

Effects of n-3 fatty acid and iron deficiency, alone and in combination, on hepcidin regulation and lipid mediators in rats

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This would not have been possible without the love and support from my husband, Coenraad. Thank you for helping me in the times when I wanted to stop as well as being my technical support. To my family and friends, I would also like to thank you for the support throughout this journey.

“As a working hypothesis to explain the riddle of our existence, I propose that our universe is the most interesting of all possible universes, and our fate as human beings is to make it so”

— **Freeman Dyson**

And then lastly, I would like to thank God, that made it possible for us to observe and study this universe with wonder, admiration and deep awe of everything it holds. Without Him I would not have started or finished this endeavour.

ABSTRACT

Background

Globally, iron deficiency is the most common nutrient deficiency and can lead to various adverse health consequences. Iron absorption, circulation and storage are regulated by the hormone hepcidin, which is expressed by the *HAMP* gene in the liver. Iron cannot be actively excreted by the human body, thus hepcidin is released to decrease iron absorption and recycling to prevent iron overload. Hepcidin expression is activated by high iron stores and by inflammation via the inflammatory cytokine interleukin-6 (IL-6). This occurs via two main pathways known as the bone morphogenetic protein–small mothers against decapentaplegic (BMP/SMAD) pathway (induced by iron levels) and the JAK/STAT3 pathway (induced by IL-6).

Long chain omega-3 (n-3) fatty acids can resolve inflammation by acting as a precursor for the synthesis of anti-inflammatory and pro-resolving lipid mediators. Low n-3 fatty acid status can lead to a decreased ability by the host to resolve inflammation and sets off pro-inflammatory signalling and increased IL-6 levels. Multiple nutrient deficiencies, such as a combined iron and n-3 fatty acid deficiency, often co-exist in populations and individuals. The presence of a combined iron deficiency with a low n-3 fatty acid status may lead to an inability to effectively resolve inflammation and increased circulating IL-6 levels, therefore potentially leading to the upregulation of hepcidin in an already iron deficient state, further hindering mechanisms to improve iron status.

Aim and Objectives

Therefore, the aim of this study was to determine the effects of n-3 fatty acid and iron deficiency, alone and in combination, on hepcidin regulation and lipid mediator (docosahexaenoic acid (DHA)-, eicosapentaenoic acid (EPA)- and arachidonic acid (ARA)- derived) signalling in rats.

The objectives of this study were to investigate the effects of n-3 fatty acid and iron deficiency, alone and in combination, on

- A) Expression of the hepcidin regulatory pathway genes *HAMP*, *BMP2* and *TFR2* in rat liver.
- B) ARA-derived 5-, 8-, 11-, 12- and 15- hydroxyeicosatetraenoic acid (HETE); EPA-derived 12-hydroxyeicosapentaenoic acid (HEPE) and DHA-derived 17-hydroxydocosahexaenoic acid (HDHA) lipid mediators in rat plasma.
- C) To determine the correlations of *HAMP*, *BMP2* and *TFR2* expression with plasma lipid mediator concentrations in rats.

Design

Female Wistar rats were fed a standard or n-3 fatty acid deficient (FAD) diet. The female rats were mated, and 39 male rats were included in the study with a 2x2 factorial. At weaning (postnatal day 21), the male rats were fed either a 1) control, 2) iron deficient (ID), 3) n-3 FAD or 4) ID + n-3 FAD diet for five weeks. Expression of *HAMP* and the *HAMP* regulatory genes *BMP2* and *TFR2* was analysed in hepatic tissue using quantified real time polymerase chain reaction (RT-qPCR). Peripheral lipid mediators such as ARA-derived pro-inflammatory lipid mediators, 5-, 8-, 11-, 12- and 15-hydroxyeicosatetraenoic acid (HETE), EPA-derived anti-inflammatory 12-hydroxyeicosapentaenoic acid (HEPE), and DHA-derived pro-resolving lipid mediator 17-hydroxydocosahexaenoic acid (HDHA) were measured in plasma using liquid chromatography-tandem mass spectrometry (LCMSMS).

Results

HAMP expression was significantly upregulated by ID, whilst *BMP2* and *TFR2* expression were both downregulated. There was a significant effect of ID for higher ARA-derived 15-HETE, but lower 5-HETE. Furthermore, there was a significant effect of ID for higher DHA-derived 17-HDHA, whereas 17-HDHA was lowered by n-3 FAD. When compared to the control group, 17-HDHA was significantly higher in the ID group but lower in the n-3 FAD group. 17-HDHA concentrations in the ID+n-3 FAD group did not differ from the control group ($p = 0.99$). *HAMP* expression correlated positively with 15-HETE and 17-HDHA and negatively with 5-HETE.

Conclusion

As expected, both iron level-dependent genes *BMP2* and *TFR2* were downregulated by ID. However, the upregulation of *HAMP* expression by ID was unexpected and suggestive of inflammation. The presence of inflammation in an ID state was confirmed by the effect of ID for increased ARA-derived pro-inflammatory lipid mediators and DHA-derived inflammation-resolving 17-HDHA levels. N-3 FAD alone did not induce *HAMP* expression, but may compromise the inflammation-resolving capacity of rats with a concomitant ID.

Key terms

Hepcidin, *HAMP*, Iron, Iron deficiency (ID), Omega-3 polyunsaturated fatty acids (n-3 PUFA), Fatty acid deficiency (FAD), Lipid mediators, Inflammation, Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA).

ABBREVIATIONS

ACD	Anaemia of chronic disease
ALA	Alpha-linolenic acid (18:3n-3)
ALOX	Arachidonate lipoxygenase
ARE	Antioxidant response element
ARA	Arachidonic acid (20:4n-6)
BALV	Bronchoalveolar lavage fluid
BMPs	Bone morphogenetic proteins
BMPR	Bone morphogenetic protein receptor
BMP-SMAD	Bone morphogenetic protein– small mothers against decapentaplegic
CN2	Dipeptidase
cDNA	Complementary DNA
CRP	C-reactive protein
COX	Cyclooxygenase
CyPh	Cytochrome hydrolase
CYP450	Cytochrome P450
D	Desaturases
D5D	$\Delta 5$ -desaturase
D6D	$\Delta 6$ -desaturase
Dcytb	Duodenal cytochrome b
DHA	Docosahexaenoic acid (20:6n-3)
DHO	Dehydrogenases
DMT1	Divalent metal transporter 1
DPA	Docosapentaenoic acid (22:5n-3)
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid (20:5n-3)
EPO	Erythropoietin
ERFE	Erythroferrone
FA	Fatty acid
FAD	Fatty acid deficient
Fe ²⁺	Ferrous iron
Fe ³⁺	Ferric iron
GGT	γ -glutamyl transferase
GPT	Glutathione transferase
GPX	Glutathione peroxidases

Hb	Haemoglobin
HD	Haemodialysis
HDHA	Hydroxydocosahexaenoic acid
HETE	Hydroxyeicosatetraenoic acid
HEPE	Hydroxyeicosapentaenoic acid
HIF2- α	Hypoxia inducible factor 2 α
HFE	Human hemochromatosis protein
HJV	Hemojuvelin
HRE	Hypoxia-response element
ID	Iron deficient / iron deficiency
IDA	Iron deficient anaemia
IFN- α	Interferon- α
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-22	Interleukin 22
IRE	Iron response element
Iron-TF	Transferrin-bound iron
IRPs	Iron regulatory proteins
KO	Knock out
LA	Linoleic acid (18:2n-6)
LCMSMS	Liquid chromatography tandem mass spectrometry
LCPUFAs	Long chain polyunsaturated fatty acids
LEAP-1	Liver-expressed antimicrobial peptide
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTs	Leukotrienes
Mb	Myoglobin
MMP	Metalloproteinases
n-3	Omega-3
n-6	Omega-6
n-3 FAD	Omega-3 fatty acid deficiency
NCOA4	Nuclear receptor coactivator 4
NF-KB	Nuclear factor kappa-B
NRF2	Nuclear erythroid factor 2
PC	Phosphatidylcholine
PCTF	Proton-coupled folate transporter
PCBP1	Poly (rC)-binding protein 1

PE	Phosphatidylethanolamine
PGH	Hydroperoxidase
PGs	Prostaglandins
PGE2	Prostaglandin E2
PGE3	Prostaglandin E3
PGsy	Prostaglandin synthases
PL	Phospholipase
PLA2	Phospholipase A2
PPARs	Peroxisome proliferator-activated receptors
PUFAs	Polyunsaturated fatty acids
RT-qPCR	Quantified real time polymerase chain reaction
RBC	Red blood cell
ROS	Reactive oxygen species
R-SMADs	Regulatory small mothers against decapentaplegic
RvD1	Resolvin D1
RvD2	Resolvin D2
RvE1	Resolvin E1
RvE2	Resolvin E2
sHE	Soluble epoxide hydrolases
STAT3-RE	STAT3 responsive element
TFR1	Transferrin receptor 1
TFR2	Transferrin receptor 2
TFRs	Transferrin receptors
TNF- α	Tumour necrosis factor-alpha
TMPRSS6	Transmembrane serine protease 6
TXsy	Thromboxane synthase
USF2	Upstream stimulatory factor 2
UTR	Untranslated regions
WHO	World Health Organization

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	I
ABSTRACT	II
ABBREVIATIONS.....	IV
LIST OF TABLES	X
LIST OF FIGURES.....	XI
CHAPTER 1: INTRODUCTION.....	12
Background and rationale	12
Aim, objectives and hypothesis	15
Aim	15
Objectives	15
Hypotheses	15
Structure of the dissertation.....	15
Research team.....	16
CHAPTER 2 LITERATURE REVIEW.....	18
2.1 Iron metabolism.....	18
2.2.1 Iron absorption	18
2.2.3 Cellular and plasma iron regulation	19
2.2.4 Ferroportin and iron homeostasis	21
2.2 The role of hepcidin in systematic iron homeostasis	22

2.2.1	Hepcidin regulation via iron availability: the bone morphogenetic protein– small mothers against decapentaplegic pathway	23
2.2.2	Bone morphogenetic protein 2 and 6	24
2.2.3	Bone morphogenetic proteins and Hemojuvelin	25
2.2.4	Transferrin receptor2 and Human hemochromatosis protein	25
2.2.5	Regulatory small mothers against decapentaplegic	26
2.2.6	Hepcidin inhibition in the bone morphogenetic protein– small mothers against decapentaplegic pathway	26
2.2.7	Hepcidin regulation via inflammation: The JAK/STAT pathway	27
2.2.8	Pro-inflammatory cytokines and the JAK/STAT pathway	27
2.2.9	STAT3 and <i>HAMP</i> activation	28
2.3	The role of omega-3 polyunsaturated fatty acids in inflammation.....	28
2.3.1	Polyunsaturated fatty acid synthesis, dietary sources	29
2.3.2	Polyunsaturated fatty acids and the cell membrane	30
2.3.3	Eicosapentaenoic acid, Docosahexaenoic acid and Arachidonic acid, lipid mediators involved in inflammatory processes	32
2.3.4	PUFAs – derived lipid mediators and altered gene expression	36
2.4	Interactions between iron and omega-3 polyunsaturated fatty acids.....	37
2.5	Effects of combined iron and fatty acid deficiencies on hepcidin expression.	39
CHAPTER 3:	MANUSCRIPT	40
CHAPTER 4:	OVERALL CONCLUSION	65
Summary		65
Strengths		66

Future research	67
BIBLIOGRAPHY	69
ANNEXURES.....	95
Annexure A: Methodology and Ethical Considerations	95
Annexure B: Ethics approval of North-West University for the affiliated study	102
Annexure C: Author guidelines for the Journal of nutritional Biochemistry	104
Annexure D: Editor's certificate	118

LIST OF TABLES

Table 1-1: Individual contributions of the research team members to this dissertation.....	17
Table 2-1: Enzymes involved in the activation of prostaglandins, thromboxanes and leukotrienes ¹	31
Table 3-1: Composition of diet.....	45
Table 3-2: Weight gain and food intake of male rats fed an ID, n-3 FAD, ID + n-3 FAD or a control diet for 5 weeks ¹	50
Table 3-3: Iron status and erythrocyte total phospholipid fatty acid composition of male rats fed and ID, n-3 FAD, ID+ n-3 FAD or a control diet for 5 weeks ¹	52
Table 3-4: Plasma lipid mediators and expression of iron metabolism genes in male rats fed an ID, n-3 FAD, ID + n-3 FAD or a control diet for 5 weeks ¹	53
Table 3-5: Pearson correlation coefficient for <i>TFR2</i> , <i>BMP2</i> and <i>HAMP</i> gene expression, ARA-derived 15-HETE and EPA- derived 17-HDHA across the Control, ID, n-3 FAD and ID + n-3 FAD.	55

LIST OF FIGURES

<i>Figure 1-1: Metabolic elongation pathways with responsible enzymes of linoleic acid (n-6) and α-linolenic acid (n-3) to long chain polyunsaturated fatty acids (PUFAs)</i>	13
<i>Figure 2-1: The main tissues involved in the regulation of iron homeostasis.....</i>	19
<i>Figure 2-2: Schematic presentation of intracellular iron uptake, utilisation and export</i>	20
<i>Figure 2-3: Cellular iron homeostasis in iron deficient cells (left) and cell repletion of iron (right)</i>	22
<i>Figure 2-4: Major mechanisms for hepcidin regulation via iron availability (BMP/SMAD pathway) and inflammation (JAK/STAT3 pathway).....</i>	24
<i>Figure 2-5: Metabolic elongation pathways with responsible enzymes of linoleic acid (n-6) and α-linolenic acid (n-3) to long chain polyunsaturated fatty acids (PUFAs)</i>	29
<i>Figure 2-6: Outline of the pathway of conversion of arachidonic acid into eicosanoids via CytP450, LOX and COX to synthesise lipid mediators, prostaglandins and thromboxanes involved in inflammation.</i>	32
<i>Figure 2-7: Biosynthesis of resolvins from EPA and DHA (E-series and D-series) via 18-HEPE and 17-HDHA.</i>	33
<i>Figure 2-8: An overview of the main pathways involved in the production of polyunsaturated fatty acid-derived lipid mediators</i>	34
<i>Figure 2-9: n-3 PUFAs reduces the expression of pro-inflammatory cytokines via PPARγ activation.....</i>	37
<i>Figure 3-1: Flow diagram of the experimental design; n indicates the numbers in each group.....</i>	44

CHAPTER 1: INTRODUCTION

Background and rationale

The global anaemia prevalence in 2011 was 32.9%, remaining a major public health burden. Iron deficiency (ID) is the main cause of anaemia and an estimated 25% of the world's population is suffering from iron deficiency anaemia (IDA) (WHO, 2015). Low- and middle- income countries have the highest prevalence (Kassebaum *et al.*, 2014, Poggiali *et al.*, 2014; Zimmerman & Hurrell, 2007). Iron is crucial to multiple biological functions in the body. Inadequate iron intake and availability could lead to impaired oxygen transport to tissue via haemoglobin (Hb), oxygen storage (as myoglobin) and erythropoiesis (synthesis of red blood cells (erythrocytes)). Iron is also a component of various enzymes involved in metabolic processes, such as electron transport, DNA synthesis and repair, as well as cell proliferation (Haas and Fairchild, 1988, Aisen & Wessling-Resnick, 2001; Hentze *et al.*, 2010, Kroot *et al.*, 2011; Puig *et al.*, 2017). Conversely, increased iron levels can also be detrimental, resulting in increased reactive oxygen species (ROS) that can oxidise lipids, proteins and DNA, leading to cell damage. Therefore, iron metabolism is strictly regulated to prevent iron deficiency (ID) or iron overload (Ganz, 2013).

ID can develop due to inadequate dietary intake and/or absorption. The master regulator of iron absorption and storage was found to be hepcidin, previously known as liver-expressed antimicrobial peptide (LEAP-1) (Krause *et al.*, 2000; Weinstein *et al.*, 2002; Schibli *et al.*, 2002; Park *et al.*, 2001; Flemming & Sly 2001). The hormone hepcidin decreases iron availability by inhibiting intestinal absorption of dietary sources as well as the release of recycled iron already available (from macrophages) resulting in reduced iron delivery to bone marrow for erythropoiesis (Nicolas *et al.*, 2001; Nicolas *et al.*, 2002). Hepcidin is expressed by the *HAMP* gene and expression is mediated by several processes, including iron levels and inflammation. Hepcidin regulation mediated by inflammation (pro-inflammatory cytokines) is conducted via JAK/STAT3 signalling, whereas regulation via iron status (iron-mediated) in hepatocytes occurs via the bone morphogenetic protein-small mothers against decapentaplegic (BMP-SMAD) pathway (Hunter *et al.*, 2002; Nicolas *et al.*, 2001).

Similar to iron, studies also found that dietary intake of long chain polyunsaturated fatty acids (LCPUFAs) are lower than the recommended adequate intake in low- and middle- income countries (Briend *et al.*, 2011; Forsyth *et al.*, 2016; WHO/FAO, 1994). Omega-3 (n-3) LCPUFAs play vital roles in the structure and function of cell membranes, brain and nervous system development and functioning, as well as the production of bioactive lipid mediators (Brenna *et al.*, 2009; Davis & Kris-Etherton, 2003; Calder, 2015a). As illustrated in figure 1-1, Eicosapentaenoic

acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 20:6n-3) are metabolically related and can be synthesised from plant-derived alpha-linolenic acid (ALA; 18:3n-3), but this does not appear to happen effectively in humans (Brenna *et al.*, 2009; Burge & Calder, 2006; Adkins & Kelley, 2010). Therefore, dietary intake of sea food sources (specifically fatty fish) are important to ensure EPA and DHA requirements are met. Diets high in omega-6 (n-6) fatty acids (FAs), namely plant-derived linoleic acid (LA; 18:2n-6), above the recommended 10% of total energy, can decrease the conversion rate of ALA to DHA. This was observed in several animal studies and notably occurs even though dietary intake of ALA is above the recommended level. Thus, due to competition at the desaturase level to convert n-6 LA to arachidonic acid (ARA; 20:4n-6) or n-3 ALA to EPA and DHA, a diet very high in n-6 FAs can lead to an EPA and DHA deficiency (Brenna, 2011; Gibson *et al.*, 2011; Lands *et al.*, 1990; Baker *et al.*, 2016).

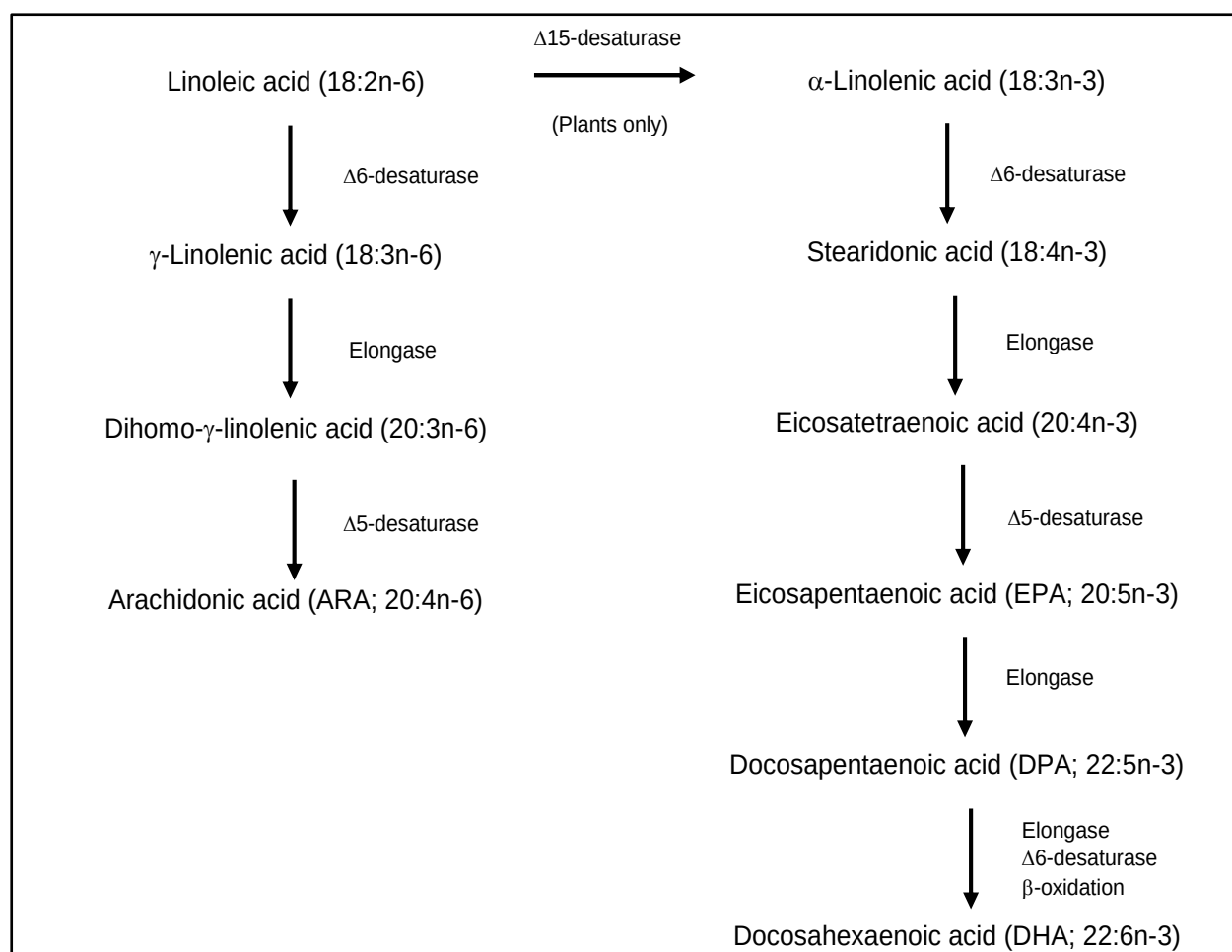


Figure 1-1: Metabolic elongation pathways with responsible enzymes of linoleic acid (n-6) and α-linolenic acid (n-3) to long chain polyunsaturated fatty acids (PUFAs)

(Calder, 2015b). Printed with permission from the original author.

The n-3 LCPUFAs, EPA and DHA are involved in resolving inflammation as precursors in the synthesis of bioactive lipid metabolites that function as inflammation antagonists with potent anti-inflammatory properties (Serhan *et al.*, 2000; Calder, 2008; Khalfoun *et al.*, 1997; Groeger *et al.*, 2010). An experiment analysing erythrocyte composition of rats fed a diet rich in ALA vs a diet poor in ALA found that DHA was inversely correlated with plasma levels of pro-inflammatory cytokines interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF- α) and C-reactive protein (CRP) (McNamara *et al.* 2010). Multiple studies found the same inverse correlation between serum n-3 FA levels and serum pro-inflammatory cytokine levels (Ferrucci *et al.*, 2006; Micallef *et al.*, 2009). This can be due to altered inflammatory resolving in an n-3 fatty acid deficient (FAD) state.

Pro-inflammatory cytokine treatment of hepatocytes, specifically with IL-6, results in the transcriptional induction of hepcidin (*HAMP*) expression via the JAK/STAT3 pathway (Nemeth *et al.*, 2003; Nemeth *et al.*, 2004; Nicolas *et al.*, 2001). Lee and colleagues found that the combination of IL-1 and IL-6 further enhanced hepcidin expression significantly in hepatocytes (Lee *et al.*, 2005). In low- and middle-income countries, individuals can present with multiple nutrient deficiencies simultaneously (Müller & Krawinkel, 2005). Individuals might present with ID and n-3 FAD concurrently, which might lead to altered hepcidin expression in an iron deficient state due to increased plasma cytokine levels. Furthermore, the combination of deficiencies can also interact due to the role that iron plays in the enzymes (co-factor of the catalytic sites) responsible for the synthesis of EPA-and DHA-derived lipid mediators. Thus, ID may lead to impaired synthesis of these inflammation regulatory modulators (Gilbert *et al.*, 2011; Kuhn *et al.*, 2005; Nakamura & Nara, 2004).

To our knowledge, the investigation of the effect of n-3 fatty acid deficiency (FAD), alone and in combination with iron deficiency (ID), on hepcidin regulation is novel. Inadequate n-3 FA status may lead to a potential upregulation of hepcidin due to increased levels of circulating pro-inflammatory cytokines or a decrease of EPA-and DHA-derived anti-inflammatory lipid mediators potentially further hindering mechanisms to improve iron availability. In this study, we will further evaluate whether an n-3 FA deficiency may also lead to the upregulation of hepcidin in an already ID state, where downregulated hepcidin transcription is expected.

Aim, objectives and hypothesis

Aim

The aim of this study was to determine the effects of n-3 fatty acid and iron deficiency, alone and in combination, on hepcidin regulation and lipid mediator (docosahexaenoic acid (DHA)-, eicosapentaenoic acid (EPA)- and arachidonic acid (ARA)- derived) signalling in rats.

Objectives

To investigate the effects of n-3 fatty acid and iron deficiency, alone and in combination, on

- A) Expression of the hepcidin regulatory pathway genes *HAMP*, *BMP2* and *TFR2* in rat liver.
- B) ARA-derived 5-, 8-, 11-, 12- and 15- hydroxyeicosatetraenoic acid (HETE); EPA-derived 12-hydroxyeicosapentaenoic acid (HEPE) and DHA-derived 17-hydroxydocosahexaenoic acid (HDHA) lipid mediators in rat plasma.
- C) To determine the correlations of *HAMP*, *BMP2* and *TFR2* expression with plasma lipid mediator concentrations in rats.

Hypotheses

- A) n-3 fatty acid deficiency, alone and in combination with iron deficiency, will lead to upregulated *HAMP* in rat liver tissue.
- B) n-3 fatty acid deficiency, alone and in combination with iron deficiency, will decrease DHA and EPA-derived lipid mediators whilst increasing ARA-derived lipid mediators in rat plasma. Due to the role that iron plays as cofactor in the synthesis of n-3 and n-6 derived lipid mediators, ID rats will show decreased DHA, EPA and ARA-derived lipid mediators.
- C) EPA-and-DHA-derived lipid mediator concentrations will be inversely correlated, whilst ARA-derived lipid mediator concentrations will be positively correlated with the expression of *HAMP* in the hepcidin-regulatory pathways in liver tissue in rats.

Structure of the dissertation

This dissertation is presented in the form of four chapters and relevant annexures. Chapter One serves as an introduction and Chapter Two consists of a literature review focussing on the iron metabolism and the regulating pathways of hepcidin. In addition, the role of inflammation on hepcidin expression, as well as the role of various mechanisms leading to the anti-inflammatory/inflammation resolving potential of n-3 LCPUFAs and their lipid mediators are

presented. Lastly, the potential interactive mechanisms of iron and n-3 PUFAs on hepcidin expression are introduced.

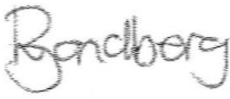
Chapter Three is a manuscript entitled: “The effects of n-3 fatty acid and iron deficiency, alone and in combination, on hepcidin pathway gene expression and plasma lipid mediators in rats”, prepared according to the author guidelines of the Journal of Nutritional Biochemistry. Chapter Three is written and referenced in accordance with the journal’s guidelines.

Chapter Four will summarise the main findings, as well as the implications and recommendations for future research. Chapters One, Two and Four are written in accordance with the technical, language and referencing requirements stipulated by the North-West University.

Research team

The primary research team consisted of Mrs Anneen Middel, Dr Lizelle Zandberg (Supervisor) and Dr Jeannine Baumgartner (Co-Supervisor). By signing below, each contributor accepted their indicative involvement as true and approved the inclusion of this resultant manuscript in this dissertation.

Table 1-1: Individual contributions of the research team members to this dissertation

Mrs Anneen Middel	
Centre of Excellence for Nutrition at the North-West University	
MSc student, responsible for the planning, implementation and execution of affiliated study; laboratory analyses of samples; data capturing and statistical analyses as well as reporting on the findings.	
Dr Lizelle Zandberg	
Centre of Excellence for Nutrition at the North-West University	
Supervisor of the student responsible for the planning and implementation of experimental work; Responsible for the molecular data generation, statistical analyses as well as reporting on the findings.	
Dr Jeannine Baumgartner	
Institute of Food, Nutrition and Health, ETH Zürich, Switzerland	
Co-supervisor of the student, responsible for conceptualising and planning of this study, implementation, and statistical analysis, manuscript writing and critical review of findings	

CHAPTER 2 LITERATURE REVIEW

2.1 Iron metabolism

Iron is an essential element for human beings as it plays a vital role as a cofactor for many haemoproteins and non-haem iron-containing proteins. Haemoproteins include haemoglobin (Hb) and myoglobin (Mb), which are found in enterocytes and muscle cells respectively, and are responsible for the binding and transport of oxygen. Iron is found within the haem group and binds oxygen. Other haemoproteins include enzymes that are involved in oxygen metabolism, as well as cytochromes, which are involved in electron transport and mitochondrial respiration. Non-haem iron-containing proteins play a role in DNA synthesis, cell proliferation and differentiation, gene regulation, drug metabolism, and the synthesis of steroid (Pantopoulos *et al.*, 2012; Hentze *et al.*, 2010; Kroot *et al.*, 2011; Puig *et al.*, 2017).

2.2.1 Iron absorption

A 70-kilogram adult male has roughly 4 g iron in his body. Most of which, is the iron found in erythrocytes, whereas 600 mg is found in macrophages and 300 mg in myoglobin. Iron from erythrocytes and macrophages are recycled (about 20 - 25 mg/day) whilst only 1-2 mg of the daily iron supply is derived from dietary intake via absorption in the intestine. Serum iron binds the glycoprotein transferrin, where it is prioritised for erythropoiesis in bone marrow whilst excess iron is stored in the liver (Gudjoncik *et al.*, 2014; Rochette *et al.*, 2015; Ganz, 2013).

Body iron levels are strictly regulated according to iron availability. The main tissues that form part of the regulation of iron are illustrated in figure 2-1. The duodenum plays an important role in the homeostasis of iron. Cells responsible for the absorption of iron obtained from the diet (duodenal enterocytes) are found mostly in the duodenum, whilst lesser amounts are absorbed in the jejunum (Yiannikourides *et al.*, 2019; Munoz *et al.*, 2011). Only approximately 10% of dietary iron is absorbed (Munoz *et al.*, 2011). Dietary iron from plant sources, non-heme iron, mostly occurs in the ferric form (Fe^{3+}) that is insoluble and not easily absorbed. Fe^{3+} needs to be reduced via membrane ferrireductase enzyme, duodenal cytochrome b (Dcytb), and dietary reducing agents (low pH) to the ferrous form (Fe^{2+}) before absorption by duodenal enterocytes occurs (Gulec *et al.*, 2014; McKie *et al.*, 2001). Animal sources of iron, namely haem iron, are more easily absorbed than non-heme iron and absorbed through a proton-coupled folate transporter (PCTF) localised in the membrane of duodenal enterocytes (Shayeghi *et al.*, 2005; Qui *et al.*, 2006). Iron is transported into the enterocyte through divalent metal transporter 1 (DMT1), which is transcribed by the SLC11A1 gene, where it is stored by ferritin (Hentze *et al.* 2010). DMT1

expression is regulated by hypoxia inducible factor 2- α (HIF2- α). HIF2- α expression is regulated by the *EPAS1* gene. DMT1 production is increased in a hypoxic gut or environment (low oxygen saturation) to increase the transport of iron into enterocytes (Mastrogiannaki *et al.*, 2013). Even though iron cannot be actively excreted by the body as there is no mechanism, approximately 1-2 mg of iron is lost every day through enterocyte and skin desquamation, blood loss as well as parasitic infections (Waldvogel-Abramowski *et al.*, 2014).

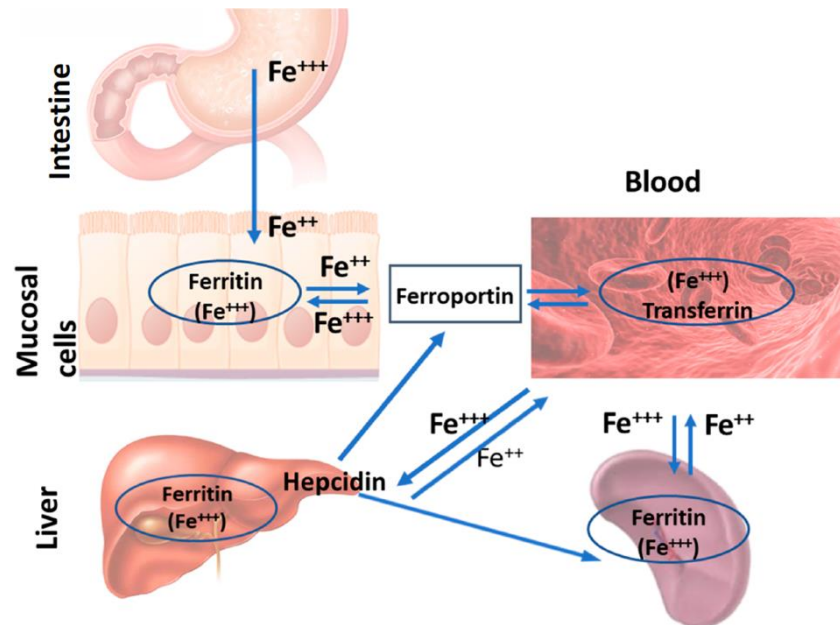


Figure 2-1: The main tissues involved in the regulation of iron homeostasis.

Illustrated from absorption of dietary intake in the intestine via the mucosal cells to transport via transferrin in the blood and storage in the liver (Yiannikourides *et al.*, 2019). Printed with permission from the original authors.

2.2.3 Cellular and plasma iron regulation

As seen in figure 2-2, cellular iron uptake, utilisation and export are mediated via multiple intracellular processes as well as extracellular stimuli. After iron is absorbed into the enterocyte and stored by ferritin it is released into circulation via ferroportin (iron export protein) where it binds transferrin (McKie *et al.*, 2001; Gudjoncik *et al.*, 2014; Munoz *et al.*, 2011;). Poly (rC)-binding protein 2 (PCBP2) assists in iron transport after receiving iron from DMT1 or addresses iron to ferroportin for cell export. PCBP2 also aids the release of iron from haem by binding haem oxygenase 1 (Yanatori *et al.*, 2017; Yanatori *et al.*, 2016).

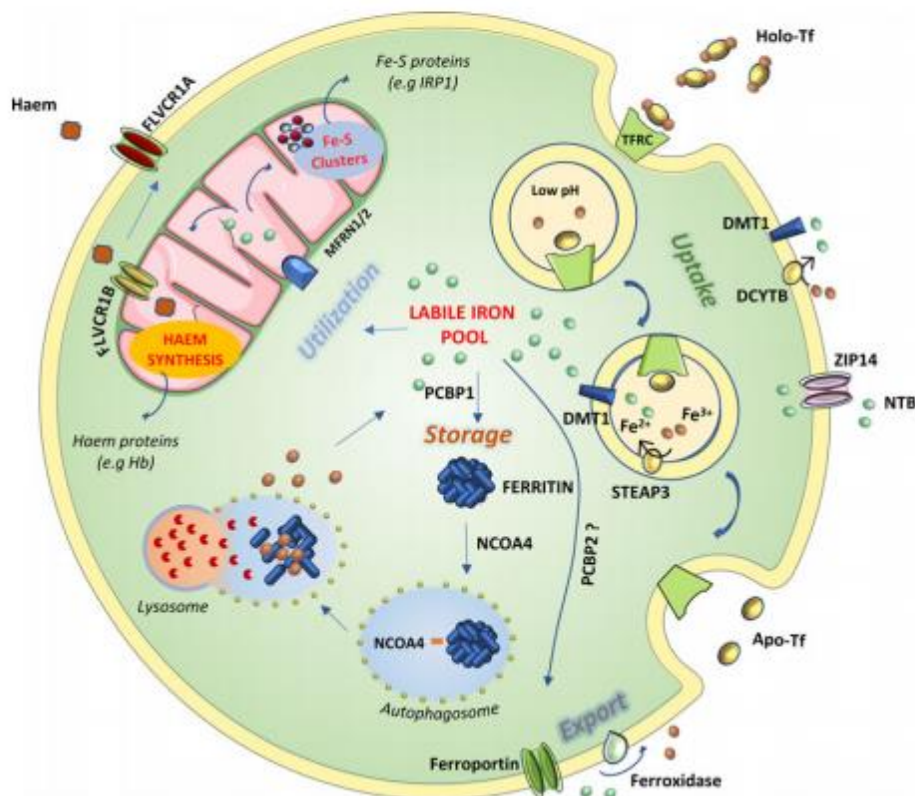


Figure 2-2: Schematic presentation of intracellular iron uptake, utilisation and export

Iron moves into the cell through DMT1, after which it is stored in the cytoplasm via ferritin or can be utilised in the mitochondria. Stored iron can also be exported by the cell via ferroportin (Camashella & Pagani, 2018). Printed with permission from the original authors.

Abbreviations: DMT1: divalent metal transporter 1

Iron is stored by the protein ferritin in the body. Ferritin is found in the cytoplasm (primarily stored in the cytoplasm), nucleus and mitochondria (uses metabolically active iron) of cells (Alkhateeb & Connor, 2013; MacKenzie *et al.*, 2008). Poly (rC)-binding protein 1 (PCBP1) found in the cytoplasm aids in protecting the cell against redox activity by delivering excess iron to ferritin to store (Ryu *et al.*, 2017; Camashella & Pagani, 2018). The nuclear receptor coactivator 4 (NCOA4) plays a role in the release of iron from ferritin via degradation by autophagosomes (ferritinophagy) (Dowdle *et al.*, 2014; Mancias *et al.*, 2014). This process is essential in ID states to release stored iron in order to maintain cellular requirements, specifically by macrophages responsible for iron recycling. NCOA4 expression is upregulated during erythropoiesis (enterocyte synthesis) (Ryu *et al.*, 2017). In iron excess states NCOA4 is transported to and degraded by cellular proteasomes by the E3 ubiquitin ligase HERC2.

Iron homeostasis in the cell is upheld by the synchronised expression of various proteins including ferritin and transferrin. Cells have transferrin receptors (TFRs) that mediate cellular iron metabolism. In the cells (other than erythrocytes) iron is transported into the mitochondria inner membrane by mitoferrin-1. Iron can then be used to synthesise haem and iron sulphur clusters that form part of the prosthetic groups of enzymes or essential proteins. Iron can also be exchanged from the endosomes to mitochondria through direct inter-organelle contact (Paradkar *et al.*, 2009; Hamdi *et al.*, 2016).

2.2.4 Ferroportin and iron homeostasis

Ferroportin mediates the iron released into plasma from the duodenal enterocytes responsible for iron absorption obtained from the diet, from macrophages responsible for recycling iron, from senescent enterocytes as well hepatocytes responsible for storing iron (Nemeth *et al.*, 2004). The ferroportin promoter consist of an antioxidant response element (ARE) supressed by BACH, BTB and CNC homology protein and activated by nuclear erythroid factor 2 (NRF2). Ferroportin is highly expressed in enterocytes, hepatocytes and macrophages (Drakesmith *et al.*, 2015). Haem increases expression through the suppression of BACH. Whereas inflammation suppresses the expression of ferroportin. Expression in duodenal enterocytes is also enhanced by HIF2- α binding a 5' Hypoxia-response element (HRE) (Mastrogiannaki *et al.*, 2013). Serum iron bound to transferrin after it is exported by ferroportin is prioritised for erythropoiesis (Hb synthesis in erythrocytes) in bone marrow whilst the liver stores excess iron (Gudjoncik *et al.*, 2014; Rochette *et al.*, 2015; Ganz, 2013).

As illustrated in figure 2-3, ferroportin, ferritin (light and heavy chain) and HIF-2 α are regulated via transcription. This occurs via the 5' untranslated region (UTR), the iron responsive element (IRE) and iron regulatory proteins (IRPs). With the binding of IRPs on the IREs located in the UTR of the mRNAs (5' and 3' region) encoding for specific iron regulating proteins, post transcriptional regulation helps to maintain cellular iron homeostasis. In ID, ferroportin protein synthesis is suppressed by active IRP1 and IRP2 that binds IRE in the 5' UTR of mRNAs to inhibit translation. IRPs bound to the 3' UTR protects mRNA from degradation via RNase of TFR1 and DMT1. With iron overload, IRP1 forms a FeS-cluster, thus cannot bind with IRE. IRP2 is degraded by mitochondrial aconitase. When iron availability increases the lack of IRPs binding IRE and allows for the mRNA translation of ferroportin, ferritin as well as HIF-2 α (Anderson *et al.*, 2013; Ghosh *et al.*, 2013; Aschmeyer *et al.*, 2018; Anderson *et al.*, 2012).

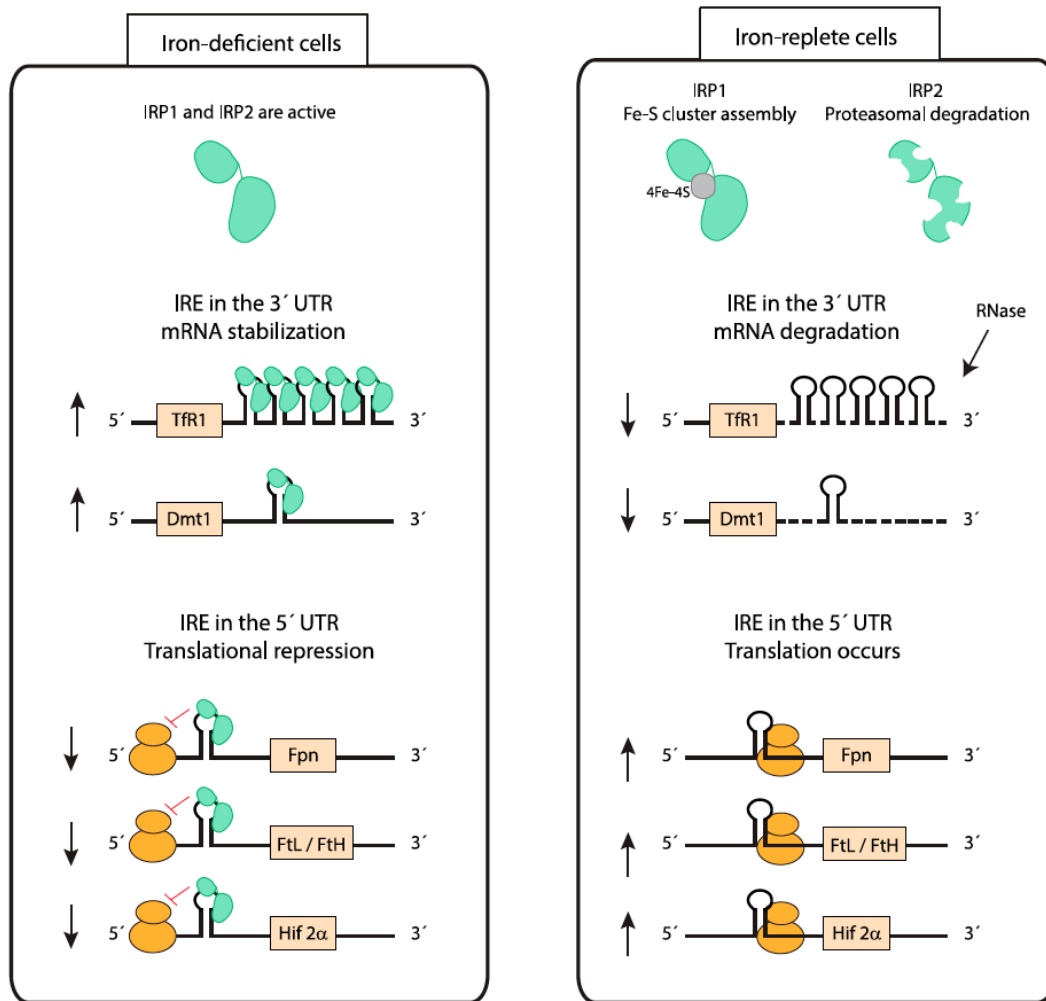


Figure 2-3: Cellular iron homeostasis in iron deficient cells (left) and cell repletion of iron (right)

IRP1 and IRP2 are active in iron deficient state and bind with the IRE in the 3'UTR to increase TFR1 and DMT1 transcription, whilst inhibiting the transcription of Fpn, FtL/FtH and HIF-2α by binding the 5'UTR. During iron repletion IRP1 forms a FeS-cluster and IRP2 is degraded to, thus the IRE in the 3'UTR is open causing the degradation of TFR1 and DMT1. However, the IRE in 5'UTR is open to increase Fpn, FtL/FtH and HIF-2α transcription (Neves *et al.*, 2019). Printed with permission from the original authors.

Abbreviations: DMT1: divalent metal transporter; Fpn: ferroportin; FtL: ferritin light chain; FtH: ferritin heavy chain; HIF-2α: hypoxia inducible factor 2-α; IRE: iron responsive element; IRP1: Iron regulatory protein1; IRP2: Iron regulatory protein2; TFR1: transferrin 1; UTR: untranslated region.

2.2 The role of hepcidin in systematic iron homeostasis

Iron is not only stored in the liver, iron absorption is also regulated by the liver via hepcidin and ferroportin expression (Nemeth *et al.*, 2004). Hepcidin (25 amino acid peptide) is predominantly transcribed by hepatocytes and secreted into circulation to regulate dietary uptake of iron in a negative feedback loop. Iron homeostasis is maintained with a feedback loop stimulated by iron

stores on hepatic hepcidin, whilst iron plasma levels are regulated by the stimulation of hepcidin production by transferrin-bound iron feedback (Park *et al.*, 2001; Kemna *et al.*, 2007; Kroot *et al.*, 2010, Anderson & Shah, 2013). Both the transferrin-bound iron as well as the concentration of iron stores, regulates hepcidin production via the bone morphogenetic protein receptor (BMPR) pathway. Further amino-terminal processing of the 25 amino acid peptide results in two smaller hepcidin isoforms of 22 and 20 amino acids (Jordan *et al.*, 2009). Hepcidin-25 mediates iron homeostasis by binding with the iron exporter ferroportin (encoded by the gene *SLC40A1*), which induces internalisation and degradation of ferroportin in the membrane of macrophages and hepatocytes, as well as the basolateral site of enterocytes. This leads to decreased intestinal iron absorption as well as macrophage iron recycling (Nemeth *et al.*, 2004; De Domenico *et al.*, 2005; Delaby *et al.*, 2005; Aschemeyer *et al.*, 2018; Ramey *et al.*, 2010). Hepcidin further physically blocks the site of iron export in the central cavity of ferroportin to reduce iron availability (Aschemeyer *et al.*, 2018). The smaller isoforms (hepcidin-20 and hepcidin-22) do not cause a similar hypoferraemic response via ferroportin (Gerardi *et al.*, 2005; Farnaud *et al.*, 2006).

An increased need for circulating iron (as in the condition of ID and anaemia, as well as hypoxia) leads to increased erythropoietic activity downregulating hepcidin expression in a healthy model, whereas decreased circulating hepcidin results in the release of stored iron (increased recycling rate) as well as an increase in iron absorption. In fact, in ID states iron absorption by enterocytes can increase up to ten-fold (Hentze *et al.*, 2010; Finch, 1994). Impaired iron homeostasis (excess iron and ID) leads to cellular and organ dysfunction. ID decreases erythropoiesis leading to decreased Hb synthesis and consequently anaemia. ID also influences the synthesis of ferroproteins which may lead to muscle weakness and an increased epithelia cell turnover rate (Ganz, 2013). Conversely iron overload, as seen in patients with hemochromatosis (mutation in *HFE* gene), leads to plasma iron levels greater than the iron binding capacity of transferrin thus non-transferrin bound iron (NTBI). NTBI moves into and accumulates in hepatocytes and other parenchymal cells potentially leading to tissue injury and increases the risk and severity of infections with gram-negative organisms (Roetto *et al.*, 2003; Nicolas *et al.*, 2001, Feder *et al.*, 1996; Ganz, 2013; Stefanova *et al.*, 2018).

2.2.1 Hepcidin regulation via iron availability: the bone morphogenetic protein-small mothers against decapentaplegic pathway

Iron-mediated hepcidin regulation in hepatocytes occurs via the bone morphogenetic protein-small mothers against decapentaplegic (BMP/SMAD) pathway, specifically the extracellular modulators hepatocyte transferrin receptor 1 (TFR), TFR2, human hemochromatosis protein

(HFE), hemojuvelin (HJV), bone morphogenetic protein 6 (BMP6), BMP2 and sensing transferrin-bound iron (iron-TF) concentration (see figure 5 for illustration of the BMP/SMAD pathway) (Kawabata *et al.*, 2005; Niederkofler *et al.*, 2005; Meynard *et al.*, 2009; Spasić *et al.*, 2008).

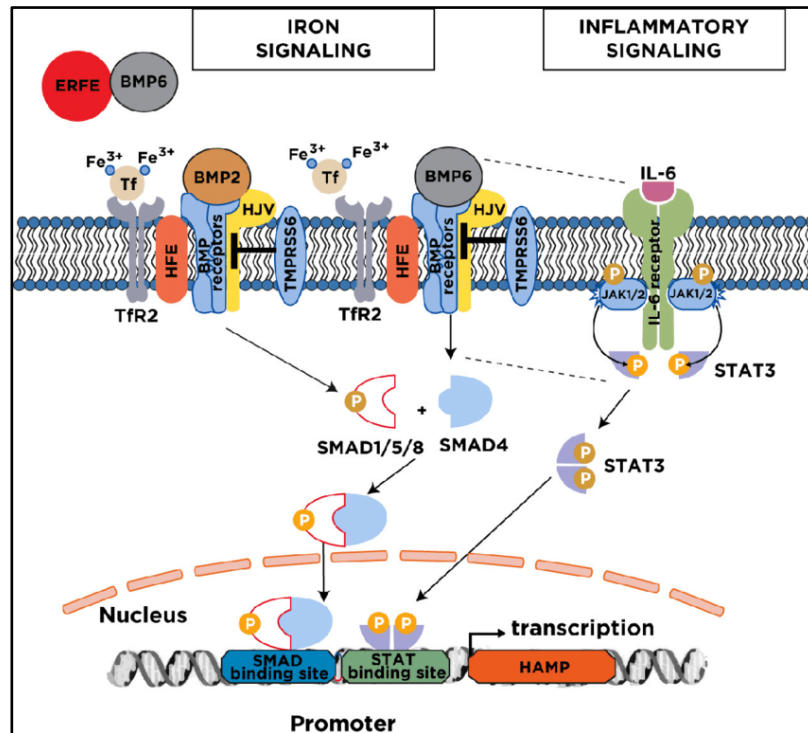


Figure 2-4: Major mechanisms for hepcidin regulation via iron availability (BMP/SMAD pathway) and inflammation (JAK/STAT3 pathway).

Iron mediated signalling of *HAMP* expression is activated by Tf binding *TFR2* as well as BMP2 and BMP6 binding to the BMP receptors. This leads to SMAD 1/5/8 phosphorylation allowing SMAD4 to bind to SMAD 1/5/8. The SMAD 1/5/8 and SMAD 4 complex binds to the SMAD binding site to induce *HAMP* transcription. Inflammatory signalling occurs by IL-6 binding the IL-6 receptor and phosphorylates STAT3 to activate the STAT3 binding site and induce *HAMP* transcription (Katsarou & Pantopoulos, 2018). Printed with permission from the original authors.

Abbreviations: BMP2: bone morphogenetic protein 2; BMP6: bone morphogenetic protein 6; BMP/SMAD: bone morphogenetic protein-small mothers against decapentaplegic; IL-6: interleukin-6; SMAD: small mothers against decapentaplegic; Tf: transferrin; TFR2: transferrin receptor 2.

2.2.2 Bone morphogenetic protein 2 and 6

Several BMPs are transcribed in the liver, but only BMP2 and BMP6 (mainly transcribed in the liver sinusoidal endothelial cells (LSECs)) have been shown to play a fundamental role in the regulation of hepcidin *in vivo* (Zhang *et al.*, 2011; Canali *et al.*, 2017; Rausa *et al.*, 2015). BMPs (various forms are known) are dimeric ligands that act on activin receptor-like kinases (ALK) and are serine-threonine kinase receptors (classified as type 1 (BMPR1) and type 2 (BMPR2))

receptors). BMPR1 needs to be activated by BMPR2 through phosphorylation (Yang *et al.*, 2014). Two BMPR 1 and two BMPR 2 receptors, as well as a BMP ligand, is needed to activate signalling within the cell (Antebi *et al.*, 2017).

BMP6 production is increased by tissue iron loading, binding BMPR1 and BMPR2 to activate hepcidin transcription factors (Canali *et al.*, 2017; Wang & Babitt, 2016). Acute iron loading (without tissue loading) does not increase BMP6 expression although the downstream cascade is still signalled to induce hepcidin expression (Corradini *et al.*, 2011a).

BMP2 also plays an important role in hepcidin regulation. Babitt and colleagues (2007) found the treatment of mice with BMP2 increased the liver hepcidin levels. BMP2 expression seems to be regulated by chronic dietary iron modifications, although less so compared to BMP6. Wang and colleagues (2019) found BMP2 mRNA significantly reduced in mice treated with a low iron diet, and expression increased in mice treated with a high iron diet although liver BMP2 protein was not increased. The authors noted this might be due to rapid protein secretion or utilisation in the liver under iron loading conditions. LPS decreases BMP2 liver expression as well as BMP6 (Wang *et al.*, 2019).

2.2.3 Bone morphogenetic proteins and Hemojuvelin

As illustrated in figure 2-4 HJV (expressed in multiple tissues including hepatocytes) acts as a BMP co-receptor with a high binding affinity for the BMP receptors. HJV binding to the BMP receptors (mainly by BMP2 and BMP6) upregulates hepcidin transcription via BMP/SMAD signalling. Notably, BMP6 can also signal for increased hepcidin expression in the absence of HJV (Babitt *et al.*, 2006; Wu *et al.*, 2012; Xia *et al.*, 2008). *In vitro*, the cleavage of HJV is triggered by hypoxia and ID to down regulate hepcidin expression. Neither BMP2 nor BMP6 have redundant function; knock out (KO) studies show each ligand compensated to some degree for the lack of the other, although both are required for optimal hepcidin induction (Wang *et al.*, 2019). In basal conditions, BMP2 is expressed in noteworthy higher levels than BMP6 (Canali *et al.*, 2017).

2.2.4 Transferrin receptor2 and Human hemochromatosis protein

Together with HJV, HFE and TFR2 (also found in the membrane and expressed in hepatocytes) play a role in the activation of hepcidin via the regulation of SMAD1/5/8 phosphorylation. HFE and TFR2 respond to acute iron loading sensing serum diferric transferrin, inducing hepcidin expression via the BMP/SMAD pathway (Gao *et al.*, 2009; Corradini *et al.*, 2011b). Mutations in

HFE and TFR2 leads to a host iron overload, although this is less severe than mutations in HJV (Brissot *et al.*, 2018). Similar to HJV, BMP6 inactivation in HFE KO and TFR KO mice lead to further downregulated hepcidin compared to HJV KO indicating that BMP6 can activate hepcidin expression without the three co-regulators, thus neither are redundant in the BMP-SMAD pathway (Latour *et al.*, 2016; Latour *et al.*, 2017).

2.2.5 Regulatory small mothers against decapentaplegic

Regulatory small mothers against decapentaplegic (R-SMADs), namely SMAD1, SMAD5 and SMAD8, are directly phosphorylated by BMPR1 when activated (phosphorylated by BMPR2 in response to the BMP ligand) and acts as a link between BMPR1 and activation specific transcription in the cell nucleus (Massaguè & Wotton, 2000; Kretzschmar *et al.*, 1997; Yang *et al.*, 2014). Notably SMAD8 (also known as SMAD9) acts as a weak activator and Tsukamoto and colleagues found that it acts as a dominant negative by binding SMAD1 and reducing its transcriptional activation strength (Tsukamoto *et al.*, 2014). Wang and colleagues investigated the role of SMAD4 in hepcidin expression and found that by conditionally inactivating SMAD4 in hepatocytes, hepcidin expression was inhibited causing systemic iron overload (Wang *et al.*, 2005). The study also concluded that both BMPs and Transforming Growth Factor- β (TGF- β) participated in the regulation of hepcidin expression. SMAD4 translocates to the nucleus in a complex with the R-SMADs to induce expression of a wide range of target genes (including *HAMP*) regulated by BMP-responsive elements (BRE) whilst SMAD6 and SMAD7 (also BMP responsive genes) inhibits the R-SMADS in a self-limiting negative feedback loop (Yang *et al.*, 2014; Anderson & Darshan, 2008). Although the role of SMAD6 is not yet well understood, both SMAD6 and SMAD7 are also activated by BMPs (BMP2, BMP6 and BMP7) as well as TGF- β ligands to decrease BMP-SMAD signalling (thus decreasing hepcidin transcription) in response to iron availability by inhibiting SMAD4 binding to SMAD responsive motifs on the hepcidin promotor (Mleczko-Sanecka *et al.*, 2010; Lai *et al.*, 2018). Transferrin concentration can modulate the sensitivity of the receptors to the ligands (mainly BMP2 and BMP6) whilst iron stores in the liver influence the effective amount of the ligands (Corradini *et al.*, 2011a; Canali *et al.*, 2017; Koch *et al.*, 2017; Ramos *et al.*, 2011).

2.2.6 Hepcidin inhibition in the bone morphogenetic protein-small mothers against decapentaplegic pathway

Hepcidin is downregulated in ID states by transmembrane serine protease 6 (TMPRSS6). Erythropoietin (EPO), hypoxia and acute ID activate TMPRSS6 transcription (Lee, 2009). TMPRSS6 is mainly expressed in hepatocytes and inhibits the BMP-SMAD pathway by cleaving

the membrane HJV, although Wahedi and colleagues found overexpression of TMPRSS6 *in vitro* cleaves multiple other substrates including HFE, TFR2, BMPR1 and BMPR2 (Silvestri *et al.*, 2008a; Silvestri *et al.*, 2008b; Wahedi *et al.*, 2017). Notably the genes encoding HFE and TFR2 are found upstream from TMPRSS6 and do not affect TMPRSS6 *in vivo* (Finberg *et al.*, 2011; Nai *et al.*, 2014). Schmidt and colleagues demonstrated that the treatment of HFE KO mice with TMPRSS6 siRNA increased hepcidin expression tapering iron loading (Schmidt *et al.*, 2013).

Hepcidin expression is also suppressed by erythroid-released hormone, erythroferrone (ERFE) (Kautz *et al.*, 2014; Camashella & Pagani, 2018). Erythropoietin (EPO) and ERFE inhibits hepcidin expression by decreasing SMAD 1/5/8 phosphorylation in hepatocytes. However, hepcidin expression was not inhibited by EPO and ERFE in SMAD1/5 conditionally knockout (CKO) mice (Wang *et al.*, 2017b).

2.2.7 Hepcidin regulation via inflammation: The JAK/STAT pathway

Infection and inflammation can lead to an iron deficiency, resulting in anaemia of inflammation. Inflammation causes a hostile environment for pathogens making it a key part of the host's defence mechanism and as mentioned earlier hepcidin was initially discovered as an antimicrobial peptide linked to this defence (Krause *et al.*, 2000; Weinstein *et al.*, 2002; Park *et al.*, 2001; Flemming & Sly 2001). Iron is necessary for microbial growth and studies suggest that bacteria form biofilms without much difficulty if iron is readily available, thus reductions in plasma iron brought on by hepcidin aids in static pathogen growth and improves the host defence (Jurado, 1997; Collins, H.L., 2003; De Domenico *et al.*, 2010; Gangaidzo *et al.*, 2001). Hepcidin regulation is also strongly influenced by an abundant outer membrane component present on Gram-negative bacteria namely lipopolysaccharide (LPS) which stimulates the production of several inflammatory cytokines (Kanamori *et al.*, 2017; Lee *et al.*, 2005; Nemeth *et al.*, 2003). Excess hepcidin transcription in chronic inflammatory diseases leads to iron restricted erythropoiesis and the development of ACD (Wang & Rabitt, 2016).

2.2.8 Pro-inflammatory cytokines and the JAK/STAT pathway

Hepcidin was initially discovered as an anti-microbial peptide as transcription increases as pro-inflammatory cytokines are released in response to infections (Hentze *et al.*, 2010). Chronically increased hepcidin expression contributes to the development of anaemia of inflammation, which is characterised by inhibiting the release of iron from macrophages, decreasing the absorption of iron as well as iron restricted erythropoiesis, to ultimately decrease iron availability (Weiss & Goodnough, 2005). Shortly after an infection leading to inflammatory stimuli, plasma iron

concentrations decrease (Pigeon *et al.*, 2001; Nicolas *et al.*, 2002). This response includes the expression and secretion of IL-6, which was found to be essentially responsible for the upregulation of hepcidin (Nemeth *et al.*, 2004b; Nemeth *et al.*, 2003; Lee *et al.*, 2005; Inamura *et al.*, 2005). Nemeth and colleagues conducted a study where they treated human hepatocyte culture with IL-6, IL-1 and TNF- α and only found transcriptional induction of hepcidin with exposure to IL-6 (Nemeth *et al.*, 2003). IL-6 mediated regulation of hepcidin expression is hepatocyte specific (Wrighting & Andrews, 2006).

The JAK/STAT pathway was also later found to be used by other cytokines (such as oncostatin M, Interleukin-22 (IL-22) or Interferon- α (IFN- α) for hepcidin induction (Lee *et al.*, 2005; Armitage *et al.*, 2011; Ryan *et al.*, 2012). The general inflammatory response to an infection (pathogen) or local tissue injury is also induced by interleukin 1 (IL-1) responsible for activating the nuclear factor κ B (NF- κ B) family of transcription factors. IL-1 studies also found that IL-1 induces hepcidin transcription in primary hepatocytes for both wild-type and IL-6 KO mice indicating that IL-1 may play an IL-6-independent role in the upregulation of hepcidin by inflammation. Lee *et al.* (2005) also found that the combination of IL-1 and IL-6 enhanced hepcidin expression significantly in primary hepatocytes.

2.2.9 STAT3 and HAMP activation

As illustrated in figure 2-4 the expression of hepcidin induced by IL-6 is conducted via JAK/STAT3 signalling. IL-6 is recognised and binds to GP130 complexes initiating JAK 1/2-mediated phosphorylation of the transcription factor STAT3 which binds to a STAT3 responsive element (STAT3-RE) in the proximal *HAMP* promoter activating hepcidin transcription (Pietrangelo *et al.*, 2007; Falzacappa *et al.*, 2007; Wrighting & Andrews, 2006). Optimal induction of hepcidin expression via inflammation also requires BMP and SMAD4 signalling (Wang *et al.*, 2005).

2.3 The role of omega-3 polyunsaturated fatty acids in inflammation

EPA and DHA were mainly thought in the past to primarily have an anti-inflammatory effect due to the role they play compositionally in the cell membrane phospholipids, specifically of the cells involved in inflammation like lymphocytes, macrophages or neutrophils. But throughout the past year's, multiple other mechanisms have been described in research (Burdge & Calder, 2015; Calder, 2008; Peterson *et al.*, 1998; Chapkin *et al.*, 1991).

2.3.1 Polyunsaturated fatty acid synthesis, dietary sources

As described in figure 1, n-6 and n-3 FA are derived from linoleic acid (LA:18:2n-6) and ALA, respectively, which are found in plant sources like seeds, nuts and plant oils (sunflower oil and canola oil are some of the sources). Neither LA nor ALA can be synthesised by the human body and therefore they are essential fatty acids (EFA) (Burge & Calder, 2015). Fatty fish sources like salmon, herring and mackerel are dietary sources of EPA and DHA. DHA is also considered a conditional EFA, specifically during a child's early developmental years (Gibson *et al.*, 2011). This pathway of desaturation and elongation to covert LA and ALA into their respective metabolites occurs mainly in the liver. The conversion of ALA to EPA involves three enzymes namely, delta-6 desaturase (D6D), elongase-5 and delta-5 desaturase (D5D) (shown in figure 2-5). D5D and D6D are transcribed mostly in the brain, liver, heart and lung and encoded by the FADS1 and FADS2 genes respectively (Cho *et al.*, 1999; Lemaitre *et al.*, 2011; Tanaka *et al.*, 2009). Activity of the enzymes are regulated by nutritional status, hormones (insulin) as well as a feedback inhibition, by the end-products (Brenner, 2003; Calder, 2017). The enzymes are shared (and compete) between the parallel n-3 and n-6 biosynthetic pathway converting ALA and LA to EPA and ARA respectively (Calder, 2015b).

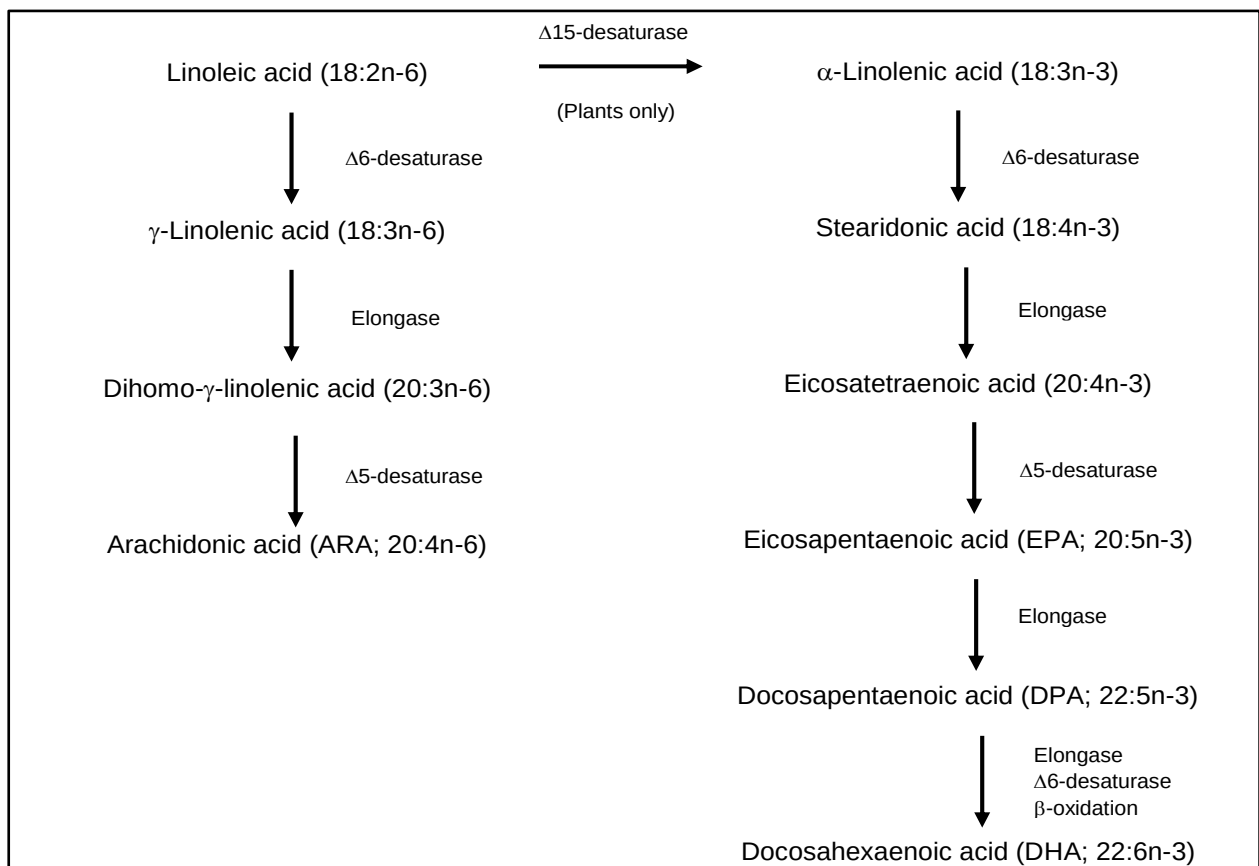


Figure 2-5: Metabolic elongation pathways with responsible enzymes of linoleic acid (n-6) and α-linolenic acid (n-3) to long chain polyunsaturated fatty acids (PUFAs)

(Calder, 2015b). Printed with permission from the original author.

2.3.2 Polyunsaturated fatty acids and the cell membrane.

The cell membrane is a functional barrier between the subcellular compartments, the cell and its environment. Cellular membranes consist largely of phospholipids with a hydrophilic head and a hydrophobic tail. The phospholipids are arranged in a lipid bilayer composed of hydrophobic tails turned inwards leaving the heads turned outwards towards the aqueous region, the outer cell region, namely interstitial fluid and the cytoplasm within the cell membrane. Lipids have numerous functions in the cells apart from the membrane structural component, including signalling molecules, protein recruitment and post-translational protein-lipid modification (Nakamura *et al.*, 2014; Shimizu, 2009; Saliba *et al.*, 2015). Apart from the phospholipids the membrane also contains imbedded proteins like receptors, ion channels and transporters (Lodish, 2008).

Changes in the lipid composition of the membrane results in changes in the membrane properties and functionality. Lipids in the membrane also affect the imbedded proteins as well as additional proteins not found in the membrane with an affinity for specific lipids. Different cell membranes contain a specific ratio of the phospholipid species and cell-specific proteins incorporated into the membrane. This depends on the cell type and function. Lipids influence the physiochemical properties of the membrane. The saturation and order of the lipids incorporated in the membrane influences the functionality and fluidity of the membrane. Saturated fatty acids decrease the fluidity and make the membrane thick due to stronger lipid-lipid interactions. Conversely, due to the acyl-chain kinks unsaturated lipids prevent tight packaging leading to an increased membrane permeability (Van Meer *et al.*, 2008; Sezgin *et al.*, 2017). Different lipids are also vulnerable to different modifications. The ratio of fatty acids influence the functionality of the cell. PUFAs are more prone to oxidation which can lead to ferroptosis, which is a non-apoptotic cell death triggered by over accumulation of peroxidised lipids (Stockwell *et al.*, 2017). Ferroptosis is dependent on iron (Dixon *et al.*, 2012).

n-3 and n-6 PUFAs form an important part of membrane phospholipids with biological activities that influence the function and responsiveness of cell membranes as well as tissue metabolism in response to hormonal and other signals. The functions of n-3 and n-6 PUFAs include the regulation of membrane structure and function, regulation and actions of intracellular signalling pathways, regulation of gene expression as well as the regulation of the production of bioactive lipid mediators (Calder, 2015a). In most cell types the n-6 PUFA ARA is the most abundant PUFA found in the membrane but studies found that by increasing EPA and DHA intake, ARA decreases whilst EPA and DHA found in the membrane composition increases (Walker *et al.*, 2015).

Phospholipase (PL) enzymes are responsible for the hydrolysis of the phospholipid by the release of free fatty acid and lysophospholipid. PUFAs are moved from the cell membranes into the cytosol by phospholipase A2 (PLA2) (Dennis, 2000). ARA released from cell membrane acts as a mediator for the cyclooxygenase (COX) pathway, lipoxygenase (LOX) pathway, cytochrome P450 (CYTP450) pathway and hydroxyeicosatetraenoic acid (HETE) pathway enzymes (figure 2-6) (Lewis *et al.*, 1990; Tilley *et al.*, 2001; Kalinski, 2012). Notably, in addition to these, the enzymes listed in table 2-1 can also be involved. These enzymes lead to the activation of eicosanoids including prostaglandins (PGs), thromboxanes and leukotrienes (LTs). Thus, fatty acid membrane composition modifications brought on by the increased dietary intake of EPA and DHA also modifies the production of lipid mediators which are generated from membrane phospholipids and decreases the efficiency of the arachidonic cascade (Buczynski *et al.*, 2009; Funk, 2001; Lee *et al.*, 1985; Yaqoob *et al.*, 2000; Healy *et al.*, 2000; Rees *et al.*, 2006; Faber *et al.*, 2011; Browning *et al.*, 2012).

Table 2-1: Enzymes involved in the activation of prostaglandins, thromboxanes and leukotrienes¹.

Enzymes	Abbreviations
Cytochrome Hydrolase	CyPh
Cytochrome Epoxygenase	CyPe
Desaturases and Elongases	D & -
Prostaglandin Synthases	PGsy
Thromboxane synthase	TXsy
Glutathione Peroxidases	GPX
Glutathione Transferase	GPT
Dehydrogenases	DHO
Soluble Epoxide Hydrolases	sEH
γ-Glutamil Transferase	GGT
Dipeptidase	CN2
Hydroperoxidase	PGH

¹Compiled from Dasilva *et al.*, 2019

The metabolic functions of membrane phospholipid EPA are similar to that of ARA and can also be used as a substrate for the COX, LOX and CYTP450 enzymes that produce eicosanoids less biologically effective than the ARA-derived eicosanoids (Goldman *et al.*, 1983; Lee *et al.*, 1984; Bagga *et al.*, 2003). This was illustrated in a study conducted by Bagga and colleagues who treated macrophages with prostaglandin E2 (PGE2) derived from ARA and prostaglandin E3 (PGE3) derived from EPA and found that although both stimulate the secretion of IL-6, PGE3

induction was significantly less effective compared to PGE₂ (Bagga *et al.*, 2003). This is due to eicosanoid receptors typically having a lower affinity for EPA-derived mediators than that derived from ARA (Wada *et al.*, 2007).

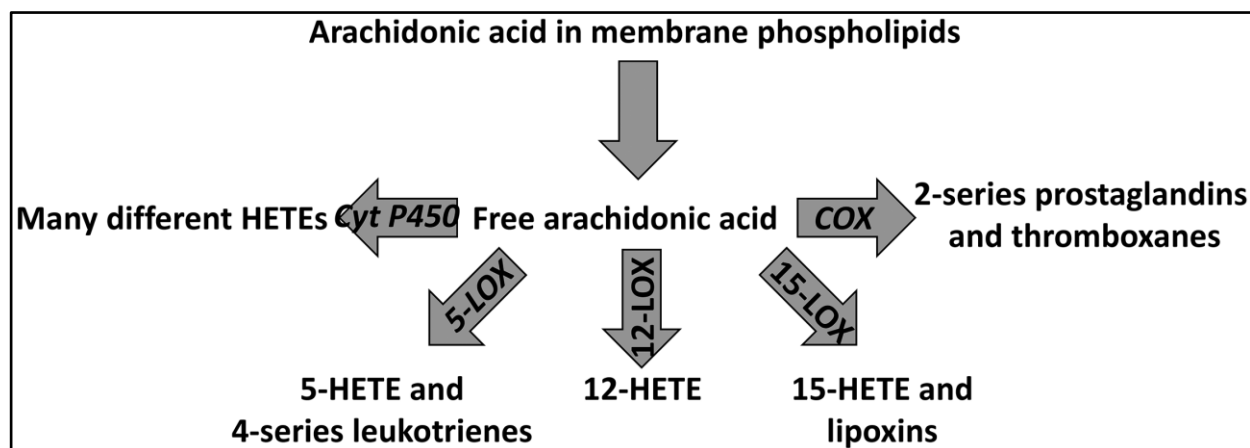


Figure 2-6: Outline of the pathway of conversion of arachidonic acid into eicosanoids via CytP450, LOX and COX to synthesise lipid mediators, prostaglandins and thromboxanes involved in inflammation.

(Calder, 2017). Printed with permission from the original authors.

Abbreviations: COX: Cyclooxygenase; LOX: Lipoxygenase; HETE: Hydroxyeicosatetraenoic acid.

2.3.3 Eicosapentaenoic acid, Docosahexaenoic acid and Arachidonic acid, lipid mediators involved in inflammatory processes

Lipid mediators (eicosanoids and docosanoids) have been linked to various processes including regulating inflammatory processes, cell signalling and gene expression as well as lipid metabolism (Massey & Nicolau, 2013; Waddington *et al.*, 2001, Serhan *et al.*, 2006; Gao *et al.*, 2006). There are multiple subfamilies of eicosanoids, including most prominently the prostaglandins, thromboxanes, leukotrienes and lipoxins. Bioactive lipid mediators are categorised by the specific oxidation and functional group. Each group contains multiple isomers (Serhan *et al.*, 1996). This study will focus on lipid mediators derived from ARA, EPA and DHA.

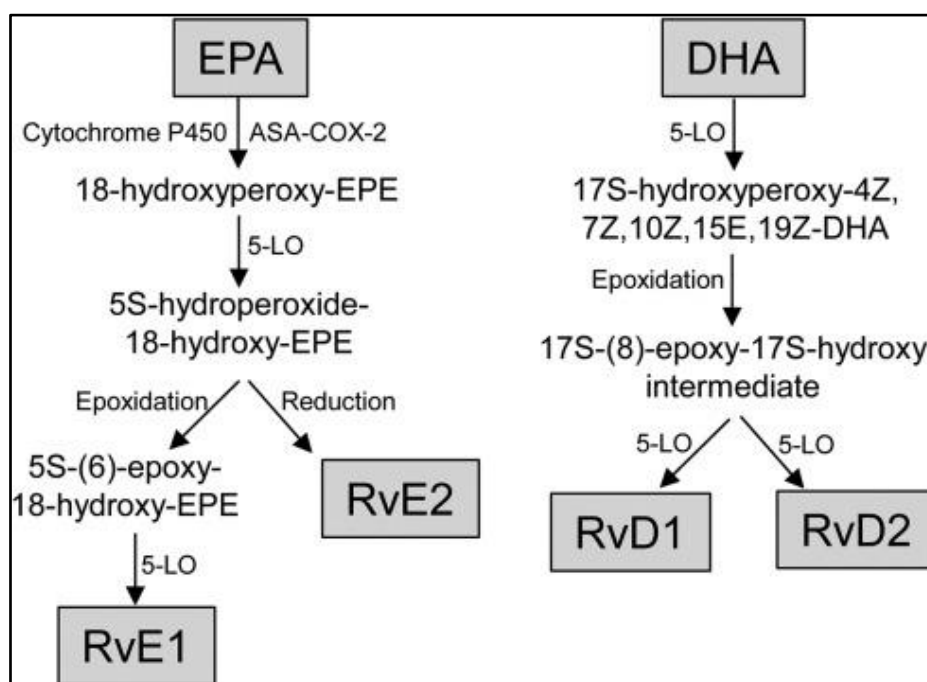


Figure 2-7: Biosynthesis of resolvins from EPA and DHA (E-series and D-series) via 18-HEPE and 17-HDHA.

Resolvins responsible for resolving inflammation is derived from EPA and DHA (Echeverría *et al.*, 2019). Printed with permission from the original authors.

Abbreviations: DHA: Docosahenoic acid; EPA: Eicosapentanoic acid; HDHA: Hydroxydocosahexaenoic acid; HEPE: hydroxyeicosapentaenoic acid; RvD: Resolvins D-series; RvE: Resolvins E-series

As seen in figure 2-7 the lipid mediators derived from ARA include leukotrienes, prostaglandins and thromboxanes as well as 5-- hydroxyeicosatetraenoic acid (5-HETE), 8-HETE, 11-HETE, 12-HETE and 15-HETE. The lipid mediators derived from ARA have been associated with conditions like cardiovascular diseases and type-2 diabetes related to chronic inflammation (Gopaul *et al.*, 1995; Sears *et al.*, 2012). ARA-derived lipid mediators can link to membrane receptors coupled to membrane G-protein and activate the cAMP complex pathway. This leads to higher transcription levels of cytokines involved in inflammation as well as decreased phagocytosis of microorganisms (pathogens) by leukocytes (Hirai *et al.*, 2001; Aronoff *et al.*, 2006; Aronoff *et al.*, 2004). These molecules are also known to play an important role in metabolites leading to artery vasodilation and vasoconstriction. The parallel EPA-derived metabolites, PGE3 and TXA3. LTB6, 5-hydroxyeicosapentaenoic acid (HEPE), 11-HEPE, 12-HEPE, 15-HEPE and 18-HEPE. 18-HEPE which compete with ARA for the same COX-enzyme have been shown to be less harmful than the n-6 derived mediators (Fitzgerald, 2003; Medina *et al.*, 2012)

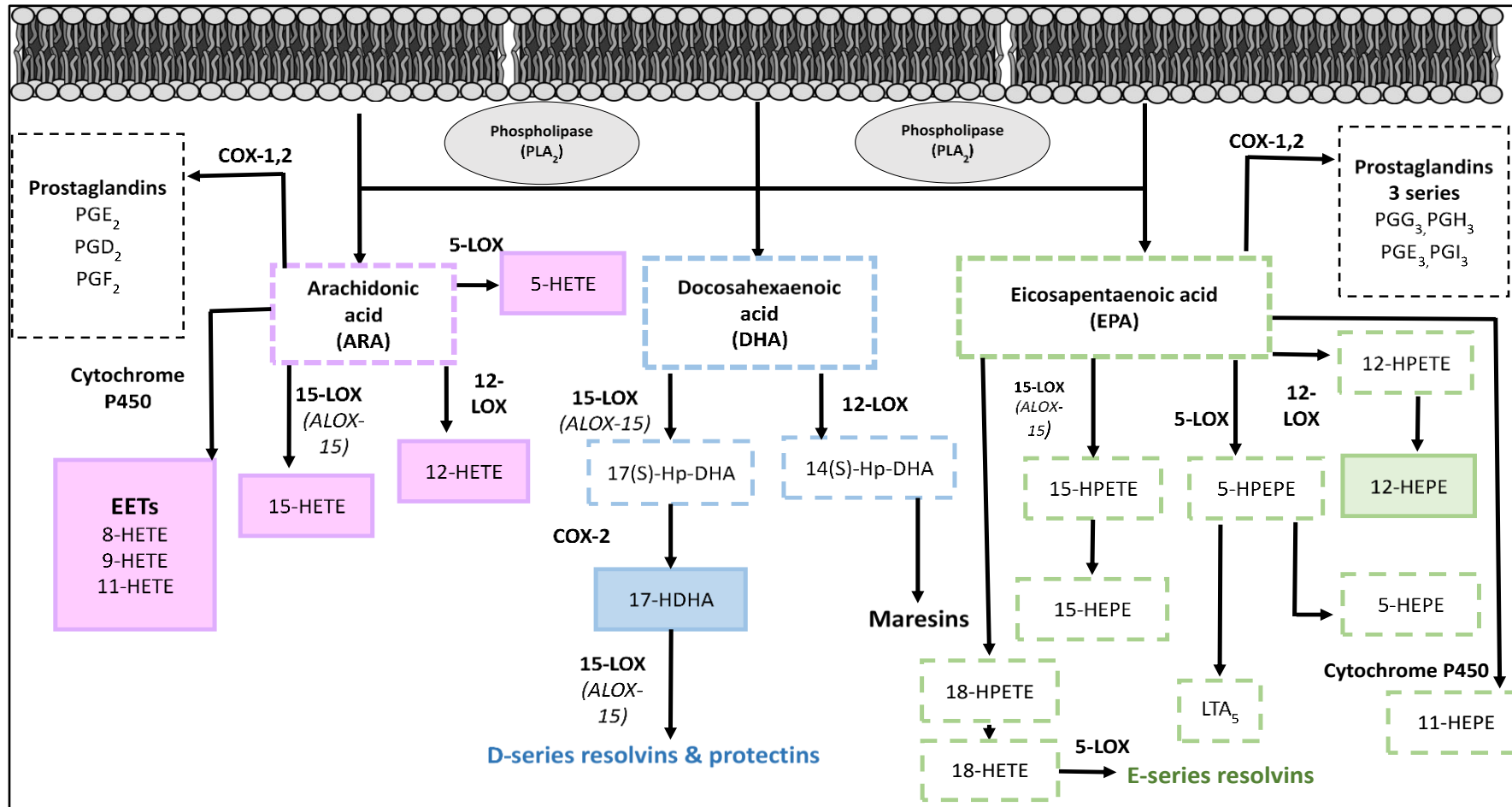


Figure 2-8: An overview of the main pathways involved in the production of polyunsaturated fatty acid-derived lipid mediators

ARA, DHA and EPA are released from the cell membranes into the cytosol by PLA₂ where they act as a precursor for the COX, LOX and CYTP450 enzymes (indicated with the arrows) that produce various lipid mediators (Zandberg et al., 2018). Printed with permission from the original author.

Abbreviations: ALOX: arachidonate lipoxygenases; COX: cyclooxygenase; HDHA: hydroxy docosahexaenoic acid; HEPE: hydroxyeicosapentaenoic acid; HETE: hydroxy-eicosatetraenoic acid; LOX: lipoxygenase; LT: leukotriene; PG: prostaglandin; TX: thromboxane)

It should be noted that not all n-6 lipid mediators are pro-inflammatory. ARA lipoxins also play a role in the resolution of inflammation by competing with the enzymes that synthesise leukotrienes and inhibits interleukins (produced by leukocytes for regulating inflammatory response) and the pro-inflammatory cytokine TNF- α (Serhan, 1994, József *et al.*, 2002; Pouliot & Serhan, 1999). Iron also play a role in COX and LOX enzymes (co-factor of the catalytic sites) thus ID may lead to impaired synthesis of these regulatory modulators (Gilbert *et al.*, 2011; Kuhn *et al.*, 2005).

EPA and DHA were later found to be biosynthesised into lipid mediators with potent actions in controlling the resolution of inflammatory substrates (illustrated in figure 2-7 and 2-8) (Serhan *et al.*, 2000; Serhan *et al.*, 2002; Hong *et al.*, 2003). These EPA and DHA metabolites/lipid mediators are known as resolvins (E-series and D-series derived from EPA and DHA respectively), protectins and maresins. E-series and D-series resolvins can further be categorised as resolvin E1 (RvE1), resolvin E2 (RvE2), resolvin D1 (RvD1) and resolvin D2 (RvD2) as described in figure 2-8. 18-HEPE is the precursor used to synthesise (via epoxidation and reduction) the E-series resolvins. DHA-derived lipid mediators include 17- Hydroxydocosahexaenoic acid (17-HDHA) which act as the precursor of the D- series resolvins and protectins, and 14- HDHA, which is the precursor of the maresins (Calder, 2015b).

Resolvins actively facilitate the resolving phase of acute inflammation, by actively stimulating the recruitment of non-inflammatory monocytes. The synthesis of resolvins, protectins and maresins also involve the COX and LOX pathways (Serhan, 2007; Bannenberg & Serhan, 2010; Serhan & Chiang, 2013). Hudert and colleagues found a diet rich in marine FA increased resolvin synthesis in mice (Hudert *et al.*, 2006). This was also found in the serum taken from humans consuming increased levels of n-3 FA (Mas *et al.*, 2012).

The effects of EPA and DHA, on the above-mentioned cytokines (TNF- α , IL-1) and specifically IL-6 has been the focus of multiple studies. Various studies have shown resolvins, protectins and maresins to be anti-inflammatory and inflammation resolving. Examples include RvE1, RvD1 and protectin D1 all inhibited trans endothelial migration of neutrophils, so preventing the infiltration of neutrophils into sites of inflammation whilst resolvin D1 inhibited IL-1 β production; and protectin D1 inhibited TNF- α and IL-1 β production (Bannenberg & Serhan, 2010; Serhan *et al.*, 2008a; Serhan *et al.*, 2008b; Serhan & Chiang, 2013). Recently a study conducted by Xia *et al.*, (2019) studying the effects of RvD1 on ventilator-induced lung injury in mice found that RvD1 decreased the levels of IL-1 β , TNF- α , IL-6 found in bronchoalveolar lavage fluid (BALF) cells.

2.3.4 PUFAs – derived lipid mediators and altered gene expression

n-3 and n-6 derived lipid mediators (eicosanoids and pro-resolving lipids) target transcription factors to regulate the expression of genes involved in inflammatory responses and diseases. As shown in figure 2-8 fatty acids can target the transcription factor nuclear factor kappa-B (NF- κ B), that regulates numerous genes involved in inflammatory processes namely cytokines, chemokines, adhesion molecules, and matrix metalloproteinases (MMP). NF- κ B is inhibited by the protein I κ B in the cell cytosol. When I κ B is phosphorylated and degraded into the proteasome, free NF- κ B can translocate into the cell nucleus and target and induce pro-inflammatory genes (Hassan *et al.*, 2010; Marion- Letellier *et al.*, 2015). Diets rich in ALA inhibited NF- κ B activation as well as adhesion molecules in rats presenting with colitis rats (Hassan *et al.*, 2010; Ibrahim *et al.*, 2012).

This process was investigated in numerous studies using Fat-1 mice (Hudert *et al.*, 2006; Liu *et al.*, 2013; Bellenger *et al.*, 2011; Kim *et al.*, 2012). Although the conversion of n-6 to n-3 fatty acids cannot occur in mammals due to the lack of enzyme expression (gene), some lower life forms such as the roundworm *C. elegans* has a gene called fat-1, which encodes an enzyme, n-3 fatty acid desaturase. The enzyme can introduce a double bond into n-6 fatty acids at the n-3 position of their hydrocarbon chains to form n-3 fatty acids leading to endogenously n-3 PUFA rich mice, even with an n-6 FA rich/n-3 FA poor diet (Spychalla & Kinney, 1997; Kang *et al.*, 2001; Kang *et al.*, 2004; Kang, 2007). Hudert and colleagues (2006) found fat-1 mice with chemical colitis induction presented with decreased NF- κ B activation and consequently decreased cytokine expression (IL-1 β and TNF α) in the colon.

The nuclear receptor family PPAR are activated by fatty acids, endocannabinoids and their derivatives PPAR γ , which inhibits NF- κ B and consequently exerts an anti-inflammatory effect (Hou *et al.*, 2012). Chen and colleagues (2014) found PPAR γ activation decreased the expression of pro-inflammatory cytokines, decreased I κ B degradation, and therefore decreased NF- κ B activation.

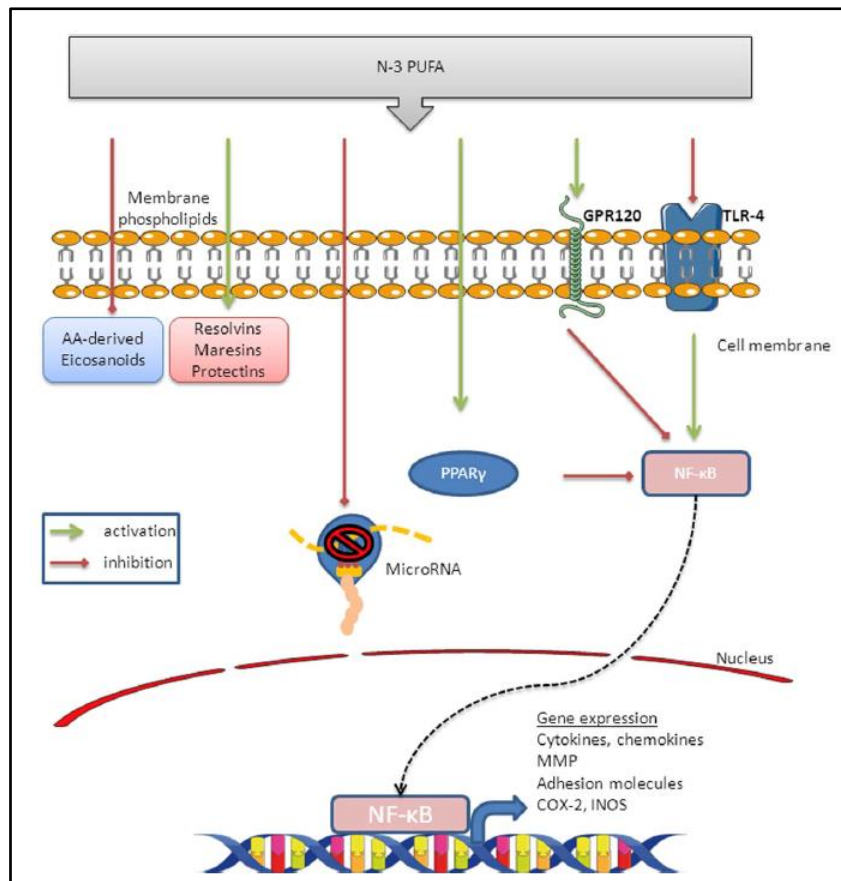


Figure 2-9: n-3 PUFAs reduces the expression of pro-inflammatory cytokines via PPAR γ activation.

PPAR γ activation decreases I κ B degradation therefore inhibits NF- κ B translocation into the nucleus leading to the activation of cytokine expression. (Marion-Letellier *et al.*, 2015). Printed with permission from the original authors.

Abbreviations: NF- κ B; Nuclear factor kappa-B; PPAR γ : Peroxisome proliferator-activated receptor γ

2.4 Interactions between iron and omega-3 polyunsaturated fatty acids

Multiple animal studies have found ID alters lipid metabolism (Sherman *et al.*, 1982; Tichelaar *et al.*, 1997; Kwik-Urbe *et al.*, 2000). As described above n-3 LCPUFA synthesis from the precursor LA is dependent on desaturase enzymes. Iron acts as a cofactor for the desaturases thus ID can further exacerbate a pro-inflammatory state in individuals with n-3 FAD. ID was shown to specifically impair the conversion of ALA to EPA via D5D and the conversion of DPA to DHA via D6D (Smuts *et al.*, 2005; Krajcovicova-Kudlackova *et al.*, 2004).

Cunnane and colleague found rats fed mildly ID diets for 12 weeks had decreased ARA plasma phospholipid levels whilst the LA and triacylglycerol was increased compared to groups with higher dietary iron intake. These changes were not replicated in the liver fatty acid and lipid

composition analysis. The authors concluded the data shows mild ID leads to significant essential FA metabolism changes (Cunnane and McKadoo, 1987).

Stangl and colleague also investigated the effects of an ID diet for 5 weeks on the FA metabolism in rat serum and liver tissue. The authors found that moderately iron deficient rats also presented with a decreased serum phospholipid level, whilst hepatic phosphatidylcholine (PC) and phosphatidylethanolamine (PE) levels were higher compared to rats fed higher iron diets. The PC and PE fatty acid composition also differed significantly with moderate ID which the authors concluded, point to compromised desaturation by Δ -9 and Δ -6 desaturases of saturated and essential fatty acids (Stangl & Kirchgessner, 1998).

Human studies investigating the effects of n-3 FA supplementation on iron status found different functional outcomes. Perunicic- Pekovic and colleagues found daily n-3 FA supplementation of 2.4 g for 8 weeks in patients undergoing haemodialysis (HD) significantly increased blood haemoglobin levels (Perunicic- Pekovic *et al.*, 2007). Conversely, Donnelly and colleagues found daily supplementation of 3.6 g n-3 fatty acids in patients with HD for 4 weeks resulted in no significant alteration of blood haemoglobin concentration (Donnelly *et al.*, 1992). Notably these results may be influenced by the role the kidneys play in RBC synthesis.

Similar studies on healthy and diseased humans of various age groups were also conducted, measuring the effects of supplementing EPA together with DHA on plasma inflammatory biomarkers (IL-6, TNF- α and CRP) found an association between low EPA and DHA levels and circulatory cytokine levels present (Itariu *et al.*, 2012; Rangel-Huerta *et al.*, 2012; Haghiac *et al.*, 2015).

Jamieson and colleagues observed Canadian Inuit women who commonly present with ID and anaemia although animal derived iron sources are traditionally consumed. They found the participants presented with a high prevalence of *H. pylori* exposure as well as adequate dietary iron intake, thus the ID and anaemia are likely caused by hepcidin expression via inflammation. Furthermore, increased LCPUFA percentage of red blood cells was associated with a decreased risk of iron depletion among food insecure women. The authors concluded the findings indicated the anti-inflammatory effects of LCPUFAs may improve iron availability in this population (Jamieson *et al.*, 2013).

The effects of n-3 LCPUFA supplementation, using a 500 mg fish oil capsule containing 45 mg EPA and 225 mg DHA, in obese children with low grade inflammation was investigated by Sidiartha and colleagues in a double-blind randomised clinical control trial. Serum IL-6, hepcidin,

iron, ferritin and transferrin saturation levels were investigated compared to 500 mg coconut oil. Apart from the supplementation no dietary changes were implemented but dietary intake was analysed using a three-day food record. Supplementation with EPA and DHA for 8 weeks lead to significantly decreased serum IL-6, hepcidin and ferritin. Serum iron and transferrin saturation were increased (Sidiartha *et al.*, 2017).

Furthermore, the membrane fluidity influenced by dietary FA intake can influence iron uptake by cells. This was investigated by Rodriguez and colleagues in rats to determine whether dietary FA intake affects iron absorption in relation to lipid peroxidation in the erythrocyte membranes. Rats were divided into three dietary groups (8 rats per group) and fed either a low n-3 PUFA/high LA diet (control), high n-3 PUFA diet (sardine oil) or a diet high in saturated FAs (tripalmitin). Radiolabelled ferrous sulphate (FeSO_4) was used to monitor iron status and lipid peroxidation. Iron absorption was significantly higher in the rats fed sardine oil, compared to the control diet. However, compared to the control group, iron absorption was significantly lower in the rats fed the tripalmitin diet (Rodriguez *et al.*, 1996).

2.5 Effects of combined iron and fatty acid deficiencies on hepcidin expression

To our knowledge studies investigating the effects of the combination of FAD and ID on hepcidin regulation are scarce. Studies focusing on cognitive outcomes have found accumulative effects of the combined deficiencies on cognitive outcomes. Baumgartner and colleagues investigated the effects of combined ID and FAD on cognitive outcomes in an animal model (Baumgartner *et al.*, 2012a; Baumgartner *et al.*, 2012b; Baumgartner *et al.*, 2012c). The combined deficiencies in male rats altered brain monoamine metabolism and caused a memory deficit. The effects of the combined deficiencies were greater than ID or n-3 FAD alone (Baumgartner *et al.*, 2012a). In this model, we will study the effects of both ID and n-3 FAD as well as the combined deficiencies on the expression of hepcidin.

CHAPTER 3: MANUSCRIPT

To be submitted to the Journal of Nutritional Biochemistry. Please see author guidelines in Annexure C.

The effects of n-3 fatty acid and iron deficiency, alone and in combination, on hepcidin pathway gene expression and plasma lipid mediators in rats

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Key words: Hepcidin, *HAMP*, n-3 fatty acids, iron deficiency, lipid mediators, inflammation.

Abstract

Background: Hepcidin is the master regulator of iron absorption and storage. Hepcidin is encoded by the *HAMP* gene and the expression in the liver is regulated by two main pathways activated by iron levels (BMP/SMAD pathway) and inflammation (JAK/STAT3 pathway). *HAMP* transcription is downregulated by iron deficiency (ID) and upregulated by inflammation. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) act as precursors for the synthesis of anti-inflammatory and inflammation-resolving lipid mediators. It is known that inadequate n-3 fatty acid intake can lead to a decreased ability by the host to resolve inflammation and sets off pro-inflammatory signalling. This may lead to the induction of upregulated *HAMP* expression due to pro-inflammatory signalling. Therefore, a combined n-3 fatty acid deficiency (n-3 FAD) and ID may lead to the upregulation of *HAMP* transcription via inflammation, although downregulated transcription is expected in an ID state.

Study design: Thirty-nine male Wistar rat pups born to females fed n-3 FAD or control diet, were included into the 2x2 factorial study and fed either a 1) control, 2) ID, 3) n-3 FAD or 4) ID + n-3 FAD diet. *HAMP*, *BMP2* and *TFR2* expression was analysed using quantitative real-time polymerase chain reaction (RT-qPCR). Lipid mediator concentrations in plasma were analysed using liquid chromatography-tandem mass spectrometry (LCMSMS).

Results: *HAMP* expression was significantly upregulated by ID, whilst *BMP2* and *TFR2* expression were both downregulated. There was a significant effect of ID for higher ARA-derived 15-HETE, but lower 5-HETE. Furthermore, there was a significant effect of ID for higher DHA-derived 17-HDHA, whereas 17-HDHA was lowered by n-3 FAD. When compared to the control group, 17-HDHA was significantly higher in the ID group but lower in the n-3 FAD group. 17-HDHA concentrations in the ID+n-3 FAD group did not differ from the control group ($p = 0.99$). *HAMP* expression correlated positively with 15-HETE and 17-HDHA and negatively with 5-HETE.

Conclusion: We showed that ID upregulated *HAMP* expression seemingly via inflammation, as the expression of *BMP2* and *TFR2* responsible for inducing *HAMP* transcription via host iron availability was downregulated. The presence of an inflammatory state was also reflected in the increased pro-inflammatory 15-HETE and pro-resolving 17-HDHA levels, as a need for resolving lipid mediators occurs in a response to inflammation. N-3 FAD alone did not induce *HAMP* expression, but may compromise the inflammation-resolving capacity of rats with a concomitant ID.

1. Introduction

Iron deficiency (ID) is the most common nutrient deficiency globally. Young children have a higher risk for the development of ID due to increased needs for growth and development [1-2]. ID in early childhood can lead to impaired physical and cognitive development, as well as altered immune function [3-5]. Inadequate intake of iron from dietary sources, poor and or altered iron absorption, blood loss, as well as haemoglobinuria (iron loss through urine) can lead to the development of ID [6].

The presence of inflammatory cytokines can alter tissue iron availability. Iron absorption is influenced by the presence or absence of the regulatory hormone hepcidin. Hepcidin is synthesised in the liver to decrease iron availability by inhibiting absorption in the gut and inhibiting the release of the already absorbed iron available from macrophages through recycling. Hepcidin is encoded by the *HAMP* gene (previously known as *LEAP-1*) and its expression is regulated via iron levels, inflammation, hypoxia or erythropoiesis (erythrocyte synthesis) by two pathways, namely JAK/STAT3 and BMP/SMAD. If iron stores are adequate and with a lack of hypoxic stimulation, *HAMP* transcription is upregulated to prevent iron overload via the BMP/SMAD pathway. Conversely, host inflammation signalling also upregulates *HAMP* transcription via the JAK/STAT3 pathway [7-9]. Iron mediated signalling occurs by *TFR2*, *BMP2* and *BMP6* bound receptors leading to SMAD 1/5/8 phosphorylation allowing SMAD4 to bind to SMAD 1/5/8. Hereby, *HAMP* transcription occurs via the SMAD binding site [10-11]. *HAMP* transcription can also be activated via the STAT3 binding site of the JAK/STAT3 pathway where interleukin 6 (IL-6) binds to the IL-6 receptor and phosphorylates STAT3 [12-15].

In turn, the long chain polyunsaturated fatty acid (LCPUFA) status can affect IL-6 signalling cascades. The n-3 LCPUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are derived from the precursor n-3 PUFA, α -linolenic acid (ALA) [16]. Both EPA and DHA act as precursors for bioactive lipid metabolites and acts as anti-inflammatory mediators by inhibiting the production of cytokines and decreases the recruitment of leukocytes. Some of the lipid mediators derived from EPA include 5-hydroxyeicosapentaenoic acid (HEPE), 11-, 12 -, 15 - and 18-HEPE. 17- hydroxydocosahexaenoic acid (17-HDHA) is derived from DHA. Whereas mainly pro-inflammatory hydroxyeicosatetraenoic acid (HETE) lipid mediators are derived from arachidonic acid (ARA) (metabolite of omega-6 (n-6), Linoleic acid (LA)) [17-22]. n-3 PUFAs plays an antagonising role in the n-6 metabolism by competing for the same enzymes. Inclusion of EPA and DHA in the phospholipid membrane also occurs at the expense of ARA, thus decreases the

availability of ARA as a precursor for pro-inflammatory lipid mediator production, therefore attenuating inflammation [22].

McNamara and colleagues found the DHA erythrocyte composition of rats fed a diet rich in or a diet deficient in ALA was inversely correlated with plasma cytokines including IL-6. Thus, an n-3 FA deficiency (FAD) presented with significantly increased pro-inflammatory cytokine levels, whilst a diet rich in (sufficient in) n-3 decreased cytokine levels [23]. Consequently, inadequate n-3 FA status may lead to a potential upregulation of *HAMP* expression due to increased levels of circulating pro-inflammatory cytokines. Furthermore, iron plays an important role as a co-factor of the catalytic sites of the enzymes responsible for synthesis of EPA-and DHA-derived bioactive lipid mediators potentially decreasing the resolving of inflammation [24-26].

Nutrient deficiencies rarely occur in isolation [27], thus ID and n-3 fatty acid deficiency (FAD) may occur within the same population or individual. It is not known whether n-3 FAD alone can lead to the upregulation of hepcidin, and if so, whether this upregulation would remain in an already ID state where downregulated hepcidin transcription via the BMP/SMAD pathway is expected. Using a 2x2 design, we therefore determined the effects of n-3 FAD and ID, alone and in combination, on hepcidin regulation and lipid mediator signalling in rats.

2. Materials and methods

All experimental procedures were approved by the Veterinary Office of the Department of Health of the Canton of Zürich (No:60/2008) as well as the AnimCare Ethics Committee of the Faculty of Health Sciences of the North-West University (NWU-00587-19-A5) [28].

2.1 Animals, housing, diets and study design

Female Wistar rats (3wk old) were fed a standard (Teklad Global Diet 2018S) or an n-3 FAD diet at a commercial animal breeder (RCC Ltd. Biotechnology and Animal Breeding Division, Füllinsdorf, Switzerland). The female rats were mated at 11 weeks old with male breeders (12 weeks old) of the same strain. The pregnant females continued to consume their respective diet and the rat pups were kept with their dams until weaning. In total, 39 male rat pups were included into the study at 21 ± 3 days of age (postnatal day (PND) 21). All rats were housed individually in stainless steel cages at $21 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ relative humidity, and at a 12-h reversed light/dark-cycle (lights off 08:00-20:00). The rats were handled daily to acclimatise them to human touch and body weights were recorded 3 times/wk. Individual food intake was recorded daily by subtracting the amount of food left in the food bowl from the amount of food provided the previous day, considering the amount of food spilt on a paper, lined underneath the wired cage floor.

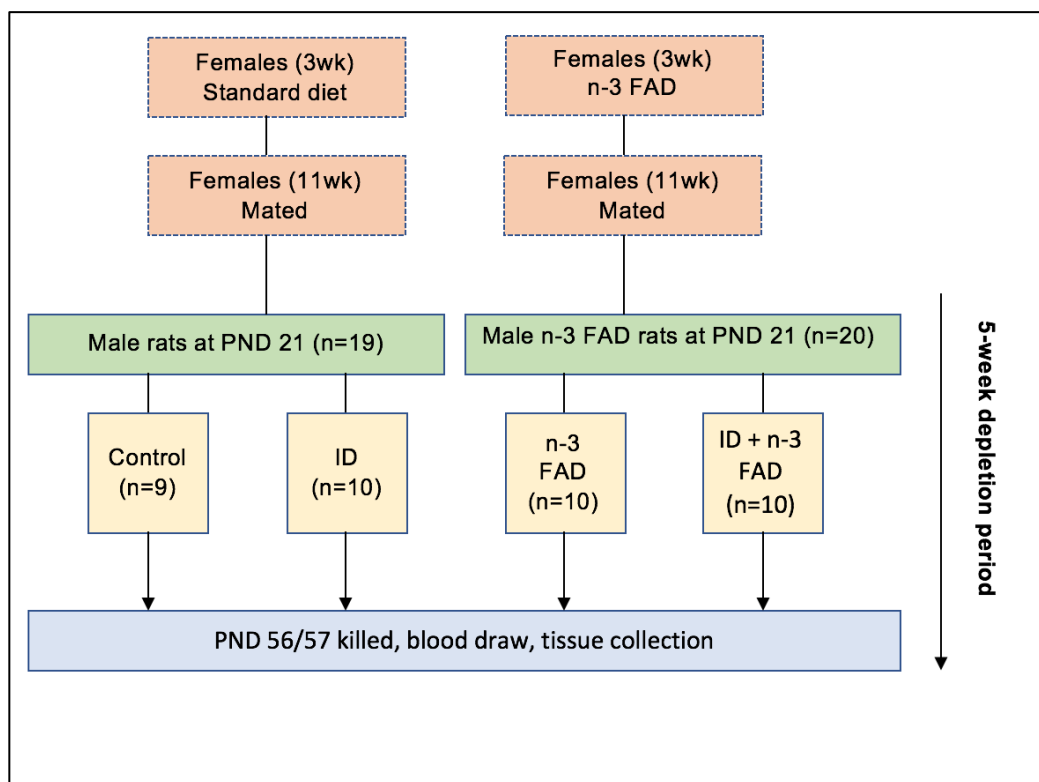


Figure 3-1: Flow diagram of the experimental design; n indicates the numbers in each group.

Boxes in dashes indicate that these parts of the experiment were done at a commercial animal breeding centre. (n - 3) FAD, (n - 3) fatty acid deficient; ID, iron deficient.

Abbreviations: FAD: fatty acid deficient; ID: iron deficient; PND: postnatal day.

The study had a 2 x 2 design and, as described by Baumgartner and colleagues (2012), male Wistar rats born to females receiving a standard diet (n = 19) were randomly allocated to a control (n = 9) and iron deficient (ID) (n = 10) group (28). Illustrated in figure 3-1, male rats born to females receiving the n-3 FAD diet (n = 20) were randomly allocated into two groups either receiving an n-3 FAD (n = 10) or an ID + n-3 FAD diet (n = 10).

Commercially (Dyets Inc., Bethlehem, USA) obtained purified experimental diets were based on the AIN-93G [29] formulation (Table 3-1) with modifications in iron content and fat source. The basal AIN-93G formulation included soybean oil at 70 g/kg diet and hydrogenated coconut oil at 30 g/kg diet, thus adding up to 10% fat. The n-3 FAD diets also contained 10% fat, but included 81 g/kg diet hydrogenated coconut oil and 19 g/kg diet safflower oil. Based on the FA composition of the oils in the basal and n-3 FAD diet, the basal diet (control and ID) was estimated to provide

35 g/kg diet LA and 5 g/kg diet ALA, and the n-3 FAD diet 15 g/kg diet linoleic acid and 0.04 g/kg diet ALA. The iron-sufficient diets contained 35 mg Fe/kg and the ID diets during the depletion period contained 3.0 mg Fe/kg. Spot samples were used to confirm the iron concentration in the different diets using atomic absorption spectrometry. Individual food intake was recorded. Their respective rat diet and Millipore water (Milli-Q UF Plus, Millipore) was provided *ad libitum*.

Table 2-1: Composition of diet

Ingredients¹		g/kg diet
Casein, vitamin free		200.0
Carbohydrate		600.0
	Cornstarch	368.0
	Sucrose	100.0
	Dextrose	132.0
	Cellulose	50.0
Mineral mix ²		35.0
Vitamin mix		10.0
L-Cysteine		3.0
Choline bitartrate		2.5
TBHQ		0.02
Fat		100.0
	n-3 FAD³	n-3 FA sufficient⁴
Coconut oil (hydrogenated)	81.0	30.0
Soybean oil	0.0	70.0
Safflower oil	19.0	0.0

¹Experimental diets were based on the AIN-93G formulation with modifications in iron content and fat source.²The mineral mix provided 35 mg iron/kg diet in the iron sufficient diets and 3 mg iron/kg diet in the iron deficient diets. ³Providing approximately 15.1 g/kg diet linoleic acid (LA) and 0.04 g/kg diet α -linolenic acid (ALA).⁴Providing approximately 35.7 g/kg diet LA and 4.8 g/kg diet ALA.

At the end of the depletion period (PND 56 and 57), the rats were exposed to CO₂ to induce unconsciousness for blood collection by cardiac puncture and then killed by decapitation. Blood was collected in EDTA coated vacutainers and separated by centrifugation. Plasma, red blood

cells and buffy coat were collected and stored for analysis. Tissue samples, such as liver, were collected and snap frozen with liquid nitrogen. Amongst other, liver and plasma aliquots were shipped to the North-West University on dry ice in smaller aliquots allocated to the different analysis and stored at -80°C until use. A study conducted by Andreasson and colleagues found that long term storage of tissues in -80°C for nearly three decades does not adversely affect the quality of the RNA extracted from the stored tissues [30].

2.2 Iron status and red blood cell total phospholipid fatty acid composition

2.2.1 Iron status

Haemoglobin concentrations were measured in whole blood directly after euthanasia using a Scil vet abc counter (Scil Animal Care Company, Viernheim, Germany).

2.2.2 Total phospholipid fatty acid composition

Red blood cell total phospholipid fatty acid analyses were performed with gas chromatography-tandem mass spectrometry (GC/MS/MS). In brief, phospholipids were extracted from RBC with chloroform: methanol (2:1 vol: vol; containing 0.01% butylated hydroxytoluene) by using a modification of the method described by Baumgartner and colleagues as well as Folch and colleagues [28,31]. Lipid extracts were concentrated, and thin-layer chromatography was used to separate the neutral lipids from the phospholipids (silica gel 60 plates, 10x 20 cm; Merck) and eluted with diethyl ether: petroleum, ether: acetic acid (30:90:1 vol: vol: vol). The lipid bands containing the phospholipids were removed from the thin-layer chromatography plate and transmethylated with methanol: sulphuric acid (95:5 vol: vol) at 70 °C for 2 hours to yield fatty acid methyl esters (FAMES).

The resulting FAMES were extracted with water and hexane. The organic layer was evaporated and re-dissolved in hexane. FAMES were analysed with chemical ionization on an Agilent Technologies 7890A Gas Chromatograph system equipped with an Agilent Technologies 7000GC/MS triple quad mass selective detector (Agilent Technologies, Santa Clara, United States of America). The gas chromatography separation of FAMES was carried out on an HP88 capillary column (100 m x 0.25 mm x 0.20 µm; Agilent) by using helium as the carrier gas at a flow rate of 2.2 mL/min. The gas chromatography injector was held at a temperature of 270 °C, and the mass spectrometry source at 250 °C. The injection volume of the sample solution was 1 µL by using a split ratio of 1:10. The oven temperature was programmed to rise from 50 °C to 170

°C at 30 °C/min, then from 170 °C to 215 °C at 2 °C/min, and lastly at 4 °C/min to 230 °C. After that, the temperature was held isothermally at 230 °C for 7 min. The total analysis time was 38.25 min. Mass spectrometry was carried out in multiple reactions monitoring mode, with at least two transitions per compound. Quantification of FAMES was performed with Masshunter (B.06.00). FAME peaks were identified and calibrated against a standard reference mixture of 33 FAMES (Nu-Check-Prep) and two single FAME standards (Larodan Fine Chemicals AB). Relative percentages of fatty acids were then calculated by taking the concentration of a given fatty acid derivative as a percentage of the total concentration of all fatty acids identified in the sample.

2.3 Gene expression analysis

2.3.1 RNA isolation and quality assessment

Liver tissue samples of all rats from the four diet groups were homogenised and digested using a method as described by Erikson and colleagues [32]. Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer prescription. In short, 100 mg tissue in 500 µl Trizol samples were kept on ice. Chloroform (100 µl) was added and the mixture was vigorously mixed for 5 seconds followed by 5 min incubation (on ice) and centrifugation at 12 000 g for 5 minutes at 4 °C. The supernatant was carefully transferred to a clean 1.5 ml Eppendorf tube followed with the addition of 200 µl ice cold isopropanol. The content was mixed through inversion and then centrifuged for 20 min at 12 000 g and 4 °C. The supernatant was carefully discarded, and the pellet was washed twice, firstly with 80 % ethanol and secondly with 70 % ethanol and centrifugation. The supernatant was discarded, and the pellet was air-dried after which it was suspended in 50 µl nuclease-free water. The concentration and quality of the RNA was assessed with spectrophotometry using a ND-1000 spectrophotometer (NanoDrop Products, Wilmington, USA). Good quality isolated total RNA with a 260:280 ratio of 2 was included for downstream analysis.

2.3.2 cDNA synthesis

cDNA was synthesised using the M-MLV reverse transcriptase (Promega) as prescribed by the manufacturer. Briefly, 1 µg of good quality total RNA from each sample was reverse transcribed using 50 µM Oligo d(T)_{20mer}, and nuclease-free water in a total of 12 µl. The reaction was heated at 65 °C for 5 minutes and cooled on ice for 5 minutes. The reverse transcription reaction total volume of 25 µl was prepared by mixing 5 µl 5X SIV Buffer, 1 µl 100mM DTT, 0.2 µl (200 Units) RNaseOUT™ Recombinant RNase Inhibitor and 0.2 µL Promega® M-MLV Reverse

Transcriptase (200 U/μl). The reverse transcription reaction was added to the annealed RNA. This reaction mix was then incubated at 50–55 °C for 10 minutes and inactivated by incubation at 80 °C for 10 minutes.

2.3.3 Gene expression analysis

Gene expression was analysed with independent quantitative real-time PCR (qPCR) analysis using the Bio-Rad CFX96 cyclor and TaqMan® hydrolysis probe chemistry-based gene expression assays. Relative expression was done for three target genes coding for *hepcidin* antimicrobial peptide (*HAMP*;Rn00584987_m1), bone morphogenetic protein 2 (*BMP2*;Rn00567818_m1) and transferrin receptor (*TFR2*;Rn01056398_m1) to the reference genes mammalian 18S ribosomal RNA (*18S rRNA*;Hs99999901_s1) and beta actin (*actB*; Rn00667869_m1). The analysis was conducted following the standard Taqman® protocol. In short, 100 ng cDNA was analysed in duplicate of 10 μl reactions. The assay 10 μl reaction mix consists of 0.2μL 20X TaqMan® Gene Expression Assay, 5 μl 2X TaqMan® Gene Expression Master Mix and 5 μl RNase-free water. The cycling conditions were as per the manufacturer's recommendations.

Relative gene expression ratios were normalised to the average of the reference genes of 18S rRNA and beta actin (*actB*) and relative mRNA expression were represented as ΔCT [33].

2.4 Lipid mediator extraction and analysis by LCMSMS

Plasma samples were prepared and analysed with liquid chromatography tandem mass spectrometry (LCMSMS) at the North-West University. Plasma concentrations of 17-hydroxydocosahexaenoic acid (17-HDHA); 12-hydroxyeicosapentaenoic acid (HEPE); 5-, 8-, 11-, and 12- hydroxyeicosatetraenoic acid (HETE) were measured.

Lipid mediators were extracted from plasma with solid phase extraction (SPE) using Strata-X (Phenomenex, Torrance, United States of America) as described by Malan and colleagues [34]. The plasma samples (1 ml) were diluted with water and adjusted with 15 % methanol to a final volume of 1 ml. 12-HETE-d8 (500 pg of each), was added to each sample for internal standards. After incubation (30 min on ice) the samples were centrifuged at 3 000 rpm for 8 min to remove any precipitated proteins. The resulting clear supernatants were acidified with 1 N hydrochloric acid to pH 3.0 and immediately applied to SPE cartridges that had been pre-conditioned with 2 ml methanol and 4 ml water. The cartridges were then washed with 10 ml 15% (v/v) methanol, 10

ml water, and 4 ml hexane in succession. Lastly, the lipid mediators were eluted with 6 ml methyl formate. A vacuum manifold (Phenomenex) was used to perform the SPE and the vacuum was regulated by the visibility of single drops from each cartridge. Methyl formate was evaporated under a fine stream of nitrogen and the residue was dissolved in 20 μ l acetonitrile, flushed with nitrogen and stored, at the most, for four days at -20°C before LCMSMS analysis.

Samples were analysed with an Agilent Technologies 6410 MSMS, coupled to an Infinity 1260 HPLC pump (Agilent Technologies, Santa Clara, United States of America). The mass spectrometer used had a mass range of 15–1650 m/z , minimum multiple reaction monitoring (MRM) dwell time of 5 ms with 10 MRMs and a maximum of 99 MRM transitions per time segment. The instrument was operated in negative electrospray ionisation mode. At least two transitions were monitored for each compound, using a dynamic MRM method. The collision and fragment or voltages were optimised with flow injection analysis. The gas temperature and flow was set at 350°C and 12 l/min respectively, and the capillary voltage at 4000 V. Chromatographic analyses was performed on a C18 column (Poroshell, 2.7 μ , 100 x 2.1 mm^2 ; Agilent Technologies) with a flow rate of 0.4 ml/min and column temperature of 50°C . Sample injections were performed with an Agilent G1367B autosampler. The sample chamber temperature was set at 5°C and the injection volume at 15 μ l, subsequent to mixing 5 μ l of sample (in acetonitrile) with 10 μ l water in the autosampler, just before injection. The analysis was performed using an acetonitrile-based system with a flow program, by mixing two solvents (A and B) with programmed ratio changes for optimal separation of compounds. Solvent A was water/glacial acetic acid, 99:1 (v/v) and solvent B 100% acetonitrile. Data were quantified with Masshunter B05 02, using external calibration for each compound and two internal standards to correct for losses and matrix effects during sample preparation and analysis.

2.5 Statistical analysis

Goodness of fit to normal distributions was visualised using both Q-Q plots and histograms. Non-normally distributed data were transformed prior to inferential statistical analysis. Data are presented as mean $\pm$ standard error (SE).

To determine the effects of ID and n-3 FAD, as well as their interactions, on DHA-, EPA- and ARA-derived lipid mediators a 2 x 2 ANOVA (iron [sufficient vs. deficient] x n-3 FA [sufficient vs. deficient]) was performed. In the presence of a significant main effect or interaction, the between-group differences were determined using one-way ANOVA with Tukey's post hoc tests. These

steps were repeated to evaluate the effects of n-3 FAD and ID, alone and in combination, on the expression of *HAMP*, *BMP2* and *TRF2* genes in rat liver tissue. The dCT values were used as the gene expression variables in a 2x2 factorial design.

The Pearson correlation test was used to evaluate the correlations between the log transformed plasma lipid mediator concentrations and the expression of *HAMP*, *BMP2* and *TRF2* genes in rat liver tissue

Data were analysed by using the Statistical Package for the Social Sciences (SPSS) version 25.0 (IBM Corp, Armonk, NY, USA and Excel Office 2016 (Microsoft Corp, Redmond, Washington, USA). Type-1 error (α) was set at 0.05 (5%).

3. Results

The following observations was made as a result of the experimental procedures described above.

Weight gain and food intake

There was a main effect for both ID and n-3 FAD for lower food intake and body weight gain across the 35-day experimental period (Table 3-1). However, only the ID and ID+n-3 FAD groups had significantly lower food intake and body weight gain when compared to the control group.

Table 3-3: Weight gain and food intake of male rats fed an ID, n-3 FAD, ID + n-3 FAD or a

					<i>P</i> value ²		
	Control	ID	n-3 FAD	ID+n-3 FAD	ID	n-3 FAD	ID+n-3 FAD
Total body weight gain (g/35d)	222 ± 9.7 _a	149 ± 3.6 _b	200 ± 7.6 _a	131 ± 6.5 _b	<0.001	0.008	0.82
Mean food intake (g/35d)	564 ± 15.4 _a	423 ± 9.6 _b	512 ± 15.4 _a	402 ± 11.3 _b	<0.001	0.050	0.14

control diet for 5 weeks¹

¹Two-way ANOVA was used to test effects of dietary iron (deficient vs. sufficient) and dietary n-3 FA (deficient vs. sufficient), and iron x n-3 FA interactions and significance indicated by the *p*-value.

²Between-group differences were determined using one-way ANOVA followed by Tukey's post hoc test) and significant differences indicated by superscript lowercase letters.

All data expressed as means ± SEM.

Abbreviations: g/35d: gram/35 days; ID: iron deficiency; n-3 FAD: omega-3 fatty acid deficiency.

Iron status and red blood cell total phospholipid fatty acid composition

We found a significant interaction of ID and n-3 FAD with haemoglobin concentrations (Table 3-2). Both, the ID and ID + n-3 FAD groups had significantly lower Hb concentrations than the

controls, with even lower Hb concentrations in the ID + n-3 FAD compared to the ID group. ID significantly reduced plasma iron levels and increased total iron binding capacity. ID resulted in lower transferrin saturation, resulting in significantly lower transferrin saturation in the ID and ID + n-3 FAD groups compared to the control. Transferrin saturation was also significantly lower in the n-3 FAD group compared to the controls, but did not result in a further decrease in the ID+n-3 FAD group.

ID resulted in significantly lower red blood cell composition of ARA ($p < 0.001$), whilst n-3 FAD showed a tendency towards higher ARA ($p = 0.08$). Both ID and n-3 FAD resulted in significantly lower red blood cell composition of EPA and DHA.

Lipid mediators

There was a significant effect of ID for higher ARA-derived 15-HETE, but lower 5-HETE (Table 3-3). There was a significant effect of n-3 FAD for lower EPA-derived 12-HEPE concentrations. There was a significant effect of ID for higher DHA-derived 17-HDHA, whereas 17-HDHA was lower for n-3 FAD. When compared to the control group, 17-HDHA was significantly higher in the ID group but lower in the n-3 FAD group. 17-HDHA concentrations in the ID+n-3 FAD group did not differ from the control group ($p = 0.99$).

Table 3-4: Iron status and erythrocyte total phospholipid fatty acid composition of male rats fed and ID, n-3 FAD, ID+ n-3 FAD or a control diet for 5 weeks¹

					<i>P</i> value ²		
	Control	ID	n-3 FAD	ID+n-3 FAD	ID	n-3 FAD	ID + n-3 FAD
Iron status							
Hb (g/dl)	148 ± 2.5 _a	56 ± 0.8 _b	146 ± 2.2 _a	45 ± 1.6 _c	<0.001	0.001	0.028
Plasma iron (µmol/L)	52 ± 2.0 _a	15.3 ± 1.0 _b	47 ± 2.8 _a	16 ± 0.7 _b	<0.001	0.26	0.10
TIBC (µmol/L)	119 ± 5.6 _b	204 ± 10 _a	127 ± 4.8 _b	199 ± 15 _a	<0.001	0.86	0.53
TS (%)	45 ± 2.9 _a	7.6 ± 0.5 _c	37 ± 2.4 _b	8.6 ± 0.7 _c	<0.001	0.093	0.03
Erythrocyte total phospholipid fatty acid composition (% of total fatty acids)							
n-6 PUFA							
Arachidonic acid	31.3 ± 0.6 _{a,b}	27.1 ± 0.8 _b	34.6 ± 1.7 _a	27.9 ± 1.3 _b	<0.001	0.08	0.28
n-3 PUFA							
Eicosapentaenoic acid	0.410 ± 0.020 _a	0.15 ± 0.0 _b	0.007 ± 0.006 _c	0.007 ± 0.007 _c	<0.001	<0.001	<0.001
Docosahexaenoic acid	3.48 ± 0.10 _a	1.77 ± 0.21 _b	0.34 ± 0.09 _c	0.03 ± 0.02 _c	<0.001	<0.001	<0.001

¹Two-way ANOVA was used to test effects of dietary iron (deficient vs. sufficient) and dietary n-3 FA (deficient vs. sufficient), and iron x n-3 FA interactions and significance indicated by the *p*-value.

²Between-group differences were determined using one-way ANOVA followed by Tukey's post hoc test) and significant differences indicated by superscript lowercase letters.

All data expressed as means ± SEM.

Abbreviations: Hb: Haemoglobin; ID: iron deficiency; n-3 FAD: omega-3 fatty acid deficiency; TIBC: Total iron binding capacity; TS: Transferrin saturation.

Table 3-5: Plasma lipid mediators and expression of iron metabolism genes in male rats fed an ID, n-3 FAD, ID + n-3 FAD or a control diet for 5 weeks¹

					<i>P</i> value ²		
	Control	ID	n-3 FAD	ID+n-3 FAD	ID	n-3 FAD	ID + n-3 FAD
Plasma lipid mediators: ARA- derived							
15-HETE	1.06 ± 0.06	1.25 ± 0.11	0.99 ± 0.07	1.27 ± 0.13	0.019	0.83	0.63
12-HETE	1.99 ± 0.10	2.11 ± 0.08	1.98 ± 0.09	2.07 ± 0.09	0.23	0.76	0.91
11-HETE	0.99 ± 0.12	0.87 ± 0.18	0.69 ± 0.09	0.93 ± 0.21	0.71	0.43	0.26
8-HETE	0.58 ± 0.14	0.61 ± 0.17	0.40 ± 0.16	0.62 ± 0.21	0.46	0.61	0.58
5-HETE	2.86 ± 0.04 _a	2.67 ± 0.03 _b	2.83 ± 0.04 _a	2.70 ± 0.02 _b	<0.001	0.99	0.45
Plasma lipid mediators: EPA- derived							
12-HEPE	1.40 ± 0.04 _a	1.33 ± 0.06 _a	1.02 ± 0.05 _b	0.93 ± 0.01 _b	0.08	<0.001	0.82
Plasma lipid mediators: DHA- derived							
17-HDHA	1.39 ± 0.05 _b	1.90 ± 0.14 _a	1.03 ± 0.05 _c	1.43 ± 0.11 _b	<0.001	<0.001	0.54
Expression of iron metabolism genes							
<i>HAMP</i>	-9.05 (± 1.16) _b	0.11 (± 1.01) _a	-8.02 (± 0.74) _b	2.40 (± 1.52) _a	<0.001	0.19	0.61
<i>BMP2</i>	10.18 (± 0.76)	7.18 (± 1.35)	9.85 (± 0.68)	8.00 (± 1.40)	0.050	0.84	0.63
<i>TFR2</i>	7.54 (± 0.84) _{a,b}	5.51 (± 0.87) _b	9.16 (± 0.80) _a	6.91 (± 0.77) _{a,b}	0.020	0.09	0.90

¹Two-way ANOVA was used to test effects of dietary iron (deficient vs. sufficient) and dietary n-3 FA (deficient vs. sufficient), and iron x n-3 FA interactions and significance indicated by the *p*-value.

²Between-group differences were determined using one-way ANOVA followed by Tukey's post hoc test) and significant differences indicated by superscript lowercase letters.

Data expressed as means ± SEM

³All the lipid mediators were measured in picogram (pg) per microliter (μL) normalised to 50 milligram (mg) plasma [pg / μL / 50 mg tissue]

Gene expression expressed as ΔCT.

Abbreviations: HDHA: Hydroxydocosahexaenoic acid; HEPE: Hydroxyeicosapentaenoic acid; HETE: Hydroxyeicosatetraenoic acid; ID: iron deficiency; n-3 FAD: omega-3 fatty acid deficiency

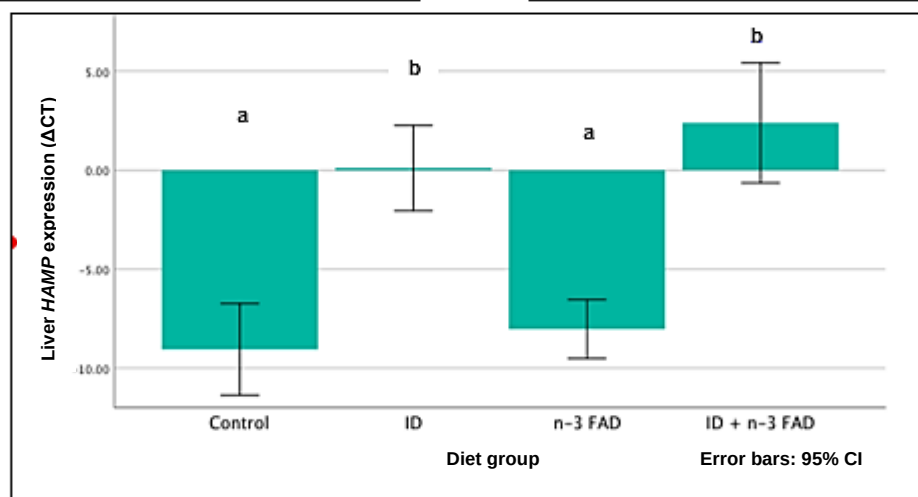
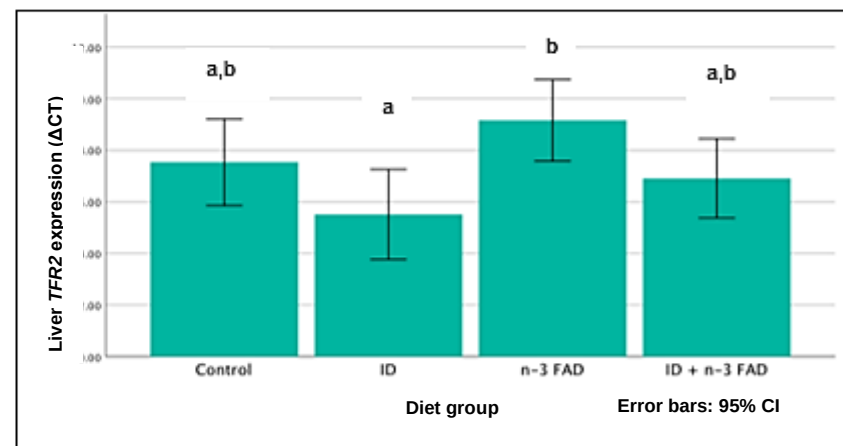
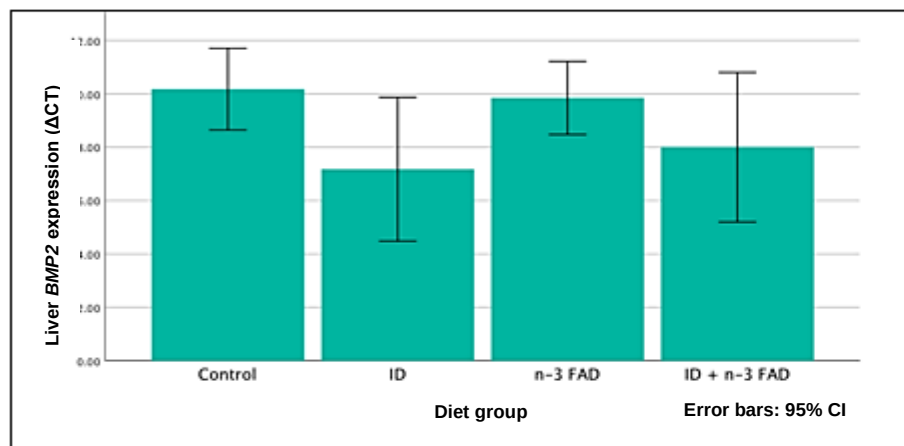


Figure 3-2: Bar chart of the gene expression for the 4 diet groups a) HAMP; b) BMP2; c) TFR2 in liver tissue

¹Two-way ANOVA was used to test effects of dietary iron (deficient vs. sufficient) and dietary n-3 FA (deficient vs. sufficient), and iron x n-3 FA interactions and significance indicated by the p-value.

²Between-group differences were determined using one-way ANOVA followed by Tukey's post hoc test) and significant differences indicated by superscript lowercase letters.

All data expressed as means ± SEM.

Gene expression expressed as ΔCT.

Abbreviations: ID: iron deficiency; n-3 FAD: omega-3 fatty acid deficiency.

Gene expression

There was a main effect of ID for significantly higher *HAMP* expression (Figure 3-2, $p < 0.001$). *HAMP* expression was significantly higher in both the ID and ID + n-3 FAD groups compared to controls. Furthermore, there was a main effect of ID for lower expression of *TFR2* ($p = 0.020$), whereby the ID alone group had lowered *TFR2* compared to the n-3 FAD alone group, but did not differ significantly from the control and ID + n-3 FAD group. There was also a trend for ID to lower *BMP2* expression ($p = 0.050$). There was no significant effect of n-3 FAD on gene expression.

Table 3-6: Pearson correlation coefficient for *TFR2*, *BMP2* and *HAMP* gene expression, ARA-derived 15-HETE and EPA- derived 17-HDHA across the Control, ID, n-3 FAD and ID + n-3 FAD.

Gene expression and plasma lipid mediator concentration		Correlation coefficient (r)	p-Value
<i>TFR2</i>	17-HDHA	-0.485	0.007
	<i>BMP2</i>	0.513	0.001
<i>HAMP</i>	15-HETE	0.381	0.041
	17-HDHA	0.430	0.017
	5-HETE	-0.543	0.002
15-HETE	17-HDHA	0.745	<0.001

Abbreviations: ARA: Arachidonic acid; EPA: Eicosapentanoic acid; HDHA: Hydroxydocosahexaenoic acid; HEPE: Hydroxyeicosapentaenoic acid; HETE: Hydroxyeicosatetraenoic acid; ID: iron deficiency; n-3 FAD: omega-3 fatty acid deficiency

Correlations between lipid mediators and gene regulations

Across diet groups *TFR2* expression correlated positively with *BMP2* expression ($r = 0.513$, $p < 0.001$), whilst correlating negatively with 17-HDHA plasma levels ($r = -0.485$, $p < 0.001$) (Table 3-4). *HAMP* expression correlated positively with 15-HETE and 17-HDHA plasma levels ($r = 0.381$, $p < 0.05$ and $r = 0.439$, $p < 0.05$ respectively) and negatively with 5-HETE plasma levels ($r = -0.543$, $p < 0.01$). 15-HETE correlated positively with 17-HDHA plasma levels ($r = 0.745$, $p < 0.001$).

4. Discussion

To our knowledge, this is the first study investigating the effects of ID and n-3 FAD, alone and in combination, on *HAMP* regulation and plasma lipid mediators. We expected ID to downregulate *HAMP* expression via the BMP/SMAD pathway and hypothesized that n-3 FAD may upregulate *HAMP* expression via the JAK/STAT3 pathway by inducing a pro-inflammatory state. However, in contrast to our hypothesis, we found that the provision of an ID diet for five weeks to growing male rats resulted in a significant upregulation of *HAMP* expression, both alone and in combination with n-3 FAD. However, the expression of *BMP2* and *TFR2* were both downregulated by ID. The upregulation of *HAMP* may therefore be explained by the presence of inflammation, which may have led to the upregulation of *HAMP* expression via the JAK/STAT3 pathway [35-36]. We were able to confirm the presence of an inflammatory state in the ID groups by showing that ID resulted in significantly higher plasma levels of ARA-derived and pro-inflammatory 15-HETE, as well as inflammation-resolving 17-HDHA derived from DHA in response to inflammation [37]. This is in agreement with a previous study by Pagani and colleagues who found that ID in mice caused a pro-inflammatory state indicated by high white blood cell count and increased expression of inflammatory cytokines (IL-6 and TNF- α) [38]. In our study, both 15-HETE and 17-HDHA correlated positively with *HAMP* expression. 15-HETE and 17-HDHA also correlated positively with each other therefore, as the pro-inflammatory 15-HETE increased, 17-HDHA synthesis is also increased in response to resolve inflammation.

Several studies have investigated the effects of ID on immune function, more specifically cellular immune function. Apart from the role iron plays in LOX enzymes synthesis, ID also impairs lymphocyte activation and proliferation as well as macrophage differentiation [39-40]. In this study as shown by the haemoglobin concentration, the rats in the ID group were severely anaemic which could compromise the oxygen availability thus leading to a hypoxic state. Hypoxia can regulate the expression of various genes by hypoxia-inducible transcription factor (HIF) and studies have shown localised hypoxia activates the transcription factor Nuclear factor kappa B (NF-kB), which in turn increases the presence of inflammatory cytokines leading to an inflammatory state [41-42]. Due to the increased metabolic needs (oxygen) during inflammatory processes studies also show localised hypoxia in areas with inflammation, further increasing the need for oxygen [43-44].

Conversely to our study, Stoffel and colleagues investigated the effects of inflammation on the serum hepcidin levels in healthy women and women with iron deficient anaemia (IDA). Acute

inflammation was introduced with an influenza vaccine and serum IL-6 levels were measured. They found mild acute inflammation did not increase serum hepcidin in women with IDA, suggesting low iron status and a need for erythropoiesis offsets the upregulation of hepcidin via inflammation (JAK/STAT3 pathway) [45]. Although our study found different results, the rats in our study were severely iron deficient anaemic (haemoglobin < 60g/L) [46]. This may indicate that the need for erythropoiesis (and inhibition of hepcidin via the BMP/SMAD pathway) may be overpowered by the inflammatory stimuli to increase hepcidin expression further down in the development of ID to severe IDA. Thus, indicating the dynamic mechanism of hepcidin regulation via two main pathways interact with different stimuli. In our study, ID was also introduced over a 5-week period thus the inflammatory response was not acute. It should also be noted that hepcidin serum levels are not only dependant on *HAMP* mRNA synthesis (transcription) but the translation of the protein and activity as well, that is dependent on non-transcriptional events, such as protein expression and modifications. Therefore, although our study found an increase in *HAMP* expression this may not necessarily indicate serum hepcidin levels were also increased.

We were not able to show that n-3 FAD alone leads to inflammation and a subsequent upregulation of *HAMP* via the JAK/STAT3 pathway. However, n-3 FAD resulted in lower plasma levels of EPA-derived 12-HEPE and inflammation-resolving 17-HDHA, which can be explained by the markedly reduced EPA and DHA levels in red blood cells. This may compromise the response to inflammatory stimuli, such as ID. We showed here that, while ID resulted in significantly higher plasma 17-HDHA concentrations compared to the control group, this increase seemed to be attenuated in the ID + n-3 FAD group despite the presence of increased 5-HETE levels, indicating a poor inflammation resolving capacity. Between group differences also showed *HAMP* expression was increased in the ID+ n-3FAD group.

Similar to previous studies, this study also found that ID plays a role in the fatty acid erythrocyte composition [47-48]. ID resulted in statistically lower red blood phospholipid levels of n-6 derived ARA and n-3 derived EPA and DHA. Although all the diets maintained the same n-6 PUFA intake, the incorporation of ARA was still decreased in the ID group compared the control group. This may be explained by the role that iron plays as a cofactor in the elongase and desaturase enzyme activity responsible for ARA synthesis from LA ($\Delta 5$ -desaturase and $\Delta 6$ desaturase) [19, 23].

A limitation of this study was that HJV acts as a BMP co-receptor with a high binding affinity (mainly by BMP2 and BMP6) upregulating *HAMP* through BMP-SMAD although BMP6 can also signal in the absence of HJV. TMPRSS6 is also expressed in response to iron deficiency and responsible for inhibiting the BMP-SMAD pathway by cleaving the membrane HJV. Thus, BMP6

and *TMPRSS6* expression might give a better indication of *HAMP* expression in the ID states. Furthermore, IL-6 and other markers of acute and chronic inflammation were not measured. Therefore, it was not possible to confirm the presence or absence of inflammation in the different diet groups indicated by the analysis of lipid mediators. Furthermore, the analysis of the expression of genes active in the JAK/STAT3 pathway would have enabled us to confirm whether *HAMP* upregulation by ID was indeed regulated via the JAK/STAT3 pathway induced by inflammation.

In conclusion, although n-3 FAD may lead to a compromised inflammation resolving capacity, it did not seem to induce inflammation and subsequent upregulation of *HAMP* expression. *HAMP* expression was, however, significantly increased in the ID group with decreased *BMP2* and *TFR2*, although decreased *HAMP* expression was expected and increased expression was expected in the combined deficiency group. This can, however, mean that the effect of inflammation on *HAMP* transcription (via the JAK/STAT3 pathway) is stronger than that of iron levels (via the BMP/SMAD pathway) induced by *TFR2* and *BMP2*. Inflammation as a response to iron deficiency will also further complicate intervention strategies to improve iron status as the increased hepcidin levels lead to the inhibition of ferroportin which is responsible for releasing absorbed iron into circulation.

Future research can investigate this mechanism further by analysing genes involved in the JAK/STAT3 pathway, as well as IL-1 and IL-6 plasma levels. Future studies can also investigate the effects of mild IDA compared to severe IDA as severe hypoxia can further exacerbate the inflammatory effect of iron deficiency. mRNA levels present only gives an indication of *HAMP* transcription thus, measuring serum hepcidin levels might also give additional insight into the activity of hepcidin in an already ID state or in the presence of n-3 FAD.

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CHAPTER 4: OVERALL CONCLUSION

Summary

The overall aim of this study was to determine the effects of n-3 fatty acid and iron deficiency, alone and in combination, on hepcidin regulation and lipid mediator (docosahexaenoic acid (DHA)-, eicosapentaenoic acid (EPA)- and arachidonic acid (ARA)- derived) signalling in rats.

More specifically we investigated the effects of n-3 fatty acid and iron deficiency, alone and in combination, on A) expression of the hepcidin regulatory pathway genes *HAMP*, *BMP2* and *TFR2* in rat liver; B) ARA-derived 5-, 8-, 11-, 12- and 15- hydroxyeicosatetraenoic acid (HETE); EPA-derived 12-hydroxyeicosapentaenoic acid (HEPE) and DHA-derived 17-hydroxydocosahexaenoic acid (HDHA) lipid mediators in rat plasma and C) determined the correlations of *HAMP*, *BMP2* and *TFR2* expression with plasma lipid mediator concentrations in rats.

Conclusion

In contrast to our hypothesis n-3 FAD, alone and in combination with ID did not upregulate *HAMP*. Our study did, however, find that ID increases the expression of *HAMP* whilst decreasing the expression of *BMP2* and *TFR2*. This leads to the assumption that *HAMP* expression was activated by the JAK/STAT3 pathway in response to a pro-inflammatory state. To further strengthen our findings JAK/STAT3 activation should be investigated in future studies. A probable ID induced pro-inflammatory signalling is supported by the increased synthesis of the pro-inflammatory ARA-derived 15-HETE as well as pro-resolving DHA-derived 17-HDHA. 17-HDHA synthesis is activated in response to inflammation. This is also reflected in the results by the positive correlation between 15-HETE and 17-HDHA. Therefore, as the pro-inflammatory 15-HETE increases, 17-HDHA synthesis increased in response to resolve inflammation. n-3 FAD presented with decreased 17-HDHA levels; however, the combined deficiencies attenuated the effects of ID and n-3 FAD in isolation on 17-HDHA synthesis.

As hypothesised, n-3 FAD alone did decrease DHA-and EPA-derived lipid mediators; however, ARA-derived lipid mediators were not significantly increased. Furthermore, we hypothesised that EPA-and DHA-derived lipid mediator concentrations will be inversely correlated with *HAMP* expression, which did not occur, but ARA-derived pro-inflammatory 15-HETE did significantly correlate with *HAMP* expression, further supporting the probable pro-inflammatory state induced by ID.

These results give novel information about the regulation of *HAMP*. Although the JAK/STAT3 pathway is dependent on BMP and SMAD4 signalling for optimal *HAMP* transcription induction (Wang *et al.*, 2005), *HAMP* expression was still increased with decreased *BMP2* and *TFR2* expression. Thus, inflammation induced by severe ID anaemia may have had a stronger effect on the downregulation of hepcidin than ID anaemia had on its upregulation, which would make interventions to improve iron availability more challenging.

Strengths

The strengths of this study include the 2 x 2 factorial design investigating the effects of ID and n-3 FAD both alone and in combination, on hepcidin, and regulatory genes (*HAMP*, *BMP2* & *TFR2*) involved in *HAMP* expression as well as fatty acid derived lipid mediators. This study design makes it ideal to study the main effects of both ID and FAD as well as the interactions between the two deficiencies.

The effects were studied using a rat model, as *in vitro* models would not consider the complex physiological and biochemical nature of the *HAMP* gene cascade. *HAMP* and the regulatory genes involved in *HAMP* expression (*TFR2* & *BMP2*) are primarily transcribed in the liver, and to obtain human liver tissue would not be possible. Inducing nutrient deficiencies will also not be ethical in human trials.

The use of RT-qPCR is a recognised method for gene expression (RNA) analysis. This study used two verified reference genes (*Actb* & *Rn18s*) that are deemed stable in hepatic tissue to measure *HAMP* and the regulatory genes involved in *HAMP* expression (*BMP2* & *TFR2*) (Svingen *et al.*, 2015). LCMSMS is an advanced technique to measure lipid mediators derived from PUFAs (Jónasdóttir *et al.*, 2015; Le Faouder *et al.*, 2013). The red blood cell phospholipid composition data contributes to the lipid mediator measurements as PUFAs that are released from cell membranes into the cytosol by PLA₂ acts as a substrate (precursor) for the COX, LOX and CYTP450 enzymes that produce lipid mediators (Dennis, 2000; Tilley *et al.*, 2001; Goldman *et al.*, 1983; Bagga *et al.*, 2003). Therefore, it gives additional perspective to the lipid mediator level measurements (increased or decreased) as it gives an indication of the variety of precursor availability between the diet groups.

Limitations

Limitations in this study include that only male rats were included, as well as the severe iron deficient anaemia that was induced (haemoglobin < 60g/L), which may not be translatable to the human situation (Davis *et al.*, 2012).

It is known that gene expression in the liver may vary between male and female rats (Laz *et al.*, 2004). Kong and colleagues investigated the differences in iron status as well as hepcidin expression between male and female rats (up to the age of 4 months) and found hepcidin expression was lower in male rats compared to female rats. This occurred with higher TS levels and hepatic iron storage in the females that may be responsible for the increased hepcidin expression (Kong *et al.*, 2014). This might be explained by the study conducted by Ikeda and colleagues that found that the female sex hormone, oestrogen, stimulates hepcidin expression (via the BMP/SMAD pathway) whereas the male sex hormone, testosterone, inhibits hepcidin expression (Ikeda *et al.*, 2012; Guo *et al.*, 2013). Therefore, the use of female rats might give different results from that found in male rats.

FAD was introduced in the mother whilst iron deficiency was only introduced later after birth in the offspring. Serum inflammatory markers were not measured and can also give insight to the symptomatic effect of altered lipid mediator concentrations. Although hepcidin expression is regulated by BMP/SMAD and JAK/STAT, the regulatory genes analysed only form part of the BMP/SMAD pathway. Thus, induction by inflammation through the JAK/STAT pathway cannot be confirmed, only assumed. Apart from the mRNA levels present, hepcidin activity is also dependent on non-transcriptional events, such as protein expression and modifications. Therefore, the measurement of serum hepcidin levels can also give more insight into the activity of hepcidin in an already ID state or in the presence of n-3 FAD.

Future research

This MSc affiliated study was nested within a larger rat project with the aim to determine the effects of maternal iron and n-3 FA depletion and repletion, and potential iron and n-3 FA interactions, on offspring neurodevelopment. The effect of repletion on hepcidin expression and lipid mediator concentrations might give more insight into the role of iron in lipid mediator signalling and hepcidin regulation. The mRNA expression of both enzymes can also give insight to the effect of ID on altered expression.

Genes analysed were only from the BMP/SMAD pathway, and therefore future research should also analyse genes in the JAK/STAT3 pathway considering the role that ID plays in lipid mediators responsible for resolving inflammation. Regarding the JAK/STAT3 pathway serum IL-6 tests can provide more information about an acute inflammatory state and the possible role of inflammation in the expression of *HAMP* in an ID state. Measurement of serum hepcidin levels can also give additional insight into the activity of hepcidin in an already ID state.

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ANNEXURES

Annexure A: Methodology and Ethical Considerations

As described in the original study (Baumgartner *et al.*, 2012), all experimental procedures were approved by the Veterinary Office of the Department of Health of the Canton of Zürich as well as the ethical committees of North-West University. This annexure was included to give more in depth information regarding the methodology and ethical considerations.

Animals and housing

Female Wistar rats (3wk old) were fed a standard (Teklad Global Diet 2018S) or an n-3 FAD diet at a commercial animal breeder (RCC). The female rats were mated (11 weeks old) with the same strain male breeders (12 weeks old). The females continued to consume their respective diet and the rat pups were kept with their dams until weaning.

The male rat pups born were brought to ETH Zürich animal facility (Schwerzenbach) and were included into the study at 21 ± 3 days of age (postnatal day (PND) 21). The rats were individually housed in stainless steel cages, kept under a 12-h reversed light/-dark-cycle (lights off 08:00–20:00h) at $21 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ relative humidity. To acclimatize them to human touch, the rats were handled daily and body weights were recorded 3 times/wk. Individual food intake was recorded daily by subtracting the amount of food left in the food bowl from the amount of food provided the previous day, considering the amount of food spilled on a paper-lined underneath the wired cage floor.

Male Wistar rats born to females receiving a standard diet and was randomly divided into a control (n=9) and iron deficient (ID) (n=10) group. 20 male rats born to females receiving the (n-3) FAD diet (n = 20) were also randomly allocated into 2 groups receiving an n-3 FAD (n = 10) or an ID + n-3 FAD diet (n = 10). Commercially obtained purified experimental diets (Dyats, Bethlehem, PA USA) were based on the AIN-93G formulation (Reeves *et al.*, 1993) with modifications in the iron content and fat source. The basal AIN-93G formulation included 70 g/kg soybean at and hydrogenated coconut oil at 30 g/kg diet, thus adding up to 10% fat. The n-3 FAD diets also contained 10% fat, but this included 81 g/kg hydrogenated coconut oil and 19 g/kg safflower oil (Salem *et al.*, 2001; Harauma *et al.*, 2010; Moriguchi *et al.*, 2013). The estimated FA composition of both the basal and FAD diet was estimated to provide 35 g/kg diet LA and 5 g/kg diet ALA and 15 g/kg diet linoleic acid and 0.04 g/kg diet ALA respectively. The iron-sufficient diets contained 35 mg Fe/kg and the ID diets during the depletion period contained 3.0 mg Fe/kg (Erikson *et al.*,

2002; Fitsanakis *et al.*, 2009). Spot samples were used to confirm the iron concentration in the different diets using atomic absorption spectrometry (AAS). To avoid lipid oxidation all diets were custom prepared in powdered form and vacuum packed in bags of 2 kg, after which it was stored at - 20°C until use. Diets were thawed in a refrigerator at 4 °C (in batches as needed) and kept in an airtight container once opened. The rats had *ad libitum* access to their respective rat diet and Millipore water (Milli-Q UF Plus, Millipore, Bedford, USA).

Study design, tissue collection and sample storage

The study was a 2 x 2 Design (n=19) during a 5 week period the Wistar rats born to females receiving a standard diet (described above/of ek kan 'n table in sit) were randomly divided into a control (n=9) and ID (n=10) group. The male rat pups born to females receiving the (n-3) FAD diet (n = 20) were also randomly allocated into 2 groups receiving an n-3 FAD (n = 10) or an ID + n-3 FAD diet (n = 10) (Ricci *et al.*, 2019).

The rats were exposed to CO₂ to induce unconsciousness for blood collection by cardiac puncture and then killed by decapitation at the end of the depletion period (PND 56 and 57) for tissue collection. Liver tissue were collected and snap frozen with liquid nitrogen and stored at - 80°C. The frozen samples were transported and stored at North-West University in smaller aliquots allocated to the different analysis.

Gene expression analysis

RNA isolation

A study conducted by Andreasson and colleagues found that long term storage of tissues in - 80°C for nearly three decades does not adversely affect the quality of the RNA extracted from the stored tissues (Andreasson *et al.*, 2013).

Liver tissue samples of all rats from the four diet groups were homogenized and digested using a method according to Erikson and colleagues (Erikson *et al.*, 1997). 100 mg liver tissue was homogenized in 500 µL Trizol reagent using the Qiagen TissueLyser II and glass beads. Homogenization occurred at 25 Hz for 10 minutes. Samples were kept on ice between steps, at all times. After, the tissue was completely homogenised, chloroform (100 µL) was added and the mixture was vigorously mixed for 5 seconds followed by 5 min incubation on ice. Phase separation was achieved by centrifugation at 12000 g for 5 minutes at 4 °C. The supernatant was carefully transferred to a clean 1.5 ml Eppendorf tube followed with the addition of 200 µL ice cold

isopropanol. The content was mixed through inversion. Centrifuged for 20 min at 12 000 g and 4 °C, until a small transparent/white pellet was visible in the bottom of the tube. The supernatant was carefully discarded without disrupting or losing the pellet. The pellet was washed twice, firstly with 80 % ethanol and secondly with 70 % ethanol and centrifugation. The supernatant was discarded and the pellet was air-dried after which it was suspended in 50 µL nuclease-free water.

RNA quality assessment

The concentration and quality of the RNA was assessed with spectrophotometry using a ND-1000 spectrophotometer (NanoDrop Products, Wilmington, USA).

cDNA synthesis

cDNA was synthesized using the M-MLV reverse transcriptase protocol (Promega, Madison, WI, USA) as prescribed by the manufacturer. The concentration derived from spectrophotometry was used to calculate the concentration for master mix 1. Briefly, 1 µg of total RNA of each sample was reverse transcribed by adding and thoroughly mixing 1 µl 50 µM Oligo d(T) 20 primer, 10 pg Template RNA per 5 µg total RNA and 12 µl nuclease-free water. The reaction was heated at 65 °C for 5 minutes and cooling it down on ice for 5 minute.

Thereafter, the reverse transcription reaction was prepared by mixing 5 µl SIV Buffer, 1 µl 100mM DTT, 0.2 µl RNaseOUT™ Recombinant RNase Inhibitor and 0.2 µl Promega® M-MLV Reverse Transcriptase (200 U/µl). The 5X SSIV Buffer was vortexed before use. The reaction tubes were mixed and centrifuged briefly. The reverse transcription reaction was added to the annealed RNA. This reaction mix was then be incubated at 50–55°C for 10 minutes and inactivated by incubation at 80°C for 10 minutes.

qPCR analysis

HAMP (Rn00584987_m1), *BMP2* (Rn00567818_m1) and *TFR2* (Rn01056398_m1), was analysed using independent quantitative real-time PCR (qPCR) analysis. The analysis was performed using the Bio-Rad CFX96 cycler (Bio-Rad, Hercules, Ca, USA) and TaqMan® (Thermofisher, Waltham, MA, USA) hydrolysis probe chemistry-based gene expression assays according to the supplier's specifications.

1.0µL of the cDNA samples were used and each reaction was done in duplicate. 1 µL 20X TaqMan® Gene Expression Assay, 10 µL 2X TaqMan® Gene Expression Master Mix and 5 µL RNase-free water as added per 1.0µL cDNA sample to a 1.5 mL nuclease free micro-centrifuge

tube, mixed thoroughly and centrifuged briefly. The qPCR TaqMan Gene Expression Assays (20x) was thawed on ice and mixed by gently swirling the tube. The 9µL reaction mix of each sample was loaded onto 96-well PCR plates, sealed and loaded into the instrument. The cycling conditions applied was as per the manufacturer's recommendation.

Quantification cycle (Cq) was determined for each sample using the comparative Cq method (Pfaffl, 2001). Relative gene expression ratios were normalized to the average of the reference genes such as 18S rRNA and beta actin (*actB*). Relative mRNA expression was represented as the fold change of the target group (intervention group) over the calibrator sample (control group). The fold change calculations or gene expression ratio was calculated using the well- established $\Delta\Delta CT$ method (Livak & Schmittgen, 2001). The differential expression between groups was tested with the Bonferroni multiple comparisons post-hoc test. Differential gene expression was considered statistically significantly when adjusted $P < 0.05$ was obtained.

Lipid mediator extraction and analysis using LCMSMS

Samples were prepared and analysed with liquid chromatography tandem mass spectrometry (LCMSMS) at the North-West University. Tissue17-hydroxydocosaheptaenoic acid (17-HDHA); 12-hydroxyeicosapentaenoic acid (HEPE) and 5-, 8-, 11-, and 12- hydroxyeicosatetraenoic acid (HETE) were measured.

Lipid mediators were extracted from plasma with solid phase extraction (SPE) using Strata-X (Phenomenex, Torrance, CA, USA) as described by Malan and colleagues (Malan *et al.*, 2016). The plasma samples (1 ml) were diluted with water and adjusted with 15% methanol to a final volume of 1 ml. 12-HETE-d8 (500 pg of each), was added to each sample for internal standards. After incubation (30 min on ice) the samples were centrifuged at 3000 rpm for 8 min to remove any precipitated proteins. The resulting clear supernatants were acidified to pH 3.0 with 1 N hydrochloric acid and immediately applied to SPE cartridges, preconditioned with 2 ml methanol and 4 ml water. The cartridges were then washed with 10 ml 15% (v/v) methanol, 10 ml water, and 4 ml hexane in succession. Lastly, the lipid mediators were eluted with 6 ml methyl formate. A vacuum manifold (Phenomenex) was used to perform the SPE and the vacuum was regulated by the visibility of single drops from each cartridge. Methyl formate was evaporated under a fine stream of nitrogen and the residue was dissolved in 20 µl acetonitrile, flushed with nitrogen and stored, at the most, for four days at -20°C before LCMSMS analysis.

Samples were analysed with an Agilent Technologies 6410 MSMS, coupled to an Infinity 1260 HPLC pump (Santa Clara, United States of America). The mass spectrometer used had a mass

range of 15–1650 m/z, minimum multiple reaction monitoring (MRM) dwell time of 5 ms with 10 MRMs and a maximum of 99 MRM transitions per time segment. The instrument was operated in negative electrospray ionisation mode. At least two transitions were monitored for each compound, using a dynamic MRM method. The collision and fragment or voltages were optimised with flow injection analysis. The gas temperature and flow was set at 350 °C and 12 l/min respectively, and the capillary voltage at 4000 V. Chromatographic analyses was performed on a C18 column (Poroshell, 2.7 µ, 100 x 2.1 mm²; Agilent Technologies) with a flow rate of 0.4 ml/min and column temperature of 50 °C. Sample injections were performed with an Agilent G1367B autosampler. The sample chamber temperature was set at 5 °C and the injection volume at 15 µl, after mixing 5 µl of sample (in acetonitrile) with 10 µl water in the autosampler, just before injection. The analysis was performed using an acetonitrile-based system with a flow program, by mixing two solvents (A and B) with programmed ratio changes for optimal separation of compounds. Solvent A was water/glacial acetic acid, 99:1 (v/v) and solvent B 100% acetonitrile. Data were quantified with Masshunter B05 02, using external calibration for each compound and two internal standards to correct for losses and matrix effects during sample preparation and analysis.

Statistical analysis

Goodness of fit to normal distributions will be visualised using both Q-Q plots and histograms. Normally distributed data will be presented as mean ± standard deviation (SD) whereas; data not normally distributed will be transformed for analysis and to improve normality and will be reported as medians (25th - 75th percentiles).

To determine the effects of ID and n-3 FAD, as well as their interactions, on DHA-, EPA- and ARA-derived lipid mediators will perform a 2 x 2 ANOVA (iron [sufficient vs. deficient] x n-3 FA [sufficient vs. deficient]). In the presence of a significant main effect or interaction, between-group differences will be determined using one-way ANOVA with Bonferroni's post hoc tests. These steps will be repeated for objective 2 to evaluate the effects of n-3 FA and ID, alone and in combination, on the expression of hepcidin and its regulatory genes in rat liver tissue. The delta Cq values will be used as the gene expression variable in a 2x2 factorial design.

To determine the correlations between *HAMP*, *TFR*, *BMP2* and the lipid mediators Pearson correlation was used.

Data were analysed by using the Statistical Package for the Social Sciences (SPSS) version 25.0 (IBM Corp, Armonk, NY, USA) and Excel Office 2016 (Microsoft Corp, Redmond, Washington, USA). Type-1 error (α) was set at 0.05 (5%).

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Annexure B: Ethics approval of North-West University for the affiliated study



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**North-West University Animal Care, Health and
Safety Research Ethics Committee (NWU-
AnimCareREC)**

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Email: Ethics-AnimCare@nwu.ac.za (for animal
studies)

24 November 2019

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 24/11/2019, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Effects of n-3 fatty acid and iron deficiency, alone and in combination, on hepcidin regulation, and lipid mediators in rats																														
Principal Investigator/Study Supervisor/Researcher: Dr L Zandberg																														
Student: A Middel - 25196812																														
Ethics number:	<table border="1"><tr><td>N</td><td>W</td><td>U</td><td>-</td><td>0</td><td>0</td><td>5</td><td>8</td><td>7</td><td>-</td><td>1</td><td>9</td><td>-</td><td>A</td><td>5</td></tr><tr><td colspan="3">Institution</td><td></td><td colspan="5">Study Number</td><td></td><td colspan="2">Year</td><td colspan="2">Status</td></tr></table>	N	W	U	-	0	0	5	8	7	-	1	9	-	A	5	Institution				Study Number						Year		Status	
N	W	U	-	0	0	5	8	7	-	1	9	-	A	5																
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Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation																														
Application Type: Single study	Risk: <table border="1"><tr><td>Category 0</td></tr></table>	Category 0																												
Category 0																														
Commencement date: 24/11/2019																														
Expiry date: 30/11/2020																														
Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of November annually until completion.																														

General conditions:
<i>While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:</i>
• <i>The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:</i> – <i>Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and</i> – <i>without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.</i> • <i>The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal</i>

without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.

- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
 - *request access to any information or data at any time during the course or after completion of the study;*
 - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
 - *withdraw or postpone approval if:*
 - *any unethical principles or practices of the study are revealed or suspected;*
 - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
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- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,



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Annexure C: Author guidelines for the Journal of nutritional Biochemistry



THE JOURNAL OF NUTRITIONAL BIOCHEMISTRY

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

• Description	p.1
• Audience	p.1
• Impact Factor	p.1
• Abstracting and Indexing	p.2
• Editorial Board	p.2
• Guide for Authors	p.5



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