

Effects of n-3 polyunsaturated fatty acid and iron deficiency, alone and in combination, during early development on colonic inflammation in rats

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PREFACE

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To my loving family - thanks for allowing me to do what my heart desires, for your cheers, love and inspiration. It was a roller coaster ride, but we did it. You are the best.

ABSTRACT

Background:

It has been shown that poor nutrition during early development has a causal relationship with adverse pregnancy outcomes, morbidity, mortality and the increased risk of short- and long-term adverse health outcomes for the child and future generations. Furthermore, a mother's nutritional status has an impact on the unborn baby, possibly through epigenetic mechanisms.

In developing countries, women are reliant on poor-quality diets. In these circumstances, nutrient deficiencies do not occur in isolation, and it is likely that in pregnant women, iron deficiency (ID) and inadequate omega-3 polyunsaturated fatty acid (n-3 PUFA) status coexist. Both deficiencies play a role in the development of colon inflammation. Inflammation in the gut and colon can lead to dysbiosis of the microbiota, compromised tight junctions, increased gut permeability, and pathogens to enter the bloodstream. The result is low-grade systemic inflammation contributing to the development of gastrointestinal diseases such as irritable bowel syndrome and also to the development of adult diseases such as diabetes, obesity, cancer, and neuro-pathologies. Therefore, optimum nutrition before and during pregnancy is crucial for the gut development and future health of the offspring. Apart from being reliant on nutrients from the mother's diet during gestation, offspring in the human situation will most likely also continue consuming a similar diet to that of their mother. Data on the combined ID and n-3 PUFA deficiency ((n-3)FAD), specifically in relation to gut inflammation, is scarce.

Aim: Therefore, in this study, the pre-and postnatal effects of ID and (n-3)FAD, alone and in combination, on colon lining lipid mediator concentrations, which indicates colon inflammation, will be investigated in rats.

Methods: Fifty-six female Wistar rats were allocated to one of four diets: 1) Control, 2) ID, 3) (n-3)FAD, or 4) ID + (n-3)FAD and were maintained on the respective diets throughout pregnancy and lactation. Offspring (n=96) continued on the respective diets after weaning until postnatal day 42-45. Concentrations of lipid mediators were analysed in homogenized colon lining tissue from the offspring, with liquid chromatography-tandem mass spectrometry (LCMSMS). Pro-inflammatory lipid mediators, 5-, 8-, 11-, 12- and 15-hydroxyeicosatetraenoic acid (HETE), and pro-resolving lipid mediators, 17-hydroxydocosahexaenoic acid (HDHA); and 11-, 12-, 15- and 18 hydroxyeicosapentaenoic acid (HEPE) were measured.

Results: ID resulted in higher colonic pro-inflammatory lipid mediator concentrations in offspring derived from arachidonic acid (ARA), including 5-, 12-, 15- and 8-HETE (all $p < 0.005$). Omega-3 PUFA deficiency resulted in higher 12-HETE ($p = 0.010$). ID resulted in higher pro-resolving lipid

mediators derived from docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), including 17-HDHA, 12- and 15-HEPE (all $p < 0.001$), whereas (n-3)FAD, resulted in lower pro- resolving lipid mediators (17-HDHA, 12-, 15-, 18- and 11-HEPE, all $p < 0.005$). Furthermore, a significant ID x (n-3)FAD interaction synergistically resulted in higher pro-inflammatory lipid mediators (12- and 15-HETE, both $p < 0.050$). For 12-HEPE, however, the effect of ID for higher 12-HEPE was attenuated by (n-3)FAD ($p = 0.049$).

Conclusion: This study found that pre- and postnatal combined deficiency of iron and n-3 PUFA in rats increased inflammation in the colon of offspring in early adolescence more than the single deficiencies alone. Omega-3 FAD furthermore attenuated the resolving of inflammation which is still possible with ID alone, resulting in a pro-inflammatory profile that could not be resolved in double-deficient rats.

Key terms:

Inflammation, colon, iron (Fe), omega-3 polyunsaturated fatty acid (n-3 PUFA), lipid mediators; hydroxyeicosapentaenoic acid (HEPE) ;hydroxyeicosatetraenoic acid (HETE); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA).

ABBREVIATIONS

ARA	arachidonic acid
ALA	alpha-linolenic acid
ALOX	arachidonate lipoxygenases
ANCOVA	analysis of covariance
ANOVA	analysis of variance
CD	Crohn's Disease
CEN	Centre of Excellence for Nutrition
COX	cyclooxygenase
DHA	docosahexaenoic acid
dL	decilitre
EPA	eicosapentaenoic acid
FA	fatty acid
FAD	fatty acid deficient / fatty acid deficiency
Fe	iron
FAME	fatty acid methyl ester
FPN	ferroportin
g	gram
GC-EI-MS	gas chromatography electron ionization mass spectrometry (GC-EI-MS)
GD	gestational day
HDHA acid	hydroxydocosahexaenoic acid
HPCSA	Health Professions Council of South Africa
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ID	iron deficient / iron deficiency
IL	interleukin
kg	kilogram
LA	linoleic acid
LCMSMS	liquid chromatography tandem mass spectrometry
LCPUFA	long chain polyunsaturated fatty acid
LOX	lipoxygenase
Lp	lipoxin
LPS	lipopolysaccharides

MaR	maresins
MRM	minimum multiple reaction monitoring
mg	milligram
ml	milliliter
MUFA	monounsaturated fatty acid
n	number / amount
n-3	omega-3
n-6	omega-6
n-3 PUFA	omega-3 polyunsaturated fatty acid
NEC	necrotizing enterocolitis
NWU	North-West University
PAWS	Potchefstroom Animal Welfare Society
PCDDP	Preclinical Drug Development Platform
PD	protectins
PG	prostaglandin
PMN	polymorphonuclear neutrophils
PND	postnatal day
PUFA	polyunsaturated fatty acid
RBC	red blood cell
Rv	resolvins
SAVC	South African Veterinary Council
SCFA	short chain fatty acid
SD	standard deviation
SEM	standard error of the mean
SOP	standard operating procedure
SPM	super-family of specialised pro-resolving mediators
Spp.	species
SPPS	Statistical Program for Social Sciences
SPE	solid phase extraction
WHO	World Health Organisation

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CHAPTER 1: INTRODUCTION

1.1 Background and motivation

Nutritional health during early development, including conception, gestation, and lactation, has been proven to be critical for optimal development and growth of offspring (Adu-Afarwuah et al., 2017; Grundlingh et al., 2013; Lowensohn et al., 2016). The first 1000 days, - the period from conception until two years of age, is believed to be a very sensitive period for development (Unger et al., 2007; McCann and Ames, 2007). Recently, however, nutritional intervention before conception (pre-conception), has become an area of focus in order to improve maternal and child health, as well as to reduce the growing burden of non-communicable diseases (Stephenson et al., 2018).

A mother's nutritional status has an impact on the unborn baby, as hypothesised by Barker (2004). Early-life programming is becoming a recognised concept that states that the environment during early development affects health and disease in adulthood, probably via epigenetic mechanisms (Barker, 2004). Accumulating evidence suggests nutrition during pregnancy and early postnatal life to be one of the most important environmental cues that programs metabolic and immunologic development in offspring (DeBoer et al., 2012; Nauta et al., 2013).

Meeting the increased nutritional demand during pregnancy in developing countries is particularly challenging, since women are likely to be reliant on poor-quality diets, low in essential nutrients and high in nutrient absorption inhibiting molecules; and live in settings with poor hygiene, associated with a high prevalence of infections (Adu-Afarwuah et al., 2017). It has been shown that poor nutrition during early development, has a causal relationship with negative pregnancy outcomes, morbidity, mortality, and the increased risk of short- and long- term adverse health outcomes for mother, child and future generations (Black et al., 2008; Black et al., 2013; DeBoer et al., 2012; Jones et al., 2010; Nauta et al., 2013). Apart from being reliant on nutrients from the mother's diet during gestation, offspring in the human situation will most likely also continue consuming a similar diet to that of the mother.

1.1.1 Iron deficiency in a public health context

Iron deficiency (ID) is the most prevalent nutrient deficiency globally, particularly in developing countries (Zimmermann and Hurrell, 2007; Vos et al., 2016). Due to blood loss from menstruation, and an inadequate dietary intake of iron in low income groups, women of

childbearing age are at a high risk of iron deficiency (Scholl, 2005; Stevens et al., 2013, WHO 2015). In Southern Africa, 28% of women 15-49 years of age had anaemia and 1.2% severe anaemia in 2011 (Black et al., 2013). Women who have iron deficiency anaemia during pregnancy experience an increased risk of death during delivery accounting for at least 20% of maternal mortality (Black et al., 2008), where IDA can also lead to still births (Allen, 2001, Christian, 2010, Rasmussen and Stoltzfus, 2003). The prevalence of IDA (serum ferritin < 12.0 ng/mL and haemoglobin levels < 11.5 g/dL) amongst African children under the age of 5 years, was 20.2% in 2011 (Black et al., 2013).

In an observational cohort study, Patel et al. (2016) found that severe anaemia was associated with an increased risk of necrotising enterocolitis (NEC) amongst very low birth weight infants (Patel et al., 2016). Anaemia may result in a decreased oxygen delivery to the gastrointestinal tract (intestinal ischemia), allowing for an increased susceptibility to mucosal injury and necrosis (Patel et al., 2017). It is also known that maternal iron deficiency is associated with babies with low weight (<2500 g) at birth (Black et al., 2013).

1.1.2 The role of iron deficiency in gut inflammation

Iron deficiency was previously shown to increase gut inflammation in humans. The mechanism involves a decrease in commensal gut microbiota which alters the production of short chain fatty acids (SCFAs), e.g. butyrate (Dostal et al., 2012; Dostal et al., 2013). Butyrate provides the colonic epithelial cells with energy (Roediger, 1982), and keeps the colon lining healthy. It also reduces intestinal permeability, improves the integrity, and act as an anti-inflammatory agent for the colon mucosa (Ferreira et al., 2012).

Low serum iron was also associated with a decrease in the tight junctions in the intestinal epithelium, higher gut permeability, as well as increased inflammation in pigs (Li et al., 2016). Adequate iron reserves assist in maintaining the mucosal function, where it can be argued that enteropathy is not only associated with those younger in age, but also with malnutrition (Hossain et al., 2016).

On the contrary, the beneficial lactic acid bacteria (*Lactobacillus* spp.) as well as *Bifidobacterium* thrive in an iron-poor environment (Ganz and Nemeth, 2015). For this reason, iron deficiency has been shown to benefit the host by increasing beneficial *Lactobacilli* spp (Dostal et al., 2013, Tompkins et al., 2001), and *Bifidobacterium* (Werner et al., 2010). A higher *Lactobacilli* count is associated with higher high-density lipoprotein (HDL) levels, improved insulin sensitivity, as well as lower C-reactive protein (CRP), which is a marker for increased inflammatory activity (Rajkumar et al., 2014). This may be related to the central role that *Lactobacilli* play in improved

barrier function and decreased paracellular permeability in the gut (Ahrne and Hagslatt, 2011). Iron-deficient anaemic women between 18-25 years of age, however, showed low lactobacilli counts (Balamurugan et al., 2010); which stands in contrast to the studies in animals (Tompkins et al., 2001, Werner et al., 2010) and in vitro studies (Dostal et al., 2013).

However, ID may not only increase gut inflammation, but gut inflammation can also lead to ID, which may create a perpetuating cycle. During infection and inflammation hepcidin concentrations are greatly increased, through IL-6 and C-reactive protein (CRP) (Drakesmith and Prentice, 2012). High concentrations of hepcidin blocks ferroportin. Iron is thus trapped in cells and sequestered, withholding it from the circulation and decreasing serum iron, which can lead to hypoferramia and decreased erythropoiesis. The prolonged activation of this mechanism is referred to as the anaemia of inflammation. This process makes iron unavailable to pathogens that need iron for survival. It therefore works as an anti-pathogenic mechanism (Cherayil, 2011, Ganz and Nemeth, 2015)

Similarly, chronic inflammatory disorders, including rheumatologic disorders and inflammatory bowel disease (IBD) like ulcerative colitis and Crohn's disease, are associated with anaemia of inflammation, where an increase in the cytokines IL-6 and interferon (IFN) stimulates hepcidin. These patients have decreased intestinal iron absorption, and are therefore usually resistant to oral iron therapy (Cherayil, 2011, Ganz and Nemeth, 2015).

In low- and middle-income countries, environmental enteric dysfunction (EED) is a common phenomenon. It is marked by an increase in intestinal permeability, a compromised gut immune function, inflammation, and malabsorption. This in part explains why there is such a poor response to nutritional therapy in malnourished children in developing countries. In 99% of children worldwide, nutritional interventions decrease stunting (low height for age) by only 33%. Furthermore, oral vaccines including those for polio and rotavirus are less effective in these children, indicating that EED alter the mucosal immunity in this group (Korpe and Petri, 2012) compromising general health and wellbeing.

1.1.3 The intake of n-3 PUFA in developing countries

Besides adequate iron status, an adequate intake of n-3 polyunsaturated fatty acids (n-3 PUFA) in pregnant women is also crucial to ensuring uncomplicated pregnancies, as well as normal growth and development of the foetus (Cetin and Koletzko, 2008). Even though biochemical cut-offs and dietary intakes indicative of n-3 fatty acid (FA) deficiency (n-3)FAD are not defined (Innis and Friesen, 2008), populations with a low consumption of fish, and/or a high intake of fat and oils rich in n-6 PUFA but low in n-3 PUFA, are at risk of inadequate n-3 FA intake (Briend et

al., 2011). Particularly in developing countries, intakes of alpha-linolenic acid (ALA, 18:3n-3) and docosahexaenoic acid (DHA, 22:6n-3) are often low among pregnant and lactating women (Huffman et al., 2011). However, suboptimal n-3 PUFA status is not limited to low-income countries. Throughout the 20th century, the intake of n-6 PUFA in developed countries has increased sharply, which is reflected in the shift from almost equal n-6 to n-3 PUFA intakes to n-6/n-3 ratios of 15-25/1 in western diets (Simopoulos, 2011).

1.1.4 The role of n-3 PUFA in inflammation

The influence of fatty acids on inflammation involves their incorporation into cell membrane phospholipids (Calder, 2015b). Cell membranes, including the intestinal mucosa (Wang and Colgan, 2017), require unsaturated fat for their structure, fluidity and proper functioning. These fatty acids cannot be synthesised in the body, and must be consumed through food (Calder, 2015a, Teitelbaum and Walker, 2001, Whitney and Rolfes, 2013). Unsaturated fat can be divided into two main groups, namely monounsaturated fat (MUFA), and polyunsaturated fat (PUFA); with the latter being classified as n-6 (omega-6) and n-3 (omega-3), depending on the location of the first double bond from the methyl end of the fatty acid (Calder, 2015b, Teitelbaum and Walker, 2001).

Alpha-linolenic (ALA) in plant oils like flaxseed, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in marine oils are the three types of n-3 fatty acids present in food. (Whitney and Rolfes, 2013). In animals, small amounts (about 8%) of ALA can be converted into EPA and EPA can again be converted to DHA (Calder, 2006) (Teitelbaum and Walker, 2001, Whitney and Rolfes, 2013), through the process of desaturation, elongation and oxidation, as illustrated in Figure 1-1 on the right. Arachidonic acid (ARA) derived lipid mediators are considered to be predominantly pro-inflammatory, whereas EPA-and DHA-derived mediators are believed to either oppose or attenuate this effect, and act as inflammation resolving bioactive compounds (Calder, 2006, Serhan et al., 2017)

Arachidonic acid plays an important role in the production of the lipid mediators prostaglandin (PGE₂) and leukotriene (LTB₄), as well as hydroxyeicosatetraenoic acids (HETEs) and lipoxins. The enzyme involved in the conversion from ARA to PGE₂ as well as thromboxane (TXA₂) is cyclooxygenase (COX), while the enzyme lipoxygenase (LOX) is central in the conversion of ARA to LTB₄ (Calder, 2006, Calder, 2015a, Massey and Nicolaou, 2013). LTB₄ is pro-inflammatory. It increases vascular permeability, enhances local blood flow and increases the production of the pro-inflammatory cytokines TNF, IL-1 and IL-6 (Calder, 2015b, Calder, 2006). PGE₂ can also have a pro-inflammatory effect in the body by increasing vascular permeability and vasodilatation. It furthermore induces pain, causes fever, and increases IL-6. But PGE₂ can also have anti-

inflammatory effects by inhibiting the production of TNF and IL-1, by inhibiting the production of leukotrienes and increasing the production of lipoxin, an anti-inflammatory lipid mediator, or rather pro-resolving lipid mediator as it is also referred to (Calder, 2015b, Calder, 2006).

EPA generates hydroxyeicosapentaenoic acids (HEPEs) and E-series resolvins (RvE's); while docosahexaenoic acid (DHA) produces docosanoids, including hydroxy-docosahexaenoic acids (HDHAs), maresins, D-series resolvins (RvD's), and protectins (PDs) (Massey and Nicolaou, 2013, Serhan et al., 2017). An active role is played by n-3 PUFAs in resolving inflammation via their metabolites - the specialised pro-resolving lipid mediators (SPMs) - resolvins, maresins, and protectins (Serhan et al., 2017). SPMs are involved in restoring the barrier function in the gut, and also control inflammation by regulating the immune system and microbial environment (Wang and Colgan, 2017).

Vilaseca et al. (1990) have compared the effect between n-6 PUFA (sunflower oil) and n-3 PUFA (cod liver oil) supplementation in the development of chronic granulomatous lesions in the colon of rats with induced colitis. They found that the formation of prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) peaked by Day 3, and thereafter declined; whilst thromboxane B₂ (TXB₂) continued to increase from Day 3 to 20 in n-6 PUFA fed rats – a change that was diminished in cod liver animals (Vilaseca et al., 1990).

This proves similar to the findings of Bosco et al. (2013), who found there to be a significant reduction of colonic pro-inflammatory lipid mediators (PGJ₂, PGJ₈, PGJ₉-EET and PGE₂) and a significant increase in the anti-inflammatory lipid mediators (PGE₃, TxB₃, LTB₅ and 5-HEPE as well as 17, 18 EEP) in mice, following a fish oil diet, where comparison was drawn to a control diet containing corn oil (Bosco et al., 2013).

Dietary n-3 PUFA can modulate substrate availability for cyclooxygenases (COXs) and lipoxygenases (LOXs), thus modulating downstream eicosanoid formation. Neilson et al. (2012) found that the administration of fish oil reduced the amount of free ARA in mice colonic mucosa, compared to a diet rich in olive oil (oleate diet). The fish oil diet generally reduced the synthesis of ARA derived LTB₄ and increased the synthesis of EPA-derived LTB₅ relative to the oleate diet. The fish oil diet also significantly reduced the levels of 12- and 15-HETEs in colonic mucosa, and the same trend was observed for the minor product 5-HETE (Neilson et al., 2012).

In humans, 15-hydroxy-eicosatetraenoic acid (15-HETE) was the predominant product formed in all the colonic biopsies from patients with active ulcerative colitis. Therefore, the role of 15-HETE as a mediator in ulcerative colitis should, be considered in addition to the effects of known modulators such as leukotriene B₄ (LTB₄) and PGE₂ (Zijlstra et al., 1992a, Zijlstra et

al., 1992b).

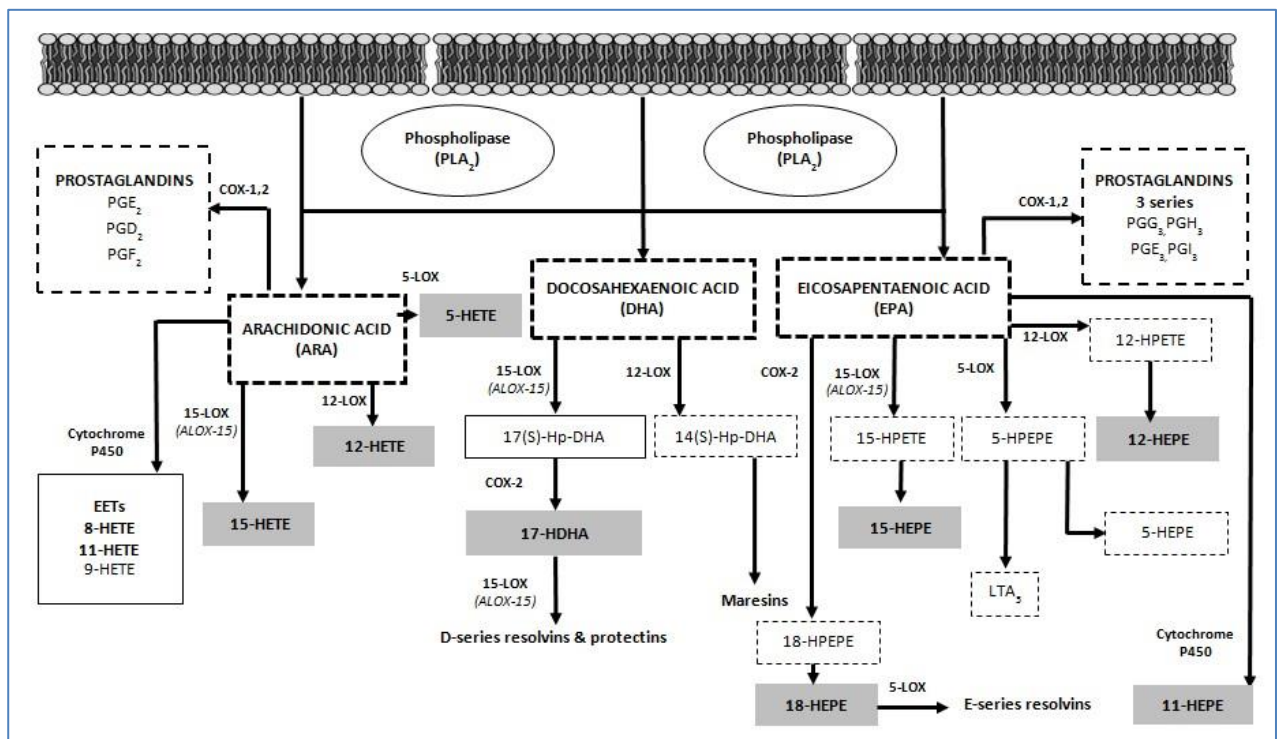


Figure 1-1: An overview of the main pathways involved in the production of polyunsaturated fatty acid-derived lipid mediators (Adapted from Zandberg et al 2018). Abbreviations: ALOX: arachidonate lipoxygenases; ARA: arachidonic acid; COX: cyclooxygenase; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; HDHA: hydroxy docosahexaenoic acid; HEPE: hydroxyeicosapentaenoic acid; HETE: hydroxy-eicosatetraenoic acid; LOX: lipoxygenase; LT: leukotriene; PG: prostaglandin; TX: thromboxane. Published with permission from the original author

1.1.5 The role of n-3 PUFA deficiency in gut inflammation

A deficiency in n-3 PUFA results in the reduced ability to resolve inflammation, and therefore also result in increased pro-inflammatory cytokine signaling (Calder, 2015b). Red blood cell (RBC) total phospholipid fatty acid composition is used as a proxy for the rest of the body's cell membrane fatty acid status, and the RBC n-6 and n-3 PUFA should therefore correlate with the pro-and anti-inflammatory lipid mediators, respectively (Brenna et al., 2018).

No studies thus far have investigated the role of n-3 PUFA deficiency in the eicosanoid profile in colonic tissue. However, in certain animal studies, n-3 PUFA supplementation proved to increase villus height and intestinal barrier function and decrease gut inflammation (Calder, 2015b, Liu et al., 2012, Myles et al., 2014). This anti-inflammatory effect was carried over from the mother to the offspring, possibly through the inheritance of an altered microbiota (Gibson et al., 2015, Myles et al., 2013, Myles et al., 2014). On the contrary, n-3 PUFA deficiency in mice lowered the

diversity of the caecal microbiota composition in the dams, which was not seen in the offspring (Robertson et al., 2017).

Furthermore, n-3 PUFA deficiency also significantly reduced the production of butyrate. A deficiency of butyrate producing microbiota is associated with a weakened colon lining, higher intestinal permeability, and inflammation in the colon mucosa. This suggests that n-3 PUFA play a crucial role in the composition of the microbiota and that n-3 PUFA deficiency can lead to an imbalance in the gut microbiota, which may contribute to impaired production of SCFAs (Robertson et al., 2017).

Compromised tight junction (TJ) function, which could be caused by inflammation (Wang and Colgan, 2017) leads to increased gut permeability and causes pathogens (like lipopolysaccharides, which is highly inflammatory) to enter the bloodstream. This can lead to low-grade systematic inflammation, and contributes to the development of irritable bowel syndrome (IBS) (Piche, 2014, Piche et al., 2009). Indeed, children between seven and ten years of age with IBS had increased gut permeability as well as low-grade inflammation, with the latter relating to the degree to which pain interferes with activities (Shulman et al., 2008).

Increased gut permeability and low-grade inflammation is also linked to diabetes, obesity, cancer and neuro-pathologies (Andrade et al., 2015; Bischoff, 2011; Bischoff et al., 2014; Ghoshal et al., 2012; Minihane et al., 2015). Thus, it can be argued that increased intestinal permeability, which could be caused by n-3 PUFA deficiency, is associated with metabolic disturbances and inflammation (Fasano, 2012, Moreno-Navarrete et al., 2012).

1.1.6 The effect of combined iron and n-3 PUFA deficiency on inflammation

It is likely that in pregnant women, particularly of lower socioeconomic status, ID and inadequate n-3 PUFA status coexist. In a rat model the combined deficiencies of iron and n-3 PUFA, these were shown to disrupt brain-monoamine metabolism and produce greater deficits in reference memory than ID or (n-3)FAD alone (Baumgartner et al., 2012). This indicates that there is a direct interaction between iron and n-3 PUFA. Even though this interaction is not completely understood, it has been shown that iron is a cofactor for desaturase and elongase enzymes required for the conversion of essential fatty acids to EPA and DHA, as well as to arachidonic acid (Nakamura and Nara, 2004).

It is generally accepted that ID may lead to a reduced activity of these enzymes and an attenuated conversion of the essential fatty acids to their longer chain metabolites. On the other hand, it has also been found that n-3 PUFA supplementation attenuated systemic inflammation

caused by iron supplementation in 7-11-year-old children in South Africa (Malan et al., 2016). However, data on potential interactions between ID and low n-3 PUFA status, specifically in relation to gut inflammation, are scarce.

1.1.7 Translatability to humans

The murine model is most frequently used in intestinal research, because their intestinal development and physiology is relatively similar to that of humans; they have many of the same genes, and a similar immune response (Chinwalla et al., 2002). Many research teams have also used mice or rats in n3-PUFA interventions (Myles et al., 2014; Myles et al., 2013; Robertson et al., 2017; Vilaseca et al., 1990; Bosco et al., 2013; Neilson et al., 2012); while mice or rats were used in iron studies (Dostal et al., 2012; Dostal et al., 2014; Werner et al., 2010; Tompkins et al., 2001). The rat model has the additional benefit that it is larger than mice allowing the harvesting of larger samples for analysis (Jiminez et al., 2015).

1.2 Aim and objectives

The aim of this study was to investigate the effects of n-3 PUFA and iron deficiency alone, and in combination, during early development on colonic inflammation at postnatal day (PND) 42-45.

The objectives for this study were to investigate whether iron and n-3 PUFA deficiency, alone, as well as in combination during early development, had an effect on:

- lipid mediators: 17-hydroxydocosahexaenoic acid (17-HDHA); 11-, 12-, 15 & 18-hydroxyeicosapentaenoic acid (HEPE); 5-, 8-, 11-, 12- and 15-hydroxyeicosatetraenoic acid (HETE); in colon lining
- the red blood cell total phospholipid fatty acid composition (including ratios indicating desaturase activity) and iron status (haemoglobin, ferritin and transferrin receptor).

1.3 Study design

This MSc project is a sub-study of a larger project aiming to investigate the effects of maternal iron and n-3 FA depletion and repletion – both alone and in combination – on the development and health of offspring. The animal trial was conducted at the vivarium of the Preclinical Drug Development Platform (PCDDP) of the North-West University (NWU), Potchefstroom, SA.

In the larger study, a total of 56 female Wistar rats at 21 ± 3 days of age were housed in pairs and randomly allocated to one of four diet groups. The diet groups included a control group, ID,

(n-3)FAD or ID+(n-3)FAD. Figure 1-2 gives an indication of the study design and a more detailed description can be found in Chapter 3. At PND 42-45, 24 offspring from each group were euthanised, and samples were collected. Colon tissue and samples were further analysed for this sub-study.

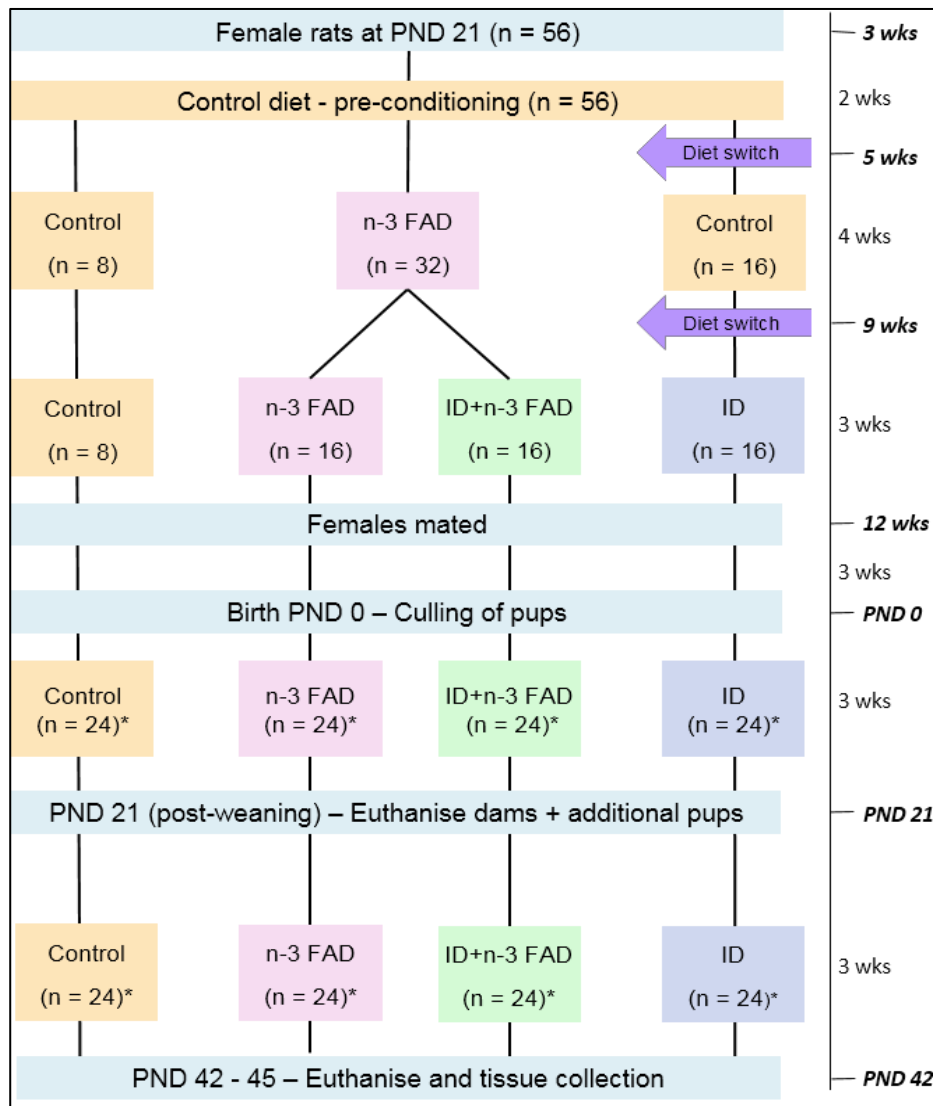


Figure 1-2: Flow diagram of the main study

Abbreviations: FAD: fatty acid deficiency; ID: iron deficiency; n: number / amount; n-3: omega-3; PND: post-natal day.

1.4 Research team and author's contribution

Project head (PI) large study:

Prof. Marius Smuts, Centre of Excellence for Nutrition (CEN), NWU Potchefstroom

Project Supervisor (Co-PI) large study

Prof. Jeannine Baumgartner, CEN, NWU Potchefstroom

Study leader of this sub-study and supervisor of MSc student:

Dr. L. Malan, CEN, NWU Potchefstroom

Co-study leader of this sub-study:

L. Zandberg, CEN, NWU Potchefstroom

PhD student on large project:

Ms. Erna Kemp, CEN, NWU Potchefstroom

MSc student:

Mrs. Venessa Schoeman

The MSc student was involved in planning of the sub-study, and was responsible for quantitation of lipid mediators, data quality assurance, statistical analysis, and the writing of the mini-dissertation.

1.5 Other study contributors

Professional supervisors and animal technicians:

Mr. Cor Bester, Vivarium of PCDDP, NWU Potchefstroom

Ms. Antoinette Fick, Vivarium of PCDDP, NWU Potchefstroom

Mr. Kobus Venter, Vivarium of PCDDP, NWU Potchefstroom

Dr. Stallone Terera (BVSc), Vivarium of PCDDP, NWU Potchefstroom

1.6 Structure of this mini-dissertation

This mini-dissertation is presented in article format according to the NWU's guidelines for postgraduate students, where the main outcomes are presented in Chapter 3, as an article prepared for publication in an accredited journal. Four chapters are included in this mini-dissertation. All relevant references are provided at the end of each chapter.

Chapter 1 serves as a brief introduction and explains the rationale for conducting this study. The study design derived from the larger study is provided in brief, along with the consequent aim and objectives. The research team and all contributors are acknowledged.

Chapter 2 includes an exhaustive literature review on the metabolism of iron and n-3 PUFA in mammals, as well as the effect of these nutrients on inflammation, and more specifically colon health, providing background information and further explaining the rationale of this study. The

methods used to determine the lipid mediators as indicator of inflammation are also explained.

Chapter 3 provides the key data findings to be submitted for publication in an academic journal titled “Effects of n-3 polyunsaturated fatty acid and iron deficiency, alone and in combination, during early development on colonic inflammation in rats.”

Chapter 4 consists of a summary and conclusion based on the specific objectives provided in Chapter 1. Limitations and recommendations for future research are also included.

Annexures attached include the ethics approval certificate, Guidelines of the Journal of Nutrition and editor’s certificate.

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CHAPTER 2: LITERATURE REVIEW

2.1 Iron and n-3 polyunsaturated fatty acid deficiencies in developing countries

Nutrition during early development, including conception, gestation, and lactation, has been proven to be critical for optimal development and growth of offspring (Adu-Afarwuah et al., 2017; Grundlingh et al., 2013; Lowensohn et al., 2016). The first 1000 days, the period from conception until two years of age is believed to be a very sensitive period for development (Unger et al., 2007; McCann and Ames, 2007). However, recently nutritional intervention before conception (pre-conception), has become an area of focus in order to improve maternal and child health, as well as to reduce the growing burden of non-communicable diseases (Stephenson et al., 2018).

Meeting the increased nutritional demand during pregnancy in developing countries is challenging, since women are likely reliant on poor-quality diets low in essential nutrients, high in nutrient absorption inhibiting molecules and live in settings with poor hygiene associated with a high prevalence of infections (Adu-Afarwuah et al., 2017). In low and middle-income countries, enteropathies are common, and severe malnutrition is associated with more severe enteropathy, intense microbial translocation, and severe systemic inflammation, that can even lead to death in children (Amadi et al., 2017).

It has been shown that poor nutrition during early development, has a causal relationship with negative pregnancy outcomes, morbidity, mortality and the increased risk of short- and long- term adverse health outcomes for mother, child and future generations (Nauta et al., 2013; Black et al., 2008; Black et al., 2013; Jones et al., 2010; DeBoer et al., 2012). Apart from being reliant on nutrients from the mother's diet during gestation, offspring in the human situation will most likely also continue consuming a similar diet to that of their mother.

2.1.1 Iron deficiency in developing countries

Iron deficiency (ID) is the most prevalent nutrient deficiency globally, particularly in developing countries (Zimmermann and Hurrell, 2007; Vos et al., 2016). ID occurs when the physiological requirements are not met by iron supply from the diet. In developing countries, where plant-based diets are common, iron bioavailability is low, leading to a higher incidence of iron deficiency (Zimmermann and Hurrell, 2007; Adu-Afarwuah et al., 2017). According to the World Health Organisation (WHO), 29% of non-pregnant women, 38% of pregnant women, and 43%

of children younger than five years old worldwide were anaemic in 2011, with half presenting with iron deficiency anaemia (IDA) (Stevens et al., 2013; WHO, 2015).

The prevalence of IDA (serum ferritin < 12.0 ng/ mL and haemoglobin levels < 11.5 g/dL) amongst African children under the age of five years was 20.2% in 2011, while the prevalence in pregnant women was 20.3 %. This was not only attributable to the low consumption of food rich in iron and poor absorption from the diet, but also due to blood loss from parasitic infection and intestinal worms during pregnancy (Black et al., 2013).

In young children, the peak prevalence of IDA occurs at around 18 months of age (Black et al., 2008). Children have high iron requirements, due to their high growth rate, and the rapid expansion of their red cell mass. Iron is also crucial in cognitive and motor skill development, and it plays an important role in the immune function (Rao and Georgieff, 2007). ID is therefore associated with a lower intelligence quotient (Black et al., 2008) and a higher susceptibility to infection, especially upper respiratory tract infections (Zimmermann and Hurrell, 2007).

Due to blood loss from menstruation and inadequate dietary intake of iron in low-income groups, women of childbearing age are also at high risk of iron deficiency (Scholl, 2005, Stevens et al., 2013; WHO, 2015). In Southern Africa, 28% of women 15-49 years of age had anaemia, and 1.2% severe anaemia in 2011 (Black et al., 2008; Black et al., 2013). Low iron stores before and during pregnancy can lead to severe anaemia during pregnancy. Women who have iron deficiency anaemia during pregnancy have an increased risk of death during delivery, accounting for at least 20% of maternal deaths. IDA can also lead to stillbirths (Rasmussen and Stoltzfus, 2003; Christian, 2010; Allen, 2001).

The mother's nutritional status also has an impact on the unborn baby, as hypothesised by Barker (Barker, 2004). Early-life programming, as it is referred to, is becoming a recognised concept that states that the environment during early development affects health and disease in adulthood, probably via epigenetic mechanisms. Accumulating evidence suggests that nutrition during pregnancy and early postnatal life is one of the most important environmental cues that programs metabolic, and immunologic development (De Boer et al., 2012; Nauta et al., 2013).

Many public health interventions to prevent and control iron deficiency and IDA in children have been conducted and are still underway. However, the efficacy and effectiveness of these strategies and regimens in improving iron status and the associated non-haematological outcomes, such as growth and cognitive development, are variable. Furthermore, the safety of iron interventions remains uncertain, especially in infants and children from low-income areas with poor sanitary conditions, and a high infectious disease burden (Baumgartner and Barth-Jaeggi, 2015).

Preschool children receiving iron and folic acid supplementation during the Pemba trail in Zanzibar during 2006, experienced an increased risk of severe illness and death, causing the trail to be stopped prematurely. This trail was conducted in an area where the occurrence of malaria was high. The authors concluded that routine supplementation with iron and folic acid in preschool children with high rates of malaria could be harmful – especially if the children are not iron deficient (Sazawal et al., 2006). This led to a change in the WHO recommendations for iron interventions in malaria regions, from universal to targeting only those who are iron deficient (Baumgartner and Barth-Jaeggi, 2015).

In 6-14-year-old African children in Côte d'Ivoire, Zimmerman et al. found that iron fortification (biscuits, which contained 20 mg Fe/d, 4 times/wk. as electrolytic iron for 6 months) did not alter iron status, anaemia or hookworm prevalence. After six months, there was also a significant increase in the number of enterobacteria and a decrease in lactobacilli in the iron group when compared to the control group. The increase of enterobacteria correlated with a mean increase of the faecal calprotectin concentration – a marker of gut inflammation. Thus, in this study, iron fortification produced a potentially more pathogenic gut microbiota profile associated with increased gut inflammation (Zimmermann et al., 2010). Similar results were seen in Kenyan infants receiving maize porridge fortified with two different types of iron (Jaeggi et al., 2015).

A study amongst South African schoolchildren, where ferrous sulphate was given for 4 days/week, however, did not change the gut microbiota, nor did it affect inflammation (Dostal et al., 2014a). It was concluded that due to poor hygiene at the beginning of the Côte d'Ivoire and Kenyan studies, the high burden of pathogenic intestinal bacteria lead to the adverse effect of the iron, considering that iron is essential for the survival of almost all pathogenic bacterial groups including *E.coli*, *Salmonella* and *Shigella*. Bacterial dysbiosis leads to intestinal inflammation, and vice versa; which creates a vicious cycle that is detrimental to overall health (Baumgartner and Barth-Jaeggi, 2015).

In South-Africa, pregnant women are routinely supplemented with a high dose of ~60 mg per day. Nevertheless, a recent study showed that despite ~85% compliance, the prevalence of iron deficiency still increased by ~20% from 18 to 36 weeks gestation (unpublished data). This may be the result of a high inflammatory background (~50% with C-reactive protein > 5 mg/ml), which reduces iron absorption in the gut (Cherayil, 2011).

Therefore, in public health interventions, iron supplementation and fortification should only be done in individuals with iron deficiency; intermittent iron administration should be considered; and great care must be taken in areas where malaria is prevalent, and where there is a high

burden of pathogenic intestinal bacteria, due to poor sanitation (Baumgartner and Barth-Jaeggi, 2015).

Unfortunately, the use of all known nutritional interventions, showed that in 99% of these children worldwide, the intervention decreased stunting (low height for age) with only 33% (Bhutta et al., 2008). Furthermore, oral vaccines including those for polio and rotavirus are less effective in these children (Korpe and Petri, 2012). One reason for this could be the presence of environmental enteric dysfunction (EED), which is a common phenomenon in low- and middle-income countries. EED is established during infancy and is defined as inflammation of the small intestine related to the quality of the environment. Poor sanitation, toxins, micronutrient deficiencies, malnutrition, bacterial overgrowth in the small intestine and a skewed gut microbiota all play a role in causing gut inflammation in infants and adults in this group (Crane et al., 2015). Interestingly, diarrhoea is not necessarily present, and individuals can be seemingly asymptomatic (Korpe and Petri, 2012; Campbell et al., 2003). EED is marked by increased intestinal permeability, a compromised gut immune function, inflammation, and malabsorption. This in part explains why there is such a poor response to nutritional therapy in malnourished children in developing countries. EED alters the mucosal immunity compromising general health and wellbeing.

2.1.2 Omega-3 polyunsaturated fatty acid deficiency in developing countries

The body cannot synthesise n-3 and n-6 polyunsaturated fatty acids (PUFA), which are essential nutrients that have to be consumed in the diet. The WHO recommends a daily dietary intake of 1-2% of total energy with a recommended daily intake of 250-500 mg docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Flock et al., 2013). Two portions of fatty fish per week for healthy adults would also provide an adequate dose (Flock et al., 2013). Even though biochemical cut-offs and dietary intakes indicative of n-3 FA deficiency ((n-3)FAD) are not defined (Innis and Friesen, 2008), populations with a low consumption of fish, and/or a high intake of fat and oils rich in omega-6 (n-6 PUFA) but low in n-3 PUFAs, are at risk of inadequate n-3 FA intake (Briend et al., 2011).

In developing countries, intake of alpha-linolenic acid (ALA, 18:3n-3) and docosahexaenoic acid (DHA, 22:6n-3) are often low among pregnant and lactating women (Huffman et al., 2011). An inadequate intake of n-3 PUFA in this group of women could lead to complications during pregnancy, and it could impair the normal growth and development of the foetus (Cetin and Koletzko, 2008). An overwhelming body of evidence has shown how n-3 PUFA deficiency during pregnancy and/or lactation influences the visual, cognitive and behavioral health of the offspring (Brenna, 2011). Improving the intake of these fatty acids will also protect the mothers

as well as their offspring from infection and inflammation (Prentice and Van der Merwe, 2011).

Myles et al. (2013) have shown that parental exposure to a high n-3 PUFA diet resulted in an altered microbiome in the offspring and a decreased inflammatory response when compared to mice fed a Western diet (Myles et al., 2013). The Western diet is characterised by an increased intake of saturated and n-6 PUFA and a low intake of n-3 PUFA. This diet is associated with increased gut permeability; induces endotoxemia; and also changes the microbiota, resulting in low-grade inflammation (Knight-Sepulveda et al., 2015). Myles found that the mother's fat consumption can leave a "lard legacy" that impacts the immunity of the offspring, and suggest that inheritable microbiota may contribute to the development of inflammatory disorders (Myles et al., 2013, Myles et al., 2014).

Observational studies furthermore showed that an association exists between high fish consumption and increased duration of pregnancy, as well as higher birthweight (Olsen et al., 1986). A number of randomised controlled trials followed these findings. A systematic review of these RCTs indicated that about 3 g of n-3 PUFA as marine oil supplement during the second half of pregnancy resulted in higher mean birthweights (approximately 50 g) and higher mean birth lengths (~0.48 cm) in the marine oil groups, when compared to controls. It was concluded that these effects were mediated by marine oils extending the length of gestation by approximately 2.5 days (Makrides et al., 2011). Most of these studies were, however, conducted in first world countries, and the only two in developing countries – Bangladesh (Tofail et al., 2006) and Mexico (Ramakrishnan et al., 2010), failed to show an increase in birth weight and length with marine oil supplementation. However, in Mexican women who had a very low intake of dietary DHA, and who were pregnant for the first time, prenatal DHA supplementation resulted in increased birth size (Ramakrishnan et al., 2010). Growth in infancy and early childhood in low-income populations is strongly linked to infectious load, but not many studies have investigated this outcome (Prentice and Van der Merwe, 2011).

Information about the fatty acid composition of diets in low-income countries is scarce (Michaelsen et al., 2011). In low-income countries, total fat intake may be low, which indicate that the consumption of PUFA may also be limited (Briend et al., 2011). Furthermore, the higher availability and affordability of oils rich in the n-6 PUFA precursor, linoleic acid (LA) like sunflower oil, may compromise the conversion of α -linolenic acid (ALA) from the n-3 PUFA series, seeing that the same enzymes are involved. The competition for the same enzyme pathway can also lead to lower production of EPA and DHA in the body and an inadequate n-3 PUFA status in low- and middle-income groups (Briend et al., 2011). Therefore, suboptimal n-3 PUFA status is not limited to low-income countries. Throughout the 20th century, the intake of n-6 PUFA in developed countries has increased sharply, which is reflected in a shift from almost

equal n-6 to n-3 PUFA intakes to n-6/n-3 ratios of 15-25/1 in western diets (Simopoulos, 2011).

In South Africa, the eating patterns of many different ethnic groups, as well as their socio-economic status all influence the intake of fatty acids (FA). In a study conducted by Ford et al. (2016) in South Africa, the dietary fat intake of preschool children and their caretakers in three different geographical areas (two urban and one rural) were assessed. The type of fat, as reflected in the red blood cell (RBC) membrane phospholipids, were also measured. In women (32-34 years) the total fat intake as a percentage of total energy intake was 32% and 37% in urban Northern Cape and urban Western Cape, respectively, which is higher than the recommended 30%. This could be related to a Westernised dietary pattern. In rural Limpopo Province, the total fat intake was only 16% amongst women, which is much lower than recommended 30% (Ford et al., 2016). In adults, a minimum of 15% is required to ensure sufficient energy and essential fatty acid intake, as well as to assist with the absorption of fat-soluble vitamins (Whitney and Rolfes 2013; Jequier. 1999.). The women in all three regions were, however, overweight, with their mean body mass index (BMI) ranging between 27 and 29. The combined intake of EPA and DHA in women was 87 mg, 103 mg, and 32 mg, respectively, which is also much lower than the recommended 250 mg per day (Ford et al., 2016).

In children (3-4 years of age), a similar pattern was seen, where children in urban areas consumed 34% - 33% of their total energy from fat, while for children in rural Limpopo Province, the percentage was only 20% (Ford et al., 2016). In children, a low fat intake (25-30%) is associated with growth failure and a lower intake of fat-soluble vitamins (Uauy, 2009). Interestingly, 40% of children in the urban Northern Cape (who had the highest intake of fat) were stunted, while only 16% in urban Western Cape and rural Limpopo Province were stunted. In the urban areas, combined EPA and DHA consumption were 50 mg and 55 mg for urban Northern Cape and urban Western Cape, respectively, but only 3 mg for rural Limpopo. These results indicate that even though fat consumption might be high in urban areas, the consumption of EPA and DHA was low in all communities. Children in rural Limpopo Province had the lowest intake of long-chain essential fatty acids, but reflected the highest red blood cell DHA profile, suggesting that they had a more sufficient conversion from ALA to DHA (Ford et al., 2016).

2.1.3 Iron and n-3 polyunsaturated fatty acid deficiencies in combination in developing countries

Data on the combined effect of iron and n-3 PUFA deficiencies is scarce and mainly performed in cognitive and brain development studies, using rats. Baumgartner and colleagues did, however,

find that simultaneous ID and low n-3 PUFA status were prevalent in children in a rural population of low socioeconomic status in KwaZulu-Natal with a monotonous plant-based diet low in animal products and seafood (Baumgartner et al., 2012b). Seeing that both iron and n-3 PUFA have a profound effect on the cognitive development of children, combined deficiency could have far-reaching consequences; not only in brain development, but also in immunity, infection, and inflammation (Calder, 2015a; Cherayil, 2011).

2.2 Gut and colon physiology, and inflammation

2.2.1 Normal gut and colon physiology

The gastro-intestinal (GI) tract is a complex, multilayer system, serving as a barrier between the contents in the lumen and the rest of the body (Martini et al., 2015). It consists of the mucosa, submucosa, muscle layer (muscularis) and the serosa. It has a surface of about 400m², uses 40% of the body's daily energy, and is renewed approximately every five days in humans (Martini et al., 2015; Bischoff et al., 2014). While absorbing nutrients, electrolytes and water the intestinal wall must also keep pathogens, commensal bacteria, and bacterial products such as lipopolysaccharides (LPS) and toxins from entering the system, by forming a physical barrier between antigens and the host (Turner, 2009; Wang and Colgan, 2017). The mucosa therefore has a distinct architecture.

The intestinal epithelium is a single layer of polarised, stratified cells that is permeable (Martini et al., 2015). Substances can infiltrate the epithelium by two pathways -. transcellular and paracellular (Turner, 2009). Nutrients like iron and hydrophobic molecules, for example lipids, are absorbed mainly via the transcellular pathway in enterocytes. Epithelial cells, however, also permit a small amount of bacteria to cross the epithelium through the same pathway via endocytosis. This process is known as bacterial translocation. Low levels of bacterial translocation are normal and activate the host's immune system (Andrade et al., 2015).

Water, ions, and small molecules can enter the body via the paracellular route, which are the spaces between epithelial cells. A protein network between epithelial cells regulates the flow of molecules into the body, while at the same time, preventing the absorption of harmful substances (Andrade et al., 2015). The junctions connecting the cells are known as TJs (Turner, 2009).

Compromised TJ function, which may be caused by inflammation (Wang and Colgan, 2017) leads to increased gut permeability, and causes pathogens (like LPS, which is highly inflammatory) to enter the bloodstream. This can lead to low-grade systematic inflammation, and contributes to the development of irritable bowel syndrome (Piche, 2014, Andrade et al.,

2015, Minihane et al., 2015, Ghoshal et al., 2012), diabetes, obesity, cancer and neuropathologies (Bischoff et al., 2014). Thus, it can be argued that increased intestinal permeability in the small intestine is associated with metabolic disturbances and inflammation (Moreno-Navarrete et al., 2012; Fasano, 2012).

Enterocytes are the most abundant epithelial cell, and apart from absorption, also secrete mucins (via Goblet cells), immune mediators, antibacterial peptides (via Paneth cells that secrete α -defensins), immunoglobulin A, secretory immunoglobulin A, chemokines, cytokines, IFN- γ , TNF, and interleukin (IL) 4, 5, 9 and 13. This forms part of the first line of defence, when a pathogen enters the body via the gastrointestinal tract and forms a physical or external barrier (Bischoff et al., 2014). This not only protects the mother, but also the unborn baby.

The intestinal epithelium is central to coordinating both inflammation and the resolution thereof. Excessive trans-epithelial migration of polymorphonuclear neutrophils (PMN) disrupts the physical barrier by causing epithelial damage, which perpetuates inflammation, as seen in active inflammatory disease (Sumagin and Parkos, 2015).

Lipoxins (Lp), resolvins (Rv), protectins (PD) and maresins (MaR), referred to as special pro-resolving mediators (SPMs) in the gut, is crucial for maintaining homeostasis. These mediators are derived from n-6 and n-3 PUFA. It is involved in restoring the barrier function and also controls inflammation by regulating the immune system and microbial environment. During acute inflammation, PMNs migrate into the tissue. SPMs limit the neutrophil infiltration, promote phagocytosis of cellular debris by macrophages, and stimulate the production of anti-inflammatory cytokines (Wang and Colgan, 2017).

In addition to the termination of neutrophil infiltration, the resolution of intestinal inflammation also depends on the apoptosis of the accumulated neutrophils and the clearance of the cellular debris. Although SPMs are highly effective in halting the migration of PMNs, SPMs also limit the inflammatory actions of the PMNs and promote the clearance of these activated PMNs from the inflamed tissue (Wang and Colgan, 2017). The intestinal epithelial cells play an active and integral role in maintaining homeostasis via SPMs, and guarantee that the immune system will only be activated when absolutely necessary (Wang and Colgan, 2017).

2.2.2 The physiology of the colon

The colon has a much higher bacterial load than the small intestine, and the immune cell composition is also noticeably different. The colon doesn't have any villi, only crypts, and there are no Peyer's patches or Paneth cells. This implies that the enterocytes have a crucial

contribution to make in antimicrobial peptide production, in the absence of defensins secreted by Paneth cells in the small intestine. However, there is a high prevalence of goblet cells secreting mucus. The mucus forms dual layers, with a thick, largely sterile inner layer and a thinner outer layer, as seen in Figure 2-1. While the immune cell types present are similar to those found in the small intestine, it is likely that there may be at least subtle differences. In particular, natural killer T cells are found more frequently, and have a more significant role in the colon (Cader and Kaser, 2013).

Whilst most of the absorption of nutrients and micronutrients take place in the small intestine, the large intestine (colon) reabsorbs water, bile salts, and vitamins (Martini et al., 2015) as well as a small amount of iron (Blachier et al., 2007) The large intestine also plays a crucial role in the breakdown of fibre and producing short-chain fatty acids (acetate, butyrate & propionate). The highest density of microbiota (bacteria) is also found in the terminal ileum and colon (Flint et al., 2012).

The microbiota in the gut consists of more than 100 trillion cells, and includes bacteria, fungi and viruses, as well as other eukaryotic species (Belkaid and Hand, 2014) that are commensal with the human intestinal tract. The highest densities are found in the terminal ileum and colon, characterised by slow flow rates and a neutral to mildly acidic pH (Flint et al., 2012). Each individual carries up to a few hundred species of intestinal bacteria, but most fall into the two dominant phyla: Firmicutes (includes the genus *Lactobacilli* and *Roseburia*) and Bacteroidetes (include *Bacteroides* and *Prevotella*) (Arumugam et al., 2011); with Actinobacteria (includes the genus *Bifidobacterium*), Proteobacteria (includes the genus *Enterobacteria* and *Escherichia*), and Verrucomicrobia also present in lower numbers (Flint et al., 2012, Gritz and Bhandari, 2015).

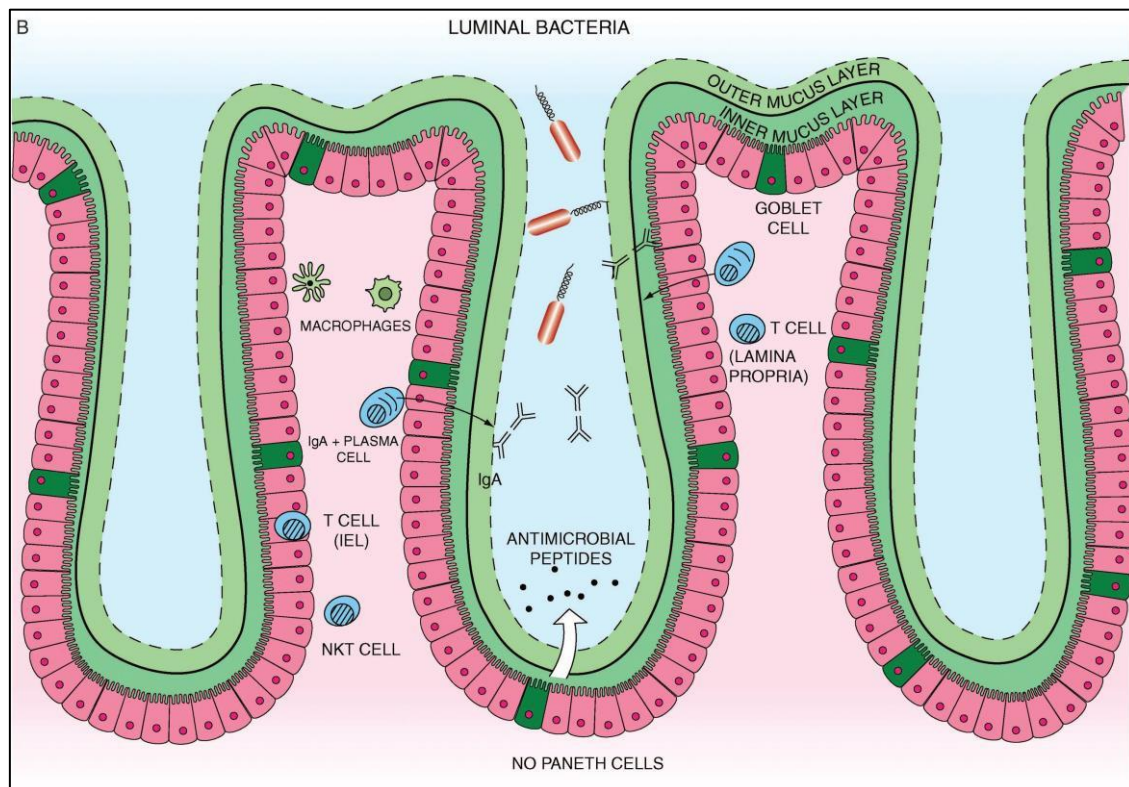


Figure 2-1: The mucosal immune system of the colon (large intestine) (Cader and Kaser, 2013)

Abbreviations: IgA: Immunoglobulin A; IEL: intra-epithelial lymphocytes; NKT: Natural killer T cells
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In the normal symbiotic state, the intestinal microbiota is involved in several processes:

- **Fermentation of unabsorbed faecal matter, such as fibre, through bacterial action.**

During this process, the short chain fatty acids acetate, propionate and butyrate are formed as waste products. Butyrate provides the colonic epithelial cells with energy (Roediger, 1982) and keeps the colon lining healthy. It also reduces intestinal permeability, improves the integrity, and acts as an anti-inflammatory agent for the colon mucosa (Ferreira et al., 2012). Low concentrations of butyrate cause differentiation of mammalian cells, as well as colon carcinoma cells (Lupton, 2004; Augenlicht et al., 2002). Acetate and propionate influences cholesterol synthesis (Wolever et al., 1991), whilst propionate is involved in the gluconeogenic process.

The microbiota is also involved in the production of:

- **Vitamin K**, which is used by the liver for synthesising blood clotting factors. The microbiota produces half of the daily vitamin K requirement (Martini et al., 2015).
- **Biotin**, which is essential for glucose metabolism. It is known that pregnant and lactating women have an increased requirement for biotin. During pregnancy, this might be caused

by the accelerated catabolism of biotin, but the reason for increased demand in lactation has yet to be elucidated (Bowman and Russell, 2006; Martini et al., 2015). The amount produced by the microbiota is, however, very small (Whitney and Rolfes, 2013).

- **Vitamin B₅** or pantothenic acid, involved in more than 100 different steps in the synthesis of lipids, haemoglobin steroid hormones, and neurotransmitters (Whitney and Rolfes, 2013; Martini et al., 2015).
- **Host defence against toxins and pathogens, as well as the shaping of the immune system.**

The microbiota can directly interact with pathogens and immune cells that enter the system via the gastrointestinal tract. The result of this interaction can either cause or prevent disease or infection (Belkaid and Hand, 2014).

The relationship between the microbiota and its mammalian host can either be mutualistic (where both benefit from the activity of the other); parasitic (where the parasite benefits at the expense of the host); or commensal (where the microbiota obtains food or other benefits without harming or benefitting the host). This will depend on the host's diet and lifestyle (Singh et al., 2017).

The natural microbiota can protect its host in different ways. Firstly, it competes with pathogens and parasites for nutrients in the digestive tract (Kamada et al., 2013) thereby inhibiting their growth (Kamada et al., 2013; Belkaid and Hand, 2014). Furthermore, it stimulates the epithelial cells to produce antimicrobial peptides and reinforce tight junctions. Butyrate is particularly important in the maintenance of the intestinal barrier. In inflammatory bowel disease, it has been shown that a deficit in butyrate causes tight junction lesions, which increase intestinal permeability and bacterial translocation. Pathogen survival and growth can also be inhibited by the antimicrobial peptides that are secreted by commensals (Bischoff et al., 2014).

Finally, commensals can modulate innate and adaptive immunity (Molloy et al., 2012). The protective role of the microbiota during acute injury in the gut was illustrated when toll-like receptors (TLR) were activated by commensals to promote tissue repair and host survival. Toll-like receptors (TLRs) are transmembrane protein receptors that play an important role in the innate immune system. They are usually found on macrophages and dendritic cells, recognising pathogens to protect the host against microbial infection (Rakoff-Nahoum and Medzhitov, 2009).

When commensals translocate across the intestinal epithelial cell barrier macrophages that reside in the lamina propria, a layer of cells under the epithelium (which contains blood vessels, myofibroblasts, nerves, and different immune cells) consume and eliminate it rapidly or it is carried alive by dendritic cells. Dendritic cells sample commensals associated with the epithelium,

and interact with B and T cells in the Peyer's patches (also called aggregated lymphoid nodules) to produce secretory immunoglobulin A (Macpherson and Uhr, 2004). These lymphoid nodules are most abundant in the terminal ileum, near the entrance to the colon, to protect the small intestine from bacteria that normally inhabit the large intestine (Martini et al., 2017). T cells also play a critical role in intestinal homeostasis in producing IL-22, which regulates the microbiota composition and maintains epithelial barrier integrity (Prendergast and Kelly, 2016). Other cytokines including IL 4, -5, -6 and -10, as well as the transforming growth factor β , all play a role in the intestinal stimulation of secretory immunoglobulin A (Mantis et al., 2011).

The microbiota can also control the production of IL-1 β , a cytokine that is involved in host defence. The microbiota aids the homeostatic production of pro-IL-1 β by resident macrophages, priming cells to respond rapidly to enteric infections by converting pro-IL-1 β to mature active IL-1 β (Belkaid and Hand, 2014).

The highest numbers of immune cells are resident in the gastrointestinal tract, and are aimed at controlling the host's relationship with the microbiota. This is achieved by minimising the contact between microorganisms and the epithelial cell surface, and thus limiting bacterial translocation and tissue inflammation (Belkaid and Hand, 2014). An imbalance or dysregulation can be caused by a defect in the barrier function, causing inflammation, and can lead to irritable bowel syndrome, colitis, and inflammatory bowel diseases in humans, like ulcerative colitis, and Crohn's disease (Piche, 2014, Shulman et al., 2008, Xavier and Podolsky, 2007).

Interestingly, Koren et al. (2012), found that pregnant women in the third trimester (T3) had higher levels of Actinobacteria and Proteobacteria populations, when compared to the first trimester (T1) of pregnancy. This was associated with higher cytokine levels in stool, which are biomarkers of inflammation in the gut (Saiki et al., 1998). Levels of the pro-inflammatory cytokines IFN- γ , IL-2, IL-6, and TNF- α were significantly higher in T3 than they were in T1, relating to gut inflammation. The data presented in this study were thus indicative of low-grade inflammation of the mucosal surfaces in the GI tract during the third trimester.

The authors initially suggested that an increase in body fat during pregnancy also reduces insulin sensitivity, which has been correlated with changes in the immune system during pregnancy. This includes elevated cytokines (TNF α and IL-6) that are thought to drive obesity-associated metabolic inflammation. However, they found that during pregnancy, in contrast to normal obesity, the excess adiposity and attenuated insulin sensitivity support the growth of the foetus and prepare the mother's body via the host-microbial interaction. The dramatic changes in the gut microbiome during pregnancy may drive metabolic changes, such as insulin resistance and weight gain, experienced by expecting mothers. This actually assists in the development of the foetus and energy demands during lactation (Koren et al., 2012). In a study by Jost et al.

(2014), low-grade intestinal inflammation measured by increased faecal calprotectin levels were also observed during the third trimester of pregnancy. Low-grade inflammation may reduce epithelial barrier integrity, which would support the hypothesis of increased bacterial translocation during pregnancy, and the presence of a bacterial pathway between mother and foetus; not only during breast-feeding as previously believed, but also during gestation (Jost et al., 2014).

2.2.3 Necrotizing enterocolitis - an example of the effects of excessive colon inflammation

Necrotizing enterocolitis is a serious disease of the intestine that mostly occurs in premature infants with a birth weight of less than 1 500 g, usually after oral feeding commences. It is associated with a mortality of between 15% and 30%, and may cause long-term neurodevelopmental disease (Lin and Stoll, 2006; Pammi et al., 2017; Neu and Walker, 2011; Fitzgibbons et al., 2009). As illustrated in Figure 2-2, NEC is a multifactorial disease, and even though the pathogenesis is not clear, intestinal ischemia, microbial dysbiosis, and excessive inflammation have been implicated (Nanthakumar et al., 2000).

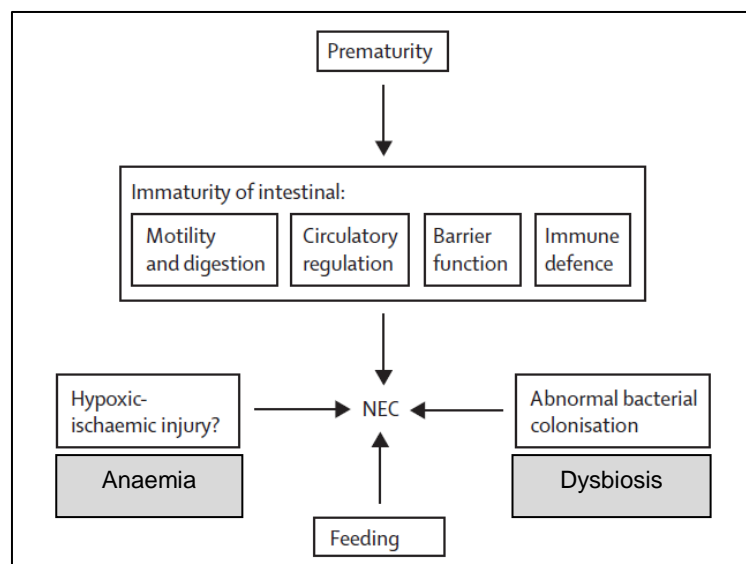


Figure 2-2: Necrotising enterocolitis is a multifactorial disease, but anaemia and dysbiosis are potential risk factors. (Modified from Lin and Stoll, 2006)

Abbreviations: NEC: – necrotising enterocolitis.

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In an observational cohort study, Patel et al. (2016) found that severe anaemia was associated with an increased risk of NEC amongst very low birth weight infants (Patel et al., 2016). Anaemia may result in decreased oxygen delivery to the gastrointestinal tract (intestinal ischemia), allowing for an increased susceptibility to mucosal injury and necrosis (Patel et al., 2017). It

is also known that maternal iron deficiency is associated with babies with low weight (<2500 g) at birth (Black et al., 2013).

Furthermore, the intestinal microbiota of preterm infants has fewer bacterial species, less diversity, and more potential pathogens, when compared to full-term infants (Carlisle and Morowitz, 2013). In a systematic review and meta-analysis, Pammi et al. (2017) found that intestinal dysbiosis associated with a decrease in the phyla Firmicutes (including lactobacilli) Actinobacteria (including Bifidobacterium) and Bacteroidetes, and an increase in Proteobacteria, precedes NEC in preterm infants (Pammi et al., 2017). The microbial dysbiosis preceding NEC could lead to intestinal inflammation, and may weaken the barrier effect of the gut, which again can cause systemic inflammation (Lin and Stoll, 2006; Turner, 2009; Bischoff et al., 2014). In a pooled meta-analysis, Patel and Underwood (2018) concluded that probiotics preparations containing either lactobacillus alone or in combination with Bifidobacterium, decrease NEC and death (Patel and Underwood, 2018), which is in accordance with the Cochrane review (AlFaleh and Anabrees, 2014).

Studies in animal models suggest that n-3 PUFA may also play a significant role in attenuating intestinal inflammation and reducing the risk of NEC (Caplan et al., 2001; Ohtsuka et al., 2011; Wijendran et al., 2015). In a systematic review, these results were confirmed in neonates younger than 32 weeks, where a reduction in the risk of NEC was observed with n-3 LCPUFA administration (Zhang et al., 2014). This could be due to the integral role that n-3 PUFAs play in the epithelial integrity, reducing the translocation of bacteria and endotoxins, as well as the anti-inflammatory and pro-resolving properties of EPA and DHA (Serhan et al., 2018; Serhan and Petasis, 2011).

2.3 The effect of iron on gut and colon inflammation

Even though inflammation is a normal response in the body in reaction to injury and infection, unresolved inflammation can also lead in later life to tissue damage and disease (Calder, 2006) including diabetes, obesity, cancer, and neuropathologies (Bischoff et al., 2014). It is known that diet and nutrition play a crucial role in the development and resolving of inflammation, and in this study, we will specifically look at the effect of iron and n-3 PUFA deficiency on gut inflammation as two deficiencies that are common in low- and middle-income countries.

2.3.1 Normal iron metabolism

Iron is a vital nutrient for virtually all organisms, forming an essential part of haemoglobin in erythrocytes (red blood cells) that carry oxygen from the lungs to other tissue. Haemoglobin

accounts for about 80% of the body's iron (Whitney and Rolfes, 2013). Seeing that iron can switch between two ionic states in the body, ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}), it can also serve as a cofactor to enzymes involved in oxidation-reduction reactions (Whitney and Rolfes, 2013). For example, iron is a co-factor for the desaturase enzymes involved in the synthesis of long-chain polyunsaturated fatty acids, as well as of the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, which are involved in the production of lipid mediators (Kuhn et al., 2005).

Only about 10% (1-2 mg per day) of the iron consumed from food is absorbed by the enterocytes in the duodenum (Balamurugan et al., 2010; Cherayil, 2011; Martini et al., 2015). Non-haeme iron generally exists in the ferric form, and needs to be reduced to the ferrous form in order for absorption to take place (Whitney and Rolfes, 2013). As shown in Figure 2-3, the iron transporter divalent metal transporter 1 (DMT 1), also known as natural resistance-associated macrophage protein 2 (Nramp 2), assists with the active absorption into the enterocyte, where the iron is bound to ferritin, or transported to the blood through ferroportin (FPN). FPN is a transmembrane protein that moves iron from the inside of cells to the outside of the cell into the plasma. In the blood, it is then oxidised and incorporated into transferrin as shown in Figure 2-3 (Cherayil, 2011).

Transferrin delivers iron to the bone marrow, where large quantities of red blood cells (RBC) are produced. Surplus iron is stored as ferritin in the liver (hepatocytes), spleen and bone marrow. Ferritin is rapidly made and broken down in order to provide adequate iron for the body's needs (Whitney and Rolfes, 2013).

Iron absorption does not only take place in the small intestine. Evidence from experimental animals suggests that it can also be absorbed from the proximal colon. In pigs, the colon contributes approximately 14% of the duodenal iron absorption (Blachier et al., 2007). The iron transporters DMT1 and FPN are both present in the colon of humans and animals, and may be expressed during iron deficiency (Takeuchi et al., 2005; Blachier et al., 2007). In the proximal colon, short chain fatty acids, produced when non-digestible disaccharides are fermented, assist in iron absorption, and could prevent iron deficiency (Asvarujanon et al., 2005; Shiga et al., 2006).

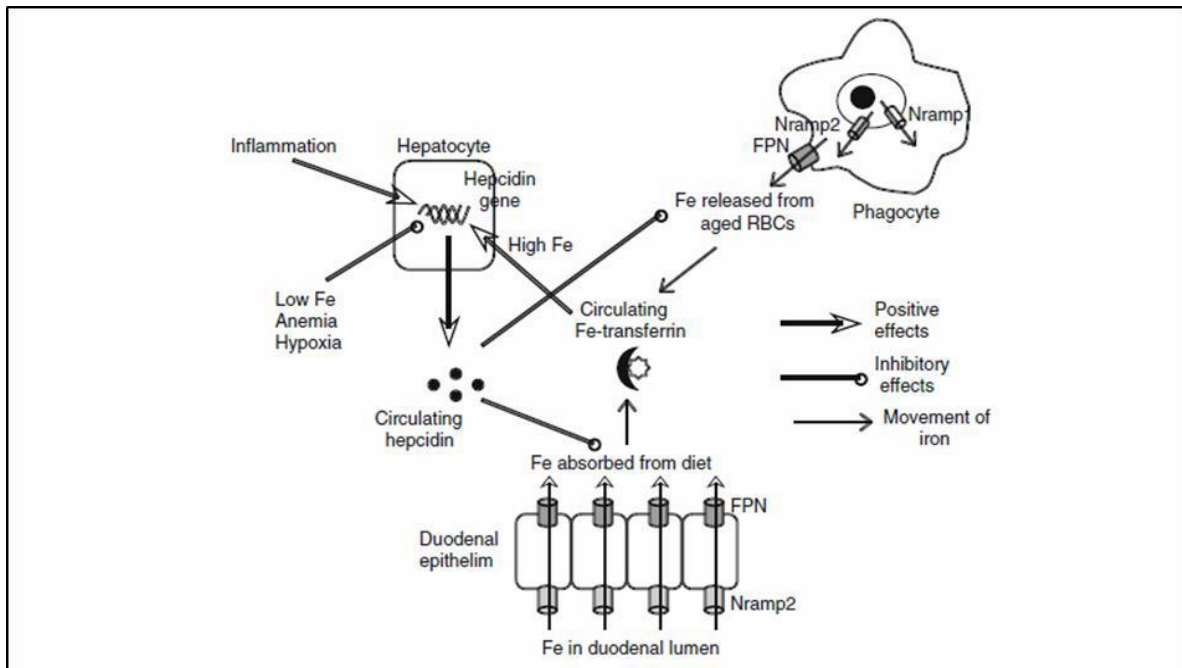


Figure 2-3: Iron homeostasis is regulated by hepcidin and ferroportin in mammals (Cherayil, 2011)

Abbreviations: Fe: iron; FPN: ferroportin; Nramp2: natural resistance-associated macrophage protein 2; RBC: red blood cell.

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The daily loss of iron is relatively small, but considering the low absorption rate from food (1-2 mg/day), it is crucial for the body to recycle and reutilise iron. For erythropoiesis for example, the bone marrow utilises approximately 20 mg of iron daily. Macrophages in the liver, spleen, and bone marrow play an important role in the recycling of iron. They engulf the senescent erythrocytes (at approximately 120 days), degrade it through the process of haemolysis, and then catabolise the haemoglobin to release free haeme iron (Fe^{++}). The free haeme iron is then again transported in the blood by transferrin and delivered to the bone marrow, where new RBCs are formed. The production of RBCs is regulated by the hormone erythropoietin (EPO), which is stimulated by hypoxia caused by anaemia, low blood flow to the kidneys, and high altitude, amongst others (Martini et al., 2015)

When there is a large surplus of iron, the liver converts some ferritin to hemosiderin, which releases iron more slowly than does ferritin. This process is important to protect the body against the damage that free iron can cause, where it acts as a free radical damaging cell lipids, protein and DNA (Whitney and Rolfes, 2013). With a low absorption rate, consumed iron passes through to the colon, where it may feed entero-pathogenic microbes (Drakesmith and Prentice, 2012), increasing the pathogen abundance, disturbing the normal gut microbiome, and causing inflammation (Zimmermann et al., 2010; Jaeggi et al., 2015; Kortman et al., 2012). Interestingly, the beneficial lactic acid bacteria, *Lactobacillus* species (spp.) as well as *Bifidobacterium*, thrive in an iron-poor environment, and these organisms may be displaced by more pathogenic species

when iron is in abundance (Ganz and Nemeth, 2015).

In mammals, the iron regulatory hormone hepcidin, secreted by the hepatocytes, is crucial in iron homeostasis. Hepcidin helps to maintain blood iron within the normal range by limiting absorption from the small intestine and controlling the release from hepatocytes, spleen and bone marrow. This is done by the degradation of FPN. The result is iron sequestration, decreased iron transfer to transferrin, and a decreased iron flow into the plasma. Therefore, under normal circumstances, hepcidin production decreases during iron deficiency and hypoxia, and increases in iron overload as illustrated in Figure 2-3 (Cherayil, 2011; Ganz and Nemeth, 2012). Hepcidin is, however, not only influenced by serum iron levels, but the inflammatory cytokines, like IL-6 can also upregulate its expression (Cherayil, 2011).

2.3.2 How inflammation causes iron deficiency

During infection and inflammation, hepcidin concentrations are greatly increased, through IL-6 and C-reactive protein (Drakesmith and Prentice, 2012). High concentrations of hepcidin blocks FPN. Iron is thus trapped in cells and sequestered, withholding it from the circulation and decreasing serum iron, which can lead to hypoferraemia and decreased erythropoiesis. As illustrated in Figure 2-4 (left diagram), low iron leads to low hepcidin levels, and an increase in FPN levels (Cherayil, 2011). Less iron in cells inhibits bacterial growth, and there is a subsequent decrease in the production of inflammatory cytokines. In the diagram on the right, the opposite is shown. High intracellular iron promotes infection and/or inflammation. This increases inflammatory cytokine production and hepcidin levels. At the same time, FPN decreases and the efflux of iron into the serum is lowered (Cherayil, 2011).

The prolonged activation of this mechanism is referred to as the anaemia of inflammation. This process makes iron unavailable to pathogens in need of iron for survival. It therefore works as an anti-inflammatory mechanism, where the host is protected from further inflammation by circulating iron (Ganz and Nemeth, 2015; Cherayil, 2011). Chronic inflammatory disorders including rheumatologic disorders and inflammatory bowel disease (IBD) are associated with anaemia of inflammation, where an increase in the cytokines IL-6 and interferon (IFN) stimulates hepcidin production through the JAK-STAT 3 pathway. These patients have decreased intestinal iron absorption and are thus usually resistant to oral iron therapy (Ganz and Nemeth, 2015; Cherayil, 2011).

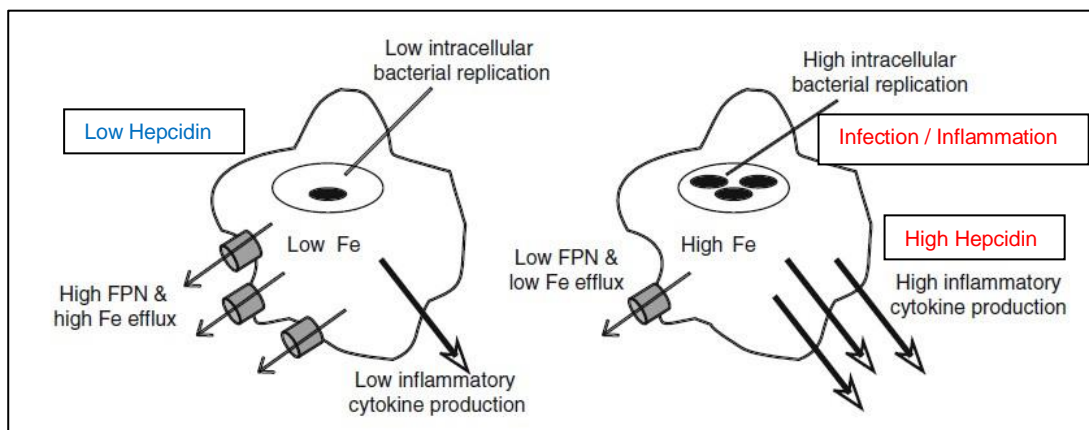


Figure 2-4: The levels of ferroportin and hepcidin in macrophages influence the intracellular bacterial growth and the expression of inflammatory cytokines (Cherayil, 2011).

Abbreviations: Fe:– iron; FPN:– ferroportin.

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2.3.3. The relationship between iron deficiency and gut inflammation – a summary of studies

Iron deficiency can also influence gut inflammation, but results are conflicting. No studies have measured gut lipid mediators as an indicator of gut inflammation or inflammatory signaling in iron deficiency. Most of the studies summarised here, measured bacterial groups in the gut and faecal short-chain fatty acid concentrations. Both these indicators are affected by gut inflammation and can be used as indications of inflammatory status, but could also affect gut inflammation indirectly (Table 2-1; Table 2-2 and Table 2-3).

Some studies included the measurement of gut permeability (via lactulose: mannitol test and trans-epithelial electrical resistance/TER) (Li et al., 2016, Hossain et al., 2016), but the only two direct measurements for intestinal inflammation were gut cytokine expression and neutrophil infiltration in the gut mucosa (Dostal et al., 2012; Li et al., 2016). Neutrophils infiltrating the mucosa secrete a peptide, calprotectin. Faecal calprotectin can therefore be used as a non-invasive biomarker in the diagnosis of IBD (Costa et al., 2003; Dostal et al., 2014b).

In a study by Dostal et al. (2012), iron depletion in male Sprague-Dawley rats caused a decrease in the butyrate-producing bacteria (Dostal et al., 2012). Butyrate is mainly produced by the members of Clostridium clusters like Faecalibacterium prausnitzii, Eubacterium hallii and Roseburia spp. (Dostal et al., 2014b; Dostal et al., 2014a). The decrease in Roseburia spp. was associated with a decrease in the production of butyrate. (Dostal et al., 2012). It is known that butyrate has anti-inflammatory properties, and that a deficiency causes TJ lesions, and impairs intestinal barrier function (Bischoff et al., 2014).

In the same study by Dostal et al. (2012), Enterobacteriaceae spp. (pathogens), which have the

potential to increase inflammation in the gut, increased (Dostal et al., 2012). These findings were confirmed in an in vitro study by the same researchers, where iron deficiency decreased not only Roseburia spp, but also Faecalibacterium Prausnitzii, which converts acetate to butyrate, as well as the Clostridium Cluster IV, which is involved in butyrate production (Dostal et al., 2013). In female Fischer rats, iron supplementation increased gut microbial butyrate concentration up to 6-fold in comparison with iron depletion, and did not affect histological colitis scores (Dostal et al., 2014b). Furthermore, there was a greater neutrophil infiltration of the colonic mucosa in male Sprague Dawley rats that received a diet containing 20 mg iron sulphate, compared to the iron deficient rats; indicating that free iron in the colon could increase gut inflammation associated with pathogen colonisation and inflammatory bowel disease (Dostal et al., 2012; Dostal et al., 2014b).

In male pigs, iron depletion increased the gene expression of IL-1B as well as IL-6, which are both pro-inflammatory, while the numbers of mucosal neutrophils increased during iron depletion. The authors concluded that iron deficiency not only increased inflammation, but also increased gut permeability (Li et al., 2016).

The same trend was seen in Bangladeshi children in the age range of 13.2 ± 5.2 months, in a study conducted by Hossain et al. (2016). When intestinal permeability was measured using the lactulose: mannitol test, where low serum iron was associated with a decrease in the tight junctions in the intestinal epithelium and higher gut permeability. Interestingly, recent diarrhoea as well as fever, was also associated with impaired intestinal integrity. This suggests that adequate iron reserves may help in maintaining the mucosal function and that enteropathy is not only associated with younger age, but also with malnutrition at an older age (Hossain et al., 2016).

In contrast, iron deficiency has been shown to benefit the host by increasing beneficial Lactobacilli spp. and Bifidobacterium. The beneficial lactic acid bacteria (Lactobacilli spp.) and Bifidobacterium thrive in an iron-poor environment (Ganz and Nemeth, 2015). Werner et al. (2010) studied the association between luminal iron sulphate, systemic iron, the gut microbiota and the development of chronic ileitis in a murine model of Crohn's disease. The mice received either an iron sulphate containing diet (180 mg Fe/kg), or iron sulphate-free diet (<10 mg Fe/kg), for 11 weeks after weaning. They found that the iron-free diet induced a significant increase in the abundance of Bifidobacterium, which attenuated inflammation, and prevented the onset of Crohn's disease-like ileitis. A proposed mechanism for this is that a decrease in luminal iron exposure significantly reduces endoplasmic reticulum (ER) stress in ileal epithelial cells (Werner et al., 2010).

Also, Tompkins et al. (2001) showed that mild iron deficiency increased lactobacilli significantly in the colons of iron deprived mice (Tompkins et al., 2001). A study in rats by (Dostal et al., 2012) as well as an in-vitro study with human cells by the same author, confirmed that iron deprivation increased lactobacilli in the colon (Dostal et al., 2013). A higher lactobacilli count is associated with higher HDL levels, improved insulin sensitivity, as well as lower inflammation (C-reactive protein) (Rajkumar et al., 2014). This may be related to the central role that lactobacilli play in improved barrier function and decreased paracellular permeability in the gut (Ahrne and Hagslatt, 2011).

In contrast to animal studies, a case-control study in Indian women 18-25 years of age, revealed that those with anaemia had low lactobacilli counts, while there was no significant difference between any of the other bacterial species measured (including Bifidobacterium genus, Bacteroides–Prevotella–Porphyromonas, Eubacterium rectale and Clostridium leptum) from faeces of anaemic and non-anaemic women (Balamurugan et al., 2010). The authors argued that this was due to the enhanced absorption of iron in the colon during the anaemic state. DMT 1 and ferroportin are expressed in the colon of mammals and expression can be increased during iron deficiency, while the absorption of iron is also enhanced by SCFAs like propionic acid, produced by the bacterial fermentation of fibre in the colon (Balamurugan et al., 2010).

Table 2-1: The effect of iron depletion on the gut – A summary of randomised controlled trials in animals

Author, year	Type of animal & age	Duration of study	Iron (in controls)	Dose (low iron)	Inflammation markers Microbiota Short chain fatty acids	Notes
Tompkins et al. (2001)	Male Swiss-Webster mice 32 days old	12 weeks	Ferric chloride	Iron deficient/base diet* < 2 mg iron/kg <i>*Prior to the introduction of the iron-controlled diets, all of the mice received a diet very rich in iron (350 mg/kg) and would, therefore, possess significant iron reserves</i>	Microbiota ↑ concentrations of all bacterial types ↑ presence of coliform bacteria in the jejunum ↑ lactobacilli in colon ↑ concentrations of all bacterial types, except the coliforms, in the colon	With the exception of coliforms, all examined bacterial groups (anaerobes, micro-aerophiles, lactobacilli, and enterococci) were significantly ($p < 0.05$) elevated in the colons of iron-deprived mice. Although the lactic acid bacteria were found at all sites in higher numbers in iron-deprived mice than in the other groups, there was little statistical evidence that a deficiency of iron in the diet encouraged the growth of enterococci and lactobacilli. The iron-deficient diet used in the current study contained enough iron to support normal levels of coliforms and other iron-requiring bacteria; a preparation that more rigorously excluded iron might have greater effect.
Werner et al. (2010)	Wild-type and heterozygous TNF [Δ ARE/WT] mice 49 days old	18 weeks	Iron sulphate free diet or injections	Iron sulphate free diet <10 mg iron/kg	Inflammation ↓ stress response in endoplasmic reticulum contribute to ↓ inflammation Diet free of iron sulphate prevents the onset of severe Crohn's disease like-ileitis. Microbiota ↓ Desulfovibrio species (↓ toxic sulphides) ↑ Bifidobacterium (prevent inflammation)	Major alterations in microbial composition were induced by the iron sulphate-free diet; while host genotype, inflammatory response, and systemic iron application had a smaller effect. Interestingly, Desulfovibrio species, which decreased through the iron sulphate-free diet, produce toxic sulphides, which have been suggested to contribute to pathogenesis in IBD, and which have been shown to be increased in patients with ulcerative colitis. An iron sulphate-free diet induced a significant increase in the abundance of bifidobacteria, which are often used as probiotics, and have been shown to prevent inflammation in murine models of IBD.
Dostal et al. (2012)	Male Sprague-Dawley rats 21 days old	24 days The rats were depleted of	Iron sulphate	Iron depletion diet 2.6 mg iron/kg	Microbiota ↓ Roseburia spp ($p < 0.001$) ↓ Bacteroides spp. ($p < 0.001$) ↑ Enterobacteriaceae (pathogens)	Inflammation Greater neutrophil infiltration of the colonic mucosa was found in the rats of the 20 mg iron sulphate group, compared to the Fe deficient

Author, year	Type of animal & age	Duration of study	Iron (in controls)	Dose (low iron)	Inflammation markers Microbiota Short chain fatty acids	Notes
		iron for 24 days			could increase inflammation (p = 0.021) ↑ Lactobacillus/Pediococcus and Leuconostoc spp. (significantly higher) Short chain fatty acids ↓ Butyrate ↓ Propionate	rats. Neutrophil infiltration of the gut mucosa is a marker of inflammation that may be associated with pathogen colonisation and inflammatory bowel disease. Microbiota Iron depletion caused significant changes in the microbiota, with a decrease in the beneficial species Roseburia, which produces butyrate that has anti-inflammatory properties. A significant increase in Enterobacteriaceae was associated with iron depletion. These organisms have developed mechanisms, including siderophores, to acquire iron in competition with other bacteria and the host. Higher numbers of these pathogens could increase inflammation. Iron depletion also increased Lactobacillus spp. Short chain fatty acids Compared to the iron sufficient group the caecal concentration of butyrate, the main metabolite of Roseburia spp. /E. rectale, was 87% lower and propionate was 72% lower (P = 0.05). Fe repletion with both Fe compounds partially restored the butyrate and propionate concentrations; iron sulphate was more effective.
Li et al. (2016)	Male pigs 21 days body weight 5.5 ± 0.4 kg	32-36 days	Iron sulphate supplement (dry matter basis),	Low iron diet 20 mg iron/kg	Inflammation ↑ expression of IL1-B and IL 6 (P < 0.1) / pro-inflammatory ↑ numbers of mucosal neutrophils/pro-inflammatory (P < 0.05) ↑ in paracellular permeability	Both a deficiency and excess of dietary iron had substantial deleterious impacts on intestinal morphology, inflammation, and function. Inflammation With the low iron diet, there were increased (P < 0.05) numbers of mucosal neutrophils observed when compared with that of pigs fed the adequate iron diet. Pigs fed the low iron diet had a >3-fold higher neutrophil number in the duodenal mucosa,

Author, year	Type of animal & age	Duration of study	Iron (in controls)	Dose (low iron)	Inflammation markers Microbiota Short chain fatty acids	Notes
						<p>compared to pigs fed an adequate iron diet.</p> <p>Permeability</p> <p>Duodenal TER (intestinal permeability measurement) was reduced in pigs fed the low iron diet ($P < 0.05$), when compared with pigs fed the adequate iron diet.</p> <p>In agreement with the TER measurement, the duodenum from pigs fed the low iron diet exhibited increased mucosal to serosal fluxes of 3H-mannitol compared with those fed the adequate iron diet ($P < 0.05$), thus confirming an increase in paracellular permeability associated with iron deficiency.</p>
<p><i>Abbreviations: IBD: Inflammatory bowel disease; IL: interleukin; mg: milligram; kg: kilogram; spp.: species; TER: trans-epithelial electrical resistance</i></p>						

Table 2-2: The effect of iron depletion on the gut – A summary of human case-control studies

Author, year Type of study	Group	Duration	Type of iron	Iron intake	Inflammation markers Short chain fatty acids Microbiota	Notes
Balamurugan et al. (2010)	Indian women 18-25 years Anaemic (n=8) Non-Anaemic (n=26)	N/A	Iron from the diet was almost exclusively non-haem iron from plant sources & eggs	Anaemic 8.8mg/d Non-anaemic 8.9 mg /d	Microbiota ↓ Faecal L. acidophilus in anaemic women	Faecal levels of L. acidophilus group bacteria were significantly lower in anaemic women. No difference between the two groups with respect to any of the other bacteria that were examined.
Hossain et al. (2016)	Bangladesh children Male & Female 13.2 ± 5.2 months underweight VS well nourished	NA	NA	NA	Permeability Low serum iron associated with ↑ permeability Low serum iron associated with ↓ TJ in intestinal epithelium Enteropathy (high L:M) / high permeability is associated with: <ul style="list-style-type: none"> - younger age; - undernutrition; - low vitamin A and iron status; and - infection particularly diarrhoea and fever. Recent diarrhoea associated with impaired intestinal integrity & absorption. Recent fever history associated with ↓ intestinal integrity.	Adequate iron reserves may help in maintaining normal intestinal mucosal function and permeability. Enteropathy (high L:M) is associated with younger age, undernutrition, low vitamin A and iron status, and infection particularly diarrhoea and fever. Recent history of diarrhoea was associated with impaired intestinal integrity and absorption. Probably due to lack of maturation of the intestinal cellular structure at younger age, the L:M is comparatively higher than that when the children are grown up. High L:M is found to be also associated with undernutrition and low iron status. Lower body iron status is associated with a defect in the tight junctions in intestinal epithelium (as reflected by more para-cellular absorption and higher urinary lactulose recovery). This is the first study reporting that a recent history of fever is also associated with impaired intestinal integrity and absorption.

Abbreviations: n: amount; L:M: Lactulose/Mannitol Test (as measurement for gut permeability); VS: versus.

Table 2-3: The effect of iron depletion on the gut – An in vitro study

Author, year Type of study	Cells	Duration	Type of iron	Dose	Inflammation markers Short chain fatty acids Microbiota	Notes
Dostal et al. (2013)	6-10 year old child gut microbiota	70 days	Iron sulphate	<ul style="list-style-type: none"> • No Fe (1.56 ± 0.1 mg Fe/L) to mimic iron deficiency [+ Chelex decreased iron to 0.9 ± 0.2 mg Fe/L] • Low Fe (3.91 ± 0.1 mg Fe/L) • Normal Fe (8.13 ± 1.8 mg Fe L/1) • High Fe (26.5 ± 2.2 mg Fe/L) 	Inflammation ↑ Lactate Low iron conditions Microbiota ↓ F. Prausnitzii ↓ Blautia spp ↓ Roseburia spp ↓ Clostridium Cluster IV ↑ Enterobacteriaceae spp - ↑ Lactobacillus spp ↑ Bifidobacterium Short chain fatty acids ↓ Butyrate ↓ Acetate ↓ Propionate	Iron deficiency leads to dysbiosis in the gut microbiota - impacting gut health Low iron conditions were associated with: Decrease in: - F. Prausnitzii - can also convert acetate to butyrate - Blautia spp. - Roseburia spp. - Clostridium Cluster IV - Bacteroides spp. - Butyrate (-84%) - Propionate (-55%) Increase in: - Enterobacteriaceae spp. - Lactobacillus spp (don't need iron for survival) / Pediococcus, Leuconostoc spp - Bifidobacterium - Lactate, where the accumulation of lactate in faeces has been correlated with inflammatory bowel disease and ulcerative colitis (Vernia et al., 1988; Hove et al., 1994). Thus, low-iron and no-iron conditions (in vitro) can lead to negative impacts on gut health. The strong dysbiosis of the gut microbiota together with a decrease in main gut microbiota metabolites observed with very low iron conditions could weaken the barrier effect of the microbiota and negatively impact gut health.
Abbreviations: F: Faecalibacterium; Fe: iron; spp.: species.						

2.4 The relationship of n-3 polyunsaturated fatty acids with gut and colon inflammation

2.4.1 The role of fatty acids and lipid mediators in inflammation and gut health

The influence of fatty acids on inflammation involves the incorporation of it into cell membrane phospholipids (Calder, 2015b). The Western diet contains high amounts of saturated fat, but cell membranes, including the intestinal mucosa require unsaturated fat for their structure, fluidity and proper functioning (Calder, 2015b; Wang and Colgan, 2017). Polyunsaturated fatty acids cannot be synthesised in the body and have to be consumed through food (Calder, 2015b; Teitelbaum and Walker, 2001; Whitney and Rolfes, 2013).

Unsaturated fat can be divided into two main groups – monounsaturated fat (MUFA) and polyunsaturated fat (PUFA), with the latter being classified as n-6 (omega-6) and n-3 (omega-3), depending on the location of the first double-bond from the methyl end of the fatty acid (Calder, 2015b; Teitelbaum and Walker, 2001). Alpha-linolenic acid (ALA) in plant oils like flaxseed, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in marine oils are the three types of n-3 fatty acids present in food (Whitney and Rolfes, 2013). In animals, small amounts (about 8%) of ALA can be converted into EPA and EPA can again be converted to DHA (Calder, 2006, Teitelbaum and Walker, 2001, Whitney and Rolfes, 2013) through the process of desaturation, elongation and β -oxidation as illustrated in Figure 2-5 on the right.

A similar series of reactions convert linoleic acid to arachidonic acid (ARA), as seen in Figure 2-5 on the left. ARA plays an important role in the production of the lipid mediators prostaglandin (PGE_2) and leukotriene (LTB_4). The enzyme involved in the conversion from ARA to PG_2 as well as thromboxane (TXA_2) is cyclooxygenase (COX), while the enzyme lipoxygenase (LOX) is central in the conversion of ARA to LTB_4 (Calder, 2015b; Calder, 2006). LTB_4 is pro-inflammatory. It increases vascular permeability, enhances local blood flow and increases the production of the pro-inflammatory cytokines TNF, IL-1 and IL-6 (Calder, 2015b; Calder, 2006). PGE_2 can also have a pro-inflammatory effect in the body by increasing vascular permeability and vasodilatation. It also induces pain, causes fever and increases IL-6. However, PGE_2 can have anti-inflammatory effects as well by inhibiting the production of TNF and IL-1, by inhibiting the production of leukotrienes and increasing the production of lipoxin, an anti-inflammatory lipid mediator (Calder, 2015b; Calder, 2006).

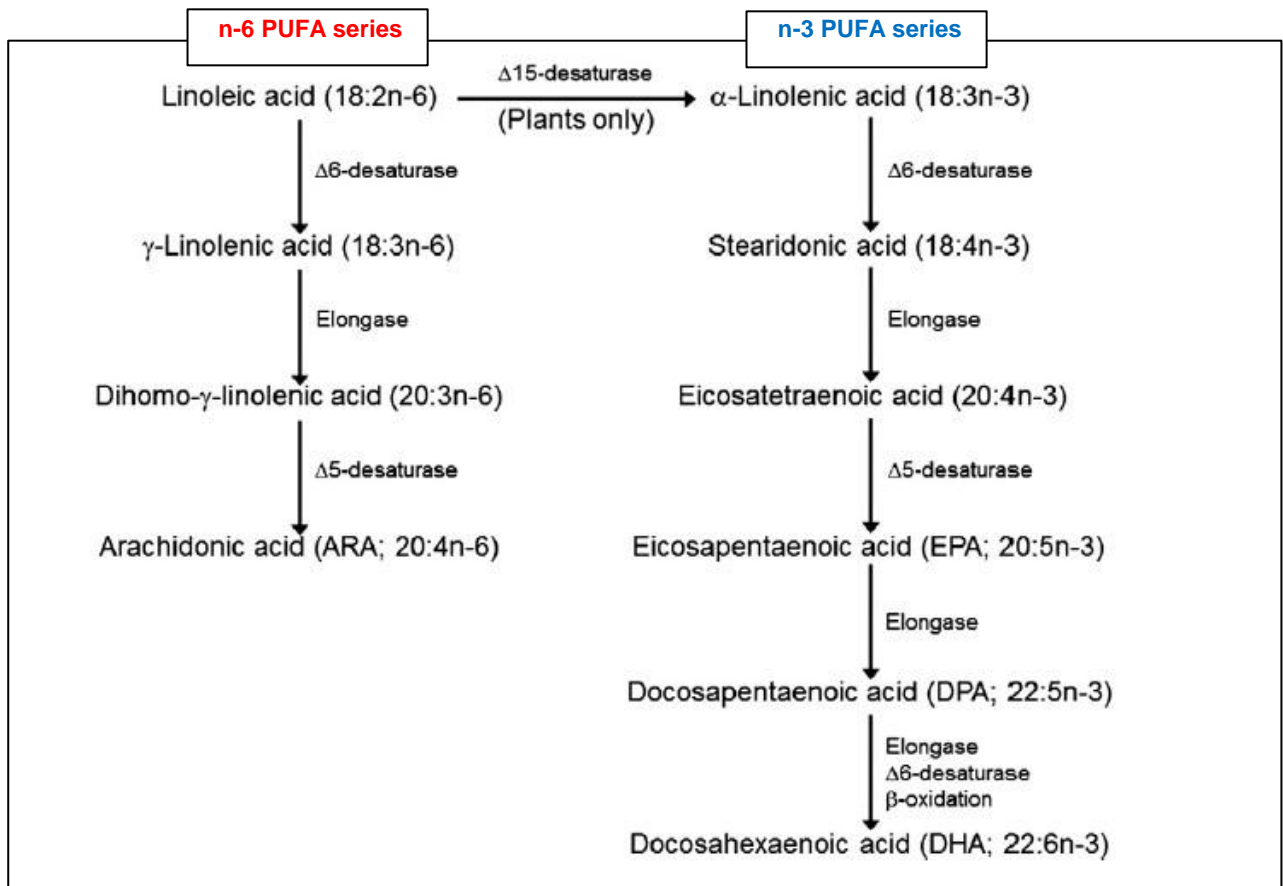


Figure 2-5: The biosynthesis of n-6 and n-3 PUFA. These two pathways compete for the same enzymes for elongation, desaturation, and oxidation in mammals (Calder, 2015b).

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The Western diet contains plentiful amounts of n-6 or linoleic acid (LA), because it is present in most plant oils, for example sunflower oil, which is freely available, accessible and cheap, especially in a country like South-Africa. During the past 100 years, the intake of n-6 PUFA has increased dramatically in relation to the n-3 PUFA consumption from a ratio of about 1:1 to more than 15-25/1 in Western diets currently (Simopoulos, 2011). This imposes a health risk, due to the pro-inflammatory nature of the n-6 PUFAs. On the other hand, n-3 PUFAs has an anti-inflammatory effect and resolves inflammation, as illustrated in Figure 2-6. This is due to the fact that n-3 PUFAs has an impact on antagonising the arachidonic metabolism as well as triggering the pro-inflammatory response.

2.4.1.1 Omega-3 polyunsaturated fatty acids antagonises arachidonic acid metabolism

The initial substrate for lipid mediator production is a membrane phospholipid. The mediators from EPA are anti-inflammatory, whereas the mediators from ARA are generally pro-inflammatory. An increased consumption of EPA and DHA results in a higher proportion

of these fatty acids in the phospholipids of cells to the expense of lipid mediators derived from ARA. Less substrate is available for the eicosanoid synthesised from ARA, leading to a decreased production of PGE₂, LTB₄ and thromboxane. For this reason, it can be argued that one of the mechanisms of the anti-inflammatory action of n-3 PUFA lies in antagonising arachidonic acid metabolism, not only by competing for the same enzymes (elongase, desaturase) in the conversion of n-3 PUFA to EPA and DHA, but also by limiting the substrate in phospholipids for pro-inflammatory lipid mediator production from ARA - Figure 2-5 (Calder, 2015b).

2.4.1.2 Super-family of specialised pro-resolving mediators (SPM)

Previously, it was believed that inflammation is a passive process, but new evidence proved that the resolution of inflammation is an active process triggered by a novel super-family of pro-resolving mediators (Serhan et al., 2018). The n-3 PUFAs act as substrates for these specialised lipid mediators (SPM) including resolvins (Rv), maresins (MaR) and protectins (PD), derived from EPA and DHA, which play an integral role in resolving inflammation (Serhan et al., 2018; Serhan and Petasis, 2011; Calder, 2006).

The lipid mediators formed from EPA include PGE₃ and TXA₃. LTB₆, 5-hydroxy-eicosapentaenoic acid (HEPE), 11-HEPE, 12-HEPE, 15-HEPE and 18-HEPE. 18-HEPE is the precursor of the E-resolvins. 17-HDHA is produced from DHA and is the precursor of the D-series resolvins and protectins, as illustrated in Figure 2-6. DHA is also the precursor of 14-HDHA, which is the precursor of the maresins (Calder, 2015b; Massey and Nicolaou, 2013).

The D-series resolvins as well as protectins are produced via a 17-lipoxygenation reaction from DHA (17 HDHA), while maresins are produced via 14-lipoxygenation of DHA, as seen in Figure 2-6. The resolvins are potent in controlling viral infection and autoimmunity and they are also involved in regulating pain and depression. Maresins engage in healing and tissue regeneration, while it also reduces inflammation (Serhan et al., 2018).

As illustrated in Figure 2-6, EPA is the precursor of the E-series resolvins, which are biosynthesised via COX-2 to 18-HEPE (18-hydroxyeicosapentaenoic acid); via 5-lipoxygenase converted to RvE₁ and RvE₂; and via 15 lipoxygenase to RvE₃. All the resolvins are potent in stopping polymorphonuclear leukocyte infiltration, thereby resolving inflammation (Serhan et al., 2018).

The resolvins and maresins furthermore also stimulate the adaptive immune system, which influences the secretion of IL-10 (increases), IL-17 (reduces) TNF- α (reduces) and IFN- γ

(reduces) by T-cells (Serhan et al., 2018). IL-10 is an essential immunoregulator in the intestinal tract and has anti-inflammatory properties (Kühn et al., 1993, Gravaghi et al., 2011), while IL-17 and TNF- α works pro-inflammatory (Jovanovic et al., 1998). Furthermore n-3 PUFA (and especially EPA), has an anti-chemotactic effect, which decreases the infiltration of neutrophils and monocytes toward serum, bacterial peptides and LTB₄. Supplementation with n-3 PUFA in humans also decreased the production of reactive oxygen species (Calder, 2006).

Therefore, it can be argued that n-3 PUFAs exhibit general anti-inflammatory properties and that it is also actively involved in resolving inflammation, not only generally, but also in the intestine. A dose of 2 g/d in humans has been proved to provide effective inflammation resolving capacity (Calder, 2015b).

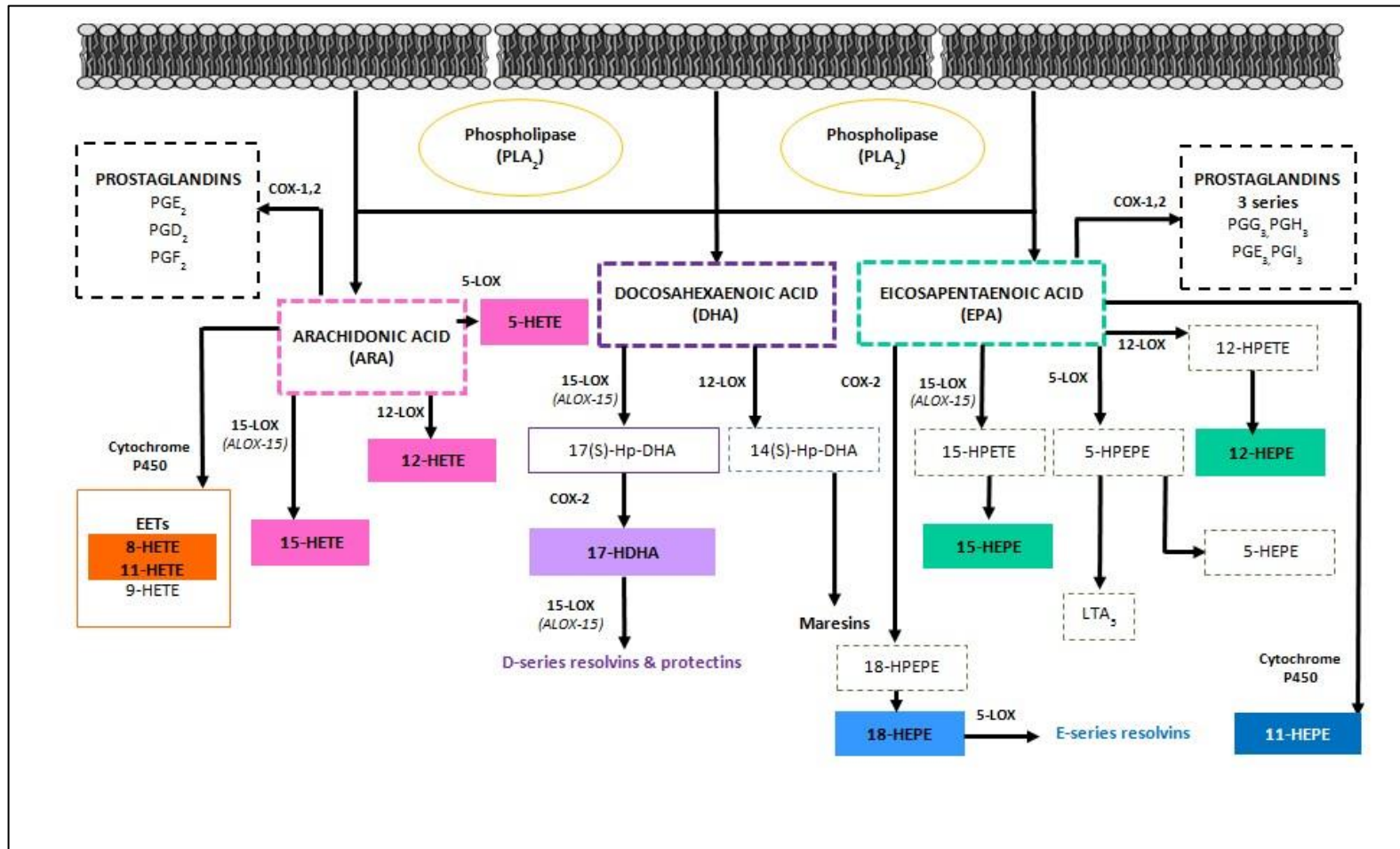


Figure 2-6: An overview of the main pathways involved in the production of polyunsaturated fatty acid-derived lipid mediators
 (Adapted from Zandberg et al 2018)

Abbreviations: ALOX: arachidonate lipoxygenases; ARA: arachidonic acid; COX: cyclooxygenase; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; HDHA: hydroxy docosahexaenoic acid; HEPE: hydroxyeicosapentaenoic acid; HETE: hydroxy-eicosatetraenoic acid; LOX: lipoxygenase; LT: leukotriene; PG: prostaglandin; TX: thromboxane
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2.4.2 The role of n-3 polyunsaturated fatty acids in intestinal inflammation

The effect of n-3 PUFAs and its supplementation in gut inflammation has been a topic of great discussion in recent years. Since very little data is available on n-3 PUFA deficiency, discussion will first turn to the effects of the opposite - n-3 PUFA supplementation. Teitelbaum & Walker (2001), reviewed the possible mechanisms by which n-3 PUFA could affect intestinal inflammation (Figure 2-7), but for the scope of this study, only the role of n-3 PUFAs on lipid mediators will be discussed in more detail (Teitelbaum and Walker, 2001). Even though microbiota is not an outcome measured in this study, it does seem as if n-3 PUFA deficiency can lead to an imbalance in the gut microbiota, which may contribute to impaired SCFA production (Robertson et al., 2017), which has a direct impact on intestinal health and inflammation.

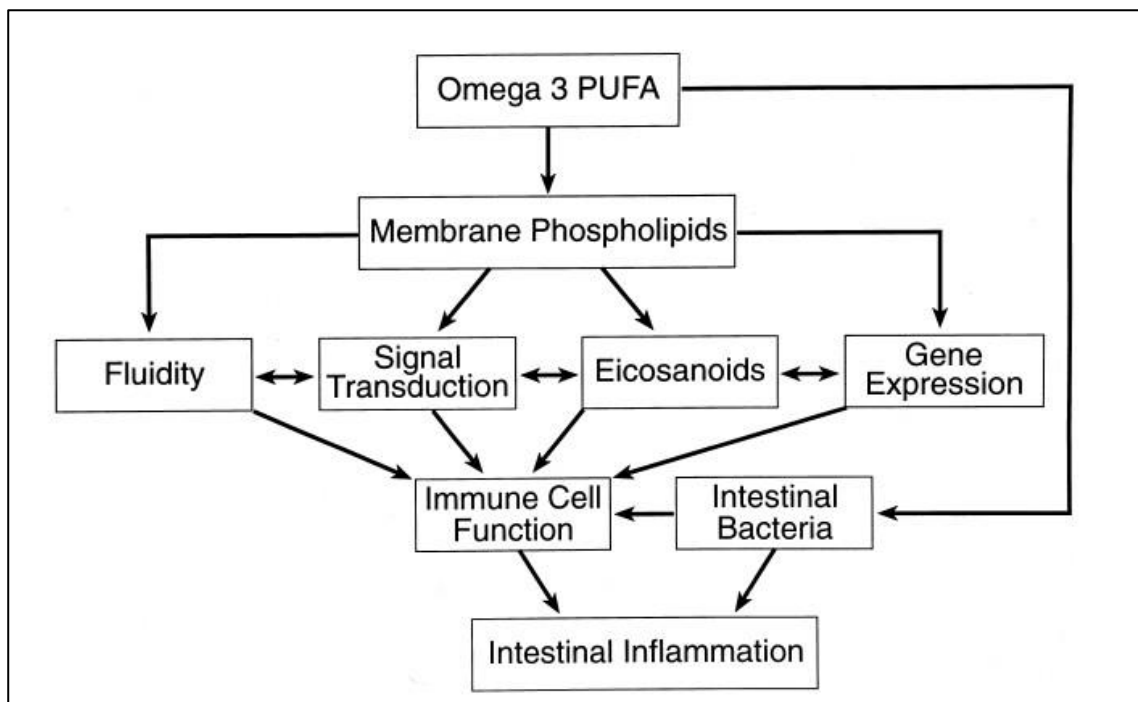


Figure 2-7: The role of n-3 PUFA in intestinal inflammation (Teitelbaum and Walker, 2001)
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Most gut inflammation studies focused on inflammatory bowel disease (IBD) – the archetypal form of inflammation of the gastrointestinal system. IBD is divided into Crohn’s disease, affecting any part and layer of the gastro-intestinal tract and ulcerative colitis ; mainly the mucosal layers of the colon and rectum. Both conditions are chronic, and involve immune dysregulation. There is also a genetic component to IBD, but that is beyond the scope of this literature review (Teitelbaum and Walker, 2001).

In inflammatory bowel disease, an imbalance in the mucus layer causes higher permeability and

an increased uptake of pathogens. This activates the dendritic cells and macrophages to synthesise pro-inflammatory cytokines (Barbalho et al., 2016). In human IBD, the inflamed tissue of the intestinal mucosa contains a large infiltration of neutrophils, as well as inflammatory lipid mediators derived from arachidonic acid such as LTB₄, PGE₂, TXB₂ and 15-hydroxyeicosatetraenoic acid (15-HETE) (Calder, 2015b). 15-HETE and PGE₂ are proportionally linked to the histologically obtained inflammation score in the colon of patients with UC (Zijlstra et al., 1992a). The high concentrations of tumor necrosis factor- α (TNF- α), IL-1 and IL-6 are particularly destructive, and also play an important role in the pathogenesis of inflammatory bowel disease (Calder, 2006).

There is evidence that the gastrointestinal mucosa is highly responsive to long-chain n-3 PUFA (Barbalho et al., 2016). In a review (Calder, 2015b) it was concluded that supplementation with n-3 PUFAs in animal models decreased chemically induced colonic damage and inflammation compared to a diet rich in n-6 PUFAs. The protective role in all these studies was associated with a reduction in the amount of pro-inflammatory lipid mediators including PGE₂, TXB₂ and LTB₄ in the colonic mucosa (Calder, 2015b; Barbalho et al., 2016).

2.4.2.1 The supplementation of n-3 polyunsaturated fatty acids and inflammation – a summary of studies

Vilaseca et al. (1990) compared the effect of n-6 PUFA (sunflower oil) and n-3 PUFA (cod liver oil) supplementation in the development of chronic granulomatous lesions in the colon of rats with induced colitis. They found that the formation of PGE₂ and LTB₄ peaked by Day Three, and thereafter declined; while thromboxane B₂ (TXB₂) continued to increase from days three to twenty in n-6 PUFA (sunflower oil) fed rats, whereas this change was diminished in cod liver animals. Twenty, thirty and fifty days after the induction of colitis the rats were killed. Colonic lesions were scored macroscopically, and histologically. In cod liver animals, the damage score was markedly reduced by day thirty, and inflammation and ulceration were almost absent after fifty days. These authors concluded that a fish oil diet prevents the increase in thromboxane in the chronic state of inflammation, and shortens the course of the colonic disease by diminishing both the severity of the lesions and their progression to chronicity. This implies that EPA supplements may have a beneficial effect on human colonic ulcerative colitis (Vilaseca et al., 1990). It has to be noted that cod liver oil does however also contain vitamin A and D which could have contributed to the effects observed in this particular study.

Dietary n-3 fatty acids can modulate the substrate availability for COXs and LOXs, thus modulating downstream lipid mediator formation in colonic mucosa. Neilson et al. (2012) compared the effect of an olive oil diet to a fish oil diet in mice. Similar to the results of Vilaseca

et al. (1990) they found that fish oil reduced the amount of free ARA in colonic mucosa. Subsequently, the production of LTB₄ was reduced and there was an increased synthesis of EPA-derived LTB₅ in the fish oil group. Furthermore, the fish oil diet also significantly reduced the levels of 12- and 15-HETEs in the colonic mucosa, and the same trend was observed for the minor product 5-HETE (Neilson et al., 2012). Interestingly, in an ex-vivo model, 12-HETE in both inflamed and healthy colonic mucosa of rodents were more abundant than 15-HETE, and the latter was more abundant than 5-HETE. In humans, 15-HETE was the more abundant molecule in colonic mucosa when compared to 12-HETE, whereas 5-HETE was present in low concentrations (Zijlstra et al., 1992b).

Bosco et al. (2013) aimed to increase cellular levels of EPA and DHA in a mouse model, with a fish oil enriched diet, while maintaining ARA levels, in order to ascertain whether this will prevent colitis through altered gene expression. They found that there was a significant reduction of colonic pro-inflammatory lipid mediators (PGJ₂, PGJ₈- and PGJ₉, EET and PGE₂) and a significant increase in the anti-inflammatory lipid mediators (PGE₃, TxB₃, LTB₅ and 5-HEPE as well as 17- and 18-EEP) in the mice following the fish oil diet, when compared to the control diet. In this study, however, the fish oil diet did not change the colonic gene expression signature, nor did it reduce inflammatory bowel disease scores. This indicated that fish oil consumption did not prevent experimental colitis. The authors reported that the overall, n-3 PUFA-derived lipid mediators were ten to hundred fold lower than their n-6 counterparts, and that this important bias in n-3:n-6 lipid mediator stoichiometry might explain why colitis amelioration was not achieved despite the substantial EPA and DHA precursor dose provided within the diet (Bosco et al., 2013).

In patients with IBD, n-3 PUFA is incorporated into the gut mucosa, resulting in a decrease in leukotriene production by neutrophils and colonic mucosa, and a decrease in prostaglandin and thromboxane production in colonic mucosa (Calder, 2006). Patients with ulcerative colitis, where persistent colonic inflammation is present, a 12-fold reduction of lipoxin was seen. It was associated with a decreased protein expression of 15-LOX-2. This suggests that there is a defective biosynthesis of lipoxin in these patients, which may contribute to their inability to resolve colonic inflammation (Mangino et al., 2006). A n-3 PUFA dose of between 2.5 and 6 g per day, with an average of 4 g per day reported clinical benefits (Calder, 2006). However, not all studies showed the same results. In a Cochrane systematic review and meta-analysis from placebo-controlled studies, it was concluded that there are insufficient data to recommend the usage of n-3 PUFAs for the maintenance of remission in CD and UC (Turner et al., 2007; Lev-Tzion et al., 2014).

Table 2-4: The effect of n-3 PUFA supplementation on the gut

Author, year	Type of animal	Duration of study	Aim & Intervention	Method Inflammation markers Microbiota Short chain fatty acids Lipid mediators	Notes
Vilaseca et al. 1990 [RCT]	Male Sprague-Dawley rats	50 days	<p>To investigate the effect of dietary fish oil supplementation on the development of chronic inflammatory lesions in the bowel.</p> <p>A standard low-fat diet that was supplemented either with sunflower (omega-6) vs. cod liver oil (omega-3) to determine the development of chronic granulomatous lesions in the rat colon. [isocaloric]</p>	<p>Lipid mediators/radioimmunoassay of intracolonic dialysis fluid Colonic lesions/scored macroscopically</p> <p>Lipid mediators Sunflower fed rats -Before the induction of colitis, luminal release of PGE₂, TXB₂, and LTB₄, as measured by intracolonic dialysis, was higher in sunflower fed animals; -prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) peaked by day 3 and thereafter declined; and -thromboxane B₂ (TXB₂), continued to increase from day 3 to 20; whereas this change was blunted in cod liver animals. Hence, significant differences between sunflower and cod liver group occurred on days 20 and 30. -LTB₄ was similar in both groups of rats.</p> <p>Lesions In cod liver animals, the damage score was markedly reduced by day 30, and inflammation and ulceration were almost absent by day 50. Thus, the initial injury was similar in both groups of rats, but the development of chronic inflammatory lesions in the colon was mitigated by the cod liver diet.</p>	<p>In conclusion, a fish oil diet prevents the increase in thromboxane in the chronic stage of inflammation, and shortens the course of the colonic disease by diminishing both the severity of the lesions and their progression to chronicity.</p>

Author, year	Type of animal	Duration of study	Aim & Intervention	Method Inflammation markers Microbiota Short chain fatty acids Lipid mediators	Notes
Bosco et al 2013 [RCT]	Wild-type (WT) or Rag2 -/- C57BL/6 breeder mice / female mice 8-12 weeks old In vivo	8 weeks: animals were fed: control or experimental diet 4 weeks prior to colitis induction, and under the same diet for 4 additional weeks.	To assess the impact of increased fish oil intake on colonic gene expression, eicosanoid metabolism and development of colitis in a mouse model of IBD. [Aimed to increase cellular levels of EPA and DHA in a mouse model, with a fish oil enriched diet, while maintaining ARA levels in order to ascertain whether this will prevent colitis, through altered gene expression]	Lipid mediators / HPLC-MS/MS Fish oil diet: ↓ colonic pro-inflammatory lipid mediators: - [*PGJ ₂ , 8,9 -EET and PGE ₂] - ↑ anti-inflammatory lipid mediators: - [PGE ₃ , TxB ₃ , LTB ₅ & - 5-HEPE as well as 17, 18 EEP] - ...vs. control diet. However, neither alteration of colonic gene expression signature nor reduction in IBD scores was observed under FO diet.	Increased intake of dietary FO did not prevent experimental colitis. These data highlight the importance of the nature of the anti-inflammatory lipid metabolites but also their relative amounts in target tissue. Lipid mediators not only play a role on inflammation, they could also support mucosal pathogenic Th17 cells generation. In our experimental condition, we neither observe alteration in helper T cell numbers and subsets nor detect resolvins in line with the lack of efficacy of our dietary FO treatment. Overall n3-derived lipid metabolites were 10–100 fold lower than their n-6 counterparts. This important bias in n-3:n-6 eicosanoid stoichiometry might explain why colitis amelioration was not achieved despite the substantial EPA and DHA precursor dose provided within the diet. In that respect, new tricks that could be translated to human clinics to improve n3-derived lipid metabolites generation or n3:n6 ratio in vivo deserve further investigations. <i>Based on an average mouse body weight of 20 g and 4 g of daily food intake, mice consumed about 10 mg per day of EPA + DHA. This dose is equivalent to ~41 mg/kg/day in humans according to the human equivalent dose formula (HED) calculated as: HED (EPA+DHA) = animal dose in mg/kg × animal weight in kg/human weight in kg</i>
Neilson et al 2012	Healthy mice 3 groups: -Wild-type mice, -COX-1 null mice and -COX-2 null mice.	9-11 weeks	To evaluate to what extent COX- and LOX-derived products could be modulated by dietary fish oil in normal colonic mucosa, and to evaluate the role of COX-1 and COX-2 in the formation of these products. Mice were fed:	Lipid mediators / LC-MS Level of ARA 2-3 x greater in mice fed oleate diet vs. fish oil diet for COX 1 and COX 2 null mice No difference for WT mice between diets. Level of EPA was 7-16 x higher in mice fed fish oil diet vs. an oleate diet. Free ARA in colonic mucosa	Administration of fish oil reduced the amount of free ARA in colonic mucosa: Similar to the prostanoids, the fish oil diet generally: - ↓ synthesis of ARA-derived LTB ₄ ; - ↑ synthesis of EPA-derived LTB ₅ relative to the oleate diet; and - ↓ the levels of 12- and 15-HETE and 5-HETE in colonic mucosa. In rodent mucosa:

Author, year	Type of animal	Duration of study	Aim & Intervention	Method Inflammation markers Microbiota Short chain fatty acids Lipid mediators	Notes
			<p>Oleate diet (olive oil / high in n-9 MUFA) or A diet high in fish oil (n-3 PUFA)</p>	<p>ARA higher with oleate [* in COX 2 null] Genotype did not significantly affect free ARA levels in mice fed fish oil PGE₂ PGE₂ 3-4 x higher, with oleate diet vs. fish oil [*WT and COX2 null mice] PGE₃ < PGE₂ on oleate diet [all groups] PGE₃ increased with fish oil [WT and COX 2] LTB₄ and LTB₅ LTB₄ 3-5 x higher, with oleate diet vs. fish oil [genotype no effect] LTB₅ levels increased 2-10 x on fish oil diet vs. oleate diet [WT & COX 1 null] Diet did not affect LTB₅ levels in COX 2 null mice. 12 HETE Most abundant of all lipid mediators in colonic mucosa 12 HETE > 15 HETE > 5 HETE > 13 HODE > PGE₂ & LTB₄ LTB₃ 12-HETE 7- to 20-fold greater with oleate diet [every genotype / * COX 1 null] 15 HETE 15-HETE 4-8 x higher on oleate diet [every genotype / * COX 1 null] 5 HETE 5-HETE generally greater with oleate diet [*COX 1 null] COX-1 & COX-2 null mice fed the oleate diet had slightly elevated 5-HETE vs. wild-type mice fed the oleate diet.</p>	<p>12-HETE > 15-HETE > 5-HETE. In human colonic mucosa: 15-HETE levels are 2-100 x higher than 12-HETE in both normal and inflamed mucosa, with 5-HETE being present at much lower concentrations [8,46–49]. These differences between rodents and humans are likely due in part to the fact that 12- and 15-HETEs are produced by a 12/15-LOX in rodents (which produces predominantly 12-HETE), and by distinct enzymes in humans, with 15-HETE predominating [50,51]. Therefore, in humans, the relative levels of 12- and 15-HETEs are likely to be reversed from those seen in mice. The low levels of 5-HETE relative to the other HETEs may be due to the low expression of 5-LOX in normal mucosa [33] The large changes in eicosanoid profiles were accompanied by relatively small changes in colonic crypt proliferation, but such changes in eicosanoid formation might have greater biological impact upon carcinogen challenge. These results indicate that in normal colon, inhibition of COX-2 would have little effect on reducing PGE2 levels.</p>

Author, year	Type of animal	Duration of study	Aim & Intervention	Method Inflammation markers Microbiota Short chain fatty acids Lipid mediators	Notes
Van der Merwe et al 2013 [RCT]	172 Rural Gambian infants 3-9 months old	9 months	Tested whether early n-3 LC-PUFA supplementation improves infant intestinal integrity, growth, and cognitive function. 2ml of highly purified fish oil, which supplied 200 mg DHA and 300 mg EPA/d vs. Olive oil (control)	Gut integrity [lactulose:mannitol ratio] mucosal inflammation :fecal calprotectin n-3 PUFA supplementation resulted in a significant increase in plasma n-3 LC-PUFA concentrations (for both DHA and EPA)	Fish-oil supplementation successfully increased plasma n-3 fatty acid status. The intervention failed to improve linear growth, intestinal integrity, morbidity, or selected measures of cognitive development.
<p>Abbreviations : ARA: arachidonic acid; COX: cyclooxygenase; DHA: docosahexaenoic acid; - EPA: eicosapentaenoic acid; HEPE: hydroxyeicosapentaenoic acid; HETE: hydroxyeicosatetraenoic acid ; HODE: hydroxyoctadecadienoic acid; LOX: lipoxygenase; LT: leukotriene; PG: prostaglandin; PUFA: polyunsaturated fatty acid; RCT: randomised controlled trial; TX: thromboxane.</p>					

2.4.2.2 Omega-3 polyunsaturated fatty acid deficiency and inflammation – A summary of studies

A deficiency in n-3 PUFA results in the reduced ability to resolve inflammation, and therefore also result in increased pro-inflammatory cytokine signaling (Calder, 2015b). Red blood cell (RBC) total phospholipid fatty acid composition is used as a proxy for the rest of the body's cell membrane fatty acid status, and the RBC n-6 and n-3 PUFA should therefore correlate with the pro-and anti-inflammatory lipid mediators, respectively (Brenna et al., 2018).

Not many studies have been conducted to examine the effect of n-3 PUFA deficiency in colitis or gut inflammation. A study in mice by Robertson et al. (2017) investigated the impact of n-3 PUFA deficiency on the caecal microbiota composition of mothers and their male offspring. They found that the diversity was lower in both the n-3 PUFA deficiency group as well as the n-3 PUFA supplemented dams compared to the control group. The group deficient of n-3 PUFA furthermore displayed significantly lower microbiota diversity when compared to the supplemented group. In the offspring, no significant differences in diversity were observed. In the n-3 PUFA deficient group, a significant reduction in the production of butyrate and acetate, as well as total short-chain fatty acids were also seen, in comparison to the control group. These results suggest that n-3 PUFA play a crucial role in the composition of the gut microbiota and that n-3 PUFA deficiency can lead to an imbalance in the gut microbiota, which may contribute to impaired SCFA production (Robertson et al., 2017).

Thus, besides the classical pro-resolving effect of n-3 PUFAs in the phospholipid membrane of cells via anti-inflammatory lipid mediators, it also actively play a role in influencing the composition of the gut microbiota, which may affect SCFA production. A deficiency of butyrate-producing microbiota could lead to a weakened colon lining, higher intestinal permeability, and inflammation in the colon mucosa

Table 2-5: The effect of n-3 PUFA deficiency on the gut

Author, year	Type of animal	Duration of study	Aim & Intervention	Inflammation markers: Microbiota / Short-chain fatty acids / Lipid mediators	Notes
Robertson et al. (2017) [RCT]	Mice (C57BL/6) 8 weeks	12 weeks	To investigate the impact of n-3 deficiency and n-3 supplementation on the caecal microbiota composition of mothers and their male offspring. Diets: -control -n-3 supplemented -n-3 deficient during gestation + their male offspring continue on the same diets for 12 weeks [adulthood]	Microbiota – offspring / SCFA - offspring Fatty analysis in erythrocytes n-6 ARA content was significantly higher in n-3 deficient mice and significantly lower in n-3 supplemented mice VS control Caecal microbiota alpha diversity significantly lower in both n-3 supplemented and n-3 deficient mothers vs. control group <u>n-3 deficient group</u> also displayed significantly lower diversity compared with the n-3 supplemented group No significant differences in alpha diversity observed in any of the offspring The overall phylum level distribution similar between mothers and their offspring Firmicutes & Bacteroidetes - most abundant phyla in each group in both mothers and offspring n—3 supplemented mothers displayed greater proportional abundance of Bacteroidetes and a lower Firmicutes:Bacteroidetes ratio These differences were not observed in offspring; SCFA <u>n-3 deficient group:</u> ↓ total SCFA VS control * ↓ acetate production * ↓ butyrate production * <u>n-3 supplemented group:</u> ↓ butyrate production VS control No significant differences between groups for propionate or isobutyrate	Deficiency of essential dietary n-3 PUFA induces a state of compositional and functional disturbance to the gut microbiome and metabolic phenotype, which may have implications for subsequent metabolic disorders. Therefore, these data suggest that a certain proportion of dietary PUFA reach the caecum and interact directly with the caecal microbiota differently, so as to the lower colonic microbiota or alternatively exert differing indirect effects on the intestinal environments of these two regions; which modifies microbial composition. The observed disruptions to SCFA production in n-3 deficiency were not due to differences in abundance of other well-known butyrate producers such as Roseburia and Pseudobutyvibrio.

Abbreviations: ARA: arachidonic acid; n-3: omega 3; RCT: randomised controlled trial; SCFA: short chain fatty acid.

2.5. The relationship of combined iron and n-3 polyunsaturated fatty acid deficiency with gut and colon inflammation

It is likely that in pregnant women, particularly of lower socioeconomic status, ID and inadequate n-3 PUFA status coexist. In a rat model, the combined deficiencies of iron and n-3 PUFA were shown to disrupt brain-monoamine metabolism and produce greater deficits in reference memory than ID or n-3(FAD) alone (Baumgartner et al., 2012a). On the other hand, it has also been found that n-3 PUFA supplementation attenuated the systemic inflammation caused by iron supplementation in 7- to 11-year-old children in South Africa (Malan et al., 2016). This indicates that there is a direct interaction between iron and n-3 PUFA.

The possible interactions between iron and n-3 PUFA deficiencies can be summarised as follows:

- Iron is a cofactor for desaturase and elongase enzymes required for the conversion of essential fatty acids to EPA and DHA, as well as to arachidonic acid (Nakamura and Nara, 2004). Therefore it is generally accepted that ID leads to a reduced activity of these enzymes and an attenuated conversion of the essential fatty acids to their longer chain metabolites.
- Iron is also part of the COX and LOX enzymes, which are involved in the production of lipid mediators (Kuhn et al., 2005).
- Iron could also be involved in the preferential incorporation of n-3 PUFAs into cell membranes; there is some evidence that iron deficiency affects the n-3 PUFAs more than the n-6 PUFAs. Thus, iron deficiency could lead to less n-3 PUFAs in cell membranes and potentially a higher incorporation of saturated fatty acids (Smuts et al., 1995).
- Diets high in unsaturated fatty acids may reduce iron retention and promote the development of iron deficiency (Miret et al., 2003).
- A depletion of DHA in the phospholipid bilayer of cell membranes may adversely affect the membrane's fluidity, thickness and deformability. This could also adversely affect the receptors involved in iron uptake, for instance DMT-1 (Schuchardt et al., 2010).
- A low status of n-3 PUFAs can furthermore lead to the inability to resolve inflammation, consequently leading to the prolonged existence of pro-inflammatory cytokines, like IL-6. Chronic inflammation has been shown to induce the expression of hepcidin, which inhibits iron absorption (Ganz and Nemeth, 2012).

To our knowledge, no studies have been conducted to examine the effect of a combined iron and n-3 PUFA deficiency on gut inflammation.

2.6 The rat model and its translation to humans

The use of tissue samples from humans would provide the most reliable data in investigating inflammatory bowel disease, but there are different challenges when it comes to this methodology. A limited sample size, the genetic variability between individuals, as well as the ethical issues surrounding the collection of human tissue are but some of these impediments (Jiminez et al., 2015). Therefore, animal models that adhere to scientific and ethical criteria are crucial for IBD research (Zak and O'Reilly, 1993). Different animal models have been used to investigate the aetiology and mechanisms underlying acute and chronic intestinal inflammation. These models include rodents, insects, fish, pigs and non-human primates. Other models that have been used less frequently include dogs, sheep, cattle, rabbits and guinea pigs (Jiminez et al., 2015).

Mice are most frequently used, because their intestinal development and physiology are relatively similar to that of humans; and they have many of the same genes and a similar immune response (Chinwalla et al., 2002). Many research teams have also used mice in n-PUFA interventions (Myles et al., 2013; Myles et al., 2014; Robertson et al., 2017); while mice or rats were used in iron studies (Uritski et al., 2004; Lobo et al., 2014; Dostal et al., 2012; Dostal et al., 2014b). The rat model has the additional benefit that it is larger than mice allowing the harvesting of larger samples for analysis (Jiminez et al., 2015). Kalmokoff et al. (2015) also recently found that fermentation does not only take place in the rat cecum, but that a considerable amount of fermentation occurs in the colon as well, which is similar to what is seen in humans (Kalmokoff et al., 2015). In Table 2-6, the advantages and disadvantages of using a murine model (rats and mice) as animal model in IBD research are summarised.

Table 2-6*. Advantages and disadvantages of using a murine model in acute and chronic intestinal inflammation research (Adapted from Jiminez et al 2015; Hedrich 2004; Chinwalla et al. (2002))

Advantages	Disadvantages
The gastrointestinal tract is anatomically and functionally similar to humans.	The lesions in mice, after the exposure to chemical agents, are not identical to the lesions in people with IBD.
Mice and rats (to a lesser extent) can be genetically modified to study specific aspects of intestinal inflammation and disease; and to elucidate mechanisms	Coprophagy, or the nocturnal consumption of faeces in mice and rats, is crucial for the re-ingestion of nutrients, and can affect the balance of the diet, microbial populations, and could potentially influence intestinal health. Coprophagy is not normal in humans, and the extrapolation of dietary effects on intestinal inflammation from rodents to humans may lead to inaccurate interpretations.
The murine and human genomes are similar (90% of human and mouse genes are shared).	
The murine and human intestinal communities exhibit the same diversity of species within Firmicutes, Bacteroidetes, and Proteobacteria phyla.	
The murine and human immune response fare similar	In the innate immune system there are differences between the murine and human expression of toll-like receptors
The small size of animals makes housing and space required smaller, and more cost-effective.	
Relatively short oestrous cycle, gestation and large litter sizes reduce the time and cost of experiments.	
Less expensive compared to other animals. Most cost-effective model with regard to purchasing, breeding, and husbandry.	
Many commercially available diagnostic biomolecules and analysis techniques have been developed to analyse and study inflammation in rodents.	

In this research project, Wistar rats were used as an animal model. This model was chosen because it is one of the most cost-effective and sophisticated animal models in which inflammation in the gut can be studied, while it also adheres to the 3R principals to meet ethical considerations (Russell et al., 1959).

Replacement: The lowest hierarchical biological animal that best represents the human intestine was chosen.

Reduction: The minimum number of animals will be used to obtain scientifically valid results; the different organs will be used for different studies so as to further minimise the number of animals.

Refinement: Literature showed that the rat model is an appropriate species to investigate intestinal inflammation.

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CHAPTER 3: MANUSCRIPT

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Effects of n-3 polyunsaturated fatty acid and iron deficiency, alone and in combination, during early development on colonic inflammation in rats

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Abbreviations ARA: arachidonic acid; COX: cyclooxygenase; LA: linoleic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; FA: fatty acid; FAD: fatty acid deficient; GLA: gamma linolenic acid; HDHA: hydroxydocosahexaenoic acid; ID: iron deficient/deficiency; LCMSMS: liquid chromatography-tandem mass spectrometry; LOX: lipoyxygenase; (n-3)FAD: omega-3 fatty acid deficient/deficiency; PND: postnatal day PUFAs: polyunsaturated fatty acids, RBC: red blood cell,

Abstract

Background: Poor nutrition during early development causes adverse pregnancy outcomes and long-term child-health outcomes. In developing countries, women rely on poor-quality diets and it is likely that iron deficiency (ID) and inadequate n-3 PUFA status coexist. Both deficiencies affect colon inflammation, which can lead to dysbiosis of microbiota, compromised tight junctions, increased gut permeability, and pathogens to enter the bloodstream, resulting in low-grade systemic inflammation and associated diseases. Furthermore, children will most likely also continue consuming a similar diet as their mothers.

Aim: The pre-and postnatal effects of ID and n-3 PUFA deficiency (FAD), alone and in combination, on colon lining lipid mediator concentrations, which indicates colon inflammation, were investigated in rats.

Methods: Fifty-six female Wistar rats were allocated to one of four diets: 1) Control, 2) ID, 3) (n-3)FAD, or 4) ID+(n-3)FAD and were maintained on the respective diets throughout pregnancy and lactation. Offspring (n=96) weaned and continued on the diets until postnatal day 42-45. Lipid mediators were analysed with liquid chromatography-tandem mass spectrometry (LCMSMS).

Results: ID resulted in higher colonic pro-inflammatory lipid mediator concentrations including 5-,12-,15- and 8-HETE (all $p < 0.005$). Omega-3 PUFA deficiency resulted in higher 12-HETE ($p = 0.010$). ID resulted in higher pro-resolving lipid mediators, including 17-HDHA, 12- and 15-HEPE (all $p < 0.001$), whereas (n-3)FAD, resulted in lower pro-resolving lipid mediators (17-HDHA, 12-,15-,18- and 11-HEPE, all $p < 0.005$). Furthermore, a significant ID **plus** (n-3)FAD interaction synergistically resulted in higher pro-inflammatory lipid mediators (12- and 15-HETE, both $p < 0.050$). For 12-HEPE, however, the effect of ID for higher 12-HEPE was attenuated by (n-3)FAD ($p = 0.049$).

Conclusion: This study found that pre- and postnatal combined deficiency of iron and n-3 PUFA in rats increased inflammation in the colon of offspring in early adolescence more than the single deficiencies alone. Omega-3 FAD furthermore attenuated the resolving of inflammation which is still possible with ID alone, resulting in a pro-inflammatory profile that could not be resolved in double-deficient rats.

3.1 Background

The colon reabsorbs water, bile salts, vitamins [1], as well as a small amount of iron [2]. It also plays an essential role in the breakdown of fibre while producing the short chain fatty acids acetate, butyrate, and propionate. The highest density of microbiota (bacteria) is found in the terminal ileum and colon [3]. The distinct architecture of the intestinal wall must keep pathogens, commensal bacteria, and bacterial products, such as lipopolysaccharides (LPS) and toxins, from entering the system by forming a physical barrier between antigens and the host [4, 5].

Inflammation in the gut can lead to dysbiosis, compromised tight junctions [5], increased gut permeability and pathogens to enter the bloodstream. The result is low-grade systemic inflammation contributing to the development of irritable bowel syndrome [6, 7]. Increased gut permeability and low-grade inflammation are also linked to diabetes, obesity, cancer and neuro-pathologies [8-12]. Iron deficiency (ID) was previously shown to increase gut inflammation in humans [13, 14]. The mechanism was shown to involve a decrease in commensal gut microbiota which alters the production of short chain fatty acids, in particular, butyrate ([13, 14].

Butyrate provides the colonic epithelial cells with energy [15]. and keeps the colon lining healthy. It also reduces intestinal permeability, improves the integrity, and act as anti-inflammatory agents for the colon mucosa [16]. Low serum iron was also associated with a decrease in the tight junctions in the intestinal epithelium, higher gut permeability as well as increased inflammation in mammals [17].

However, ID may not only increase gut inflammation, but gut inflammation could also lead to ID, leading to a perpetuating cycle. During infection and inflammation, hepcidin concentrations are significantly increased by the cytokine, interleukin -6 [18]. High concentrations of hepcidin blocks the iron transporter, ferroportin. Iron is thus trapped in cells and sequestered, withholding it from the circulation decreasing serum iron. The prolonged activation of this mechanism is referred to as the anaemia of inflammation. This process makes iron unavailable to pathogens which need iron for survival. Therefore it works as an anti-pathogenic mechanism [19, 20].

The role of n-3 PUFA in the gut involves the incorporation of it into cell membrane phospholipids [21]. Cell membranes, including the intestinal mucosa [5], require unsaturated fatty acids for structure, fluidity and proper functioning. These fatty acids cannot be synthesised in the body and have to be consumed through food [22-24]. The n-3 PUFAs also play an active role in resolving inflammation via their metabolites, the specialised pro- resolving lipid mediators (SPMs) - resolvins, maresins, and protectins [25]. The SPMs are derived from 17-HDHA and HEPEs (Figure 3.1 b) and are involved in restoring the barrier function in the gut and also in controlling inflammation by regulating the immune system and microbial environment [5]. The lipid mediators from n-6 PUFAs, mostly arachidonic acid (ARA), are considered to be predominantly pro-inflammatory [25, 26]. A deficiency in n-3 PUFA or a high intake of n-6 PUFA results in the reduced ability to resolve inflammation, and therefore also result in increased pro-inflammatory cytokine signaling [21].

It has been shown that poor nutrition during early development, has a causal relationship with adverse pregnancy outcomes, morbidity, mortality and the increased risk of short- and long-term adverse health outcomes for mother, child and future generations [33-36].

Furthermore, a mother's nutritional status has an impact on the unborn baby. Early-life programming is becoming a recognised concept that claims that the environment during early development affects health and disease in adulthood, possibly via epigenetic mechanisms [37, 38]. Apart from being reliant on nutrients from the mother's diet during gestation and lactation, offspring in the human situation will most likely also continue consuming a similar diet to that of their mother.

In developing countries, it is challenging to meet the increased nutritional demand during

pregnancy since women are reliant on poor-quality diets [39]. In these circumstances, nutrient deficiencies do not occur in isolation, and it is likely that in pregnant women, particularly of lower socioeconomic status, ID and inadequate n-3 PUFA status coexist [40, 41].

In a rat model, the combined deficiencies of iron and n-3 PUFA were shown to disrupt brain-monoamine metabolism and produce more significant deficits in reference memory than ID or (n-3)FAD alone [42]. This indicates that there is a direct interaction between iron and n-3 PUFA. Even though this interaction is not entirely understood, it has been shown that iron is a cofactor for desaturase and elongase enzymes required for the conversion of essential fatty acids to EPA and DHA, as well as to arachidonic acid [43]. It is generally accepted that ID may lead to a reduced activity of these enzymes and an attenuated conversion of the essential fatty acids to their longer chain metabolites. On the other hand, it has also been found that n-3 PUFA supplementation attenuated systemic inflammation caused by iron supplementation in 7-11 yr. old children in South Africa [44]. However, data on potential interactions between ID and low n-3 PUFA status, specifically in relation to gut inflammation are scarce. Therefore, in this study the pre-and postnatal effects of ID and (n-3)FAD, alone and in combination, on lipid mediator concentrations, were investigated in rats

3.2 Materials and methods

This study was a sub-study of a larger project aiming to investigate the effects of maternal iron and n-3 FA depletion and repletion – both alone and in combination – on the development and health of offspring. The animal trial was conducted at the vivarium of the Preclinical Drug Development Platform (PCDDP) of the North-West University (NWU), Potchefstroom, SA.

In the larger study, a total of 56 female Wistar rats at 21 ± 3 days of age (postnatal day [PND] 21) were housed in pairs and randomly allocated (in pairs) to one of four diet groups, as shown in Figure 1-2. The diet groups were: 1. Control (n = 8); 2. ID (n = 16); 3. (n-3)FAD (n = 16); or 4. ID+(n-3)FAD (n = 16).

3.2.1 Animals, housing and diets

In this research project, the pups (n = 96) of 56 female Wistar rats were used. Animals were housed at the Vivarium of the Preclinical Drug Development Platform of the North-West University (NWU), Potchefstroom, South Africa. The rats were kept at a temperature of $22 \pm 1^\circ\text{C}$ and $55 \pm 10\%$ relative humidity, under a reversed 12/12h light/dark cycle (lights on at 6:00). All the animals were paired-housed in a standard solid floor cage on Alpha-Dri bedding (LBS Biotech,

United Kingdom). Chewing devices as well as plastic tubes and nesting material (Kimtek trace element free paper) were provided.

Ethical approval was obtained from the AnimCare Ethics Committee of the Faculty of Health Sciences of the North-West University (NWU-00335-15-A5-01)

The purified experimental diets were obtained commercially from Dyets Inc. (Bethlehem, USA) and were based on the American Institute of Nutrition (AIN) 93G purified diets for laboratory rodents formulation [46], with modifications in iron content and fat source (Table 3-1). All diets were isocaloric and contained 10% fat. The basal AIN-93G formulation (control diet) contained 35 mg iron/kg, soybean oil at 70 g/kg diet and hydrogenated coconut oil at 30 g/kg [42, 47, 48]. The (n-3)FAD diets contained hydrogenated coconut oil at 81 g/kg diet and safflower oil at 19 g/kg diet [49-52]. ID diets contained 15-18 mg Fe/kg diet [53].

All diets were custom-prepared and stored at -20°C until use. The FA composition of the diets was confirmed in spot samples from each batch of diets by using gas chromatography-tandem mass spectrometry (GC-MS-MS). See Table 3-2 for the analysis of the iron and FA composition of the different diets. The iron concentrations in the diets were confirmed in spot samples from each batch of diets by using atomic absorption spectrometry (AAS).

Table 3-1: Ingredients of experimental diets based on the AIN-93G diet

	Control	ID diet	(n-3) FAD diet	ID+(n-3) FAD diet
Cornstarch (g/kg)	397.5	397.5	397.5	397.5
Casein (g/kg)	200	200	200	200
Dyetrose –Dextrinised cornstarch (g/kg)	132	132	132	132
Sucrose (g/kg)	100	100	100	100
Soybean oil (g/kg)	70	70	-	-
Hydrogenated coconut oil (g/kg)	30	30	81	81
Safflower oil (g/kg)	-	-	19	19
Vitamin mix (g/kg)	10	10	10	10
Mineral mix (g/kg)	35	35 ¹	35	35 ¹

1: Mineral mix was modified in the ID diets to contain 15 – 18 mg iron/kg diet

Table 3-2: Iron and fatty acid analyses of the four diets (Covance laboratories)

	Control	ID diet	(n-3) FAD diet	ID+(n-3) FAD diet
Iron (ppm)	41.3	15.2	43.4	15.6
Total Fatty Acids (g/100g)	9.78	9.78	9.76	9.76
Saturated Fatty Acids (g/100g)	3.82	3.82	7.53	7.53
Monounsaturated Fatty Acids (g/100g)	1.45	1.45	0.33	0.33
Polyunsaturated Fatty Acids (g/100g)	3.95	3.95	1.32	1.32
Trans Fatty Acids (g/100g)	0.083	0.083	0.039	0.039
Omega 3 Fatty Acids (g/100g)	0.495	0.495	0.009	0.009
Omega 6 Fatty Acids (g/100g)	3.64	3.64	1.37	1.37
Omega 9 Fatty Acids (g/100g)	1.41	1.41	0.327	0.327

3.2.2 Experimental design

A total of 56 female Wistar rats at 21 ± 3 days of age (postnatal day (PND) 21) were housed in pairs and randomly allocated (in pairs) to one of four diet groups, as shown in Figure 3.2. The diet groups were: 1. Control (n = 8); 2. ID (n = 16); 3. (n-3)FAD (n = 16); or 4. ID+(n-3)FAD (n = 16). All the rats were placed on the control diet at PND 21 for a two week period of preconditioning. At the end of the preconditioning phase (at five weeks of age) the rats that had been randomly allocated to one of the (n-3)FAD diet groups ((n-3)FAD and ID+(n-3)FAD) were placed on a (n-3)FAD diet for seven weeks before mating, in order to imitate a chronic (n-3)FAD diet [54-57]. Three weeks before mating, at nine weeks of age, the female rats that had been allocated to one of the ID diet groups (ID and ID+(n-3) FAD), were placed on an ID or ID+(n-3)FAD diet [58].

At 12 weeks of age, the female rats were placed in individual cages and mated with 12-week-old male breeders of the same strain (maintained on standard laboratory chow) using monogamous breeding (one male bred with one female). The researchers accustomed males to purified AIN93G diets a few days prior to transferring them to the females' cages. Mating (conception) was confirmed by the presence of a vaginal plug (gestational day (GD) 0). After conception, the females were maintained on their pre-pregnancy diet throughout pregnancy. During the mating period, the rats were minimally handled and only during the light phase, as mating took place during the dark phase.

Dams were allowed to deliver spontaneously at approximately GD 22 (PND 0). Within three to five days after birth, the litters were culled to eight pups (to maintain nutritional adequacy), with ideally four males and four females per litter [eight pups/litter; minimum of three litters/group].

The dams stayed with their pups and were maintained on their respective experimental diets throughout lactation. At the start of the post-weaning period (PND 21), the dams were euthanized. The pups were weaned from the ID, (n-3)FAD and ID+(n-3) FAD diet groups and were randomly allocated in pairs of same-sex littermates to receive either the control diet (n = 24/group, male:female = 1:1) or maintained on their respective experimental diets (n = 24/group, male:female = 1:1) for three weeks until PND 42 - 45. During this period, the littermates were housed in pairs by sex. All offspring were weighed three times per week. At PND 42-45, 24 (12 male and 12 female) offspring from each group (n = 168) were euthanized, and samples were collected.

3.2.3 Sample collection, storage, and analyses

After decapitation, trunk blood was collected in 4 ml ethylenediaminetetraacetic (EDTA) -coated vacutainer tubes (BD, Plymouth, UK) and centrifuged within one hour at 3000 x g for 10 minutes at 4°C to separate plasma from red blood cells (RBCs). Red blood cells were washed twice with 0.15 mol/L NaCl by centrifugation at 3000 x g for 10 minutes. Plasma and RBC aliquots were stored at -80°C until analysis. The crude conglomerate of the colon tissue, were collected, and snap frozen in liquid nitrogen and stored at -80°C for further analyses. Thereafter it was homogenized in phosphate buffer solution.

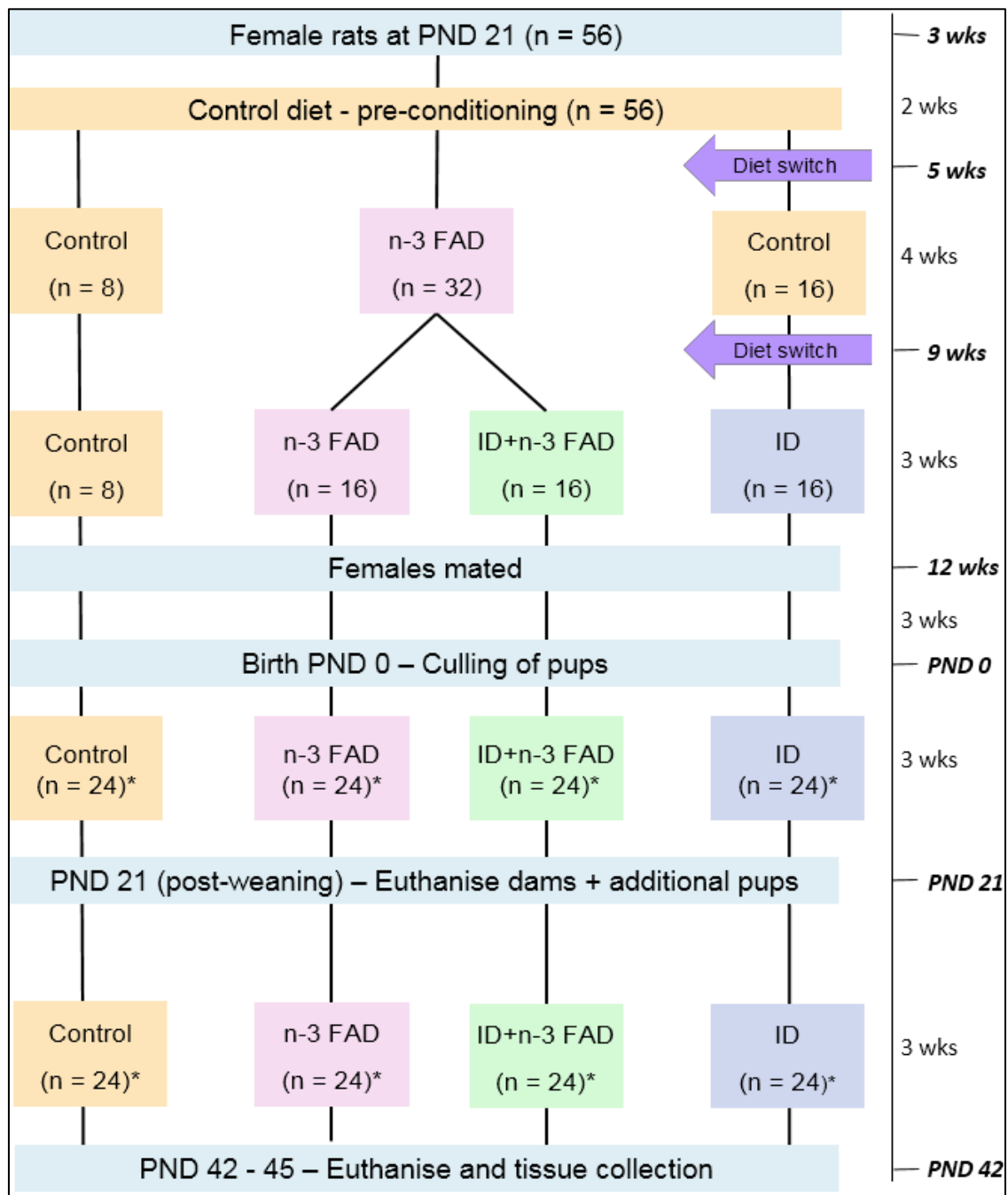


Figure 3-2: Flow diagram of the main study design

Abbreviations: FAD fatty acid deficiency; ID iron deficiency; n – number / amount; n-3 omega-3; PND post-natal day

3.2.3.1 Lipid mediator analyses

Tissue concentrations of lipid mediators were analysed in homogenized colon lining tissue. Samples were prepared as described below and analysed with liquid chromatography-tandem mass spectrometry (LCMSMS) at the North-West University. Tissue 17-hydroxydocosahexaenoic acid (17-HDHA); 11-, 12-, 15-, and 18-hydroxyeicosapentaenoic acid (HEPE) and 5-, 8-, 11-, 12-, and 15 hydroxyeicosa- tetraenoic acid (HETE) were measured.

Colon lining tissue of ~50 mg were defrosted on ice, weighed and homogenised in buffer (50

mg tissue → 500 µL homogenization buffer) using a stainless steel bead and Tissue Lyser (Qiagen Hilden, Germany). Lipid- mediators were extracted from ~50 mg homogenised tissue with solid phase extraction (SPE) using Strata-X columns (Phenomenex, Torrance, United States of America) as described by [44]. Briefly, tissue homogenates (~500 µl) were diluted with 3.3 ml cold water and 0.6 ml cold 15% methanol (v/v), were added. The internal standard, 12-HETE- d8 (50 µl of a 150 pg/µl working stock), was added to each sample. The samples were incubated on ice for 30 min and then centrifuged at 3000 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 preconditioned with 2 ml methanol followed by 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 were set at 350 °C and 12 l/min respectively, and the capillary voltage at 4000 V. Chromatographic analysis 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, subsequent to 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 will be water/glacial acetic acid, 99:1 (v/v) and solvent B 100% acetonitrile.

All samples were quantified in one batch with Masshunter (Agilent, Santa Clara, US). All compound retention times were checked against standards which were included in each run. The position of the target peak was confirmed with one or two qualifier peaks (80-120% of the validated target: qualifier ratio) at the correct retention time. Every peak was checked manually by making sure the integration start and stop at the same times for the target and qualifier and

that the peaks were integrated from the baseline.

3.2.3.2 Red blood cell total phospholipid fatty acid composition

RBC total phospholipid FA analyses were performed with gas chromatography-tandem mass spectrometry (GCMSMS). 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 of [60]. Lipid extracts were concentrated under nitrogen, and the neutral lipids were separated from the phospholipids by using thin-layer chromatography (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 band 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 2h 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). 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 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 [42].

3.2.3.3 Desaturase enzyme ratio

To estimate the activity of the $\Delta 5$, $\Delta 6$ desaturase & elongase enzymes, the ARA:LA ratio was calculated. A lower ratio was indicative of a lower enzyme activity. The GLA:LA ratio was calculated to measure the $\Delta 6$ desaturase activity. A lower ratio was indicative of a lower enzyme activity.

3.2.3.4 Iron parameter analysis

Ferritin, transferrin receptor and hepcidin concentrations were analysed using ELISA kits (ELABScience, Texas, US) according to the manufacturer's instructions. Haemoglobin concentrations were measured in whole blood directly after euthanasia using a portable HemoCue® Hb 201+ photometer (HemoCue AB, Angelholm, Sweden).

3.2.4 Statistical analyses

Statistical analyses were performed using IBM Statistical Program for Social Sciences (SPSS) Statistics software (version 25) and Excel 2016. The distribution of data was examined for normality using Q-Q plots, Shapiro-Wilk test, and the Kolmogorov-Smirnov test and by the visual inspection of histograms. The presence of outliers was identified by using boxplots. Two 5-HETE outliers in the FAD group and one 18-HEPE outlier in the ID + n-3(FAD) group were excluded from analyses. Non-normal data were log transformed (for 18-HEPE; 11-HETE; 8-HETE and n-3:n-6 PUFA ratio) prior to statistical analysis.

The ARA to LA and GLA to LA ratio were used to estimate the enzyme activities of the fatty acid metabolism, including $\Delta 5$, $\Delta 6$ desaturase & elongase and $\Delta 6$ desaturase alone, respectively.

The effects of pre- and postnatal ID and n-3(FAD), as well as their interactions, on iron status parameters, RBC fatty acid composition and colon lipid mediators at PND 42-45 were determined using 2 x 2 ANOVA. In the presence of a significant main effect or interaction, between-group differences were determined using one-way ANOVA followed by Bonferroni's post hoc tests. The results of parametric distributed data were expressed as means \pm SEM and as geometric means \pm SEM, for not normally distributed data. The significance of all tests was set at $p < 0.05$.

3.3 Results

3.3.1 Iron Status

ID resulted in lower haemoglobin concentrations at PND 42-45 (Table 3-3, $p < 0.001$), resulting in significantly lower haemoglobin in the ID and ID + (n-3)FAD groups than the control and (n-3)FAD groups. ID showed a tendency toward higher hepcidin concentrations ($p = 0.059$), whereas (n-3)FAD showed a tendency towards higher ferritin concentrations ($p = 0.073$).

3.3.2 Red blood cell total phospholipid fatty acid status

ID resulted in a lower composition of ARA whereas FAD resulted in a higher composition of ARA (both $p < 0.001$). ARA was significantly lower in the ID group compared to the other three groups (Table 3-3). ID resulted in a lower total n-6 PUFA composition ($p = 0.010$) and (n-3)FAD in a higher n-6 PUFA composition ($p < 0.001$). A significant ID x (n-3)FAD interaction on n-6 PUFA composition was also seen where ID attenuated the effect of (n-3)FAD ($p = 0.044$). The control and ID groups showed a similar n-6 PUFA composition and these percentages were significantly lower than the n-6 PUFA composition in the (n-3)FAD and (n-3)FAD + ID groups. As seen in Table 3-3, the n-6 PUFA percentage in the combined deficiency group was significantly lower than the (n-3)FAD group and significantly higher than in the ID and control groups.

ID and n-3(FAD) affected EPA significantly where ID resulted in a higher EPA composition ($p = 0.013$) and (n-3)FAD in a lower EPA composition ($p < 0.001$) (Table 3-3). A significant ID x (n-3)FAD interaction ($p = 0.018$) on EPA composition resulted in the effect of ID for higher EPA to be attenuated by (n-3)FAD. EPA in the (n-3)FAD and ID + (n-3)FAD groups was significantly lower compared to both the ID and control groups. Furthermore, the ID group had significantly higher EPA compared to the control group.

ID and (n-3)FAD both resulted in a lower DHA composition ($p = 0.002$ and $p < 0.001$ respectively). There was a significant interaction between ID and (n-3)FAD ($p = 0.001$) on DHA, and the lower DHA composition is maintained. DHA in the (n-3)FAD and ID + (n-3)FAD groups were significantly lower compared to both the ID and control groups. DHA in the ID group was also significantly lower compared to the control group, in contrast, it was significantly higher than in the (n-3)FAD and combined deficiency (ID + (n-3)FAD) groups (Table 3-3). For total n-3 PUFA, (n-3)FAD resulted in a lower total n-3 PUFA composition (Table 3-3; $p < 0.001$). Total n-3 PUFA in the (n-3)FAD group and ID + (n-3)FAD group was significantly lower than the ID and control groups. When investigating the n-6/n-3 PUFA ratio, (n-3)FAD resulted in a significant lower n-6/n-3 PUFA ratio (Table 3-3; $p < 0.001$). The total n-6/n-3 PUFA ratio in the (n-3)FAD and ID + (n-3)FAD groups were significantly higher compared to the control and ID groups.

3.3.3 Fatty acid ratios indicating estimated desaturase enzyme activities

ID resulted in a significantly lower ARA:LA ratio, which is indicative of a lower $\Delta 5$, $\Delta 6$ desaturase & elongase activity. ID also resulted in a significant lower GLA:LA ratio which implicates lower $\Delta 6$ desaturase activity. (Table 3-3; $p < 0.001$ and $p = 0.014$ respectively). The n-3(FAD) on the contrary resulted in a higher value of both these ratios ($p < 0.001$ and $p = 0.012$ respectively). An interaction between ID and (n-3)FAD for GLA:LA ratio ($p = 0.045$) was apparent, where ID

attenuated a higher GLA:LA ratio when combined with (n-3)FAD. The ID group had the lowest ARA:LA ratio compared to the other groups, and (n-3)FAD had the highest ratio. The combined deficiency group was higher than the control, lower than (n-3)FAD alone and higher than the ID group. For GLA:LA ratio the (n-3)FAD group had a significantly higher ratio than all the other groups, which values were similar.

3.3.4 Lipid mediator concentrations in colonic lining

Pro-inflammatory lipid mediators from the lipoxygenase pathway (Arachidonic acid)

ID resulted in higher pro-inflammatory lipid mediators 5-, 12- and 15-HETE concentrations in crude colonic lining homogenates (Table 3-4; $p=0.002$, $p<0.001$ and $p=0.001$, respectively). There were no between group differences for 5-HETE. For (n-3)FAD higher 12-HETE (Figure 3-3 b) was apparent. An interaction between ID and (n-3)FAD synergistically resulted in even higher 12-HETE. The main effects resulted in the control and (n-3)FAD groups to have similar 12-HETE concentrations, whereas the ID group had a significantly higher 12-HETE. The combination deficiency group had significantly the highest 12 HETE concentration. Also, a synergistic interaction between ID and (n-3)FAD resulted in higher 15-HETE in the ID+n-3 FAD group only, as illustrated in Figure 3-3 c. For 15-HETE the combined deficiency group had a significantly higher concentration compared to the control and (n-3)FAD groups. 15-HETE in the ID was however not significantly different from any of the other groups, as seen in Table 3-4.

Table 3-3: Iron status and red blood cell fatty acid composition of rat offspring at postnatal day 42-45 weaned onto the same diets as their dams fed an ID, (n-3)FAD, ID+(n-3)FAD, or a control diet¹

	Control	ID	(n-3)FAD	ID + (n-3)FAD	p value ²		
					ID	(n-3)FAD	ID x (n-3)FAD
Iron status							
Haemoglobin (g/dl) ³	13.5 ± 0.3 ^a	9.5 ± 0.3 ^b	13.4 ± 0.3 ^a	8.6 ± 0.3 ^b	<0.001	0.116	0.270
Hepcidin (pg/ml) ⁴	656 ± 83	765 ± 64	700 ± 54	845 ± 57	0.059	0.349	0.785
Ferritin (ng/ml) ⁵	119 ± 13	123 ± 8	136 ± 12	161 ± 23	0.320	0.073	0.491
Transferrin receptor (ng/ml) ⁴	6.4 ± 1.1	6.6 ± 0.5	7.7 ± 1.4	9.3 ± 1.8	0.504	0.137	0.600
Red blood cell total phospholipid fatty acid composition (% of total fatty acids)							
n-6 PUFA							
Arachidonic acid ⁶	29.7 ± 0.3 ^a	27.8 ± 0.4 ^b	30.8 ± 0.3 ^a	29.9 ± 0.5 ^a	<0.001	<0.001	0.167
Total n-6 PUFA ⁷	42.60 ± 0.18 ^c	42.37 ± 0.23 ^c	48.80 ± 0.22 ^a	47.40 ± 0.49 ^b	0.010	<0.001	0.044
n-3 PUFA							
Eicosapentaenoic acid ⁷	0.119 ± 0.009 ^b	0.161 ± 0.015 ^a	0.009 ± 0.001 ^c	0.010 ± 0.001 ^c	0.013	<0.001	0.018
Docosahexaenoic acid ⁷	4.01 ± 0.09 ^a	3.50 ± 0.11 ^b	0.48 ± 0.04 ^c	0.50 ^c ± 0.06 ^c	0.002	<0.001	0.001
Total n-3 PUFA ⁷	6.75 ± 0.23 ^a	6.50 ± 0.26 ^a	0.66 ± 0.05 ^b	0.69 ± 0.07 ^b	0.523	<0.001	0.433
Total n-6/n-3 PUFA ratio ⁸	6.38 ± 1.03 ^b	6.63 ± 1.04 ^b	73.05 ± 2.05 ^a	69.76 ± 1.14 ^a	0.956	<0.001	0.598
Fatty acid ratios to estimate fatty acid metabolism enzyme activities							
ARA:LA ratio (Δ5, Δ6 desaturase & elongase)	2.84 ± 0.44 ^c	2.45 ± 0.32 ^d	3.75 ± 0.35 ^a	3.17 ± 0.51 ^b	<0.001	<0.001	0.267
GLA:LA ratio (Δ6 desaturase)	36.4 ± 12.7 ^b	23.9 ± 8.8 ^b	81.9 ± 72.0 ^a	62.2 ± 56.9 ^b	0.014	0.012	0.045

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. Between-group differences were determined using one-way ANOVA followed by Bonferroni's post hoc test) and significant differences indicated by superscript lowercase letters. n-6:n-3 PUFA ratio was log transformed to perform ANOVA. All data expressed as means

± SEM except for RBC n-6:n-3 PUFA ratio which is expressed as geometric mean ± SEM. Values in a row with different superscripts differ significantly (p < 0.05).

²p value <0.05 is significant

³n=24 in control, ID and ID + (n-3)FAD groups; n=23 in (n-3)FAD group

⁴n=11 in control, ID and (n-3)FAD groups; n=12 in ID + (n-3)FAD group

⁵n=10 in control, (n-3)FAD and ID + (n-3)FAD groups; n=11 ID group

⁶n=23 in control and (n-3)FAD groups; n=20 in ID and (n-3)FAD groups

⁷n=23 in control and (n-3)FAD groups; n=20 in ID group and n=21 in ID + (n-3)FAD group

⁸n=23 in control; n=21 in (n-3)FAD and ID + (n-3)FAD groups and n=20 in ID group

Abbreviations: ARA: arachidonic acid; GLA: gamma linolenic acid; ID: iron deficiency, LA: linoleic acid; n-3 FAD: omega-3 fatty acid deficiency, ng: nanogram; pg: picogram;

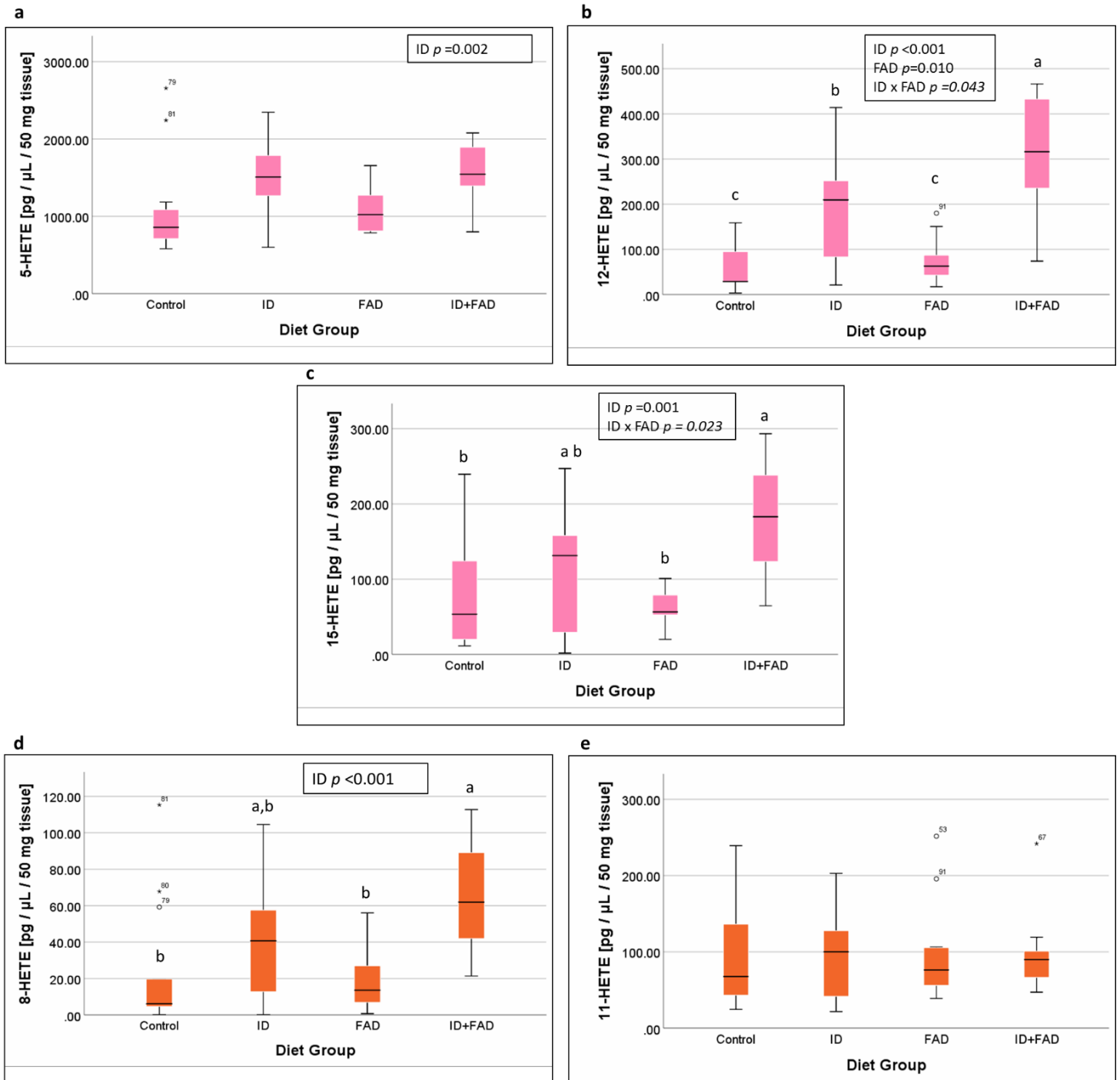


Figure 3-3 Boxplots of lipid mediators for the 4 diet groups a) 5-HETE; b) 12-HETE; c) 15-HETE; d) 8-HETE and e) 11-HETE in crude rat colonic lining homogenates

Abbreviations: FAD fatty acid deficiency; HETE hydroxyeicosatetraenoic acid; ID iron deficiency; pg picogram; μl microliter; mg milligram

Pro-inflammatory lipid mediators from the cytochrome pathway (Arachidonic acid)

ID resulted in higher 8-HETE (Table 3-4; $p < 0.001$). This main effect resulted in the combined deficiency (ID+(n-3)FAD) group to show a significantly higher concentration of 8-HETE compared to the control and (n-3)FAD groups (Figure 3-3 d). 8-HETE in the ID was however not significantly different from any of the other groups. All the pro-inflammatory lipid mediators derived from ARA (including 5-, 12-, 15-, 8- and 11 HETE) were higher in the n-3 deficiency groups ((n-3)FAD and

Table 3-4: Colon lining lipid mediator concentrations of rat offspring at postnatal day 42-45 weaned onto the same diets as their dams fed an ID, (n-3)FAD, ID+(n-3)FAD, or a control diet¹

					p-value ²		
	Control	ID	(n-3)FAD	ID+(n-3)FAD	ID	(n-3)FAD	ID x (n-3)FAD
Pro-inflammatory lipid mediators from the lipoxygenase pathway (Arachidonic acid)							
5-HETE ^{3,8}	1097 ± 176	1479 ± 131	1103 ± 91	1590 ± 107	0.002	0.663	0.693
12-HETE ³	58.3 ± 14.0 ^c	188.4 ± 30.6 ^b	73.5 ± 14.2 ^c	312.8 ± 36.0 ^a	<0.001	0.010	0.043
15-HETE ⁴	86.2 ± 21.9 ^b	111.8 ± 20.9 ^{a,b}	62.5 ± 7.1 ^b	181.8 ± 19.9 ^a	0.001	0.252	0.023
Pro-inflammatory lipid mediators from the cytochrome P450 pathway (Arachidonic acid)							
8-HETE ³	24.1 ± 9.7 ^b	41.7 ± 8.0 ^{a,b}	19.2 ± 5.1 ^b	63.9 ± 8.3 ^a	<0.001	0.292	0.103
11-HETE ³	90.6 ± 17.6	94.1 ± 15.8	96.1 ± 18.6	96.3 ± 14.6	0.912	0.818	0.922
Pro-resolving lipid mediators from the lipoxygenase pathway (Docosahexaenoic acid)							
17-HDHA ³	43.0 ± 8.1 ^{b,c}	125.0 ± 19.6 ^a	17.1 ± 3.1 ^c	73.2 ± 9.0 ^b	<0.001	0.004	0.319
Pro-resolving lipid mediators from the lipoxygenase pathway (Eicosapentaenoic acid)							
12-HEPE ³	11.6 ± 2.1 ^b	50.7 ± 10.6 ^a	2.5 ± 0.4 ^b	15.7 ± 2.8 ^b	<0.001	0.001	0.049
15-HEPE ⁵	7.6 ± 1.7 ^b	26.8 ± 6.1 ^a	0.6 ± 0.2 ^b	9.3 ± 2.1 ^b	<0.001	0.001	0.119
18-HEPE ^{6,9}	3.9 ± 0.9 ^a	3.3 ± 0.8 ^{a,b}	0.4 ± 0.1 ^c	0.9 ± 0.2 ^{b,c}	0.881	<0.001	0.418
Pro-resolving lipid mediators from the cytochrome P450 pathway (Eicosapentaenoic acid)							
11-HEPE ⁷	120.0 ± 9.6 ^a	108.6 ± 11.2 ^a	55.1 ± 5.7 ^b	60.0 ± 4.9 ^b	0.716	<0.001	0.353

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

²All the lipid mediators were measured in picogram (pg) per microliter (μL) normalised to 50 milligram (mg) colon tissue [pg / μL / 50 mg tissue]

³p value <0.05 is significant; Values in a row with different superscripts without a common letter differ significantly (p < 0.05).

⁴n=13 in control, n=15 in ID, n=12 in (n-3)FAD and ID+(n-3)FAD groups;

⁵n=13 in control, n=15 in ID, n=10 (n-3)FAD, n=12 in ID+(n-3)FAD group

⁶n=13 in control, n=12 in ID, (n-3)FAD and ID+(n-3)FAD groups

⁷n=12 in control n=15 in ID, n=12 in (n-3)FAD, n=11 in ID+(n-3)FAD groups

⁸n=13 in control, n=14 in ID, n=12 in (n-3)FAD, n=12 in ID+(n-3)FAD groups

⁹2 Missing data points at 5-HETE in the (n-3)FAD group

¹⁰1 Missing data point at 18-HEPE in the ID+(n-3)FAD group

ID+(n-3)FAD) compared to the n-3 sufficient groups (control and ID), which indicate increased inflammation (Figure 3-3 a, b, c, d & e).

The pro-inflammatory lipid mediators derived from ARA (including 5-, 12-, 15- and 8- HETE) were higher in the iron deficiency groups compared to the iron sufficient groups (control and (n-3)FAD); the values for 11-HETE were similar, which also indicate increased inflammation (Figure 3-3 a, b, c, d & e).

Pro-resolving lipid mediators from lipoxygenase pathway (Docosahexaenoic acid)

ID resulted in higher 17-HDHA ($p < 0.001$) while (n-3)FAD had a lower 17-HDHA (Table 3-4; $p = 0.004$). These main effects resulted in the n-3(FAD) group to have a significantly lower concentration of 17-HDHA compared to the ID and combined deficiency groups. The combined group had significantly lower concentrations of 17-HDHA than the ID group, whereas the control group did not differ from the (n-3)FAD or combination groups (Figure 3-4 a).

Pro-resolving lipid mediators from lipoxygenase pathway (Eicosapentaenoic acid)

ID resulted in higher concentrations of both 12-HEPE ($p < 0.001$) and 15-HEPE ($p < 0.001$) while (n-3)FAD had lower levels of 12-HEPE ($p = 0.001$), 15-HEPE ($p = 0.001$), and 18-HEPE ($p < 0.001$) as seen in Table 3-4. A significant ID x (n-3)FAD interaction ($p = 0.049$) for 12-HEPE was observed. The effect of ID for higher 12-HEPE was attenuated by (n-3)FAD when combined. The ID group had a significantly higher 12- and 15-HEPE than all the other groups. For the (n-3)FAD group a significantly lower 18-HEPE compared to the control and ID groups was observed. The control group also had significantly higher 18-HEPE than the ID+(n-3)FAD group (Figure 3-4 b, c, and d).

Pro-resolving lipid mediators from the cytochrome P450 pathway (Eicosapentaenoic acid)

In Table 3-4 the main effect of (n-3)FAD for lower 11-HEPE ($p < 0.001$) is evident. The control and ID groups have a significantly higher 11-HEPE compared to the (n-3)FAD and ID + (n-3)FAD groups (Figure 3-4 e). All the pro-resolving lipid mediators derived from DHA and EPA (including 17-HDHA, 12-, 15-, 18- and 11-HEPE) were lower in the n-3 deficiency groups ((n-3)FAD and ID+(n-3)FAD), compared to the n-3 sufficient groups (control and ID). The pro-resolving lipid mediators derived from DHA and EPA (including 17-HDHA, 12-, 15-HEPE) were higher in the iron deficiency groups (ID and ID+(n-3)FAD) compared to the iron sufficient groups (control and (n-3)FAD), while the values were similar for 18- and 11-HEPE (Figure 3-4 b, c, d and e).

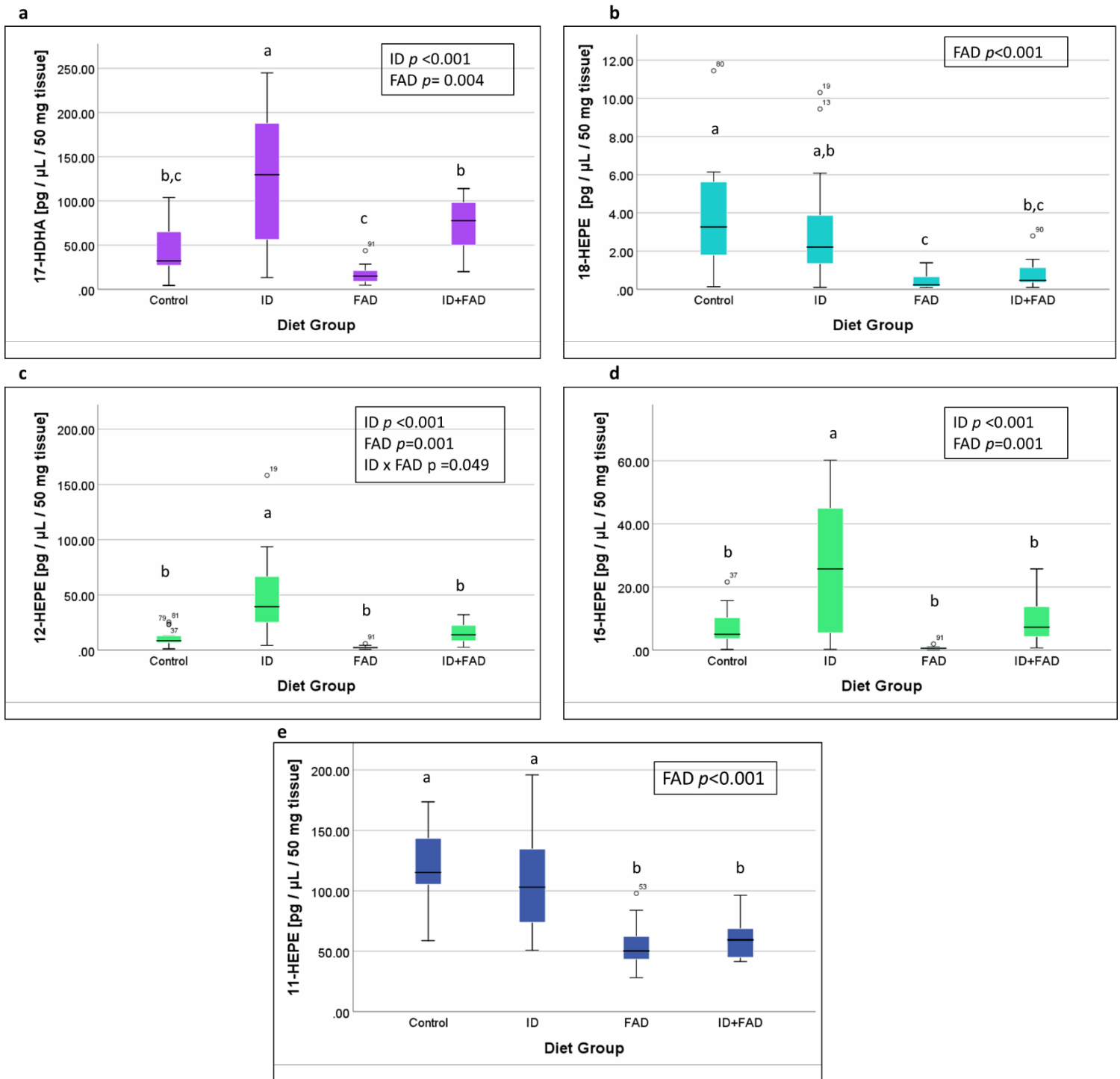


Figure 3-4 Boxplots of lipid mediators for the 4 diet groups a) 17-HDHA; b) 18-HEPE; c) 12-HEPE; d) 15-HEPE and e) 11-HEPE in crude rat colonic lining homogenates

Abbreviations: FAD fatty acid deficiency; HEPE hydroxyeicosapentaenoic acid; ID iron deficiency; pg picogram; μ l microliter; mg milligram

3.4 Discussion

To our knowledge, this is the first study to investigate the effects of pre- and postnatal ID and n-3 FAD, alone and in combination, on colonic inflammation in rats by analysing lipid mediator concentrations from crude colonic homogenates. HETE are derived from ARA and are mainly pro-inflammatory, while 17-HDHA is produced from DHA and is the precursor of the D-series resolvins and protectins, which are pro-resolving lipid mediators [21, 59], HEPE and its derivatives are derived from EPA and also have pro-resolving properties [21, 59]. This study showed that both ID and (n-3)FAD resulted in a pro-inflammatory profile. The combined deficiency of iron and n-3 PUFA in the mother's diet continuing in early development up until young adulthood is more detrimental for the resolving of inflammation in the colon than the two deficiencies alone.

Pre- and postnatal ID (15-18 mg iron/kg) resulted in significantly higher 5-, 12- and 15-HETE, derived from ARA through the LOX pathway as well as higher 8-HETE derived from ARA through the cytochrome P450 pathway, at PND 42-45 (Table 3-4 and Figure 3-3 a, b, c & d). A higher concentration of these pro-inflammatory lipid mediators is an indication of inflammation in the colonic lining epithelial [61-63], however, in our study, a conglomerate sample of colon tissue was analysed. These results are in accordance with those of other researchers, who studied the effect of iron deficiency in the gut. In pigs a low iron diet (20 mg iron /kg) resulted in higher paracellular permeability, an increased number of mucosal neutrophils as well as an increased expression of the pro-inflammatory cytokines IL-1-B and IL-6 [64]. It is known that IL-6 upregulates hepcidin as was seen in this study [19]. In Bangladeshi children, low serum iron was associated with decreased tight junctions in the intestinal epithelium and higher gut permeability, which could also activate the immune system and increase inflammation when pathogens enter the system [65]. In rats, ID (2.6 mg iron/kg) decreased the Roseburia spp in the gut, which produces the short chain fatty acid butyrate that has anti-inflammatory properties [66], also increasing inflammation. Similar results were obtained in an *in vitro* study by the same research group [14]. None of these studies, however, measured the lipid mediator concentrations within the membrane in an iron deficient state, as was done with this study, nor was the combined effect of ID and (n-3)FAD investigated.

In this study (n-3)FAD alone resulted in higher 12-HETE, which was similar to what Neilson and colleagues found [61]. It is evident from the results that (n-3)FAD led to less EPA and DHA as substrate for COX and LOX, resulting in more available ARA (Figure 3.1 a & b) and thereby the upregulation of the n-6 pathway and higher production of the pro-inflammatory lipid mediators (HETEs) derived from ARA through LOX and cytochrome P450. With the n-6 pathway being upregulated, the available iron could be utilised for the production of 15- and 12-LOX to produce 15- and 12--HETE, as well as for cytochrome (CYP3A13), to produce 8-HETE. In rats and mice, CYP3A13 is the specific cytochrome found in the intestinal epithelial cells [67]. Moreover, an

interaction between ID and (n-3)FAD synergistically resulted in higher 12-HETE and 15-HETE. Furthermore, 12-, 15- and 8-HETE concentrations were significantly higher in the ID+(n-3)FAD group compared to all the other groups, indicating that a combined deficiency upregulates inflammation in the colon even more than ID or (n-3)FAD alone. This shows that the combination of these two deficiencies can lead to an even more profound inflammatory response in the colon.

In response to inflammation, the pro-resolving lipid mediators are switched on after a certain time lapse. [68]. As expected, too little substrate was available to produce 17-HDHA from DHA via the COX-2 pathway in (n-3)FAD. In ID, on the other hand, enough substrate for the formation of 17-HDHA was available even though the iron was low (15-18 mg/kg). Iron supplies seemed to be adequate to still support the production of the enzymes involved in the COX and LOX reactions.

Furthermore, a low iron environment creates less oxidative stress which is favorable for 17-HDHA production and subsequently the production of the D-series resolvins and protectins which resolves inflammation [44]. This could also explain why the combined deficiency group had a significantly higher 17-HDHA concentration than the (n-3)FAD group. The ID group had the highest concentration of 17-HDHA, again because of the adequate n-3 PUFA substrate to produce this pro-resolving lipid mediator from DHA and because of low oxidative stress associated with low iron conditions [44]. In the combined deficiency group the tendency to a higher 17-HDHA by ID was attenuated by the (n-3)FAD, because of less substrate to produce the pro-resolving lipid mediator. Thus, even when it is crucial to resolve inflammation due to ID, with no n-3 PUFA as substrate to form 17-HDHA and subsequently resolvins and protectins, the resolution of inflammation is impaired.

Therefore, these effects were exerted most likely by that ID created inflammation in the colon (increased HETEs in combination with a tendency to higher ferritin, which indicates inflammation) and in reaction to this, the production of 17-HDHA was stimulated in order to resolve the inflammation. The question, however, remains what extent of ID is needed to activate inflammation, seeing that the dosages causing inflammation varied from 2.6 mg iron /kg [66], 3 mg iron/kg [45] and 15-18 mg iron/kg in this study for rats where Li and colleagues used 20 mg iron /kg in pigs [64].

As expected (n-3)FAD resulted in a significantly lower concentration of the pro-resolving lipid mediators 12-, 15-, and 18-HEPE derived from EPA through the LOX pathway, as well as a lower concentration of 11-HEPE derived from the cytochrome P450 pathway in the colon (Figure 3-1 b) This is due to less substrate available to produce EPA and its pro-resolving metabolites. Bosco and colleagues found that a diet high in fish oil increased the anti-inflammatory lipid mediators *in vivo* in the colon compared to the control [69]. ID, on the other hand, resulted in higher

concentrations of both 12-HEPE and 15-HEPE. This could be explained by the upregulation of pro-resolving lipid mediators in reaction to the inflammatory state caused by ID as shown by higher pro-inflammatory lipid mediators (HETE). The significant ID x (n-3)FAD interaction on 12-HEPE, where the effect of ID for higher 12-HEPE was attenuated by (n-3)FAD, is most likely due to the lower concentration of n-3 PUFA available to produce 12-HEPE.

Within the ID group, it was remarkable that the concentration of 12-, 15-, 18- HEPE, from the LOX pathway as well as 11-HEPE from the cytochrome pathway, were significantly higher compared to the (n-3)FAD and combined deficiency groups. Again, this could be contributed to the fact that the existing inflammation recruited pro-resolving metabolites to attenuate inflammation (Figure 3-1 b). Furthermore, the combined deficiency group for 12-, 15- and 11-HEPE were significantly lower compared to the ID group. This indicates that iron deficiency in combination with (n-3)FAD, is more detrimental for the resolution of inflammation, than ID alone because even if the system is triggered to resolve inflammation, it cannot due to a deficiency in n-3 PUFA, and an inability to produce the pro-resolving lipid mediators

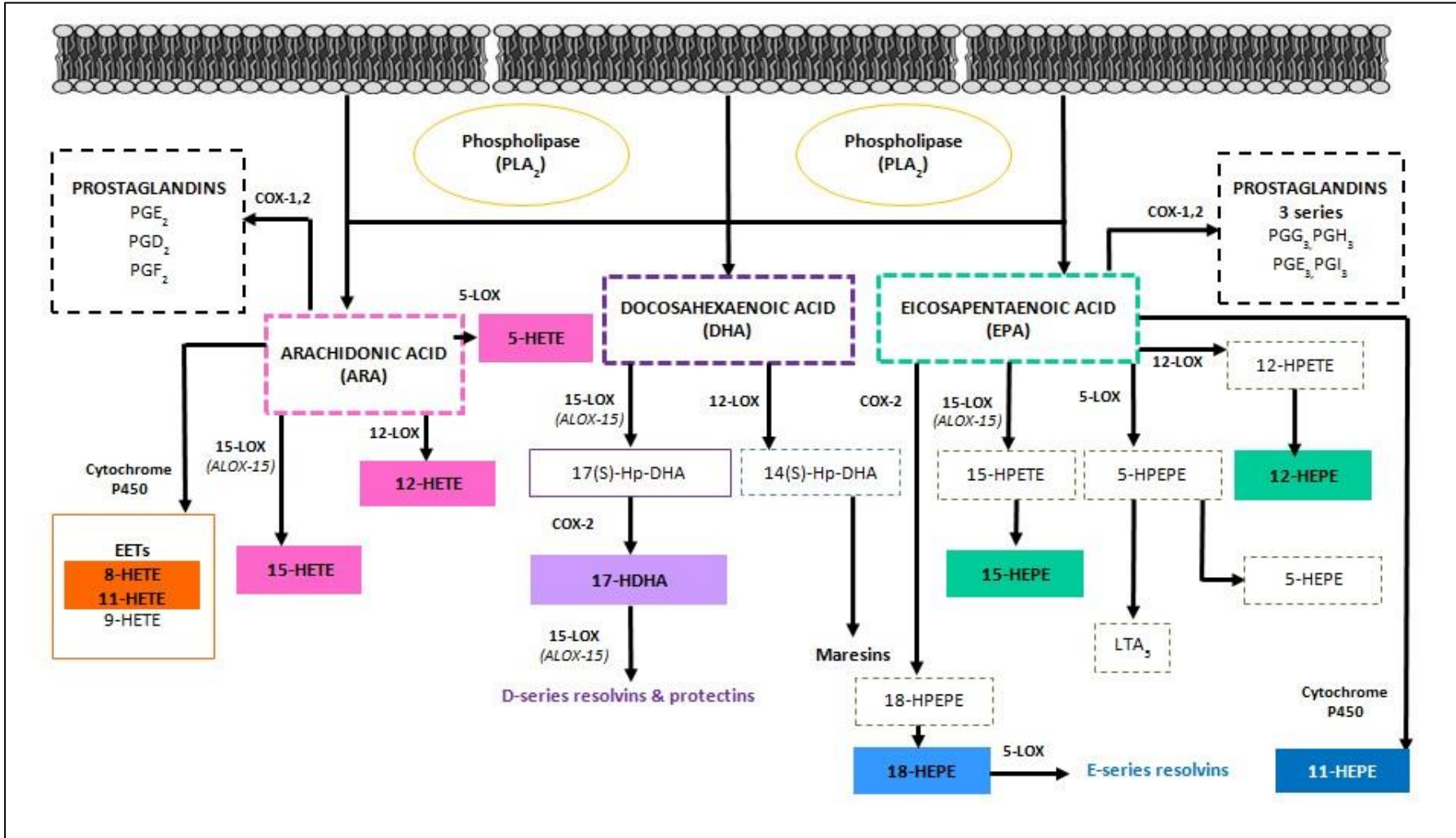


Figure 3-1 b An overview of the main pathways involved in the production of polyunsaturated fatty acid-derived lipid mediators [45]
 Abbreviations: ALOX: arachidonate lipoxygenases; ARA: arachidonic acid; COX: cyclooxygenase; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; HDHA: hydroxy docosahexaenoic acid; HEPE: hydroxyeicosapentaenoic acid; HETE: hydroxy-eicosatetraenoic acid; LOX: lipoxygenase; LT: leukotriene; PG: prostaglandin; TX: thromboxane
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Iron is a co-factor crucial for the functional activity of the enzymes $\Delta 5$ -, $\Delta 6$ -desaturase & elongase which converts LA to ARA (Table 3-4; Figure 3-1 a). A low iron availability will, therefore, decrease the enzyme activities for this reaction to take place (Table 3-4; Figure 3-1 a). The same applies to the conversion of LA to GLA where $\Delta 6$ -desaturase is active [43]. In the current study, ID resulted in lower activity of $\Delta 6$ -desaturase and also of the total fatty acid metabolism between LA and ARA, including $\Delta 5$ -, $\Delta 6$ -desaturase & elongase

The opposite is true for (n-3)FAD; inadequate substrate to form the longer chain fatty acids upregulates the pathway. Seeing that iron is available in this scenario, the enzyme activities of $\Delta 5$ -, $\Delta 6$ -desaturase & elongase which convert LA to ARA are higher. This confirms that there is indeed competition for enzymes between the n-6 and n-3 pathways. If there is not enough substrate to form the metabolites from the n-3 pathway, the enzyme production is still upregulated but consequently used to form n-6 metabolites. The significant interaction between ID and (n-3)FAD for the GLA:LA ratio implies that ID attenuates the activity of $\Delta 6$ -desaturase, even when the fatty acid metabolic pathway is up regulated because of the n-3 poor environment.

When looking at the effects of the model on iron status parameters, as expected, ID resulted in lower haemoglobin concentrations at PND 42 - 45 (Table 3-3). Low dietary iron decreases erythropoiesis and subsequently the production of haemoglobin, which makes up more than 90% of the dry weight of RBCs. Most of the iron in humans is present in haemoglobin of RBCs containing about 1 mg of iron per milliliter of erythrocytes, or about 2–3 g of total iron. Adult rodents have about 0.6–1 mg of iron in hemoglobin [70] and in rats, the normal haemoglobin range is 15-18g/dL. [71].

In this study, ID showed a tendency toward higher hepcidin concentrations. In mammals low iron is normally associated with low hepcidin concentrations. Hepcidin helps to maintain blood iron within the normal range by limiting absorption from the small intestine and controlling the release from hepatocytes, spleen and bone marrow. This is done by the degradation of the iron carrier, ferroportin. The result is iron sequestration, decreased iron transfer to transferrin and a decreased iron flow into the plasma. Therefore, under normal circumstances hepcidin production decreases during iron deficiency and hypoxia, and increases in iron overload [19, 70]. Hepcidin is however not only influenced by serum iron levels, the inflammatory cytokine, IL-6, also upregulates its expression [19]. In this study higher hepcidin concentrations are thus an indication of inflammation, and not of iron status, seeing that the n-6 pathway was upregulated together with the production of the pro-inflammatory lipid mediators (5-, 12-, 15- and 8-HETEs).

It is known that infection and inflammation increase serum ferritin [19, 24]. In this study, this is evident and can be attributed to n-3(FAD). The n-3 PUFAs are needed for the production of the pro-resolving lipid mediators, and a deficiency would cause a more inflammatory state [5, 21,

59].

Transferrin receptor was expected to be elevated in ID because in low iron conditions an upregulation of these receptors is expected to import iron from transferrin to cells by endocytosis [19]. This was however not evident in this model, probably due to the amount of iron that the ID diet contained (15-18 mg iron/kg) possibly showing a threshold sensitivity for transferrin receptor.

Red blood cell (RBC) total phospholipid fatty acid composition is used as a proxy for the rest of the body's cell membrane fatty acid status, and the RBC n-6 and n-3 PUFA status, therefore, give an indication of the model's effects on the colon tissue cell membrane fatty status [72]. As expected (n-3)FAD resulted in significantly lower percentages of EPA, DHA and total n-3 PUFA in RBCs, while it increased the pro-inflammatory fatty acid percentage of ARA and total n-6 PUFA. This was in accordance with what Robertson and colleagues found in mice which were given an (n-3)FAD deficient diet [27]. Furthermore, the n-6/n-3 PUFA ratio was also significantly increased in the (n-3)FAD group. This indicates that the (n-3)FAD diet was effective to induce a shortage of n-3 PUFA in the body's cell membrane fatty acid status as well as an increase in the n-6 PUFAs. A deficiency in n-3 PUFA results in the reduced ability to resolve inflammation and therefore also result in increased pro-inflammatory cytokine signaling [21], whereby hepcidin and ferritin can be increased, as was seen in this study.

ID resulted in a lower composition of ARA. Iron is a cofactor for $\Delta 6$ -desaturase and $\Delta 5$ desaturase as well as elongase enzymes required for the conversion of n-6 fatty acids to their longer chain metabolites like ARA [21 & 43]. Therefore, less iron will result in less enzyme activity and lower production of ARA. This will also explain why ARA was significantly lower in the ID group compared to the other three groups. The similar effect seen on total n-6 PUFA was likely driven by the effects on ARA, which is the most prominent constituent of total n-6 LCPUFA. However, ID attenuated the effect of (n-3)FAD to increase total n-6 PUFA. This could be because the ID group is not deficient of n-3 PUFA and that the available n-3 PUFA attenuated the effect of (n-3)FAD for a higher n-6 PUFA composition in the RBCs. In simple terms, the small contribution of n-3 PUFA from the ID group replaced some of the n-6 PUFA in the membrane resulting in a lower percentage of n-6 PUFA in RBCs.

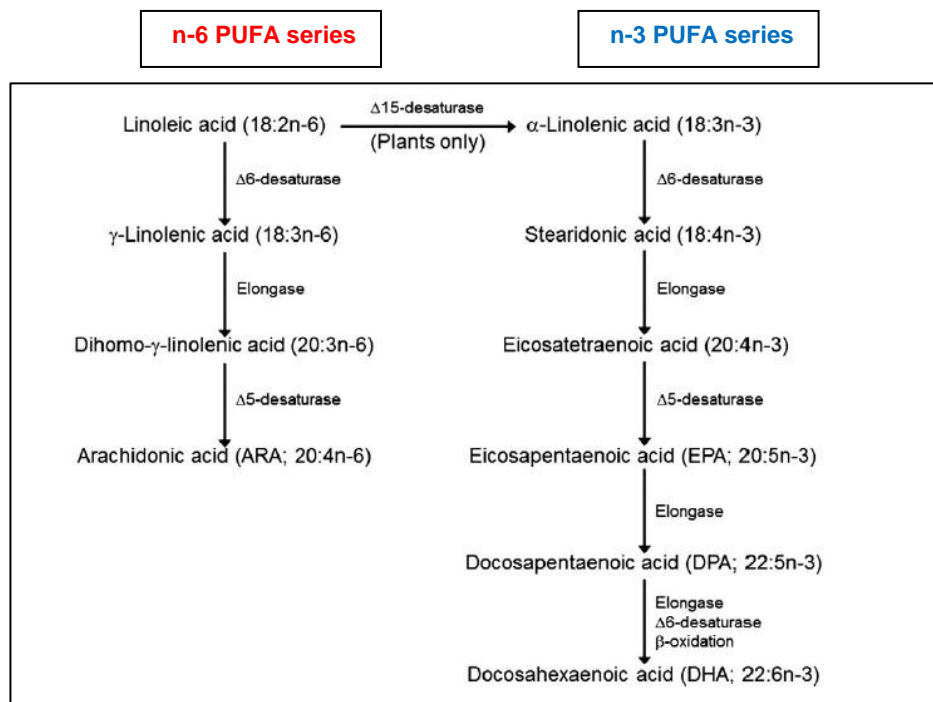


Figure 3-1 a: The biosynthesis of n-6 and n-3 PUFA. These two pathways compete for the same enzymes for elongation, desaturation, and oxidation in mammals [21]
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ID and (n-3)FAD both resulted in a lower DHA composition. In ID, this could also be explained by a decrease in the elongase and desaturase enzyme activity as in the case of ARA, since the n-6 and n-3 pathways share the same enzymes (Figure 3.1 a) [21,43]. Furthermore, there was a significant interaction between ID and (n-3)FAD, on DHA where (n-3)FAD maintained the lower DHA composition. Therefore it can be argued that the combined deficiency of iron and n-3 PUFA decreases the production of DHA even further.

ID resulted in a higher EPA composition, possibly because of the lack of iron that inhibited delta-4 desaturase which forms DHA from EPA, subsequently leading to EPA to accumulate in the RBC (Figure 3.1 a). A significant ID x (n-3)FAD interaction on EPA composition resulted in the effect of ID for higher EPA to be attenuated by (n-3)FAD, due the restoration of the balance between substrate availability and enzyme activity.

As ID did not result in a significant difference in the n-6/n-3 ratio, it shows that iron deficiency likely influenced the n-3 and n-6 fatty acid metabolisms equally. Because of the n-3 PUFA deficiency in both the (n-3)FAD and combined deficiency groups, there was a significant increase in the n-6 PUFAs, an upregulation of the n-6 pathway to produce pro-inflammatory lipid mediators and a reduced ability to resolve inflammation [21].

The model used in this study represents the human situation where children are born to mothers who have a combined iron and n-3 PUFA deficiency and at the same time consume high

amounts of n-6 PUFA. This dietary pattern increases inflammation as seen in the overall results where all the pro-inflammatory lipid mediators derived from ARA (including 5-, 12-, 15-, 8- and 11 HETE) were higher in the (n-3)FAD groups compared to the (n-3)FA sufficient groups (control and ID). The pro-inflammatory lipid mediators derived from ARA (including 5-, 12-, 15- and 8-HETE) were also higher in the ID groups compared to the iron sufficient groups (control and (n-3)FAD), whereas the pro-resolving lipid mediators derived from DHA and EPA (including 17-HDHA, 12-, 15-, 18- and 11-HEPE) were lower in the (n-3) FAD groups compared to the (n-3)FA sufficient groups (control and ID). This indicates that resolving of inflammation could not be initialised, due to too little substrate to produce the pro-resolving lipid mediators.

Taken together this profile is indicative of higher inflammation. Furthermore, the pro-resolving lipid mediators derived from DHA and EPA (including 17-HDHA, 12-, 15-HEPE) were higher in the ID groups compared to the iron sufficient groups (control and (n-3)FAD), while the values were similar for 18- and 11 HEPE. This is an indication that the system for resolving inflammation is switched on.

A limitation of this study was that calprotectin; an inflammation marker in the faeces of rats was not measured. This would have confirmed gut inflammation. The blood cytokine profile was not measured either, which could have given an indication of whether systemic inflammation occurred in association with gut inflammation and elevated IL-6 would have explained the elevated hepcidin value, despite iron deficiency. Furthermore, a conglomerate sample of colon tissue was used in the analysis. Therefore, it was not possible to determine in which layer or section of the colon the lipid mediators were most active. Comparing the effects of ID and (n-3) FAD on colonic inflammation in offspring at PND 42-45 that continued with their respective diets after weaning (our study), with offspring that switched to a control diet after weaning (PND 21), would be ideal for future research.

In a human situation, the diet pattern of the mother is most likely to continue throughout childhood into adolescence, which is exemplified in this study. The mother's nutritional status also has an impact on the unborn baby as hypothesised by Barker [37]. Early-life programming, as it is referred to, is becoming a recognized concept that claims that the environment during early development affects health and disease in adulthood, possibly via epigenetic mechanisms. Accumulating evidence suggests that nutrition before and during pregnancy as well as during early postnatal life is one of the most critical environmental cues that programs metabolic, and immunologic development [34, 36].

It can, therefore, be concluded that the combined deficiency of iron and n-3 PUFA in the mother's diet continuing in early development up until young adulthood, is more detrimental for the resolving of inflammation in the colon, than the two deficiencies alone.

Conflict of interest: None

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CHAPTER 4: SUMMARY AND CONCLUSION

4.1 Summary

This study aimed to investigate the effects of n-3 polyunsaturated fatty acid and iron deficiency, alone and in combination, during early development on colonic inflammation at PND 42-45 (representing adolescence). The objectives were to investigate whether these deficiencies, had an effect on the pro-resolving lipid mediators: 17-hydroxydocosahexaenoic acid (17-HDHA); 11-, 12-, 15 & 18- hydroxyeicosapentaenoic acid (HEPE) as well as the pro-inflammatory lipid mediators: 5-, 8-, 11-, 12- and 15- hydroxyeicosatetraenoic acid (HETE); in colon tissue. The red blood cell total phospholipid fatty acid composition (including ratios indicating desaturase activity) and iron status (haemoglobin, ferritin and transferrin receptor) were also measured.

To our knowledge, this was the first experiment to measure these outcomes in the colon tissue of Wistar rats.

4.2 Conclusion

In conclusion, this study found that pre- and postnatal combined deficiency of iron and n-3 PUFA in rats, increases inflammation in the colon of offspring in early adolescence more than the single deficiencies alone. A deficiency in n-3 PUFA furthermore attenuates the resolving of inflammation which is still possible with ID, resulting in a pro-inflammatory profile that cannot be resolved. Additional research is needed to confirm whether these effects will be sustained in the long term and whether it can be reversed when offspring are fed an iron and n-3 PUFA- sufficient diet, or if dams receive iron and n-3 PUFA supplementation during pregnancy and lactation.

4.3 Strengths and limitations

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 inflammation in the colon. Nutrient deficiencies seldom occur in isolation, and in a country like South-Africa, it is most likely that women of childbearing age suffer from both ID and (n-3)FAD simultaneously. Furthermore, the amount of iron and n-3 FA provided in the experimental diets were not too severe and representative of ID and/or n-3(FAD) similar to what can be expected in a human context. Using a rat model, made it possible to analyse the colon tissue, which is not possible in a human context. It would also not be ethical to induce nutrient deficiencies intentionally in women of reproductive age. Further to this, the use of LCMSMS is an advanced method that accurately measures lipid mediators.

Limitations in this study include the inability to follow offspring up until late adulthood, considering that this study was nested in a larger study, which required offspring to be euthanized on day 42 (adolescence). If offspring could have been followed up for a more extended time period, until late adulthood, it may have been determined whether colon inflammation resulted in low-grade inflammation and the development of IBS or even degenerative disease like diabetes mellitus, cardiovascular disease, obesity or hypertension. Chronic high-grade inflammation could have led to more severe inflammatory bowel diseases like Crohn's Disease and ulcerative colitis. Another limitation was that calprotectin; an inflammation marker in the faeces of rats was not measured. This would have confirmed gut inflammation. The blood cytokine profile was not measured either, which could have given an indication of whether systemic inflammation occurred in association with gut inflammation. Furthermore, a conglomerate sample of colon tissue was used in the analysis. Therefore, it was not possible to determine in which layer or section of the colon the lipid mediators were most active.

4.4 Recommendations on future research

Comparing the effects of ID and (n-3)FAD on colonic inflammation in offspring at PND 42-45 that continued with their respective diets after weaning (our study), with offspring that switched to a control diet after weaning (PND 21), would be the next step. As part of the main study, the samples of these offspring have already been collected, and it will be analysed in the near future. This will determine whether the effects of pre- and postnatal ID and (n-3)FAD on colonic inflammation at PND 42-45 are reversible in offspring switched to a control diet post-weaning. With equal male and female numbers, it could also be determined whether the results are sex specific.

The second experiment of the main study, which follows a repletion model, can determine whether the effects of a pre- and postnatal ID and (n-3)FAD on gut inflammation in the offspring can be reversed if dams receive supplementation during pregnancy and lactation. Possible effects on the colonic epithelial and inflammation of the dams of a pre- and postnatal ID and (n-3)FAD can also be investigated, both alone and in combination. It would be ideal to repeat the experiment and to follow the offspring up until late adulthood, to determine whether the effects of deficiency on the colon remain in the long term. The effect of ID and (n-3)FAD on inflammation in the colon from early adolescence to late adulthood can then be determined, to ascertain if it leads to low-grade inflammation (IBS) as well as other degenerative diseases like diabetes mellitus, obesity, cardiovascular disease, and hypertension. These findings would provide insight into the crucial role of optimal nutrition in early life as a strategy to prevent disease later in life.

The primary goal of this research is to translate it to the human context. Once a better understanding of the effect of iron and n-3 PUFA in colon health is obtained, human trials can investigate the role of iron and n-3 supplementation in prenatal women on the gut health of the child.

Annexure A: Ethics approval of main study



NORTH WEST UNIVERSITY
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NOORDWES-UNIVERSITEIT
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Tel: 018 299-1111/2222
Web: <http://www.nwu.ac.za>

The Applicant / Primary Investigator

Faculty of Health Sciences
Ethics Office for Research, Training and Support
Animal Care, Health and Safety in Research
Ethics Committee (AnimCare)

Tel: 018 299 2234
Fax: 018 299 2225
Email: Tiaan.Brink@nwu.ac.za

09 February 2017

Dear Dr Baumgartner

APPROVAL OF YOUR APPLICATION FOR AMENDMENT: to add new team members (MSc students) and change of animal technician

Ethics Number: NWU-00335-15-A5

Kindly use the ethics reference number provided above in all correspondence or documents submitted to the AnimCare secretariat.

Study Title: Novel interactions between iron and n-3 fatty acids: The effects of maternal depletion and repletion on offspring development and health
Study leader/Supervisor: Prof CM Smuts
Student: N/A
Application type: New Application - Large Project

Project Category (impact on animal wellbeing)	Not applicable	0	1	2	3	4	5
						X	

The abovementioned application has been reviewed and discussed by the AnimCare Animal Research Ethics Committee, Faculty of Health Sciences through the expedited review.

Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years when extension will be facilitated during the monitoring process.

After ethical review

The AnimCare, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the AnimCare, Faculty of Health Sciences prior to implementing these changes. Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form sent to Ethics-AnimCareIncident-SAE@nwu.ac.za

A monitoring report should be submitted within one year of approval of this study (or as otherwise stipulated) and before the year has expired, to ensure timely renewal of the study. A final report must be provided at completion of the study or the AnimCare committee, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimMonitoring@nwu.ac.za

Page 1 of 2

Annexure B: Ethics approval of sub-study



Dr L. Malan
Nutrition
Centre of Excellence for Nutrition

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South Africa 2520

Tel: 018 299-1111/2222
Web: <http://www.nwu.ac.za>

Health Sciences Ethics Office for Research,
Training and Support

Animal Care, Health and Safety in Research
Ethics Committee (AnimCare)
Tel: 018 299 2234
Email: Tiaan.Brink@nwu.ac.za

19 November 2018

Dear Dr Malan

APPROVAL OF YOUR APPLICATION BY THE ANIMCARE COMMITTEE OF THE FACULTY OF HEALTH SCIENCES

Ethics number: NWU-00335-15-A5-01

Kindly use the ethics reference number provided above in all future correspondence or documents submitted to the administrative assistant of the Animal Care, Health and Safety in Research Ethics Committee (AnimCare).

Study title: Effects of n-3 polyunsaturated fatty acid and iron deficiency, alone and in combination, during early development on colonic inflammation in rats

Study leader: Prof L. Malan

Student: V Schoeman-11122412

Application type: Sub-study

Project Category (impact on animal wellbeing)	NA	0	1	2	3	4	5
				X			

Expiry date: 30 November 2019 (monitoring report is due at the end of November annually until completion)

You are kindly informed that after review by the AnimCare committee, Faculty of Health Sciences, North-West University, your ethics approval application has been successful and was determined to fulfil all requirements for approval. Your study is approved for a year and may commence from 19/11/2018. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation. A monitoring report should be submitted two months prior to the reporting dates as indicated i.e. annually for Category 0-4 studies, six-monthly for category 5 studies, to ensure timely renewal of the study. A final report must be provided at completion of the study or the AnimCare committee, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimMonitoring@nwu.ac.za. Annually, a number of studies may be randomly selected for an internal audit.

The AnimCare committee, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the AnimCare committee, Faculty of Health Sciences prior to implementing these changes. These requests should be submitted to Ethics-AnimCare@nwu.ac.za with a cover letter with a specific subject title indicating "Amendment request: NWU-XXXXX-XX-XX". The letter should include the title of the approved study, the names of the researchers involved, the nature of the amendment/s being made (indicating what changes have been made as well as where they have been made), which documents have been attached and any further explanation to clarify the amendment request being submitted. The amendments made should be indicated in **yellow highlight** in the amended documents (or in the fillable MSWord format application forms where a yellow highlighter may not be visible, change the text

Annexure C: Author guidelines for Nutrition Research

GUIDE FOR AUTHORS

INTRODUCTION

Nutrition Research publishes research articles, communications, and reviews on all aspects of basic and applied **nutrition**. The mission of *Nutrition Research* is to serve as the journal for global communication of nutrition and life sciences research on **diet** and **health**. The field of **nutritional sciences** includes, but is not limited to, the study of nutrients during growth, reproduction, aging, and disease.

Articles covering basic and applied research on all aspects of nutritional sciences are encouraged, including: nutritional biochemistry and metabolism; metabolomics, nutrient and gene interactions; nutrient requirements in health and disease; digestion and absorption; nutritional anthropology and epidemiology; the influence of socioeconomic and cultural factors on nutrition of the individual and the community; the impact of nutrient intake on disease response, work performance and behavior; the consequences of nutritional deficiency on growth and development, endocrine and nervous systems, and immunity; food intolerance and allergy; nutrient drug interactions; nutrition and aging; nutrition and cancer; obesity; diabetes; and intervention programs.

A principal focus of the journal is to publish research that advances the understanding of **nutrients** and **health protectants** in food for improving the human condition. Of interest are manuscripts on the development of biomarkers for assessing how dietary components influence health status in the human.

The journal also encourages **submission** of manuscripts describing investigations in animal models and cell cultures that utilize methodologic approaches or techniques in biochemistry, immunology, molecular biology, toxicology, and physiology. Epidemiologic studies on nutrient and phytochemical intakes in human populations and novel analytical techniques for these compounds are within the scope of the mission for *Nutrition Research*.

Dr. Bruce A. Watkins, Editor-in-Chief (baw@purdue.edu or bawatkins@ucdavis.edu)
Angela Ranalli-Curtis, Managing Editor (alrcurtis@gmail.com)

Nutrition Research
Department of Nutrition
University of California, Davis
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3135 Meyer Hall
Davis, CA 95616-5270, USA

BEFORE YOU BEGIN

Ethics in publishing

Please see our information pages on **Ethics in publishing** and **Ethical guidelines for journal publication**.

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All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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Each manuscript submitted must provide a title page, list of abbreviations, abstract page, introduction, methods and materials, results, discussion, list of references, and appropriate presentation of data in tables and figures. In some cases, the results and discussion sections can be combined (e.g., communications).

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* All studies that include experimental diets must provide a table that lists the ingredients and enough detail for the nutrient content of those diets. Reference to established diets (such as AIN 93G) is appropriate when the major ingredients are listed and the premix levels are provided (actual details of each vitamin and mineral source listed is not necessary in this case). Diets that are developed with different lipid sources should provide a fatty acid compositional analysis of the lipids. In addition, studies that test a botanical or phytochemical ingredient should provide enough chemical compositional analysis as well as the amount of the active compounds.

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Examples:

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[1] Van der Geer J, Hanraads JA, Lupton RA. The art of writing a scientific article. *J Sci Commun* 2010;163:51-9.

Reference to a book:

[2] Strunk Jr W, White EB. *The elements of style*. 4th ed. New York: Longman; 2000.

Reference to a chapter in an edited book:

[3] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. *Introduction to the electronic age*, New York: E-Publishing Inc; 2009, p. 281-304.

Reference to a website:

[4] Cancer Research UK. *Cancer statistics reports for the UK*, <http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/>; 2003 [accessed 13 March 2003].

Reference to a dataset:

[dataset] [5] Oguro M, Imahiro S, Saito S, Nakashizuka T. Mortality data for Japanese oak wilt disease and surrounding forest compositions, *Mendeley Data*, v1; 2015. <https://doi.org/10.17632/xwj98nb39r.1>.

Note shortened form for last page number. e.g., 51-9, and that for more than 6 authors the first 6 should be listed followed by 'et al.' For further details you are referred to 'Uniform Requirements for Manuscripts submitted to Biomedical Journals' (*J Am Med Assoc* 1997;277:927-34) (see also [Samples of Formatted References](#)).

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