out previously that longer-term oral quercetin treatment caused iron deficiency in animals fed an iron-deficient diet. Namely, 18 hours after quercetin treatment serum iron and transferrin saturation levels were reduced significantly. This result is the same as after short-term oral quercetin treatment. Surprisingly, levels of iron in spleen and liver were significantly higher, which is not in accordance with the iron-deficient status. This occurrence was previously shown for spleen after short-term oral quercetin treatment. At this moment is it hard to explain this results. Additionally, in the same group of animals, duodenal iron was increased which confirms previously explained hypotheses that quercetin supports iron uptake, but not iron transfer across mucosa. Iron retention in the duodenum probably down-regulated DMT1 mRNA levels, as shown in this thesis, through the IRE/IRP control mechanism, such that when iron levels in tissue are high, expression of DMT1 is supressed (Muckenthaler et al., 2008). Though the same control mechanism should increase FPN levels in the duodenum, FPN mRNA levels decreased. However, it was proven that duodenal enterocytes express a FPN transcript, FPN1B, which lacks the IRE and thus is not affected by iron-excessive conditions (Zhang et al., 2009). Thus, the FPN -IRE form could mostly contribute to FPN down-regulation in the intestine under overall iron-deficient conditions. However, this is just an assumption as the mechanism of this action is hard to explain with the current limited data. Splenic hepcidin mRNA also decreased, however this occurrence is also hard to explain. However following results discussed previously, this data supports the hypothesis of different tissue-specific hepcidin isoforms. Namely, proven effects that lower

hepcidin should increase DMT1 levels in the duodenum was absent, as well as up-regulation of FPN.

After chronic ten-day-long treatment with quercetin, animals became iron-deficient, as both spleen and liver iron pools decreased. This was observed previously *in vivo*. Namely, quercetin reduced the liver iron content after an induced tissue iron-overload. This effect was attributed to the ability of quercetin to combine with non-haem iron in tissue, transport it to the bloodstream and excrete it from the body, which supported the use of quercetin and other polyphenols as medicines in iron-related disorders (Zhang et al., 2011). This issue was also confirmed in this study for both liver and spleen. This also supports the need for further research of polyphenols as potential new chelating drugs. Furthermore, the observed reduction in iron pools was most probably due to the tendency of to keep serum iron levels in balance. Surprisingly, at the same time, levels of the duodenal iron transporters decreased. Specifically, mRNA levels of DMT1, Dcytb and FPN were significantly down regulated. Additionally, in the same group of animals duodenal iron was slightly increased, which could partly explain this outcome. Namely, it is discussed previously that quercetin supports iron uptake, but not iron transfer, across mucosa. Thus, iron retention in the duodenum could down-regulate DMT1 mRNA levels through the IRE/IRP control system. However, down-regulation of Dcytb and FPN during iron deficiency is difficult to explain, particularly in the light of data showing up-regulation of the examined genes after longer-term quercetin treatment. Also, HO–1 mRNA levels were up-regulated in the liver of the same animals, which is in accordance with the established observation fact that polyphenols, particularly quercetin, increase the levels of antioxidant enzymes (Liu et al., 2012b).

Results from the group of animals that was kept on a normal diet and treated with quercetin for 18 hours, differ considerably from previous results. Namely, animals were not iron-deficient. Furthermore, levels of serum iron and transferrin saturation increased. Additionally, mRNA levels of Dcytb and FPN in the duodenum and FPN in the liver increased significantly. Up-regulation of these genes corresponds to a iron-deficient state, as was discussed earlier. Also, duodenal iron levels increased, which confirms that quercetin promotes duodenal iron uptake. The complexity of changes in parameters could be the result of an initial iron-deficient state, while after eighteen hours the animal has regained normal iron balance. By comparing results from animals fed an iron-deficient and normal diet, it is evident that the iron content of the diet greatly affects the investigated parameters of iron metabolism. In the literature it is known that differences in dietary iron content greatly affects expression of iron metabolism-related genes (Li et al., 2013). However, it looks like those animals that are kept on an iron-deficient diet are more suitable models for research in the field of iron metabolism, because they are more sensitive to applied treatments and the results are more pronounced.

Furthermore, it was proven *in vivo* that HIF–2α is a main transcriptional factor which regulates the expression of genes involved in iron uptake and is critical for compensating iron deficiency by increasing iron absorption. Namely, it was shown that HIF–2α expression is significantly induced by iron deficiency in the duodenum and that it is followed by increase in DMT1 and Dcytb levels (Shah et al., 2009). However, this view was not confirmed in this study. Specifically, there was no expression of HIF–2α mRNA in the duodenum of treated rats, regardless of iron deficiency and changes in DMT1 and Dcytb mRNA levels.

Analysis of quercetin and selected quercetin metabolites concentrations in rat serum after short-term, longer-term and chronic oral quercetin treatment by LC–MS/MS technique aimed to assess which metabolites are present in the serum and at what concentration. This was important for subsequent experiments in which THP1 cells and HepG2 cells were treated with metabolites in order to investigate their potential role in expression of iron metabolism-related genes. The result of qualitative and quantitative studies in this thesis are in agreement with previous data where one of the dominant quercetin metabolites in serum was also quercetin-glucuronide, present in similar concentrations to those shown here. Additionally, results confirmed that quercetin has a short half–life as it was not detected in serum more than 5 hours after oral treatment (Gee et al., 2004; Justino et al., 2004; Moon et al., 2008).

3.4 Conclusions

In conclusion, short-term, longer-term and chronic oral administration of quercetin caused iron deficiency in rats. Furthermore, after different time of treatments, single or double dose and different concentrations of quercetin, the disturbance in iron balance was compensated by different mechanisms. However, the main mechanism seems to be an increase of iron absorption and release of iron from liver and splenic pools.

Additionally, data confirms that quercetin increases mucosal iron uptake and inhibits iron efflux from duodenal mucosa. Still it is not clear if this effect is only due to chelation of iron by quercetin or whether quercetin, or its metabolites, has a direct effect on the expression of proteins involved in iron absorption and systemic homeostasis. Specifically, oral quercetin treatment mostly affected mRNA levels of

duodenal DMT1, Dcytb and FPN. These results also indicate that oral quercetin has a great effect on iron absorption and a minor effect on systemic iron regulation. However, the exact mechanism of the action of quercetin on iron metabolism remains to be fully elucidated.

4. MECHANISM OF THE DIRECT EFFECT OF QUERCETIN ON INTESTINAL IRON ABSORPTION IN THE DUODENUM

4.1 Introduction

Balancing systemic iron levels within narrow limits is critical for human health, as both iron deficiency and iron overload leads to serious haematological, metabolic and neurodegenerative disorders. In mammals there are no known pathways to eliminate excess iron from the body and therefore iron homeostasis is maintained by its absorption, recycling and its loss (Yehuda and Mostofsky, 2010). Therefore to maintain iron balance, especially during the growth period and pregnancy when extra iron is needed, essential iron must be provided by the food.

Nutritional iron absorption occurs primarily in the duodenum, on the apical (luminal) membrane of the enterocytes, and is tightly regulated by bioavailable iron, iron stores, erythropoietic drive and inflammation. From a common diet, average iron bioavailability rate is low. Namely, approximately 10–20 mg of iron is consumed daily by diet, from which 10% is absorbed. This amount of absorbed iron fulfils daily needs, but it can be easily reduced eventually leading to IDA. There are two types of dietary iron: non-haem iron, which is present in food from both animal or plant origin, and haem iron, which is present only in food of animal origin. Haem iron makes up only 10–15% of the total iron from the diet in meat-eating populations, but, it is estimated to contribute more then 40% of total absorbed iron. Despite its great occurrence in general diet, rate of non-haem iron absorption is much lower than it is the case with haem iron (Hurrell and Egli, 2010).

Low bioavailability of non-haem iron contributes greatly to IDA, which is the most prevalent nutritional deficiency worldwide, estimated to affect two billion people (World Health Organisation, 2007), especially in low-income countries where people's diets are based on plants and where consumption of meat is low, and consequently, availability of haem iron is minimal. On the other hand, the same problem occurs in groups eating mostly or strictly plant based diet, such as vegetarians and vegans whose popularity is rising in modern societies, or in groups which do not consume meat due to religious issues.

It is generally believed that bioavailability of non-haem iron highly depends on presence of promoters or inhibitors of dietary iron absorption. On the other hand, it is supposed that dietary factors have little effect on haem iron absorption. Among inhibitors of iron absorption, dietary polyphenols are marked as one of the most potent. Polyphenols are a group of plant secondary metabolites which include vast number of structurally diverse compounds. From a chemical point of view, they are compounds which contain one or more aromatic rings, bearing one or more hydroxyl groups, which can be esterified, etherified or glycosylated. Polyphenols are present in nearly all edible fruits, vegetables and other food of plant origin, as well as in beverages. Generally, human population consumes notable amounts of polyphenols on a daily basis, approximately 1 g and even more in the regions where diet is mainly based on plant sources, such as in developing countries. Dietary polyphenols are receiving increasing attention worldwide due to their proven health benefits for a variety of disorders (Havsteen, 2002). However, the negative impact of dietary polyphenols on non-haem iron absorption in duodenum has been highlighted previously (Cook et al., 1995; Hurrell et al., 1999; Samman et al., 2001; Kim et al., 2008; Petry et al., 2010). The exact mechanism of how polyphenols reduce

bioavailability of non-haem iron is not fully understood, but it is proposed that polyphenols have this effect as a result of their ability to chelate iron (Petry et al., 2010; Kim et al., 2011).

Bearing in mind that IDA is the most prevalent nutritional deficiency worldwide (World Health Organisation, 2007) and dietary polyphenols are consumed in increasing levels due to their health benefits, it is important to elucidate the exact mechanism by which dietary polyphenols suppress non-haem iron absorption in the duodenum. By elucidating the mechanism, polyphenol consumption could be optimised in order to contribute to overall health.

The polyphenol quercetin, a wel-known iron chelating agent, is ingested daily in great amounts (16 mg/day; Olthof et al., 2000) and thus it was considered worthwhile to investigate the chelation effect of quercetin, its methylated forms, and resveratrol, a polyphenol that do not possess iron chelation ability, on duodenal non-haem iron absorption using an *in vivo* uptake method.

4.2 Results

To investigate the effects of quercetin on duodenal non-haem iron absorption, the *in situ* duodenal loop method was carried out, where either quercetin (aglycone) or methylated quercetin isoforms or resveratrol were introduced into the rat duodenum together with radioactive iron. Afterwards, radioactive measurements of blood samples and duodenal mucosa were preformed in order to estimate mucosal iron uptake and mucosal iron transfer.

In the presence of quercetin, 3–*O*–methylquercetin, 4'–*O*–methylquercetin, 3,4'–dimethylquercetin, but not penta–methylquercetin, there was a significant increase in mucosal ^{59}Fe uptake compared with the untreated control group (see Figure 4–1). The increase in uptake was significantly higher in the presence of quercetin aglycone and 4'–*O*–methylquercetin compared with the other methylated forms (see Figure 4–1). Additionally, there was no difference in mucosal ^{55}Fe uptake when resveratrol was introduced compared with the untreated control group (see Figure 4–2).

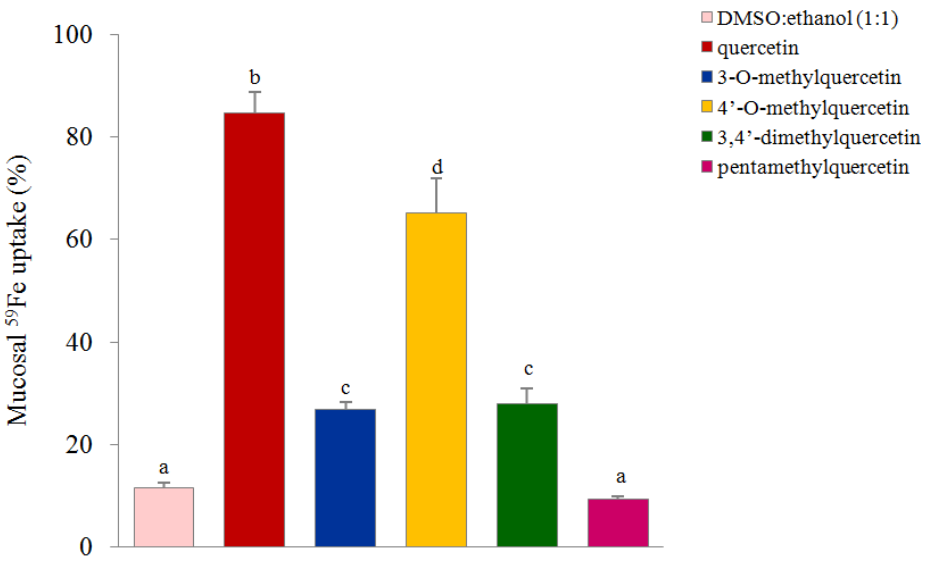


Figure 4–1 Effect of quercetin and its metabolites on mucosal iron uptake in rats

Effects of quercetin and its methylated analogues on iron transport *in vivo* were measured using the *in situ* duodenal loop method. ^{59}Fe and polyphenol were added to the lumenal uptake buffer and mucosal iron uptake was measured. Data are mean ± SEM; n=5 rats per group; groups with no common letters are significantly different from each other ($p<0.05$).

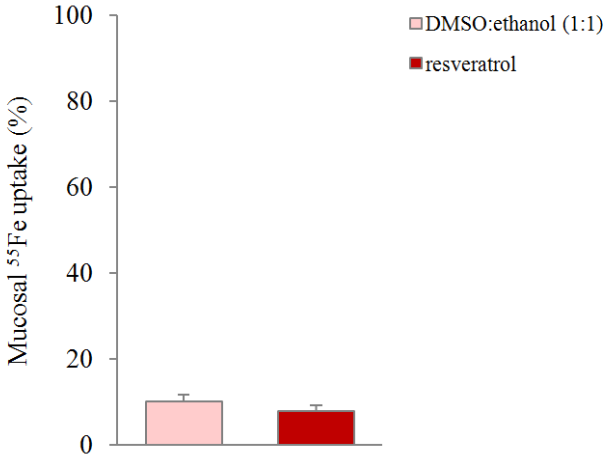


Figure 4–2 Effect of resveratrol on mucosal iron uptake in rats

Effect of resveratrol on iron transport *in vivo* was measured using the *in situ* duodenal loop method. ^{55}Fe and resveratrol were added to the lumenal uptake buffer and mucosal iron uptake was measured. Data are mean ± SEM; n=5 rats per group.

Furthermore, ^{59}Fe release from the intestinal mucosa into the blood was significantly diminished in the presence of quercetin, 3–*O*–methylquercetin, 4'–*O*–methylquercetin, 3,4'–dimethylquercetin, but not penta–methylquercetin, compared with the control group (see Figure 4–3). The decrease iron transfer was significantly emphasized in the presence of quercetin aglycone and 4'–*O*–methylquercetin compared with the other methylated forms (see Figure 4–3). In contrast, when resveratrol was introduced into the duodenum together with ^{55}Fe, iron transfer was the same as in the control group (see Figure 4–4).

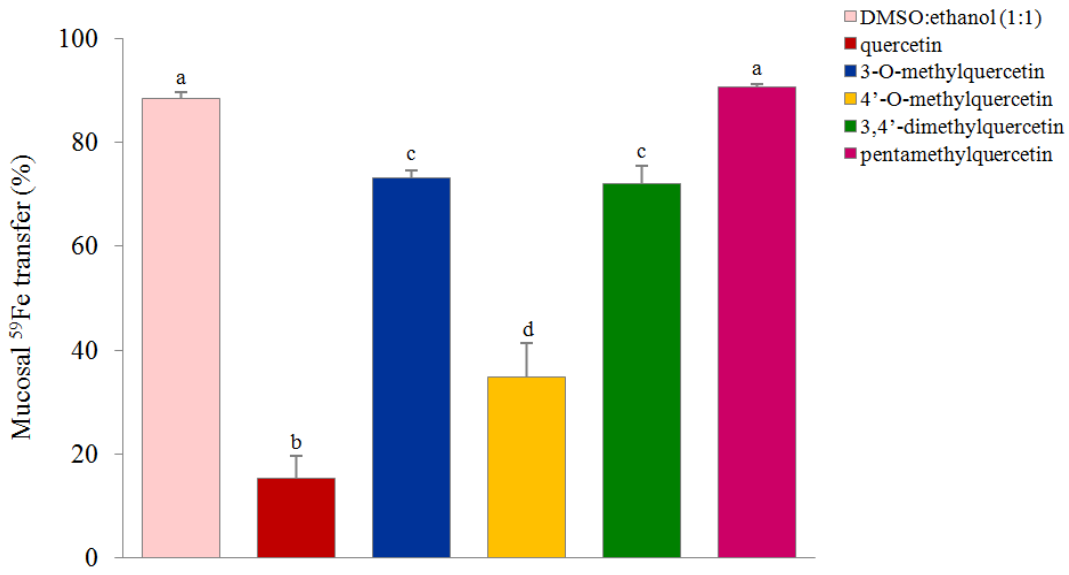


Figure 4–3 Effect of quercetin and its metabolites on mucosal iron transfer in rats
Effects of quercetin and its methylated analogues on iron transport *in vivo* were measured using the *in situ* duodenal loop method. ^{59}Fe and polyphenol were added to the lumenal uptake buffer and mucosal iron transfer was measured. Data are mean ± SEM; n=5 rats per group; groups with no common letters are significantly different from each other ($p<0.05$).

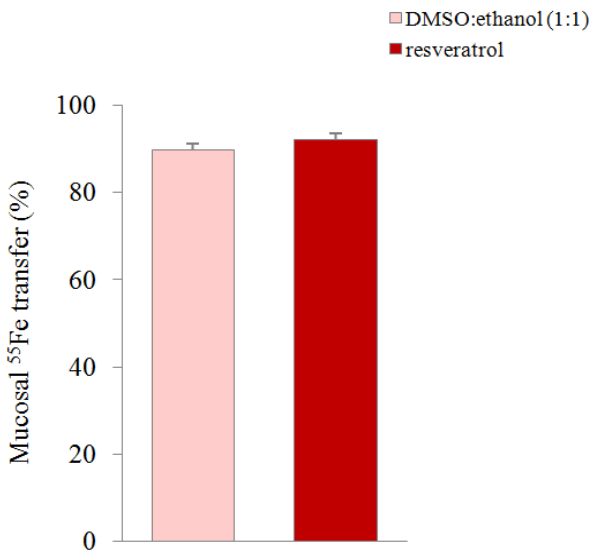


Figure 4–4 Effect of resveratrol on mucosal iron transfer in rats
Effect of resveratrol on iron transport *in vivo* was measured using the *in situ* duodenal loop method. ^{55}Fe and resveratrol were added to the lumenal uptake buffer and mucosal iron transfer was measured. Data are mean ± SEM; n=5 rats per group.

4.3 Discussion

In Chapter 3 of this thesis it was shown that quercetin increases iron uptake and retention by the duodenal mucosa *in vivo*. However, it is not clear what is the exact mechanism behind this phenomenon, but it is assumed that iron chelation by quercetin could play an important role.

Previously it has been shown that the preferred site for iron chelation by quercetin is between the 3–hydroxyl and 4–carbonyl group. However, for complexes containing one iron and one quercetin molecule, the binding strength of chelation site has an order 3–4 > 4–5 > 3'–4' (see Figure 4–5; Ren et al., 2008). Moreover, the 3–4 chelation site is also preferred for complexes which are formed between one iron and two or three quercetin molecules (see Figure 4–5; Ren et al., 2008).

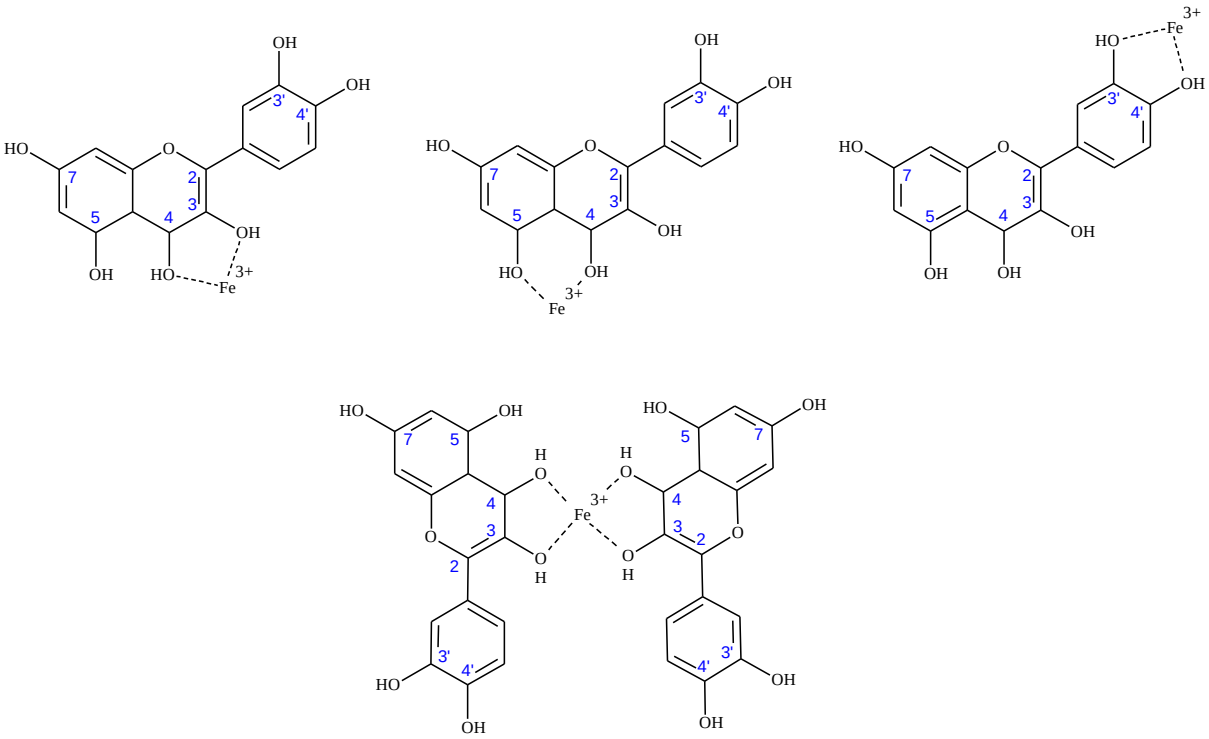


Figure 4–5 Structures of complexes between Fe^{3+} and quercetin

Therefore, in order to elucidate the importance of iron chelating by quercetin in transepithelial iron transport, *in vivo* uptake experiments were performed with quercetin aglycone and the methylated forms of quercetin, as well as resveratrol (see Figure 4–6). The main aim of this experiment was to determine whether replacing the putative iron-binding groups of quercetin would influence iron transport across the intestine. Additionally, an experiment was performed with the polyphenol resveratrol, which lacks iron chelating ability.

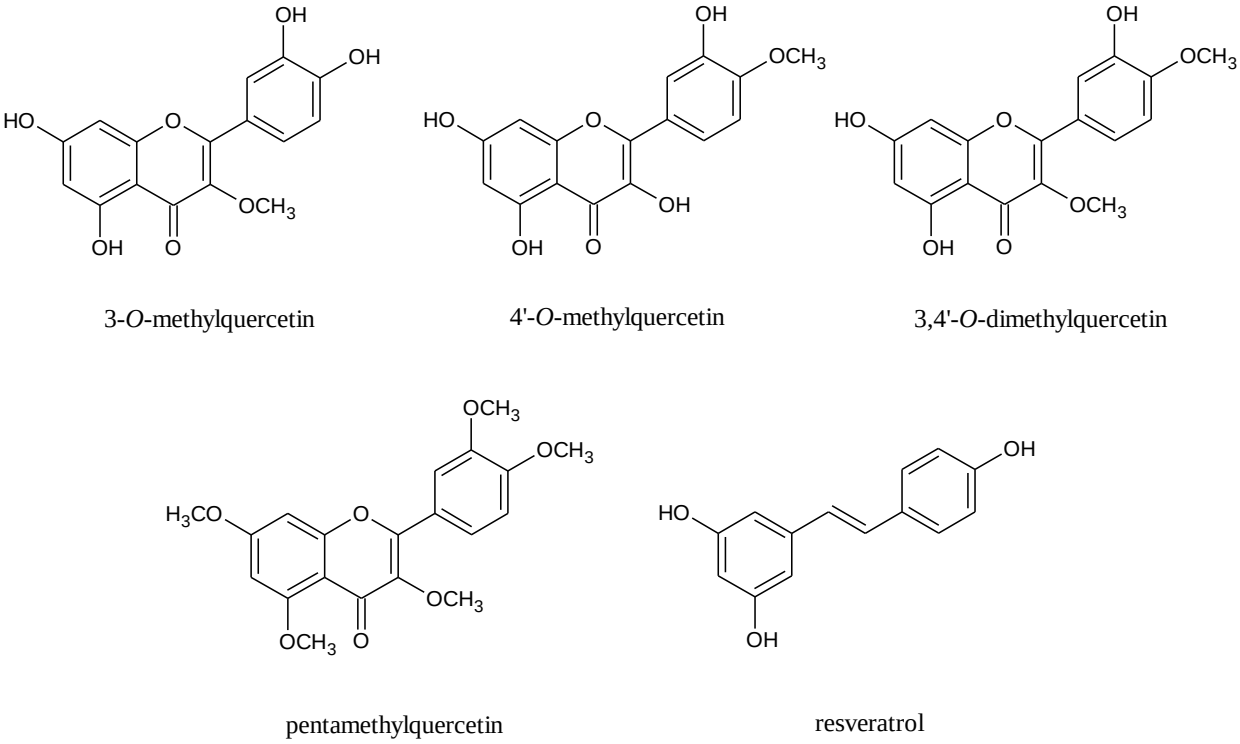


Figure 4–6 Structures of methylated forms of quercetin and resveratrol

Results in this thesis revealed that when quercetin was introduced into the duodenum together with ^{59}Fe, 85% of the total absorbed iron stayed in the mucosa, while only 15% crossed into the circulation. These results were opposite to those in the control group where no polyphenol was introduced into duodenum and 89% of total of absorbed ^{59}Fe was detected in the circulation. Additional uptake studies with the methylated quercetin forms showed that the iron chelation power of the investigated compounds was in correlation with the decrease of transepithelial iron transport. The iron chelation power of the examined compounds decrease in the order quercetin > 4'–*O*–methylquercetin > 3–*O*–methylquercetin ≥ 3,4'–dimethylquercetin > pentamethylquercetin. In the same order amount of transepithelial iron transport increased. Namely, when each polyphenol, quercetin, 4'–*O*–methylquercetin, 3–*O*–methylquercetin, 3,4'–dimethylquercetin and pentamethylquercetin, was introduced

into duodenum 15%, 35%, 73%, 73% and 91% of total of absorbed iron was detected in the circulation, respectively. In accordance with that, mucosal uptake decreased in same order and when each of listed polyphenols was introduced into duodenum, 85%, 65%, 27%, 27% and 9% of total of absorbed iron stayed in mucosa, respectively). Our data clearly indicate that the greatest increase in transepithelial iron transport was observed with compounds where 3–hydroxyl groups were methylated (3–*O*–methylquercetin, 3,4'–dimethylquercetin and pentamethylquercetin). In contrast, when the 3–hydroxyl group was present, that is in quercetin and 4'–*O*–methylquercetin, there was a decrease in transepithelial iron transport. These results demonstrate that chelation of iron by the 3–hydroxyl group of quercetin is an important determinant of iron uptake in the duodenum. In other words, transepithelial iron transport increased as the iron chelating power of the compounds decreased.

This is also confirmed with the experiment with resveratrol where inhibition of transepithelial iron transport was lacking (i.e. resveratrol does not have structural characteristics which are required for iron chelation, such as hydroxyl and carbonyl group in near vicinity or galloyl group). Resveratrol was chosen as the polyphenol abundant in a plant-based diet, particularly in red wine that is known to decrease iron absorption in intestine (Bezwoda et al., 1985; Cook et al., 1995).

The above results firmly confirm that the decrease in mucosal iron transfer is due to chelation of iron by quercetin which increases apical uptake of iron, but prevents basolateral transport. This explanation can be applied to all polyphenols that have a noticeable capacity to chelate iron, particularly those which are present in the diet and thus can directly affect iron absorption. This phenomenon was previously shown

for other polyphenols, particularly for (–)–epigallocatechin–3–gallate, but in *in vitro* conditions using Caco–2 cells as a model system (Kim et al., 2008; Kim et al., 2011). Kim et al. (2008; 2011) reported their finding as unexpected as it was common to think that polyphenols inhibit iron absorption by preventing mainly apical uptake of non-haem iron. My findings greatly contribute to a revision of this concept, especially by providing the first *in vivo* results. Further information that supports this hypothesis is the fact that the quercetin–Fe complex is considerably stable in gastrointestinal conditions. Namely, it was proven *in vitro*, by mimicking conditions that occur in the stomach, that the recovery of quercetin–Fe complex is up to 45%, which supports the importance of chelation of iron by quercetin in the human body (Escudero et al., 2014).

However, the precise place of iron chelation by quercetin is still uncertain. It is still unknown whether chelation occurs in the duodenal lumen or the cytosol of duodenal enterocytes. One explanation could be that iron is chelated by quercetin in the duodenal lumen by forming the apical–membrane–permeable quercetin–Fe complex that cannot cross the basolateral membrane of enterocyte. Despite its great size, there are *in vitro* reports that support transport of the quercetin–Fe complex across the cell membrane in both directions (Baccan et al., 2012). Furthermore, there is evidence that quercetin–Fe complex is transported by GLUTs transporters (Vlachodimitropolou et al., 2011), which could also be the case *in vivo*. Furthermore, even though it was proven that quercetin can be transported via GLUTs 1, 3 and 4 transporters, quercetin is lipophilic enough so it can easily cross lipid bilayers without interaction with transporters (Strobel et al., 2005; Cunningham et al., 2006; Vlachodimitropoulou et al., 2011). A second possibility is that that quercetin could influx into the cell and then form a complex with free iron. Additionally, it was

shown that quercetin may operate as a substrate for DcytB by increasing its reduction potential and providing more Fe^{2+} for cellular uptake by DMT1 (Vlachodimitropoulou et al. 2010). Knowing this, quercetin could firstly chemically reduce non-haem iron and thus increase apical uptake followed by formation of the quercetin–Fe complex inside the cell. However, this hypothesis would not affect the results in this study, as ascorbic acid was added to the uptake buffer which is likely to keep free iron in the Fe^{2+} form. Therefore both luminal and cytosolic iron chelation, or their combination, can provoke iron accumulation within duodenal mucosa *in vivo*. However, in both cases the quercetin–Fe complex could remain in the enterocyte due to the inhibition of FPN function or simply because the quercetin–Fe complex would be too bulky to be transported by FPN. Furthermore, the quercetin–Fe complex inside the cell could be a negative signal for the IRE/IRP system and thus destabilize FPN mRNA. Thus by chelating iron quercetin could lower free iron levels inside the cell and thus trigger the post-transcriptional IRE/IRP control system, such that when iron levels in tissue are reduced, expression of FPN is decreased (Muckenthaler et al., 2008). Furthermore, the possibility that quercetin or its metabolites have direct inhibitory effects on the function of FPN should not be discounted. Together these mechanisms could account for the increased mucosal iron retention observed in the present study. Furthermore, if a quercetin–Fe complex is formed inside the cell, it could be proposed that quercetin could affect absorption of haem iron too. Namely, quercetin could also prevent export of free iron for the haem source, after haem degradation by HO–1 which occurs in the cytosol after its absorption. This theory is only an assumption because not sufficient experimental work has been carried out to tnvestigate this further.

4.4 Conclusions

In conclusion the presented results are the first evidence that specific polyphenols inhibit *in vivo* non-haem iron absorption in the duodenum through chelation. It was shown that quercetin chelates iron within via its 3–hydroxyl group and thus prevents transepithelial non-haem iron transport across the enterocyte by increasing apical iron uptake and decreasing the basolateral iron release. However, the precise place of iron chelation by quercetin, luminal or cytosolic, is still uncertain. The ultimate aim of this research is the use of polyphenols in the future as drugs for treating iron metabolism disorders. Specifically, quercetin and other polyphenols with chelation properties could be used as a part of so-called chelato-therapies, where their ability to chelate iron can be used in iron-overload conditions to reduce iron absorption or to modify tissue iron distribution. However, it is obvious that the use of quercetin and polyphenols should be avoided in iron deficiency, especially during oral consumption of iron, either as a natural constituent of the diet or as a food supplement.

5. SYSTEMIC EFFECTS OF QUERCETIN (IP) ON IRON HOMEOSTASIS

5.1 Introduction

Polyphenols are well known for their numerous health benefits, which are mostly attributed to their ability to scavenge highly reactive free radical species or up-regulate transcription of cytoprotective enzymes (Williamson et al., 1996; Havsteen, 2002; Molina et al., 2003; Masella et al., 2005; Sharma, 2014).

Their antioxidant potential is, at least partially, associated with their ability to chelate iron. Despite iron's essential role in life, excess iron is toxic. Namely, surplus iron induces oxidative stress and reactive oxygen species, which in turn causes the oxidation of lipids, proteins and nucleic acids (Halliwell and Gutteridge, 2007). The introduction of new iron chelating drugs may ultimately improve iron-chelation therapy for patients with iron overload diseases, such as β-thalassemia and haemochromatosis. Research in the light of selecting the most effective and least toxic drug, or drug combinations, is evolving (Kontoghiorghes, 2003; Poggiali et al., 2012).

Like most other flavonoids, it was also proven that quercetin possesses a high ability to chelate iron (Leopoldini et al, 2006). Furthermore, it has been proven *in vivo* that quercetin is able to decrease intracellular iron, which makes it as valuable representative of new chelato therapeutics for iron-redistribution therapy (Zhao et al., 2005; Zhang et al., 2006; Zhang et al., 2011; Baccan et al., 2012).

Polyphenols were shown to up-regulate hepcidin along with up-regulation of transcription of a battery of cytoprotective genes, in a preliminary study in our lab using *in vitro* HepG2 culture cell model (personal communication with Henry K. Bayele and Sara Balesaria). It was reasoned that the hepcidin gene, as a main iron regulatory hormone, might be a member of the battery of genes that are involved in coordinating cellular responses to oxidative stress.

The aims of this study were to investigate the effect of quercetin on systemic iron regulation *in vivo* (in rats) and determine the mechanism of its action.

5.2 Results

5.2.1 Effects of quercetin IP administration on iron metabolism *in vivo* at various times after administration

The effect of IP quercetin on iron methabolism was studied *in vivo* in rats 0 or 2, 5, 12 and 18 hours after a single IP, or duoble IP 18 and then 5 hours before being sacrificed containing quercetin (50 mg/kg body weight) or 10% DMSO (control). After weaning rats were placed on low iron diet or regular diet for two weeks. At the end of the experimental procedure animals were killed by administering a terminal dose of pentobarbitone sodium and blood samples were removed via cardiac puncture. Subsequentlly, serum was separated and used for serum iron and transferrin saturation measurements using stadard spectrophotometric methods. Additionally, duodenum, liver, spleen and kidney were removed and subsequently used for tissue non-haem iron spectrophotometric measurements and gene expression levels determination.

5.2.1.1 Effect of quercetin on serum iron and transferrin saturation

Serum iron and transferrin saturation levels were significantly decreased five hours after a single IP treatment of quercetin (50 mg/kg) to rats fed on an iron-deficient diet, while 12 and 18 hours after the same treatment both serum iron and transferrin saturation increased significantly compared with the group that was killed right after the quercetin treatment (see Figure 5–1). Furthermore, serum iron and transferrin saturation levels decreased significantly in rats fed on an iron-deficient diet and treated with double quercetin IP treatment (see Figure 5–2; A). However, serum iron

and transferrin saturation in rats fed on normal iron diet and treated with double quercetin IP treatment showed no change (see Figure 5–2; B).

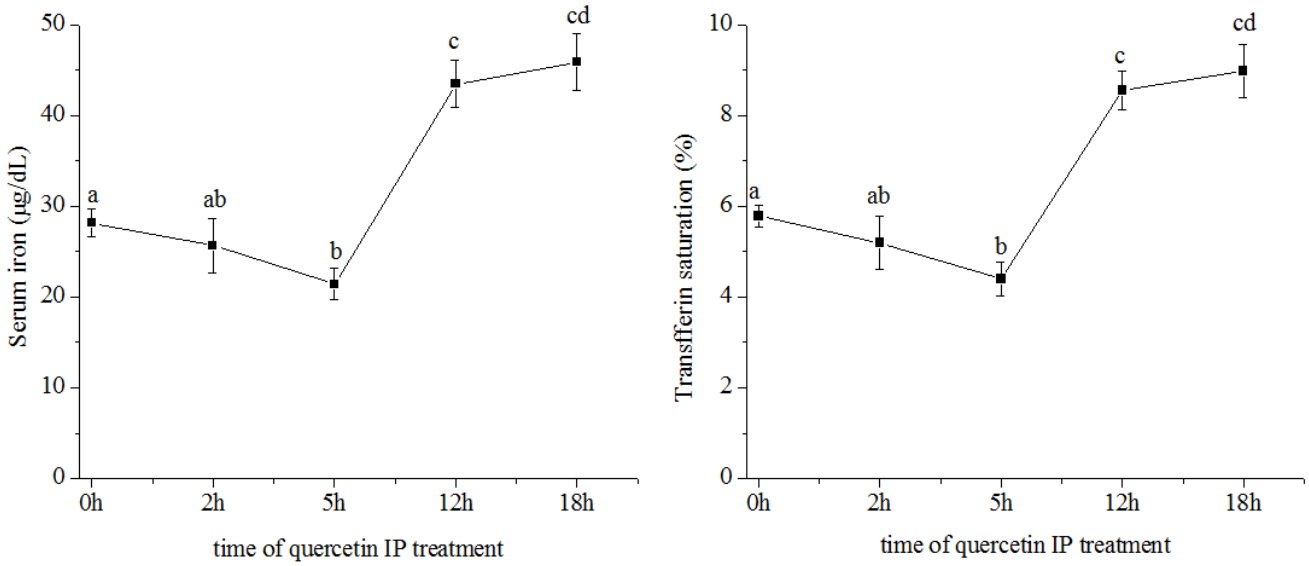


Figure 5–1 Effects of a single quercetin IP treatment on serum iron and transferrin saturation in rats at different times.
The effects of quercetin on serum iron and transferrin saturation were measured 0 or 2, 5, 12 and 18 hours after a single IP containing quercetin (50 mg/kg body weight) or 10% DMSO (control) given to rats. Data are mean ± SEM; n=4 rats per group; groups with no common letters are significantly different from each other ($p<0.05$).

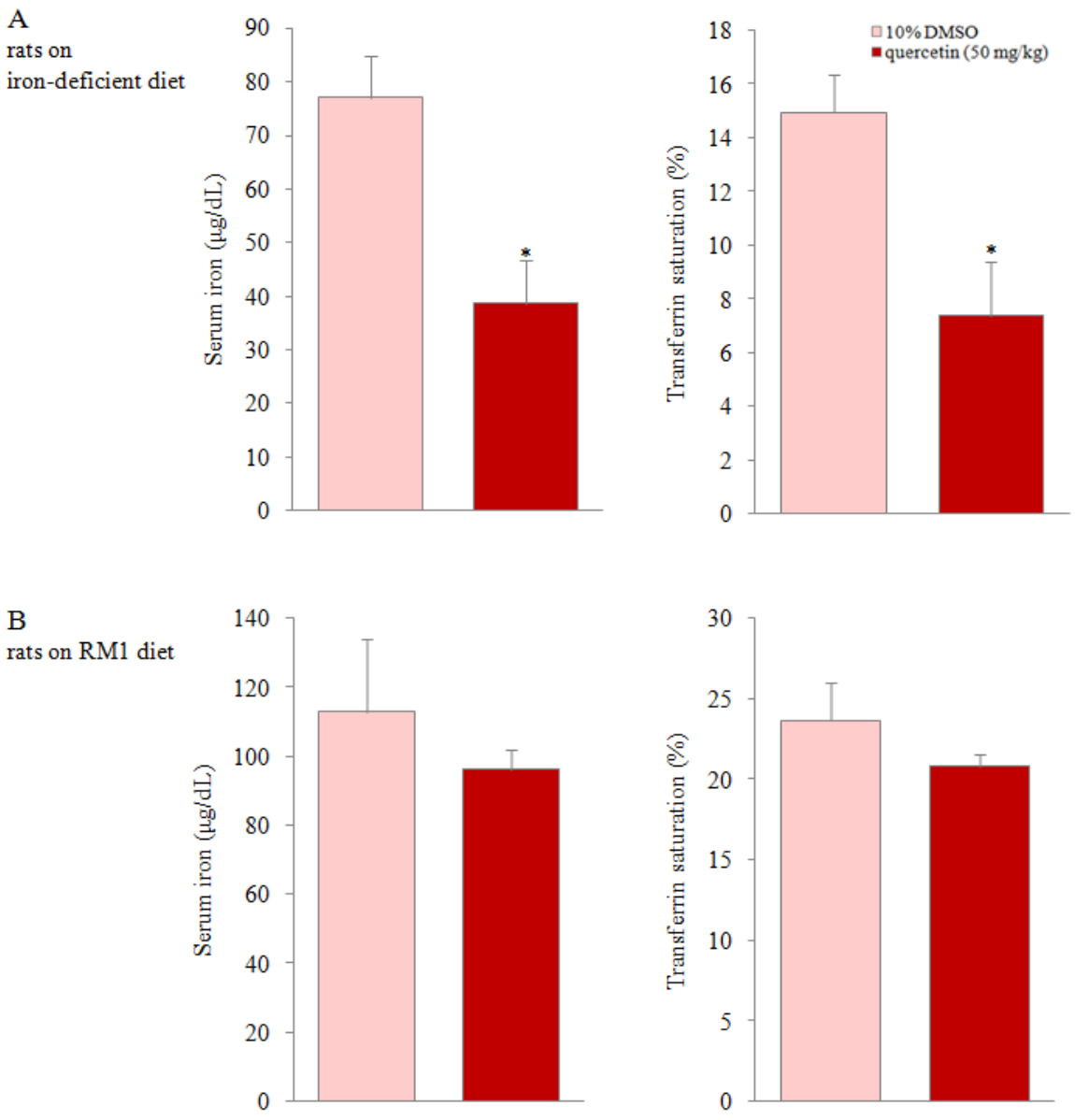


Figure 5–2 Effects of double quercetin IP treatment on serum iron and transferrin saturation in rats.

The effects of quercetin on serum iron and transferrin saturation were measured in rats that were IP with quercetin (50 mg/kg body weight) or 10% DMSO (control) eighteen and then five hours before being sacrificed. Data are mean ± SEM; n=5 (A); 4 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

5.2.1.2 Effect of quercetin on gene expression in duodenum, liver, spleen and kidney

Analysis of iron transporters (DMT1, FPN) and Dcytb mRNA expression in duodenum of rats fed on an iron-deficient diet and sacrificed at different times after IP treatment with quercetin, generally showed a significant increase of all three examined genes. DMT1 mRNA levels increased significantly 12 hours after quercetin IP treatment (2.5-fold), while levels of Dcytb and FPN mRNA levels increased notably earlier then DMT1, i.e. 5 hours after the treatment (1.6- and 1.3-fold, respectively). It should be noted, that mRNA levels of Dcytb followed the trend of a significant increase 12 and 18 hours after quercetin IP treatment (2.4- and 3.9-fold, respectively; see Figure 5–3). Furthermore, after double quercetin IP treatment with rats fed on an iron-deficient diet, analysis of the same genes in duodenum showed the opposite results. Specifically, DMT1, Dcytb and FPN mRNA levels significantly decreased (5.7-, 4.4- and 2.0-fold, respectively; see Figure 5–4; A). Rats fed on a normal iron diet (RMI) and treated with quercetin showed no significant change in examined duodenal genes (see Figure 5–4; B).

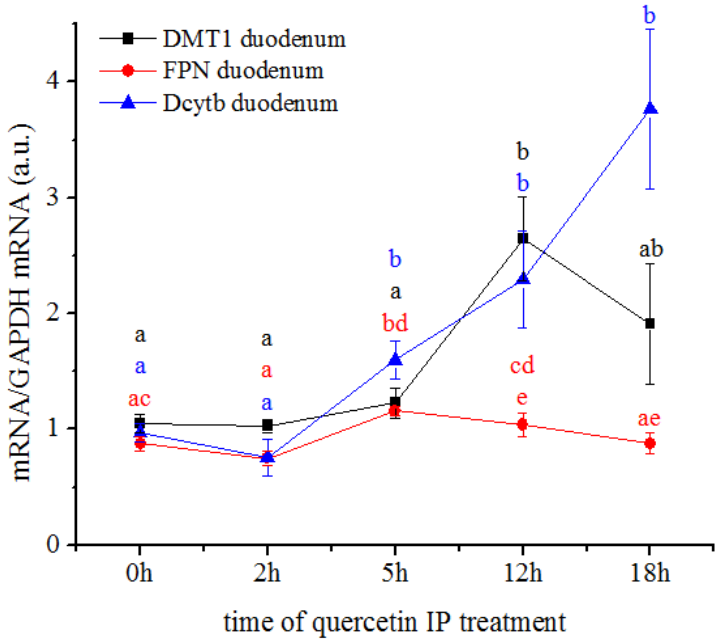


Figure 5–3 Effects of a single quercetin IP treatment on duodenal iron transporters and Dcytb gene expression in rats at different times.
The effects of quercetin on duodenal gene expression was measured 0 or 2, 5, 12 and 18 hours after a single IP containing quercetin (50 mg/kg body weight) or 10% DMSO (control) given to rats. Data are mean ± SEM; n=4 rats per group; groups with no common letters are significantly different from each other ($p<0.05$).

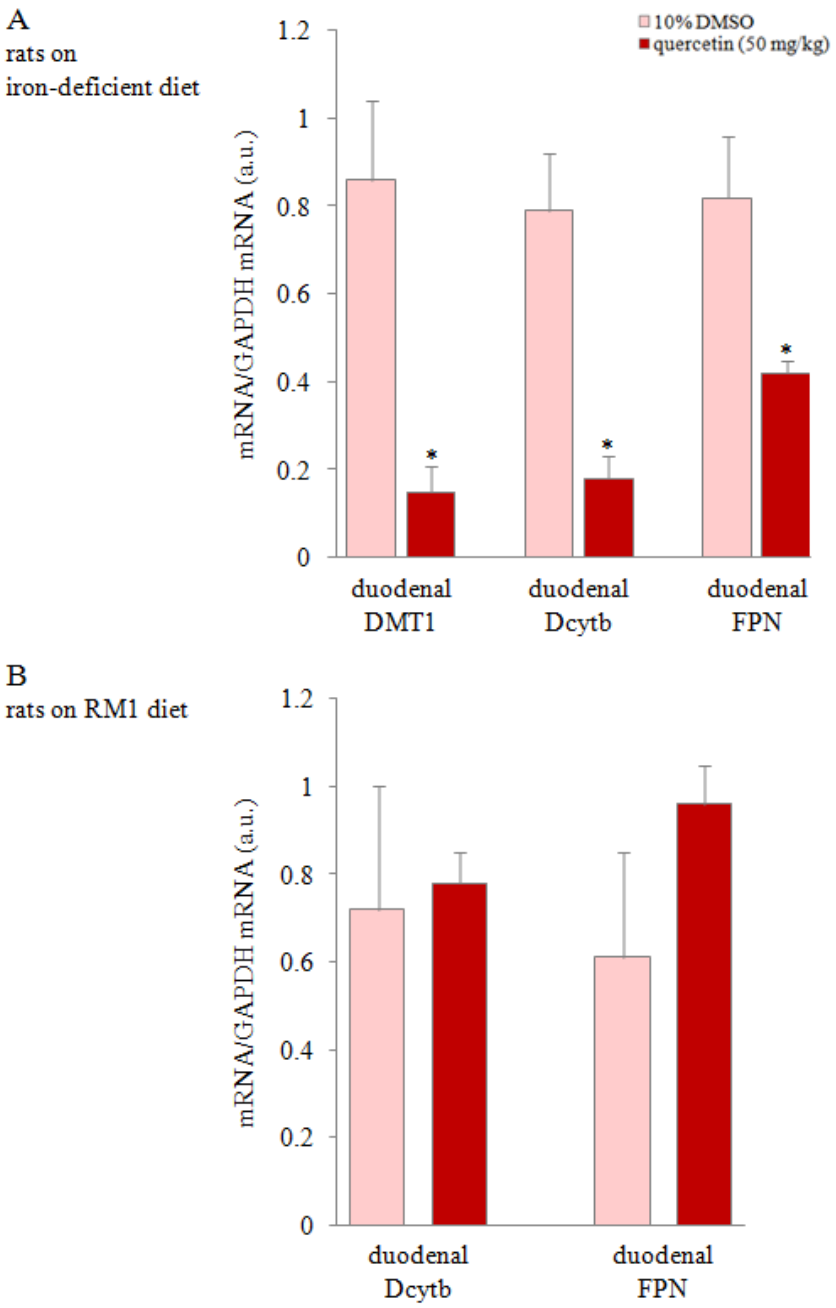


Figure 5–4 Effects of double quercetin IP treatment on duodenal iron transporters and Dcytb gene expression in rats.
The effects of quercetin on duodenal gene expression was measured in rats that were treated with quercetin (50 mg/kg body weight) or 10% DMSO (control) eighteen and then five hours before being sacrificed. Data are mean ± SEM; n=5 (A); 4 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

Analysis of hepcidin gene expression in liver, spleen and kidney of rats fed on an iron-deficient diet and sacrificed 5 hours after IP treatment with different quercetin concentrations showed a notably increase of examined gene expression in liver (see Figure 5–5). In spleen and kidney hepcidin gene levels fluctuated but not in as large range as in liver (see Figure 5–5). Namely, maximal change in hepcidin mRNA levels in liver occurred 5 hours after quercetin treatment, when levels of mRNA showed a 175-fold increase (see Figure 5–5). Additionally, maximal change in hepcidin mRNA levels in kidney occurred 12 hours after quercetin treatment, when levels of mRNA showed a 35-fold increase (see Figure 5–5). The peak change of a 2-fold increase in hepcidin mRNA levels in spleen happened 2 hours after quercetin treatment (see Figure 5–5). Furthermore, after double quercetin IP treatment with rats fed on an iron-deficient diet, analysis of the hepcidin gene in liver showed an enormous increase of 1031-fold (see ; A). Furthermore, in the same animals a vast increase of hepcidin mRNA in kidney also occurred (9.5-fold), while the same did not occur in spleen (see ; A). Double quercetin IP treatment, but with rats fed on a RM1 diet, also brought a significant change in hepcidin mRNA levels in liver (3-fold increase), however hepcidin mRNA levels in spleen and kidney decreased significantly (2.3- fold and 1.6-fold, respectively, see ; B).

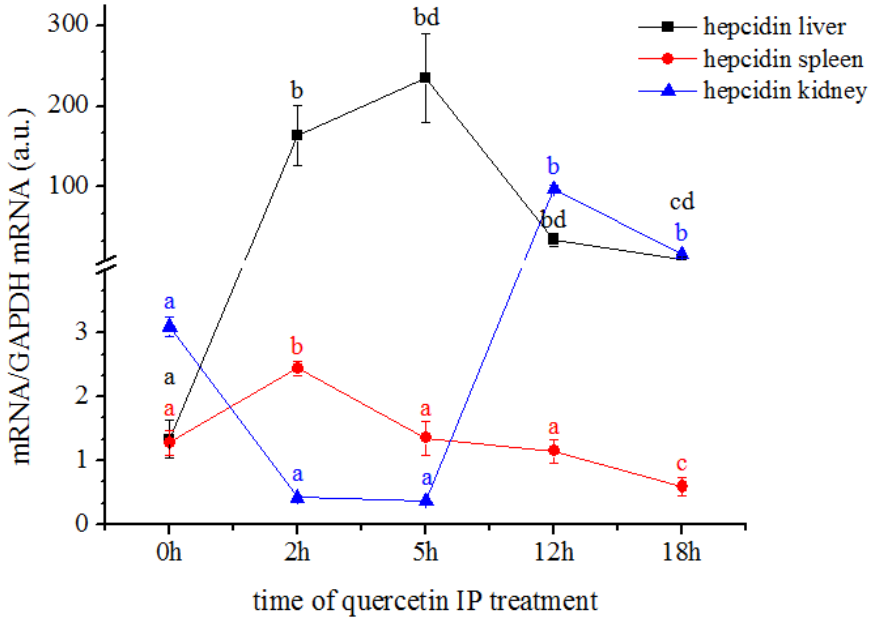


Figure 5–5 Effects of a single quercetin IP treatment on hepcidin gene expression in liver, spleen and kidney in rats at different times.
The effects of quercetin on hepcidin gene expression in liver, spleen and kidney was measured 0 or 2, 5, 12 and 18 hours after a single IP containing quercetin (50 mg/kg body weight) or 10% DMSO (control) given to rats. Data are mean ± SEM; n=4 rats per group; groups with no common letters are significantly different from each other ($p<0.05$).

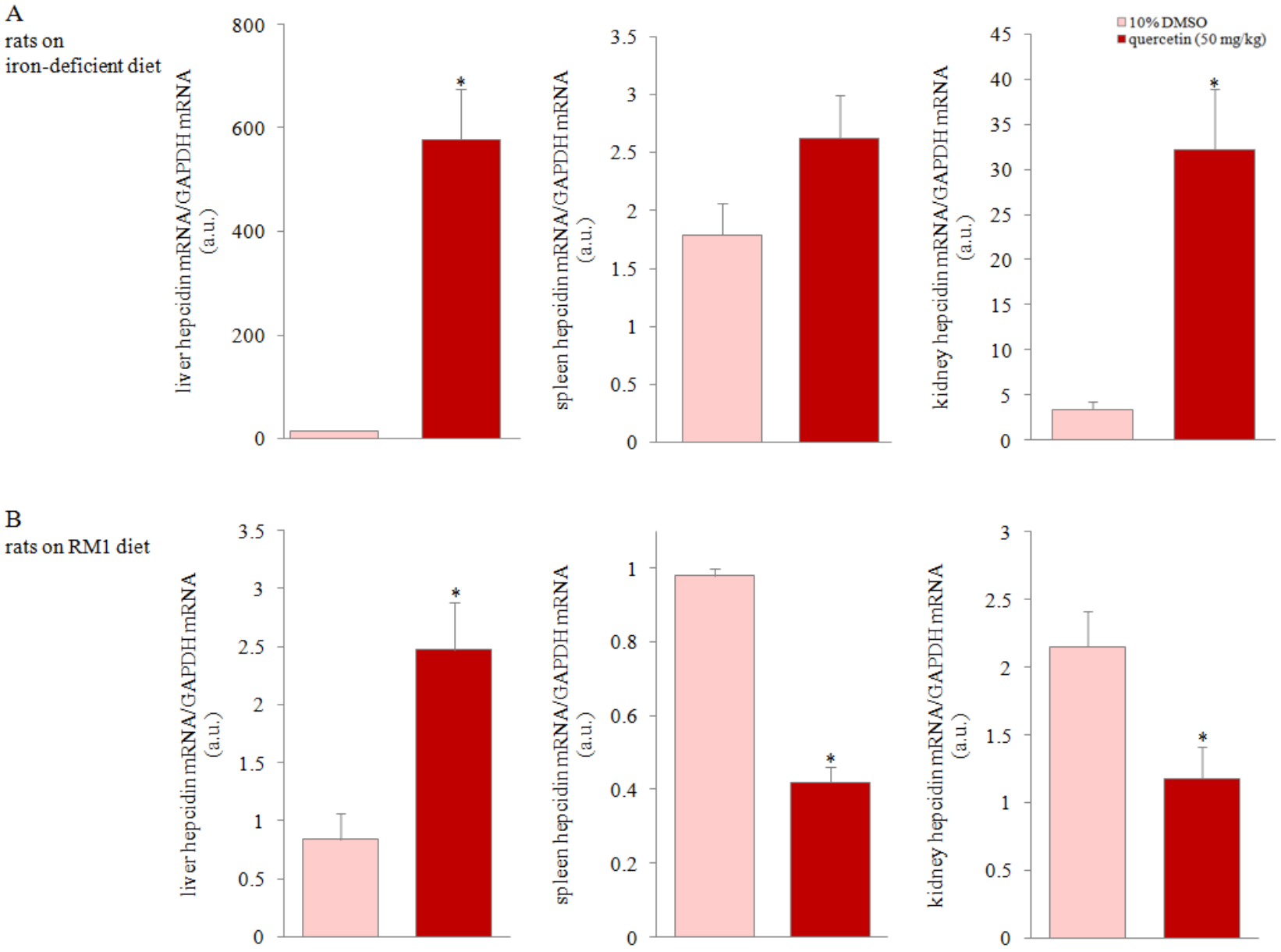


Figure 5–6 Effects of double quercetin IP treatment on hepcidin gene expression in liver, spleen and kidney in rats.

The effects of quercetin on hepcidin gene expression in liver, spleen and kidney was measured in rats that were IP with quercetin (50 mg/kg body weight) or 10% DMSO (control) eighteen and then five hours before being sacrificed. Data are mean ± SEM; n=5 (A); 4 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

Liver HO–1 gene expression notably changed in rats fed on an iron-deficient diet and sacrificed at different times after quercetin treatment (see Figure 5–7). The maximal change in HO–1 mRNA levels in liver occurred 5 hours after quercetin treatment, when levels of mRNA showed a 13.3-fold increase (see Figure 5–7). Additionally, levels of liver FPN mRNA did not change after the same quercetin treatment. In contrast to liver FPN, mRNA levels of spleen FPN fluctuated during the experiment, and reached a maximal decrease 2 and 12 hours after quercetin treatment (1.3- and 1.35-fold, respectively; see Figure 5–7). Furthermore, double quercetin IP treatment with rats fed on an iron-deficient diet, caused a significant increase in mRNA levels of HO–1, ferritin and QR in liver (11.8-, 1.8- and 1.7-fold, respectively; Figure 5–8; A). The same treatment provoked a significant decrease in FPN mRNA levels in both liver and spleen (1.5- and 1.8-fold, respectively; Figure 5–8; A). Double quercetin IP treatment, but with rats fed on RM1 diet, brought about a significant change in liver HO–1 and spleen FPN mRNA levels (3.9- fold and 1.5-fold increase, respectively; see Figure 5–8; B), while other examined genes were not affected with the treatment.

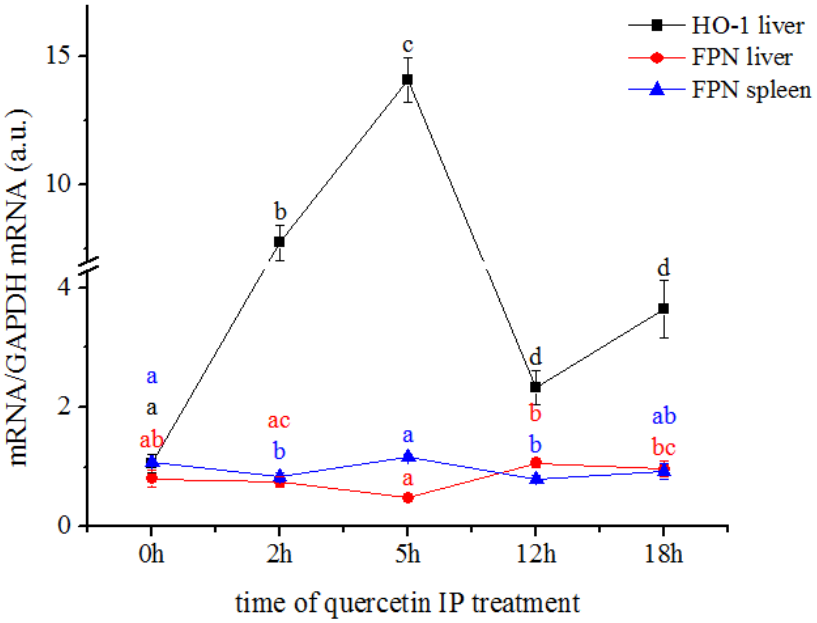


Figure 5–7 Effects of a single quercetin IP treatment on FPN and HO–1 gene expression in liver and spleen in rats at different times.
The effects of quercetin on liver and splenic gene expression was measured 0 or 2, 5, 12 and 18 hours after a single IP containing quercetin (50 mg/kg body weight) or 10% DMSO (control) given to rats. Data are mean ± SEM; n=4 rats per group; groups with no common letters are significantly different from each other ($p<0.05$).

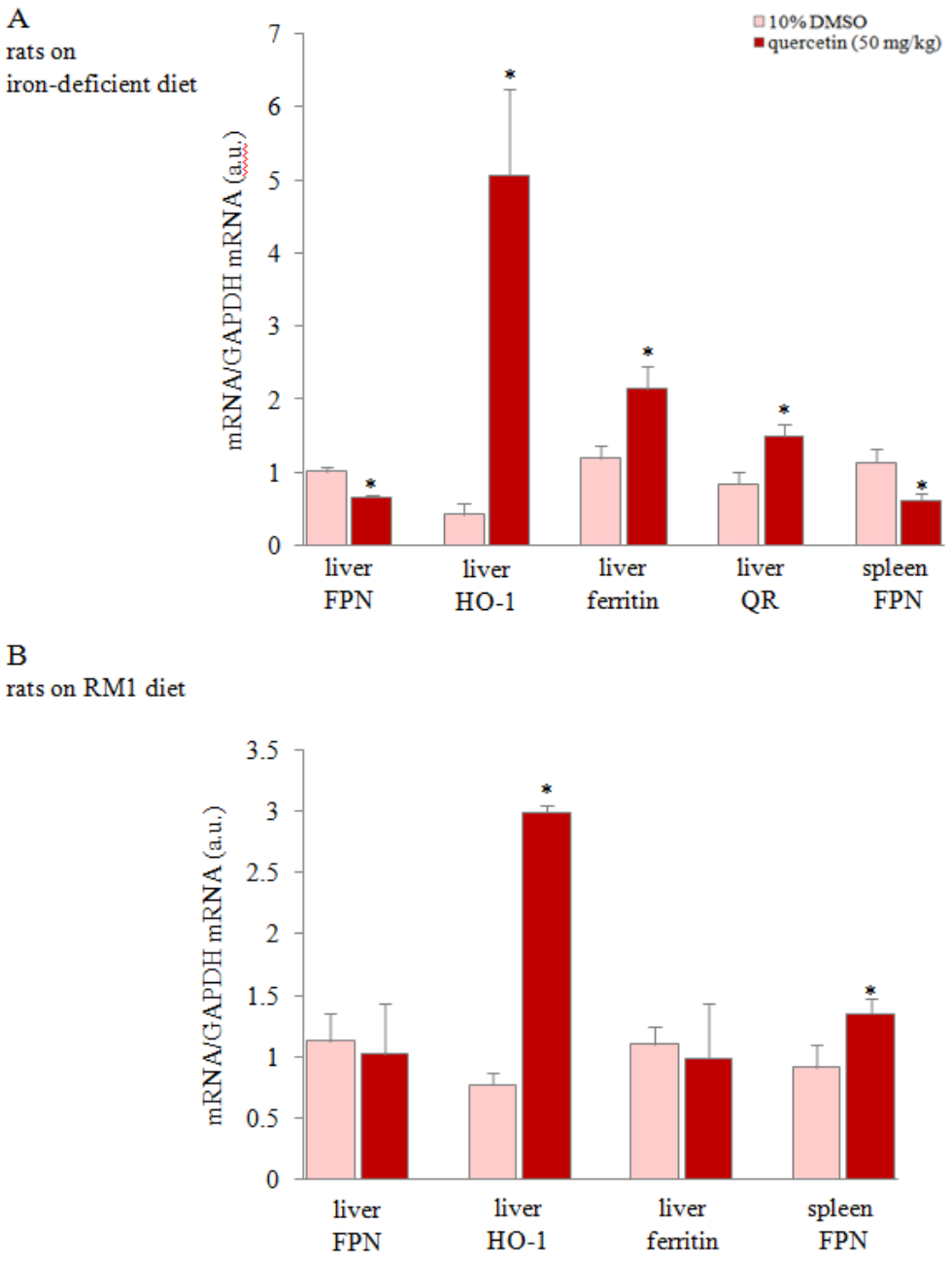


Figure 5–8 Effects of double quercetin IP treatment on relevant gene expression in liver and spleen in rats.
The effects of quercetin on liver and splenic gene expression was measured in rats that were IP with quercetin (50 mg/kg body weight) or 10% DMSO (control) eighteen and then five hours before being sacrificed. Data are mean ± SEM; n=5 (A); 4 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

5.2.1.3 Effect of quercetin on iron content in duodenum, liver and spleen

Single quercetin IP administration, during different time intervals, affected the iron content of duodenum, liver and spleen of rats fed with an iron-deficient diet (see Figure 5–9). Namely, duodenal iron levels significantly increased 2 hours after quercetin IP treatment. After that, duodenal iron followed an upward trend and reached its maximum value 12 hours after the treatment (see Figure 5–9). However, liver iron stayed the same until 18 hours after the treatment, when a significant decrease occurred (see Figure 5–9). Furthermore, spleen iron increased during the experiment and reached its maximum value 5 hours after the treatment. After 5 hours, levels of spleen iron dropped, but were still being significantly higher than at the start of experiment (see Figure 5–9).

Moreover, duodenal and liver iron levels increased significantly, while the iron levels of spleen stayed the same, in rats which were fed on an iron-deficient diet and received double quercetin IP treatment (see ; A). However, only liver iron increased significantly, while iron levels of duodenum and spleen stayed the same, after the same treatment in rats fed on a diet with a regular iron content (see ; B).

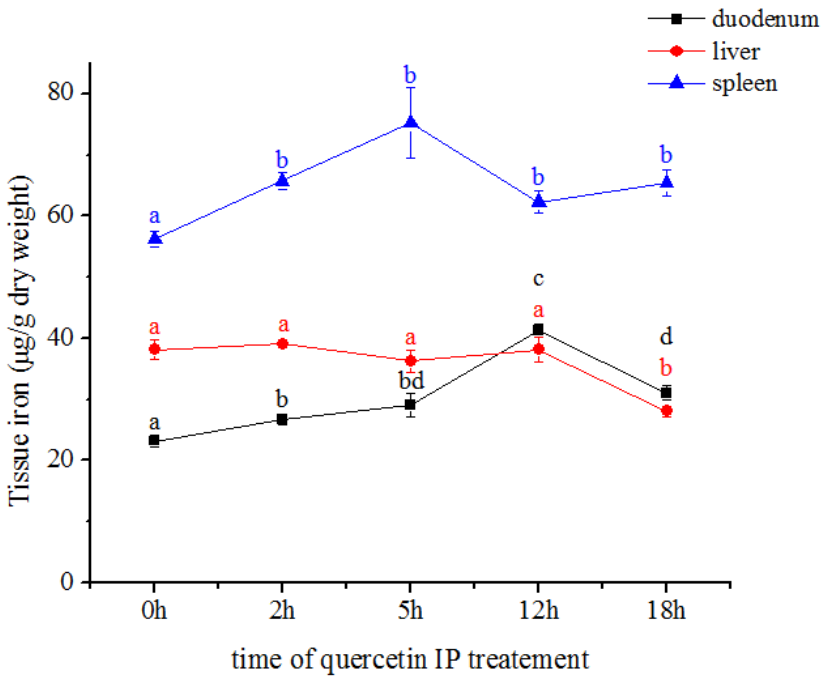


Figure 5–9 Effects of a single quercetin IP treatment on iron content in duodenum, liver and spleen in rats at different times.

The effects of quercetin on duodenal, liver and splenic iron content was measured 0 or 2, 5, 12 and 18 hours after a single IP containing quercetin (50 mg/kg body weight) or 10% DMSO (control) given to rats. Data are mean ± SEM; n=4 rats per group; groups with no common letters are significantly different from each otherp<0.05)

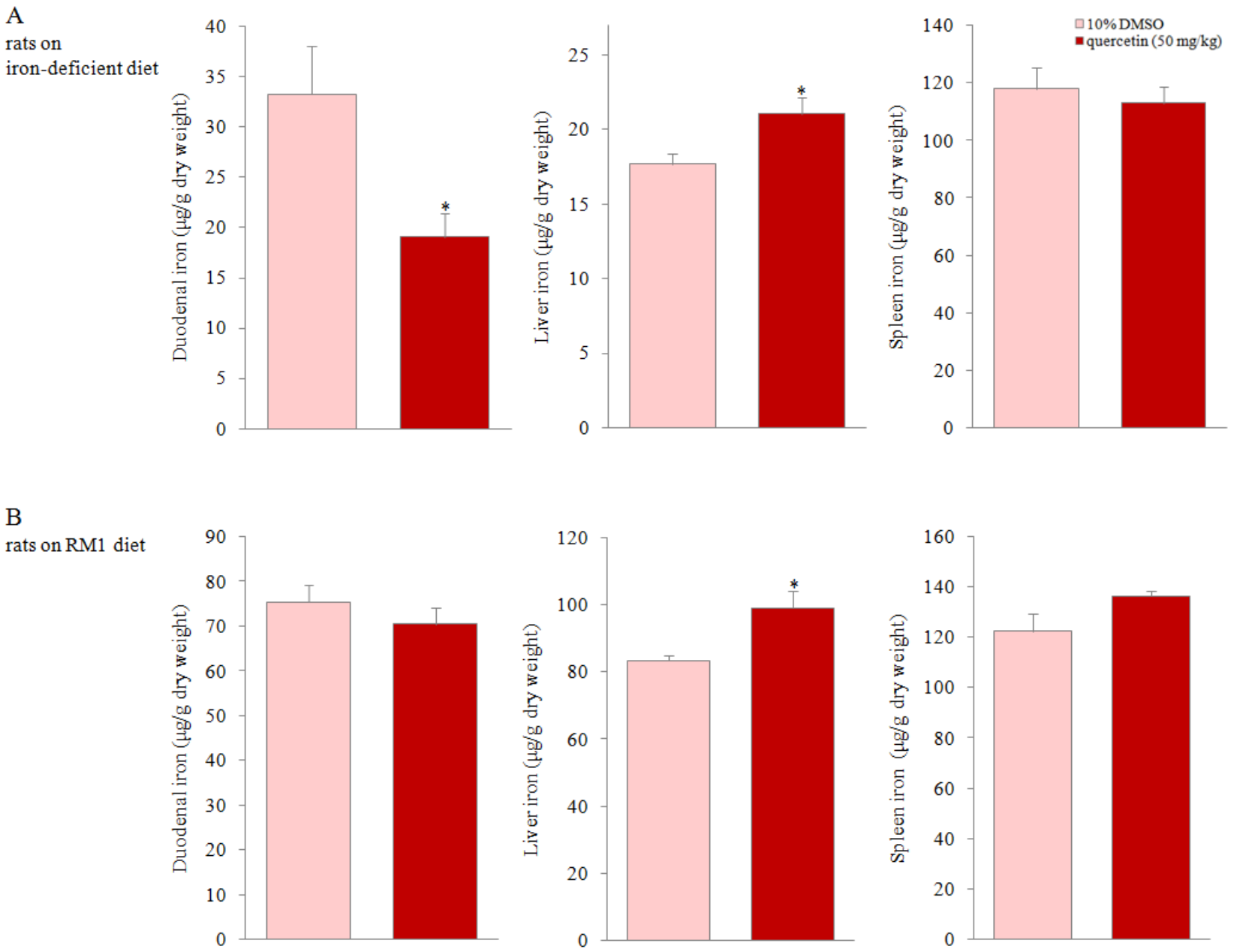


Figure 5–10 Effects of double quercetin IP treatment on iron content in duodenum, liver and spleen in rats. The effects of quercetin on duodenal, liver and splenic iron content was measured in rats that were IP with quercetin (50 mg/kg body weight) or 10% DMSO (control) eighteen and then five hours before being sacrificed. Data are mean ± SEM; n=5 (A); 4 (B) rats per group; *denotes significant difference from the 10% DMSO control group ($p<0.05$).

5.2.2 Effects of different IP dose of quercetin on serum iron metabolism *in vivo*

The effect of IP quercetin on iron methabolism was studied *in vivo* in rats 5 after a single IP containing different quercetin concentrations (0, 2, 5, 10 and 20 mg/kg body weight) or 10% DMSO (control). At the end of the experimental procedure animals were killed by administering a terminal dose of pentobarbitone sodium and blood samples were removed via cardiac puncture. Subsequentlly, serum was separated and used for serum iron and transferrin saturation measurements using stadard spectrophotometric methods. Additionally, duodenum, liver, spleen and kidney were removed and subsequently used for tissue non-haem iron spectrophotometric measurements and gene expression levels determination.

Effect of IP quercetin on iron absorption in duodenum was sudied in rats treated by a single IP 5 hours or double IP 18 hours and then 5 hours before experiment containing quercetin (50 mg/kg body weight) or 10% DMSO (control). Afterwards, uptake studies were performed where $^{59}Fe^{2+}$ was put inside the cannulated duodenum for 30 minutes while rats were anesthetized. After 30 minutes, blood samples were collected via cardiac puncture and duodenal mucosa was scraped away and subsequently gamma counted for determination of ^{59}Fe activity.

5.2.2.1 Effect of quercetin on serum iron and transferrin saturation

Serum iron and transferrin saturation levels were affected by single dose of quercetin (0, 2, 5, 10 and 20 mg/kg) given by IP five hours before being sacrificed (see Figure 5–11). Namely, after IP quercetin treatment of 5 mg/kg serum iron and transferrin

saturation levels increased significantly. Furthermore, after IP quercetin treatment of 10 and 20 mg/kg, they dropped compared with the 5 mg/kg dose group, but were significantly higher compared with the control group or the group treated with the 2 mg/kg dose of quercetin (see Figure 5–11).

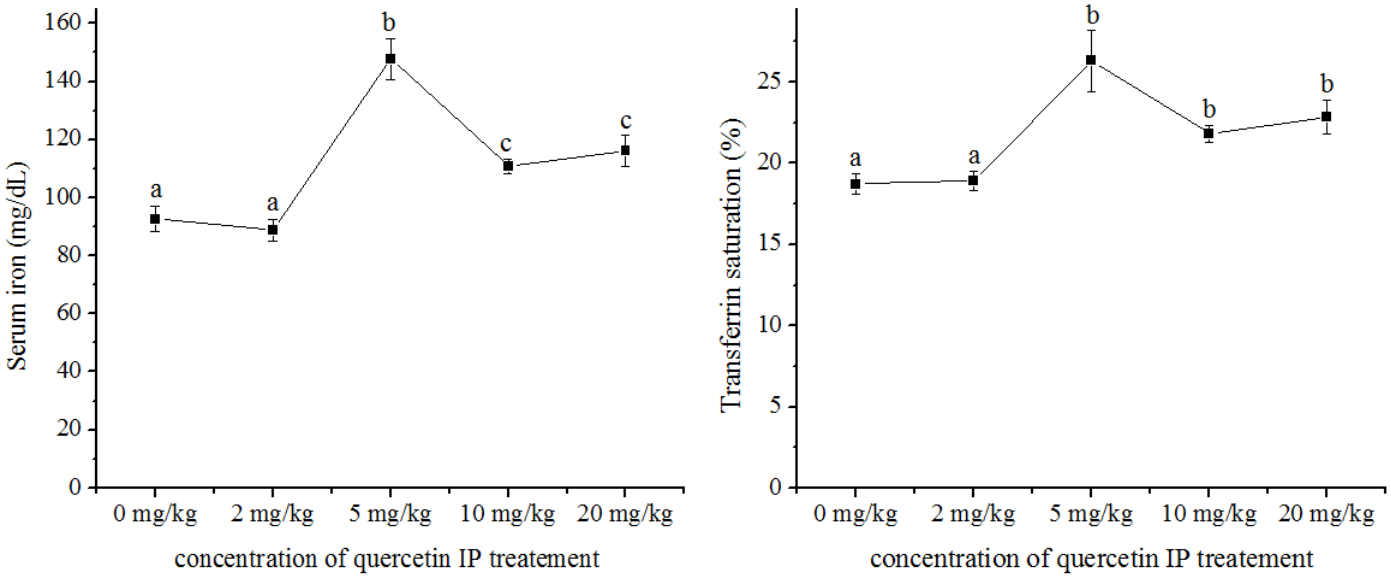


Figure 5–11 Effects of different IP dose of quercetin on serum iron and transferrin saturation in rats.
The effects of quercetin on serum iron and transferrin saturation were measured 5 hours after a single IP containing quercetin (2, 5, 10 or 20 mg/kg body weight) or 10% DMSO (control) given to rats. Data are mean ± SEM; n=4 rats per group; groups with no common letters are significantly different from each other ($p<0.05$).

5.2.2.2 Effect of quercetin on gene expression in duodenum, liver, spleen and kidney

Analysis of the mRNA expression of the iron transporters (DMT1, and FPN) and Dcytb in the duodenum of rats fed on an iron-deficient diet and sacrificed five hours after IP treatment with different quercetin concentrations generally showed a significant increase in Dcytb and FPN mRNA. Namely, a quercetin dose of 5 mg/kg and 20 mg/kg provoked the highest jump of mRNA levels (see Figure 5–12).

However, DMT1 levels either decreased significantly or stayed the same after treatment with different quercetin concentrations (see Figure 5–12).

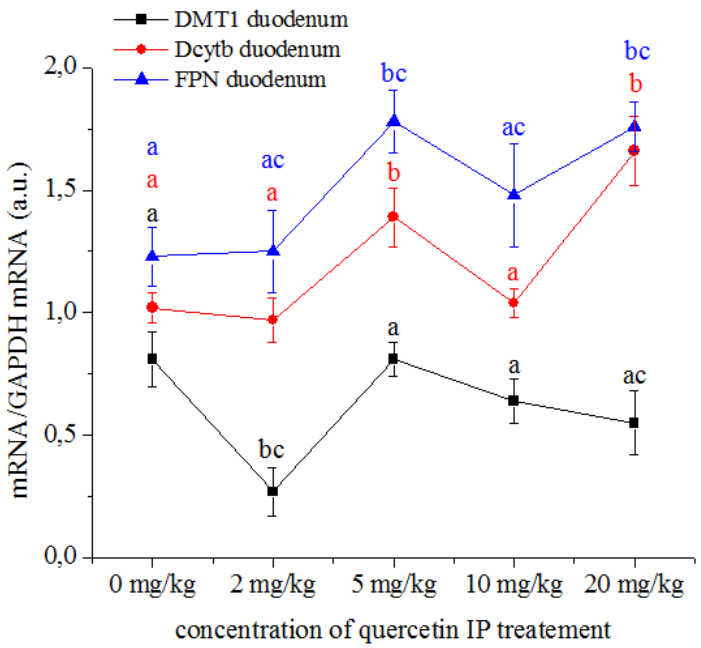


Figure 5–12 Effects of different IP dose of quercetin on duodenal iron transporters and Dcytb gene expression in rats.
The effects of quercetin on duodenal gene expression was measured 5 hours after a single IP containing quercetin (2, 5, 10 or 20 mg/kg body weight) or 10% DMSO (control) given to rats. Data are mean ± SEM; n=4 rats per group; groups with no common letters are significantly different from each other ($p<0.05$).

Analysis of hepcidin gene expression in liver, spleen and kidney of rats fed on an iron-deficient diet and treated with different quercetin IP concentration (0-20 mg/kg) after 5 hours showed a notable increase of the examined gene only in liver (see Figure 5–13). Namely, a maximal change in hepcidin mRNA levels in the liver occurred 5 hours after 10 mg/kg quercetin treatment, when levels of mRNA showed a 50-fold increase (see Figure 5–13). While after other doses, levels of liver hepcidin mRNA varied but not significantly. Generally, levels of splenic and kidney hepcidin mRNA levels fluctuated after IP quercetin but not significantly, except in spleen after IP quercetin 20 mg/kg and kidney IP quercetin 10 mg/kg, when it reduced

significantly. Additionally, after different IP quercetin concentrations splenic FPN was not altered, while the same in spleen was significantly up-regulated only after a quercetin concentration of 20 mg/kg (see Figure 5–14). Furthermore, HO-1 mRNA levels increased significantly after 10 and 20 mg/kg IP quercetin 8- and 3.2-fold, respectively, compared with control (see Figure 5–14).

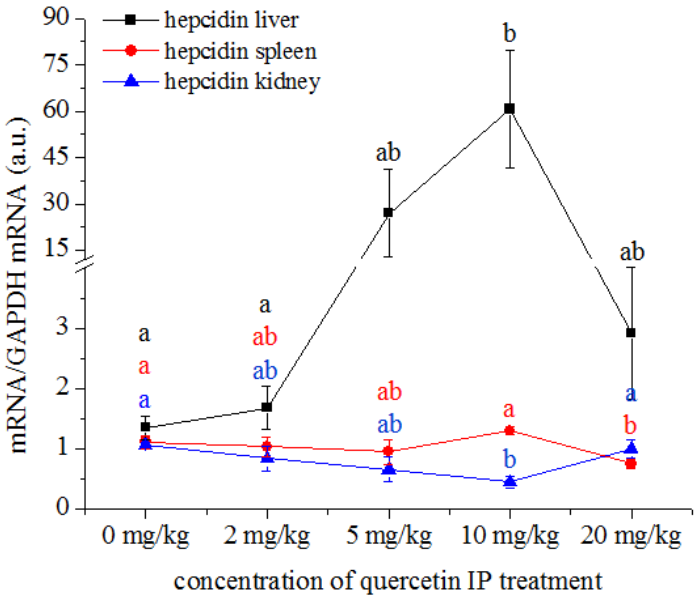


Figure 5–13 Effects of different IP dose of quercetin on hepcidin gene expression in liver, spleen and kidney in rats.
The effects of quercetin on hepcidin gene expression in liver, spleen and kidney was measured 5 hours after a single IP containing quercetin (2, 5, 10 or 20 mg/kg body weight) or 10% DMSO (control) given to rats. Data are mean ± SEM; n=4 rats per group; groups with no common letters are significantly different from each other ($p<0.05$).

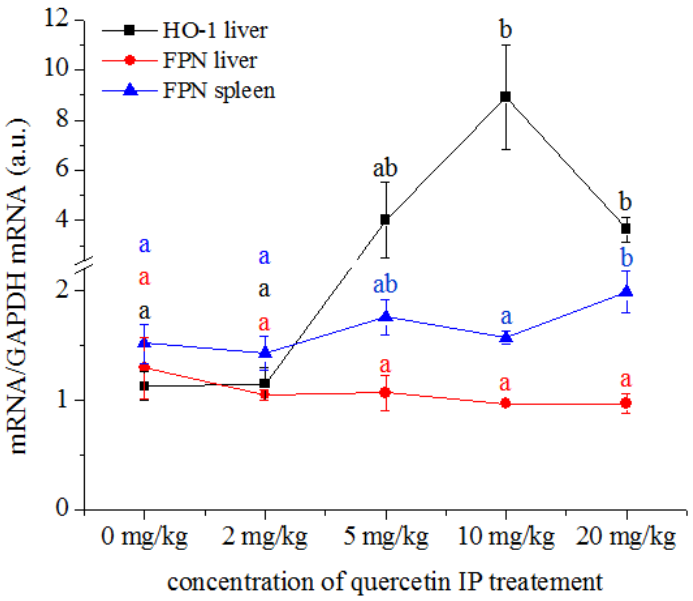


Figure 5–14 Effects of different IP dose of quercetin on FPN and HO–1 gene expression in liver and spleen in rats.
The effects of quercetin on liver and splenic gene expression was measured 5 hours after a single IP containing quercetin (2, 5, 10 or 20 mg/kg body weight) or 10% DMSO (control) given to rats. Data are mean ± SEM; n=4 rats per group; groups with no common letters are significantly different from each other ($p<0.05$).

5.2.2.3 Effect of quercetin on iron content in duodenum, liver and spleen

Different concentrations of quercetin IP treatment affected differently the iron content of duodenum, liver and spleen of rats fed with an iron-deficient diet (see Figure 5–15). Duodenal iron levels fluctuated depending on the applied quercetin concentrations. Namely, after 2 mg/kg quercetin IP treatment iron levels significantly increased, while when higher concentrations of IP quercetin was applied it exhibited decreasing trend (see Figure 5–15). Liver iron levels significantly changed only after 10 and 20 mg/kg quercetin IP treatment (1.6- and 1.2-fold decrease compared with control, respectively), while it stayed the same after other applied concentrations (see Figure 5–15). Furthermore, spleen iron levels significantly dropped after 2 mg/kg

quercetin IP treatment, while it stayed the same after other applied quercetin concentrations compared with control (see Figure 5–15).

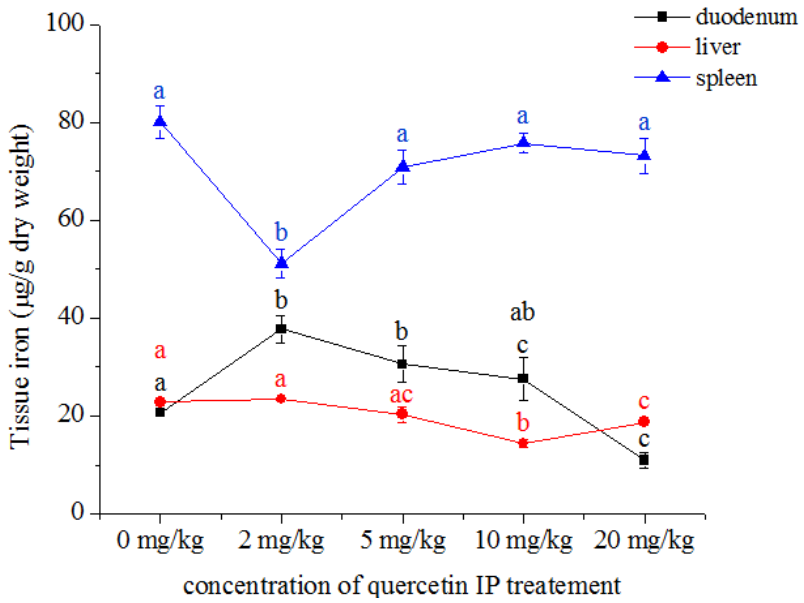


Figure 5–15 Effects of different IP dose of quercetin on iron content in duodenum, liver and spleen in rats.
The effects of quercetin on duodenal, liver and splenic iron content was measured 5 hours after a single IP containing quercetin (2, 5, 10 or 20 mg/kg body weight) or 10% DMSO (control) given to rats. Data are mean ± SEM; n=4 rats per group; groups with no common letters are significantly different from each other ($p<0.05$).

5.2.3 Distribution of quercetin metabolites in serum after quercetin IP administration

Quantitative analysis of quercetin and five selected quercetin metabolites in the serum of the rats, after different IP quercetin treatments, was performed using the LC–MS/MS technique. The contents of the determined compounds are presented in Table 5–1. Among the examined compounds only quercetin–3–*O*–glucuronide, quercetin and isorhamnetin were detected, while other examined compounds were not identified. Namely, after different time of single quercetin IP treatments (rats were treated with a single dose of quercetin (50 mg/kg) in different time periods,

from 0 to 18 hours, before being sacrificed) the concentration of quercetin–3–*O*–glucuronide declined from 90.78 to 27.77 ng/mL in serum, while after 12 hours no compound was detected. In the same group of animals, parental quercetin declined in the same way as quercetin–3–*O*–glucuronide, while isorhamnetin was detected only in animals sacrificed right away after IP quercetin treatment. In sets of animals that were treated with a double dose of quercetin, where one group was on an iron-deficient diet and the other on a normal iron content diet, quercetin–3–*O*–glucuronide, quercetin and isorhamnetin were detected in similar amounts. In the last set of animals that were treated with increasing quercetin concentrations, only quercetin–3–*O*–glucuronide (from 11.65 to 113.57 ng/mL of serum) and quercetin (from 1.42 to 25.43 ng/mL of serum) were detected.

Table 5–1 Determined concentrations of quercetin and selected quercetin metabolites in rat serum after IP quercetin treatment by LC–MS/MS technique

treatment				content of quercetin and selected quercetin metabolites (ng/mL serum)					
				quercetin–3,4'–di–*O*–glucoside	quercetin–3–*O*–glucuronide	isorhamnetin–3–*O*–glucoside	quercetin	isorhamnetin	quercetin–3,5,7,3',4'–penthamethylether
different time of quercetin treatment	0 hours before experiment	quercetin (50 mg/kg)	iron-deficient diet	nd	90.8 ± 7.23	nd	104 ± 5.98	66.7 ± 3.24	nd
	2 hours before experiment			nd	41.3 ± 3.22	nd	6.61 ± 0.45	nd	nd
	5 hours before experiment			nd	27.8 ± 1.78	nd	0.48 ± 0.01	nd	nd
	12 hours before experiment			nd	nd	nd	nd	nd	nd
	18 hours before experiment			nd	nd	nd	nd	nd	nd
	18 hours and then 5 hours before experiment	10% DMSO (control)		nd	nd	nd	nd	nd	nd
		quercetin (50 mg/kg)		nd	22.6 ± 0.94	nd	20.9 ± 1.23	3.43 ± 0.23	nd
	18 hours and then 5 hours before experiment	10% DMSO (control)	RM1 diet	nd	nd	nd	nd	nd	nd
		quercetin (50 mg/kg)		nd	99.8 ± 3.67	nd	13.3 ± 0.65	5.08 ± 0.39	nd
treatment with different dose of quercetin	5 hours before experiment	10% DMSO (control)	iron-deficient diet	nd	nd	nd	nd	nd	nd
		quercetin (2 mg/kg)		nd	16.8 ± 0.65	nd	nd	nd	nd
		quercetin (5 mg/kg)		nd	60.3 ± 3.21	nd	nd	nd	nd
		quercetin (10 mg/kg)		nd	11.6 ± 0.87	nd	1.42 ± 0.12	nd	nd
		quercetin (20 mg/kg)		nd	113 ± 2.89	nd	25.4 ± 1.23	nd	nd

nd - not detected

5.2.4 Effects of quercetin IP administration on iron absorption in duodenum

After a single or double IP quercetin treatment, mucosal uptake and transfer of ^{59}Fe remained the same. However, even though a slight increase in mucosal ^{59}Fe uptake and a decrease in mucosal ^{59}Fe transfer can be seen compared with controls, neither change was significant (see Figure 5–16; A and B).

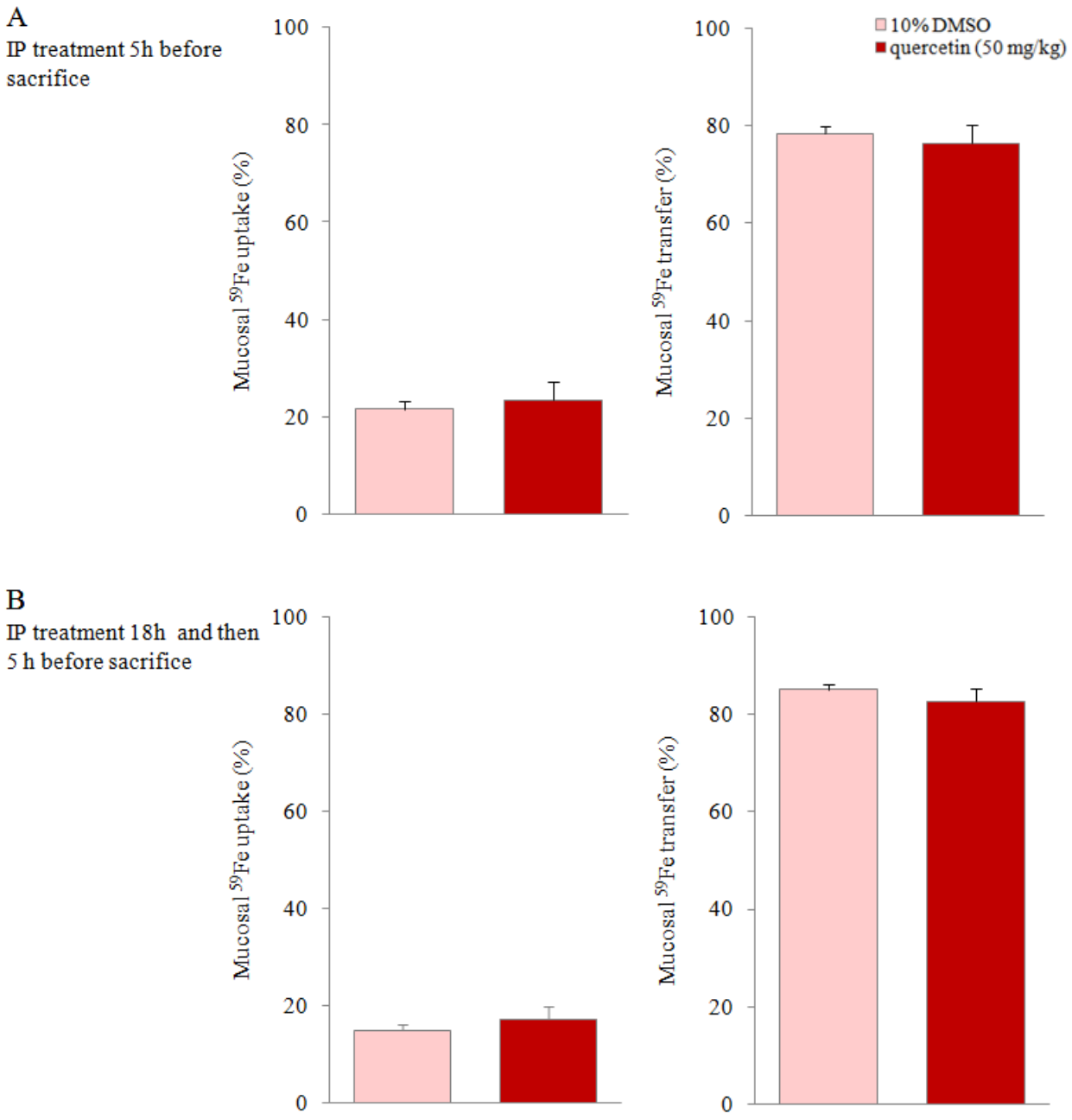


Figure 5–16 Effects of quercetin IP administration on iron absorption in rat duodenum.

The effect of quercetin on iron absorption was measured after single or double IP containing quercetin (50 mg/kg body weight) or 10% DMSO (control) given to rats. Data are mean ± SEM; n=5 rats per group.

5.3 Discussion

In this chapter the effect of quercetin given IP on iron metabolism in rats was investigated. Summarizing results obtained in this chapter, it is evident that different IP time and concentrations of quercetin had significant, but somewhat different, effects on iron homeostasis and gene expression. Table 5–2 sums all the results obtained within this study. Additionally, when comparing results from the previous two chapters (Chapter 3 and 4), where rats were treated by polyphenols orally, with results from this chapter it is evident that the route of quercetin application affects iron metabolism. Namely, IP treatment mainly affected systemic iron homeostasis, mostly by regulating hepcidin expression, while oral quercetin mainly affected iron absorption.

Specifically, after single quercetin treatment (50 mg/kg) major up-regulation of hepcidin mRNA occurred in liver and kidney. A vast increase in liver hepcidin was evident just 2 hours after quercetin treatment, reaching a peak after 5 hours (122- and 175-fold, respectively). Subsequent, hepatic mRNA hepcidin levels decreased after 12 and 18 hours, but were still significantly higher compared with the start of experiment (25- and 8-fold, respectively). Kidney mRNA hepcidin levels increased significantly 12 hours after quercetin application (35-fold), after which they went down, but still were higher than at the beginning of experiment. In the same experiment the impact of quercetin on splenic hepcidin mRNA was different from the same in the kidney and the liver. Namely, it reached a significant maximum 2 hours after quercetin treatment (2-fold), followed with attenuation, reaching significant minimum 18 hours later (2-fold).

Table 5–2 Sums of results obtained in experiments regarding effects of IP quercetin on iron homeostasis

<table>
<tr><th colspan="4">treatment</th><th>obtained results</th></tr>
<tr><td rowspan="8">different time of quercetin treatment</td><td>0 hours before experiment</td><td rowspan="5">quercetin (50 mg/kg)</td><td rowspan="6">iron-deficient diet</td><td rowspan="8">
A
<table>
<tr><th rowspan="2">tissue/serum</th><th rowspan="2">parameter</th><th colspan="5">time of quercetin IP treatment (hours)</th></tr>
<tr><th>0</th><th>2</th><th>5</th><th>12</th><th>18</th></tr>
<tr><td rowspan="2">serum</td><td>iron</td><td>a</td><td>ab</td><td>↓b</td><td>↑c</td><td>↑cd</td></tr>
<tr><td>transfferin saturation</td><td>a</td><td>ab</td><td>↓b</td><td>↑c</td><td>↑cd</td></tr>
<tr><td rowspan="4">duodenum</td><td>DMT1</td><td>a</td><td>a</td><td>a</td><td>↑b</td><td>ab</td></tr>
<tr><td>Dcytb</td><td>a</td><td>a</td><td>↑b</td><td>↑b</td><td>↑b</td></tr>
<tr><td>FPN</td><td>ac</td><td>a</td><td>↑bd</td><td>cde</td><td>ae</td></tr>
<tr><td>iron</td><td>a</td><td>↑b</td><td>↑bd</td><td>↑c</td><td>↑d</td></tr>
<tr><td rowspan="4">liver</td><td>hepcidin</td><td>a</td><td>↑b</td><td>↑bd</td><td>↑bd</td><td>↑cd</td></tr>
<tr><td>FPN</td><td>ab</td><td>ac</td><td>a</td><td>b</td><td>bc</td></tr>
<tr><td>HO–1</td><td>a</td><td>↑b</td><td>↑c</td><td>↑d</td><td>↑d</td></tr>
<tr><td>iron</td><td>a</td><td>a</td><td>a</td><td>a</td><td>↓b</td></tr>
<tr><td rowspan="3">spleen</td><td>hepcidin</td><td>a</td><td>↑b</td><td>a</td><td>a</td><td>↓c</td></tr>
<tr><td>FPN</td><td>a</td><td>↓b</td><td>a</td><td>↓b</td><td>ab</td></tr>
<tr><td>iron</td><td>a</td><td>↑b</td><td>↑b</td><td>↑b</td><td>↑b</td></tr>
<tr><td>kidney</td><td>hepcidin</td><td>a</td><td>a</td><td>a</td><td>↑b</td><td>↑b</td></tr>
</table>
</td></tr>
<tr><td>2 hours before experiment</td></tr>
<tr><td>5 hours before experiment</td></tr>
<tr><td>12 hours before experiment</td></tr>
<tr><td>18 hours before experiment</td></tr>
<tr><td rowspan="2">18 hours and then 5 hours before experiment</td><td>10% DMSO (control)</td><td rowspan="2">Serum: Iron↓*, transferrin saturation↓*
Duodenum: DMT1↓*, FPN↓*, Dcytb↓*, Iron↓*
Liver: Hepcidin↑*, FPN↓*, HO–1↑*, Ferritin↑*, QR↑*, GST↑, Iron↑*
Spleen: Hepcidin↑, FPN↓*, Iron=
Kidney: Hepcidin↑*</td></tr>
<tr><td>quercetin (50 mg/kg)</td></tr>
<tr><td rowspan="2">18 hours and then 5 hours before experiment</td><td>10% DMSO (control)</td><td rowspan="2">RM1 diet</td><td rowspan="2">Serum: Iron↓, transferrin saturation ↓
Duodenum: DMT1 no expression, Dcytb=, FPN↑, Iron↓
Liver: Hepcidin↑*, FPN=, HO–1↑*, Ferittin=, Iron↑*
Spleen: Hepcidin↓*, FPN↑*, Iron↑
Kidney: Hepcidin↓*</td></tr>
<tr><td>quercetin (50 mg/kg)</td></tr>
</table>

treatment with different dose of quercetin				
	5 hours before experiment	10% DMSO (control)	iron-deficient diet	
		quercetin (2 mg/kg)		
		quercetin (5 mg/kg)		
		quercetin (10 mg/kg)		
		quercetin (20 mg/kg)		

B

tissue/serum	parameter	concentration of quercetin IP treatment (mg/kg)				
		0	2	5	10	20
serum	iron	a	a	↑b	↑c	↑c
	transfferin saturation	a	a	↑b	↑b	↑b
duodenum	DMT1	a	↓bc	a	a	ac
	Dcytb	a	a	↑b	a	↑b
	FPN	a	ac	↑bc	ac	↑bc
	iron	a	↑b	↑b	abc	↓c
liver	hepcidin	a	a	ab	↑b	ab
	FPN	a	a	a	a	a
	HO–1	a	a	ab	↑b	↑b
	iron	a	a	ac	↓b	↓c
spleen	hepcidin	a	ab	ab	a	↓b
	FPN	a	a	ab	a	↑b
	iron	a	↓b	a	a	a
kidney	hepcidin	a	ab	ab	↓b	a

in tables A and B ↑ indicates that the parameters significantly increased compared with the group treated with quercetin 0h before experiment or with 10% DMSO, respectively

in tables A and B ↓ indicates that the parameters significantly decreased compared with the group treated with quercetin 0h before experiment or with 10% DMSO, respectively

in tables A and B groups with no common letters are significantly different from each other ($p<0.05$)

results in red and marked with * indicate that the parameters significantly differ from treated and control group

↑ indicate that parameter increased, but not significantly, between treated and control group

↓ indicate that parameter decreased, but not significantly, between treated and control group

= indicate that parameter did not change in treated group compared with control group

However, when lower concentrations of quercetin were applied, the increase in hepcidin mRNA was not as elevated as after higher quercetin concentrations. Still, hepatic mRNA was increased 45-fold after a 10 mg/kg quercetin dose, while the hepcidin mRNA in spleen and kidney stayed the same or significantly decreased after 20 and 10 mg/kg quercetin dose, respectively. The largest change in hepatic hepcidin mRNA occurred after double quercetin treatment in animals fed on iron-deficient diet. Specifically, hepatic hepcidin mRNA was up-regulated enormously, 1031-fold. The same pattern was followed by kidney hepcidin mRNA (9.5-fold), while no significant change occurred in splenic hepcidin. In the group that was on a RM1 diet, hepatic hepcidin mRNA increased, while in kidney and spleen it decreased significantly. Generally, hepatic and kidney mRNA hepcidin mainly expressed an upward trend after IP quercetin. However, differences in hepatic and kidney results are probably due to the different number of treatments (single and double), different quercetin concentrations (2–50 mg/kg) and different diets (RM1 and iron-deficient). In contrast to liver and kidney hepcidin, splenic mRNA hepcidin levels were significantly down-regulated or not altered by quercetin treatment. As mentioned in Chapter 3, the observation that hepcidin is regulated in a different way in different tissues is hitherto unknown. In the light of results from this thesis, it could be proposed that hepcidin is regulated in a tissue-specific manner by quercetin. However, based on these limited results it is very hard to give an explanation of different hepcidin expression in different tissues and additional work is needed to confirm and elucidate the mechanism of tissue-specific hepcidin transcriptional regulation.

The massive increase of hepatic and kidney hepcidin mRNA levels *in vivo* after quercetin treatment observed in this study is surprising and not easy to explain. There are only two reports from the literature that reported a similar effect of polyphenols on hepcidin expression in liver. To be exact, Tang et al. (2014), showed *in vivo* that

quercetin efficiently supports hepcidin expression by intensification of the BMP6/SMAD4 signaling pathway, both suppressed by ethanol consumption. Of note, it is known that hepcidin transcription is mainly regulated by the BMP6/SMAD4 signaling pathway (Ganz and Nemeth, 2012). Additionally, hepcidin suppression by alcohol abuse results in iron overload and predisposes the liver to more severe pathologies (Bridle et al., 2006). Tang et al. (2014) showed in mice that BMP6 and SMAD4 protein levels, as well as binding of SMAD4 to the *HAMP* promoter, were disabled by chronic alcohol exposure, directly leading to hepcidin suppression. Interestingly, quercetin treatment partially reset the ethanol effect on the BMP6/SMAD4 signaling pathway. Particularly, quercetin treatement after alcohol exposure was followed by increase in BMP6 and SMAD4 expression and SMAD4 binding activity to the *HAMP*, subsequently leading to stimulation of hepcidin expression in liver and decrease of iron overload. In Tang et al. (2014), up-regulation of liver hepcidin, on both protein and mRNA levels, was documented after a 15 week long oral treatment with quercetin (100 mg/kg). Up-regulation of mRNA hepcidin levels was around 2-fold, which is not comparable with the vast increase of hepcidin mRNA evidenced within this study. Two results are hard to compare in the sense of the intensity of hepcidin up-regulation due to the different length and way of quercetin application. Additionally, results from this thesis showed that after IP treatment the concentration of quercetin and its metabolites in serum was higher compared with the oral treatment (see Table 3–2 and Table 5–1). Thus, quercetin applied IP could have a bigger impact on cell homeostasis due to its higher serum concentration. This could be a reason for the modest hepcidin increase after a 15 weeks long oral quercetin treatment compared with the opposite vast hepcidin up-regulation after double IP treatment. Vanhees et al. (2011) also confirmed *in vivo* quercetin-mediated regulation of hepcidin. Specifically, research showed that prenatal exposure to quercetin resulted in hepcidin

induction in adult mice. Authors hypothesized that after birth, when pups were no longer exposed to quercetin, improved bioavailability of dietary iron was experienced as an iron overload. Namely, the authors suspected that animals were ”developmentally programmed” to deal with lower iron levels *in utero*. The difference between the *in utero* and postnatal life conditions resulted in activating pathways for overcoming emerging ”iron overload”, such as hepcidin up-regulation. Again, it is hard to draw a parallel between the results from Vanhees et al. (2011) and results obtained in this thesis due to the distinct experimental design. However, there are a small number of studies reporting up-regulation of hepcidin by polyphenols. Thus some results in this study are hard to discuss and only preliminary explanation could be proposed.

Apart from the suggestion that hepcidin is up-regulated by quercetin through the BMP6/SMAD4 signaling pathway, there are assumptions that it could also be regulated by the Nrf2–ARE signaling pathway. Namely, the liver, a main site of iron storage, is particularly exposed to the toxic effects of iron. The Nrf2–ARE signaling pathway plays a pivotal role in protecting the liver from disease induced by high iron. This pathway activates the transcription of a battery of cytoprotective genes encoding detoxification/antioxidant enzymes which terminate toxic iron effects. The important role of Nrf2 in inhibiting hepatic injury was shown in a couple *in vivo* models using Nrf2-null mice (Klaassen and Reisman, 2010). Furthermore, it was recently shown that Nrf2 protects mouse liver against toxicity and oxidative stress caused by iron overload (Silva–Gomes et al., 2014). Consequently, it could be proposed that Nrf2 signaling may coordinate hepcidin expression as an answer to iron overload and thus contribute to the prevention of hapatocytic cell injury. This hypothesis could be supported by parallel increases in the expression of the phase II genes HO–1 and QR recorded in this study after all the applied quercetin treatments (ranged from 13- to 2-fold). Of note, transcription of these enzymes is driven by the Nrf2–ARE signaling pathway as part of

a battery of cytoprotective machinery induced by oxidative stress (Klaassen and Reisman, 2010). These effects of quercetin are known from before (Williamson at el., 1996; Liu et al., 2012; personal communication with Henry K. Bayele and Sara Balesaria). However, up-regulation of Nrf–2 by quercetin was also confirmed (Yao et al., 2007; Granado–Serrano et al., 2012). Based on the results from these thesis and observations of others, it could be proposed that the Nrf2 transcription factor was up-regulated in the liver of examined animals by quercetin. Thus there is a great probability that the same signaling pathway induced hepcidin transcription. Furthermore, it was shown *in vitro* that HO–1 expression is also BMP6/SMAD4 dependent (Yan et al., 2009). Thus, in the same manner as with above discussed Nrf2–ARE signaling pathway, this result indicates that hepcidin up-regulation could also go through the BMP6/SMAD4 pathway. However, these both hypotheses are only a sugestion and additional work is needed to confirm the discussed issues. Nevertheless, these results confirmed the well known fact that polyphenols, particularly quercetin, increase levels of antioxidant enzymes and support the body in its fight against destructive oxidative stress.

Furthermore, an increase in hepcidin levels is expected to be followed by a reduction in FPN levels, mainly in spleen. Namely, the ”seesaw” relationship between hepcidin and FPN expression is well known (Nemeth et al., 2004; Ganz, 2011) and part of the results from this thesis are in agreement with this belief. Two and 12 hours after single quercetin treatment, levels of splenic FPN mRNA decreased while those of liver were unchanged. This confirmed previously reported views that spleen FPN is major target for hepcidin action (Chaston et al., 2008; Masaratana et al., 2011). The hepcidin effect on FPN was more pronounced after double quercetin treatment, where FPN mRNA levels declined in both liver and spleen, but in spleen more. However, in animals fed a RM1 diet this outcome was lacking. What is more, splenic FPN mRNA increased after

quercetin treatment, even though liver hepcidin mRNA increased in the same animal group. Similarly, the work of others also suggests that FPN itself may be up-regulated by antioxidants *in vivo*, which could partially explain the observed disagreement in results (Harada et al., 2011).

High hepcidin, as well as reduction in FPN levels by hepcidin, should be followed by a retention of iron in tissue, precisely in liver and spleen. This pattern was followed after a double quercetin dose where iron levels in liver increased or were unchanged in spleen. Consequently, an increase in liver iron was followed by an increase in mRNA ferritin levels. However, after a single quercetin treatment, with a dose of 50 mg/kg, spleen iron was increased but liver iron decreased. Whereas, after a lower quercetin dose (ranging from 0 to 20 mg/kg) showed reduced iron stores both in liver and spleen. As mentioned before, differences in results of tissue iron levels could be due to the different number of treatments, different quercetin concentrations and different diets. Additionally, a reduction in iron stores by quercetin could be explained by its chelato properties. Particularly, it is confirmed *in vivo* that quercetin is able to decrease intracellular iron pools by chelating iron and taking it out from the cell. It is proposed that quercetin chelates iron and takes it out from the tissue to the blood stream and finally excretes it from the body (Morel et al., 1993; Zhao et al., 2005; Zhang et al., 2006; Zhang et al., 2011; Baccan et al., 2012). These finding are of great importance for medicine as polyphenols, such as quercetin, could be use as chelator in iron redistribution therapy.

An increase in hepcidin levels is proven to be followed by a decrease of intestinal iron absorption (Laftah et al., 2004; Mena et al., 2008). Particularly, it is proven that hepatic hepcidin expression is inversely proportional to expression of intestinal DMT1, Dcytb and FPN expression *in vivo* (Frazer et al., 2004; Yamaji et al., 2004; Chung et al., 2009;

Brasse–Lagnel et al., 2011). However, this occurrence was only partially confirmed in this thesis. Namely, after single quercetin treatment (dose 50 mg/kg) levels of duodenal mRNA DMT1, Dcytb and FPN stayed the same or were up-regulated 5 and more hours after treatment. Furthermore, after a single lower quercetin dose, the same pattern of duodenal genes'. regulation was followed. These results came as a surprise, because the opposite results were expected particularly in the light of detected increased hepcidin levels. Additionally, these results were in disagreement with observed increased iron duodenal levels. Namely, it would be expected that levels of DMT1 were down-regulated by post-transcriptional the IRE/IRP control machinery, such that when iron levels in tissue are high, expression of DMT1 is inhibited (Muckenthaler et al., 2008). These contradictory results require more in-depth research in order to make clear conclusions as to why the iron transporters follow the observed pattern of expression. One of the possible explanations could be that quercetin expresses a direct effect on observed gene expression and that this mechanism could abolish the influence of hepcidin. However, after double quercetin treatment, where vast up-regulation of hepatic hepcidin occurred, duodenal mRNA levels of DMT1, Dcytb and FPN were significantly down-regulated, as well as duodenal iron levels. These results confirmed the results of earlier studies, where high hepcidin is followed by a reduction in iron transporters and absorption.

Nevertheless, it would be expected that hepcidin up-regulation is followed by a reduction in serum iron and transferrin saturation (Kemna et al., 2005). Again, this principle was to some extent confirmed within this thesis. Namely, after a double quercetin dose, serum iron and transferrin saturation levels decreased as expected. Contrary, after a single quercetin treatment serum iron and transferrin saturation levels increased. This can partially be explained by reduced liver iron levels in some animal groups. In other words, iron could leave liver tissue through unaffected FPN and thus

increase iron serum levels. Also, increase in serum iron could indicate quercetin chelato property and its ability to take iron out from tissues. Additionally, as duodenal transporters were increased despite high hepcidin levels induced by quercetin, extra iron could come through intestinal absorption.

On the other hand, uptake studies after IP quercetin treatment did not cause a change in mucosal iron uptake or transfer. These results came as a great surprise, especially after a double dose of quercetin, where quercetin enormously up-regulated mRNA hepcidin levels and affected iron transporter genes in the intestine. The results from uptake studies indicate that more in-depth studies need to be performed in order to confirm the exact affects of IP quercetin on iron absorption *in vivo*.

On top of, it is shown that quercetin administrated IP at a concentration not less than 50 gm/kg had the greatest effect on iron homeostasis 5 hours after single treatment. Additionally, double quercetin IP had a more distinct effect on iron homeostasis than single quercetin IP. Results from this Chapter, as well as from Chapter 3, point out that those animals kept on an iron-deficient diet are more suitable models for research in the field of iron metabolism, because they are more sensitive to the applied treatments and results are more pronounced.

The aim of determination of quercetin and selected quercetin metabolites in rat serum after IP quercetin treatment by LC–MS/MS technique was to see which metabolites are present in the serum and at which concentrations. As it was mentioned in Chapter 3, this was important for subsequent experiments in which THP1 cells and HepG2 cells were treated with detected metabolites in order to investigate their potential role in expression of iron-metabolism related genes. Qualitative and quantitative studies showed that right after treatment quercetin was the dominant compound, but it was quickly metabolized to

quercetin–3–*O*–glucuronide and isorhamnetin. Higher concentrations of quercetin and its metabolites were observed in serum compared with the results of study after oral quercetin treatment, which confirms that quercetin absorbed in the intestine has a short half-life. Also, these results confirm that one of the dominant quercetin metabolites in serum is quercetin–glucuronide (Gee et al., 2004; Justino et al., 2004; Moon et al., 2008).

5.4 Conclusions

In conclusion, IP administration of quercetin mainly affected systemic iron homeostasis, primary by a large up-regulation of hepatic and kidney mRNA hepcidin levels. This trend was not followed by splenic hepcidin mRNA, which suggest possible tissue-specific hepcidin transcriptional regulation by quercetin. Furthermore, analysis of the presented data and previously published results suggest that both BMP6/SMAD4 and Nrf2–ARE signaling pathways could be involved in dominant hepcidin up-regulation by polyphenols *in vivo*. Furthermore, after different administration times, single or double doses and different concentrations of quercetin, the organism reacted differently to high hepcidin levels. Generally, high hepcidin levels were followed by a reduction in mRNA FPN levels, mainly in the spleen. Additionally, it is confirmed that quercetin is able to decrease intracellular iron pools *in vivo*, possibly by chelating iron and taking it out of the cell. This fact could be particularly significant for new drug discoveries directed by natural product research targeting iron overload. Furthermore, results only partially confirmed that hepcidin reduces iron absorption. This fact points to a possible direct influence of polyphenols on the expression of duodenal genes involved in iron homeostasis. However, uptake studies after IP quercetin showed no change in iron absorption, even though significant change in genes involved in iron intestinal transport was observed. This indicates that more studies have to be done in order to explain the

quercetin effect on iron homeostasis, particularly iron absorption. Nevertheless, results confirmed the well-known fact that polyphenols, particularly quercetin, increase levels of antioxidant enzymes. This study provides a very good case for including hepcidin as an important factor involved in antioxidant effects, in addition to it being involved in the anti-inflammatory effects of quercetin. Above all, it is confirmed that polyphenols might be a new source of therapeutics for iron overload diseases and their role in iron-removal therapy is worthy of further study.

6. EFFECT OF POLYPHENOLS ON GENE EXPRESSION OF IRON-RELATED PROTEINS IN HepG2 CELLS

6.1 Introduction

The main organ for regulation of systemic iron homeostasis is the liver. Apart from being the main site of hepcidin expression, it is also one of the main sites of iron storage. Hepcidin synthesis is stimulated by iron overload. Consequently, hepcidin binds to FPN and causes its degradation in order to prevent further iron export to the circulation (Ganz, 2011; Ganz and Nemeth, 2012).

It was shown in previous chapters of this thesis that quercetin greatly affects iron metabolism *in vivo*, at the absorption stage and at the systemic level. However, it is difficult to decide whether it is quercetin or its metabolites that induce the observed effects. Furthermore, there is no literature data about a possible link between iron metabolism and quercetin metabolites. Bearing in mind the extensive metabolism of quercetin in the intestine and its short half-life (Gee et al., 2004; Justino et al., 2004; Moon et al., 2008), it was hypothesized that quercetin metabolites could be involved in changing iron metabolism. Thus, in order to get preliminary conclusions on these issues, the effect of quercetin and 6 chosen quercetin metabolites on hepcidin and FPN was studied in HepG2 cells, used as a hepatic *in vitro* model system. Furthermore, it was discussed in detail in Chapter 5 that two signalling pathways, Nrf2–ARE and BMP6/SMAD4, could regulate hepcidin expression. This is based on *in vitro* studies that show that HO–1 expression in response to polyphenol (quercetin) treatment is Nrf2–ARE- and BMP6/SMAD4-dependent (Yan et al., 2009; Klaassen and Reisman, 2010). In order to see whether polyphenols other then quercetin can affect iron systemic metabolism *in vitro*, two polyphenols of diverse structure resveratrol and epicatechin

were also included in the study. Resveratrol and epicatechin are, as quercetin, abundant in a diet of plant origin and are potent antioxidant agents (Terao, 1999; Baur and Sinclair, 2006). In Chapter 4 it was shown that resveratrol does not affect iron absorption, however it was previously confirmed *in vitro* that (–)–epigallocatechin–3–gallate and epicatechin–3–gallate, derivative epicatechin, inhibit non-haem iron absorption and decrease plasma iron (Thephinlap et al., 2007; Kim et al., 2008). A study of the effects of polyphenol of diverse structures could elucidate whether the well known fact that polyphenols inhibit iron absorption is due to their chelation or antioxidant properties, or both.

6.2 Results

In this chapter the effect of polyphenols on gene expression of iron–related proteins, such as hepcidin, FPN and HO–1, in HepG2 cells was investigated. This study included quercetin and its metabolites that are identified in serum of animals treated with quercetin orally and IP (see Chapter 3 and 5). Also, metabolites that were not detected in serum in the scope of this thesis, but are known to be present in serum after consumption of quercetin, were included in this study. Additionally, resveratrol and epicatechin, polyphenols that are frequently consumed in a plant diet, were also the subject of this investigation.

HepG2 cells were grown on 6-well plates for 24 hours and were treated with polyphenols (100 μM) for a further 5 hours. This was followed by RNA isolation and quantitative-PCR to measure changes in mRNA expression.

In general, polyphenols induced a significant decrease in hepcidin mRNA levels. Namely, quercetin–3–*O*–glucuronide, isorhamnetin, tamarixetin, 3,4'–dimethylquercetin, epicahtechin and resveratrol caused a 9-, 54-, 3.5-, 2-, 11- and 11-fold decrease compared with the corresponding control, respectively (see Figure 6–1). In contrast, among all the investigated polyphenols only quercetin induced hepcidin mRNA levels of almost 3-fold compared with the control (see Figure 6–1). There was no significant effect of 3–*O*–methylquercetin and penta–methylquercetin on hepcidin expression.

Furthermore, FPN mRNA was generaly significantly down regulated. To be exact, all investigated polyphenols, expect quercetin–3–*O*–glucuronide, 3–*O*–methylquercetin, resveratrol and penta–methylquercetin, decreased FPN mRNA levels (see Figure 6–2).

Contrary to what was expected, only epichatechin upregulated HO–1 mRNA levels in HepG2 cells, while other investigated polyphenols, such as quercetin, isorhamnetin, tamarixetin, 3,4'–dimethylquercetin and 3–*O*–methylquercetin, significantly decreased HO–1 gene expression (see Figure 6–3).

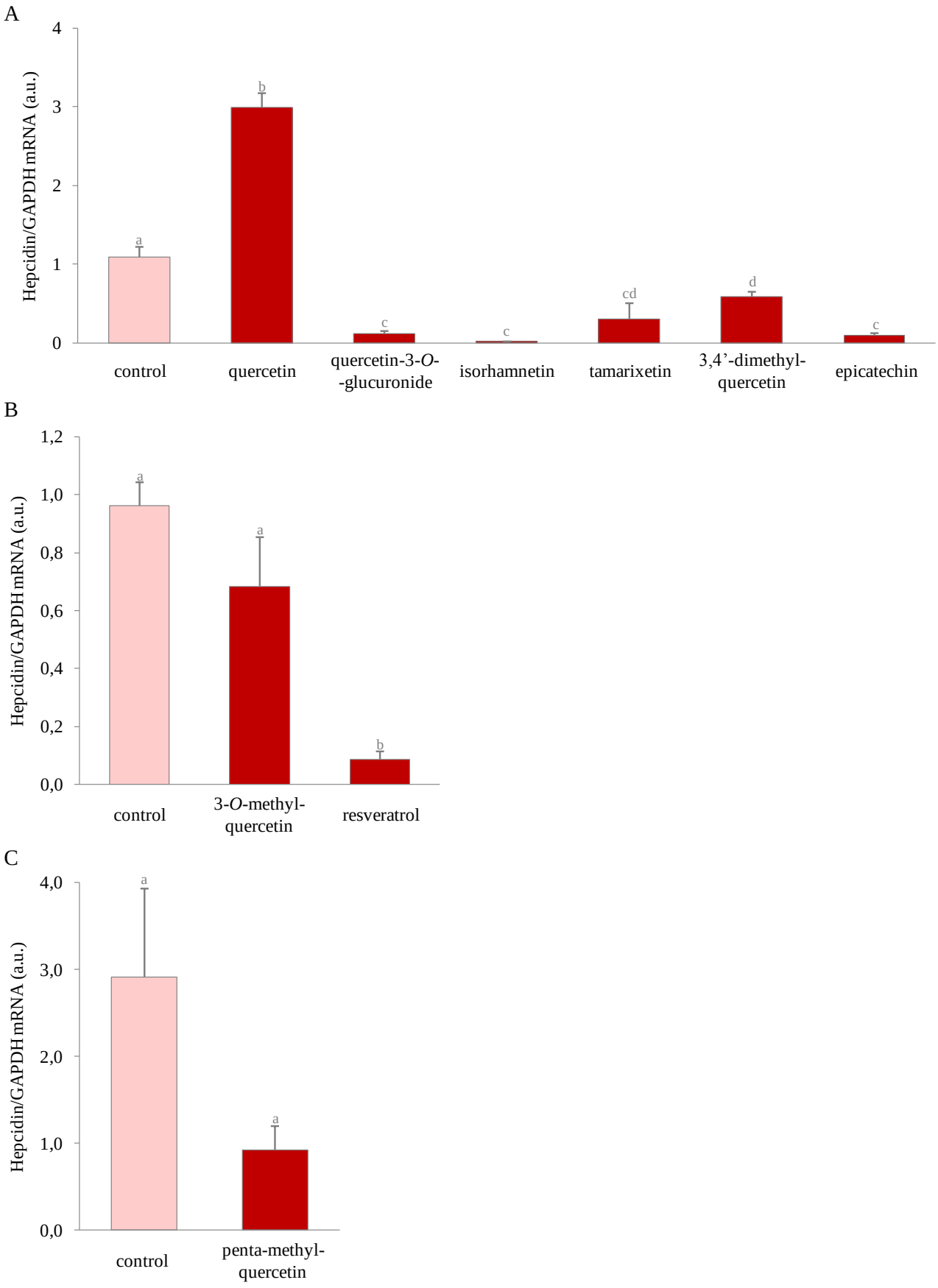


Figure 6–1 Effect of polyphenols on hepcidin mRNA expression in HepG2 cells
HepG2 cells were treated with polyphenols (100 µM) for 5 hours. Instead of compounds DMSO (A), ethanol (B) or ethanol:DMSO (1:1) were added in corresponding control wells. Changes in mRNA expression were measured by qPCR. Data normalised to GAPDH. Data are mean ± SEM; n=3; groups with no common letters are significantly different from each other ($p<0.05$).

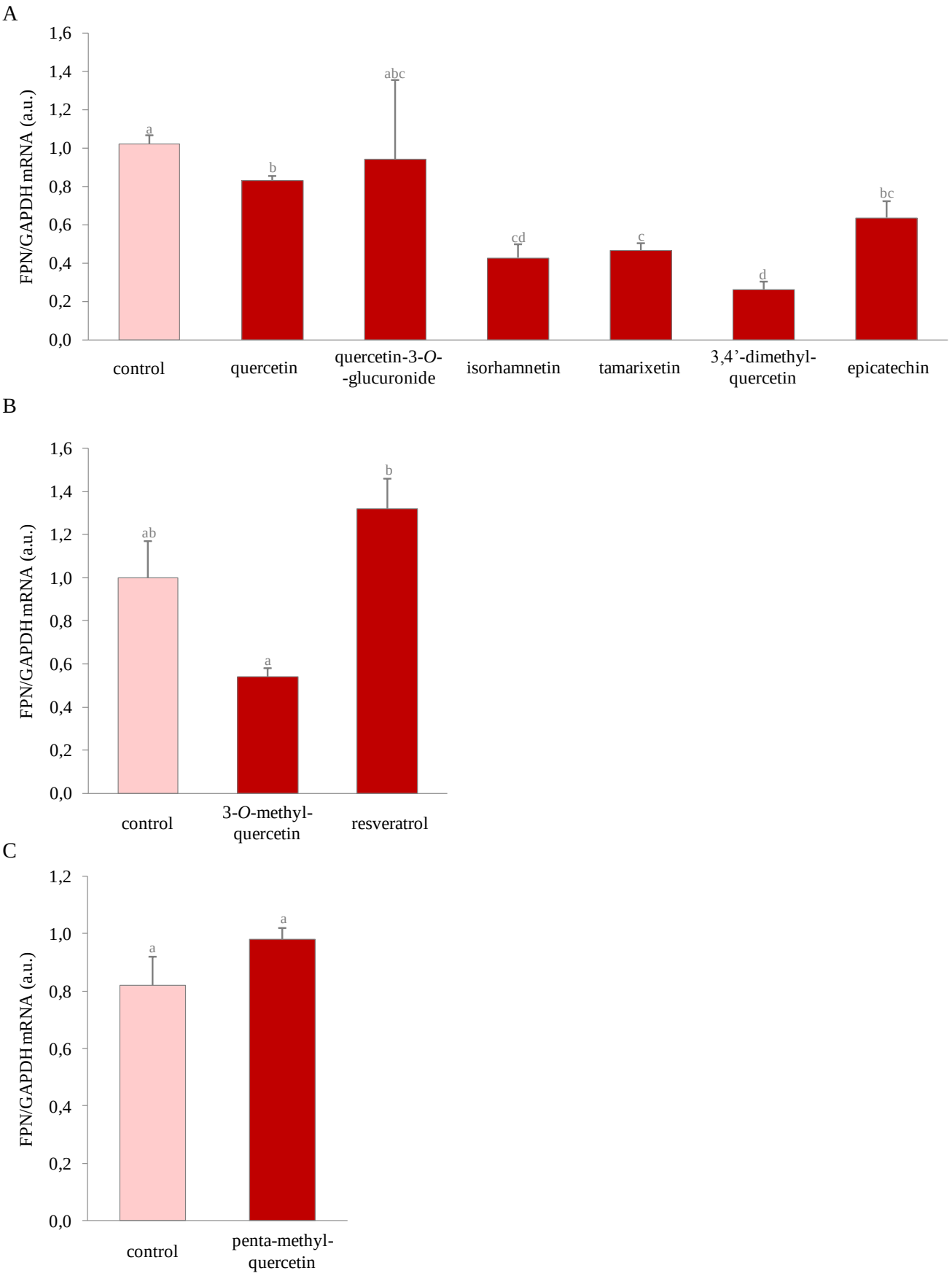


Figure 6–2 Effect of polyphenols on FPN mRNA expression in HepG2 cells
HepG2 cells were treated with polyphenols (100 μM) for 5 hours. Instead of compounds DMSO (A), ethanol (B) or ethanol:DMSO (1:1) were added in corresponding control wells. Changes in mRNA expression were measured by qPCR. Data normalised to GAPDH. Data are mean ± SEM; n=3; groups with no common letters are significantly different from each other ($p<0.05$).

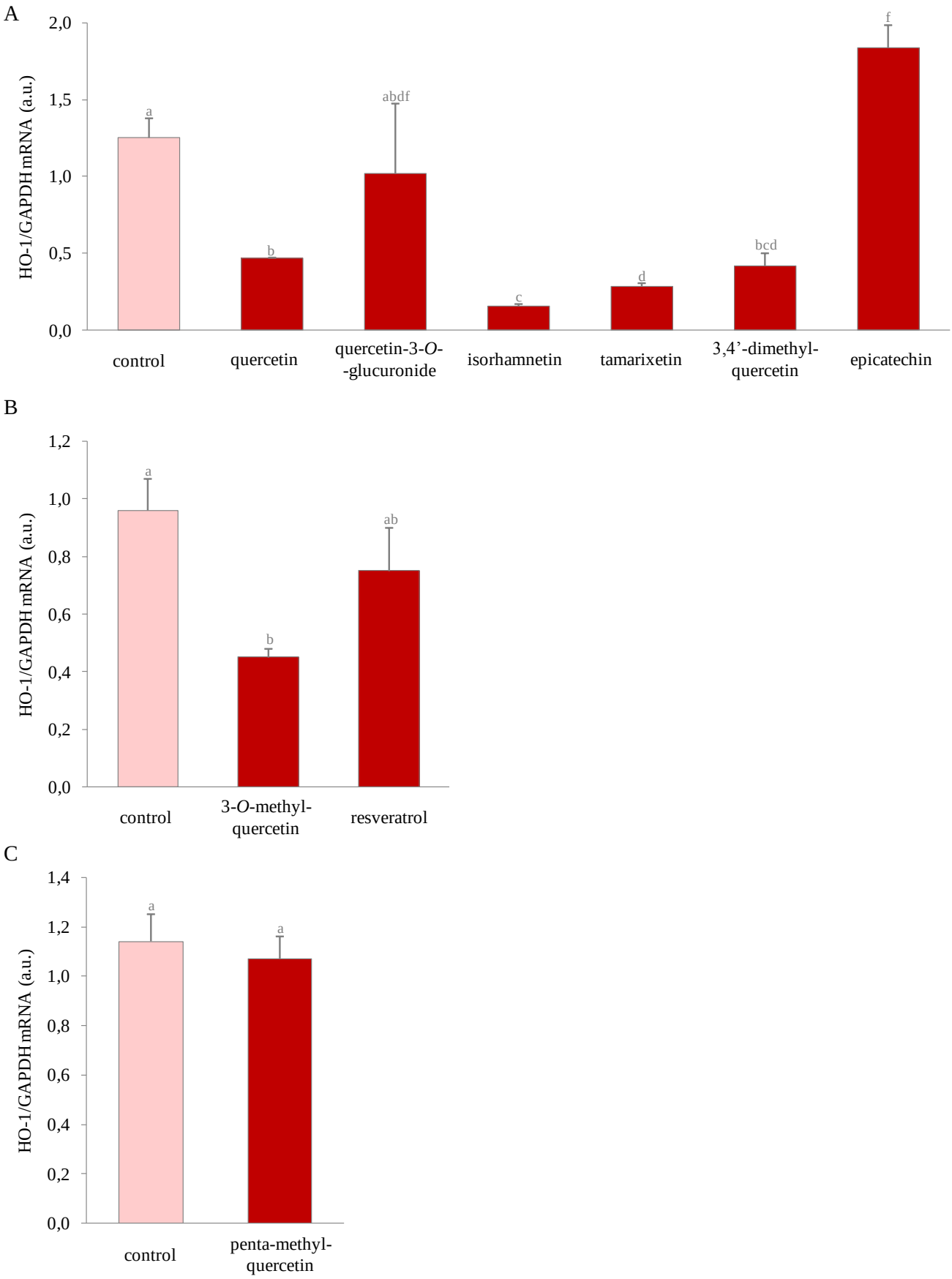


Figure 6–3 Effect of polyphenols on HO–1 mRNA expression in HepG2 cells
HepG2 cells were treated with polyphenols (100 µM) for 5 hours. Instead of compounds DMSO (A), ethanol (B) or ethanol:DMSO (1:1) were added in corresponding control wells. Changes in mRNA expression were measured by qPCR. Data normalised to GAPDH. Data are mean ± SEM; n=3; groups with no common letters are significantly different from each other ($p<0.05$).

6.3 Discussion

In this chapter the effect of quercetin, 6 quercetin metabolites, resveratrol and epichatechin on hepcidin, FPN and HO–1 expression was studied. Among the polyphenols investigated only quercetin induced a significant increase of hepcidin expression, while others mainly induced a significant decrease. It is discussed in Chapter 5 that hepcidin expression could be controlled by both the BMP6/SMAD4 and Nrf2–ARE signaling pathways. However, it is still unknown how quercetin, or other polyhenols, could affect these pathways and bring about an increase or decrease in hepcidin mRNA expression. It could be assumed that the answer could lie in the polyphenol structure. Namely, among investigated polyphenols, only quercetin has a free hydroxyl, carbonyl and galloyl group in close proximity (see Figure 6–4). This structural characteristic could be important for its interaction with signaling molecules controlling hepcidin expression and the final outcome could be dependent on it. This is only a preliminary assumption and the results and this hypothesis need to be confirmed in further research. Furthermore, this result is in accordance with previously presented results in this thesis. Namely, it was shown that liver and kidney hepcidin expression is up-regulated by IP quercetin *in vivo* (see Chapter 5.). In Chapter 5. possible ways of how quercetin could affect hepcidin expression are discussed in detail.

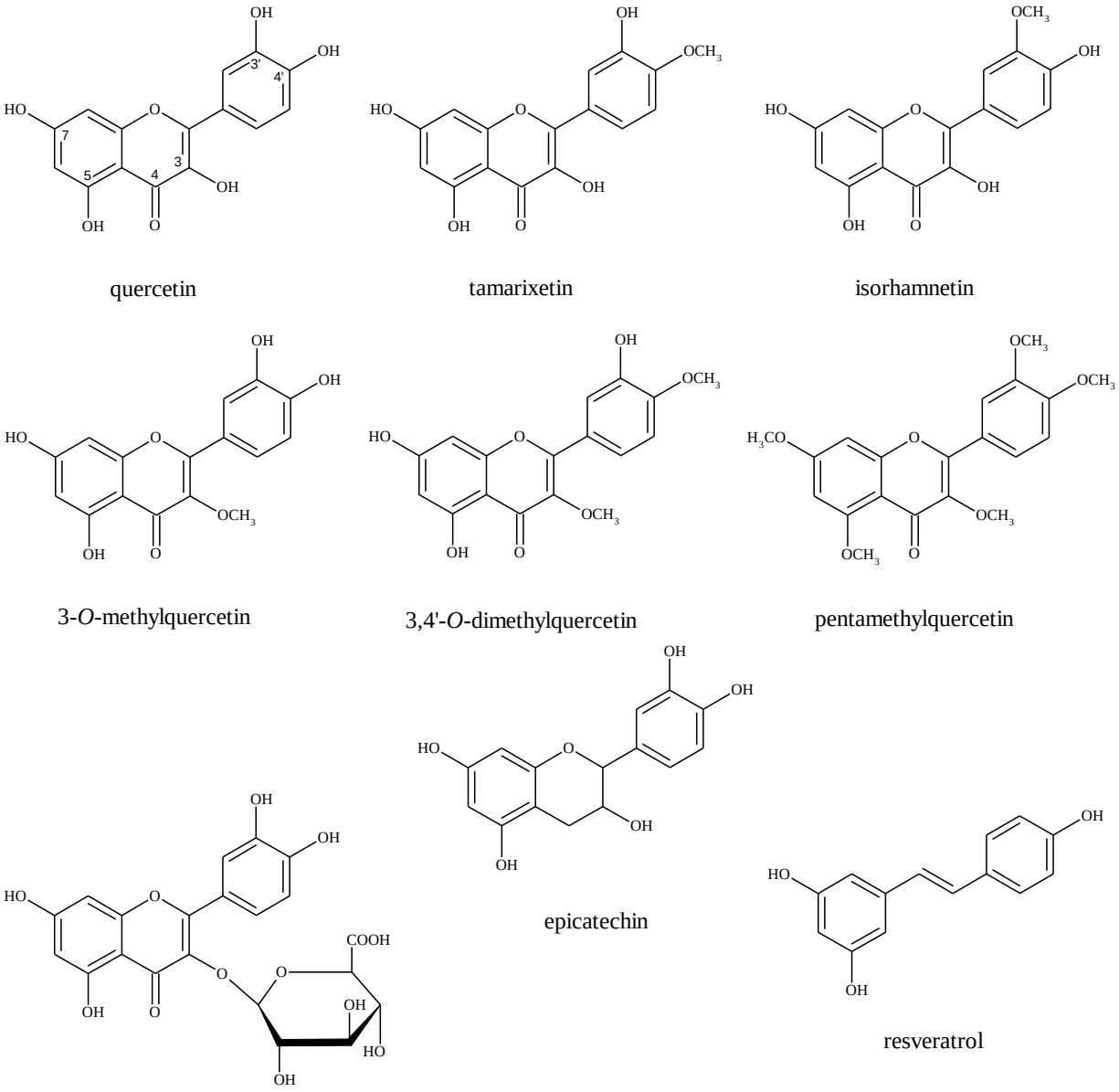


Figure 6–4 Structures of polyphenols included in this study

Moreover, FPN expression was also affected by polyphenol treatment. Namely, only resveratrol significantly increased FPN mRNA levels, while quercetin, isorhamnetin, tamarixetin, 3,4'–dimethylquercetin and epichatechin induced a significant decrease. Also, penta–methylquercetin induced an increase, while 3–*O*–methylquercetin induced a decrease, in FPN mRNA levels but it was not significant. It is difficult to explain why only resveratrol induced an increase in FPN mRNA levels. Again, structural differences

among the investigated polyphenols could have an important role in the observed increase (see Figure 6–4). Resveratrol and penta–methylquercetin are the only polyphenols, among those investigated, that could not chelate iron. This property could be important in the light of the fact that resveratrol and penta–methylquercetin cannot reduce intracellular iron. By chelating iron and lowering the intracellular liable iron pool, the IRE/IRP system could be activated in order to decrease FPN levels as the intracellular iron level is low (Muckenthaler et al., 2008). However, this is only a preliminary suggestion and this premise needs to be confirmed in further research. Furthermore, by this study again well known fact that high hepcidin induces FPN internalization is confirmed (Ganz, 2011). Namely, in HepG2 cells treated with quercetin, mRNA hepcidin was up-regulated which was followed by a reduction in FPN mRNA.

Nonetheless, HO–1 mRNA levels were also followed in HepG2 cells after polyphenol treatment. However, the results came as a surprise. Namely, almost all polyphenols, except epichatechin, provoked a decrease in HO–1 mRNA levels. This is not in agreement with the well known fact that polyphenols support up-regulation of antioxidant enzymes, such as HO–1 (Ferrándiz and Devesa, 2008; Shah, et al., 2010). Also, these results are not in accordance with previous results from this thesis, where HO–1 mRNA levels were up-regulated in liver after both IP and oral quercetin treatment *in vivo* (see Chapter 3 and 5). It is discussed in detail in Chapter 5 that up-regulation of HO–1 could be a confirmation that hepcidin expression is controlled by two pathways, Nrf2–ARE and BMP6/SMAD4. The results obtained with HepG2 cells argue against this hypothesis. However, future studies should focus on an in-depth analysis of this issue in order to determine the influence of polyphenols on hepcidin expression in hepatocytes.

6.4 Conclusions

To summarise, the results presented give a preliminary indication that quercetin metabolites could have different effects on systemic iron metabolism compared to quercetin. Namely, it is shown that quercetin metabolites induce a reduction in hepcidin expression; while it was proven that quercetin induces a large great up-regulation in hepcidin levels both *in vivo* and *in vitro*. Furthermore, quercetin metabolites affected mRNA FPN levels in the same way as quercetin. This supports the belief that polyphenols affect intracellular iron metabolism by chelating iron and affecting the IRE/IRP machinery. Furthermore, it was shown that structurally diverse polyphenols could affect iron metabolism in a different way. Namely, it could be assumed that structural characteristics that enable iron chelation, in addition to general polyphenol antioxidant power due to free hydroxyl groups, could be of great importance in controlling iron metabolism. Surprisingly, the examined polyphenols did not induce up-regulation of HO–1 mRNA levels, which is in great disagreement with previous research (Ferrándiz and Devesa, 2008; Shah, et al., 2010) and with results from this study (see Chapter 3 and 5).

7. EFFECT OF POLYPHENOLS ON GENE EXPRESSION OF IRON- AND INFLAMMATION-RELATED GENES IN THP1 CELLS

7.1 Introduction

Macrophages play a significant role in iron homeostasis. Namely, splenic and hepatic macrophages recycle senescent erythrocytes by which iron is released from haem by the action of HO–1. Iron is effluxed into the circulation in response to systemic iron requirements. The release of iron from macrophages is mainly regulated by the interaction of the hormone hepcidin with the iron exporter FPN. Furthermore, macrophages are a key agent in inflammation-induced hypoferraemia. Namely, during infection and inflammation, IL–6 and other cytokines increase hepcidin synthesis, causing iron abstraction in macrophages. The resulting hypoferraemia limit the growth and pathogenicity of invading extracellular microbes and is an important means of host defence (Chung et al., 2009; Ganz, 2012).

It was shown in previous chapters of this thesis that quercetin greatly affects iron metabolism *in vivo*, on both absorption and at the systemic level. However, it is hard to conclude whether quercetin or its metabolites induce the observed effects. In addition, there is no data in the literature about a possible link between iron metabolism and quercetin metabolites. Bearing in mind the extensive metabolism of quercetin in the intestine and its short half-life (Gee et al., 2004; Justino et al., 2004; Moon et al., 2008), it was hypothesized that quercetin metabolites could also be involved in affecting iron metabolism. In order to get preliminary data on the effect of quercetin and two chosen quercetin metabolites on macrophages regulated iron homeostasis, *in vitro* studies using THP1 cells were performed. THP1 cells are a human leukaemia monocytic cell line and

are an excellent *in vitro* model of human macrophages. In culture, these monocytes are a non-adherent cell type, but with the addition of PMA, the monocytes differentiate into macrophages and adhere to cell culture dishes. In the scope of this thesis effect of quercetin and two chosen quercetin metabolites on expression of hepcidin, FPN and inflammation related genes, such as IL–6, IL–1β, TNF–α, iNOS and COX–2, in THP1 cells was studied.

7.2 Results

The effect of polyphenols on gene expression of iron–related genes and inflammation-related genes in THP1 cells were investigated. This study included quercetin and its metabolites that are identified in the serum of animals treated with quercetin orally and IP (see Chapter 3 and 5).

THP1 cells were plated in 6-well plates for 24 hours and subsequently treated with PMA (100 nM) for a further 24 hours to promote differentiation of monocytes into macrophages. The PMA-containing medium was removed and replaced with serum-free medium for 24 hours before treatments with polyphenols. THP1 cells were treated with quercetin, quercetin-3-*O*-glucuronide and tamarixetin (30 μM) for 0, 5 and 18 hours. After treatment cells were subject to RNA isolation and gene expression analysis (hepcidin and FPN).

Other batch of THP1 cells were treated with LPS in the presence or absence of quercetin, quercetin-3-*O*-glucuronide and tamarixetin (30 μM). Namely, LPS (500 ng/mL) was added to cells 2 hours after polyphenols. Cells were treated with LPS for 18 hours. This was followed by RNA isolation and quantitative-PCR to measure changes in mRNA expression of inflammation-related genes, such as IL-6, IL-1β, TNF-α, iNOS and COX-2.

Generally, polyphenols induced a significant change in hepcidin mRNA levels. Among all the investigated polyphenols quercetin induced the greatest change in hepcidin mRNA levels in THP1 cells, with a 75-fold increase after 18 hours of treatment (see Figure 7–1). Quercetin–3–*O*–glucuronide, one of the main metabolites of quecetin, also induced an increase in the hepcidin mRNA level. However, that level was comparable with the hepcidin mRNA levels in control wells after 5 and 18 hours of treatment (see

Figure 7–1). The level of hepcidin mRNA fluctuated after tamarixetin treatment in THP1 cells. Firstly, after 5 hours it was significantly reduced (2.4-fold), and then significantly increased after 18 hours of treatment (1.4-fold) compared with untreated cells (see Figure 7–1).

Furthermore, FPN mRNA oscillated during polyphenols' treatment. In all treated wells mRNA FPN levels was significantly down-regulated after 5 hours (from 2- to 15-fold; see Figure 7–2). However, 18 hours after treatment quercetin and quercetin–3–*O*–glucuronide induced a significant increase; while tamarixetin caused a significant decrease in FPN mRNA levels compared with the start of experiment (see Figure 7–2).

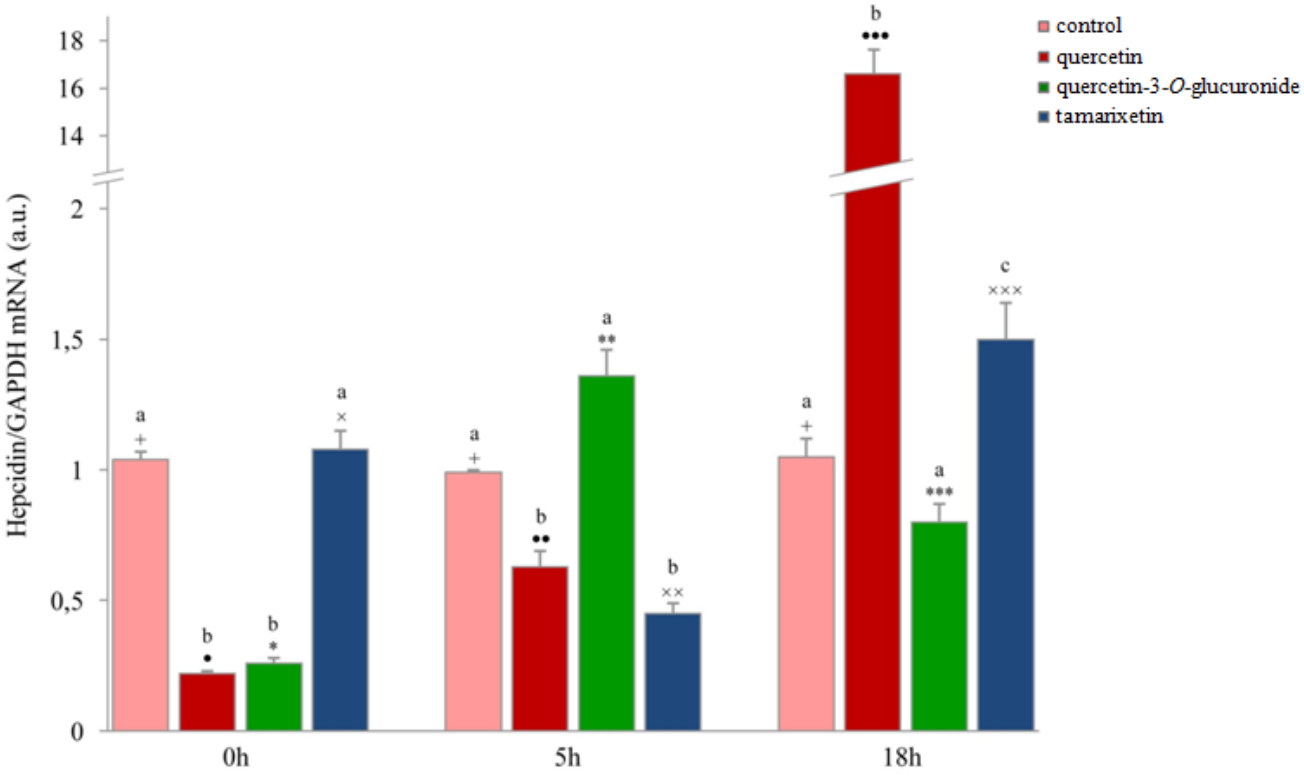


Figure 7–1 Effect of polyphenols on hepcidin mRNA expression in THP1 cells
THP1 cells were treated with polyphenols (30 µM) for 0, 5 and 18 hours. Instead of compounds DMSO was added in corresponding control wells. Changes in mRNA expression were measured by qPCR. Data normalised to GAPDH. Data are mean ± SEM; n=3; for the same treatment time groups with no common letters are significantly different from each other; for the same treatment groups with no common signs are significantly different from each other, ($p<0.05$).

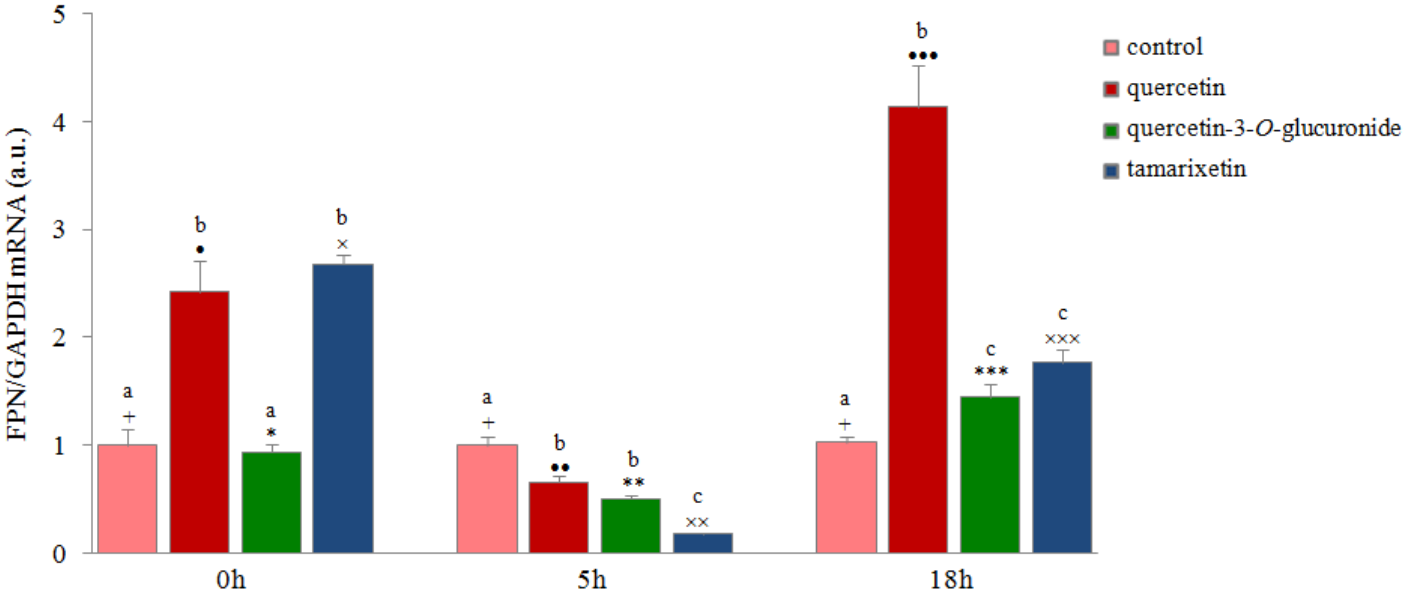


Figure 7–2 Effect of polyphenols on FPN mRNA expression in THP1 cells
THP1 cells were treated with polyphenols (30 µM) for 0, 5 and 18 hours. Instead of compounds DMSO was added in corresponding control wells. Changes in mRNA expression were measured by qPCR. Data normalised to GAPDH. Data are mean ± SEM; n=3; for the same treatment time groups with no common letters are significantly different from each other; for the same treatment groups with no common signs are significantly different from each other, ($p<0.05$).

After LPS treatment, all the investigated inflammation-related genes were significantly up-regulated in differentiated THP1 cells. However, their levels were significantly altered with different polyphenol treatments. Namely, quercetin induced a significant down-regulation of IL–6, TNF–α, iNOS mRNA levels, while it induced a significant up-regulation of IL–1β and COX–2 mRNA levels in LPS stimulated THP1 cells (see Figure 7–3 to Figure 7–7). Furthermore, quercetin–3–*O*–glucuronide induced a significant down-regulation of TNF–α mRNA level, while it induced up-regulation of IL–6, IL–1β and iNOS mRNA levels in LPS stimulated THP1 cells (see Figure 7–3 to Figure 7–7). Tamarixetin induced a significant down-regulation only of IL–6 mRNA levels, while all other investigated mRNAlevels were significantly up-regulated (see Figure 7–3 to Figure 7–7).

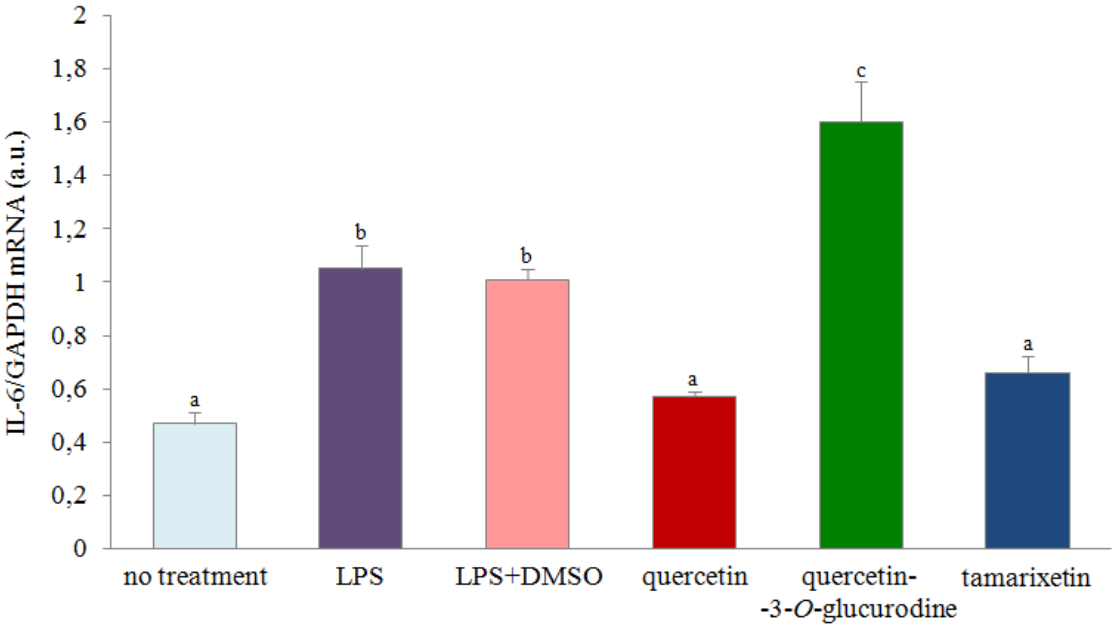


Figure 7–3 Effect of polyphenols on IL–6 mRNA expression in THP1 cells
THP1 cells were treated with LPS in the presence or absence of polyphenols (30 µM) for 18 hours. Instead of compounds DMSO was added in corresponding control wells. Changes in mRNA expression were measured by qPCR. Data normalised to GAPDH. Data are mean ± SEM; n=3; groups with no common letters are significantly different from each other ($p<0.05$).

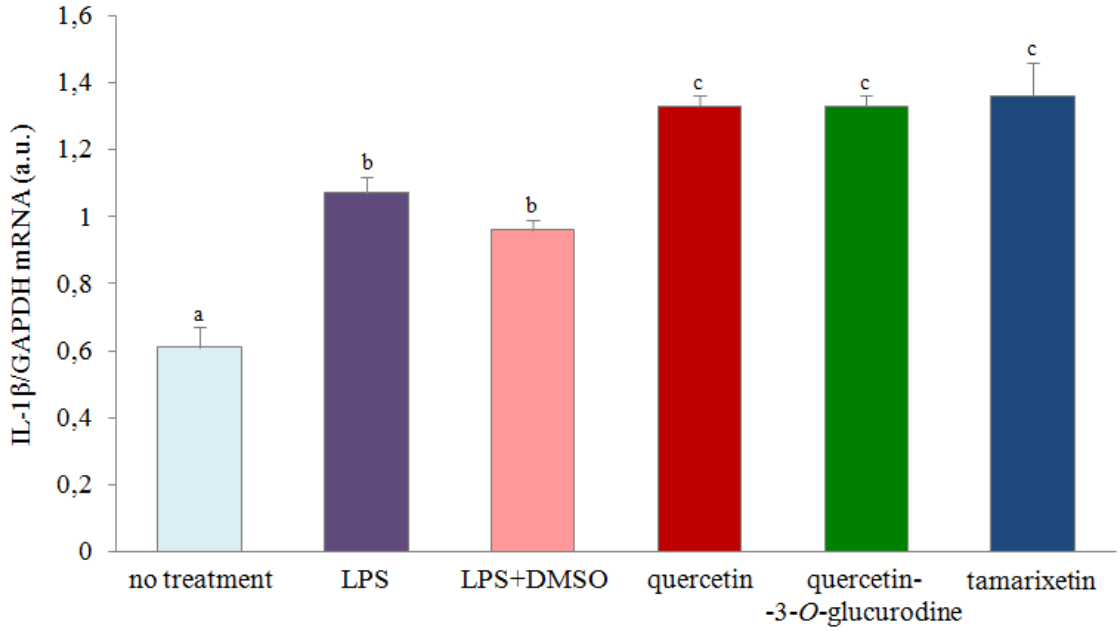


Figure 7–4 Effect of polyphenols on IL–1β mRNA expression in THP1 cells
THP1 cells were treated with LPS in the presence or absence of polyphenols (30 µM) for 18 hours. Instead of compounds DMSO was added in corresponding control wells. Changes in mRNA expression were measured by qPCR. Data normalised to GAPDH. Data are mean ± SEM; n=3; groups with no common letters are significantly different from each other ($p<0.05$).

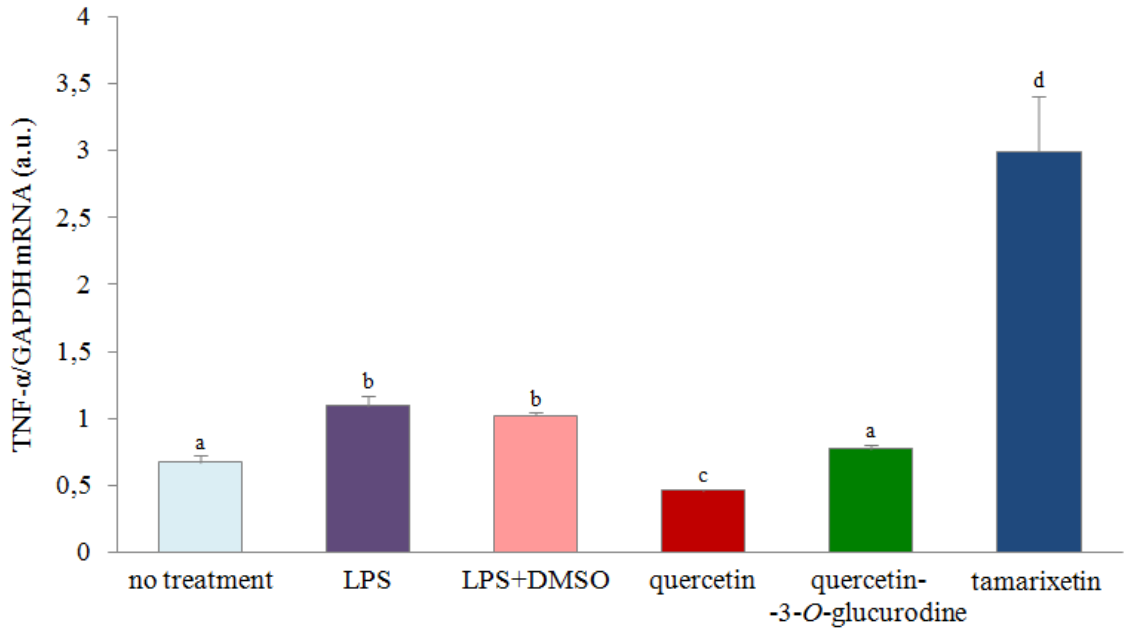


Figure 7–5 Effect of polyphenols on TNF–α mRNA expression in THP1 cells
THP1 cells were treated with LPS in the presence or absence of polyphenols (30 µM) for 18 hours. Instead of compounds DMSO was added in corresponding control wells. Changes in mRNA expression were measured by qPCR. Data normalised to GAPDH. Data are mean ± SEM; n=3; groups with no common letters are significantly different from each other ($p<0.05$).

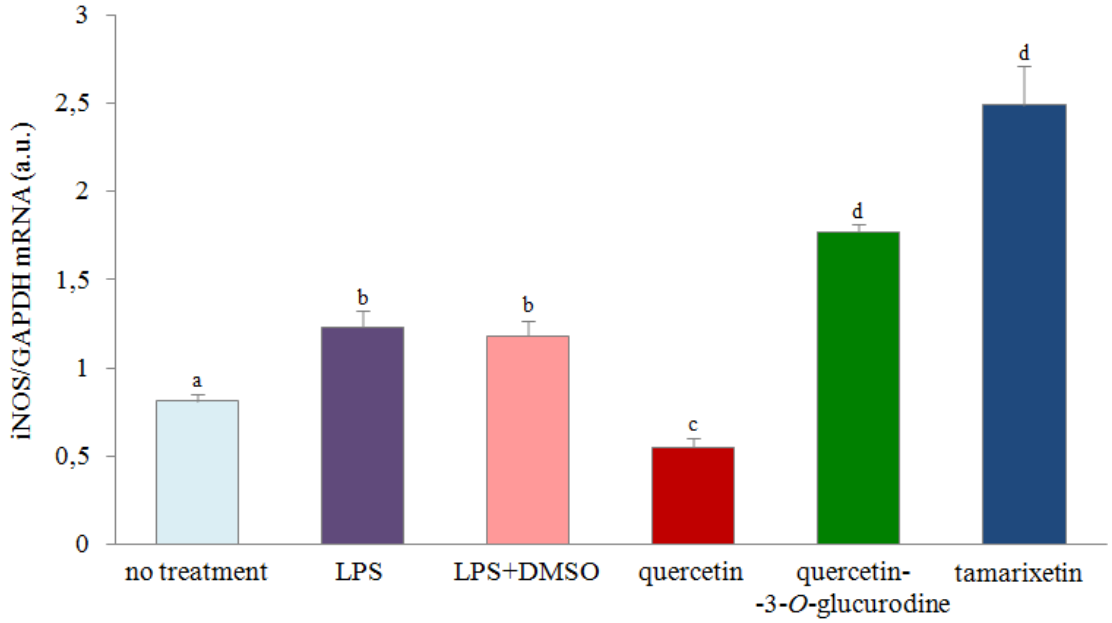


Figure 7–6 Effect of polyphenols on iNOS mRNA expression in THP1 cells
THP1 cells were treated with LPS in the presence or absence of polyphenols (30 µM) for 18 hours. Instead of compounds DMSO was added in corresponding control wells. Changes in mRNA expression were measured by qPCR. Data normalised to GAPDH. Data are mean ± SEM; n=3; groups with no common letters are significantly different from each other ($p<0.05$).

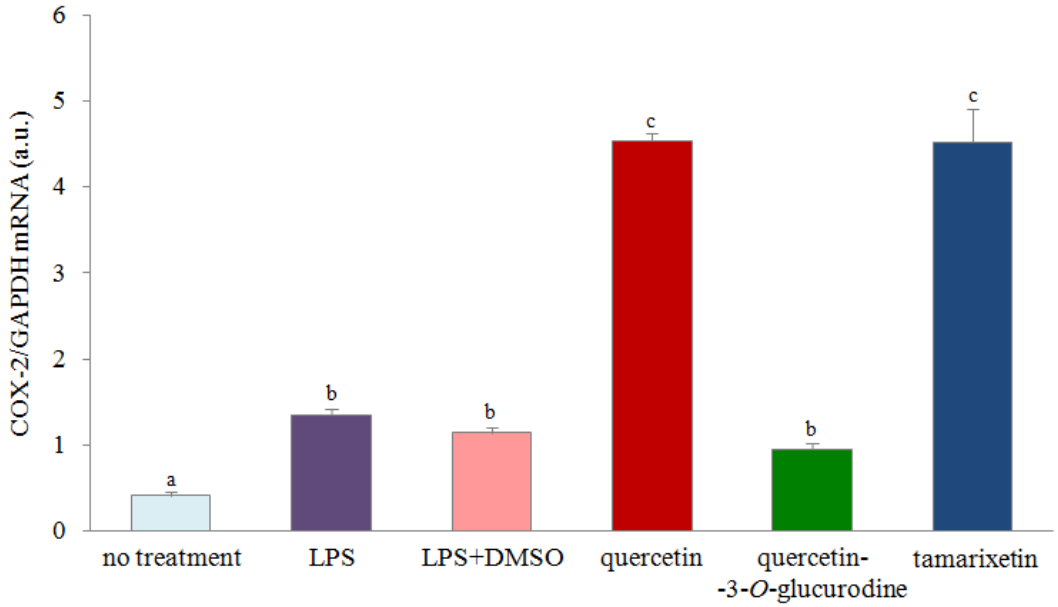


Figure 7–7 Effect of polyphenols on COX–2 mRNA expression in THP1 cells
THP1 cells were treated with LPS in the presence or absence of polyphenols (30 μM) for 18 hours. Instead of compounds DMSO was added in corresponding control wells. Changes in mRNA expression were measured by qPCR. Data normalised to GAPDH. Data are mean ± SEM; n=3; groups with no common letters are significantly different from each other ($p<0.05$).

7.3 Discussion

In this chapter the effect of quercetin, quercetin–3–*O*–glucuronide and tamarixetin on expression of hepcidin, FPN and inflammation-related genes, such as IL–6, IL–1β, TNF–α, iNOS and COX–2, in THP1 cells was studied.

Namely, all the investigated polyphenols induced significant changes in hepcidin and FPN mRNA levels. An increase in hepcidin mRNA levels after quercetin–3–*O*–glucuronide and tamarixetin treatment was followed by a reduction in FPN mRNA levels. However, after quercetin treatment, when a significant change of hepcidin mRNA levels occurred (75-fold), FPN mRNA levels were also up-regulated. This result came as no surprise as similar results were observed *in vivo* in the spleen after quercetin treatment (Chapter 3 and 5). Specifically, the results from the *in vivo* studies suggested that hepcidin originating from spleen cannot cause internalization of FPN as hepatic

hepcidin does. The results from the *in vitro* studies with THP1 cells also support this hypothesis. Namely, it could be that hepatic hepcidin, mainly expressed by hepatocytes, is different from splenic hepcidin, mainly originating from macrophages. Nevertheless, the premise that hepcidin expresses different effects on FPN from different tissues is already known (Chaston et al., 2008). Moreover, it was known from before that macrophages express hepcidin and FPN (Nguyen, et al., 2006; Sow, et al., 2007), but this is the first result providing evidence that they can be affected by polyphenols. Twenty-five mg of iron per day from red blood cells is recycled by macrophages, which make them the major contributor to body iron turnover. Factors which can regulate this turnover might be important therapeutically for treating a number of iron-related disorders. In a view of this, results which indicate that polyphenols can affect iron-related proteins in macrophages are of great importance.

During infection and inflammation, hepcidin levels are increased as a host defense mechanism in order to reduce iron levels and make it non-available to invading microorganisms. It was proven *in vivo* that IL–6 has a stimulatory effect on hepcidin transcription and that it induces hypoferraemia during inflammation (Nemeth et al., 2004; Kemna et al., 2005). Thus, it was worthwhile to make a parallel between proven hepcidin up-regulation by quercetin *in vivo* and polyphenols' effect on inflammatory-related genes in LPS-stimulated THP1 cells. In other words, the aim of this study was to investigate whether quercetin or its metabolites would induce expression of inflammatory-related genes that could be followed by an increase in hepcidin expression. Thus, expression of IL–6, IL–1β, TNF–α, iNOS and COX–2 mRNA in LPS-stimulated THP1 was followed after quercetin, quercetin–3–*O*–glucuronide and tamarixetin treatments. It was shown that all the investigated mRNAs were up-regulated at least after treatment of one of the investigated polyphenols. In general, tamarixetin- and quercetin–3–*O*–glucuronide-treatment caused greater up-regulation of the

investigated genes than quercetin. It is particularly interesting that IL–6 and IL–1β mRNA levels were greatly up-regulated by polyphenols' treatment, because it is known that these cytokines up-regulate hepcidin expression (Lee et al., 2005). These findings indicate that polpyhenols could affect iron homeostasis by inducing inflammation. However, future studies should focus on an in-depth analysis of this issue in order to determine influence of polyphenols and their metabolites on iron homeostasis driven by inflammation *in vivo*.

7.4 Conclusions

To summarise, the results presented give a preliminary indication that quercetin and quercetin metabolites could have an effect on systemic iron metabolism by changing the expression of iron- and inflammation-related proteins in macrophages. Namely, it is shown that quercetin, quercetin–3–*O*–glucuronide and tamarixetin could affect expression of hepcidin and FPN mRNA levels in THP1 cells. Moreover, they induced expression of inflammatory-related genes in THP1 cells, which are proven to up-regulate hepcidin expression (i.e. IL–6 and IL–1β). As macrophages play a significant role in iron homeostasis in health and during both infection and inflammation and iron-related disorders, verified agents which could regulate iron metabolism in macrophages are of great importance. Thus, further research on how dietary polyphenols could interact with iron recycling and storage in macrophages is fully supported.

8. CONCLUSIONS

8.1 General conclusions

This thesis investigated the mechanisms underlying the effects of dietary polyphenols, particularly quercetin, which is one of the most dominant dietary polyphenol, on systemic iron regulation and iron absorption.

From the reserach described in this thesis, it was concluded that:

1. Oral administration of quercetin caused iron deficiency in rats, which was followed by increase in iron absorption rate and decrease in iron stores of liver and spleen. Specifically, oral quercetin treatment affected mRNA levels of duodenal DMT1, Dcytb and FPN. These results also indicated that oral quercetin has a great effect on iron absorption and a minor effect on systemic iron regulation.

2. Polyphenols inhibit non-haem iron absorption *in vivo* by chelating it in duodenum. Specifically, by chelating iron quercetin prevents transepithelial non-haem iron transport across the enterocyte by increasing apical iron uptake and decreasing the basolateral iron release.

3. IP administration of quercetin mainly affected systemic iron homeostasis, primary by a vast up-regulation of hepatic and kidney mRNA hepcidin levels and decreased intracellular iron pools.

4. Different polyphenols and quercetin metabolites display different effects on the expression of iron-related genes in liver *in vitro* compared with quercetin.

5. Polyphenols express a significant effect on the expression of iron- and inflammation-related genes in macrophages *in vitro.*

8.2 Future work

This thesis has investigated, discovered and proposed mechanisms of the action of polpyhenols, particularly quercetin, on iron metabolism in a well established *in vivo* model. In future, similar studies need to be carried out in humans to establish any possible benefits for those at risk of developing iron-related disorders.

In this study, it has been shown that polyphenols, particularly quercetin, greatly affects mRNA levels of genes essential for iron absorption in the duodenum and systemic iron regulation. In further studies, western blotting of iron-related proteins in examined tissues should be performed in order to investigate changes in the examined proteins and thus confirm the proposed effects.

It this thesis it was proven that polyphenols inhibit intestinal absorption of non-haem iron. However, it is still unknown how polyphenols affect haem iron absorption. Thus, further *in vivo* research should be done in order to investigate whether absorption of iron from a haem source could be affected by dietary polyphenols.

In the present study it was proven that quercetin decreases the iron content of liver and spleen *in vivo*. Liver and spleen are the main iron storage organs, where during iron overload diseases a great increase in iron content occurs. Future research should confirm this beneficial effect of quercetin and other polyphenols in an iron overloaded mice model.

In this thesis it is observed that quercetin induces a great up-regulation of hepcidin in liver and kidney. However, the mechanism underlying this effect is not fully understood. In future, it should be investigated by which signalling pathway polyphenols, particularly quercetin, induce hepcidin up-regulation, with particular attention to the Nrf2–ARE and BMP6/SMAD4 signalling pathways.

The ultimate goals of this kind of research are:

- to examine the effects of dietary polyphenols on iron absorption and to establish a particular diet which will enhance iron absorption for patients affected with anaemia or inhibit iron absorption for patients diagnosed with iron-overload diseases;
- to examine the therapeutic effects of polyphenols as chelato therapeutics for iron-redistribution therapy in human studies, focusing on patients with iron-overload diseases.

9. REFERENCES CITED

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

Abboud, S., Haile, D. J. (2000): A novel mammalian iron–regulated protein involved in intracellular iron metabolism. *The Journal of Biological Chemistry*. 275: 19906–19912.

Adams, P. C., Barton, J. C. (2010): How I treat hemochromatosis. *Blood*. 116: 317–325.

Aisen, P., Leibman, A., Zweier, J. (1978): Stoichiometric and site characteristics of the binding of iron to human transferrin. *The Journal of Biological Chemistry*. 253: 1930–1937.

Alzaid, F., Pourvali, K., Lin, C. I., Arno, M., Aldecoa–Otalora, A. E., Sharp, P. A., Hogstrand, C., Emery, P. W., Bagchi, D., Preedy, V. R., Wiseman, H. (2010): *Summer meeting – Over– and undernutrition: challenges and approaches; Proceedings of the Nutrition Society*. 69: (OCE1) E27.

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

Armah, C. N., Sharp, P., Mellon, F. A., Pariagh, S., Lund, E. K., Dainty, J. R., Teucher, B., Fairweather–Tait, S. J. (2008): L–alpha–glycerophosphocholine contributes to meat's enhancement of nonheme iron absorption. *Journal of Nutrition*. 138: 873–877.

Armitage, A. E., Eddowes, L. A., Gileadi, U., Cole, S., Spottiswoode, N., Selvakumar, T. A., Ho, L. P., Townsend, A. R., Drakesmith, H. (2011): Hepcidin regulation by innate immune and infectious stimuli. *Blood*. 118: 4129–4139.

Arosio, P., Ingrassia, R., Cavadini, P. (2009): Ferritins: A family of molecules for iron storage, antioxidation and more. *Biochimica et Biophysica Acta*. 1790: 589–599.

Au, C., Benedetto, A., Aschner, M. (2008): Manganese transport in eukaryotes: The role of DMT1. *NeuroToxicology*. 29: 569–576.

Baccan, M. M., Chiarelli–Neto, O., Pereira, R. M. S., Breno Pannia Espósito, B. P. (2012): Quercetin as a shuttle for labile iron. *Journal of Inorganic Biochemistry*. 107: 34–39.

Ballot, D., Baynes, R. D., Bothwell, T. H., Gillooly, M., MacFarlane, B. J., MacPhail, A. P., Lyons, G., Derman, D. P., Bezwoda, W. R., Torrance, J. D., Bothwell, J. E. (1987): The effects of fruit juices and fruits on the absorption of iron from a rice meal. *British Journal of Nutrition.* 57: 331–343.

Baltussen, R., Knai, C., Sharan, M. (2004): Iron Fortification and Iron Supplementation are Cost–Effective Interventions to Reduce Iron Deficiency in Four Subregions of the World. *The Journal of Nutrition*. 134: 2678–2684.

Baur, J. A., Sinclair, D. A. (2006): Therapeutic potential of resveratrol: the *in vivo* evidence. *Nature Reviews Drug Discovery*. 5: 493–506.

Beaumont, C., Delaby, C. (2009): Recycling iron in normal and pathological states. *Seminars in hematology*. 46: 328–338.

Beecher, G. R. (2003): Overview of Dietary Flavonoids: Nomenclature, Occurrence and Intake. *The Journal of Nutrition*. 133: 3248S–3254S.

Beutler, E., Gelbart, T., Lee, P., Trevino, R., Fernandez, M. A., Fairbanks, V. F. (2000): Molecular characterization of a case of atransferrinemia. *Blood*. 96: 4071–4074.

Bezwoda, W. R., Torrance, J. D., Bothwell, T. H., Macphail, A. P., Graham, B., Mills, W. (1985): Iron absorption from red and white wines. *Scandinavian Journal of Haematology*. 34: 121–127.

Bhagwat, S., Haytowitz, D. B., Holden, J. M. (2014): *USDA Database for the Flavonoid Content of Selected Foods*. U.S. Department of Agriculture, Beltsville, USA.

Bou–Abdallah, F. (2010): The iron redox and hydrolysis chemistry of the ferritins. *Biochimica Et Biophysica Acta*. 1800: 719–731.

Bradshaw, H. D., Schemske, D. W. (2003): Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature*. 426: 176–178.

Brasse–Lagnel, C., Karim, Z., Letteron, P., Bekri, S., Bado, A., Beaumont, C. (2011): Intestinal DMT1 cotransporter is down-regulated by hepcidin via proteasome internalization and degradation. *Gastroenterology*. 140: 1261–1271.

Bridle, K., Cheung, T. K., Murphy, T., Walters, M., Anderson, G., Crawford, D. G., Fletcher, L. M. (2006): Hepcidin is down–regulated in alcoholic liver injury: implications for the pathogenesis of alcoholic liver disease. *Alcoholism: Clinical and Experimental Research*. 30: 106–112.

Brock, J. H., Mainou–Fowler, T., McGregor, S. J. (1987): Transferrins and defence against infection. *Annali dell'Istituto Superiore di Sanità*. 23: 935–941.

Cairo, G., Pietrangelo, A. (2000): Iron regulatory proteins in pathobiology. *Biochemical Journal*. 352: 241–250.

Carr, A. C., Frei, B. (1999): Toward a new recommended dietary allowance for vitamin C based on antioxidant and health effects in humans. *American Journal of Clinical Nutrition*. 69: 1086–1107.

Cavill, I. (2002): Erythropoiesis and iron. *Best Practice & Research Clinical Haematolog*. 15: 399–409.

Chaston, T., Chung, B., Mascarenhas, M., Marks, J., Patel, B., Srai, S. K., Sharp, P. (2008): Evidence for differential effects of hepcidin in macrophages and intestinal epithelial cells. *Gut*. 57: 374–382.

Chen, H., Attieh, Z. K., Su, T., Syed, B. A., Gao, H., Alaeddine, R. M., Fox, T. C., Usta, J., Naylor, C. E., Evans, R. W., McKie, A. T., Anderson, G. J., Vulpe, C. D. (2004): Hephaestin is a ferroxidase that maintains partial activity in sex–linked anemia mice. *Blood*. 103: 3933–3939.

Chen, H., Huang, G., Su, T., Gao, H., Attieh, Z. K., McKie, A. T., Anderson, G. J., Vulpe, C. D. (2006): Decreased hephaestin activity in the intestine of copper–deficient mice causes systemic iron deficiency. *The Journal of Nutrition*. 136: 1236–1241.

Chen, H., Su, T., Attieh, Z. K., Fox, T. C., McKie, A. T., Anderson, G. J., Vulpe, C. D. (2003): Systemic regulation of Hephaestin and Ireg1 revealed in studies of genetic and nutritional iron deficiency. *Blood*. 102: 1893–1899.

Cheynier, V., Comte, G., Davies, K. M., Lattanzio, V., Martens, S. (2013): Plant phenolics: recent advances on their biosynthesis, genetics, and ecophysiology. *Plant Physiology and Biochemistry*. 72: 1–20.

Chomczynski, P., Sacchi, N. (1987): Single–step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry*. 162: 156–159.

Chung, B., Chaston, T., Marks, J., Srai, S. K., Sharp, P. A. (2009): Hepcidin Decreases Iron Transporter Expression *in Vivo* in Mouse Duodenum and Spleen and *in Vitro* in THP–1 Macrophages and Intestinal Caco–2 Cells. *Journal of Nutrition*. 139: 1457–1462.

Cook, J. D., Monsen, E. R. (1976): Food iron absorption in human subjects. III. Comparison of the effect of animal proteins on nonheme iron absorption. *American Journal of Clinical Nutrition*. 29: 859–867.

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

Cook, J. D., Reddy, M. B., Hurrell, R. F. (1995): The effect of red and white wines on nonheme–iron absorption in humans. *The American Journal of Clinical Nutrition*. 61: 800–804.

Crichton, R. R. (2001): *Inorganic Biochemistry of Iron Metabolism: From Molecular Mechanisms to Clinical Consequences, 2nd edition*. John Wiley & Sons Ltd, England, UK.

Cunningham, P., Afzal–Ahmed, I., Naftalin, R. J. (2006): Docking studies show that D–glucose and quercetin slide through the transporter GLUT1. *The Journal of Biological Chemistry*. 281: 5797–5803.

Day, A. J., Gee, J. M., DuPont, M. S., Johnson, I. T., Williamson, G. (2003): Absorption of quercetin–3–glucoside and quercetin–4'–glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium–dependent glucose transporter. *Biochemical Pharmacology*. 65: 1199–1206.

DeMaeyer, E. M., Dallman, P., Gurney, J. M., Hallberg, L., Sood, S. K., Srikantia, S. G. (1989): *Preventing and controlling iron deficiency anaemia through primary health care: A guide for health administrators and programme managers*. World Health Organization, Geneva, Switzerland.

Disler, P. B., Lynch, S. R., Charlton, R. W., Torrance, J. D., Bothwell, T. H., Walker, R. B., Mayet, F. (1975): The effect of tea on iron absorption. *Gut*. 16: 193–200.

Donovan, A., Brownlie, A., Zhou, Y., Shepard, J., Pratt, S. J., Moynihan, J., Paw, B. H., Drejer, A., Barut, B., Zapata, A., Law, T. C., Brugnara, C., Lux, S. E., Pinkus, G. S., Pinkus, J. L., Kingsley, P. D., Palis, J., Fleming, M. D., Andrews, N. C., Zon, L. I. (2000): Positional cloning of zebrafish ferroportin 1 identifies a conserved vertebrate iron exporter. *Nature*. 403: 776–781.

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

Dunn, L. L., Suryo Rahmanto, Y., Richardson, D. R. (2007): Iron uptake and metabolism in the new millennium. *Trends in Cell Biology*. 17: 93–100.

Dupic, F., Fruchon, S., Bensaid, M., Loreal, O., Brissot, P., Borot, N., Roth, M. P., Coppin, H. (2002): Duodenal mRNA expression of iron related genes in response to iron loading and iron deficiency in four strains of mice. *Gut*. 51: 648–653.

Ekström, A. M., Serafini, M., Nyrén, O., Wolk, A., Bosetti, C., Bellocco, R. (2011): Dietary quercetin intake and risk of gastric cancer: results from a population–based study in Sweden. *Annals of Oncology*. 22: 438–443.

Escudero, L. B., Fusari, C. M., Altamirano, J. C., Camargo, A. B., Wuilloud, R. G. (2014): Stability of Iron–Quercetin Complexes in Synthetic Wine under In Vitro Digestion Conditions. *Journal of Food Science*. 79: C1933–C1938.

Falcone Ferreyra, M. L., Rius, S. P., Casati, P. (2012): Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Frontiers in Plant Science*. 3: 222.

Ferrándiz, M. L., Devesa, I. (2008): Inducers of heme oxygenase–1. *Current Pharmaceutical Design*. 14: 473–486.

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

Fleming, M. D., Trenor, C. C. 3rd, Su, M. A., Foernzler, D., Beier, D. R., Dietrich, W. F. Andrews, N. C. (1997): Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nature Genetics*. 16: 383–386.

Fleming, R. E. (2009): Iron Sensing as a Partnership: HFE and Transferrin Receptor 2. *Cell Metabolism*. 9: 211–212.

Fleming, R. E., Migas, M. C., Holden, C. C., Waheed, A., Britton, R. S., Tomatsu, S., Bacon, B. R., Sly, W. S. (2000): Transferrin receptor 2: continued expression in mouse

liver in the face of iron overload and in hereditary hemochromatosis. *Proceedings of the National Academy of Sciences of the United States of America*. 97: 2214–2219.

Franchini, M., Montagnana, M., Lippi, G. (2010): Hepcidin and iron metabolism: From laboratory to clinical implications. Clinica Chimica Acta. 411: 1565–1569.

Frazer, D. M., Inglis, H. R., Wilkins, S. J., Millard, K. N., Steele, T. M., McLaren, G. D., McKie, A.T., Vulpe, C. D., Anderson, G. J. (2004): Delayed hepcidin response explains the lag period in iron absorption following a stimulus to increase erythropoiesis. *Gut*. 53: 1509–1515.

Frazer, D. M., Wilkins, S. J., Becker, E. M., Vulpe, C. D., McKie, A. T., Trinder, D., Anderson, G. J. (2002): Hepcidin expression inversely correlates with the expression of duodenal iron transporters and iron absorption in rats. *Gastroenterology*. 123: 835–844.

Galy, B., Ferring, D., Minana, B., Bell, O., Janser, H. G., Muckenthaler, M., Schümann, K., Hentze, M. W. (2005): Altered body iron distribution and microcytosis in mice deficient in iron regulatory protein 2 (IRP2). *Blood*. 106: 2580–2589.

Galy, B., Ferring–Appel, D., Kaden, S., Gröne, H. J., Hentze, M. W. (2008): Iron regulatory proteins are essential for intestinal function and control key iron absorption molecules in the duodenum. *Cell Metabolism*. 7: 79–85.

Gan, L., Johnson, J. A. (2014): Oxidative damage and the Nrf2–ARE pathway in neurodegenerative diseases. *Biochimica et Biophysica Acta*. 1842: 1208–1218.

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

Ganz, T. (2012): Macrophages and Systemic Iron Homeostasis. *Journal of Innate Immunity*. 4: 446–453.

Ganz, T., Nemeth, E. (2012): Hepcidin and iron homeostasis. *Biochimica et Biophysica Acta*. 1823: 1434–1443.

Garrick, M. D., Dolan, K. G., Horbinski, C., Ghio, A. J., Higgins, D., Porubcin, M., Moore, E. G., Hainsworth, L. N., Umbreit, J. N., Conrad, M. E., Feng, L., Lis, A., Roth, J. A., Singleton, S., Garrick, L. M. (2003): DMT1: a mammalian transporter for multiple metals. *Biometals*. 16: 41–54.

Gee, J. M., Wroblewska, M. A., Bennett, R. N., Mellon, F. A., Johnson, I. T. (2004): Absorption and twenty–four–hour metabolism time–course of quercetin–3–O–glucoside in rats, *in vivo*. *Journal of the Science of Food and Agriculture*. 84: 1341–1348.

Gillooly, M., Bothwell, T. H., Torrance, J. D., MacPhail, A. P., Derman, D. P., Bezwoda, W. R., Mills, W., Charlton, R. W. (1983): The effects of organic acids, phytates and polyphenols on the absorption of iron from vegetables. *British Journal of Nutrition*. 49: 331–3423.

Girelli, D., Corrocher, R., Bisceglia, L., Olivieri, O., De Franceschi, L., Zelante, L., Gasparini, P. (1995): Molecular basis for the recently described hereditary hyperferritinemia–cataract syndrome: a mutation in the iron–responsive element of ferritin L–subunit gene (the "Verona mutation"). Blood. 86: 4050–4053.

Gkouvatsos, K., Fillebeen, C., Daba, A., Wagner, J., Sebastiani, G., Pantopoulos, K. (2014): Iron–Dependent Regulation of Hepcidin in Hjv−/− Mice: Evidence That Hemojuvelin Is Dispensable for Sensing Body Iron Levels. *PLoS One*. 9: e85530.

Gkouvatsos, K., Papanikolaou, G., Pantopoulos, K. (2012): Regulation of iron transport and the role of transferrin. *Biochimica et Biophysica Acta*. 1820: 188–202.

Gomme, P. T., McCann, K. B., Bertolini, J. (2005): Transferrin: structure, function and potential therapeutic actions. *Drug Discovery Today*. 10: 267–273.

Granado–Serrano, A. B., Martín, M. A., Bravo, L., Goya, L., Ramos, S. (2012): Quercetin modulates Nrf2 and glutathione–related defenses in HepG2 cells: Involvement of p38. *Chemico–Biological Interactions*. 195: 154–164.

Gruenheid, S., Cellier, M., Vidal, S., Gros, P. (1995): Identification and characterization of a second mouse Nramp gene. *Genomics*. 25: 514–525.

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

Gunshin, H., Starr, C. N., DiRenzo, C., Fleming, M. D., Jin, J., Greer, E. L., Sellers, V. M., Galica, S. M. (2005): Cybrd1 (duodenal cytochrome b) is not necessary for dietary iron absorption in mice. *Blood*. 106: 2879–2883.

Hallberg, L., Brune, M., Rossander, L. (1989): Iron absorption in man: ascorbic acid and dose–dependent inhibition by phytate. *The American Journal of Clinical Nutrition*. 49: 140–144.

Hallberg, L., Rossander–Hulten, L., Brune, M., Gleerup, A. (1992): Calcium and iron absorption: mechanism of action and nutritional importance. *European Journal of Clinical Nutrition*. 46: 317–327.

Halliwell, B., Gutteridge J. M. C. (2007): *Free radicals in biology and medicine, 4th edition*. University Press, Oxford, UK.

Hamdaoui, M. H., Chahed, A., Ellouze–Chabchoub, S., Marouani, N., Ben Abid, Z., Hédhili, A. (2005): Effect of green tea decoction on long–term iron, zinc and selenium status of rats. *Annals of Nutrition and Metabolism*. 49: 118–124.

Han, O., Wessling–Resnick, M. (2002): Copper repletion enhances apical iron uptake and transepithelial iron transport by Caco–2 cells. *American Journal of Physiology. Gastrointestinal and Liver Physiology*. 282: 527–533.

Hann, H. W., Stahlhut, M. W., Blumberg, B. S. (1988): Iron nutrition and tumor growth: decreased tumor growth in iron–deficient mice. *Cancer Research*. 48: 4168–4170.

Harada, N., Kanayama, M., Maruyama, A., Yoshida, A., Tazumi, K., Hosoya, T., Mimura, J., Toki, T., Maher, J. M., Yamamoto, M., Itoh, K. (2011): Nrf2 regulates ferroportin 1–mediated iron efflux and counteracts lipopolysaccharide–induced ferroportin 1 mRNA suppression in macrophages. *Archives of Biochemistry and Biophysics*. 508: 101–109.

Harland, B. F., Smikle–Williamsa, S., Oberleas, D. (2004): High performance liquid chromatography analysis of phytate (IP6) in selected foods. *Journal of Food Composition and Analysis*. 17: 227–233.

Havsteen, B. H. (2002): The biochemistry and medical significance of the flavonoids. Pharmacology & Therapeutics. 96: 67–202.

Heeney, M. M., Andrews, N. C. (2004): Iron homeostasis and inherited iron overload disorders: an overview. *Hematology/Oncology Clinics of North America*. 18: 1379–1403.

Hémadi, M., Kahn, P. H., Miquel, G., El Hage Chahine, J. M. (2004): Transferrin's mechanism of interaction with receptor 1. *Biochemistry*. 43: 1736–1745.

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

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

Hintze, K. J., Katoh, Y., Igarashi, K., Theil, E. C. (2007): Bach1 repression of ferritin and thioredoxin reductase1 is heme–sensitive in cells and in vitro and coordinates expression with heme oxygenase1, beta–globin, and NADP(H) quinone (oxido) reductase1. *The Journal of Biological Chemistry*. 282: 34365–34371.

Hollman, P. C., Bijsman, M. N., van Gameren, Y., Cnossen, E. P., de Vries, J. H., Katan, M. B. (1999): The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radical Research*. 31: 569–573.

Hoque, R., Sharp P. (2010): The effect of quercetin on intestinal iron transporter expression. *Winter meeting – Functional genomic biomarkers, nutrition and disease susceptibility; Proceedings of the Nutrition Society*. 69: (OCE8) E588.

Huang, H., Constante, M., Layoun, A., Santos, M. M. (2009): Contribution of STAT3 and SMAD4 pathways to the regulation of hepcidin by opposing stimuli. *Blood*. 113: 3593–3599.

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

Hurrell, R. F. (2004): Phytic acid degradation as a means of improving iron absorption. *International Journal for Vitamin and Nutrition Research*. 74: 445–452.

Hurrell, R. F., Lynch, S. R., Trinidad, T. P., Dassenko, S. A., Cook, J. D. (1988): Iron absorption in humans: bovine serum albumin compared with beef muscle and egg white. *American Journal of Clinical Nutrition*. 47: 102–107.

Hurrell, R. F., Lynch, S. R., Trinidad, T. P., Dassenko, S. A., Cook, J. D. (1989): Iron absorption in humans as influenced by bovine milk proteins. *American Journal of Clinical Nutrition*. 49: 546–552.

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

Hurrell, R. F., Reddy, M. B., Juillerat, M. A., Cook, J. D. (2003): Degradation of phytic acid in cereal porridges improves iron absorption by human subjects. *The American Journal of Clinical Nutrition*. 77: 1213–1209.

Hurrell, R. F., Reddy, M. B., Juillerat, M., Cook, J. D. (2006): Meat protein fractions enhance nonheme iron absorption in humans. *Journal of Nutrition*. 136: 2808–2812.

Hurrell, R., Egli, I. (2010): Iron bioavailability and dietary reference values. *The American Journal of Clinical Nutrition*. 91: 1461S–1467S.

Idzerda, R. L., Huebers, H., Finch, C. A., McKnight, G. S. (1986): Rat transferrin gene expression: tissue–specific regulation by iron deficiency. *Proceedings of the National Academy of Sciences USA*. *83*: 3723–3727.

Iwai, K., Drake, S. K., Wehr, N. B., Weissman, A. M., LaVaute, T., Minato, N., Klausner, R. D., Levine, R. L., Rouault, T. A. (1998): Iron–dependent oxidation, ubiquitination, and degradation of iron regulatory protein 2: implications for degradation of oxidized proteins. *Proceedings of the National Academy of Sciences of the United States of America*. 95: 4924–4928.

Jeong, S. Y., David, S. (2003): Glycosylphosphatidylinositol–anchored ceruloplasmin is required for iron efflux from cells in the central nervous system. *The Journal of Biological Chemistry*. 278: 27144–27148.

Johnson, J. A., Johnson, D. A., Kraft, A. D., Calkins, M. J., Jakel, R. J., Vargas, M. R., Chen, P. C. (2008): The Nrf2–ARE pathway: an indicator and modulator of oxidative stress in neurodegeneration. *Annals of the New York Academy of Sciences*. 1147: 61–69.

Justino, G. C., Santos, M. R., Canário, S., Borges, C., Florêncio, M. H., Mira, L. (2004): Plasma quercetin metabolites: structure–antioxidant activity relationships. *Archives of Biochemistry and Biophysics*. 432: 109–121.

Kato, J., Fujikawa, K., Kanda, M., Fukuda, N., Sasaki, K., Takayama, T., Kobune, M., Takada, K., Takimoto, R., Hamada, H., Ikeda, T., Niitsu, Y. (2001): A mutation, in the iron–responsive element of H ferritin mRNA, causing autosomal dominant iron overload. *The American Journal of Human Genetics*. 69: 191–197.

Kemna, E., Pickkers, P., Nemeth, E., van der Hoeven, H., Swinkels, D. (2005): Time–course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. *Blood*. 106: 1864–1866.

Kim, E. Y., Ham, S., Bradke, D., Ma, Q., Han, O. (2011): Ascorbic Acid Offsets the Inhibitory Effect of Bioactive Dietary Polyphenolic Compounds on Transepithelial Iron Transport in Caco–2 Intestinal Cells. *The Journal of Nutrition*. 141: 828–834.

Kim, E., Ham, S., Shigenaga, M. K., Han, O. (2008): The inhibiting Bioactive Dietary Polyphenolic Compounds Reduce Nonheme Iron Transport across Human Intestinal Cell Monolayers. *The Journal of Nutrition*. 138: 1647–1651.

Klaassen, C. D., Reisman, S. A. (2010): Nrf2 the rescue: Effects of the antioxidative/electrophilic response on the liver. *Toxicology and Applied Pharmacology*. 244: 57–65.

Kontoghiorghesa, G. J. (2003): Do we need more iron–chelating drugs? *The Lancet*. 362: 495–496.

Koppenol, W. H. (2001): The Haber–Weiss cycle – 70 years later. *Redox Report*. 6: 229–234.

Krause, A., Neitz, S., Mägert, H. J., Schulz, A., Forssmann, W. G., Schulz–Knappe, P., Adermann, K. (2000): LEAP–1, a novel highly disulfide–bonded human peptide, exhibits antimicrobial activity. *FEBS Letter*. 480: 147–150.

Laftah, A. H., Ramesh, B., Simpson, R. J., Solanky, N., Bahram, S., Schümann, K., Debnam, E. S., Srai, S. K. (2004): Effect of hepcidin on intestinal iron absorption in mice. Blood. 103: 3940–3954.

Lam, T. K., Rotunno, M., Lubin, J. H., Wacholder, S., Consonni, D., Pesatori, A. C., Bertazzi, P. A., Chanock, S. J., Burdette, L., Goldstein, A. M., Tucker, M. A., Caporaso, N. E., Subar, A. F., Landi, M. T. (2010): Dietary quercetin, quercetin–gene

interaction, metabolic gene expression in lung tissue and lung cancer risk. *Carcinogenesis*. 31: 634–642.

Lam–Yuk–Tseung, S., Camaschella, C., Iolascon, A., Gros, P. (2006): A novel R416C mutation in human DMT1 (SLC11A2) displays pleiotropic effects on function and causes microcytic anemia and hepatic iron overload. *Blood Cells, Molecules and Diseases*. 36: 347–354.

Lattanzio, V., Lattanzio, V. M. T., Cardinali, A. (2006): *Role of phenolics in the resistnce mechanisms of plants against fungal pathogents and insect* In *Phytochemistry: advances in research*, Ed. Imperato, F. Research Signpost, Trivandrum, Kerala, India.

Latunde–Dada, G. O., Simpson, R. J., McKie, A. T. (2008): Duodenal cytochrome B expression stimulates iron uptake by human intestinal epithelial cells. *Journal of Nutrition*. 138: 991–995.

Laufberger, V. (1937): Sur la cristallisation de la ferritine. *Bulletin de la Société de chimie biologique*. 19: 1575–1582.

Lee, H. B., Blaufox, M. D. (1985): Blood volume in the rat. *Journal of Nuclear Medicine*. 26: 72–76.

Lee, J., Mitchell, A. E. (2012): Pharmacokinetics of Quercetin Absorption from Apples and Onions in Healthy Humans. *Journal of Agricultural and Food Chemistry*. 60: 3874–3881.

Lee, P. L., Gelbart, T., West, C., Halloran, C., Beutler, E. (1998): The human Nramp2 gene: characterization of the gene structure, alternative splicing, promoter region and polymorphisms. *Blood Cells, Molecules and Diseases*. 24: 199–215.

Lee, P., Peng, H., Gelbart, T., Wang, L., Beutler, E. (2005): Regulation of hepcidin transcription by interleukin–1 and interleukin–6. *Proceedings of the National Academy of Sciences of the United States of America*. 102: 1906–1910.

Leipuviene, R., Theil, E. C. (2007): The family of iron responsive RNA structures regulated by changes in cellular iron and oxygen. *Cellular and Molecular Life Sciences*. 64: 2945–2955.

Leopoldini, M., Russo, N., Chiodo, S., Toscano, M. (2006): Iron chelation by the powerful antioxidant flavonoid quercetin. *Journal of Agricultural and Food Chemistry*. 54: 6343–6351.

Li, Y. Q., Bai, B., Zheng, Q. Q., Yan, H., Zhuang, G. H. (2013): Quantitative study of iron metabolism–related genes expression in rat. *Biomedical and Environmental Sciences*. 26: 808–819.

Lieu, P. T., Heiskala, M., Peterson, P. A., Yang, Y. (2001): The roles of iron in health and disease. *Molecular Aspects of Medicine*. 22: 1–87.

Lill, R., Muhlenhoff, U. (2006): Iron–sulfur protein biogenesis in eukaryotes: Components and mechanisms. *Annual Review of Cell and Developmental Biology*. 22: 457–486.

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

Liu, Q., Davidoff, O., Niss, K., Haase, V. H. (2012a): Hypoxia–inducible factor regulates hepcidin via erythropoietin–induced erythropoiesis. *The Journal of Clinical Investigation*. 122: 4635–4644.

Liu, S., Hou, W., Yao, P., Li, N., Zhang, B., Hao, L., Nüssler, A. K., Liu, L. (2012b): Heme oxygenase–1 mediates the protective role of quercetin against ethanol–induced rat hepatocytes oxidative damage. *Toxicology in Vitro*. 26: 74–80.

Lombardi–Boccia, G., Martínez–Domínguez, B., Aguzzi, A. (2002): Total heme and non–heme iron in raw and cooked meats. *Journal of Food Science*. 67: 1738–1741.

Ludwiczek, S., Aigner, E., Theurl, I., Weiss, G. (2003): Cytokine-mediated regulation of iron transport in human monocytic cells. *Blood*. 101: 4148–4154.

Luo, X., Hill, M., Johnson, A., Latunde–Dada, G. O. (2014): Modulation of Dcytb (Cybrd 1) expression and function by iron, dehydroascorbate and Hif–2α in cultured cells. *Biochimica et Biophysica Acta*. 1840: 106–112.

Lynch, S. R. (2005): The impact of iron fortification on nutritional anaemia. *Best Practice & Research Clinical Haematology*. 18: 333–346.

Lynch, S. R., Dassenko, S. A., Cook, J. D., Juillerat, M. A., Hurrell, R. F. (1994): Inhibitory effect of a soybean-protein-related moiety on iron absorption in humans. *American Journal of Clinical Nutrition*. 60: 567–572.

MacGillivray, R. T., Mendez, E., Shewale, J. G., Sinha, S. K., Lineback–Zins, J., Brew, K. (1983): The Primary Structure of Human Serum Transferrin; The structures of seven cyanogen bromide fragments and the assembly of the complete structure. *The Journal of Biological Chemistry*. 258: 3543–3553.

Marouani, N., Chahed, A., Hédhili, A., Hamdaoui, M. H. (2007): Both aluminum and polyphenols in green tea decoction (*Camellia sinensis*) affect iron status and hematological parameters in rats. *European Journal of Nutrition*. 46: 453–459.

Marro, S., Chiabrando, D., Messana, E., Stolte, J., Turco, E., Tolosano, E., Muckenthaler, M. U. (2010): Heme controls ferroportin1 (FPN1) transcription involving Bach1, Nrf2 and a MARE/ARE sequence motif at position −7007 of the FPN1 promoter. *Haematologica*. 95: 1261–1268.

Masaratana, P., Laftah, A. H., Latunde–Dada, G. O., Vaulont, S., Simpson, R. J., McKie, A. T. (2011): Iron absorption in hepcidin1 knockout mice. *British Journal of Nutrition*. 105: 1583–1591.

Masella, R., Di Benedetto, R., Varì, R., Filesi, C., Giovannini C. (2005): Novel mechanisms of natural antioxidant compounds in biological systems: involvement of

glutathione and glutathione–related enzymes. *The Journal of Nutritional Biochemistry.* 16: 577–586.

Mastrogiannaki, M., Matak, P., Keith, B., Simon, M. C., Vaulont, S., Peyssonnaux, C. (2009): HIF–2alpha, but not HIF–1alpha, promotes iron absorption in mice. *The Journal of Clinical Investigation.* 119: 1159–1166.

McKie, A. T. (2008): The role of Dcytb in iron metabolism: an update. *Biochemical Society Transactions*. 36: 1239–1241.

McKie, A. T., Barrow, D., Latunde–Dada, G. O., Rolfs, A., Sager, G., Mudaly, E., Mudaly, M., Richardson, C., Barlow, D., Bomford, A., Peters, T. J., Raja, K. B., Shirali, S., Hediger, M. A., Farzaneh, F., Simpson, R. J. (2001): An iron–regulated ferric reductase associated with the absorption of dietary iron. *Science*. 291: 1755–1759.

McKie, A. T., Marciani, P., Rolfs, A., Brennan, K., Wehr, K., Barrow, D., Miret, S., Bomford, A., Peters, T. J., Farzaneh, F., Hediger, M. A., Hentze, M. W., Simpson, R. J. (2000): A novel duodenal iron–regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Molecular Cell*. 5: 299–309.

Mena, N. P., Esparza, A., Tapia, V., Valdés, P., Núñez, M. T. (2008): Hepcidin inhibits apical iron uptake in intestinal cells. *The American Journal of Physiology–Gastrointestinal and Liver Physiology*. 294: G192–G198.

Merhav, H., Amitai, Y., Palti, H., Godfrey, S. (1985): Tea drinking and microcytic anemia in infants. *The American Journal of Clinical Nutrition*. 41: 1210–1203.

Meyron–Holtz, E. G., Ghosh, M. C., Iwai, K., LaVaute, T., Brazzolotto, X., Berger, U. V., Land, W., Ollivierre–Wilson, H., Grinberg, A., Love, P., Rouault, T. A. (2004): Genetic ablations of iron regulatory proteins 1 and 2 reveal why iron regulatory protein 2 dominates iron homeostasis. *EMBO Journal*. 23: 386–395.

Mims, M. P., Guan, Y., Pospisilova, D., Priwitzerova, M., Indrak, K., Ponka, P., Divoky, V., Prchal, J. T. (2005): Identification of a human mutation of DMT1 in a patient with microcytic anemia and iron overload. *Blood*. 105:1337–1342.

Mladěnka, P., Macáková, K., Filipský, T., Zatloukalová, L., Jahodář, L., Bovicelli, P., Silvestri, I. P., Hrdina, R., Saso, L. (2011): In vitro analysis of iron chelating activity of flavonoids. *Journal of Inorganic Biochemistry*. 105: 693–701

Molina, M. F., Sanchez–Reus, I., Iglesias, I., Benedi, J. (2003): Quercetin, a flavonoid antioxidant, prevents and protects against ethanol–induced oxidative stress in mouse liver. *Biological & Pharmaceutical Bulletin*. 26: 1398–1402.

Montosi, G., Donovan, A., Totaro, A., Garuti, C., Pignatti, E., Cassanelli, S., Trenor, C. C., Gasparini, P., Andrews, N. C., Pietrangelo, A. (2001): Autosomal–dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. *The Journal of Clinical Investigation*. 108: 619–623.

Moon, Y. J., Wang, L., DiCenzo, R., Morris, M. E. (2008): Quercetin pharmacokinetics in humans. *Biopharmaceutics & Drug Disposition*. 29: 205–217.

Morand, C., Manach, C., Crespy, V., Remesy, C. (2000): Quercetin 3–O–beta–glucoside is better absorbed than other quercetin forms and is not present in rat plasma. *Free Radical Research*. 33: 667–676.

Morel, I., Lescoat, G., Cogrel, P., Sergent, O., Pasdeloup, N., Brissot, P., Cillard, P., Cillard, J. (1993): Antioxidant and iron–chelating activities of the flavonoids catechin, quercetin and diosmetin on iron–loaded rat hepatocyte cultures. *Biochemical Pharmacology*. 45: 13–9.

Muckenthaler, M. U., Galy, B., Hentze, M. W. (2008): Systemic iron homeostasis and the iron–responsive element/iron–regulatory protein (IRE/IRP) regulatory network. *Annual Review of Nutrition*. 28: 197–213.

Mullen, W., Edwards, C. A., Crozier, A. (2006): Absorption, excretion and metabolite profiling of methyl–, glucuronyl–, glucosyl– and sulpho–conjugates of quercetin in human plasma and urine after ingestion of onions. *British Journal of Nutrition.* 96: 107–116.

Nairz, M., Haschka, D., Demetz, E., Weiss, G. (2014): Iron at the interface of immunity and infection. *Frontiers in Pharmacology*. 5: 152.

Nemeth, E., Rivera, S., Gabayan, V., Keller, C., Taudorf, S., Pedersen, B. K., Ganz, T. (2004a): IL–6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *The Journal of Clinical Investigation*. 113: 1271–1276.

Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., Ward, D. M., Ganz, T., Kaplan, J. (2004b): Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science*. 306: 2090–2093.

Nguyen, N. B., Callaghan, K. D., Ghio, A. J., Haile, D. J., Yang, F. (2006): Hepcidin expression and iron transport in alveolar macrophages. *The American Journal of Physiology – Lung Cellular and Molecular Physiology*. 291: L417–425.

Nicolas, G., Bennoun, M., Devaux, I., Beaumont, C., Grandchamp, B., Kahn, A., Vaulont, S. (2001): Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. *Proceedings of the National Academy of Sciences of the United States of America*. 98: 8780–8785.

Nicolas, G., Bennoun, M., Porteu, A., Mativet, S., Beaumont, C., Grandchamp, B., Sirito, M., Sawadogo, M., Kahn, A., Vaulont, S. (2002): Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. *Proceedings of the National Academy of Sciences of the United States of America*. 99: 4596–4601.

Niederau, C., Strohmeyer, G., Stremmel, W. (1994): Epidemiology, clinical spectrum and prognosis of hemochromatosis. *Advances in experimental medicine and biology*. 356: 293–302.

Nittis, T., Gitlin, J. D. (2004): Role of copper in the proteosome mediated degradation of the multicopper oxidase hephaestin. *Journal of Biological Chemistry*. 279: 25696–25702.

Oakhill, J. S., Marritt, S. J., Gareta, E. G., Cammack, R., McKie, A. T. (2008): Functional characterization of human duodenal cytochrome b (Cybrd1): Redox properties in relation to iron and ascorbate metabolism. *Biochimica et Biophysica Acta*. 1777: 260–268.

Olthof, M. R., Hollman, P. C. H., Buijsman, M. N. C. P., Amelsvoort, J. M. M., Katan, M. B. (2003): Chlorogenic acid, quercetin–3–rutinoside and black tea polyphenols are extensively metabolized in humans. *The Journal of Nutrition.* 133: 1806–1814.

Origa, R., Galanello, R., Ganz, T., Giagu, N., Maccioni, L., Faa, G., Nemeth, E. (2007): Liver iron concentrations and urinary hepcidin in beta-thalassemia. *Haematologica*. 92: 583–588.

Papanikolaoua, G., Pantopoulos, K. (2005): Iron metabolism and toxicity. *Toxicology and Applied Pharmacology*. 202: 199–211.

Park, C. H., Valore, E. V., Waring, A. J., Ganz, T. (2001): Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *Journal of Biological Chemistry*. 276: 7806–7810.

Perron, N. R., Brumaghim, J. L. (2009): A Review of the Antioxidant Mechanisms of Polyphenol Compounds Related to Iron Binding. *Cell Biochemistry and Biophysics*. 53: 75–100.

Petrak, J., Vyoral, D. (2005): Hephaestin–a ferroxidase of cellular iron export. *The International Journal of Biochemistry & Cell Biology*. 37: 1173–1178.

Petry, N. (2014): *Polyphenols and Low Iron Bioavailability* In *Polyphenols in Human Health and Disease*, Ed Watson, R. R., Preedy, V. R., Zibadi, S. 311–322, Acedemic Press, USA.

Peyssonnaux, C., Zinkernagel, A. S., Schuepbach, R. A., Rankin, E., Vaulont, S., Haase, V. H., Nizet, V., Johnson, R. S. (2007): Regulation of iron homeostasis by the hypoxia–inducible transcription factors (HIFs). *The Journal of Clinical Investigation*. 117: 1926–1932.

Piccinelli, P., Samuelsson, T. (2007): Evolution of the iron–responsive element. *RNA*. 13: 952–966.

Pietrangelo, A. (2010): Hereditary hemochromatosis: pathogenesis, diagnosis, and treatment. *Gastroenterology*. 139: 393–408,

Pietrangelo, A. (2011): Hepcidin in human iron disorders: therapeutic implications. *Journal of Hepatology*. 54: 173–181.

Pietrangelo, A., Caleffi, A., Corradini, E. (2011): Non–HFE hepatic iron overload. *Seminars* in *Liver Disease*. 31: 302–318.

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

Poggiali, E., Cassinerio, E., Zanaboni, L., Domenica Cappellini, M. (2012): An update on iron chelation therapy. *Blood Transfusion*. 10: 411–422.

Qian, Z. M., Li, H., Sun, H., Ho, K. (2002): Targeted drug delivery via the transferrin receptor–mediated endocytosis pathway. *Pharmacological Reviews*. 54: 561–587.

Recalcati, S., Minotti, G., Cairo, G. (2010): Iron Regulatory Proteins: From Molecular Mechanisms to Drug Development. *Antioxidants & Redox Signaling*. 13: 1593–1616.

Ren, J., Meng, S., Lekka, Ch. E., Kaxiras, E. (2008): Complexation of Flavonoids with Iron: Structure and Optical Signatures. *The Journal of Physical Chemistry B*. 112: 1845–1850.

Robards, K. (2003): Strategies for the determination of bioactive phenols in plants, fruit and vegetables. *Journal of Chromatography A*. 1000: 657–691.

Roetto, A., Bosio, S., Gramaglia, E., Barilaro, M. R., Zecchina, G., Camaschella, C. (2002): Pathogenesis of hyperferritinemia cataract syndrome. *Blood Cells, Molecules & Diseases*. 29: 532–535.

Roetto, A., Papanikolaou, G., Politou, M., Alberti, F., Girelli, D., Christakis, J., Loukopoulos, D. Camaschella, C. (2003): Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. *Nature Genetics*. 33: 21–22.

Rolfs, A., Kvietikova, I., Gassmann, M., Wenger, R. H. (1997): Oxygen–regulated transferrin expression is mediated by hypoxia–inducible factor–1. *The Journal of Biological Chemistry*. 272: 20055–20062.

Rossander, L., Hallberg, L., Bjom–Rasmussen, E. (1979): Absorption of iron from breakfast meals. *The American Journal of Clinical Nutrition*. 32: 2484–2489.

Roughead, Z. K. F., Zito, C. A., Hunt, J. R. (2005): Inhibitory effects of dietary calcium on the initial uptake and subsequent retention of heme and nonheme iron in humans: comparisons using an intestinal lavage method. *American Journal of Clinical Nutrition*. 82: 589–597.

Sakakibara, S., Aoyama, Y. (2002): Dietary iron–deficiency upregulates hephaestin mRNA level in small intestine of rats. *Life Sciences*. 70: 3123–3129.

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

Schmidt, P. J., Toran, P. T., Giannetti, A. M., Bjorkman, P. J., Andrews, N. C. (2008): The transferrin receptor modulates Hfe-dependent regulation of hepcidin expression. *Cell Metabolism*. 7: 205–214.

Schroeter, H., Heiss, C., Spencer, J. P., Keen, C. L., Lupton, J. R., Schmitz, H. H. (2010): Recommending flavanols and procyanidins for cardiovascular health: current knowledge and future needs. *Molecular Aspects of Medicine*. 31: 546–557.

Shah, Y. M., Matsubara, T., Ito, S., Yim, S. H., Gonzalez, F. J. (2009): Intestinal hypoxia–inducible transcription factors are essential for iron absorption following iron deficiency. *Cell Metabolism*. 9: 152–164.

Shah, Z. A., Li, R. C., Ahmad, A. S., Kensler, T. W., Yamamoto, M., Biswal, S., Doré, S. (2010): The flavanol (–)–epicatechin prevents stroke damage through the Nrf2/HO1 pathway. *Journal of Cerebral Blood Flow and Metabolism*. 30: 1951–1961.

Shander, A., Sazama, K. (2010): Clinical consequences of iron overload from chronic red blood cell transfusions, its diagnosis, and its management by chelation therapy. *Transfusion*. 50: 1144–1155.

Sharma, R. (2014): *Polyphenols in Health and Disease*. *Practice and Mechanisms of Benefits* In *Polyphenols in Human Health and Disease*, Ed Watson, R. R., Preedy, V. R., Zibadi, S. 757–778, Acedemic Press, USA.

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

Shayeghi, M., Latunde–Dada, G. O., Oakhill, J. S., Laftah, A. H., Takeuchi, K., Halliday, N., Khan, Y., Warley, A., McCann, F. E., Hider, R. C., Frazer, D. M., Anderson, G. J., Vulpe, C. D., Simpson, R. J., McKie, A. T. (2005): Identification of an intestinal heme transporter. *Cell*. 122: 789–801.

Siegenberg, D., Baynes, R. D., Bothwell, T. H., Macfarlane, B. J., Lamparelli, R. D., Car, N. G., MacPhail, P., Schmidt, U., Tal, A., Mayet, F. (1991): Ascorbic acid

prevents the dose-dependent inhibitory effects of polyphenols and phytates on nonheme-iron absorption. *The American Journal of Clinical Nutrition*. 53: 537–541.

Silva–Gomes, S., Santos, A. G., Caldas, C., Silva, C. M., Neves, J. V., Lopes, J., Carneiro, F., Rodrigues, P. N., Duarte, T. L. (2014): Transcription factor NRF2 protects mice against dietary iron-induced liver injury by preventing hepatocytic cell death. *Journal of Hepatology*. 60: 354–361.

Silvestri, L., Nai, A., Pagani, A., Camaschella, C. (2014): The extrahepatic role of TFR2 in iron homeostasis. *Frontiers in Pharmacology*. 5: 1–6.

Smith, S. R., Ghosh, M. C., Ollivierre–Wilson, H., Hang Tong, W., Rouault, T. A. (2006): Complete loss of iron regulatory proteins 1 and 2 prevents viability of murine zygotes beyond the blastocyst stage of embryonic development. *Blood Cells, Molecules & Diseases*. 36: 283–287.

Sow, F. B., Florence, W. C., Satoskar, A. R., Schlesinger, L. S., Zwilling, B. S., Lafuse, W. P. (2007): Expression and localization of hepcidin in macrophages: a role in host defense against tuberculosis. *Journal of Leukocyte Biology*. 82: 934–945.

Srai, S. K., Bomford, A., McArdle, H. J. (2002): Iron transport across cell membranes: molecular understanding of duodenal and placental iron uptake. *Best Practice and Research: Clinical Haematology*. 15: 243–259.

Storcksdieck Bonsmann, S., Hurrell, R. F. (2007): Iron-binding properties, amino acid composition, and structure of muscle tissue peptides from in vitro digestion of different meat sources. *Journal of Food Science*. 72: S19–S29.

Strobel, P., Allard, C., Perez–Acle, T., Calderon, R., Aldunate, R., Leighton, F. (2005): Myricetin, quercetin and catechin-gallate inhibit glucose uptake in isolated rat adipocytes. *Biochemical Journal*. 386: 471–478.

Stuart, K. A., Anderson, G. J., Frazer, D. M., Powell, L. W., McCullen, M., Fletcher, L. M., Crawford, D. H. (2003): Duodenal expression of iron transport molecules in untreated haemochromatosis subjects. *Gut*. 52: 953–959.

Tako, E., Beebe, S. E. Reed, S., Hart, J. J., Glahn, R. P. (2014): Polyphenolic compounds appear to limit the nutritional benefit of biofortified higher iron black bean (*Phaseolus vulgaris* L.). *Nutrition Journal*. 13: 28.

Tang, Y., Li, Y., Yu, H., Gao, C., Liu, L., Chen, S., Xing, M., Liu, L., Yao, P. (2014): Quercetin prevents ethanol-induced iron overload by regulating hepcidin through the BMP6/SMAD4 signaling pathway. *The Journal of Nutritional Biochemistry*. 25: 675–682.

Tenhunen, R., Marver, H. S., Schmid, R. (1969): Microsomal heme oxygenase. Characterization of the enzyme. *The Journal of Biological Chemistry*. 244: 6388–6394.

Terao, J. (1999): Dietary flavonoids as antioxidants in vivo: conjugated metabolites of (-)–epicatechin and quercetin participate in antioxidative defense in blood plasma. *Journal of Investigative Medicine*. 46: 159–168.

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

Theil, E. C. (2007): Coordinating responses to iron and oxygen stress with DNA and mRNA promoters: the ferritin story. *BioMetals*. 20: 513–521.

Theil, E. C. (2011): Ferritin protein nanocages use ion channels, catalytic sites, and nucleation channels to manage iron/oxygen chemistry. *Current Opinion in Chemical Biology*. 15: 304–311.

Thephinlap, C., Ounjaijean, S., Khansuwan, U., Fucharoen, S., Porter, J. B., Srichairatanakool, S. (2007): Epigallocatechin–3–gallate and epicatechin–3–gallate

from green tea decrease plasma non-transferrin bound iron and erythrocyte oxidative stress. *Medicinal Chemistry*. 3: 289–296.

Tokalıoğlu, Ş., Gürbüz, F. (2010): Selective determination of copper and iron in various food samples by the solid phase extraction. *Food Chemistry*. 123: 183–187.

Torrance, J. D., Bothwell, T. H. (1980): *Tissue iron stores*. In: Cook J. D., ed. *Methods in hematology: iron*. Churchill Livingstone, New York, USA, 90–115.

Torti, F. M., Torti, S. V. (2002): Regulation of ferritin genes and protein. *Blood*. 99: 3505–3516.

Troadec, M. B., Ward, D. M., Lo, E., Kaplan, J., De Domenico, I. (2010): Induction of FPN1 transcription by MTF–1 reveals a role for ferroportin in transition metal efflux. *Blood*. 116: 4657–4664.

Vanhees, K., Godschalk, R. W., Sanders, A., van Waalwijk van Doorn–Khosrovani, S. B., van Schooten, F. J. (2011): Maternal quercetin intake during pregnancy results in an adapted iron homeostasis at adulthood. *Toxicology*. 290: 350–358.

Viatte, L., Vaulont, S. (2009): Hepcidin, the iron watcher. *Biochimie*. 91: 1223–1228.

Vlachodimitropoulou, E., Naftalin, R. J., Sharp, P. A. (2010): Quercetin is a substrate for the transmembrane oxidoreductase Dcytb. *Free Radical Biology and Medicine*. 48: 1366–1369.

Vlachodimitropoulou, E., Sharp, P. A., Naftalin, R. J. (2011): Quercetin-iron chelates are transported via glucose transporters. *Free Radical Biology & Medicine*. 50: 934–944.

Volz, K. (2008): The functional duality of iron regulatory protein 1. *Current Opinion in Structural Biology*. 18: 106–111.

Vulpe, C. D., Kuo, Y. M., Murphy, T. L., Cowley, L., Askwith, C., Libina, N., Gitschier, J., Anderson G. J. (1999): Hephaestin, a ceruloplasmin homologue

implicated in intestinal iron transport, is defective in the sla mouse. *Nature Genetics*. 21: 195–199.

Walle, T., Otake, Y., Walle, U. K., Wilson, F. A. (2000): Quercetin glucosides are completely hydrolyzed in ileostomy patients before absorption. *The Journal of Nutrition*. 130: 2658–2661.

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

Ward, D. M., Kaplan, J. (2012): Ferroportin-mediated iron transport: Expression and regulation. *Biochimica et Biophysica Acta*. 1823: 1426–1433.

West, A. P. Jr., Bennett, M. J., Sellers, V. M., Andrews, N. C., Enns, C. A., Bjorkman, P. J. (2000): Comparison of the interactions of transferrin receptor and transferrin receptor 2 with transferrin and the hereditary hemochromatosis protein HFE. *The Journal of Biological Chemistry*. 275: 38135–38138.

Williamson, G., Plumb, G. W., Uda, Y., Price, K. R., Rhodes, M. J. (1996): Dietary quercetin glycosides: antioxidant activity and induction of the anticarcinogenic phase II marker enzyme quinone reductase in Hepalclc7 cells. *Carcinogenesis*. 17: 2385–2387.

Wolffram, S., Blöck, M., Ader, P. (2002): Quercetin–3–glucoside is transported by the glucose carrier SGLT1 across the brush border membrane of rat small intestine. *The Journal of Nutrition.* 132: 630–635.

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

World Health Organization (2002): *The World Health Report 2002. Reducing Risks, Promoting Healthy Life*. WHO Press, Geneva, Switzerland.

World Health Organization (2007): *Assessing the iron status of populations: including literature reviews, 2nd edition.* WHO Press, Geneva, Switzerland.

World Health Organization (2008): *Worldwide prevalence of anaemia 1993–2005*. WHO Press, Geneva, Switzerland.

Wyman, S., Simpson, R. J., McKie, A. T., Sharp, P. A. (2008): Dcytb (Cybrd1) functions as both a ferric and a cupric reductase in vitro. *FEBS Letters*. 582: 1901–1906.

Yamaji, S., Sharp, P., Ramesh, B., Srai, S. K. (2004): Inhibition of iron transport across human intestinal epithelial cells by hepcidin. *Blood*. 104: 2178–2180.

Yan, J., Yang, S., Zhang, J., Zhai, C., Zhu, T. (2009): BMP6 attenuates oxidant injury in HK–2 cells via Smad-dependent HO–1 induction. *Free Radical Biology and Medicine*. 46: 1275–1282.

Yao, P., Nussler, A., Liu, L., Hao, L., Song, F., Schirmeier, A., Nussler, N. (2007): Quercetin protects human hepatocytes from ethanol-derived oxidative stress by inducing heme oxygenase–1 via the MAPK/Nrf2 pathways. *Journal of Hepatology*. 47: 253–261.

Yeh, K., Yeh, M., Glass, J. (2011): Interactions Between Ferroportin and Hephaestin in Rat Enterocytes Are Reduced After Iron Ingestion. *Gastroenterology*. 141: 292–299.

Yehuda, S., Mostofsky, D. I. (2010): *Iron Deficiency and Overload: From Basic Biology to Clinical Medicine*. Humana Press, New York, US.

Ying–Wu, L., Jiangyun, W. (2013): Structure and function of heme proteins in non-native states: A mini-review. *Journal of Inorganic Biochemistry*. 129: 162–171.

Yip, R., Ramakrishnan, U. (2002): Experiences and Challenges in Developing Countries. *The Journal of Nutrition*. 132: 827S–830S.

Zacharski, L. R., Chow, B. K., Howes, P. S., Shamayeva, G., Baron, J. A., Dalman, R. L., Malenka, D. J., Ozaki, C. K., Lavori, P. W. (2008): Decreased cancer risk after iron reduction in patients with peripheral arterial disease: results from a randomized trial. *Journal of the National Cancer Institute*. 100: 996–1002.

Zakin, M. M. (1992): Regulation of transferrin gene expression. *The FASEB Journal*. 14: 3253–3258.

Zhang, D. L., Hughes, R. M., Ollivierre–Wilson, H., Ghosh, M. C., Rouault, T. A. (2009): A ferroportin transcript that lacks an iron-responsive element enables duodenal and erythroid precursor cells to evade translational repression. *Cell Metabolism*. 9: 461–473.

Zhang, Y., Gao, Z., Liu, J., Xu, Z. (2011): Protective effects of baicalin and quercetin on an iron-overloaded mouse: comparison of liver, kidney and heart tissues. *Natural Product Research*. 25: 1150–1160.

Zhang, Y., Li, H., Zhao, Y., Gao, Z. (2006): Dietary supplementation of baicalin and quercetin attenuates iron overload induced mouse liver injury. *European Journal of Pharmacology*. 535: 263–269.

Zhao, Y., Li, H., Gao, Z., Xu, H. (2005): Effects of dietary baicalin supplementation on iron overload-induced mouse liver oxidative injury. *European Journal of Pharmacology*. 509: 195–200.

Ziberna, L., Fornasaro, S., Čvorović, J., Tramer, F., Passamonti, S. (2014): *Bioavailability of Flavonoids: The Role of Cell Membrane Transporters* In *Polyphenols in Human Health and Disease*, Ed Watson, R. R., Preedy, V. R., Zibadi, S. 489–511, Acedemic Press, USA.

Zoller, H., Koch, R. O., Theurl, I., Obrist, P., Pietrangelo, A., Montosi, G., Haile, D. J., Vogel, W., Weiss, G. (2001): Expression of the duodenal iron transporters divalent-metal transporter 1 and ferroportin 1 in iron deficiency and iron overload. *Gastroenterology*. 120: 1412–1419.

Zoller, H., Theurl, I., Koch, R. O., McKie, A. T., Vogel, W., Weiss, G. (2003): Duodenal cytochrome b and hephaestin expression in patients with iron deficiency and hemochromatosis. *Gastroenterology*. 125: 746–754.