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Food Development (tech)

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TURKU, FINLAND – 2023

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ISBN 978-951-29-9462-5 (print)
ISBN 978-951-29-9463-2 (pdf)
ISSN 2323-9395 (print)
ISSN 2323-9409 (pdf)
Painosalama, Turku, Finland 2023

The true method of knowledge is experiment.

William Blake

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ABSTRACT

Triacylglycerols (TAGs) are mostly chiral and they are metabolized in the chiral environment. They are not only a source of energy but also provide essential fatty acids (FAs). Long chain omega-3 polyunsaturated fatty acids [LC (n-3) PUFAs] are derivatives of essential FA α-linolenic acid. Dietary LC (n-3) PUFAs, especially docosahexaenoic acid (DHA), have been associated with normal retinal and neural development and prevention of cardiovascular and circulatory disorders among other biological effects. Various structures of lipids in food products can modulate the release and bioavailability of FAs during digestion and subsequent metabolic fate of FAs. The current knowledge of the bioavailability of these LC (n-3) PUFAs from different TAG structures lies on the fish oil to seal oil comparisons as LC (n-3) PUFAs, mainly EPA and DHA, are located predominantly in the *sn*-2 position in fish and squid oil, but in the *sn*-1 and *sn*-3 positions in seal oil. Despite of numerous biological functions of LC (n-3) PUFAs, very little is known about the absorption of DHA from the stereospecific forms of TAGs, especially from the enantiospecific TAGs. Before recent advances in the synthesis of enantiospecific structured TAGs, the investigation of the impact of dietary TAG structure on the absorption of FAs from *sn*-1 and *sn*-3 positions of TAGs has been limited.

The aim of the current work was to investigate the effect of mild (n-3) deficiency and the effect of TAG structure on the extent of accumulation of DHA in organs, the bioavailability of DHA in plasma and feces, as well as on body weight, and organ weight. The effect of short-term first generation (n-3) deficiency was studied in a 33 day feeding trial on the lipid composition of different organs, plasma, and feces in rats. The effect of TAG structure on the FA content and composition of the different organs, plasma, and feces was investigated in a 5 day feeding trial with 360 mg/d enantiopure (> 96%) structured TAGs [*sn*-DHA-18:0-18:0 (*sn*-1 DHA) and *sn*-18:0-18:0-DHA (*sn*-3 DHA)] and their regioisomer [*sn*-18:0-DHA-18:0, *sn*-2 DHA). The lipids were extracted from organs, plasma, and feces, transformed into fatty acid methyl esters and analyzed by gas chromatography.

The (n-3) deficiency resulted in lower DHA levels in the plasma, brain, testicle, visceral fat, heart, and lungs. The DHA level of the visceral fat of (n-3) deficient animals was 14% of the visceral fat of (n-3) adequately fed animals, being most affected by the (n-3) deficient diet feeding. The brain DHA was least affected with the DHA level of the (n-3) deficient animals being 86% of the (n-3) adequate animals. The DHA level of the organs was affected in the order of visceral fat > liver triacylglycerols > lung > heart > liver phospholipids > testis > eye > brain. The effect of feeding structured TAGs for 5 days following an (n-3) deficient diet resulted in less secretion of fecal DHA from the *sn*-2 position

compared with the *sn*-1 and *sn*-3 positions suggesting superior absorption of DHA from the *sn*-2 position. A significantly higher level of DHA in the *sn*-1 DHA group compared to *sn*-3 DHA group in the liver TAG showed the difference in the absorption of DHA between the two primary positions of dietary TAG. The eye of the *sn*-1 DHA group led to a small but insignificant increase in DHA level compared to the *sn*-3 DHA group and stearic acid (18:0, STA) in liver PLs was found to be significantly higher *sn*-1 DHA group as compared to the *sn*-3 DHA group. This indicated a possible difference in the absorption of FAs from *sn*-1 and *sn*-3 positions of TAG.

This work provides the information to evaluate the conditions needed to reach mild (n-3) deficiency in the first generation of rats and to evaluate the feasibility to collect data from a variety of organs. Also, the short term feeding of structured TAGs provides evidence on the possible differences in the absorption of DHA from the primary positions (*sn*-1 and *sn*-3) of TAG in the liver TAG fraction. Together, this work shed light on the influence of the positional distribution of DHA in dietary TAGs on plasma, fecal, and organ lipids and (n-3) deficiency on the bioavailability of DHA strengthening the current understanding of lipid biochemistry.

SUOMENKIELINEN ABSTRAKTI

Triasyyliglyserolit (TAG) ovat enimmäkseen kiraalisia ja niiden aineenvaihdunta tapahtuu kiraalisessa ympäristössä. Ne eivät ole vain energialähde, vaan ne sisältävät myös välttämättömiä rasvahappoja. Pitkäketjuiset monityydyttymättömät omega-3 rasvahapot [LC (n-3) PUFA:t] ovat välttämättömän α-linoleenihapon johdannaisia. Ruokavalion LC (n-3) PUFA:t, erityisesti dokosaheksaeenihappo (DHA), on yhdistetty verkkokalvon ja hermoston normaaliin kehitykseen sekä sydän- ja verisuonisairauksien sekä verenkiertohäiriöiden ehkäisyyn, muiden biologisten vaikutusten ohella. Elintarvikkeiden erilaiset lipidirakenteet voivat vaikuttaa rasvahappojen vapautumiseen ja biosaatavuuteen ruoansulatuksen aikana sekä niiden myöhempään aineenvaihduntaan. Nykyinen tieto LC (n-3) PUFA:ien biosaatavuudesta eri TAG-rakenteista perustuu kalaöljyn ja hyljeöljyn vertailuun, koska eikosapentaeenihappo (EPA) ja DHA sijaitsevat pääasiassa *sn*-2 -asemassa kala- ja kalmariöljyssä, mutta *sn*-1 ja *sn*-3 -asemissa hyljeöljyssä. Pitkäketjuisten (n-3) rasvahappojen lukuisista biologisista toiminnoista huolimatta on hyvin vähän tietoa siitä, miten DHA:n tarkka sitoutumiskohta (*sn*-1, *sn*-2, *sn*-3) TAG-molekyylissä vaikuttaa imeytymiseen. Ennen viimeaikaista edistystä enantiospesifisten strukturoitujen TAG:ien synteesissä, ravinnon TAG-rakenteen vkutusta rasvahappojen imeytymiseen TAG:ien *sn*-1- ja *sn*-3-asemista on tutkittu hyvin vähän.

Tämän väitöskirjatyön tavoitteena oli tutkia, miten lievä (n-3) rasvahappojen puute ja TAG-rakenne vaikuttavat DHA:n kerääntymiseen eri elimiin, DHA:n biosaatavuuteen määrittämällä plasman ja ulosteen DHA-pitoisuudet, sekä koeeläimen ja sen elinten painoon. Väitöskirjatyön 33 päivää kestäneessä ruokintakokeessa tutkittiin lyhytaikaisen, ensimmäisen sukupolven (n-3) rasvahappojen puutteen vaikutusta rottien eri elinten, plasman ja ulosteiden lipidikoostumukseen. TAG-rakenteen vaikutusta eri elinten, plasman ja ulosteiden rasvahappopitoisuuteen ja -koostumukseen tarkasteltiin viisi päivää kestäneessä ruokintakokeessa, jossa 360 mg enantiopuhtaita (> 96 %) TAG-isomeereja [stereoisomeerit *sn*-DHA-18:0-18:0 (*sn*-1 DHA) ja *sn*-18:0-18:0-DHA (*sn*-3 DHA), niiden regioisomeeri *sn*-18:0-DHA-18:0, *sn*-2 DHA] sisällytettiin rottien ruokavalioon päivittäin (n-3) rasvappojen puutetta aiheuttavan jakson jälkeen. Lipidit uutettiin elimistä, plasmasta ja ulosteista. Rasvahapot muunnettiin niiden metyyliestereiksi ja analysoitiin kaasukromatografialla.

(n-3) puutos johti alhaisempiin DHA-tasoihin plasmassa, aivoissa, kiveksissä, sisäelimiä ympäröivässä rasvassa, sydämessä ja keuhkoissa. (n-3) puutoksessa eläinten sisäelinten ympärillä olevan rasvan DHA-taso laski eniten verrattuna riittävästi ruokittujen eläinten sisäelinten rasvan DHA-tasoon ollen siitä vain 14 %. Tutkituista elimistä vähiten vaikutusta ruokinnalla oli aivojen DHA:n määrään. (n-3) puutoksessa aivojen DHA-taso oli 86 % (n-3) rasvahappoja

riittävästi saaneiden koe-eläinten aivojen DHA-tasosta. Elinten DHA-tasoon (n-3) rasvahappoja puutteellisesti sisältänyt ruokinta vaikutti seuraavassa järjestyksessä: sisäelimiä ympäröivä rasva > maksan triasyyliglyserolit > keuhkot > sydän > maksan fosfolipidit > kivekset > silmät > aivot. Kun (n-3) puutteellisen ruokavalion jälkeen strukturoituja TAG:eja sisällytettiin ruokavalioon 5 päivän ajan, DHA:ta erittyi vähemmän ulosteeseen ruokavalion DHA:n sijaitessa *sn*-2-asemassa verrattuna *sn*-1- ja *sn*-3-asemiin, mikä viittaa DHA:n parempaan imeytymiseen *sn*-2-asemasta. Merkittävästi korkeammat DHA-tasot maksan TAG:eissa *sn*-1-DHA-ryhmässä verrattuna *sn*-3-DHA-ryhmään osoittivat eron DHA:n imeytymisessä ravinnon TAG:n kahden primaarisen aseman välillä. Tutkimuksessa havaittiin, että *sn*-1 DHA-ryhmän silmien DHA-pitoisuus oli vähän, mutta ei merkittävästi suurempi verrattuna *sn*-3 DHA-ryhmään. Lisäksi havaittiin, että stearііnihapon määrä maksan fosfolipideissä oli merkitsevästi korkeampi *sn*-1 DHA-ryhmässä verrattuna *sn*-3 DHA-ryhmään. Tämä viittaa mahdolliseen eroon rasvahappojen imeytymisessä TAG:n *sn*-1- ja *sn*-3-asemista.

Väitöskirjatyö antaa tieteellistä tietoa arvioitaessa olosuhteita lievän (n-3) puutteen saavuttamiseksi ensimmäisessä rottasukupolvessa sekä auttaa tulevia tutkimuksia pohdittaessa datan keräämistä useista eri elimistä. Väitöskirjatutkimus TAG-rakenteen vaikutuksesta DHA:n biosaatavuuteen tarjoaa todisteita siitä, että DHA:n imeytymisen erot TAG:n primaarisista asemista (*sn*-1 ja *sn*-3) näkyvät eroina maksan TAG:ssa. Yhteenvetona tämä työ tarjosi tieteellistä tietoa ravinnon TAG:en DHA:n paikkajakauman vaikutuksesta plasman, ulosteiden ja elinten lipideihin sekä (n-3) puutoksen vaikutuksesta DHA:n biosaatavuuteen vahvistaen nykyistä ymmärrystä lipidien biokemiasta.

LIST OF ABBREVIATIONS

Acetyl-CoA	Acetyl-Coenzyme A
ALA	α- linolenic acid
ARA	Arachidonic acid
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid (omega-3)
DPAn-6	Docosapentaenoic acid (omega-6)
DTA	Docosatetraenoic acid
EEs	Ethyl esters
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
FAMEs	Fatty acid methyl esters
FAs	Fatty acids
FFAs	Free fatty acids
GC	Gas chromatography
HDL	High-density lipoproteins
HPLC	High-performance liquid chromatography
IDL	Intermediate-density lipoproteins
LA	Linoleic acid
LC (n-3) PUFAs	Long-chain (omega-3) polyunsaturated fatty acids
LC (n-6) PUFAs	Long-chain (omega-6) polyunsaturated fatty acids
LDL	Low-density lipoprotein
LML-	Long-medium-long
MAG	Monoacylglycerol
MLM-	Medium-long-medium
NF	Normal feed
NMR	Nuclear magnetic resonance
OA	Oleic acid
PL	Phospholipid
RBCs	Red blood cells
sn-1 DHA	2,3-distearoyl-1-docosahexaenoyl-*sn* -glycerol
sn-2 DHA	1,3-distearoyl-2-docosahexaenoyl-*sn*-glycerol
sn-3 DHA	1,2-distearoyl-3-docosahexaenoyl-*sn*-glycerol
STA	Stearic acid
TAG	Triacylglycerol
TS	Tristearin
VLDL	Very-low-density lipoproteins
+/ −SD	+/ − standard deviation

LIST OF ORIGINAL PUBLICATIONS

I. Kulkarni A.; Zhao A.; Yang B.; Zhang Y.; Linderborg K.M. Tissue-specific content of polyunsaturated fatty acids in (n-3) deficiency state of rats. *Foods*, **2022**, 11(2), 208.

II. Linderborg K.M.*; Kulkarni A.*; Zhao A.; Zhang J.; Kallio H.; Magnusson J.D.; Haraldsson G.G.; Zhang Y.; Yang B. Bioavailability of docosahexaenoic acid 22:6(n-3) from enantiopure triacylglycerols and their regioisomeric counterpart in rats. *Food Chemistry*, **2019**, 283, 381; *Food Chem*istry, 411 (2023) 135430 (* Equal contribution).

III. Kulkarni A.; Linderborg K.M.; Zhao A.; Kallio H.; Haraldsson G. G.; Zhang Y.; Yang B. Influence of dietary triacylglycerol structure on accumulation of docosahexaenoic acid [22:6(n-3)] in rat organs. *Submitted manuscript*.

1 INTRODUCTION

Lipids are an essential part of the human diet. They not only supply energy but also provide nutrients like essential fatty acids (FAs) and lipophilic vitamins. Consumption of an appropriate amount of versatile dietary lipids leads to a healthy life and general well-being. However, a diet high in saturated fat increases the prevalence of heart disease, obesity, and other lifestyle-related diseases (Simopoulos, 2002). Thus, in addition to the amount of lipid intake, the quality of lipids consumed is crucial. Altogether, the reduced consumption of foods with saturated atherogenic lipids, especially lauric, myristic, and palmitic acids along with an increased intake of foods with unsaturated lipids can help to maintain a better quality of life. A higher intake of α-linolenic acid [ALA, 18:3(n-3)], long-chain (n-3) polyunsaturated FAs [LC (n-3) PUFAs] like eicosapentaenoic acid [EPA, 20:5(n-3)], and docosahexaenoic acid [DHA, 22:6(n-3)] maintain a lower (n-6) / (n-3) ratio (Meynier & Genot, 2017). Also, replacing saturated FAs with a mix of monounsaturated and polyunsaturated FAs in foods reduces low-density lipoprotein (LDL) cholesterol concentrations (EFSA NDA Panel, 2011).

Accumulated evidence gathered from the last five decades indicates that the ingestion of LC (n-3) PUFAs improves health and even contributes to preventing diseases (Calder, 2014; Swanson et al., 2012). In the body, ALA is the precursor FA of LC (n-3) PUFAs, including EPA and DHA. However, the conversion of ALA to DHA is limited (Brenna et al., 2009a; Burdge et al., 2002). The highly unsaturated LC (n-3) PUFAs are abundant in fish and other marine food sources. Therefore, the consumption of fish or fish oil in 2 to 3 servings of approximately 250 mg per day of EPA plus DHA is recommended by the EFSA NDA Panel (2010). Dietary supplements of LC (n-3) PUFAs are available in the form of triacylglycerols (TAG) (e.g., fish oil), ethyl esters (EEs), or phospholipids (PL) (e.g., krill oil) for those who wish to use them to supplement or replace fish consumption.

In the human diet, FAs are typically present in acylglycerols. TAGs are a major class of nonpolar lipids providing dietary FAs. The majority (≈ 98%) of LC (n-3) PUFAs in plant- and animal-based foods are found in the form of TAGs, followed by the polar lipids, mainly PLs. TAGs are chiral to a large extent and are metabolized in a chiral environment. In general, the seed oils are greatly enriched with PUFAs in the *sn*-2 position. LC (n-3) PUFAs, mainly EPA and DHA, are located predominately in the *sn*-2 position in fish and squid oil in general opposition to seal oil, in which they are located in the *sn*-1 and *sn*-3 positions (Ikeda et al., 1998; Mu & Høy, 2004). Indeed, the current knowledge on the effect of the positional distribution of the LC (n-3) PUFA on their

bioavailability lies in the comparison of fish oil and seal oil, i.e., FAs from the primary positions to the secondary position FA.

Nutritionally, the differences in the distribution of FAs in the 3 positions in the glycerol moiety in dietary TAGs have implications on further fat metabolism due to the positional specificity of the lipolytic enzymes. Hydrolysis of TAGs is performed by lipases by cleaving FAs from the *sn*-1 and *sn*-3 positions, while the FA at the *sn*-2 position is preserved and absorbed as monoacylglycerol (MAG). However, the digestion of FAs in the *sn*-1 and *sn*-3 positions is not equal. Lingual and gastric lipases cleave fatty acids at the *sn*-3 position faster than FAs at the *sn*-1 position (Duan, 2000; Hamosh, 1984). Pancreatic lipase hydrolyzes FAs in the *sn*-1 and *sn*-3 positions of TAGs. Porcine pancreatic lipase prefers the hydrolysis of FAs in the *sn*-3 position over FAs in the *sn*-1 position (Rogalska et al., 1990) and hydrolysis of diacylglycerol (DAG) over TAGs (Lykidis et al., 1995). Rat pancreatic lipase cleaves the ester bond from the *sn*-1 and *sn*-3 positions at equal rates (Paltauf et al., 1974), while, in contrast, dog pancreatic lipase shows a stereopreference for the *sn*-3 position in TAGs (Carrière et al., 1997). Pancreatic lipase shows a low activity towards (n-3) FAs, in particular over DHA, if located in the *sn*-3 position of TAG in whale oil under *in vitro* conditions (Bottino et al., 1967).

Evidence from the comparisons between fish oil and seal oil suggests efficient digestion, absorption, and metabolism of DHA from the *sn*-2 position as compared to the *sn*-1 and *sn*-3 positions (Christensen et al., 1995) and more assimilation of DHA from the *sn*-2 position in liver and brain (Bandarra et al., 2016) as well as in the brown adipose tissue (Lopes et al., 2017) of hamsters. However, this difference has never before been validated with TAGs with the enantiospecific positioning of DHA. Currently, very little is known about possible differences in the absorption or metabolic fate of DHA between the *sn*-1 and *sn*-3 positions. The lack of synthetic enantiospecific TAGs has hindered our understanding of the impact of the positional distribution of LC (n-3) PUFAs in dietary TAGs on the bioavailability of these valuable FAs, especially DHA, in a wide range of animal organs.

The research presented in this thesis investigated, for the first time, the effect of the enantiospecific location of dietary DHA in TAGs on FAs of rat organs. This study focused on the effect of (n-3) deficiency on the accumulation of (n-3) PUFAs in rat organs (**study I**), the effect of the structure of the dietary TAG on the bioavailability of DHA in rat plasma and feces as well as the impact on rat body weight and organ weight (**study II**), and the DHA accumulation in rat organs, including rat liver, brain, eye, kidney, testicle, and visceral fat (**study III**). To enhance the robustness of the results, the location of the other FAs was not altered, and the only difference between the three experimental lipids containing DHA was the location of the DHA. The review of the literature

introduces LC (n-3) PUFAs and the digestion and absorption of FAs and discusses the effect of (n-3) deficiency in animals and humans. Further, the effect of TAG structure on FA absorption in plasma, feces, and rat organs was reviewed.

2 REVIEW OF THE LITERATURE

2.1 Definition and structure of dietary lipids

Dietary lipids consist of a wide range of mostly non-polar organic molecules and thus contain FAs in various lipid molecules. TAGs comprise the majority of dietary fats yielding approximately 95 g of FAs from 100 g of TAG. In addition, dietary FAs are also derived from PLs and sterol esters.

TAGs consist of three FAs esterified to a glycerol backbone, and these FAs may be all different, two may be different, or all may be alike. The general chemical structure of a TAG is presented in **Figure 1 [A]**. The carbon atoms are stereospecifically numbered to designate the stereochemistry of the glycerol-containing components. The carbons are numbered 1, 2, and 3 from top to bottom when a Fischer projection is drawn with the middle hydroxyl group positioned to the left of the central carbon atom. The name of the compound with stereospecifically numbered molecules has the prefix '*sn*' immediately before the term 'glycerol' to differentiate them from conventionally numbered compounds. For example, when stearic acid (STA, 18:0) occupies positions *sn*-1 and *sn*-2 and oleic acid [OA, 18:1(n-9)] in the *sn*-3 position, the chiral acylglycerol is designated as 1,2-distearoyl-3-oleoyl-*sn*-glycerol. When two different FAs are attached to the terminal positions on the glycerol backbone, the molecule is chiral.

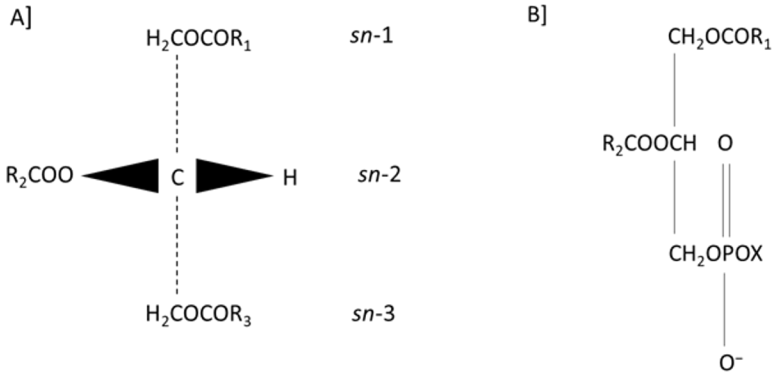


Figure 1. Chemical structures of A] triacylglycerol (TAG) where R_1, R_2, and R_3 represent different alkyl chains of FAs and B] phospholipid (PL) where X represents choline, ethanolamine, serine, or hexahydric alcohol. Adapted from Ratnayake & Galli 2009.

The glycerol backbone of a PL contains two esterified FAs at the *sn*-1 and *sn*-2 positions, while a phosphate group is esterified at the *sn*-3 position as shown in **Figure 1 [B]**. PLs are amphipathic lipids due to non-polar FAs forming a

hydrophobic head and a polar phosphate group, which contains phosphorus and a nitrogenous base or a sugar molecule, forming a hydrophilic tail. They are a primary component of cellular membranes. Although PLs constitute a small fraction of total fat in the diet, they can be important sources of essential FAs.

A FA is a carboxylic acid with an aliphatic chain and can be a saturated or unsaturated FA. A saturated FA contains a single bond in the hydrocarbon chain, whereas an unsaturated FA contains 1 or more double bonds. When a FA contains 1 double bond, it is termed a 'monounsaturated FA', while if it contains more than 1 double bond, it is called a 'polyunsaturated FA'. Unsaturated FAs can be named either by a systematic name, which indicates the double bond positions starting from the carboxyl-terminal of FA or omega-numbering, which indicates where the first double bond is positioned starting from the methyl terminal of FA. Almost all naturally occurring unsaturated FAs have double bonds in the *cis* configuration meaning that the hydrogen atoms attached to the double bonds are on the same sides. Whereas, if the hydrogen atoms are on the opposite side, it is called a *trans* configuration.

There is a wide spectrum of chain lengths ranging from 4-carbon FAs in dairy fat to 30-carbon FAs in some marine lipids. The FAO / WHO Expert Consultation classified saturated FAs as short-chain FAs that contain 3–7 carbons, medium-chain FAs with 8–13 carbons, long-chain FAs with 14–20 carbons, and very-long-chain FAs consisting of 21 carbons or more. Unsaturated FAs are divided into 3 groups according to chain lengths being short-chain unsaturated FAs of FAs with 19 or fewer carbon atoms, long-chain unsaturated FAs with 20–24 carbon atoms, and very-long unsaturated chain FAs being those with 25 or more carbon atoms (FAO/WHO, 2010).

2.2 Essential fatty acid metabolism

Both ALA and linoleic acid [LA, 18:2(n-6)] are considered nutritionally essential FAs and thus must originate from the diet. Almost all higher-order plants, algae, some fungi, and lower-order animals (e.g., *Caenorhabditis elegans*) possess Δ12-desaturases to convert OA into LA and Δ15-desaturases to convert LA to ALA (Ruiz-Lopez et al., 2015). However, mammals, including humans, lack the necessary enzymes, being Δ12 and Δ15 desaturases, to synthesize LA and ALA, and they are therefore considered 'essential' FAs for humans. Most of the crop seeds and vegetable oils, especially safflower (79% LA), sunflower (73% LA), corn (59% LA), and soybean (51% LA) oils, are good sources of LA. While walnuts (13% ALA) and oil plant seeds, including chia seeds (63% ALA), perilla oil (60% ALA), flaxseed oil (52% ALA), canola or rapeseed oil (8% ALA), and soybean oil (8% ALA) are rich in ALA (Abedi & Sahari, 2014; Gunstone et al., 2007). Green leafy vegetables, although low in fat, contain high proportions of

PUFAs at 60–70% of total FAs in the form of ALA (Kim et al., 2018; Saini et al., 2014, 2016).

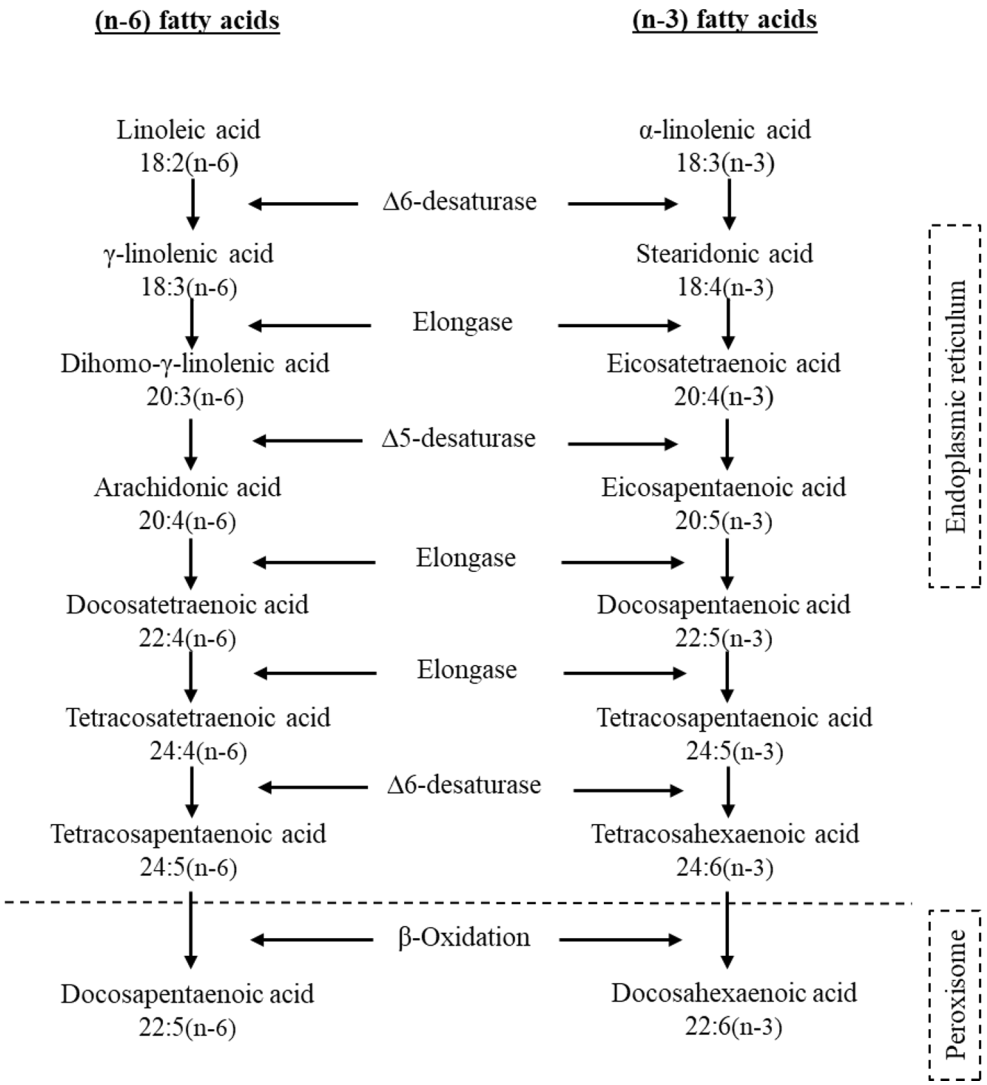


Figure 2. General overview of the (n−6) and (n−3) fatty acid elongation and desaturation pathway. Adapted from Schmitz & Ecker 2008.

These two families, (n-3) and (n-6), are defined by the position of the double bond closest to the methyl group of the FA, which emphasizes the metabolic relationship. Once ingested, the FAs of (n-3) and (n-6) families are not interconvertible because mammalian cells lack the converting enzyme being (n-3) desaturase. These two classes of essential FA are metabolically and functionally distinct, and their balance is important for good health and normal development (Schmitz & Ecker, 2008).

In animals, LA and ALA are used as such or elongated and desaturated to more highly unsaturated FAs of their respective families (**Figure 2**). The biosynthesis of PUFA starts with Δ6 desaturation by adding a double bond at the 6th C-C bond position from the -COOH end of LA and ALA that generates γ-linolenic acid [18:3(n-6)] and stearidonic acid [18:4(n-3)], respectively. Δ6 desaturation is a rate-limiting step of the pathway. Furthermore, the first part of this pathway to arachidonic acid [ARA, 20:4(n-6)] and EPA occurs by sequential steps of elongation and desaturation catalyzed by FA elongase and Δ5-desaturases, respectively. Although there is no cross reaction between (n-6) and (n-3) pathways, both pathways use the same desaturation and elongation enzymes and there is competition between LA and ALA for these enzymes. In mammals, the mechanism of final conversion to docosapentaenoic acid [DPAn-6, 22:5(n-6)] and DHA, 20 carbon FAs (ARA and EPA) undergo two successive elongation cycles to yield tetracosatetraenoic acid [24:4(n-6)] and tetracosapentaenoic acid [24:5(n-3)] and one Δ6 desaturation step to yield tetracosapentaenoic acid [24:5(n-6)] and tetracosahexaenoic acid [24:6(n-3)], respectively (D'andrea et al., 2002). These 24 carbon PUFAs are then subjected to β-oxidation in the peroxisome, where its chain is shortened by two carbons to produce the final products, DPAn-6 and DHA (Abedi & Sahari, 2014). The conversion of DPA to DHA via the peroxisomal pathway is called the 'Sprecher pathway' named after its discovery in the rat liver (Voss et al., 1991).

2.2.1 Digestion of dietary lipids

The digestion of ingested fat takes place in both the stomach and the intestine. Digestion is the process of breaking down large molecules from food into smaller components that are absorbed by the lining of the gastrointestinal tract. The digestive process requires coordinated gastric, intestinal, biliary, and pancreatic functions. Dietary fats are mainly composed of TAGs that contain various long-chain saturated and unsaturated FAs as well as a small proportion of short- and medium-chain FAs. TAGs are hydrophobic molecules and cannot be transferred in their intact form to the major diffusion barriers in the intestine, namely the unstirred water layer and the polar cell membrane (Westergaard & Dietschy, 1974). Therefore, enzymes in the gastrointestinal tract are needed to hydrolyze ester bonds and release FAs from dietary TAGs to increase solubilization.

In general, the entire fat digestion and absorption process lasts 16–24 hours if food is not consumed after the initial meal (Bisgaier & Glickman, 1983). It can vary depending on the type and amount of food consumed. In the chewing process, food mixed with saliva leads to changes in its pH, ionic strength, and temperature. Food may be acted on by the digestive enzyme, lingual lipase, secreted from the glands of the tongue called von Ebner's glands that disperses the food fat and increases the food surface area (De Hoog et al., 2006; Malone et

al., 2003a, 2003b; Silletti et al., 2007; Vingerhoeds et al., 2005). Typically, a portion of food or a beverage spends 5 to 20 seconds in the mouth before being swallowed (Sanz & Luyten, 2006). A food bolus is formed and swallowed into the stomach. The optimal pH for lingual lipase is around 4, but it is active at a pH of 6.0–6.5, and thus is also able to work in the stomach until the pH in the stomach decreases (Liao et al., 1984). In the first step of the digestion of TAG, partial hydrolysis into DAG and free FAs (FFAs) occurs in the stomach by gastric lipase, also called lingual lipase in rats. DeNigris et al. (1988) showed lingual and gastric lipase activity after incubation of homogenates of the lingual serous glands and gastric serous glands of rats, mice, rabbits, guinea pigs, and baboons, and also of the human gastric mucosa. The hydrolysis of TAGs by lingual lipase was predominant in rats and mice, whereas the lipolytic activity of gastric lipase was mainly seen in guinea pigs, rats, baboons, and humans.

Gastric lipase is secreted by the chief cells of the fundic mucosa of the stomach and is active at a pH range of 3.0–6.0 allowing the enzyme to also function in the duodenum (Abrams et al., 1988). Gastric lipolysis leads to the hydrolysis of 5–40% of ingested TAGs (Armand et al., 1994, 1996, 1999) generating mainly FFA and DAGs. When dietary lipids arrive in the stomach, they are emulsified creating a larger oil-water interface that enables the lipases to interact with water-insoluble lipid droplets (Armand et al., 1999; Favé et al., 2004). By analyzing the hydrolyzation products of the gastric sample in humans, Carriere et al. (1993) showed that gastric lipase hydrolyzed about 10% of FAs of an ingested test meal in the stomach. Gastric digestion results in short- and medium-chain FFAs that can be directly absorbed in the intestinal tract and transported directly to the liver through the portal vein. When radiolabeled FFAs of different chain lengths were injected into the rat stomach, the recovery of short- and medium-chained FAs in lymph was found to be lower than that of long-chain FAs indicating that the major portion of short- and medium-chained FAs were transported via the blood stream from its site of absorption (Bloom et al., 1951). Gastric lipase stimulates the emulsification of lipids. The formation of hydrolysis products increases the solubilization of TAGs, the binding of co-lipase, and the release of long-chain FFAs that stimulate the secretion of cholecystokinin (Armand, 2007) that facilitates fat digestion in the duodenum. However, the bulk of lipid digestion, in children and adults, occurs in the small intestine, with pancreatic lipase being the only significant source of fat-digesting lipase. In newborns, the concentration of pancreatic triglyceride lipase and bile salts is lower compared to later in life. Instead, the pancreatic triglyceride lipase-related protein 2 and bile salt-stimulated lipase in coordinated action with gastric lipase perform fat breakdown during infancy (Susanne Lindquist 2010). As the baby is weaned onto solid food, digestion occurs in the small intestine in the later

phase of life and remains the primary site of digestion into adulthood (Gurr M.I., 2009).

The partially digested food that leaves the stomach and enters the small intestine is generally referred to as 'chyme'. The entry of emulsified acylglycerols into the duodenum causes the release of cholecystokinin, gall bladder emptying, and pancreatic lipase secretion. Fats are further emulsified by bile acids, which can be released from the gall bladder or directly from the liver, resulting in an increase in the available surface for pancreatic lipase activity. The effective action of pancreatic lipase in the presence of high bile salts is co-lipase-dependent (Armand, 2007). Co-lipase binds to pancreatic lipase enabling it to bind the bile salt-covered TAG substrate (Erlanson-Albertsson, 1992). However, the presence of bile salts can promote or inhibit the activity of pancreatic lipase depending on their concentration (Bauer et al., 2005; Lowe, 2002). The pH of the gastric contents was maximum after 1 hour at a pH 4.0 and decreased regularly during the 4-hour digestion period, whereas the pH range of duodenal contents was 6.0–7.0 during the 4-hour digestion period. The synergistic actions of gastric and pancreatic lipase with bile salts lead to the formation of FFAs and 2-MAGs as previously studied by Armand et al. (1996). They incubated pancreatic lipase *in vitro* with a racemic mixture of radiolabeled alkyl diacylglycerols containing either a ^{3}H-labelled 1-alkyl moiety or ^{14}C-labelled 3-alkyl moiety and analyzed the hydrolyzation products. In the duodenum, pancreatic lipase is responsible for the hydrolysis of 40–70% of TAGs (Armand et al., 1996, 1999; Carriere et al., 1993), and gastric lipase accounts for an additional 7.5% of lipolysis (Carriere et al., 1993).

2.2.2 Absorption and transport of lipids

The mixed micelles in the small intestinal lumen promote the absorption of FAs. Once the lipid digestion products have been transported across the 'unstirred layer' adjacent to the brush border membranes, they are absorbed by the intestinal enterocytes. The micelle particle facilitates passage across a diffusion barrier that is located at the intestinal lumen-membrane interface for the uptake by the enterocytes. Uptake and transport of FAs across enterocytes can be via a protein-independent diffusion model or a protein-dependent mechanism (Mansbach & Gorelick, 2007). A protein-mediated FA uptake system is considered to be the dominant means by which FAs are taken up by membrane-associated FA-binding proteins, i.e., FA transporters on the apical membrane of enterocytes (Schwenk et al., 2010). An electron microscopic study of intestinal fat absorption was conducted by Strauss (1966). The microscopic monitoring of micelles after incubation of the hamster gut segments *in vitro* in a solution of mixed bile salt micelles showed that the uptake of micellar lipids occurs by means of diffusion. However, another *in vitro* study in rats using ^{14}C-labeled LA

showed that the predominant mechanism of absorption at a low lipid concentration is facilitated and passive diffusion at a high lipid concentration of absorbed labeled LA (Chow & Hollander, 1979). Stremmel (1988) suggested a carrier protein-mediated FA absorption when addition of certain antibody inhibited the uptake of ^{3}H-oleate in isolated rat mucosal cells and *in vivo* jejunal segment systems.

Short-chain- and medium-chain-FAs, those that are less than 12 carbons, are absorbed through the gut wall as individual FAs. They pass directly through enterocyte cells and are transported to the liver through the portal vein, where they are rapidly metabolized by β-oxidation (Aoyama et al., 2007). In contrast, other fat digestion products, e.g., 2-MAGs, long-chain (≥ 12 carbon) FAs, are reassembled into TAGs mainly through the MAG pathway (Córsico et al., 2004; Storch & Thumser, 2000). TAGs are also synthesized by a longer pathway of acylation of glycerol-3-phosphate if the supply of 2-MAGs is insufficient (Favé et al., 2004). The assembly of synthesized TAGs takes place in the endoplasmic reticulum of the enterocyte cells, packed into chylomicrons, and then transported into the systemic circulation *via* the lymph, thereby bypassing the liver (Bauer et al., 2005; Bermúdez et al., 2004; Favé et al., 2004).

The water-immiscible lipids are transported to the predominantly aqueous environment of the blood by lipoproteins. Lipoprotein particles have a hydrophilic surface consisting of apolipoproteins and hydrophilic ends of PLs and unesterified cholesterol, whereas the lipoprotein core is hydrophobic consisting of TAGs, esterified cholesterol, and fat-soluble vitamins. Lipoproteins are classified mainly by their density into chylomicrons, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL, and high-density lipoproteins (HDL). Furthermore, lipoproteins vary in their composition and structure (Segrest et al., 1994). The lipoprotein particles that are assembled in the enterocytes during fat absorption are called chylomicrons.

In the postprandial state, chylomicrons provide lipids to tissues in the exogenous pathway. Chylomicrons are assembled from apolipoproteins and absorbed resynthesized dietary lipids in enterocytes. They are secreted into the lymphatic capillaries of the intestinal villi. The lymph carries the chylomicrons to the bloodstream from where they are released into the circulation for peripheral tissue (Gurr M.I., 2009). The chylomicron TAGs are hydrolyzed by lipoprotein lipase in extra hepatic tissues and form chylomicron remnants, which are rich in cholesterol esters and removed from the plasma by the liver via LDL receptors, hepatic receptors, and the LDL receptor-related protein (Cooper, 1997). About 70–90% of the TAG is removed by the lipoprotein lipase, and the chylomicron remnant contains approximately 70% TAG, 6–7% CE, and 5–7% apolipoproteins (Jeffery & Redgrave, 1982). In the fasting state, lipids are transported to tissues in the endogenous pathway by VLDL instead of

chylomicrons (Stryer et al., 2019). TAGs are resynthesized from the FAs originating from the breakdown of chylomicron remnants by hepatic lipase in the liver and packed into VLDL, which transfers TAGs into peripheral tissues. Lipoprotein lipase is responsible for the hydrolysis of TAG-rich VLDL into denser IDL particles, which are further converted into a LDL particle that contains mostly cholesterol esters. LDL transfers cholesterol esters to peripheral tissues, whereas HDL transfers cholesterol from peripheral tissues to the liver (Mu & Høy, 2004).

2.2.3 β- oxidation and *de novo* synthesis of fatty acids

β-oxidation releases a significant amount of metabolic energy for tissues. In mammalian cells, both peroxisomes and mitochondria contain a β-oxidative pathway. Short-, medium-, and long-chain FAs are metabolized in mitochondria, which is the primary site of β-oxidation, and very-long-chain (>22 carbons) FAs are metabolized by peroxisomal β-oxidation. In yeasts and plants, FA oxidation occurs uniquely in peroxisomes (Smith et al., 2000), as mitochondria are not able to catabolize FAs. Mitochondrial β-oxidation provides acetyl-coenzyme A (acetyl CoA) in two separate pathways depending on the tissue where oxidation occurs. In skeletal and cardiac muscle, mitochondrial FA oxidation produces acetyl CoA and provides ATP for myocytes. Whereas, in hepatocytes, it provides acetyl CoA for ketone body synthesis and ATP during prolonged fasting conditions (Talley & Mohiuddin, 2022). In addition to producing energy, the carbon in acyl-CoA generated by β-oxidation of FAs can be recycled and used in the synthesis of FAs *de novo*. This may be an important process in pregnant and fetal monkeys (Greiner et al., 1996) and rats (Cunnane et al., 1994, 2003). The biosynthesis of LC PUFA was investigated in neonatal rats by monitoring the enrichment of ^{13}C labels, as 16- and 18-carbon PUFAs, using nuclear magnetic resonance (NMR) spectroscopy *in vivo*. The labeled carbons were found to be utilized in the conversion of LA to ARA and of ALA to DHA and also in the *de novo* lipid synthesis of lipids, such as cholesterol for the rat brain. Thus, in addition to elongation and desaturation to LC PUFAs, LA and ALA are partitioned to β-oxidation to be used as carbon sources in lipid synthesis (Cunnane et al., 1994). The storage of lipids in adipose tissue is preferred over β-oxidation in the postprandial state. This is facilitated by the insulin-dependent increase in lipoprotein lipase activity. Hormone-sensitive lipase releases non-esterified FAs from adipose tissue-derived TAGs in the fasting state (Burdge & Calder, 2005).

De novo synthesis of FAs is the synthetic process by which the excess dietary carbohydrates from the circulation are converted into FAs, which can further be converted into TAGs or other lipids (Song et al., 2018). *De novo* FA synthesis is regulated by two important enzymes, acetyl-CoA carboxylase and FA synthase

(Wallace & Metallo, 2020). The acetyl CoA carboxylase enzyme introduces a carboxyl group to acetyl CoA forming malonyl-CoA. Then, the enzyme fatty-acid synthase converts malonyl-CoA into the FA chain. The liver and adipose tissues are the two main sites for the production of FAs in the body. FAs synthesized in the liver are exported through lipoprotein production providing an energy source for peripheral tissues. In adipose tissue, *de novo* synthesis of FAs typically contributes to *in situ* fat deposition and long-term energy storage as TAGs (Nguyen et al., 2008). When carbohydrate intake is more than the energy requirement, the conversion of carbohydrates to FAs occurs, and this takes place in the liver. Strawford et al. (2004) showed that *de novo* synthesis contributes to 20% of new TAGs in adipose tissue of healthy humans.

2.2.4 Conversion of ALA to long-chain (n-3) polyunsaturated fatty acids

The metabolism of (n-3) FAs depends on (n-6) FAs due to the competition of (n-3) and (n-6) FAs for the same enzymes and transport systems. A large increase in the (n-6) / (n-3) ratio from 1:1 during evolution to 20:1 or even higher has been observed in the western diet during the past years (Simopoulos, 2016). A high LA intake interferes with the desaturation and elongation of ALA (Emken et al., 1988; Hagve & Christophersen, 1984). Early studies of rat liver microsomes showed that desaturation of ALA to 18:4(n-3) was inhibited by LA, and, conversely, LA conversion to 18:3(n-6) was inhibited by ALA (Brenner & Peluffo, 1966). However, Holman (1998) showed that ALA is a much stronger suppressor of (n-6) FA elongation and desaturation than LA is of (n-3) FA elongation and desaturation. In fact, 10 times more LA is required to have an equal effect on (n-3) metabolism as ALA does on LA elongation.

Although humans and animals can convert ALA to LC (n-3) PUFAs, the efficiency appears to be low especially for DHA. Tissue compositional studies in animals have demonstrated that diets rich in ALA lead to an increase in ALA in most tissues, an increase in DPA to a smaller extent, but did not increase DHA levels (Abedin et al., 1999; R. A. Bowen et al., 1999; R. A. Bowen & Clandinin, 2000; R. A. R. Bowen & Clandinin, 2005; Fu & Sinclair, 2000; Woods et al., 1996). The distribution of deuterated ALA and its metabolites in rodents showed that 16% of dietary ALA was accumulated in tissues, 6% was converted to LC PUFA, and the remaining 78% was catabolized or excreted (Lin & Salem, 2007). The outcome of most human feeding studies with ALA was similar to that of animal studies. Increased dietary intake of ALA increased the level of EPA, but no significant change was observed in the DHA level of human plasma (Bloedon et al., 2008; de Groot et al., 2004; Francois et al., 2003; Goyens et al., 2006; James et al., 2003; Kwon et al., 1991; Li et al., 1999). The conversion of ALA to LC (n-3) PUFAs appeared to be more efficient in infants than in adults. When

ALA was added to infant formulas containing the same LA levels and no LC (n-3) PUFAs, Jensen et al. (1996a) observed an increase in DHA of PLs and red blood cells (RBCs) at the highest level of ALA used, while Clark et al. (1992) found a 38% increase in total lipid DHA with a similar increase in ALA. A greater activity of the elongation/desaturation of ALA in women compared to men has been discussed by Pawlosky et al. (2003) possibly due to the importance of meeting the demand of the fetus and neonate for DHA. Further, about a 15% higher plasma DHA concentration was observed in women than men that may be due to estrogenic effects (Giltay et al., 2004). Similarly, a significantly higher DHA concentration was measured in plasma phosphatidylcholine (31%), triacylglycerol (71%), and non-esterified FA (33%) in women compared to men (Bakewell et al., 2006).

The efficiency of conversion of ALA to LC (n-3) PUFAs is an important nutritional aspect, because there is evidence that an enhanced EPA and DHA status in blood and tissues is important for optimal health. However, the limited potential to convert ALA to EPA and further conversion to DHA is shown in stable isotope tracer studies. ALA metabolism derived from plasma concentration curves in healthy men showed that the efficiency of conversion of ALA to EPA is 0.2%, of EPA to DPA is 65%, and of DPA to DHA is 37%. The overall efficiency of conversion of ALA to EPA is 0.2%, to n-3 DPA is 0.13%, and 0.05% to DHA (Pawlosky et al., 2001). Non-human primates showed an ALA to DHA conversion at 0.23% (Su et al., 1999) and 0.57% of the ^{13}C-ALA dose (Su et al., 2001). A steady-state radiotracer kinetic model indicated that approximately less than 0.2% of ALA entering the brain undergoes conversion to DHA (Demar et al., 2005). However, studies reported a significant increase in plasma DHA levels with ALA intake for a long-term period. Increased plasma levels of EPA and DHA with a decrease in ARA in plasma PLs were reported with 30 ml of flaxseed oil providing approximately 15 g of ALA for 4 weeks in healthy volunteers (Mest et al., 1983). The effect of long-term ALA intake in elderly subjects showed an increase of 21% in DHA when the daily intake of ALA increased to 3 g by substituting 55–60% of ALA of perilla oil for 7% of ALA of soy oil. DHA levels returned to the baseline DHA level at 3 months when the source of (n-3) PUFA was changed back to soy oil (Ezaki et al., 1999). These observations suggest that blood levels of DHA can be improved by the long-term intake of vegetable oils containing ALA and less LA. However, the increase in DHA is not immediate and may not be as effective as consuming preformed dietary DHA, e.g., from fish or fish oil supplements (Arterburn et al., 2006; Mori et al., 2000; Park & Harris, 2002; Woodman et al., 2002).

2.3 Long-chain (n-3) polyunsaturated fatty acids

2.3.1 Effect of LC (n-3) PUFA on health

LC (n-3) PUFAs, especially EPA and DHA, are associated with numerous health benefits. DHA is found in abundance in the human brain being approximately 5 g and 15–20% of total FAs and is in the visual elements of the retina (Innis, 2008). It is vital for the development of the fetal brain and retina and also their functioning throughout life (Lacombe et al., 2018). Maternal nutrition during pregnancy provides nutrients, including DHA, through the placenta to the fetus. Supplementation with EPA and DHA during pregnancy demonstrated better problem-solving skills (Judge et al., 2007) in infants at 9 months and better eye and hand coordination (Dunstan et al., 2008) in children at the age of 2.5 years compared to the infants and children born to mothers who did not receive DHA and EPA during pregnancy. Alzheimer's disease patients have lower serum cholesteryl ester-DHA levels than healthy people (Tully et al., 2003), and a LC (n-3) PUFA-rich diet is associated with a reduced risk of Alzheimer's disease (Ajith, 2018). Several DHA-derived mediators, such as neuroprotectins, resolvins, and maresins are produced in the brain, and they play an important role in protecting against injury, ischemia, and inflammation. LC (n-3) PUFAs have been suggested to exert an antidepressant effect, and alterations of these FAs are associated with various psychiatric disorders and neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease (Avallone et al., 2019).

In dietary interventions, LC (n-3) PUFAs have had a beneficial effect on several risk factors of cardiovascular disease. Consumption of a high OA canola oil enriched with DHA provided by algal oil improved the concentrations of HDL cholesterol and TAGs as well as blood pressure thus reducing the risk of coronary heart disease in a multicenter, randomized controlled trial (Jones et al., 2014). DHA exerts antiplatelet aggregation, a TAG-lowering effect, and an antiarrhythmic effect (Kang & Leaf, 1996). DHA and EPA have been reported to reduce the risk of cardiovascular disease by altering lipid and hemostatic factors such as reducing platelet aggregation and prolonging bleeding time (Uauy & Valenzuela, 2000). DHA and EPA can reduce inflammatory and atherogenic gene expression (Bouwens et al., 2009). An increased intake of LC (n-3) PUFAs has been associated with a reduced risk of cardiovascular morbidity and mortality (Calder, 2004). Supplementation of these FAs has also been used in non-cardiovascular conditions, such as rheumatoid arthritis (Navarini et al., 2017) and inflammatory bowel diseases (Marton et al., 2019).

An increase in cell and tissue LC (n-3) PUFA content can impact a variety of cell function mechanisms in the body. EPA and DHA are incorporated into many parts of the body including cell membranes, and they play an important role in

maintaining the structure and function of the cell membranes. Alterations in the levels of LC (n-3) PUFAs can influence the membrane fluidity and the activity of membrane proteins including receptors, transporters, ion channels, and signaling enzymes (Calder, 2014). It can also lead to changes in the pattern of lipid mediators produced (Calder, 2008; Lacombe et al., 2018). LC PUFAs are precursors of lipid mediators, eicosanoids, and docosanoids, which are involved in cell signaling pathways and gene expression. Eicosanoids derived from (n-3)-series FAs, i.e., EPA, are considered anti-inflammatory, whereas lipid mediators derived from a (n-6)-series FA, being ARA, are related to inflammatory functions (Calder, 2010). Docosanoids, such as resolvins, are synthesized from FAs with 22 carbons, especially from DHA (Dennis & Norris, 2015).

2.3.2 DHA intake recommendations

The principal (n-3) FAs being ALA, EPA, and DHA are important for optimal health and are associated with numerous physiological functions. However, due to the limited synthesis of EPA and DHA from the parent FA ALA, EPA and DHA should be consumed from food (Swanson et al., 2012). The recognition of numerous health benefits of LC (n-3) PUFAs and especially DHA has resulted in a series of recommendations by health authorities in order to increase the intake of DHA and EPA. There have been many recommendations set for different population groups, which range from 250 mg to 2 g per day of EPA + DHA (FAO/WHO, 2008). Recommendations for the minimum intake level for LC (n-3) PUFAs have been generally made for a specific set of populations, such as pregnant and lactating women, and these recommendations often account for combined EPA + DHA intake instead of only DHA.

According to the European Food Safety Authority (EFSA), a daily intake of 100 mg of DHA is recommended for infants and young children under the age of 24 months and 250 mg of EPA + DHA for adults and children over the age of 2 years (EFSA NDA Panel, 2014). An additional 100–200 mg of daily intake of DHA is recommended for women during pregnancy and lactation (EFSA, 2017). The Food and Agriculture Organization (FAO) recommends 250 mg/day for adults and 300 mg/day for pregnant and lactating women with a combined intake of EPA + DHA containing a minimum of 200 mg of DHA (FAO, 2010). The Nordic Nutrition Recommendations suggest a daily intake of 200 mg of DHA for pregnant and lactating women (Nordic Council of Ministers, 2012).

The National Health and Medical Research Council of Australia recommends 500 mg of daily intake of LC (n-3) PUFAs for healthy adults, while the American Heart Association recommendation for patients with coronary heart disease is 1 g of EPA + DHA daily. For patients with elevated plasma TAG levels, the recommendations range from 2 to 4 g of EPA + DHA per day (McKenney & Sica, 2007). The European Society of Cardiology recommends 1 g of EPA +

DHA for the secondary prevention of coronary heart disease (Van de Werf et al., 2003).

The FAO sets the highest recommended intake of EPA + DHA at 2 g per day suggesting that a higher intake may also be safe when consumed as seafood. However, a high consumption of LC (n-3) PUFAs as supplements is considered to carry a potential health risk due to increased lipid peroxidation and reduced cytokine production (FAO, 2010). Consumption of oxidized DHA and EPA appears to affect the beneficial effects of LC (n-3) PUFAs on certain blood markers. In a randomized controlled trial lasting 30 days, the less oxidized fish oil supplement reduced the level of circulating cholesterol as well as the systolic and diastolic blood pressure of the subjects compared to the more oxidized fish oil supplement, which did not influence blood pressure (García-Hernández et al., 2013). However, levels of blood cholesterol were increased (García-Hernández et al., 2013). Due to limited available data, the tolerable upper-intake levels for EPA and DHA have not been set by the EFSA. However, for the general population, the EFSA suggests that, daily, 1 g of DHA or 1.8 g of EPA supplementation, taken separately, is safe as well as up to 5 g/day combined intake of supplemental EPA + DHA (EFSA NDA panel, 2012). An intake level of 1.2 g of DHA per day during pregnancy did not show any adverse effects in meta-analyses of RCTs (Horvath et al., 2007; Szajewska et al., 2006).

2.4 Bioavailability of long-chain (n-3) polyunsaturated fatty acids

The term ‘bioavailability’ that refers to nutrition loosely describes how much of a given nutrient administered orally is then actually retained in the body after its digestion and absorption. In pharmacology, this term refers to the measurement of the rate and extent to which a drug reaches systemic circulation (Kwan, 1997). The term bioavailability for nutrition should be better defined and standardized due to its increased use in nutrition science, specifically for LC (n-3) PUFAs (Ghasemifard et al., 2014). The rate of digestion, absorption, excretion, and retention of LC (n-3) PUFAs in tissues, which is the bioavailability of LC (n-3) PUFAs, is highly variable and can be determined by factors such as the chemical binding forms (FFAs bound in EEs, TAGs, or PLs) (Schuchardt & Hahn, 2013).

The LC (n-3) PUFAs in dietary supplements are commonly available in the TAG form that are typically derived from fish or algae oil. At the upper concentration of LC (n-3) PUFAs, the FAs are cleaved and transformed into EEs. The EEs may be used to form new TAGs, or they may be included in the supplements as such. The bioavailability of DHA and EPA in the EE form has been determined to be lower than the bioavailability of DHA and EPA in the TAG form in previous, short-term postprandial studies, especially from low-fat

meals (Boustani et al., 1987; Lawson & Hughes, 1988a, 1988b). The animal studies, which have compared different forms of LC (n-3) PUFAs, also found the EE form to be less bioavailable than the TAG form (Yang et al., 1990) and the MAG form (Valenzuela et al., 2005). Lymphatic recovery of DHA at 24 hours was comparable between TAG and FFA, while it was significantly lower in the EE form (Ikeda et al., 1995). However, Hamazaki et al. (1987) reported an equal efficiency of EPA-EE and EPA-TAG to increase EPA levels in rat plasma PLs and cholesterol esters. During fat digestion, pancreatic lipase hydrolyzes the TAG resulting in two FFAs and a MAG allowing them to be easily absorbed. Whereas an additional hydrolysis step might be required for the LC (n-3) PUFAs in EE form to yield the DHA and/or EPA in the FFA form before they can be incorporated into chylomicrons for absorption (Li et al., 2021). In addition, *in vitro* studies have reported that the LC (n-3) PUFAs in the EE form are up to 50 times more resistant to hydrolysis by pancreatic lipase than LC (n-3) PUFAs in the natural TAG form (Beckermann et al., 1990; Grimsgaard et al., 1998).

Dietary fat consists predominantly of TAGs with a small portion being contributed by PLs at 3% to 6% (Ramírez et al., 2001). PL versus TAG studies have generally shown a higher incorporation of LC (n-3) PUFAs into tissues from PLs compared to the TAG form. Lemaitre-Delaunay et al. (1999) showed that the lipid form, being phosphatidylcholine versus TAG, markedly affected the kinetics and metabolic fate of DHA in a 72-hour postprandial study in humans. However, Destaillats et al. (2018) did not observe significant differences in DHA levels in neural tissues of adult rats after 60 days of feeding when DHA was provided as TAG or PL. Krill oil contains a significant portion of LC (n-3) PUFAs in PL and FFA forms, and it has been reported to have a higher absorption rate and a higher DHA accretion rate in plasma than fish oil, where DHA is mainly in the TAG form (Ghasemifard et al., 2015; Kitson et al., 2016; Rossmeisl et al., 2012). Ulven et al. (2011) reported no significant differences between krill oil and fish oil in the levels of DHA and EPA of the plasma and serum lipids. Since subjects in the krill oil group received 62.8% of the total LC (n-3) PUFAs received by subjects in the fish oil group, these findings indicate that the bioavailability of DHA from krill oil, mainly as PL, was as efficient, and possibly more so, as DHA from fish oil as TAG. On the other hand, lower DHA digestibility and the DHA accretion in the rat brain fed with krill oil were observed as compared to salmon and tuna oil even though the krill oil contained 2 to 4 times higher DHA than other oils (Tou et al., 2011). Krill oil has been reported to have different proportions of PLs (from 34% to 58%), FFAs (from 6% to 22%), and TAGs (from 23% to 50%) in several studies (Damerau et al., 2020; Schuchardt et al., 2011; Tandy et al., 2009; Winther et al., 2011).

Inconsistencies in the results could be associated with variations in the composition of krill oil, the forms of PL, i.e., phosphatidylethanolamine and

phosphatidylserine, with different contents of EPA and DHA; the doses; duration of the intervention; and different detection methods (Li et al., 2021). Detailed information on the lipid composition of krill oil used in the study would help determine how the availability of EPA and DHA differs due to the relative proportions of PLs, TAGs, and FFAs.

2.5 (n-3) deficiency status

In recent years, a low (n-3) PUFA status has been reported in humans due to the dietary shift towards more solid fats and more animal-based foods (Simopoulos, 2002, 2009). Seafood is an excellent source of (n-3) PUFAs, but the consumption of seafood varies greatly between regions and cultures and is generally lower than recommended. Reports on the global dietary intakes of LC (n-3) PUFAs estimated that less than 20% of the world population receives (n-3) PUFAs from seafood in quantities equal to or greater than 250 mg/day (Micha et al., 2014). The reliability of the EPA + DHA intake database can differ across countries based on the accurate and timely estimation of the EPA + DHA intake as well as the challenges in reporting the errors in the collection of the dietary data.

In a systematic review article by Stark et al. (2016), information from 298 studies conducted globally on blood levels of (n-3) PUFA in healthy adults was collected from prospective cohorts, case-control studies, and randomized trials. The investigation revealed that blood levels of EPA and DHA are low or very low in most of the world. The collected data were extracted from plasma total lipids, plasma PLs, and erythrocytes, and very limited data were available from the whole blood. EPA + DHA levels were considered very low if they were under 4%, low at 4–6%, moderate at 6–8%, and high if they were more than 8% of plasma erythrocyte equivalents. These categories were applied to the data of blood fractions to convert them to erythrocyte equivalents. Based on the (n-3) index which was established based on EPA + DHA level of RBCs, the authors prepared a map that illustrates different levels of EPA + DHA in blood globally. The map showed that low and very low levels of EPA + DHA are typical in areas with a western diet, while regions with a less westernized diet and a strong seafood-based culture were associated with moderate or high levels of EPA + DHA in the blood. An erythrocyte or whole-blood EPA + DHA level of less than and/or equal to 4% of total FAs may be considered a ‘state of deficiency’, as these levels increase the risk of cardiac death (Harris & Von Schacky, 2004) and have been found in patients with psychiatric disorders (McNamara et al., 2010, 2014, 2015; Pottala et al., 2012).

(n-3) deficiency has also been investigated in various animal organs by analyzing the composition of FAs in tissues. (n-3) deficiency has also resulted in several physiological effects in both animals and humans. In attempts to study

the effect of depletion of (n-3) PUFAs, especially DHA, in mammals, (n-3) deficiency has been induced in animal models (e.g., rodents, guinea pigs, and non-human primates) in different generations by dietary manipulation. A first-generation (n-3), FA-deficient animal model is generally created to evaluate the impact of an essential nutrient-deficient diet, post-weaning, in offspring born to dams who are maintained on an essential nutrient adequate diet throughout pregnancy. Achieving a severe deficiency of LC (n-3) PUFAs, particularly DHA, is difficult because LC (n-3) PUFAs, including DHA, are provided during the perinatal period and through the mother's milk during the suckling period, and DHA is efficiently maintained in the brain. Thus, second-generation model studies, in which dams are fed a diet nearly devoid of (n-3) FA sources and their offspring have been also maintained on the (n-3) FA-deficient diet, have been carried out. It takes more than two generations to reduce brain DHA by more than 80% in rats (Contreras et al., 2001; Greiner et al., 2001). Even third-generation deficiency studies have been conducted (Ahmad et al., 2002; Bourre, et al., 1989; Contreras et al., 2001), but conducting second- and third-generation studies may be limited by time and resources. Although second- or third-generational animal studies are necessary to cause a significant depletion of brain DHA leading to a loss of neural function, the multigenerational deficiency approach has the limitation in that it poorly mimics real-life human situations.

2.5.1 In rodents

In response to (n-3) PUFA deprivation, several physiological and biochemical consequences have been reported in rodents. Elevated anxiety, aggression, an increased immobility time, and vulnerability to stress have also been reported in rodents fed a low (n-3) PUFA diet as reviewed by Fedorova & Salem (2006). The biochemical and functional outcomes of (n-3) deficiency in rodents, guinea pigs, and non-human primates are listed in **Table 1**.

The first-generation (n-3) PUFA-deficient rat model exhibited a 36% reduction of DHA compared with a group kept on a diet adequate in (n-3) PUFAs in brain PLs after male rat pups at weaning were subjected to a diet deficient for 15 weeks. Simultaneously, DPAn-6 was elevated by 90%. (n-3) deficiency resulted also in increased depression and aggression in rats (DeMar et al., 2006). In another study, 15 weeks of (n-3) PUFA deprivation, starting after weaning at 21 days, reduced plasma DHA by 89% and brain DHA by 37% compared to the (n-3)-adequate diet, and these DHA concentrations did not change thereafter (DeMar et al., 2004). Dietary (n-3) PUFA deprivation for 15 weeks decreased DHA concentrations in the rat frontal cortex and was suggested to increase the susceptibility to bipolar disorder due to alterations in the enzyme expression (Rao et al., 2007). Igarashi et al. (2007a) reported a more than 90% reduction in

ALA and DHA levels in arterial plasma and liver caused by (n-3) deprivation in rats for 15 weeks, while the concentrations of ARA and DPAn-6 increased. Also, (n-3) deprivation upregulated liver conversion of ALA to DHA in rats but not in rat brain (Igarashi et al., 2007a, 2007b). Plasma DHA was significantly reduced at 1.7% of dietary ALA feeding for 15 weeks compared to the control diet containing 4.6% ALA, however, brain DHA remained unchanged (Kim et al., 2011). PUFAs contributing to tissue composition of rats were studied by Blanchard et al. (2013) in a (n-3) FA-deficient control group and two experimental groups either enriched with ALA or LA for 8 weeks. The ALA-deficient groups, being the control and LA-enriched group, had lower DHA levels in the brain and heart compared to the ALA-enriched group, whereas the LA-enriched group had lower DHA in blood, liver, and heart compared to the other groups at the end of intervention. It showed that a moderate increase in ALA intake and a parallel decrease in LA intake can lead to significant variations in the metabolism and tissue (n-3) and (n-6) PUFA content in rats.

Table 1. Animal studies of (n-3) deficiency emphasizing tissue biochemical and/ or functional outcomes.

	Reference	*Experimental*	*Control*	*Study design*	*Study samples*	*Outcome*
Rodents						
1	Tinoco *et al.* 1978	LA-ME	LA-ME + ALA-ME	Second generation	Liver, brain, kidney, spleen, heart, muscle, gastrointestinal tract, lung, ovary, testis, adrenal, plasma, erythrocytes, retina, and adipose tissue	B: DHA in all analyzed organs, brain, heart, muscle, retina, and liver were greatly reduced.
2	Bourre *et al.* 1989a	Sunflower oil	Soybean oil	Fourth generation	Lung, testis, brain cells, kidney, retina, and liver	B: Reduced DHA in studied organs. A switch in diet for 10 weeks from a sunflower oil-based diet to soybean oil-based diet in 60-day-old rats did not reach brain DHA levels the same as rats fed with soybean oil from the beginning.
3	Bourre *et al.* 1989b	9 groups containing different LA and ALA proportions		Third and following generations	Heart, lung, testis, brain cells, retina, muscle, liver, and kidney	B: Reduced DHA in studied organs. F: Anomalies in the electroretinogram and effects on learning tasks and showed an earlier mortality in response to an intraperitoneal injection.
4	Bourre *et al.* 1992	Rapeseed and peanut oil (RP diet, 220 mg / 100 g of ALA)	Sunflower oil (2 groups with 6 and 30 mg / 100 g of ALA)	Third generation	Brain, nerve endings, myelin, liver, heart, and testes	B: No difference in whole brain, myelin, or nerve endings at 31 weeks. DHA progressively declined in the liver, heart, and testes for up to 3 weeks and stabilized.

	Reference	Experimental	Control	Study design	Study samples	Outcome
5	Contreras *et al.* 2000	Coconut oil + safflower oil	Coconut oil + safflower oil + flaxseed oil	Third generation	Brain	B: DHA turnover in brain PL was reduced (30 to 80%), and DHA concentration was reduced by 90%.
6	Salem *et al.* 2001	Coconut oil + safflower oil	Coconut oil + safflower oil + flaxseed oil	Second (G2) and third (G3) generation	Brain	B: Reduced DHA by 76% and increased DPAn-6 by 47-fold. F: Poorer performance in spatial tasks (G2) and an olfactory-cued reversal learning task (G3).
7	Greiner *et al.* 2001	Coconut oil + safflower oil	Coconut oil + safflower oil + flaxseed oil	Second generation	Brain and olfactory bulb	B: 81% less brain DHA and 82% less DHA in the olfactory bulb. F: More errors in a series of olfactory-cued, 2-odor discrimination tasks.
8	Weisinger *et al.* 2002	Coconut oil + safflower oil	Coconut oil + safflower oil + flaxseed oil	Third generation	Retina	B: 55% reduced retinal DHA. F: Reduced retinal sensitivity.
9	Levant *et al.* 2004	Sunflower oil	Soybean oil	Second generation	Brain	B: 80% reduction in brain DHA at maturity. F: Catalepsy induced by haloperidol in deficient animals was 49% of that observed in control animals. In a test of locomotor activity, deficient animals exhibited 187% of the activity of control animals.
10	DeMar *et al.* 2004	Safflower oil	Safflower oil + flaxseed oil	First generation	Brain and plasma	B: Reduced plasma DHA by 89%, brain DHA by 37%, half-life of DHA was prolonged 2-fold or greater, and reduced calculated rates of DHA loss from brain.
11	DeMar *et al*. 2006	Safflower oil	Safflower oil + flaxseed oil	First generation	Brain	B: 36% of DHA reduction in brain PLs, DPAn-6 was elevated by 90%. F: Increased depression and aggression.

	Reference	Experimental	Control	Study design	Study samples	Outcome
12	Rao *et al.* 2007	Coconut and safflower oil	Coconut oil + safflower oil + flaxseed oil	First generation	Brain	B: Decreased DHA levels in the frontal cortex and an increased half-life of DHA within the brain.
13	Igarashi *et al.* 2007a	Safflower oil	Safflower oil + flaxseed oil	First generation	Plasma and liver	B: >90% reduction in ALA and DHA levels in arterial plasma and liver.
14	Igarashi *et al.* 2007b	Safflower oil	Safflower oil + flaxseed oil	First generation	Brain and liver	B: Upregulated conversion of ALA to DHA in liver but not in brain.
15	Kim *et al.* 2011	6 groups: Safflower oil + flaxseed oil (4.6%, 3.8%, 2.6%, 1.7%, 0.8%, and 0.2% ALA)		First generation	Brain, liver and plasma	B: Plasma DHA fell significantly at 1.7% dietary ALA, while brain DHA remained unchanged down to 0.8% ALA.
16	Blanchard *et al.* 2013	3 groups containing olive, rapeseed, corn, and flaxseed oil, respectively. (Control: 33.9:30:7.7:0; ALA: 37.3:22.7:5.5:17; LA: 27.3:28:27.8:0)		First generation	Red blood cells, liver, brain, and heart	B: Control and LA groups led to lower DHA levels in the brain and heart, the LA group led to lower DHA in blood, liver, and heart compared to remaining groups.
17	Bondi *et al.* 2014	Safflower oil	Safflower oil + flaxseed oil	First (G1) and second (G2) generation	Brain	B: Higher loss of brain DHA in G2 compared to G1 F: A modality-selective and task-dependent impairment in cognitive and motivated behavior. Elevated state of anxiety and hyperactivity in G1 than in G2 rats.
Guinea pigs						
18	Leat *et al.* 1986	Purified LA	Purified ALA	Third generation	Retina	B: Retinal DHA reduced from 8 to < 0.5%.
19	Weisinger *et al.* 1996a	Safflower oil	3 groups: Fish oil; canola oil; and laboratory chow	Third generation	Retina	F: Manipulation of the retinal DHA level accounted for 21–35% of the electroretinographic variability.

	Reference	*Experimental*	*Control*	*Study design*	*Study samples*	*Outcome*
20	Weisinger *et al.* 1996b	Safflower oil	Canola oil	Third generation	Retina	B: Reduced retinal PL DHA in the safflower oil group (2.5%) compared to the canola oil group (21%). F: Reductions in both peak-to-peak and a-waves of safflower oil-fed animals.
21	Weisinger *et al.* 1998	Safflower oil	Canola oil	First generation	Retina	B: Reduced retinal DHA in the safflower oil group (4.5%) compared to the canola oil group (20%). Approximately 90% of the loss occurred in the first 12 weeks compared to next 12 weeks of feeding.
22	Weisinger *et al.* 1999	Safflower oil	Canola oil	First generation	Retina	B: Reduced DHA in the safflower oil group animals was 42% of the canola oil group at 16 weeks. F: Significant losses in b- and a-waves after 6 and 16 weeks of dietary n-3 deficiency. The response amplitude to a 30-Hz flicker was reduced 42% after 16 weeks. Functional deficits were reversed after 10 weeks of dietary (n-3) repletion.
23	Abedin *et al.* 1999	Safflower oil	4 groups: Canola oil; mix vegetable oil; and mix vegetable oil + ARA (0.9% and 2.7%) and DHA (0.6% and 1.8%)	First generation	Brain, retina, liver, and heart	B: Lower DHA in the brain, retina, liver, and heart after 12 weeks in the safflower oil group. Dietary supplementation of DHA and ARA led to a pronounced increase in the tissue DHA compared to only supplementation with ALA.

	Reference	Experimental	Control	Study design	Study samples	Outcome
Non-human primates						
24	Neuringer *et al.* 1984	Safflower oil	Soybean oil	Prenatal and postnatal (n-3) deficiency	Retina and plasma	B: Plasma PL DHA reduced from 42% of control values at birth to 21% at 4 weeks, 9% at 8 weeks, and 6% at 12 weeks of age. F: Visual acuity was reduced by one-fourth at 4 weeks and by one-half at 8 and 12 weeks.
25	Neuringer *et al.* 1986	Safflower oil	Soybean oil	Prenatal and postnatal (n-3) deficiency	Retina and brain	B: DHA levels doubled in soybean oil diet-fed animals, whereas the safflower oil diet-fed animals failed to increase the DHA at 22 months of age. F: Subnormal visual acuity at 4 to 12 weeks of age and prolonged recovery time of the dark-adapted electroretinogram.
26	Reisbick *et al.* 1990	Safflower oil	Soybean oil	Prenatal and postnatal (n-3) deficiency	RBCs	B: Reduced (n-3) FA levels of RBCs in the safflower oil group. F: Home-cage drinking from the spout more frequently than controls and drank more water over 24 hours. The increase in intake was mirrored by an increased combined output of urine + feces over 24 hours.
27	Reisbick *et al.* 1991	Safflower oil	Soybean oil	Prenatal and postnatal (n-3) deficiency	RBCs	B: Reduced (n-3) FA levels of RBCs in the safflower oil group. F: Deficient monkeys drank at least twice as much of all NaCl concentrations as controls. Overall intake decreased as salt concentration increased.
28	Reisbick *et al.* 1992	Safflower oil	Soybean oil	Postnatal (n-3) deficiency	RBCs	B: Reduced (n-3) FA levels of RBCs in safflower oil group. F: Water intake over 24 hours, water intake in 15-minute tests, and excretion of combined urine and feces over 24 hours were all about twice as great in postnatal as in prenatal monkeys.

	Reference	*Experimental*	*Control*	*Study design*	*Study samples*	*Outcome*
29	Reisbick *et al.* 1994	2 groups: Safflower oil (2 – 3 y males and 4 – 5 y males and females)	2 groups: Soybean oil (2 – 3 y males and 4 – 5 y males and females)	Prenatal and postnatal (n-3) deficiency	RBCs	B: Reduced (n-3) FA levels of RBCs in the safflower oil group. F: Initiated more bouts of stereotyped behavior in their home cages, more frequent locomotion bouts, and both stereotyped behavior and the sum of all behavioral bouts were more frequent in 4 – 5-year-old than in 2 – 3-year-old monkeys.
30	Jeffrey *et al.* 2002	Safflower oil + peanut oil	2 groups: Soybean oil and DHA group (blend of lard, coconut, palm, corn, soybean and tuna oils)	First generation	Retina and RBCs	B: Elevated DHA level of RBCs in the DHA group (8.8% ± 0.7% of total fatty acids, mean ± SD), almost absent in the safflower group (0.3% ± 0.1%), and intermediate in the soybean group (1.8% ± 0.6%) F: A low ALA diet resulted in a 30% delayed rod recovery and a 5% delayed electroretinogram implicit compared to control groups.
31	Grayson *et al.* 2014	Safflower oil + peanut oil	2 groups: Soybean oil and DHA group (blend plant, animal, and fish oils)	Prenatal and postnatal (n-3) deficiency	Brain	F: Impairment in the resting-state functional connectivity among prefrontal cortical networks in the low (n-3) FA diet compared to the DHA group.

F, functional outcome; B, biochemical outcome; LA, linoleic acid; ME, methyl ester; ALA, linolenic acid; RBCs, red blood cells DHA, docosahexaenoic acid; ARA, arachidonic acid; DPAn-6, docosahexaenoic acid (n-6).

In a second-generation rodent study, brain DHA was reduced by 76% in (n-3)-deficient rats, and DPAn-6 was increased by 47-fold. The loss of DHA in the brain was associated with poorer performance in spatial tasks and an olfactory-cued reversal learning task (Salem et al., 2001). The adolescent rat model involving consecutive generations of (n-3) PUFA deficiency resulted in a greater loss of brain DHA in the second-generation compared to the first-generation deficiency model producing modality-selective and task-dependent impairment in cognitive and motivated behavior (Bondi et al., 2014). This study indicated an elevated state of anxiety and hyperactivity in the second-generation- compared to first-generation (n-3)-deficient adolescent rats. Tinoco et al. (1978) analyzed the lipid composition of the liver, brain, kidney, spleen, heart, muscle, gastrointestinal tract, lung, ovary, testis, adrenal, plasma, erythrocytes, retina, and adipose tissue of second-generation (n-3)-deficient rats and reported that the concentrations of DHA in brain, heart, muscle, retina, and liver were greatly affected due to the (n-3) deprivation as compared to other organs. Adult rats fed a diet containing only sunflower oil, which is low in (n-3) FAs, did not affect the DHA content in the whole brain, myelin, or nerve endings at 31 weeks. On the contrary, DHA progressively decreased in the liver, heart, and testes for up to 3 weeks and remained nearly stable thereafter. In this study, rats were fed a diet containing sufficient amounts of both LA and ALA for two generations and 60-day old adult rats from the third generation were subjected to (n-3) deprivation (Bourre et al., 1992). (n-3) deprivation throughout two generations led to 81% less brain DHA in rats resulting in a defect in two-odor olfactory discrimination tasks and olfactory-cued set learning (Greiner et al., 2001). Similarly, rat pups born to dams fed with a (n-3)-deficient diet, post-breeding, and raised on a (n-3)-deficient diet from birth until weaning reduced brain DHA to 80% of that of control animals at maturity (Levant et al., 2004). This study demonstrated that rats exposed to a (n-3)-deficient diet from conception produced alterations in adult behavior indicative of altered dopaminergic function. A decline in DHA level was reported in heart, lung, testis, brain cells, retina, muscle, liver, and kidney (Bourre, Francois, et al., 1989) and lung, testis, brain cells, kidney, retina, and liver (Bourre, Durand, et al., 1989) in third- or fourth-generation rats of fed a diet deficient in (n-3) FAs compared to a diet adequate in (n-3) FAs. Rats deficient in (n-3) FAs from the third generation exhibited significantly reduced retinal sensitivity and increased -wave implicit times due to a 55% of reduction in retinal DHA compared to rats fed a (n-3)-adequate diet (Weisinger et al., 2002). Another study examined DHA concentrations, incorporation, and turnover rates in brains of rats subjected to a dietary deficiency of ALA for three generations and showed that DHA turnover in brain PLs was markedly reduced by 30–80%, and the DHA concentration was reduced by 90% together with elevated levels of DPAn-6 and ARA compensation for the loss of DHA (Contreras et al., 2000).

However, in contrast, ARA turnover within brain PLs was not significantly changed by chronic (n-3) PUFA deprivation (Contreras et al., 2001).

An artificial rearing approach was established to develop a first-generation (n-3) deficiency (Hoshiba, 1986, 1996) in rodents using hand-feeding techniques with custom-designed nursing milk bottles (Hoshiba, 2004). This system was based on the use of the natural suckling behavior of rat pups together with a bottle-nipple system that can control the resistance required for milk flow. This system can be used to feed artificial milk to rats within 12 hours of birth. Lim et al. (2005) adapted the artificial rearing method and showed that a (n-3) PUFA-deficient diet feeding starting from postnatal day 2 through 15 weeks of age induced a 58% reduction in brain DHA and a 55% reduction in retinal DHA compared to a (n-3)-adequate diet in rats. This study demonstrated a loss of behavioral performance in spatial learning-related brain function due to a low intake of (n-3) FA in rats. Artificially reared rat pups fed a (n-3)-deficient formula exhibited a loss of more than 70% in the brain DHA and approximately 69% lower retinal DHA levels by 29 days of age (Moriguchi et al., 2004). The decrease in liver DHA was found to be more pronounced in the (n-3)-deficient group compared to the (n-3)-adequate group over the course of 29 days. Hussein et al. (2009) reported the application of artificial rearing in mouse nutrition for the first time. The result of a restriction of (n-3) FA intake during postnatal development of mouse pups showed markedly lower levels of the brain, retinal, liver, plasma, and heart DHA at 20 weeks of age with replacement by DPAn-6, ARA, and docosatetraenoic acid [DTA, 22:4 (n-6)]. The artificial rearing method of rodents is employed at an earlier stage in the developmental period of life as compared to first- or multi-generation animal studies that begin at weaning or afterward. The artificial rearing approach achieved more than 50% of depletion in brain DHA in the first generation-deficiency model and about 90% of reduction in brain DHA in the second-generation model (Ward et al., 1996). However, it eliminates many of the maternal interactions experienced by naturally reared pups, and the lack of these interactions may have profound effects on brain and behavioral development. Also, the substitute formula may be lacking other milk borne constituents, which may play a role in the promotion of normal development (Motouri et al., 2003; Philipps et al., 1997).

2.5.2 In guinea pigs

In order to access the (n-3) PUFA deficiency and its physiological and biochemical consequences, a (n-3)-deficiency model in guinea pigs was developed. Guinea pigs were chosen over other rodents because the timing of the guinea pig retinal growth spurt is similar to that in humans. Retinal development can be classified as prenatal, perinatal, or postnatal concerning the major retinal growth spurt. Precocial mammals such as humans, primates, and guinea pigs

have both pre- and postnatal retinal growth spurts (Dobbing & Sands, 1970; Huang et al., 1990). In contrast, altricial species, such as rats and dogs, have the greater part of retinal development occurring after birth (Dobbing & Sands, 1970). As a result of these differences in retinogenesis, the development of retinal function has been reported to be more similar in humans and guinea pigs than it is in rats (Graur et al., 1991; Huang et al., 1990; Li et al., 1992).

The effect of a variable amount of dietary ALA was found to be directly proportional to the retinal DHA content, which is consistent with the earlier work of Leat et al. (1986) resulting in 21 to 35% of electroretinogram variability in third-generation guinea pigs (Weisinger et al., 1996b). (n-3) PUFA deprivation over three generations led to significant reductions in retinal DHA (2.5%) in guinea pigs reared on safflower oil lacking (n-3) FAs compared to retinal DHA (21.0%) in animals fed canola oil containing (n-3) FAs. Safflower oil-fed animals showed significant reductions in both peak-to-peak and α-wave amplitude implying dysfunction at the photoreceptor level (Weisinger et al., 1996a). On the other hand, a first-generational study reported 4.5% of retinal DHA in the safflower oil-fed group compared to 20% of retinal DHA in the canola oil-fed group in infant guinea pigs. The results indicate that the rate of loss of DHA during the first 12 weeks of feeding was greater than that of the second 12 weeks, and approximately 90% of the loss occurred in the first 12 weeks suggesting that retinal PUFA levels in guinea pigs are readily altered by manipulation of the supply of ALA in the early weeks of life. The study also showed that an adequately supplied guinea pig mother is capable of providing the pup with enough (n–3) FAs to withstand a longer period of dietary deprivation imposed after weaning (Weisinger et al., 1998). The functional deficits in the electroretinogram were observed at 6 and 16 weeks of dietary (n-3) deficiency in first-generation guinea pigs (Weisinger et al., 1999). Low dietary ALA levels resulted in significantly lower DHA in the brain, retina, liver, and heart after 12 weeks of the (n-3)-deficient diet compared to the (n-3)-adequate diet in a single generation guinea pig study (Abedin et al., 1999).

To summarize, a substantial depletion of DHA in the retina was shown in over three successive generations of guinea pig models. However, dietary depletion over successive generations is time-consuming, expensive, and unlikely to be relevant to human nutrition.

2.5.3 In non-human primates

The neural processing of information along the visual pathways of human and rhesus monkeys is generally similar, and thus Harwerth & Smith (1985) suggested that the monkey is an excellent model for the human visual system.

Dietary (n-3) deficiency during gestation and infancy showed a depletion of (n-3) PUFAs from plasma lipids, and this depletion was associated with a

significant impairment in the development of visual acuity. In plasma PLs, DHA levels progressively decreased, falling from 42% of control values at birth to 21% at 4 weeks, 9% at 8 weeks, and 6% at 12 weeks of age (Neuringer et al., 1984). Later, Neuringer et al. (1986) examined the alterations in the FA composition of the retina and brain of rhesus monkeys produced due to (n-3) deprivation in different fetal and postnatal development. The safflower diet, which is low in (n-3) FAs, showed an 80% reduction in retinal DHA in the major membrane PLs compared to monkeys fed the soybean diet at 2 years of age (e.g., 7.3% ± 1.6% vs. 36.0% ± 2.4% of total FAs in phosphatidylethanolamine, mean ± SEM, respectively). A low level of DHA was accompanied by a compensatory increase in DPAn-6. Functionally, the depletion of retinal DHA resulted in an impairment in the development of visual acuity and delayed recovery of the dark-adapted electroretinogram. The occipital cortex is the primary visual processing lobe of the mammalian brain, and the lower DHA levels of the occipital cortex could also be associated with the effect on visual acuity. Adult rhesus monkeys receiving a low ALA diet resulted in a 30% delayed rod recovery and a 5% delayed electroretinogram compared to groups receiving 8% ALA or 0.6% DHA (Jeffrey et al., 2002). Retinal DHA levels, in term and preterm baboons after feeding formula lacking long-chain polyunsaturates, were significantly lower leading to electroretinogram abnormalities compared to the full-term breast-fed and preterm formulas that were supplemented with long-chain polyunsaturates fed baboons (Diau et al., 2003). Furthermore, impairment in the resting-state functional connectivity among prefrontal cortical networks was reported in monkeys raised on a (n-3) FA-deficient diet compared with monkeys raised on a diet fortified with fish oil (Grayson et al., 2014).

Reisbick et al. (1990) reported that the prenatal and postnatal (n-3) deficiency resulted in polydipsia where (n-3)-deficient monkeys drank more water as controls over 24 hours resulting in an increased output of urine and feces. Also, Reisbick et al. (1991) showed that (n-3)-deficient monkeys drank at least twice as much of all given NaCl concentrations as controls. They suggested that the changes in fluid intake and output may be related to direct or indirect effects on central or peripheral control mechanisms for drinking or excretion, which may be mediated by the altered composition of neural or other membranes or changes in eicosanoid metabolism. These studies demonstrated increased fluid intake in rhesus monkeys exposed to combined prenatal and long-term postnatal dietary (n-3) PUFA deficiency. Later, Reisbick et al. (1992) showed postnatal (n-3) deficiency by itself is sufficient for the effects. Perinatal (n-3) FA deficiency associated with increased home-cage stereotypy and locomotion bouts was shown by Reisbick et al. (1994). Anderson et al. (2005) reported that (n-3) deficiency limited to the prenatal period appeared to have no lasting effects on the FA composition of most tissues in rhesus monkeys after three postnatal years

on a control diet adequate in (n-3) FAs. Total (n-3) FA content of the monkeys fed with a (n-3)-deficient diet was reduced by 70–90% in plasma, erythrocytes, and tissues at birth but recovered to control values within 4 weeks in plasma, 8 weeks in erythrocytes, and 15 weeks in the cerebral cortex. The brain PL FA composition was normal at 3 years of age. However, retinal DHA recovery was 84% of controls possibly due to substantial but incomplete initial (n-3) deficiency, both biochemically and functionally.

2.5.4 In humans

Human medical research should only be conducted using strict ethical rules. Human studies cannot reduce the nutrient intake of a study group to zero and are thus limited to supplementation trials. Naturally, also multigenerational studies to induce nutrient deficiency are impossible (Brenna, 2011). Thus, the low LC (n-3) PUFA status in human plasma, serum, and chylomicrons has been considered a risk factor for possible health diseases and disorders. Globally, the EPA + DHA blood levels agree with the previous assessments of dietary intake of (n-3) PUFA from seafood (Micha et al., 2014), however, the reliability of EPA + DHA intake estimates is limited by various factors such as the accurate and timely food composition data across countries and the challenge of reporting errors in the collection of dietary data. The FA composition of blood does not have this limitation, and thus blood level targets can be better linked to specific chronic disease outcomes and events (Stark et al., 2016).

In the early 1990s, a study by Uauy et al. (1990) suggested that the retinal function varies with the supply of dietary (n-3) FAs in very-low-birth-weight human infants. They reported that infants receiving (n-3)-deficient formula had significantly lower rod electroretinogram amplitude compared to infants who were breastfed or received a (n-3) FA-rich formula. Another study found a reduction in rod α-wave amplitudes of preterm, very-low-birth-weight infants fed a low ALA corn oil-based infant formula. This study also showed differences in rod β-wave amplitude, threshold, and sensitivity. The electroretinogram changes were present at 36 weeks of gestational age, near term, but were no longer present at 57 weeks of gestational age or 4-month post-term (Birch et al. 1992). On the other hand, fish oil supplementation in pregnant mothers for 15 weeks of pregnancy until delivery did not have a significant effect on their newborn infant electroretinogram compared to the placebo, oleic acid-based diets. However, infant cord blood DHA was correlated with sensitivity, but not amplitude, of the ERG β-wave (Malcolm et al., 2003). In humans and primates, preterm delivery is associated with deficits in cortical gray matter maturation, neurocognitive deficits particularly in the realm of attention, and an increased risk of attention-deficit/hyperactivity disorder (ADHD) and schizophrenia (McNamara & Carlson, 2006) due to perinatal deficits in brain DHA accrual.

The cause of the differences in the blood FA profiles in children and adults with ADHD is unclear, and it could be due to the low intake of LC (n-3) PUFAs and/or due to LC (n-3) PUFA metabolism in ADHD. Stevens et al. (1995) reported significantly lower levels of ARA and DHA, and approximately 40% excessive thirst and skin problems in 6- to 12-year-old boys with ADHD than matched controls, as excessive thirst and skin problems are a classic sign of FA deficiency that describe (n-3)-deficient animals. Adult, college-age students with ADHD exhibited skin and thirst symptoms classically associated with essential FA deficiency and altered plasma and RBC FA profiles. The ADHD group had 53% less DHA in plasma PLs and 36% less DHA in red blood cells compared to controls (Antalis et al., 2006). Similar results of plasma fatty acid analysis using gas chromatography (GC) were shown by Chen et al. (2004), where Taiwanese children with ADHD had significantly lower DHA at 1.35% area under the curve compared to DHA in control subjects with 2.08% area under the curve in the PL of RBCs. However, the plasma DHA levels did not differ among both groups. As shown in all these studies, Stevens et al. (1996) showed that low (n-3) FA levels of plasma PLs in young boys were associated with learning and behavioral difficulties, such as hyperactive-impulsive behavior, anxiety, temper tantrums, and symptoms of conduct disorder. Several clinical trials reported in a recent review (Chang & Su, 2020) showed controversial results of supplementation of (n-3) PUFAs in children with ADHD. While some clinical trials showed an improvement in clinical symptoms, e.g., hyperactivity/impulsivity and cognitive performance, others did not find beneficial effects.

A habitual diet low in LC (n-3) PUFAs has been found to be associated with increased prevalence rates of depressive symptoms in adolescents (Allen et al., 2013). The composition of FA in the erythrocyte membranes of 217 adolescents with eating disorders showed a significantly lower proportion of DHA in depressed participants (4.32 ± 1.24) compared to DHA in non-depressed (5.14 ± 1.07) participants (Swenne et al., 2011). A shift away from fish-based to western diets in Arctic communities was associated with increased rates of seasonal affective disorder, depression, suicide, and cardiovascular disease (McGrath-Hanna et al., 2003). Cross-sectional epidemiological surveys in the general population of Finland show an inverse correlation between dietary LC (n-3) PUFA intake and the prevalence of suicidal ideation (Tanskanen et al., 2001), and a study from Belgium showed that seasonal variations in serum LC (n-3) PUFA levels coincide with the seasonal variations in violent suicide rates (De Vriese et al., 2004). The relation between plasma levels of FAs and aggression, studied by Buydens-Branchey et al. (2003), showed less DHA (16.7 ± 2.7 ng/ml) in patients with an aggressive history compared with DHA in a non-aggressive behavior history (26.9 ± 7.6 ng/ml). The study suggested that the

patient's diet before hospitalization was not optimal by being particularly deficient in (n-3) FAs and indicated a possible link between (n-3) deficiency and aggression in humans.

Previously, epidemiological studies and clinical trials assessed the impact of supplementation or dietary intake of (n-3) PUFAs on neurodegenerative diseases such as Parkinson's and Alzheimer's diseases. In a double-blind, placebo-controlled trial of 6 months, participants with mild to moderate Parkinson's disease were randomly assigned to treatment (800 mg/d DHA and 290 mg/d EPA) or placebo being an equicaloric amount of corn oil. The treatment had no statistically significant effect on the rate of change on either the Unified Parkinson's Disease Rating Scale, i.e., non-motor and motor experiences of daily living and motor complications, or the Hoehn-Yahr Scale score, i.e., progression of Parkinson's disease and level of disability. However, 75% of DHA-treated patients showed a reduced Hamilton Rating Scale for Depression total score, i.e., severity of symptoms of depression, by at least 50% compared with only 25% of patients in the placebo group (Pomponi et al., 2014). Another double-blind, placebo-controlled study analyzed the effect of fish oil supplementation being fish oil capsules containing 180 mg EPA, 120 mg DHA, and tocopherol for 3 months in Parkinson's patients. The graduation of Parkinson's symptoms measured by the Hoehn and Yahr scale did not show a significant variation during the 3 months of supplementation among treated and control groups (da Silva et al., 2008). The effect of (n-3) FA supplementation on participants with mild to moderate Alzheimer's diseases was evaluated by Freund-Levi et al. (2006). The randomized, double-blind, placebo-controlled clinical trial showed that the supplementation of daily (n-3) FAs at 1720 mg DHA and 600 mg EPA for 6 months did not show significant differences in an Alzheimer's disease assessment scale-cognitive subscale, mini-mental state examination, and clinical dementia rating compared to the control group receiving 4000 mg of corn oil that contained 2400 mg LA. The continuation of the same study showed no marked effects on neuropsychiatric symptoms (Freund-Levi et al., 2008). Despite many randomized trials failing to document a definite protective effect against diseases, (n-3) FAs were generally reported to be safe and well-tolerated, and (n-3) FAs supplementation has to take place in the early stages of the disease when a benefit may be detected (Avallone et al., 2019).

2.6 Effect of triacylglycerol structure on fatty acids absorption

2.6.1 Role of stereospecificity of lipases

The first step of TAG digestion is the partial hydrolysis into DAG and FFA, which takes place in the stomach and is performed by gastric lipase and lingual lipase. Both these lipases preferentially hydrolyze the *sn*-3 ester bond forming *sn*-1,2 DAG (Hamosh & Scow, 1973). The enzyme activity of these lipases towards the *sn*-3 ester bond is twice as high as towards the *sn*-1 ester bond (Paltauf et al., 1974). Lingual lipase from rat tongue was incubated *in vitro* with a radiolabeled racemic mixture of alkyl diacylglycerols containing a radiolabeled alkyl moiety at the *sn*-1 and *sn*-3 positions. The specificity towards the acyl group located at the *sn*-3 position was revealed using thin-layer chromatographic fractionation and radioactivity determination of extracted lipids from samples taken at time intervals. Lingual lipase incubated in gastric aspirates from human infants hydrolyzed *sn*-3 ester bonds of synthetic TAGs about 4 times faster than the *sn*-1 ester bonds of the same TAGs (Jensen et al., 1982). A similar preference was also shown by Paltauf et al. (Paltauf et al., 1974). After the desired period of incubation, the digestion products were separated by thin-layer chromatography, FAs were identified by the GC, and stereospecificity was determined by optical rotation analysis. The stereospecificity of gastric lipase, *in vivo*, during the digestion of a liquid test meal, and, *in vitro*, under simulated physiological conditions, was investigated in dogs by using prochiral triolein and a racemic mixture of diolein (Carrière et al., 1997). High-performance liquid chromatography (HPLC) lipid analysis of hydrolysates confirmed that gastric lipase prefers the *sn*-3 position over the *sn*-1 position of TAG; however, gastric lipase also hydrolyzed oleic acid [OA, 18:1 (n-1)] from the *sn*-1 position of diolein. Additionally, the activity of preduodenal lipases, being lingual and gastric, towards short-chain, medium-chain, and long-chain FAs was different. Both these preduodenal lipases preferentially attack short- and medium-chain FAs in the *sn*-3 position of the TAG (Duan, 2000; Hamosh, 1984; Jensen et al., 1982) resulting in the formation of *sn*-1,2-DAGs. The lipolytic activity of pure rabbit gastric lipase towards medium-chain TAG was three times higher than that towards long-chain TAGs (Borel et al., 1994).

Digestion of milk lipid by preduodenal lipases and milk bile salt stimulated lipase in the suckling dog showed the release of long-chain unsaturated FAs (18:1, LA, and ALA) from primary positions preferentially from the *sn*-3 position (Iverson et al., 1991). This was further supported by studying the positional specificity of gastric hydrolysis of LC (n-3) PUFAs of seal milk-derived TAGs *in vivo* in suckling pups of the California sea lion, the harp seal, and the hooded seal by analyzing lipids from the ingested milk samples collected

at time intervals from stomachs of the seals. Positional analysis of milk-derived TAG of all three species based on the Grignard hydrolysis showed that PUFAs, including DHA, were preferentially esterified at the primary positions of the TAG, and FAs located at the *sn*-2 position were not digested by gastric lipase. Thus, an ample amount of *sn*-1,2-DAGs, as a product of gastric digestion over *sn*-2-MAGs, was seen as a result of the stereospecificity of the lipase towards the *sn*-3 position of dietary TAGs (Iverson et al., 1992). In the milk-derived TAG of other species, for example, in rats, medium-chain FAs are primarily esterified at the *sn*-3 position and thus may substantially contribute to their hydrolysis by lingual lipase (Staggers et al., 1981).

The activity of pancreatic lipase has been extensively studied under *in vitro* conditions. Bottino et al. (1967) reported that lipase has a low activity towards (n-3) FAs in whale oil, in particular EPA and DHA, when located in the *sn*-3 position. Whereas DPA, which is also present at the *sn*-1 and *sn*-3 positions, was not lipase resistant. However, later observations by Ikeda et al. (1995) showed that the presence of EPA and DHA at the *sn*-1 and *sn*-3 positions of TAGs does not result in their lower recovery in lymph and that slow lymphatic recovery results from the delayed formation of TAG in the intestinal mucosa cells eventually caused by the reduced supply of *sn*-2 MAG. *In vitro* incubation of pancreatic lipase with racemic mixtures of alkyl diacylglycerols, *sn*-1,2-DAG and *sn*-2,3-DAG labeled with either a ^{3}H in the alkyl moiety or with ^{14}C in both acyl groups, were studied by Paltauf et al. (1974) to determine lipolytic actions of pancreatic lipase. Radioactivity analysis of TLC-fractionated hydrolyzation products showed that pancreatic lipase hydrolyzes ester bonds at the *sn*-1,3 positions of TAG at equal rates producing FFAs and *sn*-2-MAG. Rogalska et al. (1990) showed that HPLC separation of the diastereomeric carbamate derivatives of the diglycerides released from trioctanoin and triolein hydrolysis by porcine pancreatic lipase demonstrated the existence of a stereopreference for the *sn*-3 position. Later, the stereospecificity of dog pancreatic lipase was investigated and confirmed by Carrière et al. (1997) *in vitro* and *in vivo*. They incubated triolein and a racemic mixture of diolein and simulated the gastric conditions of hydrolysis by gastric lipase following the hydrolysis with pancreatic lipase under simulated duodenal conditions. HPLC analysis of extracted lipids suggested that pancreatic lipase has specificity towards the *sn*-3 position of TAG in addition to the *sn*-1 position of DAG.

Previous *in vitro* and *in vivo* studies on the specificities in actions of lipases towards FAs esterified to dietary and lipoprotein TAGs are summarized in **Table 2**.

Table 2. Summary of lipase specificities towards FAs located at different stereospecific locations of TAGs.

Lipase	Specificity
Lingual lipase	FA at the *sn*-3 position > FA at the *sn*-1 position of TAG[1]
	Short- and medium-chain FAs > long-chain FAs[2,3]
Gastric lipase	FA at the *sn*-3 position > FA at the *sn*-1 position of TAG[4,5,6]
	Short- and medium-chain FAs > long-chain FAs[2,3]
Pancreatic lipase	FAs at the *sn*-1 and the *sn*-3 positions of TAG[1]; FA at the *sn*-3 position of TAG[6,7] and FA at the *sn*-1 position of DAG[6]; low activity towards DHA or EPA at the *sn*-1,3 positions of TAG[8]
Lipoprotein lipase	FA at the *sn*-1 position of TAG[1,9]

[1]Paltauf et al. (1974); [2]Hamosh (1984); [3]Borel et al. (1994); [4]Jensen et al. (1982); [5] Iverson et al. (1992); [6]Carrière et al. (1997); [7]Rogalska et al.; [8]Bottino et al. (1967); [9]Morley and Kuksis (1972).

Lipoprotein lipase hydrolyses TAGs from circulating chylomicrons for peripheral tissues and has shown positional specificity towards the *sn*-1 position (Morley & Kuksis, 1972). They investigated the specificity of lipoprotein lipase originating from rat post-heparin plasma and cow's milk by incubation with radiolabeled TAGs and reported specificity towards FA esterified at the *sn*-1 position and followed by the *sn*-2 and *sn*-3 position. They suggested that the intermediate formation of the *sn*-2,3 DAG might be physiologically important in avoiding stimulation of TAG and PL biosynthesis, which proceeds exclusively through the *sn*-1,2 DAG. The stereospecificity of lipoprotein lipase towards the ester bond at the *sn*-1 position was also observed by Paltauf et al. (1974), when they incubated lipoprotein lipase with radiolabeled *sn*-1,2-DAG and *sn*-2,3-DAG containing an alkyl moiety at the *sn*-3 or *sn*-1 position *in vitro*, respectively, and observed the radioactivity of hydrolyzed lipids at time intervals.

2.6.2 Lymphatic absorption, resynthesis of TAGs, and transportation of structured TAGs

The maximum lymphatic transport of FAs is normally found between 2 and 4 hours after fat administration, which correlates well with the maximum emptying time from the stomach 1 to 2 hours before (Porsgaard et al., 2003) leaving time for hydrolysis of the dietary TAGs, absorption into enterocytes, resynthesis of TAGs in the enterocytes, and incorporation into chylomicrons secreted to the

lymph. Following maximum absorption, lymphatic transport slowly decreases toward baseline levels.

The conservation of FAs, which were esterified at the *sn*-2 position of TAG during lipolysis and absorption, was shown by Akesson et al. (1978) using a structured TAG of 12:0/12:0/18:1. They found that approximately 25% of the 12:0 FA initially located in the *sn*-2 position had migrated to the *sn*-1,3 positions during hydrolysis and absorption. This implied the conservation of about 75% of the FAs located in the *sn*-2 position. Jensen et al. (1994) estimated that about 33% of MCFAs were conserved during absorption when they were located in the *sn*-2 position compared to LCFAs. The conservation of *sn*-2 FA was also supported by Christensen & Høy (1996) using marine oil, as they showed that the structure of TAG-containing LC (n-3) PUFA was preserved in the resultant chylomicron in mesenteric lymph in rats. The resulting chylomicron TAGs were rich in LC (n-3) PUFAs located at the *sn*-2 position after fish oil feeding compared to resulting chylomicron TAGs that primarily contain LC (n-3) PUFAs at the *sn*-1,3 positions after seal oil feeding.

Once in the cell, specific binding proteins carry FAs and MAG to the intracellular site, where they are used for TAG biosynthesis. The two major FA-binding proteins, being liver and intestinal FA-binding proteins, are found in enterocytes. Most TAG biosynthesis in the enterocyte occurs along the MAG pathway, in which MAG and fatty acyl-coenzyme A are covalently joined to form DAG in a reaction catalyzed by monoacylglycerol acyltransferases. Acylation of DAG by diacylglycerol acyltransferase further leads to the synthesis of TAGs (Iqbal & Hussain, 2009). Stereospecificity of monoacylglycerol and diacylglycerol acyltransferases from rat intestine showed that neither of these enzymes have absolute stereospecificity for acylglycerols as fatty acid acceptors (Lehner et al., 1993). However, monoacylglycerol acyltransferase favored the acylation of MAGs containing a polyunsaturated fatty acyl moiety at the *sn*-2 position (Xia et al., 1993) and the formation of *sn*-1,2-DAGs. Yang et al. (1995) studied the stereospecificity of TAG resynthesis in the enterocyte in rats. Chylomicron TAG analysis showed that the FA composition at the *sn*-2 position of dietary TAG was highly (over 90%) conserved after feeding with fish oil. This indicated that the hydrolysis of TAGs to *sn*-2-MAG and resynthesis via the MAG pathway in the enterocyte is the favored mechanism of TAG absorption and resynthesis. The metabolic fate of 2-MAG and 1(3)-MAG in isolated caecal enterocytes and hepatocytes of brown trout showed that the 2-MAG was quickly resynthesized into TAG through the MAG pathway in both tissues, whereas 1(3)-MAG was processed into TAG and phospholipids at a much slower rate suggesting that 2-MAG was the preferred substrates for MGAT (Li & Olsen, 2017). TAG can also be synthesized by a separate route, if 2-MAGs from dietary TAGs are not available, by acylating glycerol-3-phosphate to phosphatidic acid,

dephosphorylating the phospholipid to DAG, and acylating the DAG to TAG. The DAG synthesized from both the pathways are metabolically unequal, as the DAG derived from the MAG pathway is directed to TAG synthesis only, whereas the DAG derived from glycerol-3-phosphate may be used to synthesize either TAG or phospholipids (Mansbach & Gorelick, 2007).

EPA and DHA incorporation into lymphatic PLs and TAGs was studied in in a postprandial study in rats by Clark & She (1995) after feeding human milk, infant formula, or infant formula fortified with fish oil. Animals infused with a formula containing fish oil resulted in an equal increase in EPA in lymphatic TAG and PLs compared to human milk and formula milk. On the contrary, the DHA content of total lymph lipids was highest in human milk, although it contained less DHA than the fish oil-treated formula. The incorporation rate of DHA in the TAG fraction exceeded the rate of incorporation into the PL fraction during the 6-hour recovery of lymph. This difference in the distribution of DHA in lymphatic PLs compared to TAG may be explained by the structure of TAG in the fish oil supplement, positional specificity of pancreatic lipase, and the pathways for lipid synthesis in the enterocyte.

The absorption of specific structured TAGs was studied by Ikeda et al. (1991) using MLM- and LML- (M: medium-chain FA and L: long-chain FA) structured TAGs in thoracic duct-cannulated rats after intragastric administration of lipid emulsions. They showed that the absorption of the 18:2 FA was higher from the 10:0/18:2/10:0 than from the 18:2/18:2/18:2 probably due to the fast hydrolysis of MCFA. However, Porsgaard et al. (2005) found a similar absorption of MLM and LML when studying the lymphatic transport of fish oil and two specific structured TAGs containing 10:0 and marine-origin (n-3) PUFAs. They also showed a higher lymphatic transport of fish oil compared with the two structured TAGs. The contrasting results of Porsgaard et al. to Ikeda et al. were related to the absorption of the MLM- and LML-type structured TAGS and could be due to differences in the purity of TAGs, as Ikeda et al. used pure TAG species, and Porsgaard et al. used a mixture of structured TAGs with MLM- and LML-type TAGs. Another possible reason could be the different FAs in the structured TAGs, as Porsgaard et al. had (n-3) FAs from fish oil, and Ikeda et al. had 18:2 (n-6). The effect of TAG structure on the lymphatic absorption of dietary DHA was studied in lymph-cannulated rats (Fujimoto et al., 1998). Administration of chemically structured TAGs of 16:0/22:6/16:0 and 16:0/16:0/22:6 or 22:6/16:0/16:0 to rats showed more efficient absorption of DHA from the *sn*-2 position than from *sn*-1 or *sn*-3 positions.

The LC (n-3) PUFA lymphatic absorption from different stereospecific positions of dietary TAGs was investigated by feeding fish oil containing EPA and DHA at the *sn*-2 location and seal oil containing EPA and DHA at the *sn*-1/3 locations to male Wistar rats. The mesenteric lymph was collected at time

intervals, and the extracted lipids were analyzed by GC. After 8 hours, lymphatic transport of LC (n-3) PUFAs was higher in fish oil-fed rats, and they were mainly located at the *sn*-2 position. However, the accumulated absorption over 24 hours did not change between fish oil- and seal oil-fed rats (Christensen et al., 1994). Christensen et al. (1995) later studied the effect of the distribution of LC (n-3) PUFAs in TAGs on the lymphatic transport of absorbed lipids in male Wistar rats and obtained similar results. The intestinal absorption and lymphatic transport of EPA and DHA was studied using synthetic structured lipids containing LC (n-3) PUFA esterified to the *sn*-2 position and decanoic acid to the remaining positions of TAG or randomized TAG with similar FA compositions. The lipids were extracted from the collected mesenteric lymph. The lipid analysis showed that the rate of lymphatic transport and the LC (n-3) PUFA content were significantly higher when EPA and DHA were located in the *sn*-2 position of the dietary TAG after the first 8 hours after administration. However, the total amount of DHA and EPA transported in the lymph did not differ between the experimental groups after 24 hours of recovery. This indicated that the LC (n-3) PUFA from the *sn*-2 position is readily absorbed in the initial period of digestion when the TAG administered had a specific intramolecular structure.

2.6.3 Plasma lipids

FAs in the *sn*-2 position of TAG in the diet are conserved during digestion, absorption, and reassembly to chylomicron TAGs (Innis & Dyer, 1997). Positional differences in SFAs were hypothesized to modulate the transport of (n-6) and (n-3) PUFAs. A rat study indicated that dietary FAs in the *sn*-2 position could influence lipemia and platelet reactivity as well as the desaturation and elongation of PUFAs. The biological effects of dietary FAs at the *sn*-2 position were evaluated by 2 to 4 months of feeding a purified diet containing native palm oil, interesterified palm oil, native lard, or interesterified lard. Interesterification in lard resulted in significantly lower plasma TAGs, and, in palm oil, increased platelet aggregation. The *sn*-2 positions of 18:2, 16:0, and 18:1 FAs were directly related to the plasma levels of 20:4 (n-6), 16:1 (n-7), and 20:3 (n-9) fatty acids, respectively (Renaud et al., 1995). Dietary TAGs with 16:0 in the *sn*-2 position increased the 16:0 FA in the *sn*-2 position of plasma and chylomicron TAGs using a formula containing synthesized TAGs containing 32% of 16:0 at the *sn*-2 position or with sow's milk compared to feeding of a formula containing synthesized TAGs containing 4.2% of 16:0 at the *sn*-2 position for 18 days. It also resulted in reduced ARA and DHA in the PL fraction and altered cholesteryl ester metabolism in piglets (Innis & Dyer, 1997). However, interesterification of the fish oil diet compared to the native fish oil diet did not have any significant

effect on plasma TAGs, cholesterol esters, free and total cholesterol as well as plasma PL FAs in rats (De Schrijver et al., 1991).

In a 7-day study in human infants, a formula containing TAGs resembling the stereoisomeric structure of human milk fat having 25.4% by wt. of the 16:0, 76.1% of which is at the *sn*-2 position, resulted in higher percentages of the 16:0 in plasma sterol esters, TAGs, and FFAs as compared to a formula containing 87.3% of the total 16:0 (25.7% by wt.) at the *sn*-1,3 positions (Carnielli et al., 1995). In contrast, when normocholesterolemic adults were fed margarine prepared from palm oil (18% *sn*-2 16:0 and 82% *sn*-1/3 16:0) or palm oil interesterified with sunflower oil (65% *sn*-2 16:0 and 35% *sn*-1/3 16:0), only men experienced significant increases in total cholesterol and LDL cholesterol concentrations but not women (Zock et al., 1995). Despite these large differences in FA configurations, only very small differences were observed in lipoprotein concentrations. Another human study showed no effect of the positional distribution of dietary palmitic acid on postprandial plasma lipids (Zampelas et al., 1994). These conflicting results might suggest that the *sn*-2 16:0 can increase lipid and lipoprotein levels in human infants and piglets, while this effect in an adult human is inconclusive.

The effects of highly purified structured TAGs on plasma and liver lipid profiles were investigated in rat studies. A diet containing structured TAGs (MLM- [M = 8:0 or 10:0, L = LA] or LML-type) and corn oil as a control diet fed for 4 weeks showed improved serum and liver lipid profiles after feeding the LML-containing diet compared to corn oil (Nagata et al., 2003). When the LCFA was replaced with EPA or DHA (8:0/EPA/8:0, EPA/8:0/8:0, 8:0/DHA/8:0, and DHA/8:0/8:0), lower serum TAG and cholesterol concentrations were found in the structured TAG groups compared with a soybean oil-fed group (Nagata et al., 2004). However, the physiological effects of the structured TAG in the above studies were due to the FAs rather than the structural specificity.

Differences in the absorption rates of EPA and DHA based on the positional distribution in plasma lipoproteins in a healthy human were studied in a prolonged intervention by Sadou et al. (1995). In fish oil TAGs, DHA is mainly at the *sn*-2 position, at about 60%, and EPA is at the *sn*-1,3 positions at 72%. The 30-day intervention consisted of a daily dose of fish oil containing 2 g EPA and 1.3 g DHA. FAs were extracted from the daily blood samples, and the FA composition was determined by GC. DHA was more efficiently incorporated into total plasma lipids during the first 2 days of intervention even though the fish oil supplementation contained almost twice as much EPA as DHA. Later, EPA and DHA levels plateaued at 15 days. The study results showed a faster incorporation in plasma lipoproteins of the DHA located at the *sn*-2 position of fish oil TAGs due to regiospecificities in gastric lipolysis and TAG resynthesis in the enterocyte. The short-term (0 to 8 h) study from this investigation showed

that a higher TAG incorporation of DHA and the higher metabolic availability of EPA compared with DHA for PL accumulation depend on their respective preferential *sn*-2/*sn*-1(3) positions in fish oil TAG.

2.6.4 Fecal lipid loss

Under normal circumstances, the digestive system can digest the TAGs and absorb them with more than 95% efficiency. A fecal loss containing greater than 5% of dietary fat, i.e., steatorrhea, indicates a state of malabsorption. Evaluation of fecal fat excretion has been used to measure fat absorption. Dietary lipids, which are not absorbed, are excreted with feces, along with other excreted lipids, such as lipids from excreted intestinal cells, a fraction of secreted bile lipids, and bacterial lipids (Mu & Høy, 2004).

The effect of the positional distribution of FA on TAG has been studied using different sources of fat in the diet. Brink et al. (1995) studied the interaction between calcium and saturated FAs. Two structured lipids, 18:0/18:0/18:1 and 18:0/18:1/18:0, were prepared by interesterification, and their absorption in rats was examined at high (1 g/100 g) and low (0.3 g/100 g) concentrations of dietary calcium. A higher concentration of dietary calcium decreased the total absorption of lipids from 92.7% to 72.6% in 18:0/18:1/18:0 fed rats and from 96.6% to 89.0% in 18:0/18:0/18:1 fed rats, respectively. The location of the 18:0 FA in the *sn*-1,3 positions increased fecal fat and calcium losses due to the formation of calcium soaps. Later, the 18:0 was replaced with a 16:0 to form the 16:0/16:0/18:1 and 16:0/18:1/16:0, and similar results were found by Aoyama et al. (1996).

The importance of the TAG structure in human milk fat for energy absorption in breastfed infants was studied with the hypothesis that the location of the 16:0 in the *sn*-2 position will be preserved, as it will be readily absorbed after pancreatic lipase hydrolysis (Filer et al., 1969). Betapol has been manufactured to possess a great similarity with human milk fat in both total FA composition and intramolecular structure. de Fouw et al. (1994) compared the intestinal absorption in rats on the Betapol diet containing TAG with 73% of the total 16:0 at the *sn*-2 position, and, in control fat, with TAG having 6% of the total 16:0 at the *sn*-2 position and examined the remaining fats in segments of the intestine. They found that the 16:0 fatty acid was rapidly absorbed from the upper segments of the intestine in the Betapol group without any effect on calcium absorption. In a 7-day study, premature newborn infants were fed two formulas that differed only in TAG configuration resulting in higher 16:0 uptake and lower calcium losses in preterm infants fed Betapol compared to control oil with the 16:0 in the *sn*-1,3 positions (Carnielli et al., 1995). An infant formula with the 16:0 at the *sn*-2 position showed less FA loss in fecal calcium in a group of term infants who were breastfed for the first two months and in a group where infants were fed with a formula containing the 16:0 at primary positions in the first

month and with a formula containing the 16:0 at the *sn*-2 position in the second month compared to a group of infants fed with a formula containing the 16:0 at primary positions for 2 months from birth (López-López et al., 2001).

Most of the studies mentioned above demonstrated that the lipid structure is an important parameter affecting lipid absorption. Saturated FAs located at the *sn*-2 position are better absorbed, and free saturated FAs released from the primary positions of TAGs during lipolysis can form calcium soaps, which are excreted in feces. However, the effect of TAG structure has not been studied for the differences it causes in absorption of LC (n-3) PUFAs from primary positions.

2.6.5 Incorporation of long-chain (n-3) polyunsaturated fatty acids into tissues

The structure of TAGs, not only the composition of FAs but also their relative order in the *sn*-1,2, or 3 positions on the TAG, can influence the metabolism of the TAG and its FAs, including lipoprotein metabolism (Amate et al., 1999; Hayes, 2001). One rationale for this possibility is that the *sn*-2 FA can be absorbed intact, i.e., as the *sn*-2 MAG, whereas the FAs at the *sn*-1,3 positions are absorbed as FFAs, which are metabolized independently.

The accumulation of LC (n-3) PUFAs in tissues from different stereospecific locations of TAGs was studied using native and randomized fish oil. A 24-day intervention study did not show differences in the accumulation of LC (n-3) PUFAs in the liver PLs with the feeding of native or randomized fish oil containing LC (n-3) PUFAs mainly at the *sn*-2 position or evenly distributed in TAG (De Schrijver et al., 1991).

Jensen et al. (1996b) investigated the influence of the dietary FA profile and the TAG structure on the FA profile and the TAG structure of milk lipids in two generations of rats. The LC (n-3) PUFA content of milk in first-generation rats was higher in those receiving fish oil, at 12.3%, compared to rats receiving seal oil at 8.0%, but not in the second generation at 8.9% and 9.1%, respectively. The EPA and DHA contents of seal oil, at 8.9% EPA and 7.5% DHA, and fish oil at 9.8% EPA and 10.3% DHA differed slightly. The seal oil and fish oil contained 20.6% and 22.7%, respectively, of total LC (n-3) PUFAs. The experiment included the Grignard degradation of oils, thin-layer chromatographic purification, and GC analysis of FAs. The results of lipid analysis showed that the *sn*-2 position of TAG contained 0.8% EPA and 1.3% DHA in seal oil-fed rats and 7.7% EPA and 19.0% DHA in fish oil-fed rats. In another study, Jensen et al. (1996c) studied the effects of dietary (n-3) PUFAs on major brain PLs in rats after fish oil or seal oil feeding throughout four generations. In the fourth rat generation, the brain PL FA composition showed similar levels of DHA incorporation in both intervention groups. However, fish oil feeding increased the brain EPA and DPA more than seal oil feeding. The fourth generation was

tested for spatial learning ability in a Morris water maze, and they found that both the fish oil and seal oil groups had similar learning abilities, which were increased compared to the vegetable oil group. Similarly, the effect of the uptake of DHA and TAG on visual acuity or mental development was evaluated by Christensen et al. (1998) in rat pups fed from birth to 12 weeks of age on a diet containing specific structured oil with approximately 56% of DHA in the *sn*-2 position, randomized oil with approximately 30% of DHA in the *sn*-2 position, or DHA-free chow as a control. After 12 weeks, the specific structured oil resulted in the highest level of DHA in the brain, whereas the level of DHA was highest in the liver of the group fed randomized oil indicating differences in the metabolism of FAs resulting from their position in the dietary TAG molecule. However, retina PLs were not affected by any of the three experimental feedings except for a low level of ARA in the treated groups, and this showed a strong tendency to maintain a high level of DHA in the retina. At 9 weeks of age, no significant differences were observed among the three groups for spatial learning in a Morris water maze. But the changes in the FA profiles caused changes in visual function shown by the higher latency of the β-wave and the lower oscillatory potential and in the auditory brainstem response shown by the generally greater amplitude of wave Ia in the group fed specific structured oil.

The effect of the early intervention with structured TAGs, either a specific structured oil with DHA mainly located in the *sn*-2 position or a randomized oil with DHA equally distributed in the TAG molecules, was studied in newborn rats (Christensen & Høy, 1997). No significant differences between the two dietary treatments were observed in the brain and liver tissues after 3 weeks of feeding. However, in adipose tissue, higher levels of DHA and EPA were found in the group fed with randomized fat. Nagata et al. (2004) studied the EPA and DHA bioavailability from structured TAGs (8:0/EPA/8:0, EPA/8:0/8:0, 8:0/DHA/8:0, and DHA/8:0/8:0) in 4-week-old rats. Feeding structured TAGs was not reflected in the structural differences in the white adipose tissue FA composition. Similarly, Kew et al. (2003) found no effect of the position of DHA in TAG on the proportion of DHA in the PL of the mouse spleen after 7 days of feeding with structured TAGs containing EPA or DHA either at the *sn*-1,3 or *sn*-2 position.

Hartvigsen et al. (2004) conducted a study where dams received an experimental diet from the eighth day of gestation to the end of their lactation. The experimental diets contained a structured oil being ALA predominantly in the *sn*-2 position at 2 or 10 mol%, a linseed oil being an ALA level of 2 or 10 mol%, or fish oil containing ALA as well as DHA and EPA. The amount of DHA in brain phosphatidyl ethanolamine and phosphatidyl serine was not affected by different diets given to dams and offspring. Whereas, a 12-week intervention with structured TAG-containing DHA at the *sn*-2 position in hamsters indicated

a more efficient incorporation of DHA in the brain, liver, and erythrocytes (Bandarra et al., 2016) and in brown adipose tissue (Lopes et al., 2017) compared to linseed oil, fish oil, and fish oil EEs.

3 AIMS OF THE STUDY

The aim of this study was to compare the difference in the bioavailability of DHA from docosahexaenoic acid [22:6(n-3), DHA] and stearic acid (18:0) from the two different enantiomers of TAG: *sn*-22:6(n-3)-18:0-18:0 and *sn*-18:0-18:0-22:6(n-3), and their regioisomeric TAG: *sn*-18:0-22:6(n-3)-18:0 by investigating the content and composition of fatty acids in the feces, plasma and organs of mildly (n-3) deficient rats subjected to five-day feeding trial. This study also aimed to determine the impact of the induced (n-3) deficiency on the fatty acids of the rat plasma and organs. The objectives of the studies were to:

I. Assess the effect of a short-term first-generation (n-3) deficiency in young adult rats on the fatty acid compositions, most importantly the contents of ARA, EPA, and DHA, of different organ tissues.

II. Investigate if the DHA originating from the *sn*-1, *sn*-2, and *sn*-3 positions and stearic acid from the remaining positions in dietary TAGs would lead to different fatty acid content and composition of rat plasma and feces, and would affect the weights of rat organs or total body.

III. To further explore the influence of dietary DHA originating from the *sn*-1, *sn*-2, and *sn*-3 positions, and stearic acid in the remaining positions, on the fatty acid compositions of rat brain, eye, liver, visceral fat, testicle, and kidney.

4 MATERIALS AND METHODS

4.1 Ethical considerations

The experimental procedure of the animal trial was approved by the Medical Ethics Research Board of the Peking University Health Science Center, China (Study identifier LA2016043).

4.2 Synthesis of regio- and enantiopure structured triacylglycerols

The chemoenzymatic synthesis of symmetrically and asymmetrically structured TAGs was conducted at the University of Iceland. For the synthesis of structured TAGs, DHA (≥ 95%) in ethyl ester form (Pronova Biocare, Sandefjord, Norway) was converted into free acid (Haraldsson et al., 2000). The synthesis of the symmetrically structured TAG that contains DHA in the *sn*-2 position and STA in the remaining *sn*-1,3 positions (**Figure 3**) was based on a previously described two-step chemoenzymatic route from glycerol using a highly regioselective immobilized *Candida antarctica* lipase from Novozymes (Bagsværd, Denmark) (Halldorsson et al., 2003). *Candida antarctica* lipase was used to act exclusively at *sn*-1, 3 positions to acylate STA residues in the first step, followed by acylation of DHA to the *sn*-2 position using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride as coupling agent. The asymmetrical enantiopure structured TAGs possessing DHA located in the *sn*-1 or *sn*-3 positions along with the STA residues occupying the remaining positions were synthesized in five steps by a fully chemical without enzyme route. Chiral precursors (R)- and (S)- solketals were used in the chemical synthesis of these TAG enantiomers *sn*-22:6(n-3)-18:0-18:0 and *sn*-18:0-18:0-22:6(n-3) respectively (Kristinsson et al., 2014).

The chemical and regioisomeric purity of all products and intermediates was excellent (> 98%). It was established by ^{1}H (400 MHz) and ^{13}C nuclear magnetic resonance (NMR) and infrared spectroscopy as well as by satisfactory high-resolution accurate mass spectrometry analyses. The lipid oxidation status of the starting materials and products was investigated by 400 MHz ^{1}H NMR. Chiral TAGs were analyzed by chiral HPLC measurements based on the use of two chiral columns connected to a sample recycling system to ensure excellent enantiopurity (> 96% enantiomeric excess) (Kalpio et al., 2015). The synthesis of the enantiopure structured TAGs possessing DHA either in *sn*-1 or *sn*-3 positions and two STA residues in the remaining positions [*sn*-22:6(n-3)-18:0-18:0 as (*sn*-1 DHA) and *sn*-18:0-18:0-22:6(n-3) as (*sn*-3 DHA)] (Kalpio et al., 2020) and their regioisomeric TAG [*sn*-18:0-22:6(n-3)-18:0 as (*sn*-2 DHA)]

(Halldorsson et al., 2003; Haraldsson et al., 2000) has been reported separately. Tristearin was prepared by treating glycerol with STA with the aid of the immobilized *Candida antarctica* lipase under a vacuum at 70–75 °C (Haraldsson et al., 2000). All chemical transformations were performed under a strict inert atmosphere (nitrogen or argon). The TAGs were stored under nitrogen at −85 °C.

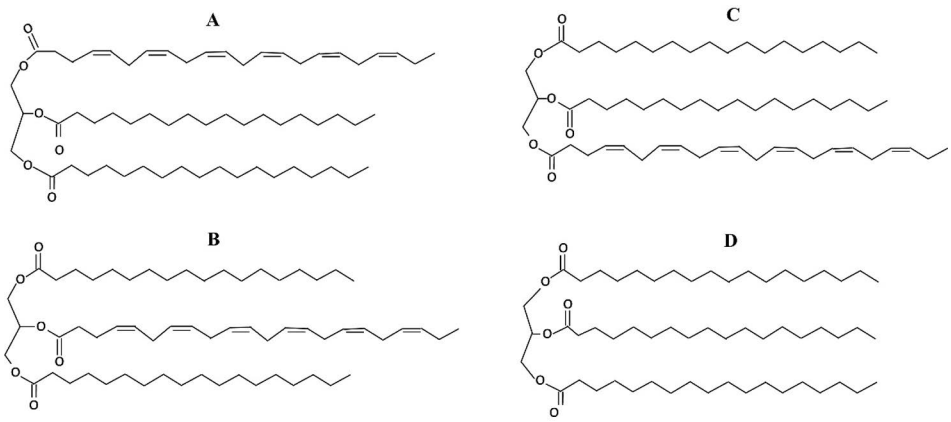


Figure 3. Experimental structured triacylglycerols A) 2,3-distearoyl-1-docosahexaenoyl-*sn*-glycerol, B) 1,3-distearoyl-2-docosahexaenoyl-*sn*-glycerol, C) 1,2-distearoyl-3-docosahexaenoyl-*sn*-glycerol, and D) tristearin.

4.3 Animal trial

The animal trial was conducted at the Peking University Health Science Center, Beijing, China.

4.3.1 Animals and diet

A total of seventy-two young male Sprague-Dawley rats (age 21 ± 2 days) were obtained from the Department of Laboratory Animal Science of Peking University Health Science Center.

Three rodent AIN-93G diets were prepared using either soybean oil, peanut oil bought in Finland, or peanut oil bought in China. Diets were matched for non-fat nutrients **(Table 3)**. The standard AIN-93G diet contained soybean oil as a source of (n-3) FA (Reeves et al., 1993). The peanut oils were analyzed to ensure the presence of low levels of (n-3) FAs and used to prepare diets deficient in (n-3) FAs. The fatty acid composition of the oils used in the feed is shown in **Table 4**. The normal feed group (**study II and III**) is also referred to as the (n-3) adequate group (**study I**). Before grouping, all rats were kept for 7 days in isolation with constant temperature and humidity to acclimate to the housing

conditions and were fed on a standard AIN-93G diet (Nuoyuan Biotechnology Co., Ltd, Beijing, China).

Table 3. Composition of diets. Reprinted from the original publication (Linderborg et al., 2019) with the permission of Elsevier.

Ingredients	Contents (g/kg)
Corn starch	397
Casein (>85% protein)	200
Corn dextrin (90-94% tetrose)	132
Sucrose	100
Oil[1]	70
Fiber	50
Minerals	35.5
Vitamins	10
L-cystinol	3
Choline bitartrate (42% choline)	2.5
Tert-butylhydroquinone	0.014

[1] Low omega-3 fatty acid diet (modified AIN-93G diet) for *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA, and TS groups contained peanut oil. Omega-3 deficient diet for (n-3) deficient group contained peanut oil. The Standard omega-3 AIN-93G diet for the NF group contained soybean oil.

Table 4. Fatty acid composition of oils used in the feed. Values are expressed as mean mass percentages of two replicates. Modified from the original publication (Linderborg et al., 2019) and (Kulkarni et al., 2022) with the permission of Elsevier and MDPI, respectively.

Fatty acids	Soybean oil[1]	Peanut oil[2]	Peanut oil[3]
16:0	10.8	9.7	10.3
18:0	4.4	3.2	3.2
18:1(n-9), OA	22.7	61.9	45.6
18:2(n-6), LA	51.9	17.9	32.3
18:3(n-3), ALA	6.3	0.1	0.2
20:0	0.4	1.4	1.4
22:0	0.4	2.5	2.9
24:0	0.1	1.3	1.4
Others[4]	3.0	2.4	2.8

[1] Soybean oil was used in the feed of the normal feed (NF) group.

[2] Peanut oil (bought in Finland) was used in the feed of the *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA, and tristearin (TS) groups.

[3] Peanut oil (bought in China) was used in the feed of the (n-3) deficient group.

[4] This category includes 16:1(n-7), 18:1(n-7), 18:3(n-6), 20:1(n-9) and 23:0. OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid.

4.3.2 Study design

The rats were allowed to adapt to housing conditions for 7 days. Thereafter the seventy-two rats were randomly divided into 6 experimental groups: 2,3-distearoyl-1-docosahexaenoyl-*sn*-glycerol (*sn*-1 DHA), 1,3-distearoyl-2-docosahexaenoyl-sn-glycerol (*sn*-2 DHA), 1,2-distearoyl-3-docosahexaenoyl-sn-glycerol (*sn*-3 DHA), tristearin (TS), normal feed (NF), and (n-3) deficient group (n= 12 in each group). A rat from the (n-3) deficient group died during the induction phase and therefore did not receive the intervention. The study design overview showing rat housing and feeding during different phases among experimental groups is described in **Figure 4**. **Study I** involved NF and (n-3) deficient groups whereas, **studies II and III** involved *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA, TS and NF groups.

During the following four-week induction phase, four rats were housed in one cage and their food intake was monitored. During this phase, four groups were fed AIN-93G containing peanut oil deficient in (n-3) FA (bought in Finland) as the only source of FAs (**Table 4**) to induce a mild (n-3) FA deficiency state, while the fifth group was on the standard AIN-93G containing soybean oil (NF group), and the sixth group on (n-3) deficient diet containing peanut oil bought in China ((n-3) deficient group), respectively. The total body weight of the rats was monitored every three days.

From the last day of the induction phase, the rats were housed individually to begin the 5-day intervention phase. Rats were weighed daily from the last day of the induction phase and throughout the 5-day feeding period. During the intervention phase, four mildly (n-3) deficient groups received *sn*-1 DHA, *sn*-2 DHA, or *sn*-3 DHA, or tristearin as the experimental fat for five days. Whereas the NF group continued on (n-3) adequate soybean oil based diet and the (n-3) deficient group continued the (n-3) deficient peanut oil based diet feeding, until the end of the intervention phase. α- Tocopherol (100 mg/100 g) was added to the experimental fats before dividing the fat into individual doses. Individual doses were stored under nitrogen at −80 °C. Pellets containing experimental fats were prepared with a daily dose of 360 mg of experimental fat or tristearin, based on previously applied doses (Ghasemifard et al., 2014; Kaur et al., 2010). The aliquots of experimental fat were melted quickly using a water bath at 40 °C. The experimental fat was added to the first pellets each morning and was embedded between two halves of a low (n-3) FA feed pellet. The experimental pellets were stored at dark +4°C overnight and fed to rats in the morning. These pellets were served as the first morning feed. Once verified that the experimental fat pellets were consumed completely, the rats were provided with the remainder of the normal daily ration. Rats were fed *ad libitum*. The rats were sacrificed on the morning of day 6.

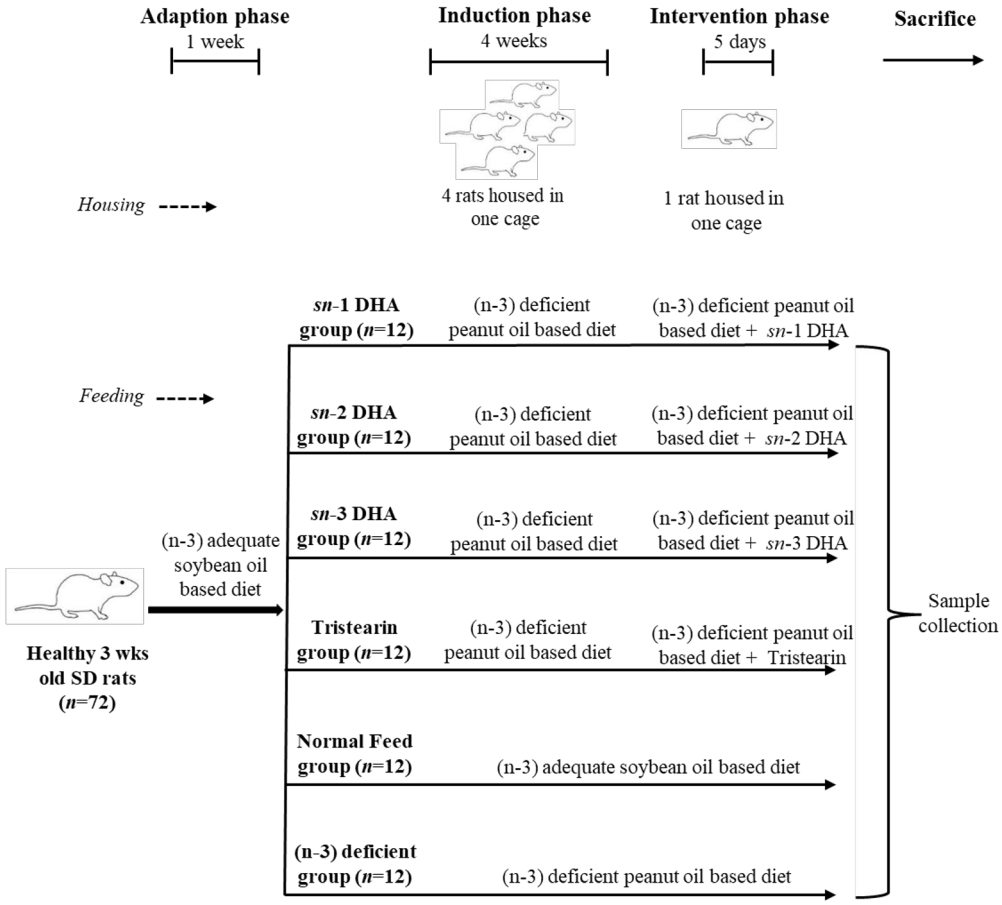


Figure 4. Overview of the study design showing the housing system of rats and distinction of groups based on the feeding during different phases of the animal trial.

4.3.3 Sample collection

Baseline fecal samples were collected on the last day of the induction phase. During the 5-day intervention period, fecal samples were collected daily. After collection and weighing, the fecal samples were immediately frozen and stored at −80 °C.

At the end of the 5-day intervention phase, while the rats were in the fasting state, they were sedated by inhaling isoflurane and sacrificed with exsanguination. After the femoral artery was cut off, blood samples were collected in dry tubes without anticoagulant. The blood samples were centrifuged, and the supernatant plasma was collected into sterile tubes and frozen at −80 °C. The brain, eye, liver, testicle, heart, lung, kidney, and visceral fat were collected

from each rat and weighed. All the samples were immediately frozen in liquid nitrogen, packed individually, and stored at −80 °C for subsequent lipid analysis.

4.4 Lipid extraction

Lipids were extracted from the plasma and feces (**study II**) and the liver, brain, eye, testicle, visceral fat, and kidney (**study III**) of *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA, TS, and NF groups while from the plasma, feces, liver, brain, eye, testicle, visceral fat, heart, and lung of (n-3) deficient and NF groups (**study I**). Lipid extraction was performed in duplicate for plasma, feces, and all the organs except the eye where lipids were extracted from only one eyeball for each rat. All of the solvents used were HPLC grade (Sigma-Aldrich, Saint Louis, MO, USA).

The frozen fecal samples were homogenized by using a mortar and a pestle. The homogenized samples from the intervention phase were pooled to form the fecal study samples. The plasma samples were weighed and directly extracted for lipids. The liver, testicle, visceral fat, kidney, lung, and heart were cut into pieces on an ice bath, and representative samples were randomly taken from similar regions of each organ tissue for lipid extraction. The frozen brain tissue samples were homogenized using the Bio-Gen PRO200 homogenizer (PRO Scientific, Monroe, CT, USA). The homogenate was divided into two portions, and one portion was stored at −80 °C for lipid analysis. The tissue samples of the liver, testicle, visceral fat, lung, heart, kidney, and brain were weighed, followed by homogenization in methanol (10 mL of MeOH for 1 g of tissue) with an Ultra-Turrax T 25 instrument (IKA Werke GmbH & Co. KG, Staufen, Germany) in four intervals for 30 sec at 8000 rpm. The homogenized tissue in methanol was stored at −80 °C. The eyeball was not homogenized as other organ tissues to avoid tissue loss, as it was challenging to recover all the tissue from the eyeball and its film from the Ultra Turrax blades. Therefore, eyeballs were weighed and directly used for lipid extraction.

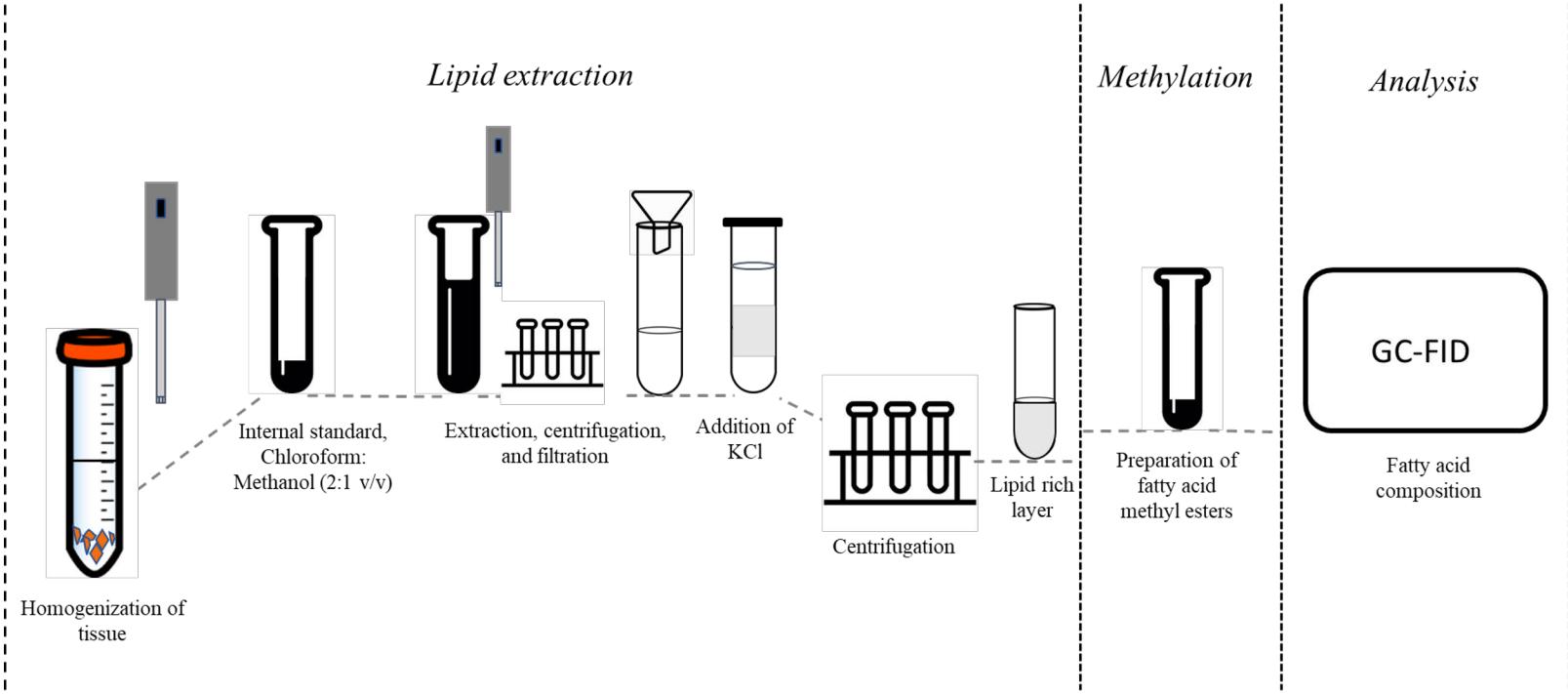


Figure 5. Overall stepwise experimental process from lipid extraction from tissues to analysis.

Total FAs were extracted with a modified Folch method using methanol, chloroform, and 0.88% potassium chloride (KCl) in milli-Q water (Christie, 1989; Folch et al., 1957) from plasma and feces (≈ 200 mg) as well as from one aliquote (tissue homogenate in solvent) of the liver, visceral fat, testicle, brain, heart, and lung (each aliquot ≈ 200 mg for the brain and kidney; ≈500 mg for other organs) and one eyeball of each rat (≈ 84- 145 mg).

For the lipid extraction of tissues, triheptadecanoin (for TAG) and dinonadecanoylphosphatidylcholine (for PL) (Larodan Fine Chemicals AB, Malmö, Sweden) were added as internal standards in the liver while triheptadecanoin only was used in the analysis of brain, eye, visceral fat, testicle, kidney, lung, and heart. 1 mL of internal standard, ≈ 1-1.3 mL of methanol (depending on the tissue), and 2 mL of chloroform were added to the homogenate of the sample in a glass tube. The mixture was homogenized using Ultra Turrax for 30 sec at 8000 rpm. The mixture was centrifuged for 4 min at 3500 rpm and the supernatant was collected in a clean glass tube (1st extraction). The second extraction was repeated by adding 3 mL of chloroform/methanol (86:14 v/v) solution. The first and second extraction supernatants were filtered using a funnel filter and filter paper (Whatman, Maidstone, UK). The filter paper was washed with 1 mL of chloroform to obtain the maximum lipid content. 1 mL of 0.88% KCl in milli-Q-water was added to the filtrate and the mixture was vortexed. The test tubes were centrifuged for 3 min at 3500 rpm to separate the layers, and the lower layer of lipid-rich chloroform was collected. Overall experimental steps for the lipid extraction from tissues until the analysis is shown in **Figure 5.**

For plasma samples, 1 mL of internal standard (Triheptadecanoin for TAG and dinonadecanoylphosphatidylcholine for PL), 2 mL of methanol and 3 mL of chloroform were added to a glass tube followed by vortexing. Then 1 mL of 0.88% KCl in milli-Q-water was added and the sample was centrifuged for 3 min at 3500 rpm. The lower lipid-rich layer was collected in a clean glass tube. Later, the extraction was repeated two more times by adding 2 mL of chloroform/methanol (86:14 v/v) solution, followed by vortexing, centrifugation, and collection of the lower lipid-rich layer.

The fecal lipids were extracted by adding 1 mL of internal standard (Triheptadecanoin), 1.5 mL of methanol and 2 mL of chloroform in a glass tube. The sample was homogenized using Ultra Turrax for 30 sec at 8000 rpm. The homogenized fecal sample was centrifuged for 4 min at 3500 rpm separating the fecal solid layer at the bottom and the upper solvent layer containing lipids. The upper layer was transferred into a clean glass tube. In the second extraction, 4 mL of chloroform/methanol (86:14 v/v) was added to the lower layer of the fecal sample and was homogenized using Ultra Turrax for 30 sec at 8000 rpm. Thereafter, both the first and second extracted lipids were filtered using Büchner funnel and filter paper (Whatman, Maidstone, UK) to get a solid free filtrate. The

filter paper was washed with 1 mL of chloroform to obtain the maximum lipid content. Then 1 mL of 0.88% KCl in milli-Q-water was added to the solvent, followed by vortexing, centrifugation (for 3 min at 3500 rpm), and collection of the lower lipid-rich layer in a clean glass tube.

After extraction, the extract was evaporated to dryness under nitrogen flow at 40 °C in a heat block. The dried extract was dissolved in 1 mL of chloroform in a test tube. The sample was immediately used for the next step or transferred to a vial to store in the freezer.

4.5 Isolation of triacylglycerol and phospholipid fractions

As plasma and liver were assumed to contain significant amounts of both neutral and polar lipids, they were analyzed after fractionation into TAG- rich fraction and PL- rich fraction with solid-phase extraction using Sep-Pak Vac 1cc silica cartridges (Waters, Dublin, Ireland).

The isolation of lipid classes in the plasma was based on Hamilton & Comai, (1988). The column was activated by eluting 1.5 mL of hexane:methyltertiarybutylether (MTBE) (96:4) followed by 4 mL of hexane. The lipid extract was dissolved into 1 mL of hexane:MTBE (200:0.8), which was then transferred into the column. The sample tube was washed with 1 ml of hexane:MTBE (200:0.8) and transferred to the column to recover all the lipids. The column was then eluted with 5 mL of hexane:MTBE (200:0.8) to remove cholesterol esters, 10 mL of a mix of hexane:MTBE (96:4) isolating TAGs, 6 mL of hexane:acetic acid (100:0.2) and MTBE:acetic acid (100:0.2) (both eluted to waste), and PLs were finally eluted with 12 mL of MTBE:methanol:water (5:32:2).

The neutral (mostly TAG) and polar (mostly PL) fractions were isolated from the extracted liver lipids according to Christie, (1989). The column was conditioned by elution with diethyl ether (5 mL). After column conditioning, lipid extract was dissolved into 1 mL of diethyl ether and applied to the column. The test tube was washed twice with 1 mL of diethyl ether for maximum recovery of extracted lipids. TAGs were eluted with 7 mL of diethyl ether. The test tube was then washed twice with 1 mL of methanol and pipetted into the column. PLs were eluted with 7 mL of methanol.

The TAG- and PL- rich fractions were evaporated under nitrogen and dissolved in 1 mL of hexane and 1 mL of chloroform, respectively, and transferred into the autosampler vials.

4.6 Formation of fatty acid methyl esters

Lipids were esterified into fatty acid methyl esters (FAMEs) by the acid-catalyzed method and sodium methoxide-catalyzed transesterification method (Christie, 2010).

Extracted lipids from the brain, eye, testicle, heart, and, lung were methylated using the acid-catalyzed method. The lipid sample was evaporated under nitrogen flow in a glass tube with ground joints and 2 mL of acetyl chloride/methanol (1:10) was added. The sealed tube was kept in the oven at 50 °C overnight. Once it was cooled down, 2 mL of 1 M potassium carbonate and 500 µL were added to it. The glass tube was vortexed for ~10 sec and then centrifuged for 3 min at 3500 rpm. The top layer containing FAMEs was recovered in an autosampler vial or a glass insert for gas chromatography analysis.

Lipids from plasma, liver TAG, liver PL, visceral fat, and kidney were evaporated under nitrogen flow and then dissolved in 1 mL of sodium-dried diethyl ether and transferred to a glass tube with ground joints. 25 µL of methyl acetate and 25 µL of 1M sodium methoxide were added and the mixture was vortexed. After 5 min of incubation at room temperature, the reaction was stopped by adding 6 µL of glacial acetic acid and vortexing. The mixture was centrifuged for 5 min at 3500 rpm and the supernatant was collected into an autosampler vial. The solvent was evaporated under nitrogen flow. FAMEs were dissolved into hexane in the vial or a glass insert and the vial was vortexed to ensure thorough mixing.

Fecal lipids were methylated with acid-catalyzed and sodium methoxide-catalyzed transesterification methods to differentiate the glycerol-bound FAs from the free FAs. Sodium methoxide-catalyzed transesterification leads to methylation of glycerol-bound FAs whereas, the acid-catalyzed methylation method involves methylation of glycerol-bound FAs and free FAs.

4.7 Gas chromatographic analysis

The FAMEs were analyzed with the GC; Shimadzu GC-2010 and Nexis GC-2030 (Shimadzu, Kyoto, Japan), equipped with AOC-20i autoinjector, flame ionization detector, and wall-coated open tubular column DB-23 (60 m × 0.250 mm I.D. × 0.25 µm film thickness, Agilent Technologies, Santa Clara, CA, USA). The carrier gas was helium, and the injection mode was splitless with an injection volume of 0.5 µL (the split was opened after 1 min). Temperatures were 270 °C for the inlet and 280 °C for the detector. The oven temperature program was: 130 °C held for 1 min, increased 6.5 °C/min to 170 °C, increased 2.75 °C/min to 205 °C and held for 18 min, increased 30 °C/min to 230 °C and held for 2 min.

The FAMEs were analyzed based on the area under the curve calculated using LabSolutions software. Fatty acid identification was based on the external standards (Supelco 37 Component FAME mix (Supelco, St. Louis, MO, USA), GLC-68D, GLC-490, GLC-566C, 22:3(n-3), 22:4(n-6), 22:5(n-6), and 11A (Nu-Check-Prep, Elysian, MN, USA)). The quantification of the liver TAG, plasma TAG, brain, eye, testicle, kidney, visceral fat, heart, lung, and feces was based on the triheptadecanoin, and that of liver PL and plasma PL was based on the dinonadecanoylphosphatidylcholine. The compositional data were calculated using the correction factors from external standards and expressed as relative % of all FAs of TAG and PL fractions of plasma and liver, brain, eye, testicle, visceral fat, lung, kidney, and heart. FAs of feces were expressed as μg/100 mg and molar percent of fecal DHA and 18:0 in relation to the fed doses were calculated.

4.8 Statistical analysis

Statistical analyses were performed with the SPSS 23 and 25 program (IBM, Armonk, NY, USA) to evaluate the significance of differences in the FA composition of lipids in the feces, brain, eye, heart, lung, testicle, kidney, and visceral fat as well as TAG and PL fractions of plasma and liver. Statistical differences in the weights of the rat body and organs among the different intervention groups were also determined.

In **study I**, differences between the (n-3) deficient and (n-3) adequate groups were evaluated with independent samples *t*-test. In **studies II and III**, all data from *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA, NF, and TS groups were checked for normality and variance homogeneity and reported as means ± standard deviation (SD). Analysis of Variance (ANOVA) was tested for significance between the intervention groups and followed by Tukey's HSD with Bonferroni corrections. Tamhane's T2 analysis was used when variances were not homogenous. Statistical significance was determined at $p < 0.05$. Non-parametric Kruskal-Wallis test followed by the Mann-Whitney *U* test was performed when the data were not normally distributed. In **study III**, differences between the *sn*-1 DHA and *sn*-3 DHA groups were evaluated with independent samples *t*-test.

5 RESULTS AND DISCUSSION

5.1 Effect of (n-3) deficiency (Study I)

In this study, mild first-generation (n-3) deficiency was induced by keeping young (age 21 ± 2 days) Sprague–Dawley male rats on a peanut oil based diet for 33 days after weaning. A soybean oil based diet was used as an (n-3) adequate control. In **study I**, the detailed FA composition of plasma, feces, liver TAG, liver PL, brain, eye, testicle, visceral fat, kidney, heart, and lung of (n-3) deficient (*n*= 11) and (n-3) adequate group (*n*= 12) was investigated. The study aimed to investigate the impact of (n-3) deficient diet in the form of peanut oil based rodent chow on the levels of LC (n-3) PUFAs in various tissues and organs in order to understand the extent of LC (n-3) PUFAs depletion as compared to (n-3) adequate diet in the form of soybean oil based rodent chow in young adult rats.

Excluded samples: One rat from the (n-3) deficient group died during the induction phase and thus did not receive the intervention.

5.1.1 Body weight and organ weight

At the end of the experiment, there were no significant differences in the organ weights between the two experimental groups.

Table 5. The organ weights and body weight (g) of the rats at the end of the experiment. Reprinted from the original publication (Kulkarni et al., 2022) with the permission of MDPI.

Groups [1]	(n-3) Deficient	(n-3) Adequate
Eye	0.3 ± 0.02	0.3 ± 0.02
Brain	2.1 ± 0.1	2.0 ± 0.2
Liver	11.7 ± 1.4	12.5 ± 1.5
Heart	1.4 ± 0.2	1.3 ± 0.1
Lung	1.6 ± 0.2	1.5 ± 0.2
Testicle	2.9 ± 0.3	3.0 ± 0.3
Visceral Fat	5.1 ± 1.7	5.2 ± 1.6
Total body weight	366 ± 26	364 ± 35

[1] The (n-3) deficient group received peanut oil based (n-3) fatty acid deficient diet and (n-3) adequate group received soybean oil based AIN-93G standard diet for 33 days. Values for organ weight and total body weight are mean (g) ± SD; *n* = 12 for (*n*-3) adequate group and *n* = 11 for (n-3) deficient group.

The total body weight of the rats did not differ between (n-3) deficient and (n-3) adequate groups at the beginning or end of the experiments (**Table 5**). The average weight gain in the rats in both groups was 269.88 ± 6.01 g.

5.1.2 Fecal lipids

The total fecal lipid and FA levels (µg/100 mg) of rat feces collected during and pooled from the last five days of the experiment are shown in **Table 6**.

Table 6. Glycerol-bound fatty acids and total (free and esterified) fatty acids (µg/100 mg) excreted in the rat feces collected during the last 5 days of the experiment. Modified from the original publication (Kulkarni et al., 2022).

Fatty Acids	Groups [1]			
	(n-3) Deficient	(n-3) Adequate	(n-3) Deficient	(n-3) Adequate
	Glycerol-Bound Fatty Acids		Total Fatty Acids	
16:0	43.12±5.74[a]	37.04±4.87[b]	365.16±100.2	388.19±80.11
18:0, STA	15.22±6.98	19.74±12.77	311.71±75.3	301.73±70.8
18:1(n-9), OA	18.84 ± 4.51[a]	12.66 ± 1.76[b]	186.96 ± 99.24[a]	83.63 ± 17.57[b]
18:2(n-6), LA	6.31 ± 0.92[a]	7.56 ± 1.25[b]	58.28 ± 28.81	63.99 ± 16.89
18:3(n-3), ALA	0.2 ± 0.1[a]	0.42 ± 0.06[b]	1.14 ± 0.84[a]	4.64 ± 1.31[b]
20:4(n-6), ARA	1.96 ± 0.29	1.72 ± 0.53	7.13 ± 1.2[a]	5.18 ± 1.24[b]
20:5(n-3), EPA	0.16 ± 0.08	0.09 ± 0.05	0.09 ± 0.05[a]	0.16 ± 0.07[b]
22:5(n-6), DPAn-6	0.71 ± 0.3	0.93 ± 0.46	1.31 ± 0.84[a]	0.44 ± 0.15[b]
22:5(n-3), DPA	0.02 ± 0.02	0.01 ± 0	0.21 ± 0.1	0.17 ± 0.09
22:6(n-3), DHA	0.13 ± 0.05	0.17 ± 0.04	0.62 ± 0.21	0.77 ± 0.26
Total (n-6)	10.25 ± 1.27	11.44 ± 1.93	79.64 ± 30.66	80.62 ± 18.93
Total (n-3)	1.19 ± 0.2	1.38 ± 0.28	4.96 ± 1.45[a]	7.62 ± 1.56[b]
Total PUFA	11.45 ± 1.24	12.83 ± 2.17	84.60 ± 31.79	88.24 ± 20.42
TFAs	141.6 ± 16.9	127.5 ± 21.5	1635.0 ± 364.0[a]	1098.0 ± 196.9[b]

[1] The (n-3) deficient group received peanut oil based (n-3) fatty acid deficient diet and (n-3) adequate group received soybean oil based AIN-93G standard diet for 33 days. Values are mean (µg/100 mg) ± SD, $n = 12$ for (n-3) adequate group and $n = 11$ for (n-3) deficient group. Values with different superscripts indicate significant differences between the (n-3) deficient and (n-3) adequate feedings ($p < 0.05$). PUFA, polyunsaturated fatty acids; TFAs, total fatty acids; STA, stearic acid; OA, oleic acid; LA, linoleic acid; ALA, α-linolenic; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPAn-6, (n-6) docosapentaenoic acid; DPA, (n-3) docosapentaenoic acid; DHA, docosahexaenoic acid.

Total fecal lipids were 8-10-fold higher than glycerol-bound FAs, and a higher amount of total fatty acids compared to glycerol-bound FAs was seen in all FAs.

Loss of fecal lipids, as total FAs, was higher in the (n-3) deficient group than in the (n-3) adequate group.

DHA levels in both experimental groups as glycerol bound- and total- FA was lower in the (n-3) deficient group compared to the (n-3) adequate group. However the statistical significance could not be reached (p= 0.082 for glycerol-bound FAs and p= 0.161 for total FAs). The similar loss of DHA in both experimental groups could be an indication that DHA in the digestive tract is more efficiently reabsorbed than EPA or ARA. Also, the ALA from the (n-3) adequate diet might not have efficiently converted to DHA. On the other hand, the EPA level of the (n-3) adequate group in total fecal FA was significantly higher than the (n-3) deficient group might suggest the conversion of ALA in the (n-3) adequate group to EPA. Differences in the microbial metabolism of the available FAs or the anatomical and physiological features of the gastrointestinal tract might have affected the result (DeSesso & Jacobson, 2001). The levels of ARA and DPAn-6 were significantly higher in the feces of the (n-3) deficient group as compared to the (n-3) adequate group. This could be an indication of compensation of (n-3) FAs by ARA in the epithelial cells.

The fecal FA composition reflected that of the feed. The saturated FAs, particularly palmitic acid and STA, were the major contributors of fecal FAs in both experimental groups, excreted in the form of calcium and magnesium soaps as described previously (Brink et al., 1995; Jandacek, 1991). The level of glycerol-bound LA was significantly higher in the feces of the (n-3) adequate group than that in the (n-3) deficient group, whereas there was no significant difference found in the total FA. The ALA content in the (n-3) adequate group was significantly higher than the (n-3) deficient group both in glycerol bound- and total FAs.

5.1.3 Plasma lipids

The FA composition of TAG- and PL- rich fractions of fasting plasma reflected the FA profile of feed, especially in the contents of OA, LA, and ALA (**Table 7**). A correlation between plasma FAs and those in the liver, heart, and quadriceps muscle has been previously reported in rats after the dietary treatment with ALA for three weeks (Tu et al., 2013). Fasting plasma contained two to three times more total FAs in PLs than in total FAs in TAGs. The total FA content of TAGs or PLs did not differ between the two groups. The levels of EPA and DHA were significantly lower in the (n-3) deficient group in fasting plasma TAGs and PLs as compared to the (n-3) adequate group. Also, the total (n-3) PUFA content in plasma TAGs and PLs of the (n-3) adequate group was significantly higher than that of the (n-3) deficient group. Whereas, the LC (n-6) PUFAs, such as ARA in the plasma, PLs, and DPAn-6 in both TAGs and PLs, were found to be significantly higher in the (n-3) deficient group as compared to the (n-3) adequate

group. The decrease in LC (n-3) PUFAs, mainly EPA and DHA, in the organs of the (n-3) deficient group was found to have been compensated with an increase in the LC (n-6) PUFAs.

Table 7. Fatty acids of the TAG- and PL- rich fractions of fasting rat plasma (% of total fatty acids) of (n-3) deficient and (n-3) adequate groups. Reprinted from the original publication (Kulkarni et al., 2022) with the permission of MDPI.

Fatty Acids	Groups [1]			
	(n-3) Deficient	(n-3) Adequate	(n-3) Deficient	(n-3) Adequate
	TAG-rich fraction		PL-rich fraction	
18:1(n-9), OA	28.32 ± 3.3[a]	19.81 ± 2.3[b]	5.18 ± 0.5[a]	3.66 ± 0.3[b]
18:2(n-6), LA	22.68 ± 1.98[a]	28.24 ± 2.87[b]	10.72 ± 1.56[a]	12.97 ± 1.52[b]
18:3(n-3), ALA	0.5 ± 0.17[a]	2.19 ± 0.35[b]	0.02 ± 0.01[a]	0.04 ± 0[b]
20:4(n-6), ARA	11.59 ± 2.64	12.39 ± 4.96	27.72 ± 1.69[a]	24.93 ± 0.91[b]
20:5(n-3), EPA	0.25 ± 0.29[a]	1.24 ± 0.3[b]	0.01 ± 0[a]	0.05 ± 0[b]
22:5(n-6), DPAn-6	0.99 ± 0.32[a]	0.33 ± 0.19[b]	1.66 ± 0.4[a]	0.32 ± 0.15[b]
22:5(n-3), DPA	0.12 ± 0.05	0.09 ± 0.1	0.02 ± 0.01	0.01 ± 0
22:6(n-3), DHA	0.73 ± 0.15[a]	2.58 ± 0.66[b]	3.20 ± 0.44[a]	5.74 ± 0.97[b]
Total (n-6) FA	36.59 ± 2.17[a]	42.10 ± 3.67[b]	41.14 ± 0.66[a]	39.67 ± 1.1[b]
Total (n-3) FA	1.61 ± 0.36[a]	6.10 ± 0.56[b]	3.27 ± 0.43[a]	5.87 ± 0.96[b]
Total PUFA	38.21 ± 2.35[a]	48.21 ± 3.74[b]	44.41 ± 0.75[a]	45.54 ± 0.68[b]
TFAs (µg/100 mg)	54.8 ± 25.1	40.5 ± 16.5	127.7 ± 16.5	125.7 ± 21.0

[1] The (n-3) deficient group received peanut oil based (n-3) fatty acid deficient diet and the (n-3) adequate group received soybean oil based AIN-93G standard diet for 33 days. Values are mean (relative % of total FAs) ± SD, n = 12 for (n-3) adequate group and n = 11 for (n-3) deficient group. Values with different superscripts indicate significant differences between the (n-3) deficient and (n-3) adequate feedings ($p < 0.05$). PUFA, polyunsaturated fatty acids; TFAs, total fatty acids; OA, oleic acid; LA, linoleic acid; ALA, α-linolenic; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPAn-6, (n-6) docosapentaenoic acid; DPA, (n-3) docosapentaenoic acid; DHA, docosahexaenoic acid.

5.1.4 Tissue (n-3) fatty acids

The average relative percentage of DHA, EPA, ALA, and total (n-3) FAs of liver TAG, liver PL, brain, eye, testicle, visceral fat, heart, and lung are shown in **Figure 6**.

All the organ tissues showed significantly higher DHA in the (n-3) adequate group than (n-3) deficient group, except the eye (**Figure 6C**). The DHA content in the organs was in the range of 0.02–16.9% of total FAs in the (n-3) deficient group and 0.01–18.7% in the (n-3) adequate group. The rat eye was the richest in DHA in both experimental groups among the studied organs, with more DHA

in the (n-3) adequate group (18.7% of total FAs) than in the (n-3) deficient group (16.9% of total FAs). However, the difference did not reach statistical significance ($p = 0.95$) between the two experimental groups, indicating that the eye DHA in the (n-3) deficient rats was maintained by the (n-3) adequate diet during pregnancy and weaning. Significantly lower retinal DHA (23%) content has been reported in guinea pigs after safflower oil based feeding (low in ALA) compared to canola oil based feeding for 24 weeks (Weisinger et al., 1998). Their study indicated that the loss of DHA in the first 12 weeks was greater than that of the second 12 weeks, implying that the retinal DHA was altered by the ALA supply in the early weeks of life. However, the observed eye DHA of both experimental groups in this study was not affected in 33 days, and a longer intervention is thus needed to achieve (n-3) deprivation in the eye of rats.

The brain tissue was the second highest after the eye in the richness of DHA, with a significantly higher level of DHA in the (n-3) adequate group (15.8% of total FAs) compared to the (n-3) deficient group (13.9% of total FAs). The visceral fat had the lowest DHA levels in both experimental groups among all rat organs. The DHA level of visceral fat of the (n-3) deficient group was 14% of the visceral fat of the (n-3) adequate group, being most affected by the (n-3) deficient diet feeding, whereas the brain DHA of (n-3) deficient group was least affected with about 86% of DHA level of the (n-3) adequate group (**Table 8**). A previous study showed that single-generation (n-3) deprivation in rats for 18 weeks reduced 36% of DHA in the brain (DeMar et al., 2006), whereas (n-3) deprivation in this study lasted only for 33 days and resulted in milder (n-3) deficiency in the brain. Another study in rats showed lower DHA in brain cells and organs after feeding sunflower oil, which is low in ALA, as compared to soybean oil (J. M. Bourre, Francois, et al., 1989). However, the dams in their study were fed either the sunflower or soybean oil based diet for two generations, and the (n-3) deficiency was studied in the following generation of rats.

The EPA level in the brain was very low and almost undetectable. The liver TAG had the highest level of EPA (0.38% of total FAs), whereas the visceral fat had the lowest EPA (0.03% of total FAs) content in the (n-3) adequate group (**Figure 6B**). The range of EPA content among the tissues in the (n-3) deficient group was 0.01–0.06% of total FAs, being the highest in the liver TAG. A significantly lower DHA level in the liver TAG, liver PL, brain, testicle, visceral fat, heart, and lung and the lower EPA levels in the liver TAG, liver PL, eye, testicle, visceral fat, heart, and lung of the (n-3) deficient group as compared to (n-3) adequate group (**Figure 6B**) can be explained by the lack of ALA in the diet of (n-3) deficient group for the EPA and DHA synthesis *in vivo* (Domenichiello et al., 2014, 2017; Igarashi et al., 2007a). The DPA in the liver TAG, heart, and lung of the (n-3) adequate group was significantly higher than in the corresponding tissues in the (n-3) deficient group, whereas, in the liver

PLs, it was non significantly higher in the (n-3) adequate group than (n-3) deficient group. The highest level of DPA was found in the rat heart tissue of both experimental groups, being 0.89% and 0.27% of total FAs in the (n-3) adequate group and (n-3) deficient group, respectively. DPA was not detected in the rat brain, eye, testicle, and visceral fat (**Table 9**).

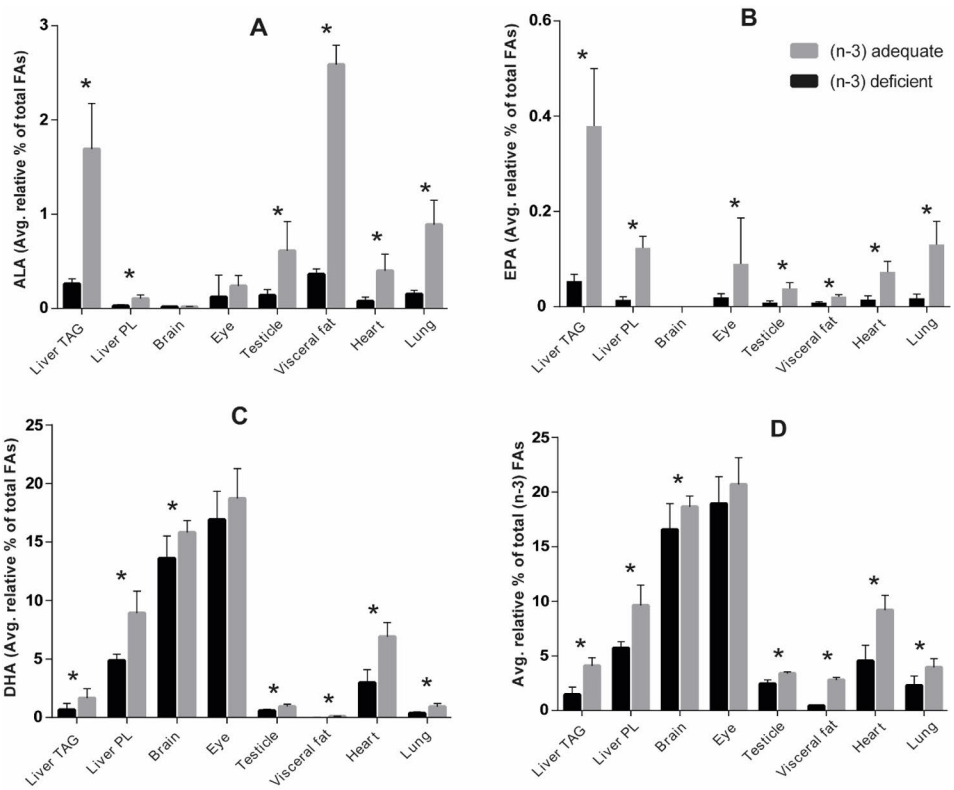


Figure 6. Average relative % (+/ −SD) of (A) ALA, (B) EPA, (C) DHA, and (D) total (n-3) fatty acids in (n-3) deficient (n = 11) and (n-3) adequate group (n = 12) of rat organs after 33 days of the feeding of (n-3) deficient or (n-3) adequate diet. The statistical difference ($p < 0.05$) between the diet groups is indicated by the asterisk. Partly modified from the original publication (Kulkarni et al., 2022).

The incorporation of ALA to rat organs was significantly higher in the (n-3) adequate group when compared to the (n-3) deficient group, except for the brain and eye. The equal ALA content of the brain and eye in both experimental groups (**Figure 6A**) suggested that ALA levels of the eyes or brains are maintained at a steady level despite acute (n-3) deficiency, at least in cases where normal levels of (n-3) were available during pregnancy and weaning. The visceral fat takes up a significant proportion of dietary ALA (McCloy et al., 2004), and this was seen in the visceral fat of the (n-3) adequate group also in this study (**Figure 6A**). The lower levels of longer-chain metabolites, EPA and DHA, of the visceral fat in

the presence of ALA in the (n-3) adequate group suggested that the visceral fat efficiently took up ALA but does not efficiently convert ALA to EPA and DHA (Poudyal et al., 2013). However, the comparatively low ALA in the (n-3) adequate group of other tissues is likely to be the result of β-oxidation of ALA (Fu & Sinclair, 2000).

Table 8. Level of DHA in organs of (n-3) deficient group, with values, expressed as a percentage of the respective values in the (n-3) adequate group. Reprinted from the original publication (Kulkarni et al., 2022) with the permission of MDPI.

Groups	**Percentage** [1]
Visceral fat	14 ± 0.03
Liver TAG	37 ± 0.34
Lung	39 ± 0.14
Heart	43 ± 0.17
Liver PL	50 ± 0.07
Testicle	56 ± 0.15
Eye	83 ± 0.13
Brain	86 ± 0.13

[1] The values are expressed as a percentage ± SD; n = 12 for (n-3) adequate group and n = 11 for (n-3) deficient group. TAG, triacylglycerol; PL, phospholipid.

Table 9. Level of DPA (% of total fatty acids) in the liver, heart, and lung of (n-3) deficient and (n-3) adequate groups.

Groups [1]	(n-3) Deficient	(n-3) Adequate
Liver TAG	0.02 ± 0.02^{a}	0.03 ± 0.02^{b}
Liver PL	0.08 ± 0.03	0.10 ± 0.05
Heart	0.27 ± 0.11^{a}	0.89 ± 0.23^{b}
Lung	0.07 ± 0.04^{a}	0.37 ± 0.15^{b}

[1] The (n-3) deficient group received peanut oil based (n-3) fatty acid deficient diet and the (n-3) adequate group received soybean oil based AIN-93G standard diet for 33 days. Values are mean (relative % of total FAs) ± SD, n = 12 for (n-3) adequate group and n = 11 for (n-3) deficient group. Values with different superscripts indicate significant differences between the (n-3) deficient and (n-3) adequate feedings ($p < 0.05$).
DPA, (n-3) docosapentaenoic acid; TAG, triacylglycerol; PL, phospholipid.

The total (n-3) FA content was significantly higher in rat liver TAG and PL, brain, testicle, visceral fat, heart, and lung of the (n-3) adequate group, whereas the eye of the (n-3) adequate group had a non-significantly higher total (n-3) content compared to the (n-3) deficient group. The eye had the highest and the

visceral fat had the lowest total (n-3) FA content in the (n-3) deficient group among all rat organs (**Figure 6D**).

5.1.5 Tissue (n-6) fatty acids

The average relative percentage of LA, ARA, DPAn-6, and total (n-6) FAs of liver TAG-and PL- rich fractions, brain, eye, testicle, visceral fat, heart, and lung are shown in **Figure 7**.

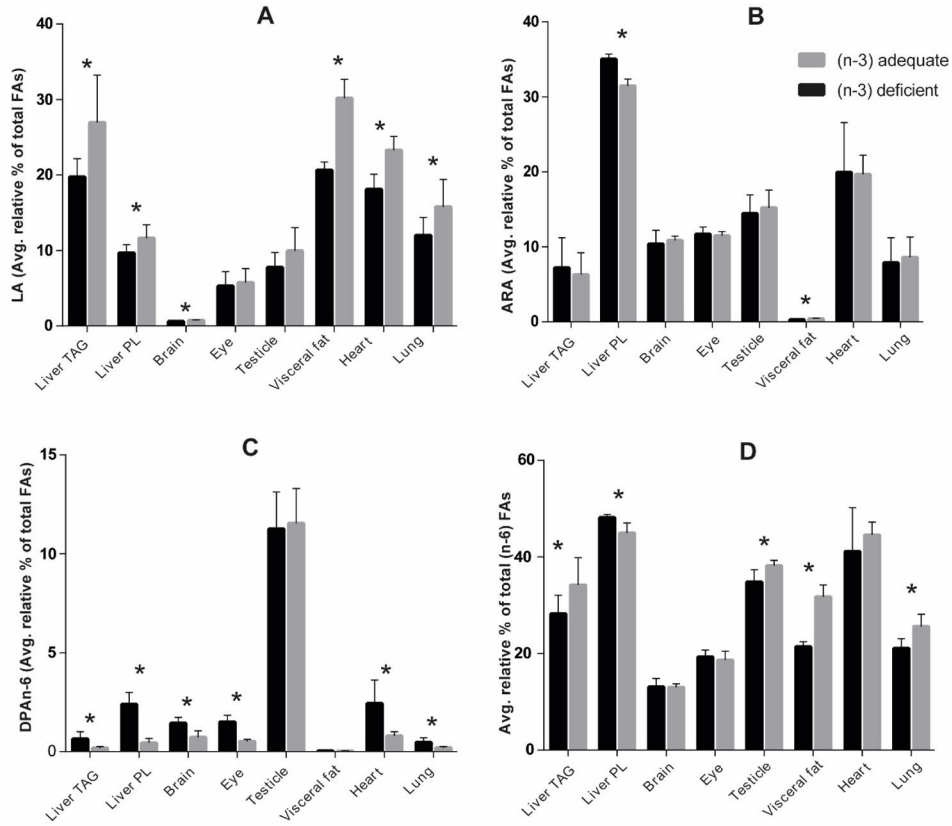


Figure 7. Average relative % (+/ −SD) of (A) LA, (B) ARA, (C) DPAn-6, and (D) total (n-6) fatty acids in (n-3) deficient (n = 11) and (n-3) adequate group (n = 12) of rat organs after 33 days of the feeding of (n-3) deficient or (n-3) adequate diet. The statistical difference ($p < 0.05$) between the diet groups is indicated by the asterisk. Partly modified from the original publication (Kulkarni et al., 2022).

In this study, the (n-3) adequate diet increased LC (n-3) PUFAs as compared to the (n-3) deficient diet as expected. However, it is known that the metabolism of (n-3) FAs depends also on the (n-6) FAs due to the competition of (n-3) and (n-6) FAs for the same enzymes (Brenna et al., 2009b). The LA level from the feed was reflected in the organs in both experimental groups **(Figure 7A)**. ARA, an LC (n-6) PUFAs, is as important as DHA in normal brain growth (Bazan,

2003). The liver PLs of the (n-3) deficient group had significantly higher ARA levels than that of the (n-3) adequate group. Visceral fat of the (n-3) adequate group had significantly higher ARA levels than that of the (n-3) deficient group (**Figure 7B**). The liver PLs had the highest ARA (31–35% of total FAs), whereas the visceral fat had the lowest ARA (0.3–0.4% of total FAs) in both the experimental groups. The higher ARA content in the liver PLs of the (n-3) deficient group compared to the (n-3) adequate group supports the idea that ALA inhibits the conversion of LA into LC (n-6) PUFAs (Smink et al., 2012). Previously perinatal dietary (n-3) FA depletion and subsequent repletion with the (n-3) FA (ALA) in rats showed that chronic (n-3) FA deficiency upregulated liver (n-6) FA biosynthesis and increased membrane ARA composition (McNamara, Jandacek, Rider, Tso, Cole-Strauss, et al., 2010). Other peripheral organs; liver TAG, brain, eye, testicle, heart, and lung; in this study had an almost equal level of ARA and were not affected by the ALA deficiency, suggesting that ARA may be relatively resistant to large changes in the ratio of (n-3) to (n-6) FAs in the diet. The higher ARA content was observed in the heart compared to the brain, eye, testicle, visceral fat, and lung suggesting greater uptake of ARA by the heart after being delivered by the liver (**Figure 7B**).

In addition to ARA, the lower levels of LC (n-3) PUFAs in the (n-3) deficient group were offset by the significant increase in the DPAn-6 in the liver, brain, eye, heart, and lung (**Figure 7C**). The observed significant increase in the LC (n-6) PUFAs of the (n-3) deficient group was also previously seen in the form of DPAn-6 of rat brain phospholipid (DeMar et al., 2006; Enslen et al., 1991), DPAn-6 of rat heart (Igarashi et al., 2008), DTA of liver of growing pigs (Smink et al., 2012), and DPAn-6 in retinal and brain of rhesus monkeys (Neuringer et al., 1986). In addition, the level of DPAn-6 in the rat testicle and visceral fat did not differ between both experimental groups. The DPAn-6 content of rat testicle was the highest (11% of total FAs) as it is one of the most abundant (n-6) PUFAs found in the rat testicle.Whereas, the visceral fat had the lowest DPAn-6 content (0.04% of total FAs) among all analyzed organs in both experimental groups (**Figure 7C**).

The total (n-6) FA content varied among rat organs, showing significantly higher levels in the (n-3) adequate group of liver TAG, testicle, visceral fat, and lung; significantly higher levels in liver PL of the (n-3) deficient group; and almost equal levels in the brain, eye, and heart of both experimental groups (**Figure 7D**).

5.2 Effect of triacylglycerol structure on the bioavailability of DHA (Study II and III)

The bioavailability of DHA was determined from the *sn*-1, -2, and -3 positions of TAG with the 5-day intervention of structured TAGs in rats. **In study II**, the FA composition of plasma and feces was investigated, and also the effect of stereospecificity of DHA on the rat body weight and organ weight was studied. **In study III**, the effect of the dietary structure of DHA in TAGs on the FA compositions of rat liver, brain, eye, testicle, visceral fat, and kidney were studied. Both studies included three DHA-fed groups (*sn*-1 DHA, *sn*-2 DHA, and *sn*-3 DHA) and two control groups (tristearin and normal feed groups, TS and NF, respectively). The NF group was referred to as the (n-3) adequate group in **study I**.

Excluded samples: **In study II**, one rat from the *sn*-1 DHA group was excluded from the body weight analysis on day 5 because of unexplained weight loss. One rat in the *sn*-1 DHA group of testis was excluded from the organ weight analysis due to its underdeveloped appearance. Two rats from *sn*-3 DHA group were excluded from plasma lipid analysis since their lipid content was standing out from the rest of the group. **In study III**, one rat in the *sn*-1 DHA group was excluded from the FA composition of testicle due to its underdeveloped appearance. The FA composition analysis of kidney excluded two rats each from *sn*-1 DHA and tristearin groups and one rat each from *sn*-2 DHA and *sn*-3 DHA groups since their lipid content was standing out from the rest of the group (> 2SD away from the mean).

5.2.1 Body weight and organ weight

The rats were weighed on the baseline day (last day of the induction phase) and daily during the 5-day intervention (**Table 10A**). There were no significant differences in the body weight of the rats at the baseline or during intervention days 1-4. The outcome was possibly due to the *ad libitum* availability of feed. On day 5, the NF group had the highest body weight among all groups (statistically significant difference from the *sn*-1 group only, $p = 0.014$) and also was the only group that gained weight at the end of the intervention. This may indicate some possible effect of the fecal fat loss which was lowest in the NF group and highest in the TS group (**Table 11**) or adaptation to the few experimental pellets before their daily ration in other experimental groups. The effect of lower weight gain in the DHA-fed groups during the relatively short period of experimental feeding might have resulted from the (n-3) PUFA's anti-obesity properties (Li et al., 2008). Such properties have been seen in rodent models by Hassanali et al. (2010) and Li et al. (2017) but not by Bandarra et al. (2016).

Table 10A. The body weight (g) of the rats during the last day of the induction phase (baseline) and during the five days of intervention. Reprinted from the original publication (Linderborg et al., 2019) with the permission of Elsevier.

	Group[1]				
	sn-1 DHA	*sn*-2 DHA	*sn*-3 DHA	Tristearin	Normal feed
Baseline[2]	325±34	336±15	346±27	336±21	326±36
Day 1	310±32	322±13	332±26	321±21	337±36
Day 2	333±34	343±14	352±24	336±22	343±34
Day 3	330±32	338±15	343±25	332±20	348±35
Day 4	342±33	354±13	359±18	348±22	349±35
Day 5[3]	328±32^{b}	339±13^{ab}	344±22^{ab}	333±22^{ab}	364±35^{a}

Table 10B. Weight of rat organs (g) after the intervention phase. Modified from the original publication (Linderborg et al., 2019).

	Group[1]				
	sn-1 DHA	*sn*-2 DHA	*sn*-3 DHA	Tristearin	Normal feed
Eyes	0.3±0.02	0.3±0.03	0.3±0.02	0.3±0.03	0.3±0.02
Brain	2.0±0.1	2.0±0.07	2.0±0.07	2.0±0.1	2.0±0.2
Liver[3]	9.8±1.2^{b}	10.4±1.02^{b}	11.0±0.9^{b}	10.2±0.9^{b}	12.5±1.5^{a}
Heart	1.3±0.1	1.3±0.1	1.4±0.2	1.3±0.2	1.3±0.1
Lungs	1.4±0.1	1.4±0.2	1.5±0.12	1.5±0.2	1.5±0.2
Testicle	3.0±0.2	3.0±0.2	3.0±0.3	3.0±0.2	3.0±0.3
Kidneys	2.7±0.3	2.7±0.2	2.8±0.3	2.8±0.2	2.8±0.1
Visceral fat	4.2±2.0	5.1±1.3	4.9±1.5	4.0±1.23	5.2±1.6
Liver to body weight ratio	0.03±0	0.03±0	0.03±0	0.03±0	0.03±0

[1] The groups *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA, and tristearin received omega-3 fatty acid deficient diet for the 4-week induction period. Thereafter, for the 5-day intervention period the *sn*-1 DHA, *sn*-2 DHA, and *sn*-3 DHA groups received structured triacylglycerols with DHA in the indicated *sn*-position and two stearic acid residues in the other positions (360 mg/day), and the tristearin group received tristearin for the 5- day intervention period, in addition to the *ad libitum* omega-3 fatty acid deficient diet. During the 4-week induction period and the 5-day intervention period, the normal feed group received soy bean oil based normal feed.

[2] Body weight after the 4-week induction phase on peanut oil (*sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA, and TS) or soy bean oil (NF) based feed.

[3]Values with different superscripts differ significantly in each column ($p < 0.05$). Values for body weight are mean (g) ± SD, n=12 except for group *sn*-1 on day 5 where n=11. Values for organ weight are mean ± SD, n=12 except for the testicle of the *sn*-1 group (n=11).

The weight of the livers in the NF group was significantly higher compared with the livers of the other groups, but the liver-to-body weight ratios did not differ significantly. The fresh weight of eyes, brain, lungs, heart, testicles, kidneys, or visceral fat did not differ between the groups (**Table 10B**).

5.2.2 Fecal lipids

The total fecal FAs, DHA, and DPA in the form of glycerol-bound FA and total FA of study and baseline samples are shown the **Table 11**.

The supplementation of DHA increased its content in the feces in all DHA-fed groups compared with the control groups (**Table 11**). The fecal DHA of about 0.1-0.5% (molar percent) of total DHA fed to the rats (**Figure 8C**) indicated an overall high extent of absorption of DHA regardless of the positional distribution of DHA in TAGs. In the study feces, the total concentration, as well as the molar percentage of DHA, was four times higher and that of the glycerol-bound DHA was more than five times higher in the *sn*-1 DHA and *sn*-3 DHA groups compared with the corresponding levels in the *sn*-2 DHA group (**Table 11, Figure 8C**). This indicated a superior extent of absorption of DHA from the *sn*-2 position compared with the *sn*-1 and *sn*-3 positions. A significant part of the DHA was excreted as free FA as the total fecal DHA level was about 2-3 times higher than the glycerol-bound DHA (**Table 11**). Chylomicron TAGs of rats fed oil have retained about 85% of the original FAs in the *sn*-2 position (Yang & Kuksis, 1991). Thus, interesterification of *sn*-2 monoacylglycerol to *sn*-1/3 monoacylglycerol and subsequent hydrolysis during digestion may explain the presence of non-glycerol bound DHA in the feces of the *sn*-2 DHA group (Couëdelo et al., 2012; Karupaiah & Sundram, 2007). No difference was found between the fecal loss of DHA from *sn*-1 and *sn*-3 positions. The *sn*-1 and *sn*-3 DHA groups showed higher DPA compared to the *sn*-2 DHA group indicating a possible retroconversion from DHA to DPA in the digestive tract.

The loss of STA cleaved from the *sn*-1 and *sn*-3 positions to feces was greater than STA of the *sn*-2 position ($p = 0.001$, $p = 0.004$ respectively, **Table 11, Figure 8B**). The saturated FAs lost to feces as calcium and magnesium soaps have been described previously (Brink et al., 1995; Jandacek, 1991; Mattson et al., 1979). Overall, the fecal fat loss in the glycerol-bound FA forms was only about 5-7% of the total lipid loss in the *sn*-1 DHA, *sn*-2 DHA, and *sn*-3 DHA groups (**Table 11**). The lower loss of the glycerol-bound STA ($p < 0.01$) in the *sn*-2 DHA group compared with the *sn*-1 DHA and *sn*-3 DHA groups (**Table 11, Figure 8A**) might indicate low absorption of distearin.

Table 11. Total lipid and DHA content excreted in feces (µg/100 mg) of different intervention groups at the baseline and during the five-day intervention feeding phase. Modified from the original publication (Linderborg et al., 2019).

Lipid	Groups[1]				
	sn-1 DHA	*sn*-2 DHA	*sn*-3 DHA	Tristearin	Normal feed
	Glycerol-bound fatty acids				
DHA in study feces[2]	8.58±3.14^{b}	1.69±0.42^{c}	10.72±2.37^{b}	0.20±0.17^{a}	0.17±0.04^{a}
DHA in baseline feces[3]	0.12±0.04^{b}	0.14±0.09^{ab}	0.14±0.05^{b}	0.12±0.04^{b}	0.22±0.05^{a}
Total FAs in study feces[4]	135.2±35.4^{ac}	121.6±19.3^{a}	149.8±23.1^{c}	2541.1±204.8^{b}	127.5±21.5^{ac}
Total FAs in baseline feces[5]	135.7±18.5^{ab}	142.74±17.0^{a}	125.8±13.7^{ab}	121.7±13.5^{b}	126.8±18.4^{ab}
DPA in study feces[2]	0.23±0.21	0.23±0.22	0.16±0.16	0.09±0.08	0.01±0
	Total fatty acids				
DHA in study feces[2]	22.41±5.55^{b}	4.82±1.82^{c}	21.08±7.23^{b}	0.62±0.21^{a}	0.77±0.26^{a}
DHA in baseline feces[3]	0.20±0.14^{ab}	0.22±0.15^{ab}	0.27±0.15^{ab}	0.16±0.1^{b}	0.36±0.14^{a}
Total FAs in study feces[4]	2056.4±232.4^{bc}	2272.2±144.2^{b}	1972.3±239.2^{c}	3904.9±332.4^{a}	1098.0±196.9^{d}
Total FAs in baseline feces[5]	1669.0±183.3^{ab}	1951.1±314.5^{a}	1675.7±411.2^{ab}	1577.0±234.5^{b}	1110.2±244.7^{c}
DPA in study feces[2]	2.33±1.45^{a}	0.61±0.55^{b}	2.04±0.98^{a}	0.26±0.11^{bc}	0.17±0.09^{c}

[1] The groups *sn*-1 DHA, *sn*-2 DHA, and *sn*-3 DHA and tristearin received (n-3) fatty acid deficient feed for the 4-week induction period. During 5-day intervention period the *sn*-1 DHA, *sn*-2 DHA, and *sn*-3 DHA groups received structured TAGs with DHA in the indicated *sn*-position and two STA residues in the other positions (360 mg/day), and the tristearin group received tristearin for the 5 day intervention period, in addition to the *ad libitum* (n-3) fatty acid deficient feed. During the 4-week induction period and the 5-day intervention period, the normal feed group received soy bean oil based normal feed. [2] DHA and DPA content in fecal samples from the 5 days of intervention feeding; [3] DHA content in fecal samples collected on the last day of the induction phase; [4] Average total fatty acid content in the feces collected during the 5 days of the intervention feeding phase; [5] Average total fatty acid content in the feces collected on the last day of the induction phase. Values are mean ± SD, n=12 in each group. Values with different letters in the same row differ significantly ($p < 0.05$).

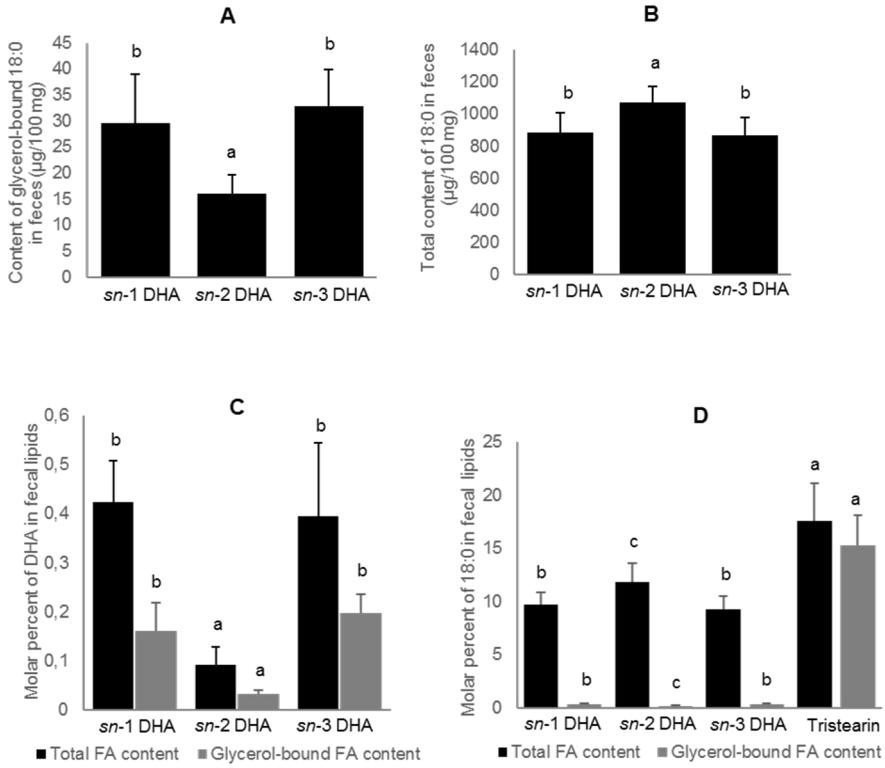


Figure 8. Fecal content of [A] glycerol-bound stearic acid (18:0) (µg stearic acid in 100 mg feces ± SD) and [B] total stearic acid (18:0) (µg stearic acid in 100 mg feces ± SD) [C] total and glycerol-bound DHA in molar percent (moles in the feces during the five day intervention period compared with the moles fed in the structured TAGs) (mean ± SD) [D] stearic acid (18:0) in molar percent (mean ± SD) during the five days of intervention in rats fed with distearoyl-docosahexaenoyl-glycerols with DHA in *sn*-1, *sn*-2 or *sn*-3 positions and tristearin. *n*=12. Bars with different letters differ from one another within the fatty acid type (total or glycerol-bound). Reprinted from the publication (Linderborg et al., 2019) with the permission of Elsevier.

Saturated FA, STA over palmitic acid, was included in the design of the synthesis of the experimental TAGs. STA has been reported to have a neutral or lowering effect on total and low-density lipoproteins (LDL)-cholesterol compared with other saturated FAs (Hunter, 2001). The possible reason that STA does not raise plasma cholesterol could be its rapid conversion into oleic acid (Bonanome et al., 1992) compared to palmitic acid. In addition, the effect of positional distribution of STA and palmitic acid in synthetic fats was studied for atherogenic potential in rabbits (Kritchevsky et al., 2000). The amount of total FAs lost to study feces of the TS group was 2 times compared with in the *sn*-1 DHA, *sn*-2 DHA, and *sn*-3 DHA groups and was 4-fold when compared with the

NF group (**Table 11**). By a rough estimation not taking into account the STA in the feed or endogenous STA, about 17 molar percent of fed STA was lost to feces in the TS group (**Figure 8D**). Whereas, about 10% of STA was lost in the *sn*-1 DHA and *sn*-3 DHA groups and about 12% of STA loss was observed in the *sn*-2 DHA group. The loss of STA esterified with glycerol was less than 0.4% molar percent in the *sn*-1 DHA, *sn*-2 DHA, and *sn*-3 DHA groups, while most (about 15%) of the total loss of STA in the TS group was bound in glycerol. While the reduced lipolysis, as well as reduced transportation to the lymph of tristearin compared with triolein, has been detected previously (Bergstedt et al., 1990), the absorption has been shown to increase when other lipids are ingested together with tristearin (Bergstedt et al., 1991).

5.2.3 Plasma lipids

The selected FAs of fasting rat plasma TAG- and PL-rich fractions are shown in **Table 12**.

The 5-day intervention with structured TAG containing DHA increased the DHA content in the plasma TAGs and PLs of DHA-fed groups compared to the TS or NF groups. Overall, DHA was efficiently absorbed in all three DHA-fed groups and thus the differences in the fecal loss of DHA between the groups fed with DHA groups were not reflected in fasting plasma. On the contrary, seal oil with DHA at *sn*-1/3 positions was shown to lower TAG DHA concentrations in rat plasma compared with fish oil containing DHA mainly at the *sn*-2 position (Yoshida et al., 1996). However, the contrast in results could be due to the longer feeding trial (3 weeks) and differences in the dietary FA composition. In another study, the positional distribution of EPA, but not DHA, affected the incorporation of these FAs into monocytes and neutrophils in mice (Kew et al., 2003). In this study, no significant differences were found in the levels of DHA, STA, or any other FAs in the fasting plasma TAGs or PLs between the *sn*-1 DHA, *sn*-2 DHA, and *sn*-3 DHA groups.

The NF group had higher DHA content compared with the TS group in both TAGs and PLs ($p = 0.008$, $p < 0.01$ respectively) indicating conversion of ALA to DHA in the NF group (**Table 12**). The significantly higher DHA levels in plasma TAG and PL of DHA-fed groups compared to the NF group indicate that the DHA dose in this study was at least adequate, if not high.

In the plasma TAG, the equal levels of EPA in the DHA-fed groups and NF group as well as higher EPA content than TS group ($p < 0.05$) suggested the retroconversion of DHA to EPA in the DHA-fed groups and conversion of ALA to EPA in the NF group. A similar trend was seen in the plasma PL. The higher LA content in the feed (**Table 4**) caused the higher level of ARA in plasma PLs of the NF group compared with the other groups. The level of ARA in plasma PLs was significantly higher in the TS group than in the *sn*-3 DHA group ($p <$

0.05). In plasma TAGs, the content of ARA in the TS and NF groups was equal and higher than ARA levels in the DHA-fed groups. This indicates compensation for the lack of DHA with ARA as detected previously (Wainwright et al., 1999).

Table 12. Fatty acids of the TAG and PL fractions of fasting rat plasma (μg/100 mg) of different intervention groups. Modified from the original publication (Linderborg et al. 2019).

Fatty acids	Group[1]				
	sn-1 DHA	*sn*-2 DHA	*sn*-3 DHA	Tristearin	Normal feed
TAG- rich fraction					
18:1(n-9), OA	14.2±6.6^{ab}	14.2±5.6^{ab}	11.7±3.2^{ab}	15.1±4.9^{a}	8.3±3.9^{b}
18:2(n-6), LA	6.16±2.66^{b}	6.36±2.33^{ab}	5.49±1.35^{b}	5.88±1.91^{b}	11.54±5.06^{a}
18:3(n-3), ALA	0.12±0.07^{a}	0.14±0.05^{a}	0.11±0.03^{a}	0.10±0.05^{a}	0.91±0.42^{b}
20:4(n-6), ARA	3.28±1.10^{ab}	2.95±0.76^{b}	3.0±0.84^{b}	4.57±1.10^{a}	4.42±1.33^{a}
20:5(n-3), EPA	0.43±0.26^{a}	0.52±0.22^{a}	0.37±0.16^{a}	0.07±0.06^{b}	0.47±0.19^{a}
22:5(n-6), DPAn-6	0.14±0.07^{a}	0.13±0.08^{a}	0.12±0.05^{a}	0.45±0.19^{b}	0.14±0.12^{a}
22:5(n-3), DPA	0.02±0.02	0.03±0.01	0.03±0.03	0.03±0.01	0.02±0.01
22:6(n-3), DHA	3.58±1.91^{a}	3.46±1.40^{a}	2.95±0.92^{a}	0.29±0.10^{b}	1.06±0.58^{c}
Total (n-6)	10.1±3.7^{ab}	9.9±3.1^{b}	8.9±2.27^{b}	11.6±3.2^{ab}	16.6±6.1^{a}
Total (n-3)	4.2±2.2^{b}	4.3±1.6^{b}	3.6±1.1^{b}	0.7±0.3^{a}	2.7±1.2^{b}
Total PUFA	14.3±5.7	14.0±4.7	12.4±3.1	12.2±3.4	19.0±7.2
Total TAG FAs	43.5±17.7	43.7±15.6	37.9±9.4	42.8±12.1	40.5±16.5
PL- rich fraction					
18:1(n-9), OA	7.70±1.64^{b}	7.71±1.38^{b}	7.77±1.73^{b}	8.40±1.52^{b}	4.60±0.85^{a}
18:2(n-6), LA	9.62±2.09^{b}	9.84±1.17^{b}	9.13±2.15^{b}	8.34±1.47^{b}	16.26±2.89^{a}
18:3(n-3), ALA	0.01±0.01^{b}	0.01±0.0^{b}	0.01±0.0^{b}	0.01±0.0^{b}	0.05±0.01^{a}
20:4(n-6), ARA	21.84±4.76^{bc}	21.84±40^{bc}	18.68±3.30^{c}	26.0±4.95^{b}	31.38±5.58^{a}
20:5(n-3), EPA	0.11±0.06^{bc}	0.12±0.05^{c}	0.08±0.03^{bc}	0.01±0^{a}	0.07±0.01^{b}
22:5(n-6), DPAn-6	0.36±0.35^{b}	0.27±0.06^{b}	0.26±0.07^{b}	2.3±0.77^{a}	0.43±0.28^{b}
22:5(n-3), DPA	0.04±0.04	0.02±0.0	0.02±0.01	0.02±0.01	0.02±0.0
22:6(n-3), DHA	9.88±2.16^{ab}	10.69±1.96^{a}	9.81±1.88^{a}	3.71±0.61^{c}	7.26±2.04^{b}
Total (n-6)	33.1±6.3^{bc}	33.5±5.0^{bc}	29.4±5.4^{c}	38.0±6.8^{b}	49.9±8.2^{a}
Total (n-3)	10.1±2.2^{ac}	10.9±2.0^{c}	9.9±1.9^{c}	3.8±0.6^{b}	7.4±2.1^{a}
Total PUFA	43.1±7.8^{b}	44.3±6.8^{b}	39.3±7.1^{b}	41.7±7.2^{b}	57.3±9.7^{a}
Total PL FAs	104.2±16.9^{ab}	106.9±15.2^{ab}	98.5±16.3^{ab}	104.7±16.1^{a}	125.7±21.0^{b}

[1] The groups *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA and tristearin received omega-3 FA deficient diet for the 4-week induction period. Thereafter, for the 5 day intervention period the *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA groups received structured triacylglycerols with DHA in the indicated *sn*-position and two stearic acid residues in the other positions (360 mg/day for each rat), and the tristearin group received tristearin (360 mg/day for each rat) for the 5 day intervention period, in addition to the *ad libitum* omega-3 FA deficient diet. During the 4-week induction period and the 5 day intervention period the normal feed group received soy bean oil based normal feed.

Values are mean ± SD, n=12 except group *sn*-3 (n=10). Values with different superscripts in same row differ significantly ($p < 0.05$). PUFA: polyunsaturated fatty acids.

Similarly, significantly higher DPAn-6 levels in plasma TAG and PL fraction of the TS group compared to others suggested that the decrease in the LC (n-3) PUFAs in the TS group was compensated with an increase in the LC (n-6) PUFAs.

The composition of plasma FAs reflected the FA profile of the feed, which is seen in the contents of oleic acid, LA, and ALA. The fasting plasma contained more than double the amount of PL FAs compared with TAGs (**Table 12**). No differences were found in the total amount of TAGs in the fasting plasma between the groups, but the plasma of the NF group had more PLs than the TS group ($p = 0.03$).

5.2.4 Tissue fatty acids

The average relative percentage of DHA, EPA, ARA, DPAn-6, STA, and ALA in the liver TAG-rich fraction, live PL-rich fraction, brain, eye, testicle, visceral fat, and kidney of *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA, TS, and NF groups are presented in **Figure 9**.

5.2.4.1 Docosahexaenoic acid

The highest DHA accumulation was found in the eye (16- 19% of total FAs) of rats of all the intervention groups, followed by the brain (13- 16 % of total FAs) (**Figure 9A**). The brains of the NF group showed significantly higher DHA level compared to the TS group. There were no significant differences found in the DHA levels of eyes between the intervention groups. The *sn*-position of DHA in the TAG molecule did not influence the accumulation of DHA in the brain or the eye of the rats. However, a slight but statistically insignificant increase in the DHA level in the eye of the *sn*-1 DHA group compared to *sn*-3 DHA was observed indicating a possible difference also between the bioavailability of DHA between the two primary positions of dietary TAGs.

In the liver lipids, a higher DHA content was found in the liver PL fraction compared to the TAG fraction in all intervention groups. The difference in the percentage of DHA between PL and TAG was approximately 3-fold in the DHA-fed groups, approximately 10-fold in the TS group, and 5-fold in the NF group. DHA feeding resulted in significantly higher ($p < 0.001$) content of DHA in both TAG (mean 3.5-4.8% of total FAs) and PL (approximately 13%) of the DHA-fed groups compared to the NF group (1.6% in TAG and 8.9% in PL) and the TS group (0.5% in TAG and 5.2% in PL).

In the liver TAG, when *sn*-1 and *sn*-3 DHA groups were compared separately (**Figure 10A**), the *sn*-1 DHA group showed significantly ($p = 0.035$) higher DHA content (4.8% of total FA) compared to the *sn*-3 DHA group (3.5% of total FA), whereas no significant differences were found in the DHA level of liver PL (**Figure 10B**). On the other hand, the significant difference for DHA among the

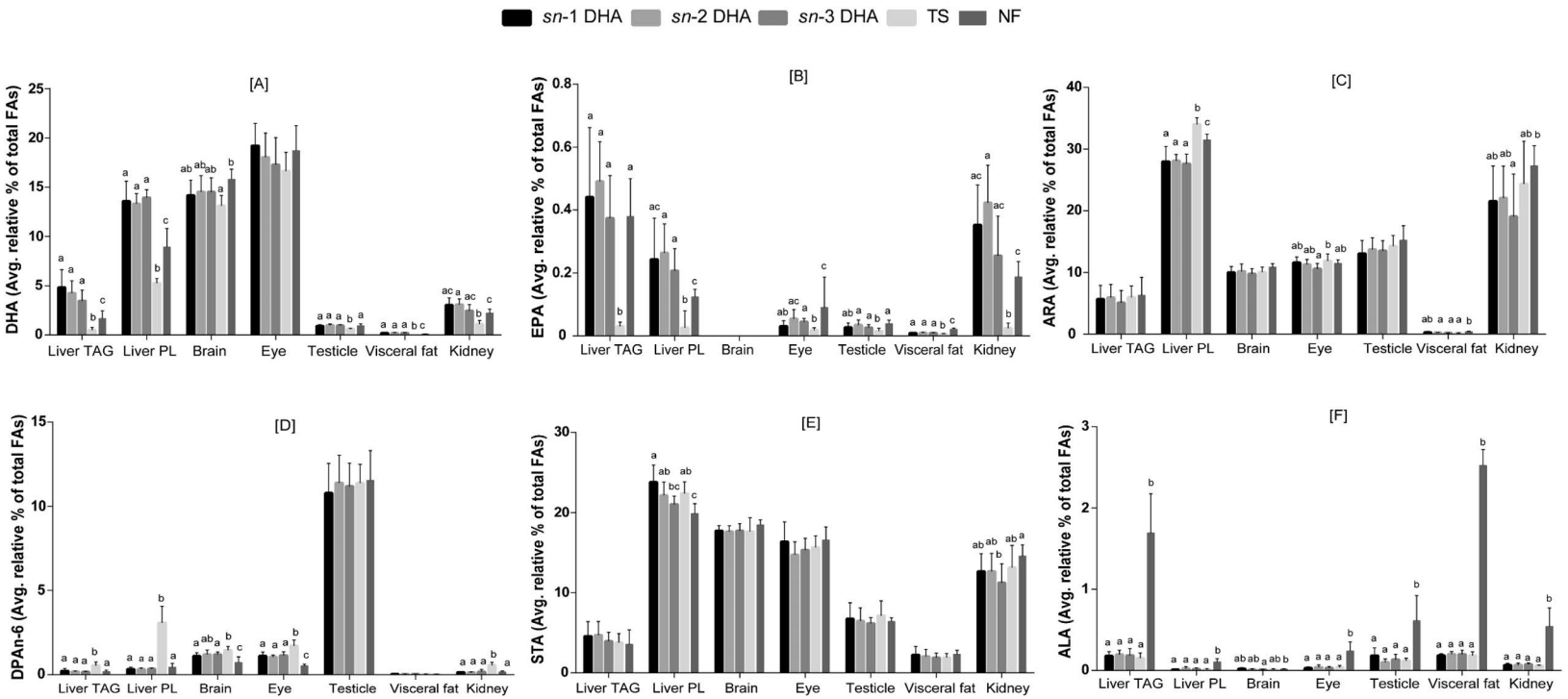


Figure 9. Average relative % (+/ −SD) of (A) DHA, (B) EPA, (C) ARA, (D) DPAn-6, (E) STA, and (F) ALA of total fatty acids in *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA, tristearin and normal feed groups of rat organs after 5-day intervention phase. *n*= 11 for testicle of *sn*-1 DHA; for rat kidney: *n*= 10 in *sn*-1 DHA group, *n*= 11 in *sn*-2 DHA group, *n*= 11 in *sn*-3 DHA group and *n*= 10 in tristearin group; and *n*= 12 for all the other organs;. Bars with different letters differ from one another within each organ for the fatty acid.

DHA-fed groups was not seen when all five intervention groups were compared with each other (**Figure 9A**). Previously, Christensen & Høy (1997) reported that the 3-week intervention feeding DHA (3.8% of dietary FAs) either as a specific structured oil with DHA located primarily in the *sn*-2 position or a randomized TAG with equal DHA distribution at *sn*-1/3 in newborn rats did not result in significant differences in DHA levels in the liver and brain. However, the two enantiospecific TAGs were not separated and the liver lipids were not fractionated into TAG and PL in their study. Therefore, the results may not be directly comparable to the findings of this study. In this study, we did not find a statistically significant difference in DHA level in the PL fraction of the liver among the *sn*-1 DHA, *sn*-2 DHA, and *sn*-3 DHA groups, which is in line with the previous findings considering the dominance of PLs in the lipid fraction of the liver.

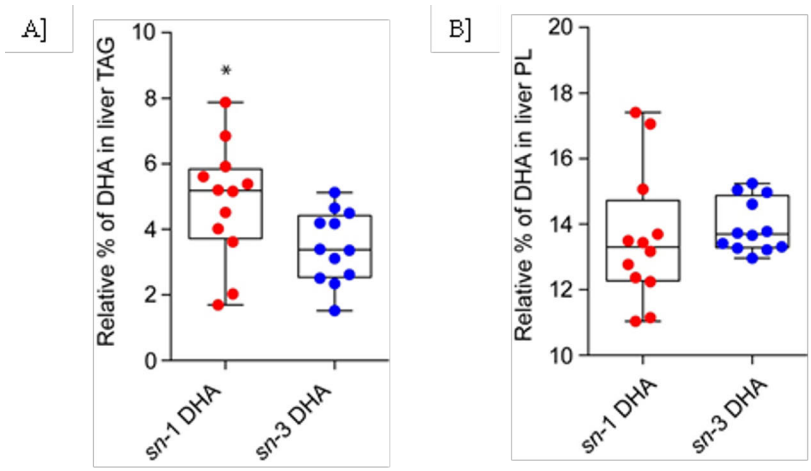


Figure 10. DHA (% of total fatty acids) in liver TAG and PL (mean ± SD) in rats after a 5-day intervention with 360 mg/day of structured TAG (n = 12 in each group). Experimental fat contained DHA either at the *sn*-1 or *sn*-3 position of TAG and 18:0 at the remaining positions (*sn*-1 DHA, and *sn*-3 DHA groups, correspondingly). Asterisk indicates statistical significance among *sn*-1 DHA and *sn*-3 DHA groups of liver TAG and PL ($p < 0.05$).

In the testicle, visceral fat, and kidney, the TS group had a significantly lower DHA content as compared to the NF group ($p < 0.001$) as well as to the DHA-fed groups ($p < 0.05$). The visceral fat DHA level in DHA-fed groups was significantly higher compared to that in the NF group ($p < 0.001$), whereas the DHA level in the testicle and kidney did not differ between the NF group and DHA-fed groups (For kidney, only significantly different to *sn*-2 DHA group).

Among the studied organs, the DHA content of visceral fat was lowest in all the intervention groups (0.01- 0.2% of total fatty acids) (**Figure 9A**).

Previous studies investigated the effect of the TAG structure on the general accretion of DHA in different organ tissues, which showed an increased level of DHA in the brain, erythrocytes, and liver (Bandarra et al., 2016) and the brown adipose tissue (Lopes et al., 2017) from a structured TAG with DHA at the *sn*-2 position as compared to linseed oil, fish oil or fish oil ethyl esters in 10-week-old hamsters. However, the effect of enantiospecific TAGs consisting of DHA was never studied before to compare the effect of DHA from *sn*-1 and *sn*-3 positions on the accumulation of DHA in the organs. In a postprandial study of lymph lipids of rats, Fujimoto et al. (1998) showed more efficient absorption of DHA from the *sn*-2 position than from the *sn*-3 position. Despite the previous evidence of higher bioavailability of *sn*-2 DHA compared to *sn*-1/3 DHA, results from this study did not confirm the higher accumulation of DHA from the *sn*-2 position as compared to the *sn*-1 or *sn*-3 positions in studied rat organs.

5.2.4.2 Eicosapentaenoic acid

The highest level of EPA was found in the liver TAG fraction of DHA-fed groups (0.4- 0.5 % of total FAs) among all studied organs. However, no significant difference was seen between the three DHA-fed groups (**Figure 9B**). EPA level in the brain is typically 250-300 times lower than DHA because of its rapid β-oxidation upon uptake in the brain PLs (C. T. Chen et al., 2009). EPA was almost undetectable in the brain samples in this study.

A higher level of EPA in the liver and kidney of the NF group compared to the TS group showed evident conversion of dietary ALA to EPA, whereas the retroconversion of DHA to EPA likely provided the main source of EPA in these organs in the DHA-fed groups on top of the (n-3) deficient base diet. On the other hand, in the (n-3) deficient TS group, the EPA level was almost completely depleted due to the lack of precursors in the diet. As the positional distribution of DHA in dietary TAG did not result in different organ content of EPA among these groups, it is interesting to notice the equal efficiency of retroconversion of DHA to EPA. Retroconversion of DHA to EPA has previously been observed in lymph (Ikeda et al., 1995) and livers of rats supplemented with DHA (Kaur et al., 2010).

5.2.4.3 Arachidonic acid

The liver PL fraction of all intervention groups had the highest ARA content (28-34 % of total FAs) among all studied organs. However, the position of DHA on the TAG did not affect the accumulation of ARA in any of the organs of DHA-fed groups (**Figure 9C**).

The DHA level in the liver PL fraction was increased in DHA-fed groups compared to the TS group and the NF group (**Figure 9A**) which was accompanied by a decrease in the ARA levels (**Figure 9C**). LC (n-3) PUFAs have an inhibitory effect on Δ6- and Δ5-desaturases, which are rate-limiting enzymes in the biosynthesis of ARA from LA (Cho et al., 1999; Garg et al., 1988) and observation from this study supports the finding. On the other hand, the higher ARA levels in liver PL of the TS group compared to the levels in the DHA-fed group and the NF group was likely a compensative mechanism to maintain the fluidity of the cell membrane (Christensen & Høy, 1997; Wainwright et al., 1999). A similar trend in the ARA levels was seen in the liver TAGs, brain, eye, testicle, and kidney, even though statistically significant differences were not reached.

5.2.4.4 Docosapentaenoic acid (n-6)

TS group showed significantly higher DPAn-6 levels compared to all other groups in the liver TAG, liver PL, eye and kidney, and also in the brain except for the *sn*-2 DHA group (**Figure 9D**). The brains of the TS group were significantly different from *sn*-1 DHA, *sn*-3 DHA, and NF groups. There were no significant differences in the DPAn-6 content of the testicle and visceral fat. However, it should be noted that the highest (10.5- 11.6% of total FAs) amount of DPAn-6 was found in the rat testicle being one of the important constituent of the organ. Similar observations were found by Lin & Salem (2007) while studying the uptake and metabolism of deuterated ALA and LA in rats.

5.2.4.5 Dcosapentaenoic acid (n-3)

DPA is an intermediate in the conversion of ALA to DHA, as well as in the retroconversion pathway from DHA to EPA. Overall, the DPA levels were low in all the organs studied, being the highest in the kidney (0.1-0.2% of total FA) and practically absent in the brain, eye, testicle, and visceral fat (**Table 13**).

The liver PL had higher DPA content than liver TAG and the DPA level in the liver PL of the TS and NF groups was 2-fold higher than in the DHA-fed groups. The DHA in the diet provided to the DHA-fed groups likely has suppressed the conversion of ALA to DPA and DHA in the liver. It has been previously shown that (n-3) deficiency upregulated the DHA synthesis in the liver from precursor ALA to meet the DHA demand of peripheral organ tissues (Igarashi et al., 2007a), which likely contributed to maintaining the levels of DPA in the (n-3) deficient TS group.

Table 13. Level of DPA (avg relative % of total fatty acids) in the liver and kidney of different intervention groups.

	Group[1]				
	sn-1 DHA	*sn*-2 DHA	*sn*-3 DHA	Tristearin	Normal feed
Liver TAG	0.03±0.02	0.02±0.02	0.02±0.01	0.02±0.04	0.03±0.02
Liver PL	0.04±0.01^{a}	0.05±0.02^{a}	0.05±0.01^{a}	0.1±0.02^{b}	0.1±0.05^{b}
Kidney	0.13±0.05^{a}	0.14±0.04^{a}	0.11±0.05^{ab}	0.07±0.02^{b}	0.24±0.04^{c}

[1] During the 5-day intervention period, three groups received structured triacylglycerols (360 mg/day for each rat) with docosahexaenoic acid (DHA) either at *sn*-1, *sn*-2, or *sn*-3 positions and two stearic acid residues in the remaining positions (*sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA groups), and the tristearin group received tristearin (360 mg/day for each rat), in addition to the *ad libitum* omega-3 FA deficient diet. The normal feed group received soybean oil based standard feed from the beginning until the end of the animal trial. Values are mean ± SD, *n*=12 in each group for liver TAG- and PL- rich fractions, for rat kidney: *n*= 10 in *sn*-1 DHA group, *n*= 11 in *sn*-2 DHA group, *n*= 11 in *sn*-3 DHA group and *n*= 10 in tristearin group. Values with different superscript letters in the same row indicate that the means are significantly different ($p < 0.05$). PUFA- polyunsaturated fatty acids.

5.2.4.6 Stearic acid

The positional distribution of DHA on the TAGs did not affect the STA in the liver TAG, brain, eye, testicle, and kidney among DHA- fed groups. Overall, the proportion of STA was increased in the liver PL fraction of the DHA-fed groups and the TS group compared to the NF group (**Figure 9E**), which likely resulted from the increased intake of STA. In this study, a greater loss of STA in the rat feces from the primary positions compared to the loss from the *sn*-2 position was observed. Despite that, the STA level in the liver TAG or PL fraction of the *sn*-2 DHA group did not differ from the STA levels of the *sn*-1 DHA and *sn*-3 DHA groups. Interestingly, a significantly higher proportion of STA in the *sn*-1 DHA group compared to the *sn*-3 DHA group in the liver PL suggested differences in the bioavailability of STA from primary positions of TAGs.

5.2.4.7 α-linolenic acid

The ALA content of TAG-rich fraction, live PL-rich fraction, eye, testicle, visceral fat, and kidney of the NF group (fed with ALA-rich soybean oil) was found to be significantly higher than the TS group (fed with ALA-deficient peanut oil) (**Figure 9F**). Especially in the visceral fat, the ALA represented 2.6%

of total FAs in the NF group whereas only 0.2% in the DHA-fed groups fed and the TS group.

The ALA is elongated and desaturated to longer chain (n-3) PUFAs, for example, DHA. The higher DHA content, particularly in the brain, of the NF group than in the TS group implies that the majority of the DHA is synthesized in the liver from ALA as the major source of (n-3) PUFA (Scott & Bazan, 1989) and the chylomicrons in the circulation are transported to the developing brain (Anderson et al., 1994). Although the brain DHA level in the DHA-fed groups was slightly higher compared to the level in the TS group, the difference was not statistically significant. This is in agreement with the previous findings that the brain DHA level is well-buffered against the variation in (n-3) FA levels, ALA, in the diet (Abedin et al., 1999; Rapoport & Igarashi, 2009). The (n-3) adequate diet during pregnancy and weaning provided a sufficient DHA pool to maintain the brain DHA of the TS group despite the four weeks of feeding on an (n-3) deficient diet. Also, the liver has shown a 100-fold higher efficiency in terms of DHA synthesis from circulating ALA compared to the brain in rats fed the (n-3) PUFA deficient diet. The efficiency was increased by 10- fold in PL and 100-fold in TAG in the liver of the rats fed with (n-3) PUFA deficient diet as compared to rats fed with high DHA during 15 weeks of post-weaning (Rapoport & Igarashi, 2009).

5.3 General Discussion

5.3.1 Strengths and limitations of the studies

Study design. The effect of 'first generation of (n-3) deficiency' was investigated in **study I** in rat organs. The study included the determination of lipid compositions of a variety of tissues, including the brain, eye, liver, visceral fat, testicle, heart, and lung as well as plasma and feces of the (n-3) deficient group (peanut oil based diet) and (n-3) adequate group (soybean oil based diet). Previously, very few studies determined the effect of (n-3) deficiency on the heart, lung, and testicle in particular. Thus, as a strength, this study offers novel information on (n-3) FA deficiency generated after pregnancy and weaning, and a comparison of a wide selection of organs. Also, the fecal samples are included in the analysis for the first time to study the (n-3) deficiency. With the fact that the metabolism of (n-3) FAs depends on the (n-6) FAs due to the competition of (n-3) and (n-6) FAs for the same enzymes, a customized diet could have had equal LA content. Instead, both the experimental groups had a different amount of LA which could be considered a limitation of the study. However, including peanut oil and soybean oil in the diet is common in real life.

In **studies II and III**, the synthetic enantiospecific structured TAGs enabled the direct comparison of the positional distribution of DHA and STA without the

interference of other FAs which has been a problem in most previous studies using interesterified mixtures or natural oils. Another strength of these studies was the inclusion of two control groups, TS (a group fed with tristearin on top of a (n-3) deficient diet) and NF (a group fed with a standard soybean oil based diet), enabling the assessment of the (n-3) deficiency status and the status of FAs in the typically fed laboratory animals. Estimation of optimal baseline (n-3) status, dose, and length of the intervention was a challenge. It might be considered a limitation for the detection of minor differences between the *sn*-1 and *sn*-3 positions in addition to the results obtained in these studies.

Analysis of samples. In previous studies, the analysis of fecal lipids was not studied to a large extent for the bioavailability trials of LC (n-3) PUFA. As a strength, the FA composition analysis of the fecal fats (**study I and II**) was carried out by two different methods which enhanced the understanding of the mechanisms behind fecal fat loss. As a limitation, the possible effect of gut microbiota on the fate of the FAs which were not absorbed in the small intestine and entered the colon was not taken into account. Another limitation of the studies was the direct extrapolation of the results obtained in rats to humans as it is reviewed earlier by DeSesso & Jacobson (2001) that the anatomical and physiological differences exist between the gastrointestinal tract of rats and humans and differences, e.g. in bile acid concentrations, can affect the digestion and absorption of saturated FAs.

5.3.2 Relevance and significance of the research

Previous studies focusing on the effect of the (n-3) deficiency were conducted mostly by inducing a severe (n-3) deficiency in animals by depleting several generations from (n-3) FAs with dietary manipulation. Additionally, the majority of the studies conducted in the past were concentrated on the analysis of a narrower selection of organs. The effect of (n-3) deficiency on the heart, lung, and testicle was not studied to large extent. Multigenerational (n-3) deficiency models in animals can induce a severe (n-3) FAs deficiency, for example in brain DHA leading to loss of neural function. Such an approach has limitation in that it poorly mimics real-life human situations. In **study I**, the first-generation (n-3) deficient rat model was studied which is clinically relevant to understand the (n-3) deficiency in young animals in conditions that could mimic the human LC (n-3) PUFA status. This study provided information on the DHA status of a wide range of organs showing the least and most affected organs due to the (n-3) FA deficiency.

This is the first study performed to investigate the effect of the position of DHA in TAGs on its bioavailability and accumulation in different organs of mildly (n-3) deficient rats by intervention with stereospecifically structured TAGs. Enzymes are essential for the digestion of fat and studies have shown

differences in the efficiency of lingual, gastric, and pancreatic enzymes towards both primary positions and type of FAs in TAG molecules during the digestion of lipids. However, previous studies have not compared the bioavailability of DHA between the two primary positions. **Studies II and III** together provided a comprehensive and systematic view of the absorption, excretion as well as accumulation, in different organs of DHA fed in the form of stereospecifically structured TAGs to mildly (n-3) deficient rats.

5.3.3 Future prospect

This PhD thesis supports the continuation of the investigation of the bioavailability of DHA and other LC (n-3) PUFAs from regio- and enantiopure TAGs in rats to answer more new study questions. In the future, a long-term intervention with structured TAGs will provide new information on the differences in the bioavailability of DHA and other (n-3) FAs from the primary positions of the TAGs.

6 SUMMARY AND CONCLUSION

This PhD research was focused on: 1) Determination of the effect of the first-generation (n-3) deficiency, after 33 days of feeding trail, on the tissue-specific content of polyunsaturated fatty acids in rats. 2) The impact of positional distribution of DHA in TAGs on its bioavailability and accumulation in mildly (n-3) deficient rats. The experimental part of this study was enabled by the synthesis of regio- and enantiopure TAGs, animal intervention trial, and lipid analyses of the rat tissues, plasma, and feces. To our understanding, this is the first study reporting findings of the bioavailability of DHA from enantiospecific TAGs in an animal model.

The results from the first-generation (n-3) deficiency provide an understanding of the extent of applied depletion of DHA levels in various rat organs. When the two experimental groups, (n-3) adequate and (n-3) deficient groups, were compared after 33 days feeding trial, the lipid composition analysis detected lower DHA content in the plasma, brain, testicle, visceral fat, heart, and lungs of the (n-3) deficient group. Whereas, the DHA content of the eye and feces did not differ between the experimental groups. The DHA content of visceral fat of the (n-3) deficient group was 14% of the (n-3) adequate group, being most affected by the (n-3) deficient diet feeding, whereas the brain DHA was least affected with the DHA content of the (n-3) deficient group being 86% of DHA level of the (n-3) adequate group. The DHA level of the organs was affected in the order of visceral fat > liver triacylglycerols > lung > heart > liver phospholipids > testicle > eye > brain. The low levels of (n-3) FAs in the liver, brain, eye, heart, and lung were offset by an increase in the (n-6) FAs, mainly ARA.

A 5-day feeding trial with TAGs with DHA either at *sn*-1, *sn*-2, and *sn*-3 positions and STA at remaining positions showed less fecal DHA excretion from *sn*-2 position compared to *sn*-1 and *sn*-3 positions. Thus, the study substantiated superior bioavailability of DHA from the *sn*-2 position compared with the *sn*-1 and *sn*-3 positions as measured by the amount of DHA lost to feces but did not substantiate differences between the two primary positions (*sn*-1 and *sn*-3). The study also supports earlier findings on the loss of stearic acid to feces especially when located in the *sn*-1 and *sn*-3 positions. The liver TAG results showed significantly higher DHA from the *sn*-1 position compared to the *sn*-3 position when only *sn*-1 DHA and *sn*-3 DHA groups were compared. Similarly, STA in liver PLs was found to be significantly higher *sn*-1 DHA group as compared to the *sn*-3 DHA group. There was a slight but insignificant increase in the DHA level in the eye of the *sn*-1 DHA group compared to the *sn*-3 DHA group. The short feeding trial increased the DHA content of plasma TAG, plasma PL, liver TAG, liver PL, testicle, visceral fat, and kidney of DHA-fed groups compared to

the tristearin fed group. The DHA levels of DHA-fed groups in the visceral fat were 2-fold higher compared to the normal feed group.

This PhD thesis provides extensive and novel information on the DHA content in various rat organs after 33 days of (n-3) deficient diet feeding and new insights on the influence of the positional distribution of DHA in dietary TAGs on the bioavailability of DHA. Overall, the results of the first generation (n-3) deficiency showed that 33 days of (n-3) deficient feeding was enough to induce DHA deficiency. The outcome of the DHA depletion in various tissues indicated that in rats adequate maternal nutrition during pregnancy and weaning does not provide enough (n-3) FAs for 33 days of an (n-3) deficient diet. Results of this study can be used also to evaluate the conditions needed to reach mild (n-3) deficiency in the first generation of rats and to evaluate the feasibility to collect data from a variety of organs or only selected ones. The observed differences in the bioavailability of DHA from the *sn*-1 position compared to the *sn*-3 position in liver TAG as well as a small and insignificant increase in the DHA content in the eye of the *sn*-1 DHA group compared to the *sn*-3 group indicate possible differences in the absorption of DHA from primary positions of TAGs. Because of the lack of regio- and enantiopure TAGs, the understanding of the effect of the TAG structure on the absorption of fatty acids has been hindered, particularly from enantiopure TAGs. These studies provide support for a longer feeding intervention to investigate the possible differences in other organs as well as confirm the results obtained in this study. The findings from this PhD thesis significantly strengthen the current understanding of lipid biochemistry and structure-function relationships of dietary lipids.

ACKNOWLEDGEMENTS

The work of this thesis was carried out at the Food Sciences, Department of Life Technologies, University of Turku under the supervision of Professor Kaisa Linderborg and Professor Baoru Yang.

I am extremely grateful for the financial support provided by the Raisio plc research foundation, the Finland – China Food and Health Network (FCFH), the Finnish Food Research Foundation, the Orion Research Foundation, the Turku University Foundation, and the Department of Life Technologies (University of Turku). I wish to thank the Doctoral Programme in Technology (DPT) and the University of Turku Graduate School (UTUGS) for supporting the completion of my doctoral studies. I would like to express my special appreciation for the financial support provided by the Doctoral Programme of Molecular Life Sciences, the Finnish Chromatography Society, and the Turku University Foundation. I would additionally like to thank the Turku University Foundation, the Doctoral Programme in Technology, and the Finnish Chromatography Association for travel grants which supported me to present my results in international conferences.

I wish to express my gratitude to my supervisors, Professor Kaisa Linderborg and Professor Baoru Yang for their excellent guidance, support, and encouragement during my journey. Baoru, I thank you from the bottom of my heart for providing me with an opportunity to be a part of your group. I sincerely appreciate your criticism and expertise during my PhD study. Kaisa, thank you for introducing me to the 'world of lipid analysis' and always inspiring me to do better. I cannot imagine reaching here without you and your support. Your encouragement has led me here today. Thank you for giving your time to me whenever I needed it.

I'm grateful to my excellent steering group members, Professor Emeritus Heikki Kallio, Adjunct Professor, University Lecturer Jukka-Pekka Suomela, and Professor Gudmundur G. Haraldsson (University of Iceland) for their advice and suggestions throughout this project work. I deeply appreciate the scientific collaboration with Professor Yumei Zhang and her team (Peking University Health Science Center, Beijing, China) and Professor Gudmundur G. Haraldsson and his team (Science Institute, University of Iceland). I wish to express my gratitude to all my co-authors involved in the articles of this project for their contribution.

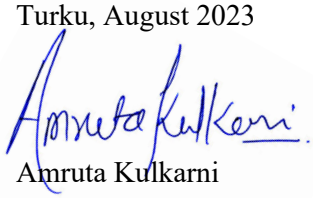
My sincere thanks also goes to the pre-examiners Associate Professor Huiling Mu and Adjunct Professor Jyrki Ågren for reviewing the thesis critically and for their valuable comments and suggestions for improving the thesis. I would also like to thank Robert M. Badeau for the language examination of this thesis.

I am grateful for having colleagues Gabriele Beltrame, Niko Markkinen, Liz Gutierrez Quequezana, Eija Ahonen, Marika Kalpio, Anu Nuora, Ye Tian, Kang Chen, Niina Kelane, Maaria Kortesniemi, Annelie Damerau, Tanja Kakko, Anna Pugganen, Ella Aitta, Salla Laito, Priscilla Ollennu-Chuasam, Raghunath Pariyani, and Johanna Jokioja in my journey. Thanks a lot for the support, friendship, and nice discussions during coffee breaks. I also want to thank and express my appreciation to PhD Marko Tarvainen for his valuable help and guidance during my research trainee period. Many thanks to all present and past colleagues at the Food Sciences unit for their help and the wonderful work environment. Also, Natasa Mantziari and Natalia Pakharukova, thank you for your friendship and support. I also want to thank Anu, Heli, Jani, Tapio, and Leena for all the administrative and technical help during my work.

Big thanks and hugs to my Indian friends whom I met when I moved to Turku: Bhagyashree, Mahesh, Poonam, Subhash, Yashu, Raghu, Aman, Hasan, Preeti, Santosh. Cheers to all the fond memories we made together. I feel very lucky to meet each one of you. I am deeply grateful to Nikita, Deepankar, Srikar, Shravani, Nitin, Avlokita, Binu, Neena, Sachin, Jyoti, Priyanka, Lav, Arjun, and Dwitiya for our get-together and events that we arranged. Thanks for the fun- and laughter-filled evenings. Justyna, thank you for being such a wonderful friend. The list of friends is long ☺ and would go on and on. I appreciate each one of you whom I met during this journey and will forever cherish the memories.

A very special thanks go to my family. My mom and dad, and my in-laws, thank you for your constant support, guidance, and love. I am grateful to my sisters and brother for always being there when I needed you. I want to thank my husband, Prasanna, for his constant encouragement, love, support, and patience. You are a gem and I learn so much from you every day. Thank you for the tons of memories we made together and we continue doing that. Finally, I want to mention our lovely daughter, Ira. You are my source of energy and joy. Life has changed for good and I thank you for choosing me.

Turku, August 2023



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DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

1. **REINO R. LINKO (1967)** Fatty acids and other components of Baltic herring flesh lipids. (Organic chemistry).
2. **HEIKKI KALLIO (1975)** Identification of volatile aroma compounds in arctic bramble, *Rubus arcticus* L. and their development during ripening of the berry, with special reference to *Rubus stellatus* SM.
3. **JUKKA KAITARANTA (1981)** Fish roe lipids and lipid hydrolysis in processed roe of certain *Salmonidae* fish as studied by novel chromatographic techniques.
4. **TIMO HIRVI (1983)** Aromas of some strawberry and blueberry species and varieties studied by gas liquid chromatographic and selected ion monitoring techniques.
5. **RAINER HUOPALAHTI (1985)** Composition and content of aroma compounds in the dill herb, *Anethum graveolens* L., affected by different factors.
6. **MARKKU HONKAVAARA (1989)** Effect of porcine stress on the development of PSE meat, its characteristics and influence on the economics of meat products manufacture.
7. **PÄIVI LAAKSO (1992)** Triacylglycerols – approaching the molecular composition of natural mixtures.
8. **MERJA LEINO (1993)** Application of the headspace gas chromatography complemented with sensory evaluation to analysis of various foods.
9. **KAISLI KERROLA (1994)** Essential oils from herbs and spices: isolation by carbon dioxide extraction and characterization by gas chromatography and sensory evaluation.
10. **ANJA LAPVETELÄINEN (1994)** Barley and oat protein products from wet processes: food use potential.
11. **RAIJA TAHVONEN (1995)** Contents of lead and cadmium in foods in Finland.
12. **MAIJA SAXELIN (1995)** Development of dietary probiotics: estimation of optimal *Lactobacillus* GG concentrations.
13. **PIRJO-LIISA PENTTILÄ (1995)** Estimation of food additive and pesticide intakes by means of a stepwise method.
14. **SIRKKA PLAAMI (1996)** Contents of dietary fiber and inositol phosphates in some foods consumed in Finland.
15. **SUSANNA EEROLA (1997)** Biologically active amines: analytics, occurrence and formation in dry sausages.
16. **PEKKA MANNINEN (1997)** Utilization of supercritical carbon dioxide in the analysis of triacylglycerols and isolation of berry oils.
17. **TUULA VESA (1997)** Symptoms of lactose intolerance: influence of milk composition, gastric emptying, and irritable bowel syndrome.
18. **EILA JÄRVENPÄÄ (1998)** Strategies for supercritical fluid extraction of analytes in trace amounts from food matrices.
19. **ELINA TUOMOLA (1999)** *In vitro* adhesion of probiotic lactic acid bacteria.
20. **ANU JOHANSSON (1999)** Availability of seed oils from Finnish berries with special reference to compositional, geographical and nutritional aspects.
21. **ANNE PIHLANTO-LEPPÄLÄ (1999)** Isolation and characteristics of milk-derived bioactive peptides.
22. **MIKA TUOMOLA (2000)** New methods for the measurement of androstenone and skatole – compounds associated with boar taint problem. (Biotechnology).
23. **LEEA PELTO (2000)** Milk hypersensitivity in adults: studies on diagnosis, prevalence and nutritional management.
24. **ANNE NYKÄNEN (2001)** Use of nisin and lactic acid/lactate to improve the microbial and sensory quality of rainbow trout products.
25. **BAORU YANG (2001)** Lipophilic components of sea buckthorn (*Hippophaë rhamnoides*) seeds and berries and physiological effects of sea buckthorn oils.
26. **MINNA KAHALA (2001)** Lactobacillar S-layers: Use of *Lactobacillus brevis* S-layer signals for heterologous protein production.
27. **OLLI SJÖVALL (2002)** Chromatographic and mass spectrometric analysis of non-volatile oxidation products of triacylglycerols with emphasis on core aldehydes.
28. **JUHA-PEKKA KURVINEN (2002)** Automatic data processing as an aid to mass spectrometry of dietary triacylglycerols and tissue glycerophospholipids.
29. **MARI HAKALA (2002)** Factors affecting the internal quality of strawberry (*Fragaria x ananassa* Duch.) fruit.
30. **PIRKKA KIRJAVAINEN (2003)** The intestinal microbiota – a target for treatment in infant atopic eczema?
31. **TARJA ARO (2003)** Chemical composition of Baltic herring: effects of processing and storage on fatty acids, mineral elements and volatile compounds.
32. **SAMI NIKOSKELAINEN (2003)** Innate immunity of rainbow trout: effects of opsonins, temperature and probiotics on phagocytic and complement activity as well as on disease resistance.
33. **KAISA YLI-JOKIPII (2004)** Effect of triacylglycerol fatty acid positional distribution on postprandial lipid metabolism.
34. **MARIKA JESTOI (2005)** Emerging *Fusarium*-mycotoxins in Finland.
35. **KATJA TIITINEN (2006)** Factors contributing to sea buckthorn (*Hippophaë rhamnoides* L.) flavour.
36. **SATU VESTERLUND (2006)** Methods to determine the safety and influence of probiotics on the adherence and viability of pathogens.
37. **FANDI FAWAZ ALI IBRAHIM (2006)** Lactic acid bacteria: an approach for heavy metal detoxification.
38. **JUKKA-PEKKA SUOMELA (2006)** Effects of dietary fat oxidation products and flavonols on lipoprotein oxidation.
39. **SAMPO LAHTINEN (2007)** New insights into the viability of probiotic bacteria.
40. **SASKA TUOMASJUKKA (2007)** Strategies for reducing postprandial triacylglycerolemia.

41. **HARRI MÄKIVUOKKO (2007)** Simulating the human colon microbiota: studies on polydextrose, lactose and cocoa mass.
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