THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Oxidation of marine oils during *in vitro* gastrointestinal digestion and its effects on stress in human intestinal Caco-2 cells

CECILIA TULLBERG

Food and Nutrition Science
Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2018

OXIDATION OF MARINE OILS DURING *IN VITRO* GASTROINTESTINAL DIGESTION AND ITS EFFECTS ON STRESS IN HUMAN INTESTINAL CACO-2 CELLS

CECILIA TULLBERG
ISBN: 978-91-7597-676-1

Doktorsavhandlingar vid Chalmers tekniska högskola
Ny serie Nr: 4357
ISSN 0346-718X

Chalmers University of Technology
Department of Biology and Biological Engineering
Food and Nutrition Science
SE-412 96 GÖTEBORG
Sweden
Telephone: + 46 (0) 31 772 10 00

Printed by Chalmers Reproservice
Gothenburg, Sweden 2018

Front cover:
Formation of aldehydes during *in vitro* digestion of marine oils using human and simulated digestion fluids, and the effect of the marine oil digests on Caco-2 cells.
Illustrated by Cecilia Tullberg

OXIDATION OF MARINE OILS DURING *IN VITRO* GASTROINTESTINAL DIGESTION AND ITS EFFECTS ON STRESS IN HUMAN INTESTINAL CACO-2 CELLS

CECILIA TULLBERG
Department of Biology and Biological Engineering
Chalmers University of Technology, Gothenburg, Sweden

ABSTRACT

Marine oils are attracting public interest due to the preventive effects, e.g., on inflammation, which are linked to the long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFAs). However, LC n-3 PUFAs are highly susceptible to oxidation, which could interfere with their positive effects. It has been shown *in vitro* that marine lipids not only oxidize during storage, but also during gastrointestinal (GI) digestion. Little is so far known about the marine lipid oxidation reaction under human GI conditions.

In this work, oxidation of marine oils during *in vitro* GI digestion was investigated targeting the highly reactive lipid oxidation products malondialdehyde (MDA), 4-hydroxy-*trans*-2-hexenal (HHE), and 4-hydroxy-*trans*-2-nonenal (HNE); all three with documented carcinogenic and genotoxic properties. Variables studied during the digestions were; source of the GI-fluids (porcine/human), presence of additional gastric lipase (from rabbit) or addition of a lipase inhibitor, type of *in vitro* model (static/dynamic),physical status (bulk/emulsified), oxidation status and origin of the marine oil, as well as additions of food-derived pro- and antioxidants. Furthermore, effects from marine oil digests related to intestinal cell stress were studied.

Aldehyde levels increased over time in the intestinal phase during digestion of cod liver oil, in a static *in vitro* digestion model with human digestive fluids (HDF) or simulated digestive fluids (SDF, i.e., electrolyte solution with enzymes and bile of porcine origin). The highest aldehyde levels were reached during the intestinal phase (t=210 min) using HDF (60 μM of MDA, 0.96 μM of HHE, and 1.6 μM of HNE). In the static model with HDF, lipolysis was found to correlate positively to lipid oxidation, as shown when adding rabbit gastric lipase or orlistat, a lipase inhibitor, to cod liver oil. Aldehydes also increased during digestion of cod liver oil in a dynamic digestion model (tiny-TIM) with SDF. Cod liver oil having a higher degree of oxidation at start of the digestion reached higher levels of aldehydes during GI conditions compared to non-oxidized oils. Pre-emulsification of cod liver oil was slightly protective in the gastric phase, but had a pro-oxidative effect during the intestinal phase. Addition of fish hemoglobin (Hb) as a pro-oxidant to emulsified cod liver oil strongly promoted aldehyde formation, while the metal chelator EDTA had a protective effect during gastric digestion. Industrially relevant levels of tocopherols (α-tocopherol, and Covi-ox® T 70 EU; 4.5 mg/g oil) were protective to cod liver oil oxidation in the static in vitro digestion model with HDF. In the same model, detected aldehyde levels in intestinal digests from four different marine oils were ranked as: cod liver oil ~ whole fish oil >> krill oil ~microalgae oil.

To study cellular effects of GI oxidation, a cultured human intestinal epithelium (Caco-2 cell line) was treated with cod liver-, fish-, and algae oil digests, and corresponding levels of pure MDA and HHE (0-90 μM). Cell viability was not affected by the digests, nor their levels of MDA and HHE. Stress-related proteins were not found to increase upon exposure to digests or aldehydes, rather the opposite.

To summarize, MDA, HHE, HNE were formed during *in vitro* GI digestion of marine oils in all the models tested; absolute levels were, however, affected by pre-treatment of the oils, and were higher with HDF than SDF. Although bulk oils digested without added pro- or antioxidants did *not* induce a stress response in the Caco-2 cells, studies in humans are needed to be able to say if the absence of stress effects from aldehydes or other oxidation products can be translated to *in vivo* conditions.

Keywords: lipid oxidation, marine oils, LC n-3 PUFAs, *in vitro* digestion, human digestive fluids, gastrointestinal, aldehydes, MDA, HHE, HNE, Caco-2 cells

LIST OF PUBLICATIONS

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

I. **Tullberg*, C.**, Larsson, K., Carlsson, N.G., Comi, I., Scheers, N., Vegarud, G., and Undeland, I., Formation of reactive aldehydes (MDA, HHE, HNE) during the digestion of cod liver oil: comparison of human and porcine *in vitro* digestion models, Food & Function, 2016, 7, 1401-1412.

II. Larsson*, K., **Tullberg*, C.**, Alminger, M., Havenaar, R., and Undeland, I., Malondialdehyde and 4-hydroxy-2-hexenal are formed during dynamic gastrointestinal *in vitro* digestion of cod liver oils, Food & Function, 2016, 7, 3458 – 3467.

III. **Tullberg*, C.**, Vegarud, G., Undeland, I., Oxidation of marine oils during *in vitro* gastrointestinal digestion with human digestive fluids – role of oil origin, added tocopherols and lipolytic activity. *Submitted.*

IV. **Tullberg*, C.**, Vegarud, G., Undeland, I., Scheers, N., Effects of marine oils, digested with human fluids, on cellular viability and stress protein expression in human intestinal Caco-2 cells. Nutrients 2017, 9, 1213.

CONTRIBUTION REPORT

Paper I: The author, Cecilia Tullberg (CT), participated in the design of the study, performed the experimental work and statistical calculations, interpreted data and drafted the writing of the manuscript.

Paper II: CT participated in a major part of the experimental work and had shared responsibility in manuscript writing.

Paper III: CT participated in the design of the study, performed the experimental work and statistical calculations, interpreted data and drafted the writing of the manuscript.

Paper IV: CT participated in the design of the study, performed the experimental work and statistical calculations, interpreted data and drafted the writing of the manuscript.

ABBREVIATIONS

APCI	Atmospheric pressure chemical ionization
BHT	Butylated hydroxytoluene
Caco-2	Caucasian colon adenocarcinoma
DAG	Diacylglycerides
DHA	Docosahexaenoic acid
DNPH	2,4-dinitrophenylhydrazine
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FA	Fatty acids
FFA	Free fatty acids
GC-MS	Gas Chromatography-Mass Spectrometry
GI	Gastrointestinal
GIT	Gastrointestinal tract
Hb	Hemoglobin
HDF	Human digestive fluid
HDuF	Human duodenal fluid
HGF	Human gastric fluid
HGL	Human gastric lipase
HHE	4-hydroxy-*trans*-2-hexenal
HNE	4-hydroxy-*trans*-2-nonenal
HPLC	High-Performance Liquid Chromatography
HSP	Heat shock protein
LC-MS	Liquid Chromatography-Mass Spectrometry
LC n-3 PUFAs	Long-Chain n-3 Poly-unsaturated Fatty Acids
LC n-6 PUFAs	Long-Chain n-6 Poly-unsaturated Fatty Acids
LMW	Low molecular weight
MAG	Monoacylglycerides
MDA	Malondialdehyde
ONE	4-oxy-*trans*-2-nonenal
PLs	Phospholipids
PV	Peroxide value
RGL	Rabbit gastric lipase
SDF	Simulated digestive fluid
SOD	Superoxide dismutase
SPE	Solid phase extraction
TAG	Triacylglycerols
TBARS	Thiobarbituric acid reactive substances
TIM	TNO Gastro-Intestinal Model
Trx	Thioredoxin

TABLE OF CONTENTS

1. INTRODUCTION

In this thesis, oxidation of marine oils during gastrointestinal (GI) digestion, and its potentially local harmful effects on the intestinal epithelium, will be addressed. Focus is on three lipid oxidation-derived aldehydes – malondialdehyde (MDA), 4-hydroxy-*trans*-2-hexenal (HHE), and 4-hydroxy-*trans*-2-nonenal (HNE) – which are highly reactive towards, e.g., proteins and DNA. These aldehydes have been suggested to counteract some of the positive effects from long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFAs) on human health[1,2].

There is a growing global demand for marine oils rich in LC n-3 PUFAs, with a steadily increasing market for human consumption of such "omega-3" oils[3]. The demand is driven by the documented preventive effects from the LC n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on, e.g., inflammation and subsequent development of cardiovascular diseases[4-7]. A problem with PUFA-rich marine oils is, however, that they are highly susceptible to oxidation. Lipid oxidation is a known cause for food deterioration by inducing changes in structure, nutritional value, color, flavor and smell of food items, leading to reduced shelf life. Therefore, the development of lipid oxidation during food storage is well studied, but indeed not yet fully understood. The growing consumption of marine oils - either as capsules or incorporated into other food items – has further increased the interest in stabilizing these particular food oils during storage. Both physical and chemical strategies are currently used to prevent oxidation during processing and storage prior to ingestion. With the use of flavored and/or encapsulated fish oil, there is a risk that consumers who are unused to ingesting these supplements do not recognize the rancid smell of marine oil that has oxidized, or that the smell is masked. The major companies producing marine oil capsules are aware of the problem with lipid oxidation and assure strict quality control of the oil, however, the booming marine oil market opens up for less serious actors. Recent studies have shown that many of the marine oil supplements found in supermarkets in New Zealand[8], Norway[9], and South Africa[10], are oxidized above the recommended limits. This raises the question of potentially toxic effects from ingesting pre-oxidized marine oil supplements, either locally in the GI tract (GIT), or systemically after uptake. Regarding local effects, it has been shown that protein-adducts can be formed in the intestinal tract after providing mice pure HHE[11], a well-known oxidation product of n-3 PUFAs. It was additionally shown that administrating mice with pre-oxidized marine oil induced an inflammatory response, and accumulation of lipid oxidation products, e.g., in plasma[11]. However, little is known about the destiny of ingesting pre-oxidized marine oil supplements in humans.

Additional to the documented instability of marine oils during process and storage, it has been shown *in vitro* that such oils can oxidize during passage through the GIT[12-14]. Pre-oxidized marine oil was shown to yield higher levels of oxidation products under gastric and intestinal conditions compared to fresh oil, and a profound stimulatory effect was seen from the oxidant hemoglobin (Hb). At the start of this thesis work, no studies, however, existed on GI-oxidation of marine oils comprising human GI fluids. It was hypothesized that the profile of enzymes, pro- and antioxidants of such fluids would differ from those found, e.g., in porcine enzyme and bile mixtures. Furthermore, limited work was done in dynamic *in vitro* GI-models, which better than the static models simulate pH-, dilution changes, and removal of digested compounds over time. Moreover, markers used to monitor oxidation were previously mainly broad spectrum measures, like peroxide value (PV) and TBA-

reactive substances (TBARS), lacking specificity. Using more realistic digestive fluids and models together with physiologically relevant oxidation markers, like MDA and α, β-unsaturated aldehydes, would help deepening the understanding of oxidation mechanisms, and also to estimate whether potentially harmful oxidation compounds could form under GI-conditions. This could aid the evaluation of adverse health effects from oxidation products, e.g., on the intestinal epithelium. It would thus be of interest to evaluate stress response and cell survival from exposure of marine oil digests using human cultured intestinal cells.

If processors of marine oils can maintain the LC n-3 PUFAs intact all the way from the fisheries step, to the site in the human body where these fatty acids could exert a positive effect, this could lead to a better utilization of our marine fish oil resources, which in turn would reduce overexploitation of the population of small pelagic fish. Currently 22 million tons of fish are used yearly in the global fish oil production, which is currently ~1 million tons[15]. To maintain a sustainable fishing industry, while still fulfilling the global demand for LC n-3 PUFA-rich oils, the use of alternative marine oil sources, like algae and krill, are interesting options which are currently increasingly explored. A previous study found that both krill and algae oil were more stable towards oxidation during storage compared to fish oil[16], thus it is possible that there are differences in stability also during GI digestion. No one has, however, to our knowledge investigated the question of oxidative stability during digestion, comparing oils of different marine oil origin.

To summarize, a few recent studies showed that oxidation of marine oil may take place in the GIT during *in vitro* digestion[12-14]. Therefore a deeper understanding of lipid oxidation mechanisms under such conditions, including the role of oil source, pre-treatment and pre-storage, as well as evaluations of biological effects was needed.

2. OBJECTIVES

The overall goal of this thesis work was to deepen the understanding of marine oil oxidation during *in vitro* GI digestion, targeting the oxidation-derived aldehydes MDA, HHE, and HNE. Focus was on mechanisms, role of food derived oxidants as well as of chemical and physical status of the oil, and on cellular responses to marine oil digests. Reaching these goals would lead to an increased knowledge on how to optimize the utilization of marine oils for human consumption. To concretize this goal, the following specific questions are addressed in this thesis:

- Will the same levels of MDA and the α,β-unsaturated aldehydes HHE and HNE be formed from marine oils during static *in vitro* digestion under physiological conditions using **human GI fluids** as with **commercial enzymes and bile**? (Study I)

- Which **levels** of the targeted lipid oxidation products (MDA, HHE, and HNE) can be formed during dynamic *in vitro* GI digestion of cod liver oil? (Study II)

- Can oxidation of cod liver oil be affected during *in vitro* digestion by different **pre-treatments of the oil**; emulsification, pre-oxidation, addition of potential pro-/antioxidants (ascorbic acid, fish-Hb, EDTA and tocopherols)? (Study II and III)

- Are the same levels of the targeted lipid oxidation products formed when digesting **different marine oils**; cod liver oil, fish oil, krill oil and algae oil? (Study III)

- Is there a relation between **lipolysis** and lipid oxidation of marine oils during digestion? (Study III)

- Is there a **cellular response** to digests of marine oils and pure lipid oxidation products in human Caco-2 cells? (Study IV)

3. BACKGROUND

3.1 Basic lipid chemistry

3.1.1 Lipid classes and fatty acids

A proposed definition for lipids is: "…fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds", and fatty acids (FA) are described as molecules with a straight chain of carbon atoms, naturally synthesized from malonyl coenzyme A[17]. Lipids can be classified into simple lipids (e.g., triacylglycerols, TAG), complex lipids, derived lipids (e.g., free fatty acids, FFA, and monoacylglycerol, MAG), and miscellaneous lipids (e.g., carotenoids)[18]. Two examples of complex lipids are phospholipids (PLs) and glycolipids. PLs are amphipathic due to their polar head group with a phosphate and functional group such as a choline group added to the *sn-3* position of the glycerol backbone (Figure 1), making them important as cell membrane constituents for increased fluidity. PLs are also believed to play an important role in cell signaling[19,20]. Glycolipids have a sugar group connected to the glycerol backbone, and are similar to PLs in that they are found in the cellular membranes and play an important role in cell signaling[19,21].

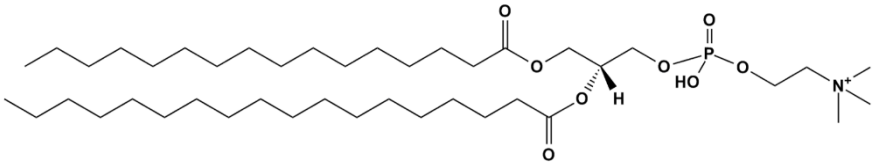

Figure 1: The chemical structures of a phospholipid (PL)
A PL with a phosphate and a choline group added to the glycerol backbone

The major part (90-95%) of the lipids ingested by humans are in the form of TAG[22]. TAG contain a glycerol backbone and three fatty acids in *sn-1*, *sn-2* and *sn-3* position (Figure 2). When TAG are hydrolyzed, the resulting lipids are either one diacylglycerol (DAG) and one FFA; one MAG and two FFA; or three FFA and the glycerol backbone. The FFA can either be long chained (LC; here >18 carbon atoms), medium-chained, or short-chained (<8 carbon atoms)[18]. FA without double bonds are referred to as saturated fatty acids (SFA), while FA with one double bond gives a monounsaturated fatty acids (MUFA), and multiple double bonds a polyunsaturated fatty acids (PUFAs). Some extra attention will be given the LC PUFAs, in the next section. The nomenclature of FA is often written in shorthand. As an example, the FA in the *sn-2* position in Figure 1 is written as C18:1 n-9, describing the number of carbon atoms from the ω-carbon to the carboxylic acid terminus (18), followed by the number of double bonds (1), and the position of the first double bond from the ω-direction (9).

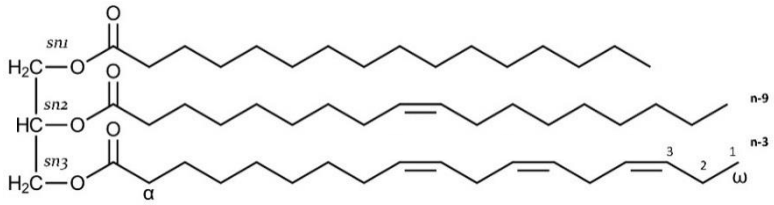

Figure 2: The chemical structure of a triacylglycerol (TAG)
An example of a TAG with a polyunsaturated n-3 fatty acid (n-3 PUFA) in the sn-3 position, a monounsaturated fatty acid (MUFA) in the sn-2 position and a saturated fatty acid (SFA) in the sn-1 position.

3.1.2 LC PUFAs

LC PUFAs belong to a group of PUFAs which are required in humans for normal development and growth. Especially LC n-3 PUFAs have attracted attention, due to their protective effects against cardiovascular and inflammatory diseases[4-7]. The main LC n-3 PUFAs are EPA (Figure 3a), docosapentaenoic acid (DPA) and DHA (Figure 3b), which are synthesized by algae[23] but also found in, e.g., krill, fish, and marine oils thereof. LC n-3 PUFAs have been found to act preventive against inflammatory diseases, such as cardiovascular diseases (CVDs)[24]. EPA has additionally been seen to have a positive effect, e.g., on Alzheimer, while DHA has been found to play an important role for the brain development in infants and act protective against cancer[24]. LC PUFAs can be synthesized in the human body, with the exception of the insertion of a double bond in the n-6 and n-3 position. To be able to synthesize n-3 and n-6 PUFAs humans need to get the essential FAs C18:2 n-6 and C18:3 n-3 through the diet. The enzymes responsible for elongation of LC PUFAs in the human bodies are, however, participating in the elongation of *both* LC n-3 and n-6 PUFAs, and the synthetization pathways are thus competing over the same enzymes[25]. The common Western diet is not balanced between n-3 and n-6, resulting in a high n-6/n-3 ratio[25], which leads to a low conversion factor of C18:3 n-3 to EPA (≈5%) and DHA (<0.5%)[26]. Therefore, most people following the Western diet has a need to enhance their dietary intake of DHA and EPA[27]. There is therefore a general recommendation of a combined daily intake of 250 mg EPA and DHA per day to contribute to a normal heart function[28,29].

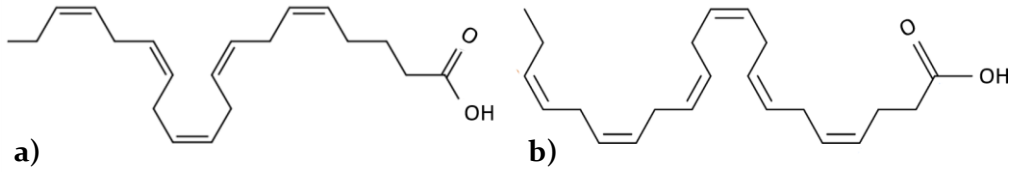

Figure 3: The chemical structures of two free fatty acids (FFA)
a) The chemical structure of the FFA eicosapentaenoic acid, EPA (C20:5 n-3), a long-chain n-3 polyunsaturated fatty acid (LC n-3 PUFA); b) The chemical structure of the FFA docosahexaenoic acid, DHA (C22:6 n-3), a LC n-3 PUFA.

3.2 Marine oils

3.2.1 Most common types of marine oils

Marine oils are as mentioned naturally rich in LC n-3 PUFAs and has historically been used as an energy source in the diet, but also as lamp oil. Since the 1920's cod liver oil has been recognized for its high content of vitamin A and D, and fish body oil (from here on called "fish oil") has been industrially produced and available for human consumption since the 1940's[23]. In the 1970-1980's the connection between fish oil and preventive effects on CVDs became clear, and marine oils have been consumed by humans as nutraceuticals since then[23]. Globally, fish oil supplements have increased with 60% in retail sales value from year 1999 to 2008[30]. Still, only approximately 24% of the fish oil produced is used for human consumption, the rest is used as feed ingredient (2010)[23], Figure 4. There is, however, a growing awareness of the global problems with overfishing and exploitation of the oceans, problems which needs to be addressed together with the increasing demand for LC n-3

PUFA-rich products of marine origin. The Food and Agriculture Organization of the United Nations (FAO) has developed a Code of Conduct for Responsible Fisheries[31], and the International Fishmeal and Fish Oil Organization (IFFO) is at the moment working with a proposed Codex Standard for Fish Oils[32], which will clarify, e.g., recommended additives and routines when handling marine oils. This could be of great help in aligning the view on how to globally maintain a sustainable fishing industry in the future. Today, the main production of marine oil is centralized to Peru, Chile and Scandinavia – where fish oil also has a tradition of being consumed – and the market is rapidly expanding[3,33], e.g., due to the fast increase of Chinese aquaculture[30,34].

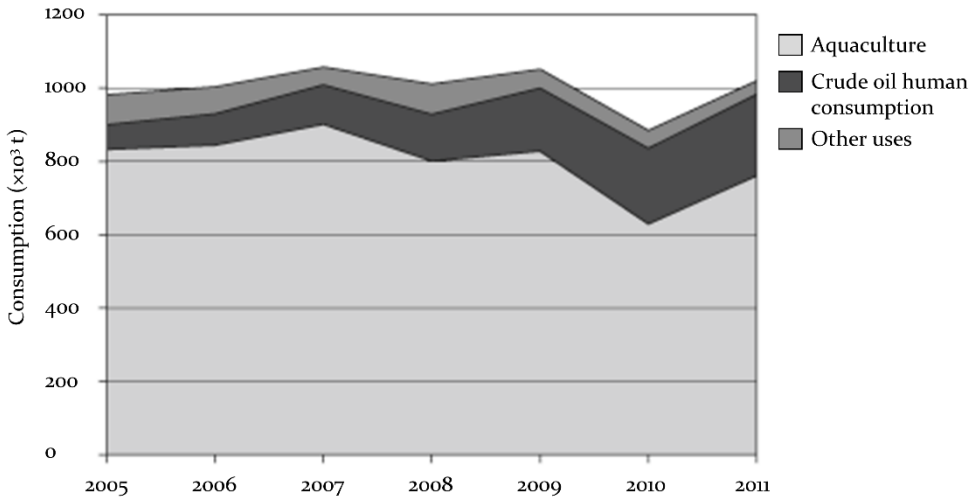

Figure 4: World consumption of fish-oil by market segment
Figure reprinted from Shepherd et al.[3], with permission from John Wiley and Sons, and is based on unpublished data from IFFO and GOED.

The major fish species used for fish oil production are small fatty pelagic fish, such as herring (*Clupea harengus*) and Peruvian anchoveta (*Engraulis ringens*)[33]. The content of LC n-3 PUFAs in the commonly consumed fish oil is 18% EPA and 12% DHA[23], however, this will vary widely with species, age and geographic location of the fish, as well as with season of catch[33]. Fish oil is commonly separated from fish liver oil, such as cod liver oil, the latter which is produced from the fatty liver from cod and is considered to be an oil of high nutritional quality[23]. Both fish oil and cod liver oil needs to be refined before human consumption, to eliminate, e.g., free fatty acids, unwanted volatiles and toxic compounds such as pollutants and heavy metals, and this is done, e.g., by addition of KOH, activated carbon, silica, water steam, or molecular distillation[35].

Nowadays there are several types of marine oils on the market except for the traditional fish oils. Some examples are concentrated fish oil, e.g., ethyl ester (EE) oil, and marine oil of alternative marine sources. The dominant natural form of lipids in fish oil is as TAG[23], which might differ in these alternative oils. In EE oils, the LC n-3 PUFA content can reach >90 % EPA and DHA, such as in the drug Omacor® (today called Lovaza®). It is also possible to trans-esterify EE enzymatically, to achieve a recovered TAG oil, which is still concentrated compared to the original fish oil, >65% EPA and DHA[36]. Another example is oil produced from microalgae, here after referred to as algae oil in this text. Today most of the industrially produced algae oil comes from the family of *Thraustochytriidae*, e.g.,

Schizochytrium sp., since they have the ability to yield an oil rich in DHA[37]. Microalgae can be grown in bioreactors on land, and the interest in this type of marine oils has increased rapidly the last years, thanks to improved yield and production cost, and due to the consumer demand for a vegetarian marine oil. In algae oil, lipid classes vary largely between species – major characteristics are that the main lipid class is TAG, but there can also be significant levels of PLs and glycolipids[38]. Another alternative marine oil which recently has been given a lot of attention is krill oil. Krill is a zooplankton, rich in PLs and the natural antioxidant astaxhantin[39]. Krill consumes microalgae and incorporates ingested FAs in PLs, rather than TAG[39]. Fish oils generally also contain small amounts of PLs, and a report stated a ratio of 1% PLs in capelin (*Mallotus villosus*) oil[40], which is to be compared with PLs being approximately 60% in krill oil[41], presented as % of total lipid detected. The impact of the delivery form of LC n-3 PUFAs is currently under discussion, and it has been suggested that delivery through PLs rather than TAG might be more efficient from a bioavailability aspect[42].

3.2.2 Stabilization and formulations

Marine raw materials contain natural antioxidants, however, during refinement of fish and cod liver oil, most of these antioxidants are removed[23]. To re-stabilize the extracted marine oils, different antioxidants and metal chelators can be added. Tocopherols work as chain-breakers in the lipid oxidation reaction, and they have been the major additives in fish oils traditionally[24]. Tocopherol mixtures containing both α-, β-, γ-, and δ-tocopherols are today commonly used by the industry, providing synergistic effects in their different ways of action[23]. There is also a wide range of synthetic compounds which have been investigated and used in fish oil products, e.g., butylated hydroxytoluene (BHT), tertbutylhydroquinone (TBHQ), and propyl gallate[23,43]. However, there is today a preference for addition of naturally derived antioxidants, such as those extracted from herbs, e.g., rosemary[43], and spices[23]. These types of plant extracts are generally rich in polyphenols, such as the flavonoid quercetin. Polyphenols act antioxidatively both by quenching singlet oxygen, and as hydrogen donors[24].

The distribution form of marine oil before consumption is commonly as bulk oil, or encapsulated, however, the oil can also be provided as liposomes or as emulsion[23]. Encapsulation and emulsion can aid both as a delivery system and as protection against lipid oxidation in case the emulsion is properly structured (e.g., as microemulsion and/or with proper antioxidants incorporated) and, for capsules, using a good coating material and encapsulation technique[23]. Microemulsions and microcapsules are common delivery systems when introducing the oils to food or drinks, while a common way to directly provide fish oil and cod liver oil as supplements is through soft-gel gelatin coated capsules[44]. These capsules commonly contain between 250-300 mg of EPA and DHA, to make sure that the daily dose of recommended intake is reached. The most common and least costly way to produce microcapsules is through spray-drying[24,45], resulting in coated particles with a size of 10-400 μM[45]. It should, however, be mentioned that these different delivery systems also could decrease the stability of the final product compared to crude bulk oil, since they commonly involve increased temperature and exposure of the oil throughout processing[45].

It is well known that emulsification of food systems can have both pro- and/or antioxidative effect on lipid oxidation[46], depending on, e.g., pH, droplet size distribution, as well as choice[46] and concentration[47,48] of the emulsifier. The emulsifier can additionally influence the accessibility of both pro- and antioxidants to the oil-water interface[49].

3.3 The lipid oxidation reaction

3.3.1 The mechanisms of lipid oxidation in marine oils

The lipid oxidation reaction is a well-studied, highly complex, reaction, with parts of its mechanisms known since the 1940's[50]. It is one of the major causes for deterioration in lipid-containing foods; especially under conditions when bacterial growth is reduced, such as during frozen storage or after drying. In recent years, the lipid oxidation reaction has been given renewed concerns, due to its possible connection to damages in biological processes *in vivo*[51,52]. Lipid oxidation involves three major parts; initiation, propagation and termination, schematically described in Figure 5.

During initiation, a lipid alkyl radical (L•) is formed, which together with O_2 can be converted into a peroxyl radical (LOO•), which in turn can generate lipid hydroperoxide (LOOH) and a new lipid radical (L•)[53]. The lipid oxidation reaction is after the first propagation step self-propagating and accelerating[54]. The peroxyl radical (LOO•) formed propagates the continuous lipid oxidation reaction, where new lipid radicals are formed[53]. LOOH continues to generate free radicals in a process called free-radical chain branching[50]. The hydroperoxide can, e.g., react with transition metals to form lipid alkoxyl radicals (LO•), generating secondary lipid oxidation products through a pathway called β scission[55].

The reaction can be initiated in several ways, e.g., by the presence of a free radical (such as in Figure 6, section 3.3.2) as in autoxidation, by photo-oxidation, or by enzymatic lipid oxidation in the presence of, e.g., lipoxygenase[50,51]. In photo-oxidation, a photosensitizer, such as chlorophyll, is activated by light and reacts with ground state oxygen, $^3O_2$, forming singlet oxygen, $^1O_2$, which then readily reacts with double bonds in

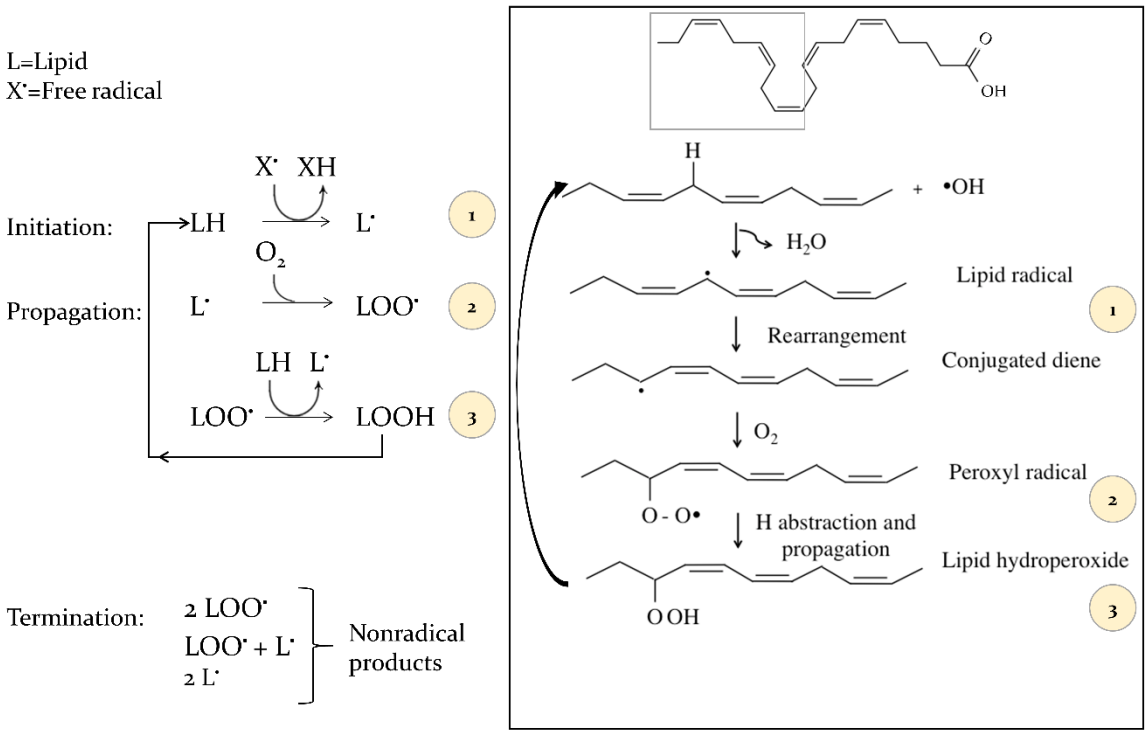

Figure 5: The lipid oxidation reaction
The three major chain sequences for lipid oxidation autoxidation, to the right the reaction pathway, including a FFA without the carboxyl group, adapted from Jairam et al.[56].

FA[55]. The lipid oxidation reaction is enhanced by several factors, such as heat, light, oxygen pressure (O_2>100 mmHg), presence of transition metals (low molecular weight – LMW – or heme-bound), free radicals, FFA formed via lipolysis, as well as humidity or extremely dry conditions (a_w>0.55 or a_w<0.15)[54]. Regarding transition metals, they can have multiple pro-oxidative roles, among which one is that iron in the form of Fe^{2+} can generate hydroxyl radicals (OH•) through the Fenton reaction. The hydroxyl radical can then initiate the lipid oxidation reaction[57]. Regarding FFA, these can be pro-oxidative due to the ability to lower the surface tension of an oil, making the oil/water interface more available to oxygen[58].

What is important to remember, is that multiple intermediates are formed simultaneously throughout the lipid oxidation reaction, and the specific reaction pathways will change with time and substrate concentration[54]. The complexity of the lipid oxidation reaction –, i.e., the pathways operating and end-products generated, will in the end depend on the specific food model system. All the lipid oxidation products generated in a specific food model system can seldom be properly described or explained, which illustrates a knowledge gap in the understanding of the lipid oxidation reaction[50]. It is generally emphasized to target many different lipid oxidation products, preferably at several stages of the lipid oxidation reaction, and preferably specific lipid oxidation target molecules, to get a good picture over the oxidation reaction[50]. Indeed it is also crucial to select oxidation markers that are of relevance for the specific aspect of lipid oxidation studied, i.e., whether it is a specific aspect of food quality, or physiological effects of lipid oxidation products.

3.3.2 Lipid oxidation products and their analysis

There are several methods commonly used when assessing lipid oxidation in food systems, targeting different lipid oxidation products. The major groups of lipid oxidation products formed through the first two stages of the lipid oxidation reaction are shown to the right in Figure 5. The primary oxidation products, the hydroperoxides, can, e.g., be determined by titration, e.g., with potassium iodine, or the spectrophotometric ferric thiocyanate method[59], providing a PV, while secondary lipid oxidation products, such as aldehydes and ketones, commonly are determined by spectrophotometric methods providing a p-anisidine value (AV) or a TBARS value of the food[60,61]. Other methods to detect secondary lipid oxidation products focus on analysis of total carbonyls following 2,4-dinitrophenylhydrazine (DNPH) derivatization, where, e.g., aldehydes and ketones react with DNPH and thereafter are detected spectrophotometrically[60]. Further, as a combined measure of primary and secondary products, conjugated dienes can be analyzed spectrophotometrically without any reagent, due to the natural UV absorption by the double bonds of conjugated dienes at 234 nm[60]. The PV and AV methods can also be combined, and is then reported as a Totox value (Totox=2*PV+AV), but the latter is mainly used for food oils. The recommendations for maximum levels of oxidation in fish oil are a PV of 5 meq/kg oil, an AV below 20, and a TOTOX value below 26[62]. There is a wide range of other more sophisticated methods available for evaluating lipid oxidation, though the ones mentioned are still the most commonly used - since they are both quick and easy[55]. However, all the methods have also received critique for being unspecific, and the interest in more precise methods for detection of targeted lipid oxidation products is growing. This is particularly since certain lipid oxidation products can have physiological impact in biological systems and since it has become clear that there are knowledge gaps in the understanding and prediction of the routes of the lipid oxidation reaction[50].

More advanced method to quantitatively analyze lipid oxidation products in foods are often based on the combination of a chromatography method; liquid chromatography (LC) or gas chromatography (GC) with a mass spectrometer (MS) to enhance the detection level and the specificity[61]. Another detection method is to target specific lipid oxidation products by nuclear magnetic resonance (NMR)[61].

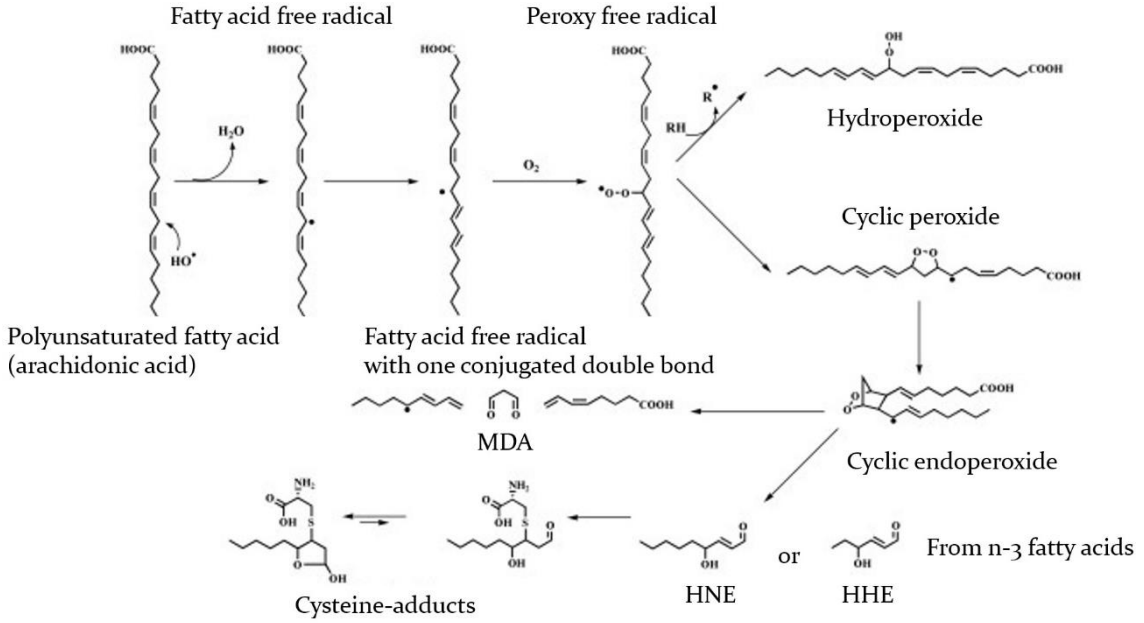

Figure 6: An example of formation of the lipid oxidation products MDA and HNE
Mechanism of formation of MDA and HNE from C20:4 n-6, including an example of how HNE can generate tertiary lipid oxidation products when reacting with amino-acid residues. A similar reaction pathway would apply to HHE from n-3 PUFA. Figure is adapted from Bocci et al.[63].

The lipid oxidation reaction is known to generate highly reactive oxidation products, which could potentially interact with DNA and proteins, leading, e.g., to various effects on cell function. Some of these oxidation products are the aldehyde MDA, and the α,β-unsaturated aldehydes HHE, HNE and 4-oxy-*trans*-2-nonenal (ONE)[64-66]. A schematic formation pathway of MDA and HNE from C20:4 n-6 is presented in Figure 6. MDA is a well-known lipid oxidation marker, which has traditionally been analyzed together with other carbonyls using the thiobarbituric acid reactive substances (TBARS) test[12,67], or by high-performance liquid chromatography (HPLC)[68]. MDA is the most abundant secondary lipid oxidation product formed, however, it can also be generated as a by-product from synthesis of prostaglandins and thromboxanes, making it unspecific[69]. Hydroxyalkenals, such as HNE, are well known lipid oxidation products and have been analyzed in biological samples since the 1960's[70]. HNE in foods was to our knowledge first analyzed by Lang *et al.* in 1985, by gas chromatography-mass spectrometry (GC-MS) after derivatization with N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA)[71]. Attention was, however, just recently given to HHE, which has been noticed due to its potentially high reactivity, and to its connection to LC n-3 PUFAs; it has been found to be the main lipid oxidation product formed from oxidation of DHA[72,73]. The toxicity of the aldehydes originates in the double bond next to the α-carbon, generating a strong electrophilic site[74]. If there are other groups connected to the α-carbon, such as in the case of α,β-unsaturated aldehydes and their hydroxyl group, the resulting aldehyde becomes even more reactive.

The α,β-unsaturated aldehydes reacts with proteins and DNA in several different ways, some of which are shown in Figure 7. HNE can for example react with amino acid side groups via 1,4-Michael adduction[75], generating different products depending on the amino acid. Lysine (-NH_2), cysteine (-SH) and histidine (-imidazole) side groups are particularly prone to Michael adduction. Additionally, there is a possibility for HNE to react with the lysine group through Schiff's base adduction, generating pyrroles (Figure 7)[75]. Several of these reactions can occur at the same time, since the carbonyl group of α,β-unsaturated aldehydes can participate in Schiff's base adduction, at the same time as the β-carbon is an electrophilic site, allowing for participation in Michael-adduction[69]. What is not shown in Figure 7 is that the tertiary lipid oxidation products formed, e.g., can react with each other, resulting in cross-linking, through reversed Michael and Schiff's base addition[75]. Since both HNE and HHE are hydroxyalkenals they are expected to react in similar ways, and their cytotoxic profiles have been shown to be similar, e.g., on rat cortical neuron cells. Some minor differences in reactivity have so far been detected, e.g., in their ability to generate glutathione adducts – HHE here being more reactive than HNE[72]. Both HNE and HHE also acts as signaling molecules and are biological active[72]. LC_{50} measured in ARPE-19 retinal pigmented epithelial cells is reported to be slightly lower for HNE (40 μM) compared to HHE (60 μM)[77]. For the protein adducts depicted in Figure 7, HNE has been found to have a reactivity towards amino acid side groups that follows the order cysteine>>histidine> lysine>> arginine[78], generating Michael adducts, and with lysine and Schiff's base adducts with arginine[69]. Comparing ONE with HNE, ONE has been shown to be more reactive towards sulfhydryl groups, but generates Michael adducts and Schiff base in similar way as HNE[69]. ONE readily crosslinks proteins and interacts with DNA bases[78]. Hence, although

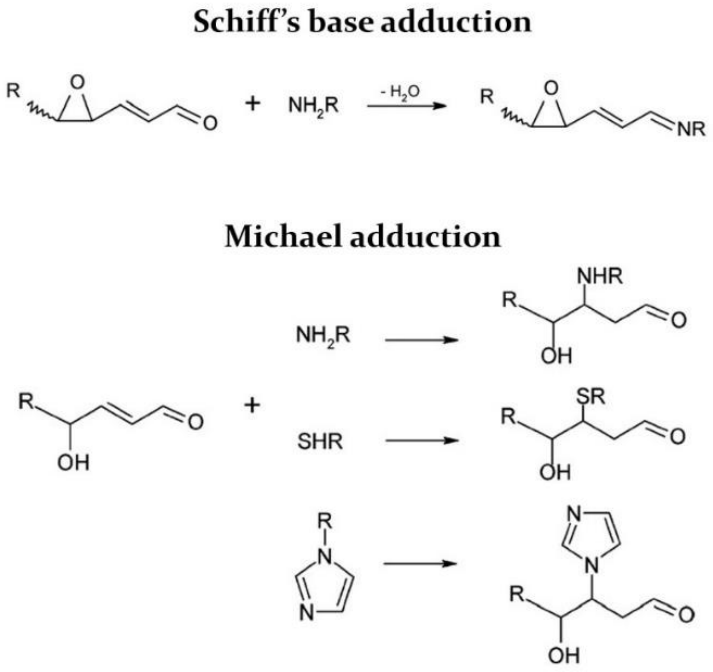

Figure 7: Schiff´s base and Michael adduction
Suggested reactions between α,β-unsaturated aldehydes and proteins, via Schiff's base or Michael adduction, generating tertiary lipid oxidation products, by Guillén and Goicoechea[64]. Reprinted with permission from Taylor & Francis.

recently discovered, targeting ONE is believed to be of importance to increase the understanding of the role of lipid oxidation in biological systems. MDA reacts in less specific ways compared to the mentioned α,β-unsaturated aldehydes; it can easily cross-links proteins and can generate two Schiff bases from its two carbonyl groups[69].

Further to the reactions listed above, There is also a connection between lipid oxidation and protein oxidation, via the Maillard reaction, a reaction which involves proteins and reducing sugars, including 2-hydroxyaldehydes, generating advanced glycation end products (AGEs)[78]. AGEs are already today commonly used as markers for, e.g., diabetes[78].

3.4 Lipid digestion and absorption

An overview of the human digestive system is shown in Figure 8. The main focus in this section will be on *lipid* digestion in the mouth, stomach, upper small intestine and absorption. An illustration of the major steps in lipid digestion is presented in Figure 9.

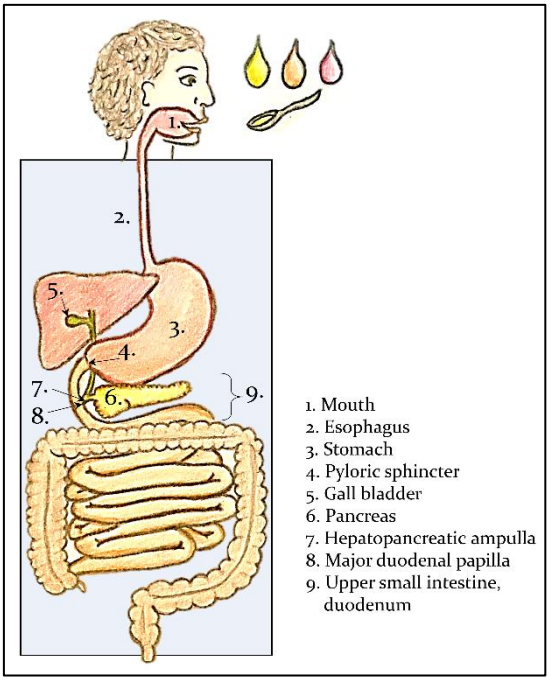

Figure 8: A schematic overview of the human digestive system

3.4.1 Mouth

Digestion of ingested food starts already in the mouth, and begins with mechanical grinding, lubrication of the food matrix by the salivary mucus, and enzymatic hydrolysis of carbohydrates by salivary α-amylase[79]. Humans produce approximately 1-1.5 L of saliva per day, mainly upon food ingestion, and saliva contains 97-99.5% water, electrolytes, mucus, lysozyme, IgA and amylase[79]. The presence of a lingual (tongue related) lipase in saliva, is questionable, since only trace activities has been measured[80], it has been reported to have an activity of 2 μmol/min/L saliva[81], and to be present at concentrations of 20-60 μM (secreted upon high fat food ingestion)[82]. Human lingual lipase is hence not believed to play a role in human lipase digestion, and it has been proposed that detected activities are of

microbial origin[80]. After grinding and lubrication in the mouth, the food is swallowed as a food bolus, and transported through the esophagus by peristalsis[83].

3.4.2 Stomach

In the gastric tract the pH of the food bolus is rapidly decreased, due to the gastric secretion of HCl from gastric parietal cells[84]. The enzymatic degradation of the food bolus continues, due to the secretion of pepsinogen - the inactive form of pepsin - and gastric lipase from the so called chief cells[84]. Approximately 2-3 L of human gastric fluid (HGF) is produced each day[84,85], depending upon fasted or fed state. The average pH in the HGF is 1.0-1.5 in fasting state[86]. The pH will differ upon the buffering capacity of the food when ingested. Emulsification occurs in the stomach due to mechanical grinding, by muscular contraction of the stomach walls, combined with the emulsifying action of, e.g., the FFA and PLs present[87]. Human gastric lipase (HGL) has been reported to contribute to 10-25% of the overall lipid digestion in humans[22,80]. The enzyme has a pH optimum at 4-5.4, but is active from approximately pH 2-7[80,86]. The stereopreference for HGL in TAG is the *sn-3* position, generating *sn-1,2* DAG, and for DAG the preference is for the *sn-1* position, generating 2-MAG[80]. The different TAG positions is shown in Figure 2, section 3.1.1. Gastric lipid digestion plays an important role in the intestinal hydrolysis of lipids, not only since lipids get pre-digested, but also because the oil-droplets formed after mechanical grinding, HCl and HGL secretion appear to be more easily available for digestion in the upper small intestine[22]. The food bolus that leaves the stomach is called "chyme", and is released at a rate of approximately 3 mL chyme per emptying, which is controlled by the pyloric sphincter[79]. This is also dependent upon the ingestion of a liquid or solid meal. The average gastric meal emptying time is 4 hours, however, a fatty meal can remain up to 6 hours in the stomach[79].

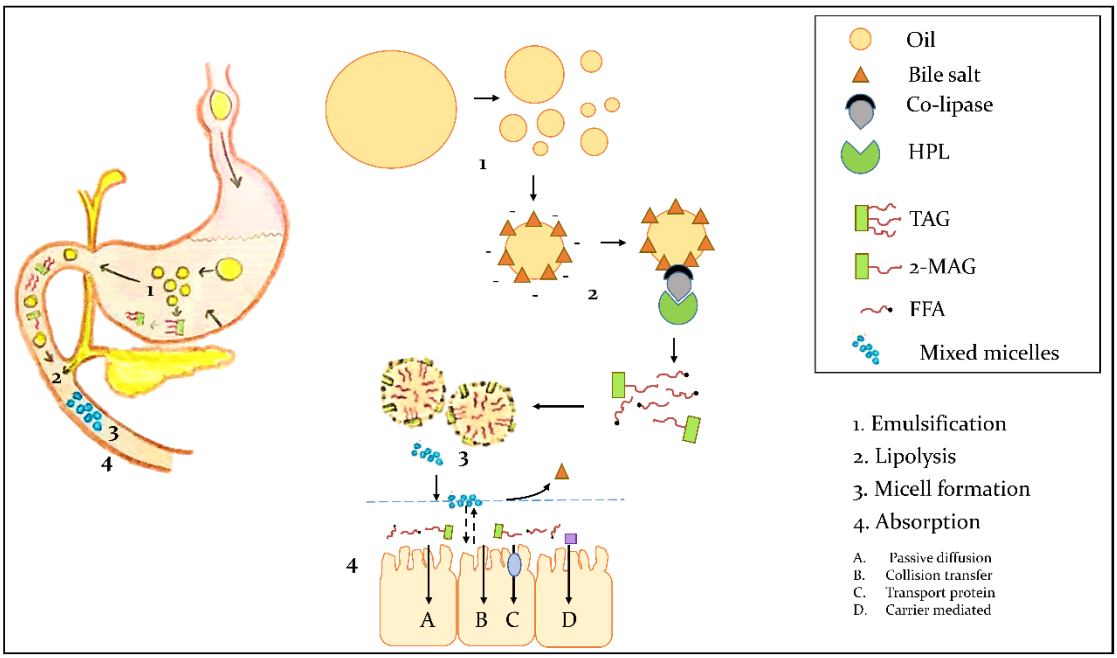

Figure 9: A schematic overview of lipid digestion in the gastric and duodenal tract

3.4.3 Upper small intestine

The main part (75-90 %) of the lipid digestion occurs in the upper small intestine[80], i.e., in the duodenum, facilitated by the human duodenal fluid (HDuF) which contains the pancreatic fluid and bile, and intestinal fluid. The intestinal epithelium is arranged into micro-villi, finger-like tentacles facilitating absorption, and crypts, pockets where epithelial cell proliferation takes place. Intestinal crypts additionally secretes 1-2 L of intestinal fluid per day, a fluid which mainly contains mucus and water[79]. Lipids in the chyme reaches the duodenum in a partly pre-emulsified form, and bile salts and other surface active agents such as PLs, will be absorbed on the surface of the droplets which can be as small as 1-50 μM[87]. The bile and pancreatic fluid are released from the gall bladder and pancreas, upon stimuli from the acidic HGF and lipid rich chyme. Some lipases are also to be found in the brush border membrane – those lipases are important signaling molecules, but are believed to play a minor role for lipase digestion[88].

The pancreas pancreatic fluid is released at a level of 1200-1500 mL daily, and contains sodium bicarbonate, zymogens, electrolytes and pancreatic enzymes[79]. Human pancreatic lipase (HPL) has a pH optimum at pH 7-7.5, however, the active field is only between pH 5-8, a field more narrow compared to HGL[80]. The major pancreatic enzyme which is involved in lipid digestion is HPL and its cofactor co-lipase. Co-lipase is secreted from the pancreas as a pro-colipase, which is cleaved by trypsin in the duodenum, generating a pro-protein hormone which has been connected with, e.g., satiety signalling[87,89]. There are a few other pancreatic enzymes that also are of importance for lipid digestion; human pancreatic lipase-related proteins (HPLRP2), with activity towards MAG, PLs and galactolipids; carboxyl ester hydrolase (CEH), with activity towards cholesterol esters, lipophilic vitamins, galactolipids and long chain acylglycerols; pancreatic phosphatide 2-acylhydrolase (PPLA2), with activity towards the *sn-2* position of 3-*sn*-glycerophospholipids[80]. The HGL continues to contribute to lipolysis during duodenal digestion (7.5 % in the upper small intestine), while HPL hydrolyses the major part of the TAG in the human diet (40-70%) when co-lipase is present[90]. The HPL is selective for *sn-1* and *sn-3* position of acylglycerols, and has a higher activity towards TAG than DAG, generating 2-MAG and FFA[80].

The gallbladder is located close to the liver and stores and concentrates bile, which is a fluid that contains cholesterol, PLs, and bile acids among others. Conjugated bile acids – bile salts – are synthesized from cholesterol, and the main human bile salts are derived from the amino acids glycine or taurine, conjugated with cholic acid into the amphiphilic bile salts glycocholate or taurocholate[22,79,87]. The release of bile is regulated by the peptide hormone cholecystokinin (CKK), which is released from the duodenal mucosa upon entrance of acidic gastric fluid and lipid-rich chyme[22,79]. Between 500-1000 mL bile is secreted daily[79,85], which results in 2-6.4 mM bile salt concentration in the fasting state, which is then increased to 6.8-16.2 mM in the fed state according to a review by Porter *et al.*[91].

Bile and pancreatic fluid enters the duodenum at the major duodenal papilla, through the hepatopancreatic ampulla (also called ampulla of Vater), controlled by the hepatopancreatic sphincter[79]. The lipolysis in the duodenal tract is facilitated by the collaboration of several compounds from the bile and pancreatic fluid. Co-lipase is of high importance for the activity of HPL, since the HPL needs to be present *at* the oil-water interface of lipid droplets to be active, and needs aid in reaching this area[92]. Bile salts

accumulated on the lipid droplet create a negative charge on its surface, which repulses HPL[22]. The co-lipase is then attracted to the lipid droplet surface by this negative charge[22]. HPL can at that point form a 1:1 complex with the co-lipase, which "anchors" and stabilizes the lipase to the interface[92]. The bile salts can further facilitate the enzymatic action of HPL by extending the surface area available for the HPL-co-lipase complex[87]. It is notable that presence of calcium ions is necessary as well, to reach the maximal activity of the HPL[22]. The TAG in the emulsified lipid droplets are in this way hydrolyzed and digested into FFA and 2-MAG[84,87]. The major role of the bile salts is to solubilize these lipolysis products formed, to facilitate the transportation of these products from the oil-water interface of lipid droplets, by the formation of mixed micelles[80,87]. Bile salts and hydrolyzed PLs (lysophospholipids) form micelles for transportation of MAG, FFA and cholesterol[84,87], and in the core of the micelles uncharged fatty acids, sterols and fat soluble vitamins can be found[22].

3.4.4 Absorption

The main part of the lipid absorption occurs in the upper small intestine. The micelles (4-6 nm in diameter) transports their content across the unstirred water layer closest to the intestinal epithelium, a layer of acidic mucosa secreted by the epithelial cells[22,92]. The bile salts are passed on in the lumen and are recycled in the entero-hepatic circulation[22], while short FFA and 2-MAG becomes protonated due to the acidic environment. The lipids can reach the micro-villi and enter the intestinal epithelium in different ways, both through passive and active transport. Active transport is, e.g., carrier mediated or facilitated by transport proteins such as plasma membrane fatty-acid-binding proteins (FABPpm), the cell surface receptor CD36 or fatty acid translocase (FAT)[22,91]. Passive transport is, e.g., passive diffusion, where the gradient decides diffusion rate. If there is a low concentration of the FFA and 2-MAG in the lumen, active transport will be the main absorption mechanism[91]. Inside the endoplasmatic reticulum of the cells, the fatty acids are resynthesized into TAG, and re-packaged into chylomicrons. Chylomicrons are lipid transporters with a core of TAG and cholesterol, and an outer layer of PLs and stabilizing apolipoprotein (apoB48). The chylomicrons are assembled in the Golgi complex and excreted into lymph vessels[22,79]. Chylomicrons are secreted to the lymphatic system and transported to the heart, before distribution in the body[22,79,84]. Most of the lipids will thus not pass by the liver before distribution in the body. The exception is shorter FAs (<C12) which can be absorbed in their native form and directly enter the portal blood vein, followed by delivery to the liver and β-oxidation[22].

3.5 Lipid oxidation in the gastrointestinal tract (GIT)

The first suggestion of a link between oxidation of lipids and digestive fluids was raised already in 1995, by Terao *et al.*, who saw that bile and pancreatic fluids from rats had a promoting effect on lipid oxidation[93]. Kanasawa and Ashida reported similar effects from gastric fluids when administering linoleic acid to rats, following post-prandial detection of aldehydes in the gastric lumen[94]. The clear hypothesis that lipid oxidation can occur in the GIT of humans was raised in the year 2000, by Halliwell *et al.*[57]. It was reasoned that the GIT fulfills several criteria, which would make it a pro-oxidative environment. The factors listed were among others the presence of pro-oxidants, such as Fe^{2+}, and Cu^{2+} ions, and free heme, as well as pre-oxidized lipids and cholesterol in the diet[57]. Other pro-oxidative factors in the GIT which later have been raised are elevated temperature in the human body, mechanical

grinding and emulsification of food lipids, as well as the presence of oxygen in the intake meal. The review by Halliwell *et al.*[57] has been followed by several studies, examining the role of different parameters *in vitro*, in animals or in humans to better understand lipid oxidation under GI conditions and subsequent effects of oxidation products on the intestinal epithelium.

3.5.1 Lipid oxidation studied during in vitro lipid digestion

3.5.1.1 Static lipid digestion models

Shortly after the review by Halliwell *et al.*[57] was published, a new study was highlighting the gastric tract as a pro-oxidative environment[95]. This after digestion of turkey meat and soybean oil *in vitro* using simulated gastric fluid (electrolyte solution with added pepsin of animal origin), reporting that hydroperoxide levels found in turkey meat was 6-fold higher after gastric digestion[95]. In this study *in vitro* digestion with simulated gastric fluid was also compared with human gastric fluid (HGF), which confirmed that hydroperoxides can form in both systems, and that acidic conditions (pH 3) promotes lipid oxidation[95]. Furthermore it was seen that the presence of metmyoglobin (6-20 μM) and addition of polyphenols from red wine had pro- and antioxidative effect, respectively[95]. *In vitro* digestion of marine lipids have since then been evaluated in a few studies. In our own lab, cod liver oil was investigated using simulated digestive fluid (SDF); electrolyte solution in combination with enzymes and bile of porcine-, and for the gastric lipase, fungal origin, in a static *in vitro* digestive system. In this study, lipid oxidation was followed through detection of TBARS and hydroperoxides[12]. Lipid oxidation was observed both in the gastric, and in the duodenal phase, however, no oxidation was seen from subjecting the oil solely to low pH and 37°C, when the digestive fluids were left out[12]. Furthermore, the addition of hemoglobin (Hb) (11.5 μM) to emulsified cod liver oil was in this study found to strongly promote lipid oxidation, while no effect was seen from the inclusion of α-tocopherol at 1 mg/g oil. Lipid oxidation has additionally been shown to occur during gastric digestion of herring oil emulsions and liposomes using HGF *versus* pure acidic solution[13]. Although detected at low levels, both PV, TBARS and oxygen uptake rate all increased, and no difference was seen between HGF and the acidic solution. Using HGF during the gastric digestion, the addition of metHb (0.16 μM) was found to be pro-oxidative, and several additives showed anti-oxidative capacity, e.g., red wine and berry juice[13]. Kenmogne-Domguia *et al.* followed lipid oxidation of two mixed oil emulsions (15% tuna oil, 13% oleic sunflower oil, and 2% sunflower oil or lecithin, w/w), during *in vitro* digestion using porcine enzymes and bile[67]. In the digestion model, 20 μM of metmyoglobin was added to the gastric fluid, to simulate heme and non-heme iron release from heme proteins during digestion of a standard meal. Both total MDA, HHE, and HNE were targeted, as well as headspace oxygen level[67]. It was found that MDA, HHE, and HNE development, as well as oxygen uptake increased over time of digestion, with levels of 240 μM MDA, 7.2 μM of HHE, and 1.6 μM of HNE being detected[67].

There are several studies reporting on lipid oxidation during static *in vitro* digestion of different meat sources. Steppeler *et al.* recently presented results from digestion of chicken, pork, beef and salmon[96]. It was shown that lipid oxidation is highly promoted by the PUFA concentration in the meat[96]. Digestion of salmon muscle resulted in the highest levels of TBARS and HHE[96]. Heme iron was also found to be pro-oxidative, and the highest level of TBARS was detected in a system where fish oil was added to minced meat from

beef[96]. The connection between meat digestion and lipid oxidation was also seen by Van Hecke *et al.* when comparing lipid oxidation in chicken, pork, and beef during digestion[97]. This research group has additionally shown that the initial lipid concentration plays a major role for lipid oxidation during digestion, and beef with increased fat content generated higher levels of lipid oxidation products during digestion[98,99]. Additionally, Van Hecke *et al.* tested several different pro- and antioxidants, in combination with the meat during digestion, and, e.g., proposed mechanisms explaining the pro-oxidative effect of ascorbic acid[98].

3.5.1.2 Dynamic lipid digestion models

Lipid oxidation of minced mullet (*Mugilidae*) was evaluated in a dynamic *in vitro* digestion model by Maestre *et al.*, using the TNO Intestinal Model-1 (TIM-1)[14]. They used SDF with enzymes and bile of porcine origin, and fungal gastric lipase, detecting conjugated dienes throughout the digestion[14]. In this study, lipid oxidation was found to occur during the whole digestion, increasing to high levels in the intestinal phase[14]. Furthermore, the addition of a polyphenol-rich extract was found to decrease lipid oxidation, both in the gastric and intestinal phase[14].

Although several studies are listed here (section 3.5.1.1 and 3.5.1.2), it should be added that at the start of this project work, no previous studies had investigated lipid oxidation *during* digestion of marine foods/oils using HGF or HDuF.

3.5.1.3 Cell models to study uptake and effects of oxidation products

The group of Maestre *et al.* incubated Caco-2 cells with oxidized and non-oxidized DHA, in order to study cell metabolism and bioaccessibility of oxidized versus non-oxidized PUFAs[14]. Using radiolabeling of DHA, they reported that the oxidized PUFAs were able to be absorbed into the cells (10% of absorbed non-oxidized DHA), after 2 hours approximately 0.01 µmol oxidized FA/mg protein was absorbed, compared to approximately 0.075 µmol non-oxidized FA/mg protein[14]. Furthermore, they noted that co-incubation of Caco-2 cells and PUFAs with polyphenol-rich extracts enhanced the cellular DHA uptake[14]. Another group used deuterium labeling of the lipid oxidation products MDA and HHE to study the basolateral transport, using a Caco-2 (TC7) cell model[11]. In this study, they saw that HHE (100 µM, 24 h) could be transferred across the cell membrane (0.2%), and additionally, they saw no adverse effect on trans-epithelial electrical resistance (TEER) after incubation with the pure aldehydes (0-100 µM, 24 h). Digests of marine oils have previously been evaluated in yeast cells (*Saccharomyces cerevisiae*) and human dendritic cells in order to study inflammatory responses[100]. The authors, partly from our lab, found that cod liver oil digests from a static *in vitro* digestion model, increased intracellular oxidation in yeast cells, measured as fluorescence of the oxidized dye 2',7'-dichlorodihydrofluorescein (DCFH2). They also found pro-inflammatory response in the human dendritic cells, measured as the ratio of the cytokines IL-12p40/IL-10[100]. Caco-2, HT-29 and HCT-116 cell lines were incubated with digests of high fat- and lean beef from a static *in vitro* model, which were evaluated based on genotoxicity and cytotoxicity[98]. The digests of high fat beef (15%) contained elevated levels of lipid oxidation products (40 µM MDA, 4 µM HHE). Caco-2 cells were then shown to be more sensitive to the digests, compared to both the HT-29 and the HCT-116 cell lines, and had a decreased cell survival correlated with incubation time[98].

3.5.2 *Systemic effects from ingestion of oxidized lipids*

3.5.2.1 Animal studies

Systemic effects of lipid oxidation products are highly interesting, since they show a combined response of uptake and distribution of the lipid oxidation products *in vivo*. There are several animal studies investigating lipid oxidation during digestion of marine oils. Pre-oxidized tuna oil was fed to mice and accumulation of lipid oxidation products (e.g., HHE) in plasma and uptake of oxidized oil was followed by Awada *et al.*[11]. They reported that administration of oxidized oil to mice led to an accumulation of HHE over time in the plasma, and that there was an increase of the inflammation markers IL-6, MCP-1 in the plasma, and of NF-κB in the intestinal mucosa[11]. Feeding the mice with pure HHE showed that HHE could be absorbed in the intestine, and led to an accumulation of protein adducts in the small intestine[11]. In another study, rats fed with a fish oil-rich (30%) high fat diet were found to have increased TBARS and decreased GSH levels, in gastrocnemius muscle and liver tissue[101]. Lipid oxidation during *in vivo* digestion has also been observed using minipigs, with administration of a Western diet based on beef and sunflower oil, and aspiration of gastric digests[102]. The accumulation of conjugated dienes and TBARS was followed in the gastric tract posterior to feeding, and for both measures, levels were elevated[102]. Gobert *et al.* furthermore demonstrated that fruits and vegetables, added in pieces or as an extract to the diet, has a protective effect on lipid oxidation in the gastric tract[102].

3.5.2.2 Human studies

It is not possible to follow lipid oxidation during digestion in humans today, due to ethical constrains, unless ileostomy is being used. However, postprandial lipid oxidation markers can still be detected, e.g., in plasma, and a few studies where oxidized oil has been provided to humans have been conducted. It is important to stress though that these studies do not reveal *where* the oxidation products are formed; in the GIT or later. In one recent study by García-Hernández *et al.*, participants received no capsules (*n=18*), fish oil capsules of low oxidation degree (PV<20 meq/kg oil, *n=17*), or high oxidation degree (PV>75 meq/kg oil, *n=17*), for 30 days, where after circulating TAG and cholesterol levels were evaluated[103]. During the study period, all study participants were advised to eat a fish-rich hypercholesterolemia diet[103]. The group which received highly oxidized fish oil had a slight non-significant increase in cholesterol levels in the plasma, while the group given low oxidized fish oil had significantly decreased circulating cholesterol levels[103]. Two studies have been published by the group of Ottestad, where supplementation of fresh (PV 4 meq/kg oil) and oxidized (PV 18 meq/kg oil) fish oil was evaluated in healthy volunteers, detecting lipid oxidation- and inflammation markers in plasma and urine[104,105]. In the studies (7 weeks; *n=17-18*/group), no differences in lipid oxidation products (e.g., HHE and HNE), nor inflammation markers (e.g., IL-6) were detected[104,105]. However, none of these studies comprised cross-over design, and the study period was relatively short. There are two additional human studies where pre-oxidized vegetable oils have been shown to increase the presence of lipid oxidation products in chylomicrons[106,107]. In the study by Staprañs *et al.* a cross-over design was used (*n=6*), however, the oil was given in fasted state and detection was done postprandial, 4 hours after administration of the oil[107].

4. METHODS AND METHODOLOGICAL CONSIDERATIONS

4.1 Study design

This thesis is based on four studies, I-IV, and a summary of their designs can be found in Figure 10. In Study I, III and IV, human digestive GI fluids were used in a static *in vitro* digestion model. In Study I, a comparison was also made to enzymes and bile of porcine origin. In Study II, a dynamic *in vitro* digestion model was used with SDF; electrolyte solution comprising physiologically relevant salts with enzymes and bile of animal and/or fungal origin, at a set pH. Human gastric and duodenal fluids were collected at Lovisenberg

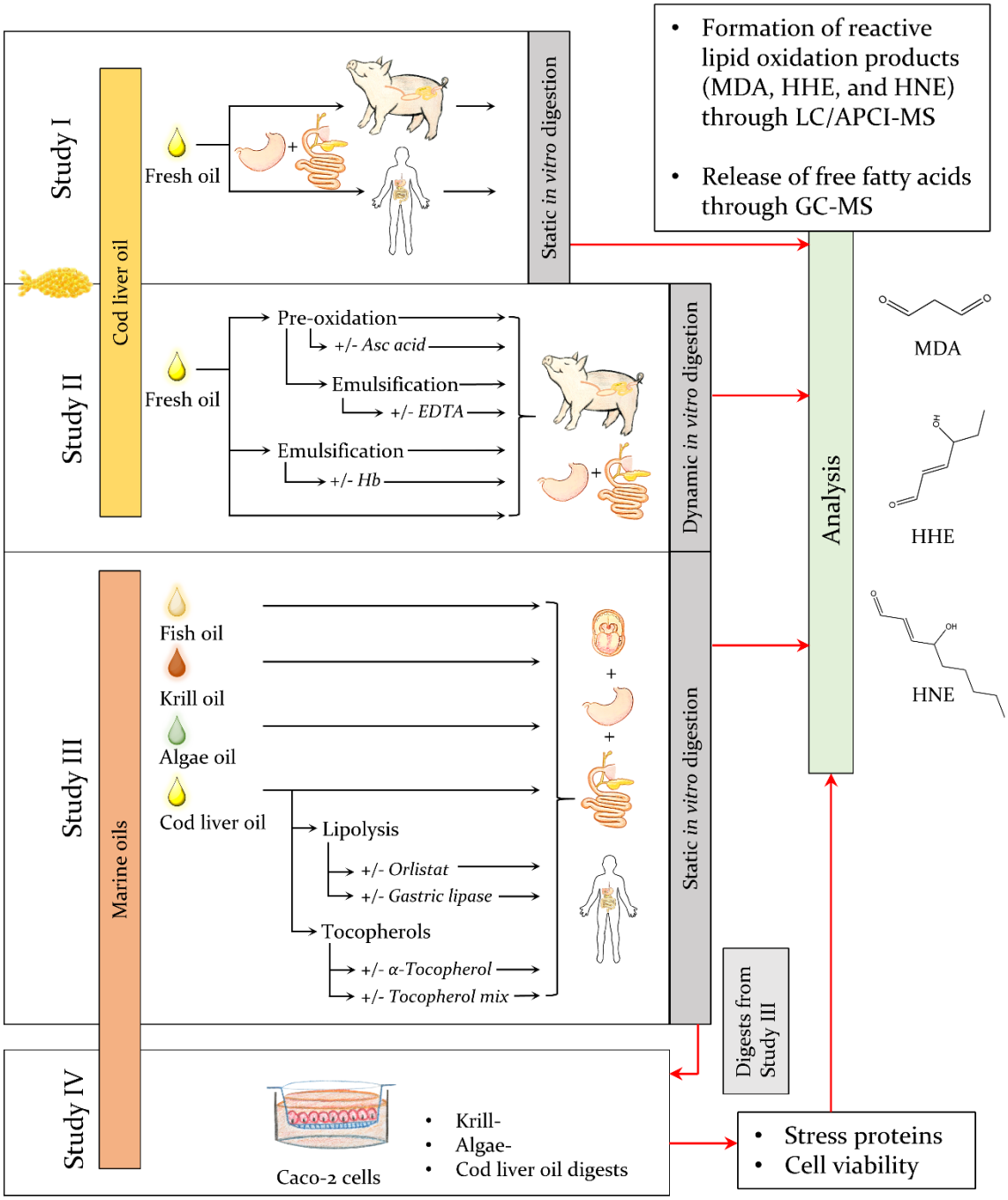

Figure 10: Schematic overview of the study design of Study I-IV

Hospital, Oslo. For each study, fluids of each type were pooled prior to use in the static *in vitro* digestion models. In each study, the digestion model was designed to mimic the intake of a fatty fish meal (Study I, II), a spoon of oil (Study II), or a capsule of oil (Study III, IV). Collection of samples were done throughout the gastric and duodenal digestion phases to investigate lipid oxidation and degree of lipolysis. The former was monitored as formation of selected aldehydes and the latter as release of free fatty acids (FFA).

4.1.1 Study I

The aim of Study I was to compare the degree of lipid oxidation and lipolysis during digestion of cod liver oil using human digestive fluids (HDF) or SDF, with added enzymes and bile of porcine origin. The absolute levels of oxidation products formed using HDF were in themselves of high interest since this had not previously been documented. Therefore, a question to be answered were *if* lipid oxidation occurs during digestion using HDF, and if so, to *what extent*?

A static *in vitro* digestion model was used to compare the different GI fluids ("human"- versus "porcine"-based digestive fluids), and a standardized digestion protocol developed by the InfoGest network was used as a basis when designing the experiment.[108] The HDF were characterized by determination of enzyme activities, calcium and bile-salt concentration prior to digestion. To study lipid oxidation during digestion, the target aldehydes MDA, HHE, and HNE were followed using a liquid chromatography atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS) method which was developed and validated for this purpose.

4.1.2 Study II

The aim of Study II was to investigate how lipid oxidation during dynamic *in vitro* digestion of cod liver oil is affected by different pre-treatments of the oils, and additions of selected pro- and antioxidants. A secondary aim was to elucidate how the levels of oxidation products reached in a dynamic digestion model differ from those reached in a static model.

The study was done using the dynamic digestion method tiny-TIM, with SDF using enzymes and bile of porcine and fungal origin. The cod liver oil to be digested was pre-oxidized and/or pre-emulsified. Cod-Hb was added prior to digestion of the oil, to study the effect of a pro-oxidant which has been proven to be very powerful in marine lipid systems during storage. The chelator ethylenediaminetetraacetic acid (EDTA) was added as a potential antioxidant to pin-point the role of LMW metal ions in GI-oxidation. Again, the reactive aldehydes MDA, HHE, and HNE were followed.

4.1.3 Study III

The aim of Study III was to compare the development of lipid oxidation and lipolysis in marine oils of different origin during static *in vitro* digestion using HDF.

Again, the HDF were characterized, prior to digestion, by recording enzyme activities and by measuring the concentration of bile-salts and trace metal ions. Endogenous Hb and ascorbic acid was also measured in the fluids. In this study, lipid oxidation was followed by detection of MDA, HHE, HNE and ONE throughout the digestion period. The relation between lipid oxidation and lipolysis was investigated by addition of the lipase

inhibitor orlistat, and by addition of an external gastric lipase: rabbit gastric lipase (RGL), to the digestive system with cod liver oil. The possibility of preventing lipid oxidation by tocopherol addition was also investigated, by addition of either α-tocopherol, or the tocopherol mix Covi-ox® T 70 EU to the cod liver oil.

4.1.4 Study IV

The aim of Study IV was to evaluate the possible cellular effects induced in cultured intestinal cells from digests of cod liver oil, krill oil and algae oil emerging from a static *in vitro* digestion model using HGF and HDuF. The digests from Study III were used for this purpose. The Caco-2 cell line was chosen as cell model to simulate the human intestinal epithelium.

The effect of digests on Caco-2 cell survival was investigated by cell viability, and the expression of proteins connected to stress was evaluated by proteome profiler arrays. The cells were also incubated with pure MDA and HHE at the same levels as found in the digests, to evaluate the specific effect of these aldehydes on stress responses and cell survival.

4.2 Materials

4.2.1 Marine oils

All the marine oils used in this work are generally recognized as safe (GRAS) according to the US Food and Drug Administration (FDA), and approved for human consumption by the European Food Safety Authority (EFSA). The initial oxidation level of each one of the oils (cod liver oil, fish oil, krill oil and algae oil) can be found in Table 3, and their lipid compositions in Figure 17, section 5.1.1.

4.2.1.1 Cod liver oil

Cod (*Gadus morhua*) liver oil, was included in all our digestion models (Study I-IV), since this is an oil with a long history of being consumed, and it is today a common "omega-3" supplement. Refined cod liver oil without added antioxidants was received from Lýsi hf (Reykjavík, Iceland). The absence of antioxidants allowed us to better follow the mechanisms of lipid oxidation in the oil, without any distraction from additives which were not part of the design for Study I-IV. The effect of the selected antioxidants and pro-oxidants could then easily be evaluated in comparison to the pure oil as control. Since many of the marine oil supplements available for consumers on the markets have been found to contain significant levels of oxidation products[9,10,109], a relevant question was how the oxidation level of the ingested supplements would affect lipid oxidation during digestion. To compare different initial oxidation levels (Study II), the cod liver oil was oxidized for 6-9 days in room temperature. This resulted in three cod liver oils with low- ("fresh"), medium-, and high oxidation levels (Table 3, section 5.1.1).

4.2.1.2 Fish oil

Refined whole fish oil from Peruvian anchoveta (*Engraulis ringens*) without added antioxidants was chosen to symbolize the intake of a fish oil capsule (Study III). The specific origin of the oil was selected since 30-35% of the global fish oil and fish meal manufacturing is based on Peruvian anchoveta[110], and since the major part of the global fish oil produced

comes from Peru[33]. As for cod-liver oil, a non-stabilized fish oil was chosen to better be able to follow reaction mechanisms connected to the oil *per se*. The fish oil was supplied by Lýsi hf (Reykjavík, Iceland).

4.2.1.3 Krill oil

The unrefined and non-stabilized krill oil Superba™ Krill Oil (Aker Biomarine Antarctic AS, Oslo, Norway) was produced from Antarctic krill (*Euphausia superba*) and provided by Sanpharm AB (Gothenburg, Sweden). The oil was the only marine oil provided in capsule form, thus a high number of capsules were cut open and pooled before being used in the digestion models. Krill oil is produced without refining since it contains very low levels of pollutants, and doesn´t need to be purified before consumption[111]. Krill is the largest biomass in the world, and has been suggested as a more sustainable source for marine oil compared to fish[111]. Krill oil has earlier been recognized for its oxidative stability, for naturally being rich in the antioxidant astaxanthin, and for having a large part of the FA bound to PLs[112]. These facts made krill oil an interesting alternative marine oil to study in the digestion- and cell models of Study III-IV.

4.2.1.4 Algae oil

The algae oil was an unrefined oil from the microalgae *Schizochytrium* sp. called Life´s DHA™ S35-CO100, supplied from DSM (Basel, Switzerland). *Schizochytrium* sp. belongs to the family of the *Thraustochytriidae*[113]. This specific oil was selected since *Schizochytrium* sp. is commonly used by the industry for production of microalgae oil, due to its beneficial lipid profile, and its fast growth rate compared to other microalgae. It belongs to the heterotrophic microalgae, which allows for fermentation during cultivation[114], resulting in high productivity and low production cost[113]. The specific oil from *Schizochytrium* sp. has been found to naturally contain sterols and Yao *et al.*[38] reported a very high content of TAG (78-93%)[38], of which the main FAs are C16:0 (60% of total FA) and C22:6 n-3 (DHA, 26% of total FA)[38]. DPA (C22:5) in n-6 form is also commonly found in this oil[114]. *Schizochytrium* oil has also been found to contain several natural antioxidants, e.g., polyphenols and tocopherols, which have been suggested to protect the oil against lipid oxidation[115]. In contrast to other microalgae, *Schizochytrium* sp. contains very low levels of glycolipids and PLs (0.07 w/w% and 0.8 w/w%, respectively), and no detectable chlorophyll[38]. The high content of DHA, and the combination of being affordable and available on the market as an alternative vegetarian marine oil, made it interesting to include *Schizochytrium* oil into Study III-IV.

4.2.2 Digestive enzymes and bile

Digestive enzymes and bile were chosen according to the recommendations by Minekus *et al.* in the static[108] and the dynamic[116] models. The commercial enzymes used in the digestion models were all purchased from Sigma-Aldrich (Schnelldorf, Germany), and were of porcine origin, with a few exceptions. In the dynamic digestion model, e.g., amylase from *Bacillus sp.*, pancreatin of porcine origin (Pfizer Animal Health), fresh porcine bile (TNO, Zeist), and bovine trypsin were used. Detailed description of the digestive enzymes used can be found in each corresponding paper (Paper I-IV).

4.2.2.1 Gastric lipase

No gastric lipase of non-human origin was included in Study I, since no suitable substitute to the human enzyme was proposed by Minekus *et al.*[117]. However, since a focus in this thesis work is the relation between lipid digestion and lipid oxidation, gastric lipase was used in Study II-III when simulating gastric fluids. For the dynamic digestion model (Study II), lipase from *Rhizopus oryzae* (F-AP, Amano Enzyme Inc) was used. This lipase was chosen due to its relatively low cost, viewed against the relatively large amounts needed for the dynamic digestion model. The use of lipase from *Rhizopus oryzae* has, however, been criticized, since it has a different specificity compared to HGL, with a preference towards the *sn-1* position of TAG, since it has a different pH optimum (pH 7-8), and since it becomes inhibited by the bile salts in the duodenal step of digestion[86]. RGL (Germe, Marseille, France) was added in the gastric step of static digestion with HDF, to boost the lipolytic activity (Study III). RGL has been proposed as a suitable substitute for HGL, due to their similar specificities, although its activity is about 2-folds higher[80,118]. The activity of HGL in the HGF was analyzed at pH 6 and was only 16 U/mL. This activity was less than what was expected based on the fact that HGL in fasted state can reach 100-120 U/mL at pH 5-6[118-120]. The addition of RGL was hereby motivated. RGL is unfortunately still quite expensive and hard to acquire, hence only a small quantity could be obtained.

4.2.2.2 Pancreatic enzymes and bile of animal origin

Porcine pancreatic lipases have been shown to have similar lipolytic actions as human pancreatic lipase, determined as release of FFA, when used in *in vitro* models[121,122]. They are therefore commonly used when simulating *in vitro* digestion. Pancreatic extract of porcine origin contains most of the important pancreatic lipases found in humans, with the exception of PLRP2[80], and has been shown to have a similar activity as HDuF on, e.g., the surface active drug delivery excipients Labrasol®[123], and Gelucire® 44/14[124]. Bile extracts of porcine origin or whole frozen porcine bile are both recommended by Minekus *et al.*[117].

4.2.3 Additives to the cod liver oil prior to digestion

4.2.3.1 Brij™ 35 as an emulsifier

Brij™ 35 (polyoxyethylene 23 lauryl ether) was used in Study II as emulsifier to create cod liver oil emulsions (20%, w/w).The oil was homogenized with 17 mM Brij™ 35 in 10 mM phosphate buffer, as described by Sasaki *et al.*[125]. The emulsifier Brij™ 35 was chosen since it is nonionic and do not contain any FA constituents, as some other emulsifiers which could interfere with the study of lipolysis and lipid oxidation. Additionally, it had previously been shown that the susceptibility towards lipid oxidation did not change with the addition of Brij™ 35 as a stabilizer in corn oil emulsions (20% w/w)[126].

4.2.3.2 Orlistat as a lipase inhibitor

To investigate the relation between lipolysis and lipid oxidation, the lipase inhibitor orlistat (Figure 11) was added to the digestion system with the intake meal (Study III). Orlistat, or tetrahydrolipstatin, found as a drug under the trade name Xenical – is produced by the bacterium *Streptomyces toxitricini* and is commonly given to patients as a way to treat obesity[90]. Orlistat is known to be a strong inhibitor of digestive lipases, and has been reported to reduce lipolysis with about 35%, by blocking the catalytic site of the lipase molecule - the serine residue[90]. Carrière *et al.* reported both inhibition of HGL and HPL

when studying the effect of orlistat *in vitro* as well as *in vivo*[127]. However, they saw that the inhibition of HPL was meal-dependent, with a greater inhibition when orlistat was given together with a solid compared to a liquid test meal, something that was explained by the fast action of HPL on emulsion systems[127]. 120 mg of Orlistat was added in the study by Carrière *et al.*[127], which corresponds to 0.24 mg Orlistat/initial meal. We wanted to include orlistat in our digestion model at the same concentration, to study the connection between lipolysis and lipid oxidation during *in vitro* digestion of cod liver oil.

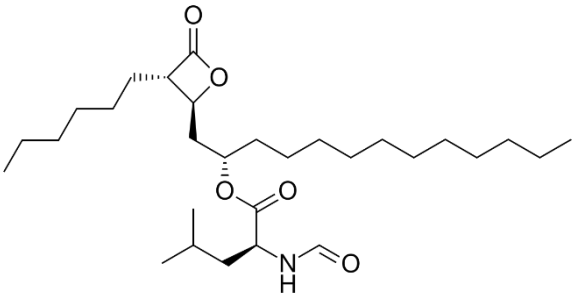

Figure 11: The chemical structure of the lipase inhibitor orlistat

4.2.3.3 Hemoglobin as a pro-oxidant

Hb, or rather the heme-bound iron, is known to be a strong pro-oxidant in lipid-based systems. As mentioned in section 3.5.1.1., the addition of Hb or pure metHb to digestion systems have both been seen to promote lipid oxidation during digestion of marine oils[12,13]. Larsson *et al.* included 11.5 µM cod-Hb in a static digestion system with emulsified cod liver oil (Study II). Hb was added as hemolysate from cod, and the level was selected to simulate a relevant amount of blood present in a fish fillet. This level also allowed direct comparison to the work of Larsson *et al.*[12] to see if a similar pro-oxidative effect of Hb was obtained in a dynamic, as in a static *in vitro* digestion system. Hemolysates were prepared according to Richards and Hultin[128], by bleeding of recently slaughtered cod followed by removal of plasma and lysis of the red blood cells. The amount of Hb in the hemolysate was quantified in its carbon monoxide form, against a bovine Hb standard.

4.2.3.4 Antioxidants

Different types of antioxidants were added to the digestion systems in Study II and III, to investigate if lipid oxidation during digestion could be retarded or not.

4.2.3.4.1 EDTA

EDTA is a well-known metal chelator, and it was added to pin-point the role of LMW metals during GI-oxidation of marine oils. However, it is an artificial additive and hence there is some resistance for using it in foods[23]. EDTA has generally been shown to work well as an antioxidant in oil-in-water emulsions, however, the protective effect has varied widely[49,129]. In our dynamic system, 50 µg EDTA/g intake meal was added, to see if this concentration was sufficient to prevent lipid oxidation during digestion of emulsified cod liver oil.

4.2.3.4.2 Tocopherols

Tocopherols have already been mentioned in section 3.2.2 as naturally occurring antioxidants, commonly added to marine oils. α-Tocopherol was earlier added as an antioxidant to cod liver oil (1 mg/g oil) prior to static *in vitro* digestion without giving any protective effect[12], thus we here wanted to investigate if an increase in the concentration would be more efficient. According to EFSA, 2-6 mg tocopherol/g oil is the recommended concentration for fish oils[130]. We decided to include 4.5 mg tocopherols/g oil, to study the effect of tocopherol addition on lipid oxidation. As mentioned by Hjaltason and Haraldsson, mixtures of tocopherols are commonly used by the industry to maximize the beneficial effect[23]. This is since the different homologues work in different ways, thanks to differences in methylation degree and where on the chromanol ring of the tocopherol the methyl groups are positioned[45]. To compare the effect of a single isomer with a mixture of isomers during static digestion of cod liver oil, we included both pure α-tocopherol, and Covi-ox® T 70 – a mixture of tocopherols containing 14% α-, 2% β-, 60% γ-, and 24% δ-tocopherol (Study III).

4.3 Methodology

4.3.1 Design of simulated in vitro digestion

There are several different models to choose from when working with digestion focused research, and depending on the research question there can be strong reasons to choose an *in vitro* GI digestion model over *in vivo* trials. Due to ethical reasons researchers aim to keep the number of animal studies at a low level. Additionally, although digestion in animals may have some similarities to human digestion, there are also many differences, e.g., in pH levels by acid and bicarbonate secretion, buffering capacity, in bile and pancreatic secretion, digestive enzymes and enzyme specificities, GI fluid physiological content and volume, surface tension, bacterial level, redox potential etc. Some of the reasons to choose *in vitro* digestion models are that they allow us to:

- Perform experiments which require ethical constrains would not be possible to conduct in humans
- Perform fast and cheap static *in vitro* digestion studies, which require minimum personnel and still can provide relevant information
- Perform studies with a mechanistic approach, where, e.g., the kinetics of a reaction can be followed
- Perform controlled factorial studies to investigate correlation between specific components and their outcome/generated products, e.g., for screening purposes
- Perform studies with high reproducibility, suitable for hypothesis testing

It is important to remember that *in vitro* models are *models* that only will simulate *in vivo* trials due to complex physiological status and individual variations. Many assumptions are made already in the design of the models, and the choice of the model will decide which questions that can be answered. There are several different *in vitro* GI digestion models in use, both static and dynamic ones. What *in vitro* GI digestion models have in common are that they normally involve electrolyte solutions, stirring, pH change, the addition of enzymes, and a set temperature at 37°C, this to simulate the human body. More precise settings vary, however, widely with the models. In this thesis work, both static

Table 1: Comparison of pros and cons of static and dynamic *in vitro* GI digestion models

	Static *in vitro* digestion model	Dynamic *in vitro* digestion model
Pros	• Cheap • Easily repeatable model • Easily scalable model • Easily available model • Parameter of interest can be adapted easily	• Better reflect human conditions • More realistic outcome • High *in-situ* control of system • Peristaltic movement • Continuous pH changes, enzyme addition and dilution • Easily interpreted data, when comparing to *in vivo* • Automatic sampling
Cons	• Over-simplified model • Might over- or underestimate outcome • Substrate-product inhibition reactions • Batch addition, could lead to a depletion, e.g., of enzymes • Fixed addition of calcium, bile, and enzymes – might result in an unrealistic high initial lipolysis • Fixed pH and dilution • Manually sampling of solid or coagulated samples may be a problem	• Expensive • Fixed factors (e.g., certain volume required) • Analytes might be too diluted over time for accurate analysis • Overconfidence in data might lead to wrongly drawn conclusions • Few dynamic models available

and a dynamic *in vitro* GI digestion models were used. An overview of pros and cons of static and dynamic *in vitro* digestion models are shown in Table 1.

Factors of importance when designing an *in vitro* model are sample state (liquid or solid), volume of digestive fluid, dosage form, vessel type, stirring speed[117]. A low stirring (30-75 rpm) has been shown to best mimic *in vivo* conditions, however, this also increases the risk of coning, especially if combined with a poor choice of vessel[117]. Other factors of importance are the substrate:enzyme ratio, the pH gradient, the dilution of input food, and, when studying lipid oxidation, the oxygen available. According to Kostewicz *et al.*, *in vitro* lipolysis models have previously been found to correlate well with *in vivo* data[117]. Human GI fluids are complex fluids and the presence of antioxidants, trace metal ions, and other constituents of the GI condition could have considerable effect on the outcome, when studying lipid oxidation during digestion. In this thesis work, aspirated HDF were used in the static models (Study III and IV), and compared to simulated GI fluids of animal origin (Study I). Simulated GI fluids are composed of electrolyte solutions that mimics the digestive fluids in humans, with corresponding animal or fungal enzymes and pH. The comparison of HGF and HDuF has, to our knowledge, never previously been used in an *in vitro* digestion model for lipid oxidation during GI digestion.

4.3.1.1 Static *in vitro* digestion model

Static *in vitro* digestion models are the most common type of *in vitro* digestion models[117]. At the start of this thesis work, it was known that a standardized static *in vitro* GI digestion protocol was about to be released by an EU Cost Action FA 1005 initiative,

InfoGest; a food digestion research network. The protocol was published by Minekus *et al.*[117] as a harmonized model with the idea that all *in vitro* digestion models should follow the same protocol, being able to keep the basic settings in the same way, adjusting only minor parts depending on their specific research focus.

At the start of this thesis work, some digestion parameters crucial for investigation of lipid oxidation during digestion were identified:

- Lipases – with the focus on gastric and pancreatic lipase
- Bile – to allow micelles to form and to optimize the lipase activity
- Calcium – important for some lipase activity and aids in removal of lipolysis products from the oil-water interface by crystallization of lipids, but can also lead to protein inhibition via soap formation[80,92,117]
- Darkness – light can act as an initiator in the lipid oxidation reaction
- Oxygen –promotes lipid oxidation and is needed throughout the oxidation reaction

The first study (Study I), was performed using a static *in vitro* digestion model, and GI enzymes based on their protein content (mg/mL) or weight (mg). Later a revised model was used, based on specific enzyme activities[117]. Traditionally, researchers have relied on the activities specified by the manufacturer, however, it has been observed that the enzyme activities differ a lot between batches. Enzyme activity may additionally decrease rapidly

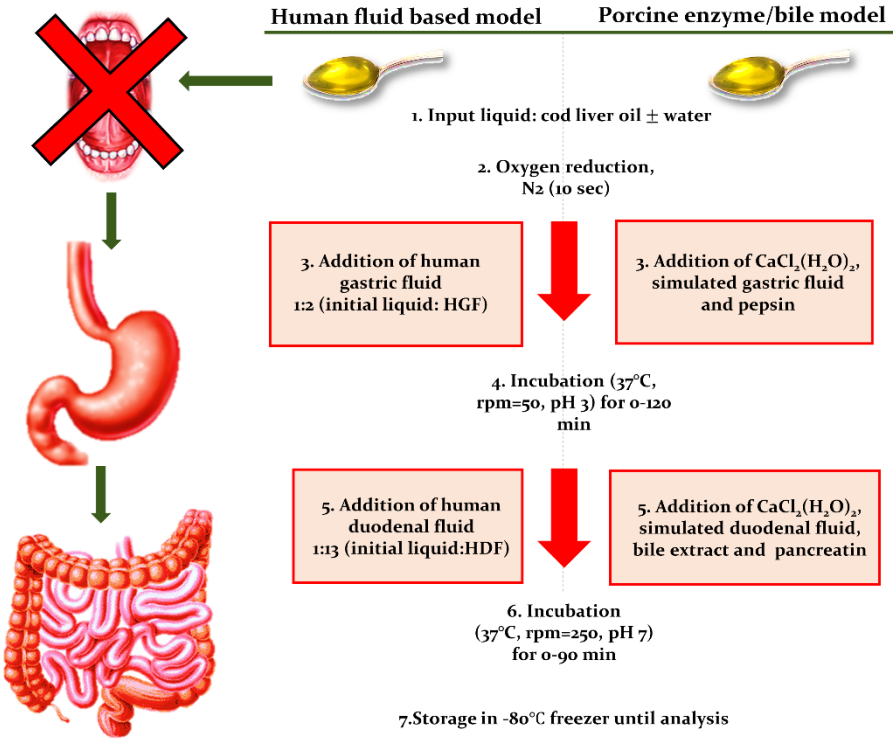

Figure 12: Comparison of models, simulated GI digestion
A schematic overview of the main parameters kept constant in the human fluid based, versus the porcine enzyme/bile based model (Study I). Sample collection was done at t= 10, 30, 60 and 120 min in the gastric phase, and at t= 10, 30, 60 and 90 min in the duodenal phase.

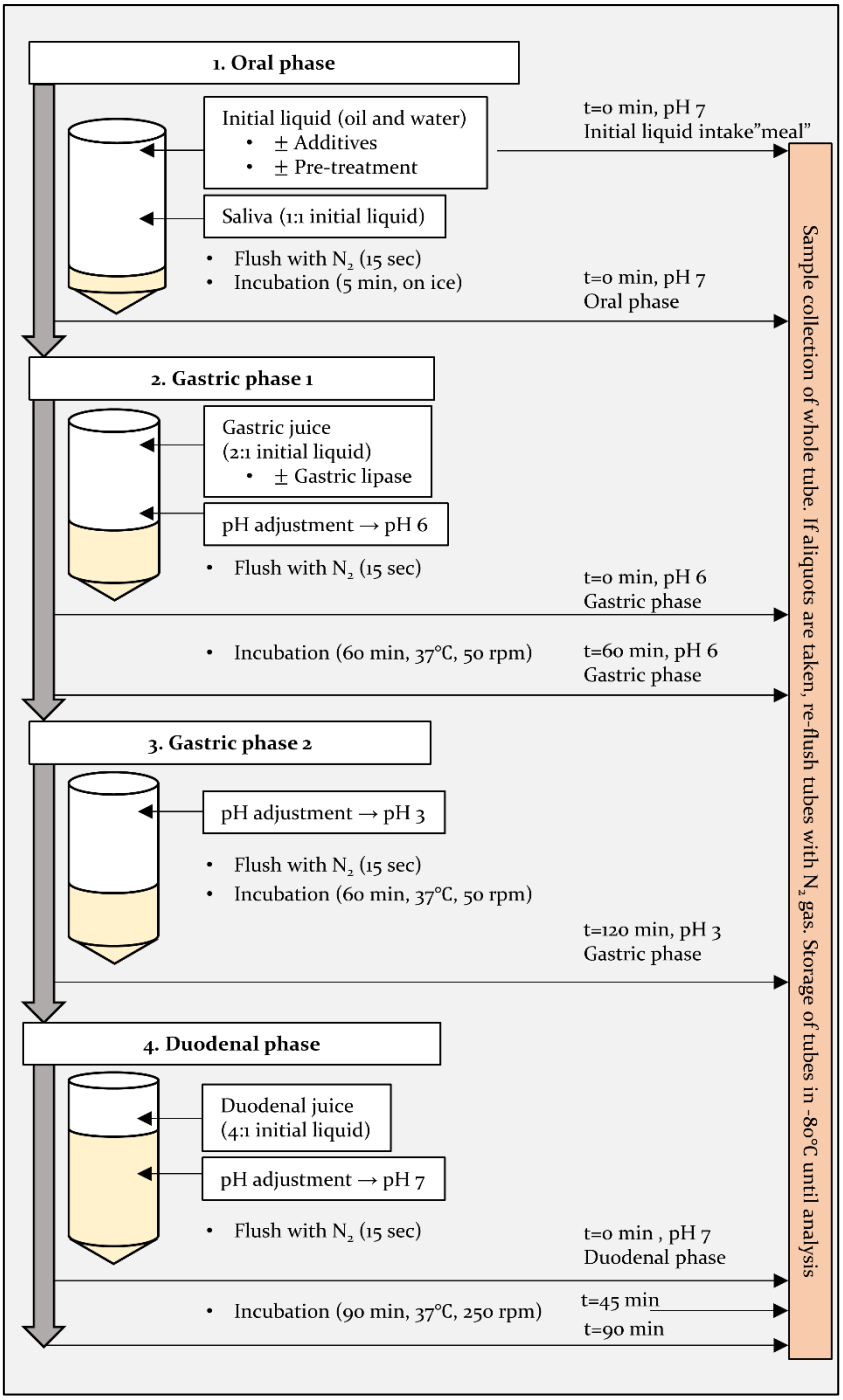

Figure 13: Flow chart showing the final study design (Study III-IV)

over time, depending on storage conditions and stability of the specific enzyme[117,131]. In Study I, two different static models using porcine enzymes and bile were developed, model I (A) and II (B). Model A was designed based on protein content (mg/mL) of the enzymes, while model B was matched towards the human GI fluids on the basis of enzymatic activities (U/mL) and volumes. Both models were included in Study I, to facilitate the comparison between these two digestion models. Thereafter, HDF and SDF ("porcine", model A and B) were used in separate digestion runs and compared (Study I, Figure 12). In the first model developed, no saliva was included since this is commonly not emphasized in digestion

models focusing on lipids. According to the study by Gorelik *et al.*[132], saliva can work both in pro- and antioxidative ways on lipid oxidation during digestion of red turkey meat, and therefore saliva was included in the following static digestion models used (Study III-IV). The specific enzyme activities and characteristics of each batch of human fluids are described in the corresponding study where they were used (Study I, Study III-IV). Gastric lipase was not in focus in the first model (Study I), since we then mainly wanted to screen for similarities between simulated porcine and human GI juices. The importance of gastric lipase in the HGF has later been emphasized (Study III-IV), which affected the pH adjustment of the HDF prior to storage (section 4.3.2.1). Pancreatic lipase activities (U/mL), bile concentration (mM) and calcium (ppm) was analyzed in all studies (Study I, III-IV). The gastric and duodenal transit time, electrolyte solutions, pH, temperature etc. were adapted from the InfoGest protocol[117]. To facilitate sampling of the inhomogeneous oil-in-water-in-HGF sample in the gastric phase, each sample point was prepared as an individual tube and further treated separately. To avoid pre-oxidation, the oils were kept on ice until start of the digestion, and to reduce the oxygen present, samples were flushed with nitrogen gas when opening the tubes. Tubes were covered by aluminum (Study I) or kept in darkness (Study III-IV) to avoid exposure to light. All oils were provided as crude oils, the krill oil received in capsules (500 mg) were cut open and pooled prior to digestion. In Figure 12, the settings in Study I, when comparing the SDF with HDF, are described. A few factors were revised in the following model, e.g., the dilution of the initial liquid, the inclusion of an oral phase, a pH change during the gastric phase, and more frequent flushing with N_2 gas throughout the digestion. The gastric pH change was inspired by the reported pH gradient change in humans during fed state[86]. The final static *in vitro* digestion model is shown in Figure 13.

4.3.1.2 Dynamic *in vitro* digestion model

The use of a dynamic *in vitro* digestion model might simulate *in vivo* digestion better than the static *in vitro* digestion models, and could provide us with physiological relevant data. In Study II, a dynamic *in vitro* GI digestion system was used, a TNO gastro-Intestinal Model (TIM, TNO, Zeist, Netherlands). This model is operated by a computer, and contains several GI compartments simulating mixing, continuous inflow of acids, enzymes, and bile, gastric emptying, and removal of excess of substances in the intestinal part of the system by filtration[117]. Filtration is done by a 50 nm pore filter for removal of mixed micelles, simulating absorption[117]. Good correlation between TIM models and in vivo data has been reported[117,133].

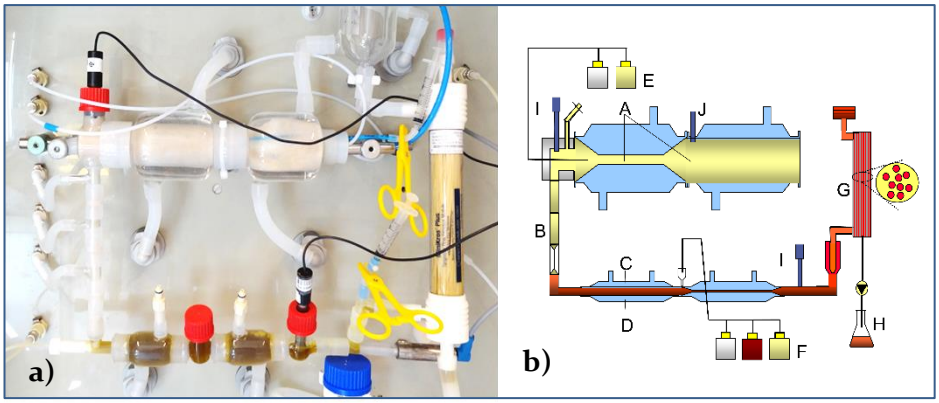

Figure 14: The tiny TNO gastro-Intestinal Model (tiny-TIM)
a) A photo of the tiny-TIM; b) A schematic overview of the tiny-TIM.

Table 2: Content of the test meals ("intake meal", 150 g) used in Study II, dynamic digestion model with simulated digestive fluids (SDF)

Meal	Cod liver oil + bread	Cod liver oil emulsion + bread/no bread	Blank + bread/no bread
Saliva (g)	20	20	20
Bread (g)	20	20/0	20/0
Water (g)	105	85/105	110/130
Oil/Emulsion (g)	5	25	0

More specifically, a tiny-TIM system (TNO, Zeist, Netherlands) was used, which is a simplified TIM system, composed of a gastric and a small intestinal compartment. A schematic overview of the tiny-TIM is shown in Figure 14.

Preparation of initial "meal" (oil and water) was done using cod liver oil, crude oil or emulsified, with or without addition of pro- or antioxidant, in combination with wheat flour bread as bulk. Bulk was needed for the gastric emptying in the tiny-TIM to work sufficiently, since the program of the tiny-TIM is set for semi-solid food digestion. The initial meals in the dynamic *in vitro* GI digestion model are described in Table 2. The initial pH was set to 6.5, and the pH in the gastric and intestinal phase were controlled for, with a gradient of approximately pH 4, 3 and 2 at t=30, 60, and 120 min in the gastric phase, respectively, and approximately pH 6.5 in the intestinal phase. The tiny-TIM continuously secretes gastric fluid (1 mL/min) in the gastric phase, and bicarbonate, pancreatic juice and bile (1 mL/min) in the intestinal phase.[134] The digestions were run for >180 min, with sampling every 30 min in the gastric phase and every 30 min in the intestinal phase. Dynamic digestion results in complex dilution of the initial meal during the gastric and duodenal digestion (Figure 15).

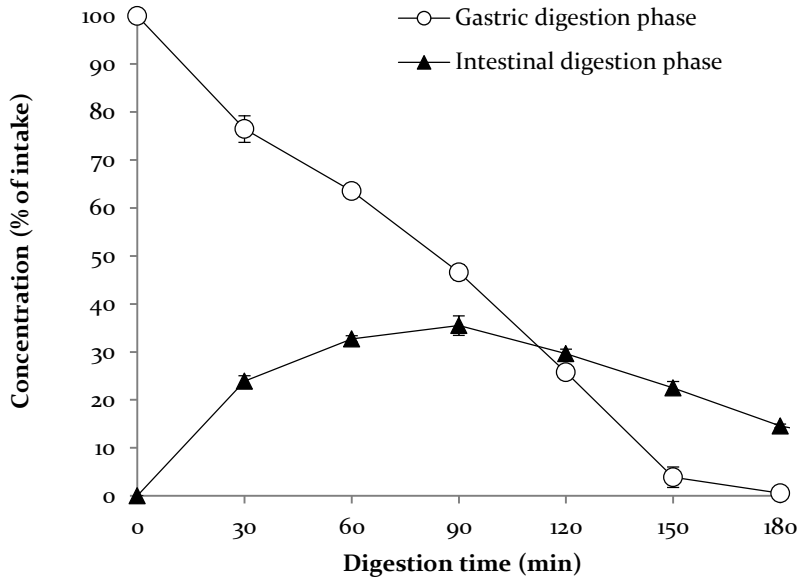

Figure 15: Dilution curve for tiny-TIM
Gastric and intestinal dilution curve for the coloring agent Patent Blue V in the different phases of dynamic in vitro digestion in tiny-TIM, error bars shown as (max-min)/2, n=2.

4.3.1.3 Source of GI fluids

4.3.1.3.1 Simulated GI fluids of non-human origin

In the dynamic model, amylase was added to the initial "meal" in the salivary step, corresponding to approximately 375 U/mL in saliva, and pH was adjusted to pH 6.5. In the gastric phase an electrolyte simulated GI fluid at pH 5 was added together with 0.5 M HCl. Pepsin (400 U/mL) and gastric lipase from *Rhizopus oryzae* (37 U/mL) were then added. The gastric enzyme solution was added to the gastric compartment of the tiny-TIM at approximately 1 mL/min. Pancreatin, fresh porcine bile, and bovine trypsin were all added continuously, at ratios 1:1:2 (mL pancreatin, mL bile, mg trypsin, respectively) in the intestinal phase. The enzyme activities and bile salt concentrations used in the dynamic model were not measured, but reported according to the manufacturers.

The simulated gastric and intestinal fluids used in the static *in vitro* digestion model were both prepared according to the InfoGest protocol[117], and the amount of the enzymes were correlated to activity (U/mL) recorded in the HDuF. The Ca^{2+} concentration is reported to change between 3-4 mM (fasted state) to 15 mM (fed state) in humans[92], however, many others have found lower levels[117]. 0.3 g/L $CaCl_2.2H_2O$ was added in the dynamic model, both in the saliva and in the gastric phase, which corresponds to 2 mM. Calcium in the static model was added as described by Minekus *et al.*[117]; Ca^{2+} was adjusted to 0.15 mM and 0.6 mM in the gastric, and the intestinal phase, respectively.

4.3.1.3.2 Human GI fluids

Saliva, gastric, and intestinal fluids were aspirated from human study participants. In saliva α-amylase was detected with an activity of approximately 40 U/mL, which is in accordance with previously found results in humans during resting conditions[135]. In Study I, no gastric lipase was detected, however, since the gastric lipase was not the main focus at that time, the pH was not adjusted prior to freezing and this could explain why the lipase in the HGF was lost. In Study III-IV, HGL was detected at 16 U/mL. Previous studies have found 100-120 U/mL in HGF at pH 5-6[119,136]. However, in the study by Ville *et al.* pentagastrin was used during collection of fluids[136], to induce secretion of HGF. In the study by Pedersen *et al.*[119], protease inhibitors were added. No addition of protease inhibitor was done in this work, since we wanted to maintain the native HGF as pure as possible, however, this might have affected the low levels of HGL present in the HGF. In the HDuF around 0.3 mM Ca^{2+} was detected, which is in line with the addition according to the InfoGest protocol[117]. The pancreatic lipase activities detected were in the range of 50-140 U/mL, which is fairly low, but realistic when comparing with data from fasted human study participants. HPL secreted during digestion of a liquid meal has been reported to be around 250 U/mL[137], and the secretion of enzymes increase during fasted state. According to Keller *et al.*, the activity during fasting state ranges between 100-400 U/mL[138]. Human bile salt composition differs highly depending on fasted or fed state, with 55% taurine conjugated and 44% glycine conjugated bile salts reported in fasted state[139]. Additionally, bile salt concentration depends on aspiration time after ingestion, ranging between 5-20 mM[140,141]. In a review by Fuchs and Dressman[142], the average bile-salt concentration in fasted HDuF was found to be 3.3 mM, however, the concentration may vary a lot between individuals. The bile salt concentrations detected in the aspirated HDuF used in Study I, III-IV varied from 2.4-2.6 mM. The average pH in the gastric phase vary highly, but in fasted state a mean pH of 1.7 could be expected[143].

The average pH in the duodenum is 6.5 during fasted state[142], however, commonly pH 7 is used in *in vitro* digestion models.

4.3.2 Analyses

4.3.2.1 Characterization of human gastrointestinal (GI) fluids

For studies presented in Study I and Study III-IV, human GI fluids were aspirated. The Declaration of Helsinki was followed[144], and all participants in the studies signed up as study participants with informed consent. Participants are anonymized in the published results, and the human GI fluids were pooled prior to being used in digestion models. Study participants got to know that the human GI fluids would be characterized in regard of enzyme activities and other relevant aspects such as bile salt concentration, and that samples were to be used to study the digestion of foods to evaluate and understand degradation and stability of nutrients. No further screening was allowed and samples were not allowed to be stored after finalizing the study.

Saliva was collected from healthy study participants within the research group, at age 20-50 years (Food and Nutrition Science, Department of Biology and Biological Engineering, Chalmers University of Technology, Sweden). The saliva was collected prior to breakfast, in the morning, by spontaneous drooling. Collection was done using sterile straw pipets for saliva, i.e., being a non-invasive method. Salivary secretion was stimulated by pictures of appetizing displays of fish meals, shown to study participants during collection.

Aspiration of human GI fluids was done according to Ulleberg *et al.*[131], based on the method described by Holm *et al.*[145], using a three-lumen tube (Figure 16a and 16b). The tube was inserted through the nose and gently moved to the stomach and the small intestine, the upper tube being placed in the canalis ventriculi of the stomach, and the lower tube close to the papilla of Vater, the location of the tube was confirmed by gastroscopy. A stimulatory solution (17.5 g/L sucrose, 450 mg/L NaCl, 800 mg/L L-phenylalanine and 575 mg/L L-valine in H_2O) was given through an inlet in the tube at 100 mL/h to stimulate the production of GI fluids, while gastric and duodenal fluid were aspirated through two inlets.

The participants were placed in a bed during aspiration. Each aspiration took 1-2 hours and was terminated after maximum 2 hours. The aspirations were done at Lovisenberg Diakonale Hospital (Oslo, Norway, October 2013 and November 2015), and the study was conducted with approval from the Norwegian Regional Ethics Committee (project no 2012/2230, Biobank no 2012/2210). Study participants were mainly students at Norwegian University of Life Sciences (NMBU), healthy and 20-40 years old. The study participants were economically compensated with NOK 800.

The pH, color and volume of the aspirates from the saliva, gastric and upper duodenum part were assayed. For the HGF the exclusion criteria were pH>4 and/or dark color of the fluids. Individual aspirates were pooled to eliminate individual effects as much as possible, and samples were stored at -80°C. For HGF (Study III), the pH was raised to pH 6 according to Ville *et al.*[136], to better preserve the activity of the HGL. Collected HDF were to be used within 4 months, to avoid loss of enzymatic activity according to Ulleberg *et al.*[131], to avoid loss of enzymatic activity. The enzymatic activities were measured in the fluids prior to being used in the digestion models.

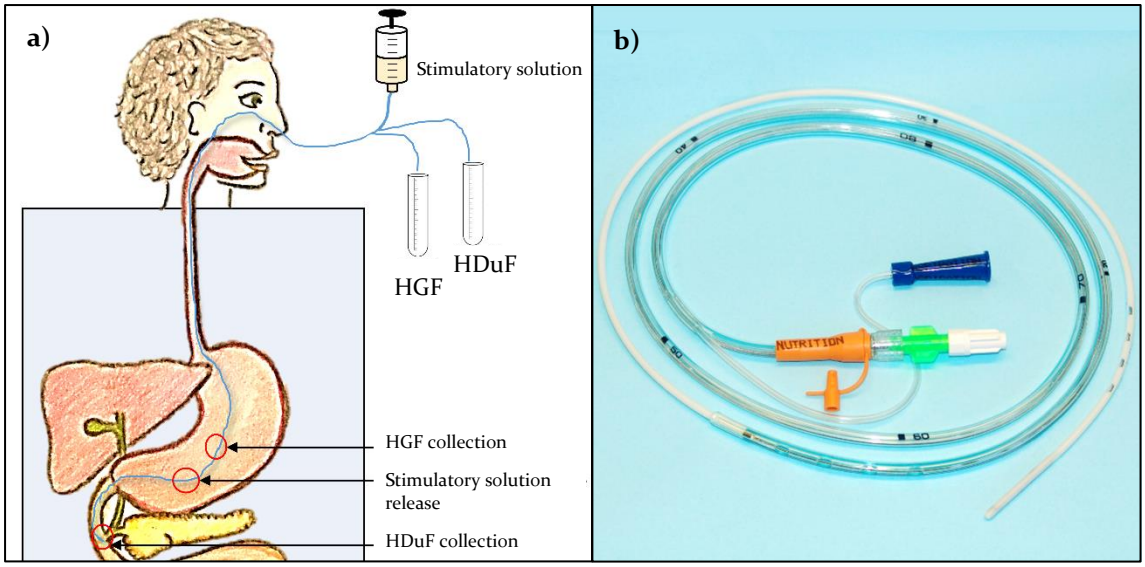

Figure 16: Aspiration of human digestive fluids (HDF)
a) Illustration of collection of human duodenal fluid (HDuF) and human gastric fluid (HGF) with a three-lumen tube; b) Photo of a three-lumen tube (Maxter Catheters, Marseilles, France).

4.3.2.1.1 Enzyme activities

Enzyme activities were measured in the human GI fluids prior to being used in the static *in vitro* GI digestion models. When comparing simulated GI fluids with HGF and HDuF in porcine model II in Study I, the added pancreatin was normalized based on the pancreatic lipase activity. The other detected digestive enzymes were analyzed for characterization reasons, but were not used as a basis for digestion model development.

Gastric lipase activity was assayed in HGF using a titrimetric protocol as described by Ville *et al.*[136], and developed by Gargouri *et al.*[121]. The assay was performed at pH 6, using tributyrin as substrate. The activity of gastric lipase activity in HGF is expressed in U/mL. Pancreatic lipase activity was assayed in HDuF using two different methods. The titrimetric protocol described by Erlanson and Borgström was used in Study I and III[146]. The assay was performed at pH 8, using tributyrin as the substrate. Activity of pancreatic lipase activity in HDuF is expressed as U/mL. For determination of the enzymatic activity of lipases, automated pH stat equipments were used in combined with a water bath, to hold 37°C. Additionally in Study I, the pancreatic lipase activity was measured using a colorimetric kit, as described by Ulleberg *et al.*[131]. The kit was based on the substrate 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6-methylresorufin) which is enzymatically hydrolyzed by pancreatic lipase, and the results are expressed as U/mL.

Salivary α-amylase activity was assayed in the pooled saliva according to Bernfeld[147], using potato starch as substrate. This was done by spectrophotometric determination of the absorbance at a wavelength of 540 nm, which detects reduced 3,5-dinitrosalicylic acid. The results are expressed as U/mL. Pepsin activity was assayed in the pooled HGF according to the method by Anson[148]. This was done by spectrophotometric determination at 280 nm, based on detection of released peptides using human Hb as substrate, and calculated to U/mL (also referred to in the literature as "Anson" or "Sigma" unit). Trypsin activity was assayed in HDuF spectrophotometrically at 247 nm, based on the method developed by Walsh and Wilcox[149]. The activity of trypsin in HDuF was expressed as U/mL, using p-Toluene-Sulfonyl-L-arginine methyl ester (TAME) as substrate.

4.3.2.1.2 Bile-salt concentration

Bile-salt concentration in the HDJ was measured with two different methods. In Study I, the formation of Thio-NADH was detected spectrophotometrically (at 410 nm) by a bile acid kit. The detection was done at the Central Laboratory of the Norwegian School of Veterinary Science (Oslo, Norway). In study III and IV, the bile-salt concentrations were detected spectrophotometrically (at 540 nm), by another bile acid kit. This kit was based on the formation of NADH and formazan, which is a dying agent. Both of these bile acid kits were based on the action of the enzyme 3α-hydroxysteroid dehydrogenase (3α HDH), and quantification was done with taurocholic acid as an external standard. The results were expressed as µmol bile salt/L. Bile-salt is made of conjugated bile acids, and the enzyme in the two bile acid kit can react both with the bile-salts and the bile acids.

4.3.2.1.3 Ascorbic acid

The reason for analyzing the presence of ascorbic acid in the HDF was due to the knowledge that ascorbic acid does occur endogenously in human digests, and can act both as a pro- and an antioxidant, depending on the concentration and the food matrix. Although ascorbic acid was added externally in Study II, it was also determined in the native HDF, by the method described by Lykkesfeldt[150], with minor in-house modifications. HDF were centrifuged, diluted 1:1 with meta-phosphoric acid (10% w/v) and disodium EDTA (2 mM), and stored at -80°C before analysis. At the time for analysis, samples were diluted 1:1 with TCEP (0.312 mM) in 1:10 McIlvaine buffer, followed by a dilution of 9:10 with phosphate buffer (pH 2.8). Detection of ascorbic acid was done as described by Scheers and Sandberg[151], using an HPLC system.

4.3.2.1.4 Trace metal ions

The detection of trace metal ions in the HDF was done as a way to screen the fluids for pro-oxidants presence. Additionally, the detected amount of Fe was compared with the detected amount of Hb, as a control for the Hb-detection methods used. The rationale behind this was that the amount Hb detected with the Hornsey method were unusually high, and interference in the Hb analysis was suspected. If high levels of Fe were detected, this would support the findings of high Hb. Additionally, all trace metal ions could hypothetically work as pro-oxidants, therefore it was of interest to examine the HDF on this matter. The detection of soluble trace metal ions was done according to the method by Fredrikson *et al.*[152]. Acidic microwave digestion of samples was followed by separation and detection using ion chromatography coupled with UV-vis, quantified with external standards, for Cu, Ni, Zn, Co, Mn, and Fe.

4.3.2.1.5 Calcium ions

Calcium ion concentrations in the HDF were measured by HPLC with a CD20 conductivity detector. Isocratic conditions were applied, using 9 mM metanesulfonic acid as a mobile phase, at 0.9 mL/min as flow rate. Injections of 20 µL per sample or standard was used at room temperature conditions. A standard curve of Ca^{2+} (as $CaCl_2$) in the range of 0.01-10 ppm, were used, and analysis showed linearity within the chosen region.

4.3.2.1.6 Hemoglobin (Hb)

Hb was measured in the HDF to observe if there was any Hb present in the gastric and/or duodenal tract. A hypothesis was that the aspiration devise, the three-lumen tube

inserted in the study participants, could have caused minor ruptures and small potential internal bleedings during the placement of the tube, which would explain the high lipid oxidation levels detected when using the HDF (Study I). If the digestive fluids were discolored they were excluded from the studies, however, minor bleedings might still have occurred, being too small to cause a color change in the aspirated fluids, but large enough to create a pro-oxidative environment. The total heme pigment content was analyzed as described by Hornsey[153], using spectrophotometric detection (at 640 nm), with bovine Hb as external standard (Study I). Hb was also analyzed using a HemoCue® Plasma/Low Hb System (Study III). In this method, disposable micro-cuvettes were used for detection, and human Hb was used as external standard. The HemoCue® method was based on Hb methylation, followed by derivatization with azid, and detection of azidmetHb.

4.3.2.1.7 Total protein

The total protein content was analyzed in the HDF (Study I), as an initial method to normalize the method for the total enzymes present in the human fluids, and the SDF with added enzymes. In Study IV, total protein content was analyzed as a way to estimate the cell content: total protein content correlates with the cell number in each well, and cell viability and normalized stress response could thereby be estimated. In Study I the total protein levels in HDF were analyzed using the Qubit Fluorometer and the Quant-ITTM protein assay kit, following the instructions by the manufacturer. Additionally, in both in Study I and Study IV, the total protein content was measured by the Pierce™ BCA Protein Assay Kit, according to the instructions from the manufacturer.

4.3.2.2 Lipolysis during digestion

Lipolysis during digestion was studied both as a way to confirm that the digestion model worked, and to detect specific FFA released, since it was believed that the latter could have a connection to the lipid oxidation reaction.

4.3.2.2.1 Lipid extraction

Tubes with oil were covered in aluminum foil and kept on ice to minimize oxidation prior to analysis. Heptadecanoic acid (C17:0) was then added as internal standard. Extraction of lipids present in the digests and the liquid intake (oil and water), was done using chloroform:methanol ($CHCl_3$:MeOH), as described by Lee *et al.*[154]. This method was developed as a combination of Bligh and Dyer and Folch *et al.* with the specific purpose of adjusting the solvent ratio depending on the fat content of the sample[155,156]. $CHCl_3$-MeOH, with BHT, in different ratios were therefore added, depending on the lipid content of the sample, see description in Study I and III. $CHCl_3$-MeOH was added to the samples at a 10:1 solvent to sample ration, followed by addition of NaCl (0.5%; 1:2.75). The chloroform phase was then collected and samples were evaporated to dryness (N_2 gas, 40°C).

4.3.2.2.2 Separation of lipid classes using SPE

For analysis of FFA, pre-separation on solid phase extraction (SPE)-columns into lipid classes was done according to Kaluzny *et al.*[157]. Prior to SPE, the evaporated extracted lipids were dissolved in a small volume of $CHCl_3$ and added on Telos NH2 SPE-, or Mega Bond Elut NH2-columns. The different lipid classes - neutral lipids, phospholipids and FFA - were then collected. It was shown in a study by Cavonius *et al.*[158], that some co-extraction of palmitic and stearic acid from the SPE-columns could occur, hence, oil-free blanks were

always included. The FFA phase was retrained and samples were again evaporated to dryness (N_2 gas, 40°C).

4.3.2.2.3 Detection of free and total fatty acids on GC-MS

After extraction and if applicable, SPE-fractionation, both total FA and FFA were analyzed on GC-MS. This was done by an in-house method, described by Cavonius *et al.* with minor modifications[159]. The lipids in the samples were methylated by addition of toluene, followed by acetic acid (HAc) in MeOH (10% v/v) and incubation (60°C, 150 min). To terminate the methylation process, H_2O was added to the samples, and extraction of the methylated FAs was done by addition of petroleum ether. The organic phase was collected and evaporated to dryness under N_2 gas at 40°C, followed by addition of isooctane. To identify the FA, the external fatty acid standard mix GLC 463 was included in the GC-MS analysis. FAs were reported as mg fatty acid methyl esters (FAME)/g oil or as area percentage of total FA detected (%).

4.3.2.3 Analysis of peroxide value (PV)

The PV levels of the four crude marine oils used in study III were measured prior to digestion, as described by Undeland, Hultin, and Richards[160], using the ferric thiocyanate method which was originally developed by Shantha and Decker[161]. The oils were diluted in $CHCl_3$:MeOH, followed by addition of an ammonium thiocyanate solution and an Fe(II)chloride solution, before incubation (24°C, 20 min). PV was measured spectrophotometrically at 500 nm, using the external standard cumene hydroperoxide for quantification.

4.3.2.4 Analysis of free aldehydes using LC/APCI-MS

4.3.2.4.1 The choice of marker aldehydes and development of analysis method

As mentioned in section 3.3.1, targeting specific oxidation products will deepen the understanding of the lipid oxidation mechanism. Aldehydes being formed as secondary lipid oxidation products are highly reactive, and thus of particular interest to target in order to understand potential toxic effects of GI-induced lipid oxidation. The aldehydes HHE, HNE and MDA are of special interest when studying lipid oxidation in marine oils (section 3.3.2). MDA since it is one of the more common secondary lipid oxidation products from PUFAs, and HHE and HNE since they are derived from n-3[73,162] and n-6[162,163] PUFAs, respectively. Although the aldehydes MDA, HHE, and HNE had earlier been analyzed, a method for simultaneous analysis of all the three analytes in marine oil digests using LC-MS, did not previously exist at the start of this thesis work. We therefore aimed to set up such a method.

MDA has historically often been analyzed by the thiobarbituric acid (TBA) test, as described in section 3.4.2. This is still a method commonly used when detecting MDA equivalents to determine lipid oxidation in foods during both storage and *in vitro* digestion[67,96,97,164]. However, the TBA test has received critique since it is unspecific and detects TBA reactive substances (TBARS) in a broad manner, meaning that not all of it is the TBA-MDA complex[70,165]. Also, the tough conditions during the TBA-test itself, such as the acidic environment and the elevated temperatures (commonly 100°C) needed for sufficient MDA-TBA complexing have been questioned, since they could promote lipid oxidation in themselves[70,165]. These factors could lead to an overestimation of MDA levels[70,165]. An alternative to the TBA-test has been detection of the MDA-TBA complex, or

MDA without pre-treatment, by HPLC[70,165]. In biological samples, HNE has been determined both by LC coupled with UV detector, as well as with GC-MS[70], the latter being a common method to determine volatile aldehydes in marine oils, when proceeded, e.g., by dynamic headspace[61]. GC-MS is the most common analysis method for oxidation derived aldehydes, but LC-MS has, e.g., been used for fish[68,166]. Both when using LC-MS and GC-MS, derivatization of the target molecules is commonly needed to create more stable compounds[167].

An existing in-house method for analyzing HHE on LC/APCI-MS by DNPH-derivatization was used as a starting point for the development of the new LC-MS method to simultaneously determine HHE, HNE and MDA[168]. The choice of LC-MS over GC-MS was based on difficulties in detecting compounds directly on GC-MS, since no dynamic headspace method was available in the lab at this time. Also, when using GC-MS, the samples are exposed for high temperatures, something that could be detrimental when studying sensitive lipid oxidation products[169]. A draw-back with using LC-MS is the disturbance from background noise that could make detection of low concentrations difficult[169]. To avoid this, a more thorough sample preparation was needed.

The chosen target aldehydes are normally found in the range 2-40 nM (MDA) and 0.5-10 nM (HNE) in exhaled breath condensates[170]. Van Hecke measured MDA equivalents and HNE in beef (15 and 0.43 µM, respectively), pork (5 and 0.22 µM, respectively) and chicken (6 and 0.05 µM, respectively)[97]. Our own previous studies had shown that aldehyde levels in fresh cod liver oil were 0.75 µM (total MDA) and 0.028 µM (HHE)[100], with a limit of detection for the method of 0.016 µM HHE[168]. Hence, we aimed to develop a method as good as, or improved, compared to the latter.

Stocks of HHE and HNE were prepared according to recommendations by the manufacturer (Cayman Chemicals, Ann Arbor, USA), with the difference that HHE was dissolved in MeOH, instead of EtOH. The stock solutions were stored in -80°C , a temperature which has previously been found to prevent changes in carbonyls in fish during 13 months[171]. It has also been shown that the HNE levels in plasma were stable for 22 months when stored in -80°C [172]. The MDA precursor 1,1,3,3-tetraethoxypropane (TEP), was used as an external standard for MDA, and hydrolyzed in H_2SO_4 (1%) according to Pilz *et al.*[173] before each new run of experiments.

4.3.2.4.2 Sample pre-treatment

The method chosen for sample pre-treatment can be found in Study I, however, the rationale behind the choice of the different pre-treatment steps will be described here. To avoid formation of lipid oxidation products during preparation of samples, the addition of EDTA and BHT, commonly used to avoid deterioration of fish oil[61], were added at ranges frequently used[68,174]. To precipitate proteins and to acidify the solution, samples were acidified by the addition of HCl[168]. Acidification with trichloracetic acid (TCA) had been used in other studies for precipitation[68,175,176]. However, when TCA was evaluated as acidification solution, the detected peaks in the LC/APCI-MS chromatograms were negatively affected, by decreasing peak size and reduced detectability, and HCl was thus chosen over TCA.

DNPH was chosen as derivatization agent, according to the results in the study by Mendes *et al.*[68] where they showed that the use of DNPH over the traditional TBA derivatization agent is superior in overall method performance. In our previous in-house

method, concentrations of DNPH in MeOH tested were 0.85 mM and 1.25 mM in the sample, the latter according to levels used by Manini *et al.*[170] However, it was observed in our trials that the crystals of DNPH in MeOH did not dissolve properly and the DNPH:MeOH solution was over-saturated. The crystals might have an interfering effect when injecting samples on the LC/APCI-MS, hence a lower concentration of DNPH in MeOH (0.5 mM in the sample) and only using the upper phase of the DNPH solution was recommended. A lower DNPH concentration did not affect the detectability on LC/APCI-MS.

Extraction was done in two steps, using dichloromethane as the extraction solution. The first extraction step was shown to extract approximately 90% of the pure standards, while a second extraction step extracted about 10% of the remaining standards. The analysis method was sensitive for the acidic solution used during DNPH-derivatization, thus the transfer of extracted analytes in dichloromethane had to be done carefully. Centrifugation was done after each dichloromethane extraction step, which was repeated twice.

After the extraction step, samples were evaporated to dryness (N2 gas, 25°C) and re-diluted in MeOH. Finally, samples were centrifuged, to assure that, e.g., undissolved DNPH crystals were removed prior to analysis on LC/APCI-MS.

4.3.2.4.3 Development of an LC/APCI-MS method for free aldehyde analysis

The LC/APCI-MS method described here is based on principles described by Grosjean *et al.*[177], Deighton *et al.*[178], Andreoli *et al.*[179] and Sakai *et al.*[166], and the detailed method can be found in Study I. An LC/APCI-MS system in APCI negative mode was used, and detailed information about the settings can be found in Paper I. Data collection was done in selected ion monitoring (SIM) modes, collecting ions at 234.0, 293.1, and 335.1 m/z, for MDA, HHE, and HNE, respectively. ONE was later also included in the LC/APCI-MS analysis, and data was then collected in SIM mode at 333 m/z (Study III).

4.3.2.4.4 Validation of the LC/APCI-MS method

According to Harris[180] samples with different concentrations should show a linearity, preferably with R2>0.995. All standards were within this range, MDA was well above (R2= 0.9993; 0.025-40 µM) as was HHE (R2= 0.999; 0.001-0.3 µM), while HNE was the standard showing the lowest linearity (R2= 0.9985; 0.005-0.1 µM). Linearity was also controlled for at increasing standard concentration; MDA, R2=0.985 (0.025-100 µM), HHE, R2=0.997 (0.01-10 µM), HNE, R2=0.997 (0.005-10 µM). LoQ was defined as a signal-to-noise ratio above 10. The precision of the method on an intra-day variation (n=9) was shown to be 0.99% standard deviations for MDA, 2.1% for HHE and 1.2% for HNE. Inter-day variation and repeatability in digested samples was within 10% for all aldehydes, which is acceptable in biological matrixes[181]. The intra-assay precision was also sufficient, no significant difference was seen between different aliquots of a given sample. Further, no significant difference was seen when two different laborants were running the method.

4.3.2.5 Effects of GI oxidation in the Caco-2 cell model

4.3.2.5.1 The choice of Caco-2 cells as a model of the intestinal epithelium

The use of cell lines as a tool to study, e.g., mechanistic pathways, toxicity, and analyte permeation and absorption is widely accepted. Human cancer cells (e.g., the Caco-2 cell line) are commonly used to simulate the human intestinal epithelium[182-185]. Human cancer cell lines may differentiate into more specialized cells, depending on which

mutations the specific cell line exhibit[186], which makes them useful when you want to study certain mechanisms or effects of a treatment on a specific cell type[186]. The disadvantages of using human cells compared to other cell models are firstly that human cell lines require a long culturing time before differentiation, secondly that they have a limited life span, and thirdly that the cells have specific requirements of growth conditions, such as media, and atmosphere. Using Caco-2 cells to simulate absorption has been suggested as an analysis tool for emulsion based systems[117,187], among others. The cell line originates from a tumor that was isolated from a white male in the 1970s[188]. When the cells grow confluent, they differentiate into an epithelial monolayer that resembles the duodenal epithelium, with villi and micro-villi, and is today widely used as a cell model of the brush border in the gut[188]. As mentioned in section 3.5.1.3, the Caco-2 cell line has previously been used to study absorption of the lipid oxidation products HHE and MDA, and oxidized DHA[11,14], however, the effect of marine oil digests in Caco-2 cells related to cell stress had to our knowledge previously not been investigated.

4.3.2.5.2 Effects of digests on oxidative stress in Caco-2 cells

Caco-2 cells (passage 19, American Type Culture Collection, Rockville, USA) were cultured according to the recommendations, and the cell work was performed in a sterile hood, with HEPA-filter. Caco-2 cells require 7.1-7.4 pH, 37°C, humid air (95%) and 5% CO_2[182,189]. Whenever working with cell cultures, the cell medium used should be pre-heated (37°C). The medium used for Caco-2 cells was EMEM, with fetal bovine serum (FBS) (10%) and the antibiotic Normocin™ (0.2%), which was changed every 2-3 day. Cells were cultured until 80% confluency was reached, before passaging the cells by trypsination. Cells were counted at the time of splitting by hemocytometer and microscopy. When the cells had reached passage 29-37 they were seeded in Transwell® plates with polycarbonate inserts (~60 000 cells/insert), or in CellBiND® polystyrene plates without inserts (~200 000 cells/insert). Cells were then cultured for another 14 days, before cell experiments took place. In all the experiments, controls (without oil) were included, as were standards with pure aldehydes. Standards added corresponded to the concentrations of MDA and HHE measured in the marine oil digests, or above to study aldehyde toxicity. A standard mix of both MDA and HHE were also included, to investigate synergistic effects. The maximum aldehyde levels tested were in the same range as in the studies by Awada *et al.*[11] (90 µM) and by Alghazeer *et al.*[190] (45 µM).

Caco-2 cells were exposed to marine oil digests, added 1:1 to the apical medium in the inserts (2 h, 37°C). Controls and standards were incubated at the same conditions. Thereafter, the medium was aspirated from the cells, and PBS was added to wash the cells before lysis. Cells were harvested by lysis with RIPA buffer with EDTA-free Pierce™ Protease and Phosphatase inhibitor, and basal medium and lysed cells were collected and stored in -80°C until analysis.

Cell viability was calculated from the measurement of total cellular protein in cell lysates, as described in section 4.3.2.1.7. Aldehydes present in the different fractions were analyzed as described in section 4.3.2.4. To evaluate the effect of marine oil digests in Caco-2 cells related to cell stress, a human cell stress array kit was used, according to the manufacturer´s instruction. Briefly, arrays spotted with 26 different antibodies directed towards stress-related proteins were incubated with the digests diluted in blocking buffer. The signals were detected by chemiluminescence, followed by image software analysis.

Expression of stress-related proteins in Caco-2 cells in response to marine oil digests were defined as % of control.

4.3.2.6 Statistical analysis

Different statistical tests were performed in each study, and a specific description can be found in each of Paper I-IV. Statistical analysis of the data was performed using SPSS statistics version 22 (IBM Corporation, New York, USA), or Microsoft Office Excel, 2013. Statistical differences over the whole digestion period between different liquid intake meals were determined by mixed model with repeated measurements, followed by Tukey's HSD *post-hoc* test. Differences between more than two groups were determined by general linear modelling. Student´s t-test was used when comparing two groups. Results are shown as mean values, and error bars represents standard deviation in cases where $n>2$, and (max-min)/2 where $n=2$. Differences were considered significant at $p\leq0.05$, and significant levels are shown in graphs and tables when applicable; *=$p\leq0.05$, **=$p\leq0.01$, ***=$p\leq0.001$.

5. RESULTS AND DISCUSSION

5.1 Characterization of marine oils and human GI fluids

5.1.1 Fatty acid composition of the marine oils and initial oxidative status

The lipid composition of the marine oils was detected by GC-MS and the comprehensive information on the whole fatty acid profile of each oil can be found in the corresponding articles where the marine oil were used. In Figure 17, the sum of detected SFA, MUFA, total PUFAs, LC n-3 PUFAs, and total FAME detected is shown. Additionally, the measured content of specific LC n-3 PUFAs are 10% DHA and 8% EPA in cod liver oil, 12% DHA and 15% EPA in fish oil, 6% DHA and 12% EPA in krill oil, and 48% DHA and 8% EPA in algae oil. It should be noted that the total amount FAME detected (mg/g) differed in the different oils. The reason for this might be that the oils were not fully methylated and/or that not all peaks shown in the chromatogram could be identified, and were thus not included in the analysis. The latter was particularly evident for the krill oil. Additionally, it should be noted that there were some losses over the column when detecting the LC n-3 PUFAs, especially with late retention times. Therefore a conversion factor was used, comparing the relations between the known amounts of FA in the external standard, with the resulting peak area ratios after analysis. This factor varied substantially for DHA (from 1.5-3) and percentage wise affected the detected content of DHA in algae oil–more than in the other oils, due to the dominance of DHA in this oil.

The described variation and its impact was noticed during a re-evaluation of the oils done in order to have them analyzed in the same GC-MS run. This re-evaluation was done

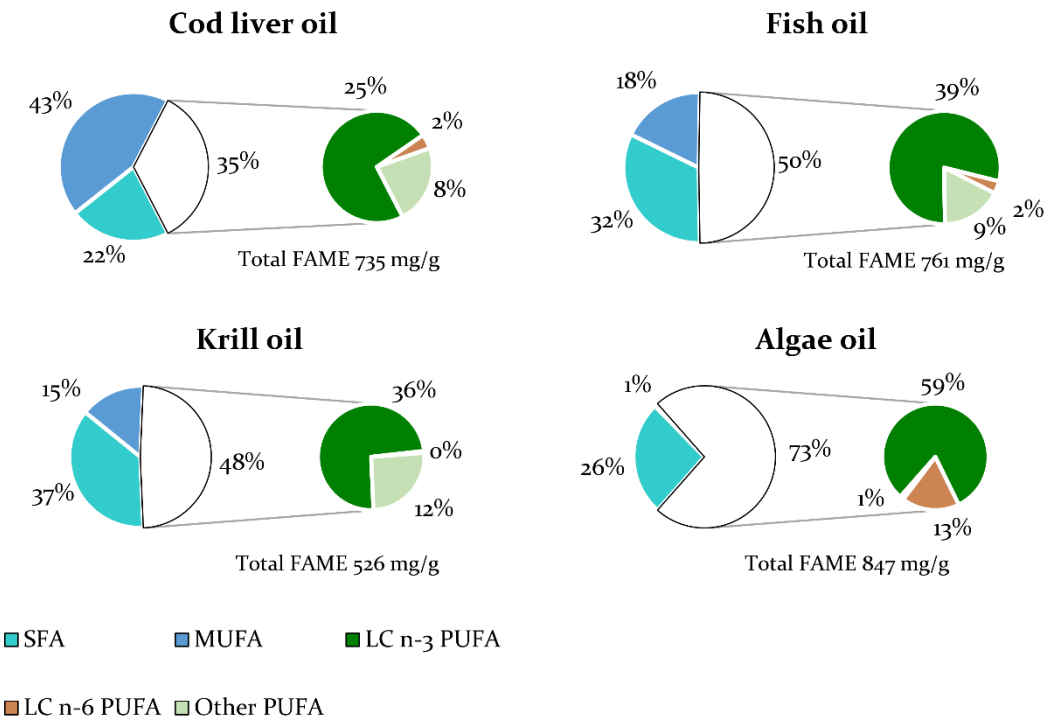

Figure 17: Fatty acid composition of marine oils
Analysis of total fatty acid methyl esters (FAME), mg FAME/g oil, for Study III-IV, n=3. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids. LC n-3 and n-6 PUFAs with chain length >C18, >1 double bond.

after the digestion trials belonging to Study III and IV were done, both in which the amounts of oils to digest were normalized on basis of DHA and EPA. It could hence be that the DHA in the oils was slightly underestimated and that more algae oil therefore was added to the digestion model than was intended. According to the last and most thorough investigation of the DHA content of the algae oil, it contained 484 mg DHA/g oil, which is similar to the value reported by the manufacturer (442 mg/g, correspondence with DSM). This result was, however, almost 85% higher compared to what was detected when designing the digestion study. Assuming our updated measurements are the correct ones, approximately 10 mg EPA and DHA was in fact added via the algae oil compared to 5 mg for the other oils. It is therefore likely that the already low levels of lipid oxidation would have been even lower if we could re-do this trial with the correct amount EPA+DHA (5mg). An internal, preferably labelled, DHA standard added to the oils would have been needed to confirm the fate of the DHA bound in the TAG of the oil.

The results of the initial oxidation level of the oils are shown in Table 3. As can be seen, the initial oxidation level varied slightly between the oils of different marine origin, however, after storing them in the lab to become pre-oxidized, the PV and aldehyde levels increased substantially. PV is expressed as mmol PV/kg oil, which is converted to meq/kg oil by multiplying the value by 2.

Table 3: Initial oxidation level in oils, with fresh or pre-oxidized oil
The initial oxidation level of the different marine oils used in this thesis work. Aldehydes are detected in free form, not bound. CO= cod liver oil.

	CO, fresh	**CO, fresh**	**Fish oil**	**Krill oil**	**Algae oil**	**CO, ox. medium**	**CO, ox. high**
Used in study	I, II	III, IV	III, IV	III, IV	III, IV	II	II
PV (mmol/kg oil)	1.3± 0.1	1.5± 0.06	0.9± 0.2	1.00± 0.3	0.18± 0.05	12.4±0.6	20.0±1.8
MDA (µmol/kg oil)	2.23±0.3	1.70± 0.8	1.80±0.6	6.56± 2	0.31± 0.4	25±1	47±8
HHE (µmol/kg oil)	1.76±0.1	2.69± 0.4	0.30±0.2	0.69±0.1	0.12±0.2	4.1±0.5	5.5±1.2
HNE (µmol/kg oil)	0.04±0.04	0.17±0.2	0.02±0.02	0.09±0.07	0.07±0.1	0.06±0.01	0.08±0.02

5.1.2 Characterization of the human gastrointestinal (GI) fluids

The human GI fluids were characterized on the basis of compounds that could act as either pro- and/or antioxidants. Enzymatic activities were also measured (Table 4), according to Minekus *et al.*[117]. Saliva was only used in Study III and IV. HGF was used both in Study I, III and IV, with very similar pepsin activities (1 255 U/mL versus 1232 U/mL). Gastric lipase was, however, only detected in the HGF used in Study III and IV. In HDuF varying levels of lipase was detected (48-140 U/mL) with the method recommended by Minekus *et al.*[117]. In Study I, the lipase activities measured by the colorimetric method were also reported. The trypsin activity was only measured in the HDuF used in Study III and IV.

Table 4: Enzyme activities in the human digestive fluids (HDF)
a) Enzyme activities in HDF used in Study I; b) Enzyme activities in HDF used in Study III-IV, n=3. Human gastric fluid, HGF; human duodenal fluid, HDuF; samples not analyzed, n.a.

Enzyme activity (U/mL)	Saliva b	HGF a	HGF b	HDuF a	HDuF b
Amylase	37	n.a.	-	n.a.	43
Pepsin	n.a.	1 255	1 232	n.a	n.a.
Trypsin	n.a.	n.a.	n.a.	-	49.2
Lipase	n.a.	-	16	140	48

Trace metal ions, ascorbic acid, hemoglobin and bile-salt concentrations detected in the human GI fluids are shown in Table 5. Low levels of all trace metal ions (iron, copper, zinc, nickel) were detected (<1 ppm). Hb was detected at high levels with the Hornsey method, however, the method was not adapted for HDF, and the high values measured were likely due to interference from, e.g., bilirubin. Levels of Hb detected with the HemoCue® devise were close to the limit of detection (0.3 g/L fluid, i.e., 4.65 µM), and the low presence of iron confirms that low levels of Hb are present in the HDF. Hb was found to be strongly pro-oxidative in our dynamic digestion study at 0.74 g/L oil (11.5 µM at intake, which corresponds to approximately 5.75 µM at t=90 min of gastric digestion). The fact that LMW iron has been found to act as a pro-oxidant in deodorized vegetable oil already at very low levels (0.1 ppm)[191], and that Hb, has shown pro-oxidant activity already at 0.06 µM in washed cod mince[192] still makes it possible that traces of Hb or liberated LMW-Fe could explain the high lipid oxidation levels reached when comparing the use of human GI fluids with simulated porcine GI fluids. The calcium levels detected corresponds to 0.6 mM (HGF) and 0.3 mM (HDuF), which is in line with levels suggested in the InfoGest protocol[117], and the levels were very similar between different batches of human GI fluid. The bile salt levels ranged from 2.4-2.6 mM, which is low, but correlates well with data reported in fasted HDuF. Trace metal ions were also tested in the stimulation solution provided to study participants during aspiration. The stimulatory solution was not a source of pro-oxidants (Table 5). From this it can be concluded that the measured trace metal ions, ascorbic acid, Hb or bile-salt in the HDF all are in line with expected values. Differences between the HDF and SDF could, however, possibly be ascribed to the presence of Hb as a pro- oxidative parameter in the human fluids.

Table 5: Characterization of the human digestive fluids (HDF)
Characterization of trace metal ions, ascorbic acid, hemoglobin and bile-salt in the human digestive fluids (HDF), n=3, Hb determined with HemoCue® and bile salt with kit. Study, P; Human gastric fluid, HGF; human duodenal fluid, HDuF; ascorbic acid, a.a.; hemoglobin, Hb; no data, n.d.; samples not analyzed, n.a.

Human GI fluid PI	Ca2+ (ppm)	Zn (ppm)	Cu (ppm)	Ni (ppm)	Fe (ppm)	A.a. (ppm)	Hb (g/L)	Bile-salt (mM)
Stimulatory solution	n.a.	0.02	n.d.	n.d.	0.02	n.d.	n.d.	n.a.
HGF	33.1	n.d.	n.d.	n.d.	0.18	n.a.	0.26	n.a.
HDuF	17.2	0.2	0.91	n.d.	0.37	n.a.	0.4	2.6
Human GI fluid PIII, PIV	**Ca2+ (ppm)**	**Zn (ppm)**	**Cu (ppm)**	**Ni (ppm)**	**Fe (ppm)**	**A.a. (ppm)**	**Hb (g/L)**	**Bile-salt (mM)**
Saliva	n.d.	n.d.	n.d.	0.01	n.d.	n.d.	n.d.	n.a.
HGF	35.5	0.50	0.74	0.12	0.92	0.32	n.d.	n.a.
HDuF	16.2	0.50	0.9	0.12	0.84	0.26	0.26	2.4

5.2 Do marine lipids oxidize during *in vitro* GI digestion?

5.2.1 *Effect of digestion model on the formation of lipid oxidation products*

5.2.1.1 Static or dynamic *in vitro* model

Different types of digestion models were used in the four studies (Study I, II, III and IV). One question was could the *choice of a static or dynamic model* affect the formation of lipid oxidation products. This question could be addressed using Study I and II where SDF of porcine origin were used. The average concentrations of MDA and HHE detected at t=60 and 180 min in these two studies are compared in Figure 18, where µmol aldehyde/g oil has been calculated. It should be noted that the amount of input oil (g), the specific enzymes used, and the dilutions during the digestions differed in the two different models. Regarding the latter, the dilution curve for the dynamic model is highly complex (see Figure 15). Thus, a direct comparison between the models is not possible. In the static model (Study I, porcine model A and B) there is approximately 2-times more oil-in-digest (absolute oil amount (g) in digest (mL)) emulsion in the gastric phase (4.2%), compared to in the dynamic model (2.1%), calculated from the initial "meal". This is reflected in the gastric HHE levels (µmol/g oil), where the static porcine model B yielded slightly higher levels compared to levels detected in the dynamic model, however, low HHE levels were detected in the static porcine model A. In the dynamic model, > 7 times more MDA was detected in the gastric phase (t=60min), compared to the static models. In the duodenal step, the difference in oil-in-digest has changed to 1.8% (static porcine model A) and 0.8% (static porcine model B) versus 0.5% (dynamic model), meaning the static model still comprises a higher oil-in-digest

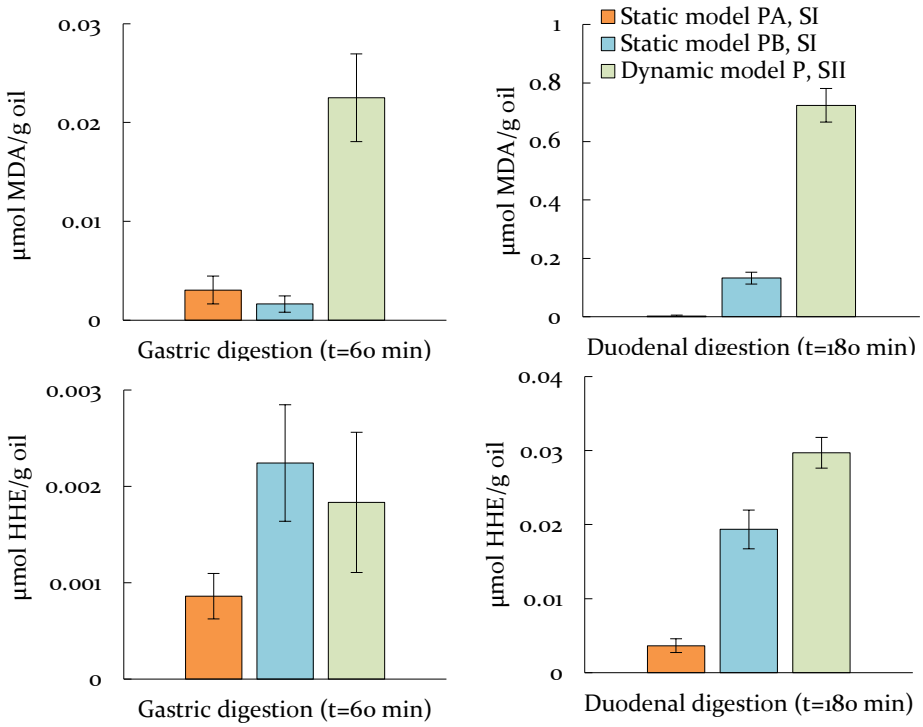

Figure 18: Comparison of aldehydes (µmol/g oil) detected during digestion
Comparison of the aldehydes MDA and HHE (µmol/g oil) in the gastric (t=60 min) and duodenal step (t=180 min) of static versus dynamic digestion. Blank values are subtracted to allow for comparison between models. S(I,II)=Study (I,II), PA=Porcine model A, PB=Porcine model B, P=Simulated GI fluids of porcine origin.

however, the detected aldehyde levels did not reflect this. The MDA and HHE levels detected (μM/digests) are shown in Figure 19. The MDA levels were higher in the dynamic model compared to the static model, throughout the whole digestion. This order might, however, have turned, if the collection period would have been prolonged. The HHE levels were similar between the models, and even higher in the static models at the end of the digestion (t=210) compared to the dynamic model. This shows that overall, higher aldehyde levels (MDA and HHE) were detected in the dynamic model, both when expressed on an oil-basis and a digest-basis, however, results depends on digestion time. Due to the higher dilution factor of the oil in the dynamic model, it was expected that the aldehyde concentration when expressed on a digest level would be higher in the static model. Further to more oil, the lack of removal of products both from lipolysis and lipid oxidation in the static model, could in itself enhance lipid oxidation. However, the higher levels of aldehydes developed in the dynamic model can be explained, e.g., by the continuous enzymatic addition and the peristaltic movement. This indicates that underestimation of the level of lipid oxidation products should be taken into account, during static digestion models.

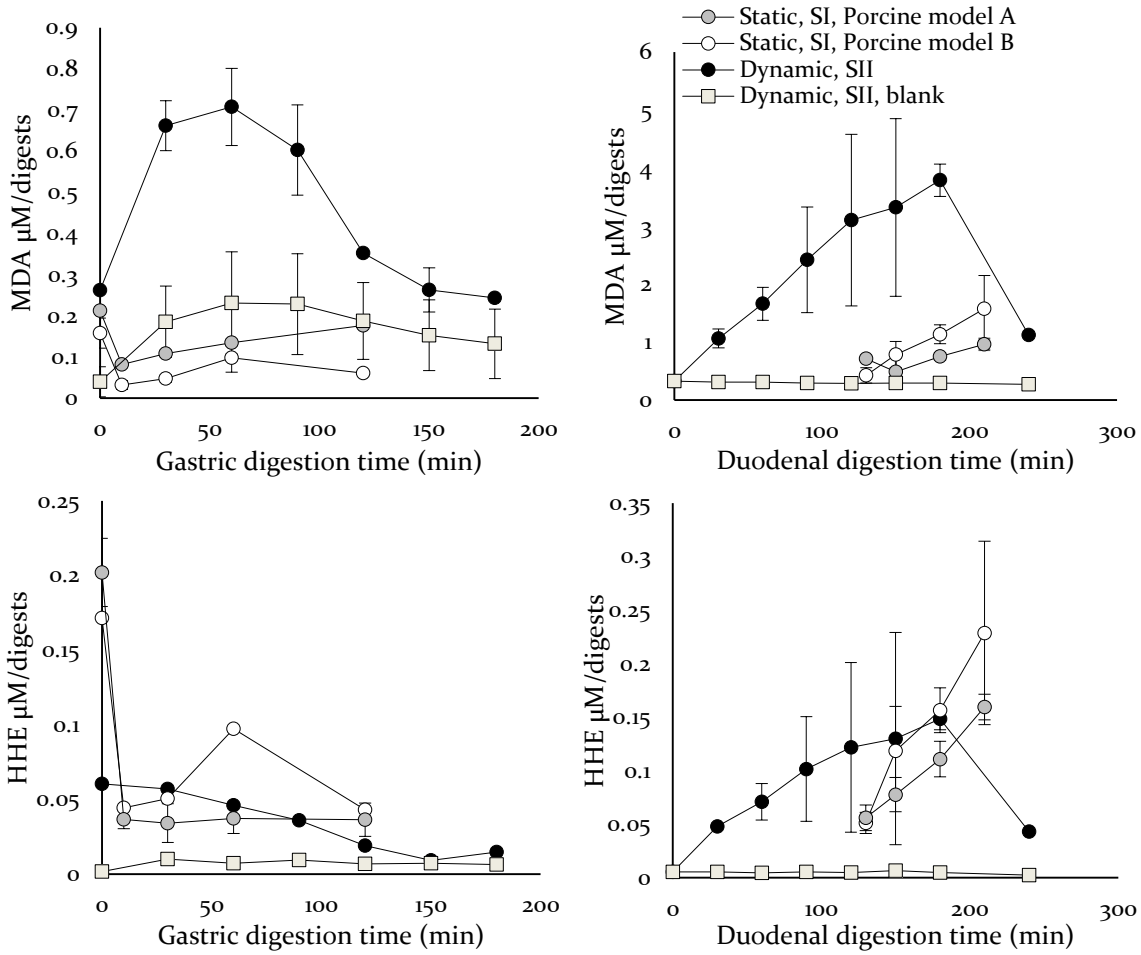

Figure 19: Aldehyde formation during static and dynamic digestion
Aldehyde (MDA and HHE) formation (μM/digests) in digests during gastric (0-120 min) and duodenal (120-210 min) digestion of fresh cod liver oil, static versus dynamic digestion models. SI=Study I, SII=Study II. Error bars are shown as standard deviations, n ≥ 3.

5.2.1.2 Human or simulated GI fluids

The main question to be answered in Study I was if there were any differences in the amount of the selected lipid oxidation products formed during static *in vitro* digestion, using human GI fluids compared to simulated "porcine" fluids. As can be seen in Figure 20, significantly higher levels of MDA, HHE, and HNE were detected when using human GI fluids, compared to simulated porcine GI fluids, in the duodenal phase. Less pronounced differences were observed in the gastric phase of digestion. Aldehyde levels were, however, ranked in the same order; MDA>HHE>HNE, with both the human and porcine GI fluids.

Some possible explanation for the observed differences in absolute levels are connected to the more intact nature of the human GI fluids, compared to the purified porcine ones in SDF. One hypothesis may be that the human specific enzymes/isoenzymes act towards different positions on the FAs compared to the porcine enzymes, as previously suggested, e.g., by Aarak *et al.*[193] and Carrière *et al.*[137]. This would result in a release of different FFA, from the very same FAs, depending on the origin and complexity of the enzymes used. In Study I we did indeed see that more EPA and DHA were released with the human GI fluids compared to the simulated GI fluids. A suggestion is that these released FFA were more susceptible to lipid oxidation, since they are highly unsaturated. Another hypothesis was that there were pro-oxidants present, e.g., trace metal ions and Hb, in the human GI fluids, which are not included when composing the simulated porcine GI fluids. Trace metal ions and Hb in the human GI fluids were detected in concentrations which could be pro-oxidative (0.18-0.92 ppm and ~0.3 g/L, respectively, Table 5), as discussed in section 5.1.2. Other potential explanations could be the presence of cell debris in HDF, which are excreted with the GI fluids.

Comparing the results of MDA, HHE, and HNE levels in Study I with Study III (cod liver oil digests), it was noted that less aldehydes/g oil was developed with the second batch of HDF during duodenal digestion. Still, however, higher levels of aldehydes were reached with HDF compared to with SDF (t=180 min Study I, t=165 min Study III). This could be

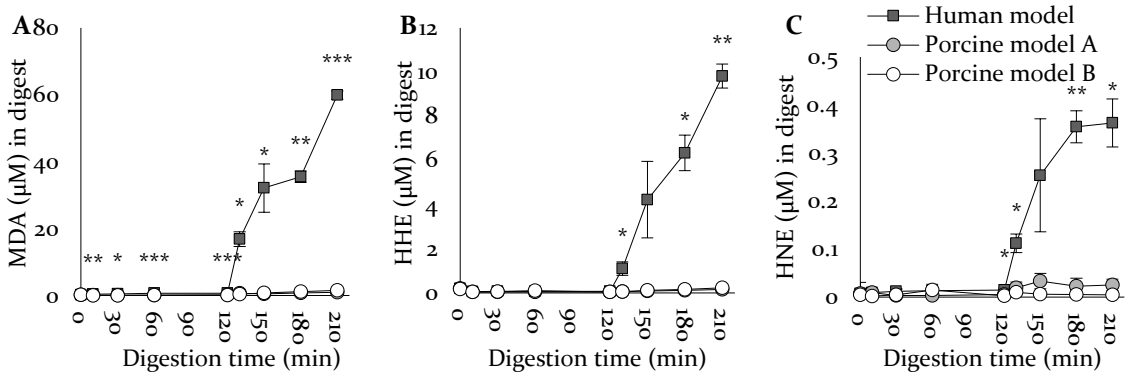

Figure 20: Aldehyde formation during static digestion with human or simulated GI fluids
*Aldehyde formation in digests during gastric (0-120 min) and duodenal (120-210 min) digestion of fresh cod liver oil with HDF and simulated fluids with pure porcine enzymes and bile. GI= Gastrointestinal; Figure A, MDA= Malondialdehyde; Figure B, HHE= 4-hydroxy-trans-2-hexenal; Figure C, HNE=4-hydroxy-trans-2-nonenal. Error bars are shown as standard deviations, n ≥ 3, except for time=130 min (Porcine model B), n=2. *=p<0.05, **=p<0.005, and ***=p<0.001.*

explained by the higher lipase activity in the human digestive fluid used in Study I (Table 4), and/or by the higher level of oxidation products in the cod liver oil used in Study I (Table 3). Additionally, the slightly longer incubation time in Study I could explain the differences in aldehyde levels detected.

5.2.1.3 Lipid oxidation during gastric versus duodenal *in vitro* digestion

Large differences were seen in all the *in vitro* models used (static or dynamic), and with both types of GI fluids used (human or simulated porcine), between the gastric and duodenal phase of digestion. The formation of aldehydes in the duodenal phase was in all cases significantly higher compared to the gastric phase, as can be seen in Figure 21. Both MDA, HHE, and HNE were analyzed and presented in each study (Study I-IV), and ONE was analyzed in Study III, however, was not detectable. The relative difference between the end of the gastric and duodenal phase is presented in Table 6. This is in line with previous *in vitro* studies, where the major part of lipid oxidation has been reported to occur in the duodenal phase[12,67,96,194]. More lipid oxidation was expected to occur to some extent during the gastric phase, as seen both by Larsson *et al.*[12] and by Kristinova *et al.*[13]. Additionally, variations in model design, such as addition of gastric lipase, reduced oxygen level by N_2-flushing, and pH settings, might be the cause of the low oxidation detected in the gastric phase. Low oxidation levels in the gastric phase were confirmed both in the static and in the dynamic model, however, there was a relative increase in lipid oxidation products formed after 30 minutes of dynamic gastric digestion (Figure 3A and B, Study II).

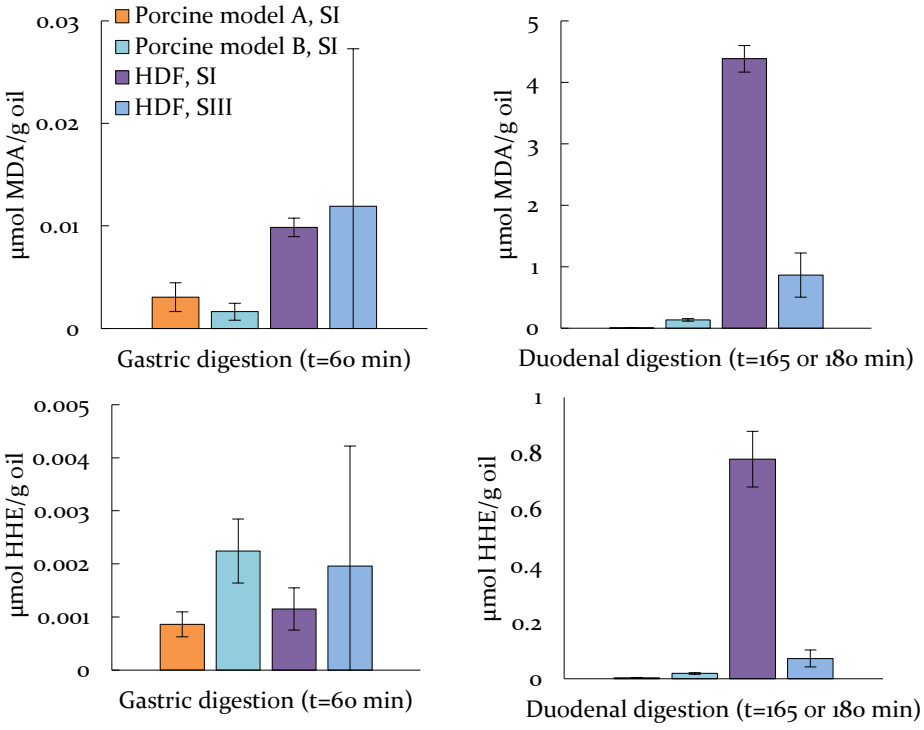

Figure 21: Aldehyde formation during gastric versus duodenal digestion
Comparison of MDA and HHE (µmol/g oil) in the gastric (t=60 min) and duodenal step, t=165 (SIII) or 180 min (SI), of static digestion using human or simulated porcine fluids. S (I,III)=Study (I, III), HDF= human duodenal fluid.

Table 6: Aldehyde formation at the end of digestion, expressed as ratio (duodenal/gastric)
The ratio (calculated as X/Y) between the MDA, HHE, and HNE levels detected at the end of gastric digestion (t=120 min, Y) and the end of duodenal digestion (t=210 min, X). CO=cod liver oil; S(I,III)=Study(I,III); P(A,B)=Porcine model (A,B) with SDF; H=Human model with HDF.

Model	CO PA, SI	CO PB, SI	CO H, SI	CO H, SIII	Fish H, SIII	Krill H, SIII	Algae H, SIII
MDA ratio	5	26	91	125	38	6	16
HHE ratio	4	5	146	15	8	3	10
HNE ratio	5	2	24	25	13	10	90

In the duodenal step, the aldehyde concentrations reached were in line with levels reported by others, as shown in Table 7. It should be noted that different initial meals and setting parameters have been used in the models compared in Table 7. Kenmogne-Domguia *et al.*[67] included for example metHb as a pro-oxidant, in their model, which could explain the 10-fold higher aldehyde levels reported, in comparison with the other results presented in Table 7.

Table 7: Aldehyde levels detected in different studies
A comparison of aldehyde concentrations (μM in digests) detected in various studies at the end of digestion. CO=cod liver oil; SDF=simulated digestive fluid; HDF=human digestive fluid; (I,II,III)=Study(I,II,III); PB=Porcine model B.

Intake meal	Beef muscle[97]	Salmon muscle[96]	Tuna:sunflower oil[67]	CO[12]	CO	CO	CO	CO
Study	Van Hecke *et al.*	Steppeler *et al.*	Kenmogne-Domguia *et al.*	Larsson *et al.*	SI, PB	SI	SII	SIII
GI fluid	SDF	SDF	SDF	SDF	SDF	HDF	SDF	HDF
Digestion model	Static	Static	Static	Static	Static	Static	Dynamic	Static
MDA (μM)	28	20[a]	240	22	1.6	60	3.8	17
HHE (μM)	-	0.5[a]	7.2	0.26	0.23	9.8	0.15	2.8
HNE (μM)	0.52	0.1[a]	1.2	-	0.005	0.36	0.02	0.18

[a]Approximate values after recalculations from μmol/kg oil into μM in the digests

There was a high standard deviation in the levels of aldehydes in cod liver oil at the end of digestion in Study III and IV, compared to Study I and II, where the same batch of cod liver oil was used. However, also others have seen high variability in development of lipid oxidation products during long time storage studies when the exponential phase is reached or passed[195]. Also, the aldehydes are highly reactive, which means that after reaction with, e.g., proteins or phospholipids they are no longer detected in free form.

5.2.2 *Effect of marine oil origin on the formation of lipid oxidation products*

Another question in this study was if different marine oils would show different susceptibility towards lipid oxidation during digestion (Study III). The fatty acid compositions of all the marine oils used were thoroughly described (Table S1, Paper III), see Figure 17 for summary. A normalization of the oil intake on the basis of EPA and DHA content was attempted, to receive approximately 5 mg of combined EPA and DHA per digestion. As can be seen in Figure 22, all marine oils tested did oxidize during static *in vitro* GI digestion. However, some of the marine oils were more susceptible to oxidation than others, and their ranking order was cod liver oil~fish oil>>krill oil~algae oil.

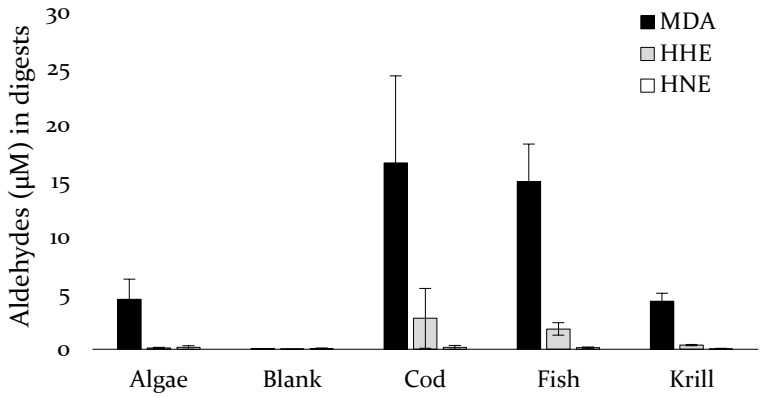

Figure 22: Aldehydes detected in different marine oil digests
Aldehydes (µM in digests) detected in the different oil digests at the end of static duodenal digestion (t=210 min), Study III and IV, n=3. The blank with pure human GI fluids is included.

Generally, levels of the specific aldehydes detected in the different oils were at the same magnitudes, with the ranking order between the aldehydes being MDA>HHE>HNE. Several factors differed in the oils, which could explain the observed variations. Naturally occurring antioxidants are reported to be present in both the krill oil and the algae oil, with krill oil containing astaxanthin and phospholipids[112], and with the algae oil being rich in polyphenols and tocopherols[115]. Both algae oil and krill oil have earlier been found to be more stable compared to fish oil during storage conditions[16], which supports our findings. This study was, however, the first to describe stability of different marine oils during digestion. Other explanations for the differences between the oils could be their initial oxidation levels. The importance of initial oil quality for lipid oxidation development during digestion was previously shown by Larsson *et al.*[12]. Cod liver oil overall had the highest initial levels of oxidation products (Table 3). The only exception from this was for MDA where the krill oil showed the highest initial levels. That all three marker aldehydes then remained low in krill oil during the digestion could be linked to that other lipid oxidation products than MDA, HHE, and HNE are formed when krill oil is digested. Previous studies have found that the lipid oxidation reaction takes different routes in the PL-rich krill oil, compared to oils mainly containing TAG, with formation of, e.g., pyrroles as a result[112]. Pyrroles have, however, been suggested to have a protective effect on lipid oxidation[196], however, the oxidative status and presence of, e.g., trace metal ions influences the stability of PL emulsions[197]. A theory is that the high PL-levels of the krill oil may act as sinks for aldehydes via Schiff base formation and further conversion to pyrroles[198]. The high initial MDA levels detected in the krill oil could be linked to the high initial level of FFA in this oil. FFA have

been shown in several studies to be more susceptible to lipid oxidation, compared to when FA are bound in TAG[199-201].

5.2.3 *Effect of oil pre-treatment on the formation of lipid oxidation products*

5.2.3.1 Effect of emulsification of the oil

Since emulsions are a common delivery system for fish oils, we were therefore interested in what effect pre-emulsification would have on lipid oxidation during GI digestion. As mentioned in section 3.4.2, some emulsification takes place already during the gastric tract of digestion, while the main part takes place in the duodenal tract. Digests used in Study III and IV, with and without marine oils, were investigated by microscopy (Zeiss axiostar plus) to confirm that some emulsification took place also during our static *in vitro*

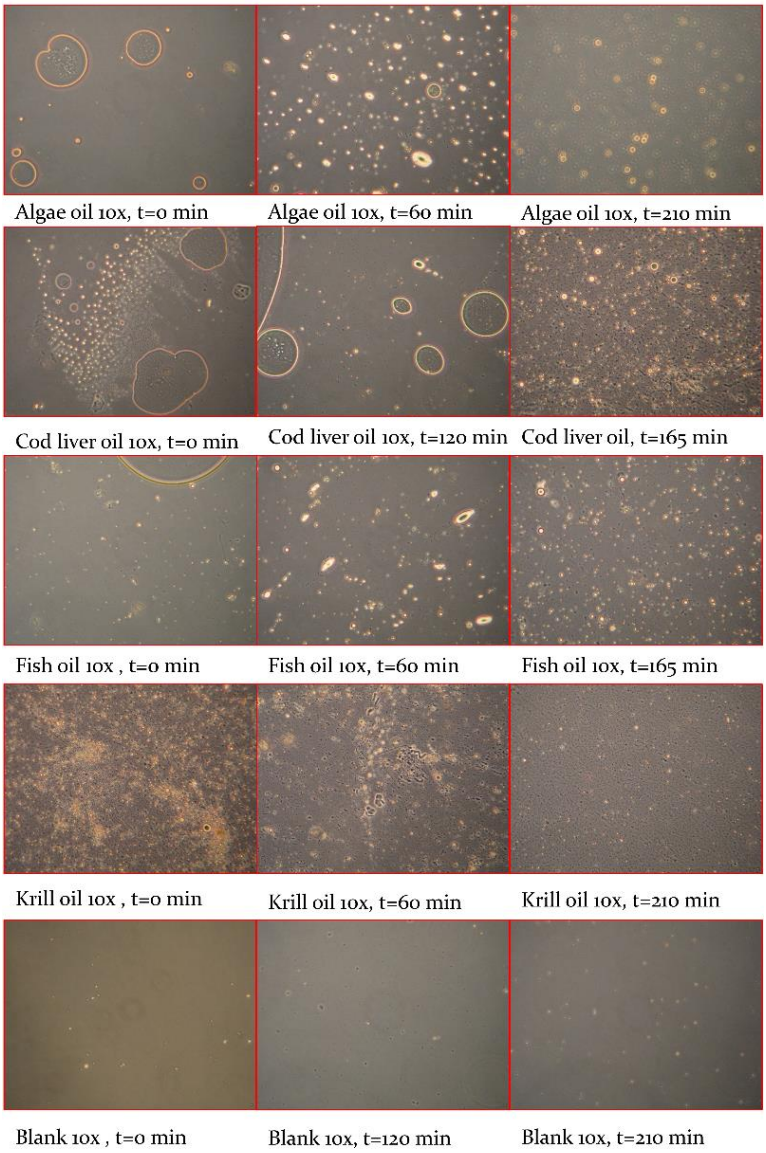

Figure 23: Degree of emulsification of digests
Pictures from analysis of oil digests with light microscopy showing the degree of emulsification of the digested oils, with a magnification of 10x.

digestion. The results are displayed as photographs with magnification 10x in Figure 23. The emulsion generally became finer throughout the digestion, with very large oil droplets at t=0, smaller ones at t=60-120 min, and the smallest droplets detected at t=180-210 min. The exception was the krill oil digests, which were a fine dispersion already at t=0 min. The method to investigate the emulsion through microscopy is not quantitative, however, from the photos obtained it is possible to see if emulsification took place at all, or not. The photos also indicates the fineness of the emulsion droplets, which could reflect how stable the emulsion is: the finer and more densely packed the droplets, the more stable the emulsion.

Emulsification before digestion was previously shown to have a pro-oxidative effect both during gastric and duodenal digestion in the study by Larsson *et al.*[194], when evaluating lipid oxidation of herring oil during dynamic GI *in vitro* digestion. In our Study II, the same dynamic *in vitro* digestion model was used to investigate GI-oxidation in fresh and highly pre-oxidized cod liver oil, with results displayed in Figure 24.

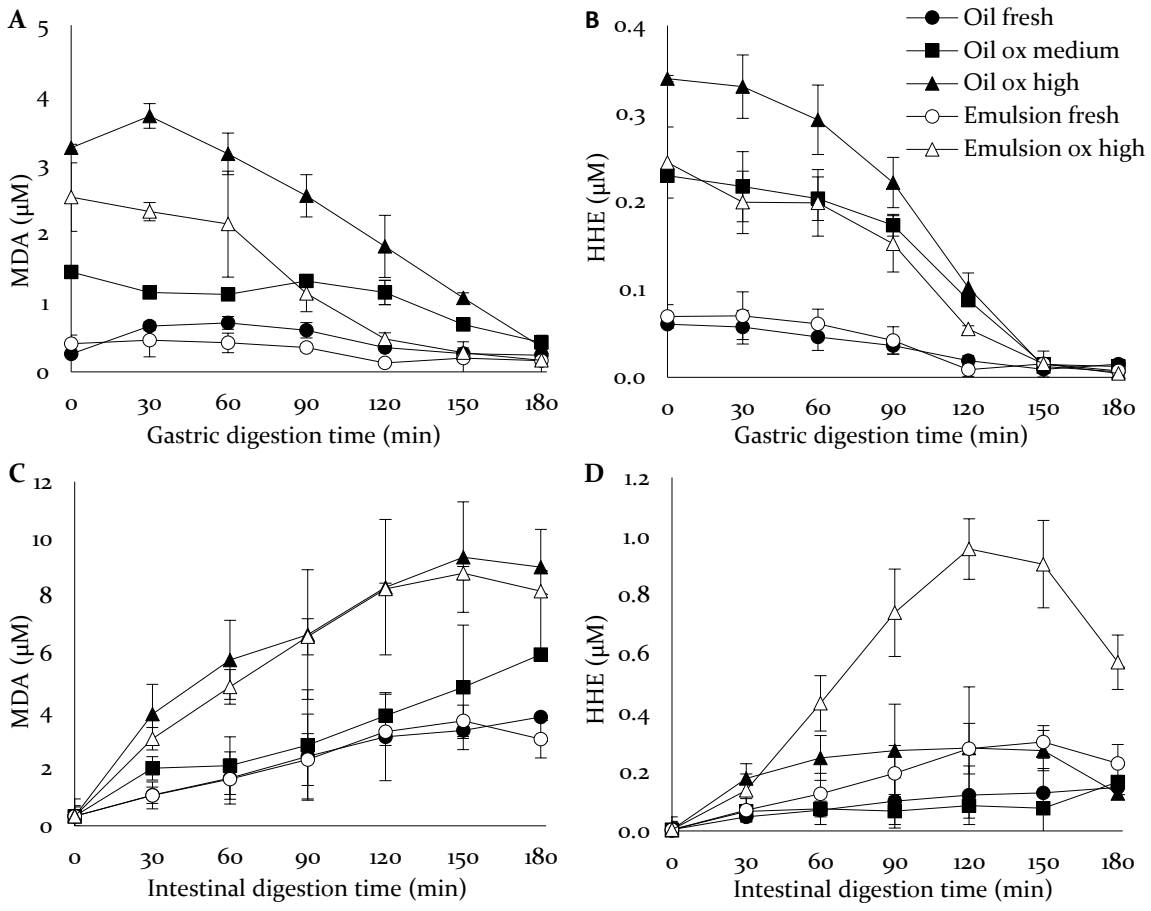

Figure 24: Aldehyde formation during dynamic digestion of emulsified and pre-oxidized oil
Aldehyde formation (μM in digests) during dynamic gastric and intestinal digestion (t=0-180 min) of fresh and oxidized cod liver oil. Error bars are shown as standard deviations, n=3, or (max-min)/2, when n=2.

During *in vitro* dynamic gastric digestion, lower levels of MDA and HHE were formed in digests from pre-emulsified oils, compared to bulk oils. This can be explained by the slightly lower initial oxidation levels detected in the pre-emulsified oil versus the bulk oil (Table 3). This difference was, however, lost during duodenal digestion where similar levels of MDA were formed in pre-emulsified oils, as in bulk oils. For HHE, the levels increased in the digests with pre-emulsified oils compared to bulk oil during duodenal digestion. The HHE levels detected were even significantly ($p<0.05$) higher in digests from the highly oxidized pre-emulsified oil, compared to highly oxidized bulk oil at the end of digestion (t=60-180 min). Mei *et al.*[126] had previously shown that an emulsion stabilized with the emulsifier Brij™ 35 was stable within the pH range 3-7, which supports the stability we observed during gastric digestion. We hypothesize that the shift in effect from pre-emulsification during duodenal digestion might be due to the introduction of bile-salts in combination with a pH-change, which could change the dominating surface active components at the droplet interface. The full mechanism(s) behind the result is still to be discovered.

5.2.3.2 Effect of initial oil quality

In Study II, cod liver oil with three levels of pre-formed oxidation products were compared (fresh oil, medium-, and high pre-oxidation), both as bulk oil and as pre-emulsified oil, in a dynamic *in vitro* digestion model. The initial oxidation levels are presented in Table 3, and the formation of MDA and HHE during dynamic *in vitro* digestion is shown in Figure 24. During gastric and duodenal digestion, the highly pre-oxidized oil was found to generate significantly ($p<0.05$) higher absolute levels of MDA and HHE at t=60-120 min, compared to medium oxidized and fresh oil. Significantly ($p<0.05$) higher aldehyde levels were also detected in the medium oxidized oil, compared to the fresh oil (t=60-120 min). When investigating the *relative* change in oxidation level – initial level versus level detected during digestion – the MDA formation was highest in the fresh cod liver oil, followed by medium- and highly oxidized oil (Figure 3, PII). The relative HHE formation during gastric digestion was independent of the pre-oxidation level. Highly oxidized pre-emulsified oil gave rise to increased levels of aldehydes compared to fresh pre-emulsified oil throughout gastric digestion. During duodenal digestion, the fresh oil (bulk and pre-emulsified) was again found to generate the highest relative rate of aldehyde formation, both for MDA and HHE, compared to the more oxidized oils. However, still significantly ($p<0.05$) higher absolute aldehyde levels were detected in the highly oxidized oils (t=60-120 min), compared to medium oxidized or fresh oils. Thus, the overall ranking of the absolute oxidation levels of the oils was kept throughout digestion, meaning a higher initial oxidation level resulted in higher oxidation level also in the digests. This finding is in line with what was found by Larsson *et al.*[12] when pre-oxidation of cod liver oil was evaluated in a static *in vitro* digestion model. To conclude, at the end of digestion, the relative oxidation rate was highest in digests with fresh cod liver oil, however, the highest absolute oxidation levels were measured in the pre-oxidized oils.

5.2.4 The role of lipolysis for lipid oxidation

5.2.4.1 The role of gastric and pancreatic lipolysis

It is nowadays believed that gastric lipase contributes to 10-25% of the total lipolysis *in vivo* in humans, as described in section 3.4.2[22,80]. FFA released from TAG have been shown

to be more susceptible to lipid oxidation, compared to the intact TAG, during storage conditions[199-201]. One suggested mechanism to explain the susceptibility of FFA to lipid oxidation, is that they can associate with pro-oxidative transition metal ions[202], bringing them closer to lipid droplet interface, where they can induce lipid oxidation. Hence, the presence of both gastric and pancreatic lipase might be of high importance when studying lipid oxidation during digestion. Previously, Larsson *et al.*[12] noted a link between the lipolysis of cod liver oil and the development of TBARS during static *in vitro* digestion, something which was also suggested by Kenmogne-Domguia *et al.*[67]. Free LC n-3 PUFAs have further been found to be more oxidatively stable, compared to non-hydrolyzed oil[67,93]. In this thesis work, we wanted to further investigate the role of lipolysis in relation to lipid oxidation in the GIT.

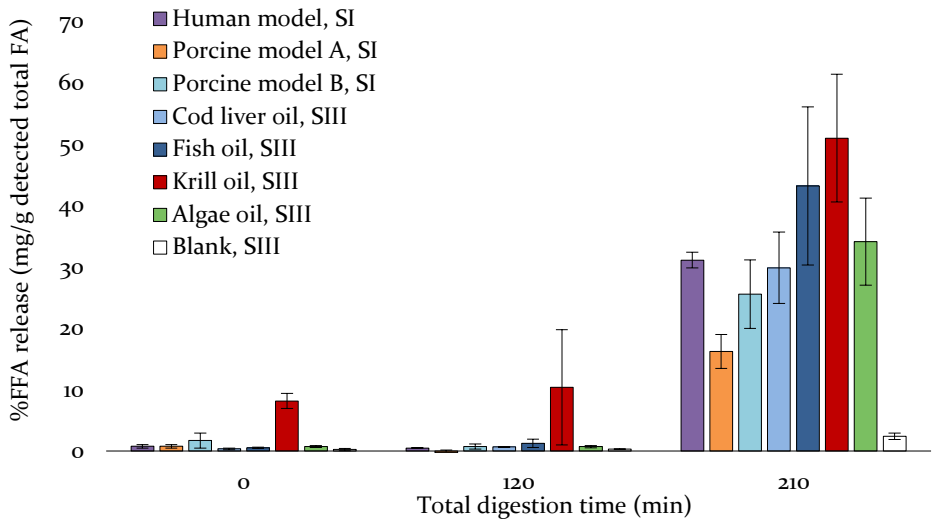

Figure 25: % FFA release during digestion
%FFA release (mg/g detected total FA), detected at the end of gastric and duodenal digestion, n=3. All samples from Study I were with fresh cod liver oil, all samples from Study III were with HDF. S(I,III)= Study (I,III). 120 min= end of gastric digestion, 210 min= end of duodenal digestion.

Figure 25 shows the degree of lipolysis in different marine oils during static *in vitro* digestion (Study I & III). All marine oils were hydrolyzed during duodenal digestion at similar levels (~30-50%), when human GI fluids were used, although the mean relative %FFA release was higher for fish- and krill oil, compared to cod liver- and algae oil. During gastric digestion, only krill oil showed a high release of FFA, and this oil had approximately 10% of the total FAs detected in free form already at start of the digestion. Krill oil was the only oil that was readily emulsified in water, which could explain the fast release of FFA. Low levels of lipolysis were detected during gastric digestion of the other oils. This is in line with the low activity of the gastric lipase detected in the HGF. The development of aldehydes during digestion generally corresponds well with the release of FFA, with barely any lipid oxidation occurring before duodenal digestion. Approximately the same levels of FFA were released with the HDF (31%) as with the fluids used in the porcine model B (26%, Figure 25). This suggests that digestions using either of the digestive fluids should be comparable based on total lipolysis. Porcine model A had lower lipolysis level, being in line with that less lipase

was added to this model (25-folds lower lipase activity) compared to the porcine model B and compared to the inherent lipolytic activity of the human GI fluids used in Study I. When comparing the levels of lipolysis at the end of duodenal digestion with the literature, others have detected 15% (fish oil, Zhu *et al.*[203]), 15-30% (salmon oil and salmon muscle, Aarak *et al.*[204]), and 40% (cod liver oil, Larsson *et al.*[12]) FFA release (w/w on an oil basis) at the end of static *in vitro* digestion with porcine digestive fluids, and, in one case, fungal gastric lipase[12]. Our results using both human and porcine enzymes and bile are hence in line with earlier reported lipolysis in marine oil/muscle based static *in vitro* digests.

The level of lipolysis was compared with the levels of aldehydes in the marine oil digests, evaluating how much aldehydes that were produced based on released FFA (Figure 26). However, no clear relation was seen between aldehyde formation and FFA release after normalization, neither for MDA, nor HHE. Hence, we can conclude that other factors than the FFA release alone has an impact on lipid oxidation in the GIT.

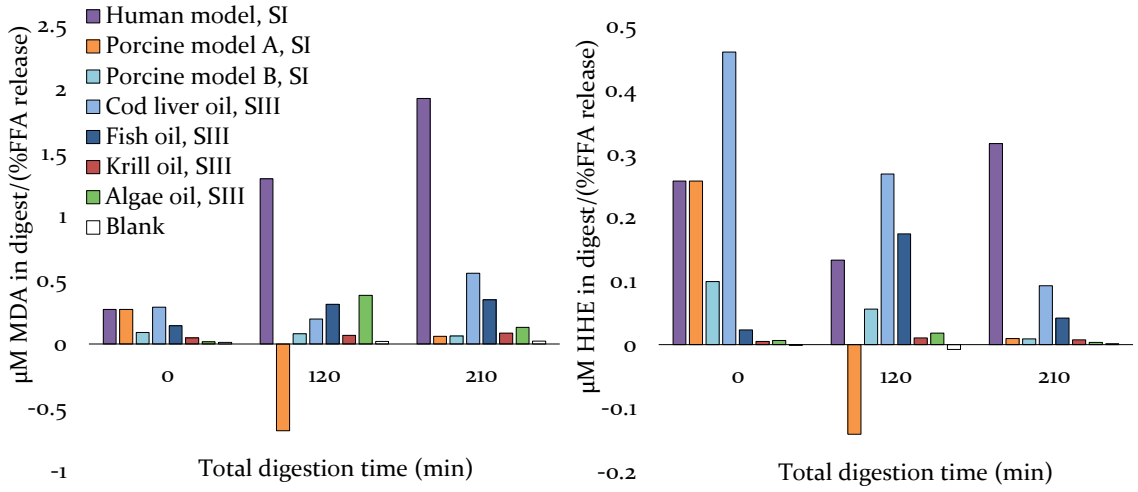

Figure 26: Detected aldehydes normalized towards %FFA release
MDA or HHE concentration (µM in digests) normalized towards %FFA release at the start (t=0 min), and end of gastric (t=120 min) and duodenal (t=210 min) digestion, n=3. All samples from Study I were with fresh cod liver oil, and all samples from Study III with HDF. S(I,III)= Study (I,III). Blank is pure HDF without oil (SIII). 120 min= end of gastric digestion, 210 min= end of duodenal digestion.

5.2.4.2 The role of specific release of EPA and DHA during digestion

One hypothesis was that the *specific* FFA released during lipolysis had an impact on lipid oxidation, with more unsaturated FFA being more susceptible to lipid oxidation. Aarak *et al.*[193] noted a difference in specific FFA released when digesting salmon oil with human- and simulated digestive GI fluids in a static *in vitro* digestion model. When analyzing the levels of released DHA and EPA after gastric digestion (t=120 min) in the human model versus in the simulated porcine model B, no differences were seen, and in all models a decrease was noted in free DHA and EPA compared to initial free DHA and EPA (Study I). A hypothesis is that part of the FFA detected in the cod liver oil at start of the digestion are consumed in the lipid oxidation reaction at a higher rate than new FFA are formed, which could explain that the concentration of FFA in the digests decrease during gastric digestion.

During duodenal digestion, the release of specific FFA differed depending on which GI fluids that were used, which confirms the findings by Aarak *et al.*[193]. Approximately 2-fold more EPA and DHA were released in the human model compared to the porcine models. However, when normalizing the formation of HHE on basis of the release of EPA and DHA (Figure 27), more HHE was formed per amount DHA and EPA in the duodenal phase with the human digestive GI fluids, compared to with SDF and compared to the other digestion steps. Hence, there seems to be other factors during duodenal digestion which affects the formation of HHE than purely the access to free LC n-3 PUFAs.

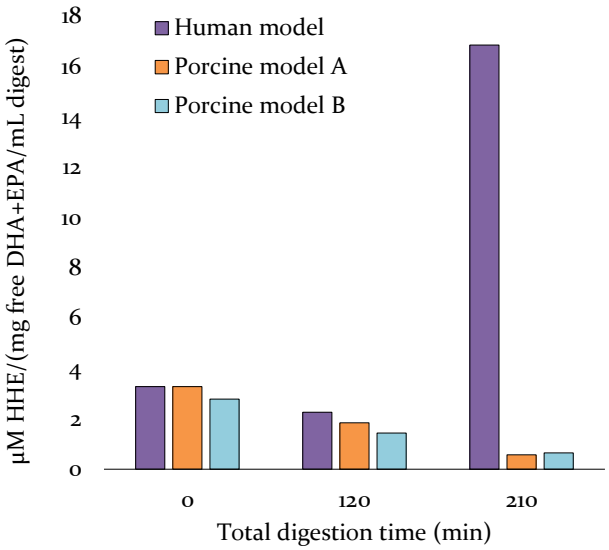

Figure 27: Formation of HHE during digestion normalized towards EPA and DHA
HHE formation (µM) normalized against detected free DHA and EPA in digests at the start (t=0 min), and at the end of gastric (t=120 min) and duodenal (t=210 min) digestion, n=3.

Comparing the release of specific FFA in marine oils of different origin during digestion, the highest release of EPA was detected in the krill and fish oils after duodenal digestion. The highest free DHA level, was detected after digestion of the algae oil, where also a majority of the FAs were found as DHA prior to digestion. After digestion, there was approximately 2-fold more free n-3 PUFAs in the algae oil, compared to what was detected after digestion of krill and fish oil, and 6.5-fold more than detected in the cod liver oil digests. Still, the algae oil digests had low levels of HHE compared to the other oil digests. The release of n-6 PUFAs from the different marine oil digests were similar among the different oils. Neither the release of free n-3 PUFAs, nor n-6 PUFAs could thus provide any explanation to the differences in lipid oxidation products detected in the different marine oils.

5.2.4.3 Effect of addition of rabbit gastric lipase (RGL) on lipid oxidation

Due to the low gastric lipase activity detected in the aspirated HGF a study with a higher content of gastric lipase effect on lipid oxidation was conducted. To date, gastric lipase has often been an enzyme neglected in digestion models. To investigate the role of its activity, RGL, shown to have similar features in activity towards different TAG[118], and similar pH optimum[205] as HGL, was added to the HGF used to digest fresh cod liver oil in the static

in vitro digestion model (Study III). The addition resulted in an increase in MDA levels at the start of the gastric digestion phase compared to non-fortified HGF, however, the difference was evened out at the end of the gastric digestion phase (Figure 28). No difference was seen in HHE formation during gastric digestion with RGL, while HNE was detected at significantly ($p<0.05$) higher levels, compared to without addition of RGL. During duodenal digestion, the only aldehyde that appeared to be affected by RGL addition was HNE, were higher levels were detected, compared to with non-fortified HGF.

The release of FFA in digests with or without RGL showed that lipolysis during digestion was not significantly affected by RGL, although the mean FFA release was 25% higher with RGL. Slightly more EPA and DHA (10 and 20%, respectively), and ~2-fold more n-6 PUFAs, were released when adding RGL, compared with non-fortified HGF (t=120 min). The latter could explain the large increase in HNE formation after RGL addition, since HNE is derived from n-6 PUFAs[162,163], and the result implies that gastric lipase could be important for lipid oxidation development, although the differences in our study were not significant. We therefore would recommend to include gastric lipase when designing static *in vitro* digestion models. This lipase should then preferably be as similar to HGL as possible, and currently RGL is regarded a good choice if there is no access to HGL[86].

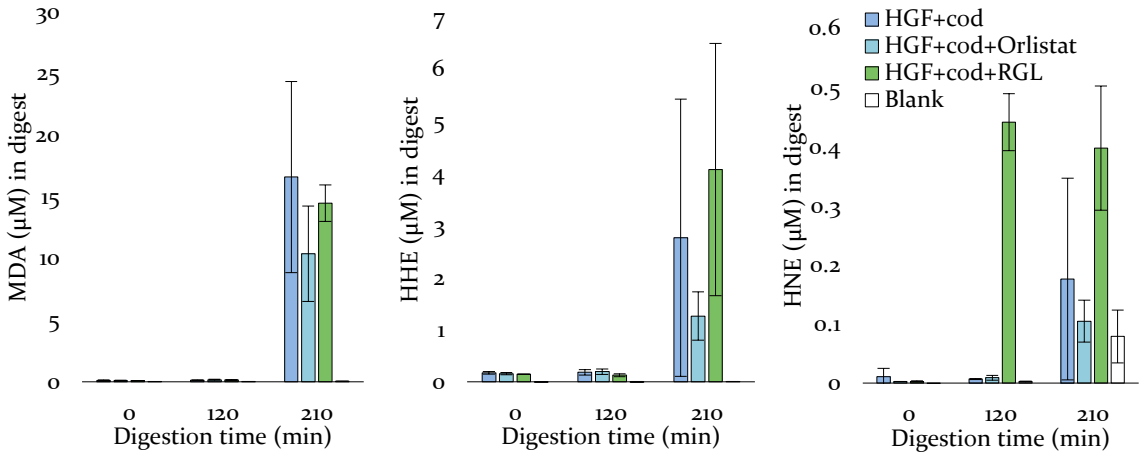

Figure 28: Aldehyde formation during digestion with addition of orlistat and rabbit gastric lipase
Aldehyde formation when adding the lipase inhibitor orlistat or rabbit gastric lipase (RGL) to human gastric fluid (HGF) to study lipid oxidation during digestion of fresh cod liver oil, n=3.

5.2.4.4 Effect of addition of the lipase inhibitor orlistat on lipid oxidation

The role of lipolysis on lipid oxidation during digestion of cod liver oil was further investigated by inhibition studies of the enzymes by orlistat, a lipase inhibitor. When adding orlistat to the oil before static *in vitro* digestion, there was an increase in MDA levels detected during gastric digestion (t=60 min), however, this effect levelled off at the end of gastric digestion (Figure 28). During duodenal digestion, the MDA levels detected with orlistat were lower, compared to levels detected in cod liver oil digests without orlistat (control, "HGF+cod" in Figure 28), however, differences were not significant. HHE and HNE

levels during gastric digestion did not differ from the control. During duodenal digestion with orlistat, lower levels of HHE and HNE were again detected compared to control, but the difference was not significant. Overall, orlistat appeared to delay the onset of lipid oxidation, although not completely preventing it. This suggests that the lipases are important for lipid oxidation, which is in line with the findings after the RGL addition (section 5.2.4.3).

The effect of orlistat addition on lipolysis *per se* during digestion was also investigated. It was seen that orlistat significantly reduced the %FFA release, compared to control, both during gastric and duodenal digestion. With orlistat, almost 30-times less EPA and 3-times less DHA were released during gastric digestion, and almost 20-times less EPA, and 15-times less DHA, were released during duodenal digestion, compared to control. In total, about 10-times, and 20-times less LC n-3 PUFAs were released with orlistat addition at the end of gastric and duodenal digestion, respectively, compared to control These results confirmed that there appear to be a relation between lipolysis and lipid oxidation, and that also gastric lipolysis is affected by the addition of orlistat.

5.2.5 *Effect of exogenous pro- and antioxidants on the formation of lipid oxidation products*

Hb from different origins have previously been found to promote lipid oxidation[206]. Hemoproteins have been connected both to the formation and decomposition of aldehydes[207] and hydroperoxides. The addition of cod derived Hb to emulsified cod liver oil had previously shown strong pro-oxidative action during static *in vitro* digestion (11.5 μM)[12]. It was therefore of interest to investigate if the same effect was seen when adding the same type of Hb to cod liver oil emulsion (11.5 μmol/L emulsion) during dynamic *in vitro* digestion.

A strong pro-oxidative effect was seen from the addition of Hb, both during gastric and duodenal digestion, resulting in increased MDA and HHE formation (Figure 29). The highest aldehyde levels were detected at 90 min in the gastric phase, and at 150 min in the duodenal phase. The results in this study were thus in line with the previously reported results by Larsson *et al.*[12]. Additionally, met-myoglobin (20 μM) was previously found to act as a pro-oxidant during static *in vitro* digestion of a marine oil containing emulsion[67], which further supports our findings. There are several mechanisms proposed which could explain the pro-oxidative action of Hb during digestion: i) Low pH could promote oxidation of Hb into its more reactive oxidized form metHb → release of hemin group[208], which has shown to act as a pro-oxidant, e.g., in fish muscle[209]; ii) Low pH could lead to an exposed heme group, due to partly denaturation of the heme protein[210,211]; iii) Proteolysis of the heme protein, making the protein less polar, could increase the interaction between peroxides and heme iron, attracting hydroperoxides to the oil-water interface[212]; iv) Proteolysis and/or denaturation of the heme protein can lead to a release of free iron, which could participate in lipid oxidation through the Fenton reaction[212,213]; v) Changes in the heme pocket due to, e.g., proteolysis or oxidation of the heme protein could attract the distal histidine to the iron in an oxidized heme protein, which would lead to the formation of Fe^{3+} hemichromes in low spin, which in turn could cause formation of superoxide radicals[208].

Human Hb present in the HDF could have an effect on the elevated aldehyde levels detected after digestion with the human model compared to the simulated porcine models (Study I). It could also explain the differences in aldehyde levels on an oil basis which were

seen in the two studies using HDF (Figure 21). As can be seen in Table 5, the levels of Hb and iron detected in the human GI fluids are not insignificant, hence the presence of Hb could explain these differences.

Ascorbic acid has been reported to be produced endogenously and has been detected in HGF at 87-89 μM[214,215]. Ascorbic acid seems to act both as a pro- and antioxidant, depending on the environment and its concentration[216]. It acts as a pro-oxidant in the presence of trace metal ions, and can, e.g., reduce Fe^{3+} to reactive Fe^{2+} ion form[216]. Previously, ascorbic acid (60-100 μM) was reported to act as a pro-oxidant in fish muscle[209], and it also showed pro-oxidative action during static gastric *in vitro* digestion of a herring oil emulsion (100 μM)[13]. However, the addition of 250 μM ascorbic acid to turkey meat, had a slightly antioxidative effect during static *in vitro* gastric digestion with HGF[217]. We wanted to investigate whether the naturally occurring levels of ascorbic acid in gastric fluid would act as a pro- or antioxidant during dynamic *in vitro* digestion of cod liver oil. Therefore, ascorbic acid (2.7 μM) was added to the gastric fluid initially present in the TIM-model prior to dynamic *in vitro* digestion (Study II). Thereafter, it was provided during dynamic *in vitro* digestion of a fresh cod liver oil by the continuous addition of gastric fluid to the digestive system (85 μM ascorbic acid, 1 mL/min). Ascorbic acid was found to have a pro-oxidative

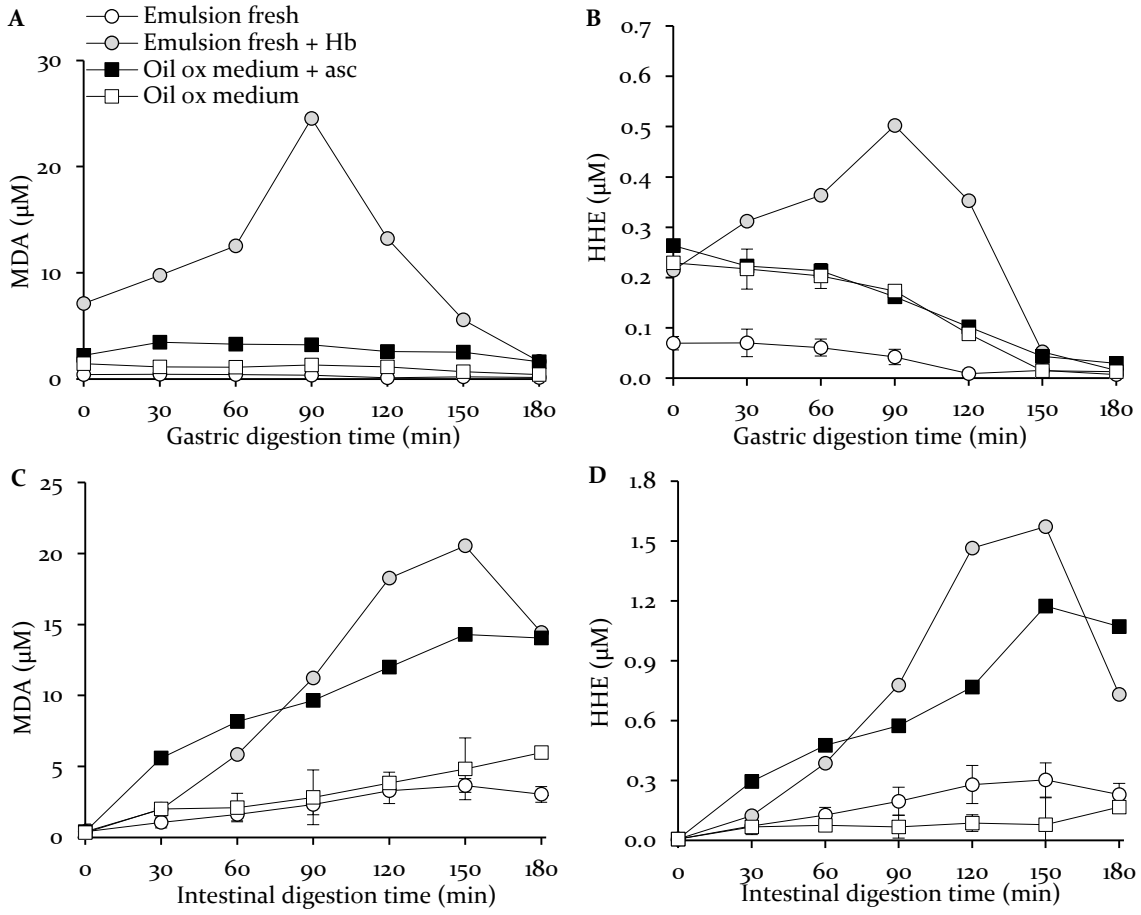

Figure 29: Aldehyde formation during dynamic digestion of cod liver oil, with oxidants and emulsified
Aldehyde formation (MDA and HHE, μM in digests) during dynamic gastric and intestinal digestion (t=0-180 min) of fresh emulsified cod liver oil with and without Hb, and medium oxidized bulk cod liver oil with and without ascorbic acid (asc), n=1-3.

effect during the dynamic *in vitro* digestion of cod liver oil, detected by increased formation of MDA (both during gastric and duodenal phase), and HHE (during duodenal digestion) (Figure 29).

No ascorbic acid was detected in the first aspirated batch of HDF (Study I), however, samples were then frozen directly in -80°C without pre-treatment, and ascorbic acid was therefore probably lost during storage. The second HDF batch (Study III and IV) was protected according to the description of Lykkesfeldt *et al.*[150], and ascorbic acid could be detected both in the gastric and duodenal fluid, at concentrations corresponding to approximately 1.5-1.8 µM (Table 5). In both batches of HDF, it is likely that some ascorbic acid was lost between the collection and the start of the storage, which could explain the low levels detected. Ascorbic acid, with its multiple roles within lipid oxidation, is a component that should be considered in *in vitro* GI models aiming to study lipid oxidation. When working with human GI-juices, care should be taken to preserve the endogenous levels.

It is likely that there are also other components present in the HDF which acts as pro-oxidants during digestion, which were not analyzed in this thesis work. Examples could be reactive oxygen species (ROS) secreted by cells involved in the immune responce[57], and the presence of pro-oxidative enzymes, such as salivary peroxidase[132]. This emphasizes that control over the digestion model system and further characterization of HDF possibly could explain some of the results obtained in this thesis work; particularly the fact that HDF were considerably more pro-oxidative than the simulated ones.

The effect of addition of potential antioxidants on lipid oxidation was investigated both in the dynamic *in vitro* digestion model (EDTA, Study II) and the static model (α-tocopherol, and the tocopherol mix Covi-ox® T 70, Study III). During static *in vitro* digestion, it was previously shown that, e.g., polyphenol-rich berry juice had a protective effect when added to herring oil before gastric digestion with HGF[13], and the addition of BHT was found to be protective already at low levels (20 ppm) when added to cod liver oil before digestion with simulated digestive GI fluids[218]. *In vivo* in minipigs, fruits and vegetables have been shown to have a protective effect against oxidation during digestion of vegetable oil[102]. Little work has, however, been done with tocopherols which are the main antioxidants used in the fish oil industry[12]. Also, studies with chelators were missing which could indicate how dominant the role of LMW metals are in GI-oxidation.

EDTA has previously been shown to work antioxidatively in emulsion systems, e.g., when being added to dressing enriched with fish oil (10-50 µg/g emulsion)[219]. It is, however, also known that the specific food system and the EDTA concentration matters for its antioxidative activity[129], and especially the ratio EDTA:iron is of importance[220,221]. In Study II, EDTA was added at 50 µg/g intake liquid "meal" (0.13 mM, ~200:1 EDTA:iron), which consisted of a highly pre-oxidized cod liver oil emulsion. This concentration should according to Alamed *et al.* be enough to yield a protective effect in fish oil emulsion with Brij™ 35 as emulsifier during heating and subsequent storage (8 days)[222]. However, in our trial (*n=1*), only a slight decrease in formation of MDA and HHE was seen from EDTA in the gastric digestion phase, and no difference in MDA formation was seen during duodenal digestion with/without EDTA. For HHE, levels even increased compared to pure cod liver oil. Frankel *et al.* showed in marine emulsified oils that equimolar ratios of EDTA:iron can have a pro-oxidative effect, while EDTA was found to act protectively when being added at

higher ratios (2:1 and 4:1)[220]. In another study, a ratio of approximately 3-4.5:1 (EDTA:iron) was, however, not protective[221]. Still, the EDTA:iron ratio in our study is well above, which indicates that it is not the LMW metals which are responsible for the major continuation of the lipid oxidation in a pre-oxidized oil system.

The addition of tocopherols was evaluated in Study III, by adding 4.5 mg tocopherols/mL fresh cod liver oil, before static *in vitro* digestion with human digestive GI fluids. Larsson *et al*.[12] did previously not see any protective effect from adding 1 mg α-tocopherols/mL fresh cod liver oil when digesting with simulated GI fluids. In a study by Van Hecke *et al*.[98] it was seen that lipid oxidation in lipid-rich beef could be increasingly prevented with increasing amount of α-tocopherol from 0 to 4.5 mg/g meat. In our study, we saw that HHE-formation could be prevented both during the gastric and duodenal phase of digestion, and MDA-formation during duodenal digestion. Furthermore, a mix of tocopherols (Covi-ox® T 70) was more efficient compared to pure α-tocopherol in preventing the formation of MDA and HHE. Both aldehydes were, however, generated at significantly lower levels with both types of tocopherol-additions, compared to pure cod liver oil. An explanation for the improved protection from adding a mix of tocopherols, compared to one single homologue, is dues to the expected synergistic effects from the mix. The tocopherols present in Covi-ox® T 70 are 14% α-, 2% β-, 60% γ-, and 24% δ-tocopherol, which should provide the mix with different possibilities of actions. An example of such differences in action is that α- tocopherol is able to reduce Cu^{2+}, while the other homologues are not[223], due to the different placement of their methyl substitutions on their chromanol ring[45]. γ-tocopherol has been found to be more active than α-tocopherol in *in vitro* systems, as opposed to in *in vivo* systems, possibly due to its ability to dimerize into molecules that also possess an antioxidative ability[224]. α-tocopherol is, however, often ranked as the homologue with the highest antioxidative activity[224], but it has also been shown to be more prone to lipid oxidation, in itself being susceptible towards lipid oxidation - all depending on the food matrix, and surrounding environment[225,226].

5.3 Effects of marine oil digests on stress in Caco-2 cells

A question addressed in this thesis work was what the effect of oxidized LC n-3 PUFAs would be on the stress response in human intestinal cells. It has previously been shown that HHE and MDA can be absorbed by intestinal cells in cellular uptake studies[11,14]. Additionally, when giving pre-oxidized LC n-3 PUFAs to mice, it was seen that there was a postprandial increase in plasma levels of inflammatory markers, e.g., NF-κB, IL-6 and MCP-1 and glutathione peroxidase[11], the very same markers (NF-κB, IL-6 and MCP-1) which were shown to decrease in plasma and spleen, when mice were provided a high fat diet with *fresh* LC n-3 PUFAs[227,228]. In another mice study, providing *oxidized* LC n-3 PUFAs resulted in increased plasma HHE levels[11], and providing pure HHE induced an inflammatory response and increased the HHE-adducts detected in the animals after administration[11]. Hence our hypothesis was that the oxidation products of the LC n-3 PUFAs could counteract the positive health benefits connected to these PUFAs. To test this hypothesis, the effects of marine oil digests on stress in Caco-2 cells were investigated together with the effects of the inherent MDA and HHE concentrations in the oil digests. In all experiments, digests with marine oil, digests without oil, and digestion blanks (e.g., pure cell medium), were included. A pilot trial regarding transport of MDA and HHE across the basal cell border was also done, both with pure HHE and with oil digests added to the Caco-2 cells (2 h). This trial showed that both MDA and HHE could be detected in the basal medium after incubation. However,

the detected aldehydes could also have been formed during incubation, and since no labelling of the aldehydes was done, no conclusions regarding transport could be drawn from this trial.

5.3.1 *Effects of marine oil digests and pure aldehydes on cell survival*

After exposing Caco-2 cells to marine oil digests (cod liver oil, krill oil and algae oil, digests from Study III, t=210 min), no reduction in cell survival was observed (Figure 30). Furthermore, cell survival was not affected by exposure to pure MDA and HHE at levels detected in the oil digests, and also not by higher MDA and HHE levels (1.4-90 µM). This was in agreement with the findings of, e.g., Awada *et al.*[11], who did not see any negative effect on cell monolayer integrity after exposing Caco-2 and T27 cells to HHE and HNE even for 24 h (0-100 µM). Our incubation time was relatively short (2 h), but was chosen in support of physiological data, which suggests varying residence time in the duodenum, with a peak lipid concentration after 3 hours of total digestion for coarse emulsions[229]. Hence 2 hours incubation time with the Caco-2 cells, to simulate the duodenal phase, should be sufficient to investigate effects of the digests on the cells. If cells would have been allowed to adjust to the exposure for a longer time period after the experiment, prior to cell lysis, the results could possibly have been different.

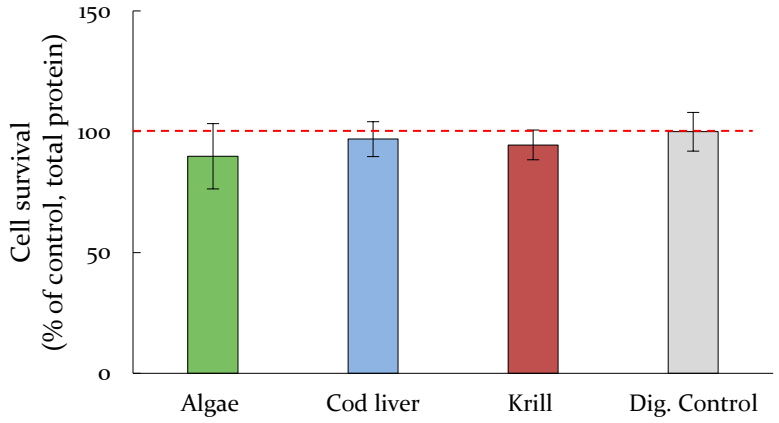

Figure 30: Cell survival as % of total protein levels compared to control (untreated cells)
Data are shown as mean % ± (max-min)/2, n=2. Dig. Control = control digests without oil.

5.3.2 *Effects of marine oil digests on expression of stress-related proteins*

Effects of marine oil digests on stress in Caco-2 cells were further investigated by the expression of stress-related proteins, using proteome profiler arrays (Figure 31). The stress-related proteins that showed a difference in the presence of marine oil digests were heat shock protein-60 and 70 (HSP-60 and HSP-70), superoxide dismutase 2 (SOD2), and thioredoxin-1 (Trx-1). HSP-70, Trx-1 and SOD-2); the levels of these proteins generally decreased.

HSP-70, Trx-1 and SOD-2 are all part of the anti-oxidative stress defense and are all generally increased when the cells are exposed to oxidative stress[230,231]. The chaperon HSP-70 is involved in the oxidative defense by prevention of protein aggregation and unfolding,

the oxidoreductase Trx-1 reduces oxidized proteins, and the superoxide dismutase SOD-2 is involved in superoxide radical scavenging. Thus, in contrary to what was expected, the levels of stress-related proteins did not *increase* at exposure to marine oil digests, nor at high levels of aldehydes present, the only exception being HSP-60. This suggests that the presence of marine oil digests did not induce a stress response, rather the opposite. Cod liver oil digests were the most oxidized of all digests, while the algae and krill oil digests were the least oxidized, see Figure 22. The decrease in stress response seen based on the HSP-60, HSP-70, and Trx-1 levels, was especially pronounced after exposure to the krill- and algae oil digests. The reduction was less pronounced with cod liver oil digests. This could be due to the higher aldehyde levels in the cod liver oil digests, but the results could also be related to the natural antioxidants present in the krill- and algae oils[39,232,233], something that we did not investigated further in this study. Another difference between the oil digests was the expression of SOD-2, which was reduced with both the cod liver oil digests and algae oil digest, but not with the krill oil digests. Krill oil differed from the other oils both in its content of astaxanthin, and that a majority of its LC n-3 PUFAs are found bound in phospholipids, opposed to TAG. Undigested extracts of FFA from krill oil have been shown to induce apoptosis when added to cancer cell lines, e.g., Caco-2 cells[234]. This suggests a protective effect from krill oil, however, the mechanism behind the effect was not investigated in the study by Jayathilake *et al.*[234]. That krill oil digests did not affect SOD-2 levels, while it reduced HSP 60/70 and Trx-1, point at the possibility that different compounds in the various marine oil digests are involved in the observed suppression of stress-related protein release. All in all, none of the oil digests showed any cytotoxic, nor stress-inducing effect in Caco-2 cells.

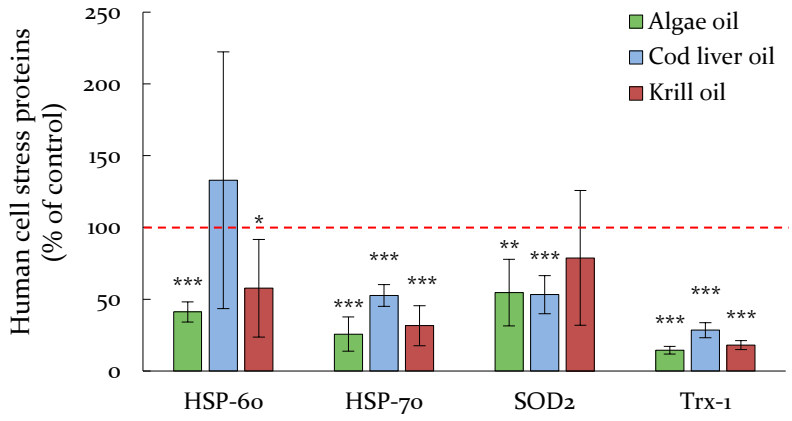

Figure 31: Effects of marine oil digests on expression of stress-related proteins
The expression of those human stress-related proteins that showed a difference in the presence of marine oils digested by human GI fluids. Data are shown as % of control (cells with digest without oil), error bars are shown as SD, n=3.

5.3.3 *Effects of pure aldehydes on expression of stress-related proteins*

Investigating the release of stress-related proteins in response to pure MDA and/or HHE on Caco-2 cells, it was observed that the presence of aldehydes barely had any impact on the cells when added in the same levels as detected in the oil digests (8.3 µM MDA, and 1.4 µM HHE, Figure 32). For MDA, 8.3 µM, reduced the expression of SOD2, and for HHE,

1.4 µM reduced the expression of HSP-60 and Trx-1. When increasing the aldehyde levels, the same effect as for the low aldehyde levels was seen overall. However, the protein Trx-1 was down-regulated in the presence of a mix of pure HHE and MDA (45 µM each). The same mix (HHE and MDA, 45 µM each) increased the detection of the protein HSP-60, as did high levels of pure MDA (90 µM). Despite the latter two observations the pure aldehydes (1.4-90 µM), just like the digests, generally had decreasing or no effects on stress in Caco-2 cells.

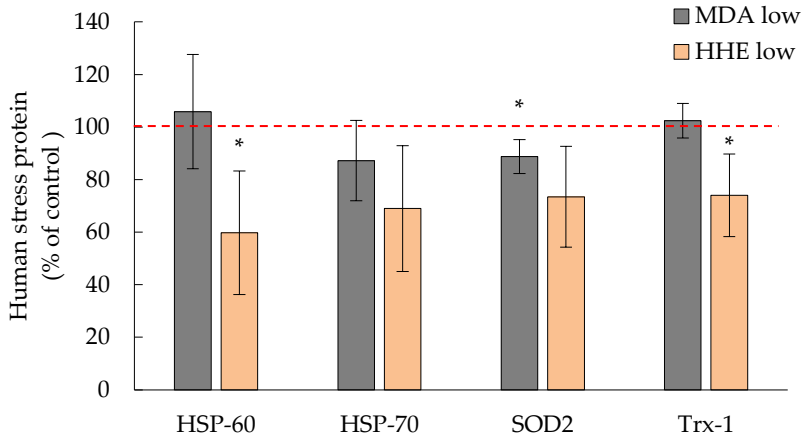

Figure 32: Effects of aldehydes on expression of stress-related proteins
The expression of those human stress-related proteins that showed a difference in the presence of low aldehyde levels (8.3 µM MDA; 1.4 µM HHE; i.e., the levels found in cod liver oil digests). Data are shown as % of control (cells with digest without oil), and presented as means ± (max-min)/2, n=2.

5.3.4 *Explanation of the stress-response results*

The results from exposing Caco-2 cells to marine oil digests and pure aldehydes suggest that physiological levels of the studied aldehydes are *not* harmful to the intestinal cells. This is different from the findings in the study by the group of Awada *et al.*[11], which revealed harmful effects while subjecting Caco-2 cells to the aldehydes HHE and HNE. Awada *et al.*[11] detected a significant increase in cellular protein carbonylation (24 h incubation) and in Michael adduct formation (2 h incubation), after exposing Caco-2/ TC7 cells to HHE and HNE (50-100 µM). They also detected an increase in the enzyme gastrointestinal glutathione peroxidase 2 (GPx2) and in glucose-regulated protein 78 (GRP78) expression after exposure to 100 µM HHE and HNE for 24[11], both considered indicators for increased cellular stress. Van Hecke *et al.*[98], noted a decrease in cell survival as measured by cytotoxicity (xCelligence RTCA) profiles, after digesting beef with different lipid content, followed by incubation of the digests with Caco-2 cells (1-48 h); cell survival decreased with increasing incubation time. Digests contained approximately 40 µM MDA and 1 µM HNE. However they saw no DNA damaging effect (measured by γ-H2AX fluorescence intensity), which is in line with the results in our study. Other studies have reported outcomes which could further help explaining our results. Ishikado *et al.* described a connection between increased intracellular levels of HHE and Nrf2 activation, showing that HHE could lead to protective effects when studied in human umbilical vein endothelial cells (HUVEC)[235]. Studies have also connected oxidized EPA with anti-oxidative effects in endothelial cells[236], and described the double edged role that ROS and aldehydes have as signalling molecules at low concentrations, but that they can also induce apoptosis if the levels become too high[11,237].

5.4 Relevance of the levels of lipid oxidation products used here to study cellular response

Oxidized lipids have been proposed to be causing inflammatory responses *in vivo* in several studies, and the intake of oxidized vegetable oils has resulted in increased lipid peroxide levels in the plasma from healthy human study participants[107,238]. In a recent study by Garcia-Hernandez *et al.*, it was reported that oxidized fish oil capsules increased blood cholesterol levels in healthy study participants (n≥17)[103]. According to Surh and Kwon[239], Koreans have an average daily intake of 16.1 µg 4-hydroxy-2-alkenals through the food, which could be correlated to the results in Study III, where 5 mg EPA+DHA generated 2.8 µM HHE and 0.18 µM HNE in the intestinal digests. If recalculating this based on digest volume and assuming a daily intake of 250 mg EPA+DHA, the total amount of 4-hydroxy-2-alkenals present in digests would be 34.8 µg/day, approximately the double amount of what is expected to already be the daily intake via foods in Korea. Marine oils are, however, normally stabilized with antioxidants, and the levels calculated here simulate a worst case scenario.

As mentioned in section 5.3.4, aldehydes – such as the ones in focus in this thesis work (MDA, HHE, and HNE) – are believed to play dual roles *in vivo*. At low levels they can act as signaling molecules, however, at increased concentrations they might interact with proteins and DNA, and might be both genotoxic and cytotoxic to biological systems[66]. For HHE and HNE, LC_{50} values reported are in the range 20-60 µM after toxicity studies using different animal- and human cells, according to the review by Pillon and Soulage[66]. The corresponding value for MDA was a lot higher, ≥600 µM[66]. These levels were all determined after long term exposure (>16 hours), and at shorter incubation times (<4 hours), low levels of cell damage was detected[66]. Still, short incubation times are highly interesting if one wants to study acute cellular responses. The toxicity of aldehydes can be studied in several ways, as described by Long *et al.*[76], some of which are through depletion of glutathione, generation of reactive oxygen species (ROS), and generation of aldehyde-protein-adducts. In neuronal cells, it was seen that the LC_{50} value for HHE was 23 µM, and 18 µM for HNE, measured as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) activity after 30 min incubation (0-50 µM aldehydes)[76]. In human retinal pigmented epithelial cells it was seen that the LC_{50} for HHE was 60 µM, and for HNE 40 µM[77]. HNE is well studied, since it has been appointed to be the most commonly found cytotoxic aldehyde formed from lipid oxidation[240]. HNE has indeed been found to be more toxic compared to HHE, e.g., when it comes to reactivity towards the phospholipids, which are constituents of the cellular membrane[241]. Among the aldehydes generated during lipid oxidation, HNE is one of the more studied ones. HNE has been detected at low levels in, e.g., human plasma (0.28-0.68 µM)[70], while levels increase upon stress[242,243]. Vandemoortele *et al.* recently showed that the combination of lipids together with MDA had a catalytic effect on the reactivity of MDA during static *in vitro* digestion[244], indicating that the reactivity of the aldehydes might differ during digestion of foods, compared to when studied isolated. Comparing the aldehydes of main focus in this thesis work – MDA, HHE, HNE – with, e.g., ONE, it is seen that ONE has a higher reactivity compared to HNE in some aspects, such as reactivity towards Lys ε-amino groups, resulting in Schiff base formation[78,245]. However, the two studies by Ottestad *et al.* showed no negative effect on oxidative stress markers in plasma, after administrating oxidized fish oil (8 g/day) to healthy humans[104,105], and as mentioned previously – aldehydes have been seen to act in protective ways as well, while present at low levels.

Absorption of lipid oxidation products have been demonstrated *in vivo* in rats in several studies[11,94,246]. Among these, one study supports the ability to transport lipid oxidation products through the lymphatic system in rats[246], and another study connects plasma circulation of LOOH *in vivo* in humans to intake of a high fat diet[247]. Staprañs *et al.*[107] saw that lipid oxidation products (conjugated dienes) detected in chylomicrons in human plasma could be related to dietary intake of oxidized lipids. Gorelik *et al.*[248] reported that administration of 250 g turkey meat significantly increased the level of plasma MDA in human volunteers (*n=10*), however, when marinating the meat in red wine prior to cooking, no increase in plasma MDA was seen. This showed both that plasma aldehyde levels could be linked to the diet, and that formation of MDA could be prevented[248]. Aldehyde levels in healthy human plasma varies; 1-6 µM have been reported for MDA,0.006-0.09 µM for HHE, and 0.007-11 µM for HNE[66].

During static *in vitro* digestion, the highest aldehyde levels detected were 60 µM MDA, 9.8 µM HHE, 0.36 µM HNE (Study I); these levels were with fresh cod liver oil and HDF. In the dynamic digestion model, Study II, the highest levels detected were 24.5 µM MDA, 1.6 µM HHE and 0.04 µM when digesting cod liver oil enriched with fish-derived Hb. Digesting pre-oxidized cod liver oil without any compounds added, 9.4 µM MDA, 1.0 µM HHE, and 0.07 µM HNE were detected. With fresh cod liver oil in the dynamic digestion model, 3.8 µM MDA, 0.15 µM HHE, and 0.07 µM HNE were detected. When relating these levels to detected aldehyde levels in the literature, the HHE-level is quite close to the reported cellular LC_{50} values, while both MDA and HNE are well below these concentrations. The overall low HNE values are in line with the low LC n-6 PUFA concentration of marine oils compared to other edible oils. However, the low levels could also be related to the very high reactivity of HNE, suggesting that only a fraction of formed HNE remain in free form.

With two exceptions, no adverse effects on cellular responses were observed from the MDA and HHE levels tested (0-90 µM) (Study IV). The results from this thesis work thus suggest that the MDA and HHE formed during digestion may not lead to acute stress for the cells in the intestinal tract, provided that the results could be directly translated to humans. However, long-term effects were not investigated, and although no negative effects were seen on cell viability and stress protein expression, other cellular factor might still be affected. The literature shows ambiguous results on the role and harmfulness of the chosen aldehydes, supporting the theory of a dual role of MDA, HHE, and HNE. Many studies suggest high toxicity of the aldehydes, and cellular apoptosis and cell growth inhibition has been reported already at 1 µM HNE in Caco-2 cells[249], and at 10 µM HHE in human epithelial renal proximal tubular cells[250]. RDI-values have, however, not been clarified for MDA, HHE, and HNE in foods, and therefore, precautions should still be taken. It is also clear that dynamic versus static digestion models generated different aldehyde levels, and that HDF yielded differences in lipid oxidation product formation, compared to SDF. Further investigations are needed to establish that no local damages occur *in vivo* in the intestine, and to investigate aldehyde levels tolerable for human consumption.

6. CONCLUSIONS

The aim of this thesis work was to investigate the mechanisms of marine lipid oxidation during *in vitro* GI digestion and also how to prevent it. In addition, we wanted to know if the oxidized digests were harmful to human intestinal cells. From the obtained results, it was clear that lipid oxidation occurs during *in vitro* digestion, both when using static and dynamic models. Results also revealed that there is a value in using HDF over SDF, when designing digestion models. Initial studies of the effects of marine oil digests on stress in epithelial cells revealed no such effects. More precisely, the results showed that:

- Higher aldehyde levels were formed during static *in vitro* digestion using human GI fluids compared with commercial enzymes and bile under physiological conditions, something which could be due to the complex matrix of HDF, or the presence of, e.g., Hb. This should be considered when designing and evaluating future *in vitro* digestion models, where lipid oxidation is in focus

- The levels of the targeted aldehydes formed during dynamic *in vitro* GI digestion of fresh cod liver oil were 3.8 µM MDA, 0.15 µM HHE, and 0.07 µM HNE, which were higher than the levels detected when using the static model (µmol/g input oil). This indicates that we might underestimate the level of aldehydes when using the latter digestion systems to study lipid oxidation

- The aldehyde levels formed in all models were lower compared to toxic levels reported in the literature

- Oxidation of cod liver oil during *in vitro* digestion was enhanced by the tested levels of cod-Hb and ascorbic acid. No effect was observed from the addition of EDTA. Commercially relevant types and levels of tocopherols were found to be protective against oxidation, with a mix of tocopherols, Covi-ox® T 70 EU, being superior to α-tocopherol

- The susceptibility of lipids towards oxidation during static GI-conditions varied with the marine oil origin. Cod liver oil and fish oil were shown to be more prone to oxidize compared to krill oil and algae oil. A possible explanation for this is the presence of endogenous antioxidants, e.g., astaxanthin and phenols, in the krill oil and the algae oil

- Lipolysis played an important role for the development of lipid oxidation during digestion. The addition of a lipase inhibitor, orlistat, reduced lipid oxidation, while the addition of rabbit gastric lipase led to changed lipid oxidation routes, increasing the formation of HNE, but not the other aldehydes. This indicates that FA in the free form generally were more susceptible to oxidation under static GI-conditions compared to esterified FA

- No adverse effect on Caco-2 cell survival was observed after cellular exposure to digests from cod liver oil, krill oil and algae oil. The levels of cellular stress proteins were reduced after exposure to the digests, and the same was seen for a mixture of pure MDA and HHE, corresponding to levels found in the digests

7. FUTURE PERSPECTIVES

A number of new tracks for investigations have been raised by the findings of this thesis work, which could be addressed in future studies:

- To **deepen the understanding of the mechanism during digestion**, it would be highly interesting to expand the lipid oxidation products targeted when studying oxidation during digestion further. Lipid oxidation is highly complex and the targeted aldehydes chosen in this thesis work (MDA, HHE, and HNE) can only give an indication of what happens during the reaction. They were all detected as free aldehydes in the digests, and are all secondary lipid oxidation products. Other compounds of interest would be pyrroles, which are expected to develop from lipid oxidation of PLs[198], and aldehyde-protein adducts, which would indicate the amount of bound aldehydes in the digests

- **Further characterization of the marine oils** could provide us with valuable insight in the major antioxidants acting as protecting agents in the oils. This could explain the low oxidation levels in the krill- and algae oil further, and possibly show us new naturally occurring antioxidants which could be used as protective additives in foods. Good experiments to do would be to spike, e.g., cod liver oil with the astaxanthin levels present in krill oil, or with specific phenols present in the algae oil, to see if the stability of the cod liver oil would become the same as for the other oils

- **Confirm the protective effects of tocopherols in the presence of pro-oxidants and with pre-oxidized oil.** The addition of tocopherols (4.5 mg/g oil) was shown to inhibit lipid oxidation in fresh cod liver oil. It would be interesting to see whether the same effect could be reached if tocopherols were added together with Hb, which was shown to act as a strong pro-oxidant during digestion, or if the oil was pre-oxidized. This would indicate if tocopherol addition is sufficient or if further protection of the oil is needed. The answer to this question could aid the fish oil industry on how to maximize protection of their oils, and for governments developing dietary recommendations

- **Matrix effects should also be investigated**, simulating the intake of marine oil supplement in combination with different meals. The result from this thesis work indicate that intake of marine oil with, e.g., a bloody beef meal might not be a good combination. This needs to be confirmed in other studies

- **Other ways of protecting marine** oil during digestion than the use of different additives should be investigated, e.g., the possibility of using different encapsulation techniques as barriers to lipid oxidation

- **Investigation of the reason for the pro-oxidative environment in the HDF.** With a deeper understanding of the major pro-oxidative mechanisms acting upon marine oil during digestion with HDF, these mechanisms could be targeted when developing defense barriers for the oil. Knowledge about HDF pro-oxidative mechanisms could thus aid both our understanding for GI-oxidation *per se*, and the

optimization of marine oil protection systems to be used by the fish oil industry. To fully rule out Hb as an actor, the Hb level in the HDF needs to be confirmed in a method with a lower limit of detection, and addition of low levels of iron (<1 ppm) should be evaluated in our digestive system

- **Further characterization of the HDF in general** could help optimizing the existing simulated GI digestive fluids further. HDF should preferably be used in the future when studying lipid oxidation during digestion, however, this is not always possible due to economical constrains and limited availability of freshly aspirated human GI fluids. There is ongoing work with the standardized digestion protocol by Minekus *et al.*[117], both in providing suggestions on how the protocol can be adapted if focusing on lipid digestion, as well as clarifying the procedure in the original protocol. This type of network collaboration is of high importance for the development of future digestion models. Standardized protocols are needed both for the comparison between studies, and to assure that the studies conducted hold a high scientific level

- **The role of gastric lipase could be further investigated**. We could unfortunately only add a small amount of RGL (48 U RGL/mL HGF) in this thesis work, and it is possible that the impact on lipid oxidation would have been more pronounced if we could have boosted the HGF further. RGL has been reported to have an activity twice as high as HGL[118]. This shows that the activity in our RGL-boosted system should still be relevant, and further boosting of the digestive system could confirm these findings. Additionally, the way HDF is handled in order to maintain as much of the gastric lipase as possible could be improved in future aspirations, e.g., by shortening the time between the aspiration and freezing of the samples

- **Evaluation of the effects of the lipid oxidation products on cellular stress levels**. Cellular studies with Caco-2 cells indicated no direct effects of MDA and HHE on the intestinal epithelium, however, there may still be potentially harmful effects of lipid oxidation products formed during digestion. Aldehyde protein adducts with MDA have for example been connected with coronary artery disease development[251]. The findings from the cell study in this thesis work should therefore be confirmed with other stress markers

- **Confirm the findings of this thesis work with *in vivo* studies**. The findings in this thesis work are all based on *in vitro* studies, and optimally the results should be confirmed by human *in vivo* trials. This could, e.g., be done with ileostomy patients given marine oils, where samples could be collected throughout ileal digestion. The cell studies could then be confirmed by GI biopsies. Another interesting study would be to provide study participants with marine oil or a fish meal prior to aspiration of HDF

8. ACKNOWLEDGMENTS

There are so many people I want to thank for accompanying me on this journey:

First of all, my dear supervisors: Ingrid Undeland for your patience and your honesty, your open door and for always being up for a discussion - I have learnt so much from you these years. It has through times been tough, but it has never been boring and you have always encouraged and challenged me. Nathalie Scheers, for sharing your knowledge and advices – and for all the mud baths while trail running and for the climbing sessions, a highly important part of these past years! Gerd Vegarud, who has never felt far away, although located in Ås, Norway. Thank you for your support also in moments of crisis, you have always been encouraging and positive and it has been a true pleasure working with you.

Ann-Sofie Sandberg, Bo Ekstrand and Ulf Svanberg for appreciated and constructive feedback on this thesis. Marie Alminger, thank you for valuable scientific discussions, great company at conferences and research trips, and for introducing me to LiFT.

The rest of the people at Food & Nutrition Science, who have been such a big part of my past years: Annette and Nisse – you have been true friends in need in so many ways – thank you for all your valuable advices and for sharing your knowledge. Thank you Mi-Lo, Anna B, Jenny and Helena for being so helpful and for sorting out things I did not even know of. The trailblazer and dear friend Karin L – thanks for your good advices and help, for your awesome humor and for giving me new ideas for bike tours. Hanna, thank you for being a close friend in anything from lab work – especially during long and crazy lab days – to mental support, as well as being a great roomie. To my other roomies Karin J and Nikul; Karin you have saved my days several times with chocolate and exciting discussions, and Nikul thank you for being an awesome back cracker and for all the graphical and programming related help. To the three of you: I am truly grateful for having had the honor to share this friendly, warm and supporting office with you. To my new roomie Mohsen, thank you for your smiles and positive words, and for bearing with me during my stressful final writing stage. Linnea thank you for always being there, for helping out with anything from lab work to finding chocolate hideouts, and for being a great friend and travel companion. Hanna and Linnea, a special thanks for all the support in the preparation for that special day, getting me dressed and taking care of me! Niklas, it has been very nice to learn and take courses together, and thank you for keeping my motivation up for training. Thank you Andy for challenging me on my knowledge in organic chemistry, for great scientific talks and for your friendship. Otto and Gabriel, thank you for persistently struggling with me to find teammates for our innebandy team. Graziele, thank you for the fun moments at the courses abroad! Lin, Izabella and Mads - for the extra help with statistics and for making me laugh. A special thanks to Malin Östensson at National Bioinformatics Support (NBIS) and Calle for statistical support in certain questions. Mehdi, Bita and Lillie – for extra help with anything from analysis to fruitful scientific discussions. Cecilia M, Gustaf and Malin – thank you for always being so positive and cheerful. Finally, thank you Marcus H, for being incredibly efficient, positive and self-going, it was a pleasure having you as a Master's Thesis student.

Irene – you were my cornerstone at the lab in Ås, Norway, thank you for all the laughter and fun moments, and for your hard work with getting study participants and collecting samples. This project would not have been the same without you. A big thank you all researchers who have been part of my favorite Norwegian research group: Kristi, Ellen,

Ragnhild, Tora, Tove… it has been a pleasure! To the whole team in Norway: thanks for the warmheartedly welcome you gave me, letting me be part of your great group.

Thank you Marcus Persson at AAK, my mentor the last years, for supportive discussions – it has been truly inspiring to have you as mentor. Our meetings have always left me energized and motivated. Also a big thank you Anders Borde and Laban at Astra Zeneca for interesting scientific discussions and great company at conferences, and for giving me the opportunity to connect with your group. To all of you, thank you for providing me with valuable insights in the oil and pharma industry.

Thank you my incredible friends outside office: Anna, David, Elin, Johan E, Sara D with family, Sofie, Jenny, Johan A, Kristina R, Malin, Hanna A, Helena, Sommis, Sara K, Hanna L with family, Daniel, Lina… and all the amazing people at my choir Gustavikören who have been my second family in Gothenburg – especially Emily, Kristina F, Johanna, Liisa, and Marianne. Thank you for filling my life with joy and for being part of my life.

Finally, a special thank you goes to my whole fantastic family for always supporting me, even when I have been working weekends and holidays in the summer cottages - in Nybrostrand as well as in Västervik. I love you. To my dear parents and great sister and brother for cheering all the way. To my grandmother Siw for being a close friend and great source of inspiration. Thank you also for helping out with proofreading and for all our lovely evening talks. To my husband Kolle, for your unwavering support and for being an exquisite listener and problem solver. Your advices on this thesis have been precious. Thank you for putting up with me also during the hard times, and for all your help and love. You are the dearest of all to me.

This work was financially supported by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas, Project no. 222-2012-1331). Foundation from Nils Pihlblads stipendiefond has additionally financially supported the research visits in Ås.

9. REFERENCES

1 Turner, R., McLean, C. H. & Silvers, K. M. Are the health benefits of fish oils limited by products of oxidation? *Nutrition Research Reviews* **19**, 53-62 (2007).

2 Albert, B. B., Cameron-Smith, D., Hofman, P. L. & Cutfield, W. S. Oxidation of marine omega-3 supplements and human health. *BioMed Research International* **2013**, 8 (2013).

3 Shepherd, C. J. & Jackson, A. J. Global fishmeal and fish-oil supply: inputs, outputs and marketsa. *Journal of Fish Biology* **83**, 1046-1066 (2013).

4 Calder, P. C. Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology? *Br J Clin Pharmacol* **75**, 645-662 (2013).

5 Lavie, C. J., Milani, R. V., Mehra, M. R. & Ventura, H. O. Omega-3 polyunsaturated fatty acids and cardiovascular diseases. *J Am Coll Cardiol* **54**, 585-594 (2009).

6 Delgado-Lista, J., Perez-Martinez, P., Lopez-Miranda, J. & Perez-Jimenez, F. Long chain omega-3 fatty acids and cardiovascular disease: a systematic review. *Br J Nutr* **107 Suppl 2**, S201-213 (2012).

7 Calder, P. C. n–3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *The American Journal of Clinical Nutrition* **83**, S1505-1519S (2006).

8 Albert, B. B., Derraik, J. G., Brennan, C. M., Biggs, J. B., Garg, M. L., Cameron-Smith, D., Hofman, P. L. & Cutfield, W. S. Supplementation with a blend of krill and salmon oil is associated with increased metabolic risk in overweight men. *The American Journal of Clinical Nutrition* (2015).

9 Halvorsen, B. L. & Blomhoff, R. Determination of lipid oxidation products in vegetable oils and marine omega-3 supplements. *Food & Nutrition Research* (2011).

10 Cameron-Smith, D., Albert, B. B. & Cutfield, W. S. Fishing for answers: is oxidation of fish oil supplements a problem? *Journal of Nutritional Science* **4**, e36 (2015).

11 Awada, M., Soulage, C. O., Meynier, A., Debard, C., Plaisancie, P., Benoit, B., Picard, G., Loizon, E., Chauvin, M. A., Estienne, M., Peretti, N., Guichardant, M., Lagarde, M., Genot, C. & Michalski, M. C. Dietary oxidized n-3 PUFA induce oxidative stress and inflammation: role of intestinal absorption of 4-HHE and reactivity in intestinal cells. *J Lipid Res* **53**, 2069-2080 (2012).

12 Larsson, K., Cavonius, L., Alminger, M. & Undeland, I. Oxidation of cod liver oil during gastrointestinal *in vitro* digestion. *J Agric Food Chem* **60**, 7556-7564 (2012).

13 Kristinova, V., Storrø, I. & Rustad, T. Influence of human gastric juice on oxidation of marine lipids – *in vitro* study. *Food Chem* **141**, 3859-3871 (2013).

14 Maestre, R., Douglass, J. D., Kodukula, S., Medina, I. & Storch, J. Alterations in the intestinal assimilation of oxidized PUFAs are ameliorated by a polyphenol-rich grape seed extract in an *in vitro* model and Caco-2 cells. *The Journal of nutrition* **143**, 295-301 (2013).

15 Jackson, A. in *Omega -3 Summit, Bruges 4th March 2011* (IFFO, http://www.iffo.net/system/files/IFFO%20Omega%203%20summit%20Bruges%281%29.pdf, 2011).

16 Ryckebosch, E., Bruneel, C., Termote-Verhalle, R., Lemahieu, C., Muylaert, K., Van Durme, J., Goiris, K. & Foubert, I. Stability of omega-3 LC-PUFA-rich photoautotrophic microalgal oils compared to commercially available omega-3 LC-PUFA oils. *Journal of Agricultural and Food Chemistry* **61**, 10145-10155 (2013).

17 Christie, W. W. & Han, X. in *Lipid Analysis* Ch. 1, 3-19 (Woodhead Publishing, 2012).

18 Cunnane & Griffin. in *Introduction to human nutrition* (eds Gibney;, Vorster;, & Kok) (Blackwell Science Ltd., 2007).

19 Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P. *Molecular biology of the cell*. Vol. 4th (Garland Science, 2002).

20 Piomelli, D., Astarita, G. & Rapaka, R. A neuroscientist's guide to lipidomics. *Nature Reviews Neuroscience* **8**, 743 (2007).

21 da Costa, E., Silva, J., Mendonça, S., Abreu, M. & Domingues, M. Lipidomic approaches towards deciphering glycolipids from microalgae as a reservoir of bioactive lipids. *Marine Drugs* **14**, 101 (2016).

22 Gurr, M., Harwood, J. & Frayn, K. *Lipid Biochemistry: An Introduction , 5th Edition*. (Wiley-Blackwell, 2002).

23 Hjaltason, B. & Haraldsson, G. G. in *Modifying Lipids for Use in Food* (ed F.D. Gunstone) Ch. 4, 56-79 (Woodhead Publishing, 2006).

24 Arab-Tehrany, E., Jacquot, M., Gaiani, C., Imran, M., Desobry, S. & Linder, M. Beneficial effects and oxidative stability of omega-3 long-chain polyunsaturated fatty acids. *Trends in Food Science & Technology* **25**, 24-33 (2012).

25 Harnack, K., Andersen, G. & Somoza, V. Quantitation of alpha-linolenic acid elongation to eicosapentaenoic and docosahexaenoic acid as affected by the ratio of n6/n3 fatty acids. *Nutrition & Metabolism* **6**, 8-8 (2009).

26 Plourde, M. & Cunnane, S. C. Extremely limited synthesis of long chain polyunsaturates in adults: implications for their dietary essentiality and use as supplements. *Applied Physiology, Nutrition, and Metabolism* **32**, 619-634 (2007).

27 Simopoulos, A. P. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. *Biomedicine & Pharmacotherapy* **60**, 502-507 (2006).

28 EFSA. Commission Regulation (EU) No 432/2012 of 16 May 2012 establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to children's development and health Text with EEA relevance. *Official Journal of the European Union* **55**, L 136 (2012).

29 EFSA. Regulation (EC) No 1924/2006 of the european parliament and of the council of 20 December 2006 on nutrition and health claims made on foods. *Official Journal of the European Union*, L 404 (2006).

30 Hjaltason, B. & Haraldsson, G. G. in *Modifying Lipids for Use in Food* (ed Frank D Gunstone) Ch. 24, 587-602 (Woodhead Publishing, 2006).

31 FAO. Code of conduct for responsible fisheries 41 p. (FAO, Rome, 1995).

32 FAO/WHO. Report of the twenty forth session of the codex committee on fats and oils, proposed draft standard for fish oils. (FAO/WHO, Melaka, Malaysia, 2015).

33 Pike, I. H. & Jackson, A. Fish oil: production and use now and in the future. *Lipid Technology* **22**, 59-61 (2010).

34 IFFO. *Fish oil market to reach US$1.7 billion by 2018 due to flourishing aquaculture industry in China*, http://www.iffo.net/industry-news?page=4 (2015).

35 Haraldsson, G. G. & Hjaltason, B. in *Modifying Lipids for Use in Food* (ed Frank D Gunstone) Ch. 14, 336-368 (Woodhead Publishing, 2006).

36 Breivik, H. in *Long-Chain Omega-3 Specialty Oils* (ed Harald Breivik) Ch. 5, 111-140 (Woodhead Publishing, 2012).

37 Mühlroth, A., Li, K., Røkke, G., Winge, P., Olsen, Y., Hohmann-Marriott, M., Vadstein, O. & Bones, A. Pathways of lipid metabolism in marine algae, co-expression network, bottlenecks and candidate genes for enhanced production of EPA and DHA in species of chromista. *Marine Drugs* **11**, 4662 (2013).

38 Yao, L., Gerde, J. A., Lee, S.-L., Wang, T. & Harrata, K. A. Microalgae lipid characterization. *Journal of Agricultural and Food Chemistry* **63**, 1773-1787 (2015).

39 Tou, J. C., Jaczynski, J. & Chen, Y. C. Krill for human consumption: nutritional value and potential health benefits. *Nutr Rev* **65**, 63-77 (2007).

40 Opstvedt, J. in *Ifoma Technical Bulletin* Vol. 22 1-27 (International Fishmeal & Oil Manufacturers Association UK, 1985).

41 Ramprasath, V. R., Eyal, I., Zchut, S., Shafat, I. & Jones, P. J. H. Supplementation of krill oil with high phospholipid content increases sum of EPA and DHA in erythrocytes compared with low phospholipid krill oil. *Lipids in Health and Disease* **14**, 142 (2015).

42 Burri, L., Hoem, N., Banni, S. & Berge, K. Marine omega-3 phospholipids: metabolism and biological activities. *International Journal of Molecular Sciences* **13**, 15401-15419 (2012).

43 Rubio-Rodríguez, N., Beltrán, S., Jaime, I., de Diego, S. M., Sanz, M. T. & Carballido, J. R. Production of omega-3 polyunsaturated fatty acid concentrates: a review. *Innovative Food Science & Emerging Technologies* **11**, 1-12 (2010).

44 Hjaltason, B. & G. Haraldsson, G. in *Long-Chain Omega-3 Specialty Oils* (ed Breivik) Ch. 11, 263-289 (Woodhead Publishing, 2007).

45 Muggli, R. in *Long-Chain Omega-3 Specialty Oils* (ed Harald Breivik) Ch. 7, 165-195 (Woodhead Publishing, 2012).

46 Jacobsen, C. in *Oxidative stability and shelf life of foods containing oils and fats* (ed Hu and Jacobsen) Ch. 8, 287-312 (AOCS Press, 2016).

47 Nielsen, N. S., Horn, A. F. & Jacobsen, C. Effect of emulsifier type, pH and iron on oxidative stability of 5% fish oil-in-water emulsions. *European Journal of Lipid Science and Technology* **115**, 874-889 (2013).

48 Kargar, M., Spyropoulos, F. & Norton, I. T. The effect of interfacial microstructure on the lipid oxidation stability of oil-in-water emulsions. *Journal of Colloid and Interface Science* **357**, 527-533 (2011).

49 Waraho, T., McClements, D. J. & Decker, E. A. Mechanisms of lipid oxidation in food dispersions. *Trends in Food Science and Technology* **22**, 3-13 (2011).

50 Schaich, K. M. in *Lipid Oxidation* (eds Amy Logan, Uwe Nienaber, & Xiangqing Pan) Ch. 2, 53-128 (AOCS Press, 2013).

51 Shahidi, F. & Zhong, Y. Lipid oxidation and improving the oxidative stability. *Chemical Society Reviews* **39**, 4067-4079 (2010).

52 Porter, N., Caldwell, S. & Mills, K. Mechanisms of free radical oxidation of unsaturated lipids. *Lipids* **30**, 277-290 (1995).

53 Schaich, K. M. in *Lipid Oxidation* (eds Uwe Nienaber & Xiangqing Pan) 1-52 (AOCS Press, 2013).

54 Schaich, K. M. in *The Wiley Encyclopedia of Packaging Technology* (ed K.L. Yam) 659-674 (John Wiley and Sons Inc., 2009).

55 Hu, M. in *Oxidative Stability and Shelf Life of Foods Containing Oils and Fats* xvii-xxxviii (AOCS Press, 2016).

56 Jairam, V., Uchida, K. & Narayanaswami, V. in *Lipoproteins - Role in Health and Diseases* (eds Sasa Frank & Gerhard Kostner) Ch. 16 (InTech, 2012).

57 Halliwell, B., Zhao, K. & Whiteman, M. The gastrointestinal tract: a major site of antioxidant action? *Free Radical Research* **33**, 819-830 (2000).

58 Aubourg, S. P. Fluorescence study of the pro-oxidant effect of free fatty acids on marine lipids. *Journal of the Science of Food and Agriculture* **81**, 385-390 (2001).

59 Mihaljević, B., Katušin-Ražem, B. & Ražem, D. The reevaluation of the ferric thiocyanate assay for lipid hydroperoxides with special considerations of the mechanistic aspects of the response. *Free Radical Biology and Medicine* **21**, 53-63 (1996).

60 Shahidi, F. & Zhong, Y. in *Bailey's Industrial Oil and Fat Products* (John Wiley & Sons, Inc., 2005).

61 Mozuraityte, R., Kristinova, V., Standal, I., Carvajal, A. & Aursand, M. in *Oxidative Stability and Shelf Life of Foods Containing Oils and Fats* (eds Min Hu & Charlotte Jacobsen) Ch. 5, (Elsevier, 2016).

62 GOED. *GOED voluntary monograph version 5: global organisation for EPA and DHA omega-3*, http://goedomega3.com/index.php/files/download/350 (2015).

63 Bocci, V. A., Zanardi, I. & Travagli, V. Ozone acting on human blood yields a hormetic dose-response relationship. *Journal of Translational Medicine* **9**, 66-66 (2011).

64 Guillén, M. D. & Goicoechea, E. Toxic oxygenated α,β-unsaturated aldehydes and their study in foods: a review. *Critical Reviews in Food Science and Nutrition* **48**, 119-136 (2008).

65 Catalá, A. Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chemistry and Physics of Lipids* **157**, 1-11 (2009).

66 Pillon, N. J. & Soulage, C. O. in *Lipid Peroxidation* (ed Angel Catala) Ch. Lipid Peroxidation by-Products and the Metabolic Syndrome, (InTech, 2012).

67 Kenmogne-Domguia, H. B., Moisan, S., Viau, M., Genot, C. & Meynier, A. The initial characteristics of marine oil emulsions and the composition of the media inflect lipid oxidation during *in vitro* gastrointestinal digestion. *Food Chemistry* **152**, 146-154 (2014).

68 Mendes, R., Cardoso, C. & Pestana, C. Measurement of malondialdehyde in fish: a comparison study between HPLC methods and the traditional spectrophotometric test. *Food Chemistry* **112**, 1038-1045 (2009).

69 Domingues, R. M., Domingues, P., Melo, T., Pérez-Sala, D., Reis, A. & Spickett, C. M. Lipoxidation adducts with peptides and proteins: deleterious modifications or signaling mechanisms? *Journal of Proteomics* **92**, 110-131 (2013).

70 Esterbauer, H., Schaur, R. J. & Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* **11**, 81-128 (1991).

71 Lang, J., Celotto, C. & Esterbauer, H. Quantitative determination of the lipid peroxidation product 4-hydroxynonenal by high-performance liquid chromatography. *Analytical Biochemistry* **150**, 369-378 (1985).

72 Long, E. K. & Picklo Sr, M. J. Trans-4-hydroxy-2-hexenal, a product of n-3 fatty acid peroxidation: make some room HNE.... *Free Radical Biology and Medicine* **49**, 1-8 (2010).

73 Van Kuijk, F. J., Holte, L. L. & Dratz, E. A. 4-Hydroxyhexenal: a lipid peroxidation product derived from oxidized docosahexaenoic acid. *Biochim Biophys Acta* **1043**, 116-118 (1990).

74 LoPachin, R. M. & Gavin, T. Molecular mechanisms of aldehyde toxicity: a chemical perspective. *Chemical Research in Toxicology* **27**, 1081-1091 (2014).

75 Barrera, G., Pizzimenti, S., Ciamporcero, E. S., Daga, M., Ullio, C., Arcaro, A., Cetrangolo, G. P., Ferretti, C., Dianzani, C., Lepore, A. & Gentile, F. Role of 4-hydroxynonenal-protein adducts in human diseases. *Antioxidants & Redox Signaling* **22**, 1681-1702 (2014).

76 Long, E. K., Murphy, T. C., Leiphon, L. J., Watt, J., Morrow, J. D., Milne, G. L., Howard, J. R. H. & Picklo, M. J. Trans-4-hydroxy-2-hexenal is a neurotoxic product of docosahexaenoic (22:6; n-3) acid oxidation. *Journal of Neurochemistry* **105**, 714-724 (2008).

77 Choudhary, S., Xiao, T., Srivastava, S., Zhang, W., Chan, L. L., Vergara, L. A., Van Kuijk, F. J. G. M. & Ansari, N. H. Toxicity and detoxification of lipid-derived aldehydes in cultured retinal pigmented epithelial cells. *Toxicology and Applied Pharmacology* **204**, 122-134 (2005).

78 Sayre, L. M., Lin, D., Yuan, Q., Zhu, X. & Tang, X. Protein adducts generated from products of lipid oxidation: focus on HNE and ONE. *Drug Metabolism Reviews* **38**, 651-675 (2006).

79 Saladin, K. S. *Anatomy and physiology: the unity of form and function* 6th edn, (McGraw-Hill, 2012).

80 N'Goma, J.-C. B., Amara, S., Dridi, K., Jannin, V. & Carrière, F. Understanding the lipid-digestion processes in the GI tract before designing lipid-based drug-delivery systems. *Therapeutic Delivery* **3**, 105-124 (2011).

81 Stewart, J. E., Feinle-Bisset, C., Golding, M., Delahunty, C., Clifton, P. M. & Keast, R. S. J. Oral sensitivity to fatty acids, food consumption and BMI in human subjects. *British Journal of Nutrition* **104**, 145-152 (2010).

82 Kulkarni, B. & Mattes, R. Evidence for presence of nonesterified fatty acids as potential gustatory signaling molecules in humans. *Chemical Senses* **38**, 119-127 (2013).

83 Tortora & Derrikson. *Introduction to the human body the essentials of anatomy & physiology*. 7th edn, (John Wiley and Sons, 2007).

84 Marieb, E. N. & Hoehn, K. *Human & anatomy & physiology*. 5th edn, (Pearson Benjamin Cummings, 2010).

85 Vander, A., Sherman, J. & Luciano, D. in *Human Physiology: The Mechanisms of Body Function* Ch. 17, (McGraw-Hill Higher Education, 2001).

86 Sams, L., Paume, J., Giallo, J. & Carriere, F. Relevant pH and lipase for *in vitro* models of gastric digestion. *Food & Function* (2016).

87 Bauer, E., Jakob, S. & Mosenthin, R. Principles of physiology of lipid digestion. *Asian-Australasian Journal of Animal Sciences* **18**, 282-295 (2005).

88 McConnell, R. E., Benesh, A. E., Mao, S., Tabb, D. L. & Tyska, M. J. Proteomic analysis of the enterocyte brush border. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **300**, G914-G926 (2011).

89 Lowe, M. E. Structure and function of pancreatic lipase and colipase. *Annual Review of Nutrition* **17**, 141-158 (1997).

90 Armand, M. Lipases and lipolysis in the human digestive tract: where do we stand? *Current Opinion in Clinical Nutrition & Metabolic Care* **10**, 156-164 (2007).

91 Porter, C. J. H., Trevaskis, N. L. & Charman, W. N. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. *Nature Reviews. Drug Discovery* **6**, 231-248 (2007).

92 Thomas, N., Holm, R., Rades, T. & Müllertz, A. Characterising lipid lipolysis and its implication in lipid-based formulation development. *The AAPS Journal* **14**, 860-871 (2012).

93 Terao, J., Ingemansson, T., Ioku, K., Yuki, H. & Ito, Y. Effects of rat bile-pancreatic juice on Fe2+ induced peroxidation of phospholipids. *Bioscience, Biotechnology, and Biochemistry* **59**, 55-58 (1995).

94 Kanazawa, K. & Ashida, H. Dietary hydroperoxides of linoleic acid decompose to aldehydes in stomach before being absorbed into the body. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **1393**, 349-361 (1998).

95 Kanner, J. & Lapidot, T. The stomach as a bioreactor: dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants. *Free Radical Biology and Medicine* **31**, 1388-1395 (2001).

96 Steppeler, C., Haugen, J.-E., Rødbotten, R. & Kirkhus, B. Formation of malondialdehyde, 4-hydroxynonenal, and 4-hydroxyhexenal during *in vitro* digestion of cooked beef, pork, chicken, and salmon. *J Agric Food Chem* **64**, 487-496 (2016).

97 Van Hecke, T., Vanden Bussche, J., Vanhaecke, L., Vossen, E., Van Camp, J. & De Smet, S. Nitrite curing of chicken, pork, and beef inhibits oxidation but does not affect N-nitroso compound (NOC)-specific DNA adduct formation during *in vitro* digestion. *Journal of Agricultural and Food Chemistry* **62**, 1980-1988 (2014).

98 Van Hecke, T., Wouters, A., Rombouts, C., Izzati, T., Berardo, A., Vossen, E., Claeys, E., Van Camp, J., Raes, K., Vanhaecke, L., Peeters, M., De Vos, W. H. & De Smet, S. Reducing compounds equivocally influence oxidation during digestion of a high-fat beef product, which promotes cytotoxicity in colorectal carcinoma cell lines. *Journal of Agricultural and Food Chemistry* **64**, 1600-1609 (2016).

99 Van Hecke, T., Vossen, E., Vanden Bussche, J., Raes, K., Vanhaecke, L. & De Smet, S. Fat content and nitrite-curing influence the formation of oxidation products and NOC-specific DNA adducts during *in vitro* digestion of meat. *PLoS ONE* **9**, e101122 (2014).

100 Larsson, K., Istenic, K., Wulff, T., Jonsdottir, R., Kristinsson, H., Freysdottir, J., Undeland, I. & Jamnik, P. Effect of *in vitro* digested cod liver oil of different quality on oxidative, proteomic and inflammatory responses in the yeast Saccharomyces cerevisiae and human monocyte-derived dendritic cells. *J Sci Food Agric* **95**, 3096-3106 (2015).

101 Feillet-Coudray, C., Aoun, M., Fouret, G., Bonafos, B., Ramos, J., Casas, F., Cristol, J. P. & Coudray, C. Effects of long-term administration of saturated and n-3 fatty acid-rich diets on lipid utilisation and oxidative stress in rat liver and muscle tissues. *British Journal of Nutrition* **110**, 1789-1802 (2013).

102 Gobert, M., Remond, D., Loonis, M., Buffiere, C., Sante-Lhoutellier, V. & Dufour, C. Fruits, vegetables and their polyphenols protect dietary lipids from oxidation during gastric digestion. *Food & Function* **5**, 2166-2174 (2014).

103 García-Hernández, V. M., Gallar, M., Sánchez-Soriano, J., Micol, V., Roche, E. & García-García, E. Effect of omega-3 dietary supplements with different oxidation levels in the lipidic profile of women: a randomized controlled trial. *International Journal of Food Sciences and Nutrition* **64**, 993-1000 (2013).

104 Ottestad, I., Hassani, S., Borge, G. I., Kohler, A., Vogt, G., Hyötyläinen, T., Orešič, M., Brønner, K. W., Holven, K. B., Ulven, S. M. & Myhrstad, M. C. W. Fish oil supplementation alters the plasma lipidomic profile and increases long-chain PUFAs of phospholipids and triglycerides in healthy subjects. *PLoS ONE* **7**, e42550 (2012).

105 Ottestad, I., Retterstøl, K., Myhrstad, M. C. W., Andersen, L. F., Vogt, G., Nilsson, A., Borge, G. I. A., Nordvi, B., Brønner, K. W., Ulven, S. M. & Holven, K. B. Intake of oxidised fish oil does not affect circulating levels of oxidised LDL or inflammatory markers in healthy subjects. *Nutrition, Metabolism and Cardiovascular Diseases* **23**, e3-e4 (2013).

106 Sutherland, W. H. F., de Jong, S. A., Walker, R. J., Williams, M. J. A., Murray Skeaff, C., Duncan, A. & Harper, M. Effect of meals rich in heated olive and safflower oils on oxidation of postprandial serum in healthy men. *Atherosclerosis* **160**, 195-203 (2002).

107 Staprãns, I., Rapp, J. H., Pan, X. M., Kim, K. Y. & Feingold, K. R. Oxidized lipids in the diet are a source of oxidized lipid in chylomicrons of human serum. *Arteriosclerosis, Thrombosis, and Vascular Biology* **14**, 1900-1905 (1994).

108 Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D. J., Menard, O., Recio, I., Santos, C. N., Singh, R. P., Vegarud, G. E., Wickham, M. S., Weitschies, W. & Brodkorb, A. A standardised static *in vitro* digestion method suitable for food - an international consensus. *Food Funct* **5**, 1113-1124 (2014).

109 Albert, B. B., Derraik, J. G. B., Cameron-Smith, D., Hofman, P. L., Tumanov, S., Villas-Boas, S. G., Garg, M. L. & Cutfield, W. S. Fish oil supplements in New Zealand are highly oxidised and do not meet label content of n-3 PUFA. *Sci. Rep.* **5** (2015).

110 IFFO. The production of fishmeal and fish oil from Peruvian anchovy. 1-10 (IFFO, United Kingdom, 2009).

111 Maresz, K., Meus, K. & Porwolik, B. Krill oil: background and benefits. *Int Sci Health Found*, 1-11 (2010).

112 Thomsen, B. R., Haugsgjerd, B. O., Griinari, M., Lu, H. F. S., Bruheim, I., Vogt, G., Oterhals, Å. & Jacobsen, C. Investigation of oxidative degradation and non-enzymatic browning reactions in krill and fish oils. *European Journal of Lipid Science and Technology* **115**, 1357-1366 (2013).

113 Ratledge, C. & Hopkins, S. in *Modifying Lipids for Use in Food* (ed Frank D Gunstone) Ch. 5, 80-113 (Woodhead Publishing, 2006).

114 Martins, D. A., Custódio, L., Barreira, L., Pereira, H., Ben-Hamadou, R., Varela, J. & Abu-Salah, K. M. Alternative sources of n-3 long-chain polyunsaturated fatty acids in marine microalgae. *Marine Drugs* **11**, 2259-2281 (2013).

115 Lv, J., Yang, X., Ma, H., Hu, X., Wei, Y., Zhou, W. & Li, L. The oxidative stability of microalgae oil (*Schizochytrium aggregatum*) and its antioxidant activity after simulated gastrointestinal digestion: relationship with constituents. *European Journal of Lipid Science and Technology* **117**, 1928-1939 (2015).

116 Minekus, M., Marteau, P. & Havenaar, R. A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. *Alternatives to Laboratory Animals* **23**, 197-209 (1995).

117 Kostewicz, E. S., Abrahamsson, B., Brewster, M., Brouwers, J., Butler, J., Carlert, S., Dickinson, P. A., Dressman, J., Holm, R., Klein, S., Mann, J., McAllister, M., Minekus, M., Muenster, U., Müllertz, A., Verwei, M., Vertzoni, M., Weitschies, W. & Augustijns, P. *In vitro* models for the prediction of *in vivo* performance of oral dosage forms. *European Journal of Pharmaceutical Sciences* **57**, 342-366 (2014).

118 Capolino, P., Guérin, C., Paume, J., Giallo, J., Ballester, J.-M., Cavalier, J.-F. & Carrière, F. *In vitro* gastrointestinal lipolysis: replacement of human digestive lipases by a combination of rabbit gastric and porcine pancreatic extracts. *Food digestion* **2**, 43-51 (2011).

119 Pedersen, P. B., Vilmann, P., Bar-Shalom, D., Müllertz, A. & Baldursdottir, S. Characterization of fasted human gastric fluid for relevant rheological parameters and gastric lipase activities. *European Journal of Pharmaceutics and Biopharmaceutics* (2013).

120 Carrière, F., Barrowman, J. A., Verger, R. & Laugier, R. *Carriere F, Barrowman JA, Verger R & Laugier R: Secretion and contribution to lipolysis of gastric and pancreatic lipases during a test meal in humans. Gastroenterology 105, 876-888*. Vol. 105 (1993).

121 Gargouri, Y., Pieroni, G., Lowe, P. A., Sarda, L. & Verger, R. Human gastric lipase. *European Journal of Biochemistry* **156**, 305-310 (1986).

122 Verger, R. in *Lipases* (ed HL Brockmann B Borgstrom) 83–150 (Elsevier, 1984).

123 Fernandez, S., Jannin, V., Rodier, J.-D., Ritter, N., Mahler, B. & Carrière, F. Comparative study on digestive lipase activities on the self emulsifying excipient Labrasol®, medium chain glycerides and PEG esters. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1771**, 633-640 (2007).

124 Fernandez, S., Rodier, J.-D., Ritter, N., Mahler, B., Demarne, F., Carrière, F. & Jannin, V. Lipolysis of the semi-solid self-emulsifying excipient Gelucire® 44/14 by digestive lipases. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1781**, 367-375 (2008).

125 Sasaki, K., Alamed, J., Weiss, J., Villeneuve, P., López Giraldo, L. J., Lecomte, J., Figueroa-Espinoza, M.-C. & Decker, E. A. Relationship between the physical properties of chlorogenic acid esters and their ability to inhibit lipid oxidation in oil-in-water emulsions. *Food Chemistry* **118**, 830-835 (2010).

126 Mei, L., McClements, D. J., Wu, J. & Decker, E. A. Iron-catalyzed lipid oxidation in emulsion as affected by surfactant, pH and NaCl. *Food Chemistry* **61**, 307-312 (1998).

127 Carrière, F., Renou, C., Ransac, S., Lopez, V., De Caro, J., Ferrato, F., De Caro, A., Fleury, A., Sanwald-Ducray, P., Lengsfeld, H., Beglinger, C., Hadvary, P., Verger, R. & Laugier, R. Inhibition of gastrointestinal lipolysis by Orlistat during digestion of test meals in healthy volunteers. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **281**, G16-G28 (2001).

128 Richards, M. P. & Hultin, H. O. Contributions of blood and blood components to lipid oxidation in fish muscle. *Journal of Agricultural and Food Chemistry* **50**, 555-564 (2002).

129 Jacobsen, C., Let, M. B., Nielsen, N. S. & Meyer, A. S. Antioxidant strategies for preventing oxidative flavour deterioration of foods enriched with n-3 polyunsaturated lipids: a comparative evaluation. *Trends in Food Science & Technology* **19**, 76-93 (2008).

130 EFSA, P. o. B. H. B. Scientific opinion on fish oil for human consumption. 1874 (EFSA, 2010).

131 Ulleberg, E. K., Comi, I., Holm, H., Herud, E. B., Jacobsen, M. & Vegarud, G. E. Human gastrointestinal juices intended for use in *in vitro* digestion models. *Food digestion* **2**, 52-61 (2011).

132 Gorelik, S., Kohen, R., Ligumsky, M. & Kanner, J. Saliva plays a dual role in oxidation process in stomach medium. *Archives of Biochemistry and Biophysics* **458**, 236-243 (2007).

133 Verwei, M., Minekus, M., Zeijdner, E., Schilderink, R. & Havenaar, R. Evaluation of two dynamic *in vitro* models simulating fasted and fed state conditions in the upper gastrointestinal tract (TIM-1 and tiny-TIM) for investigating the bioaccessibility of pharmaceutical compounds from oral dosage forms. *International Journal of Pharmaceutics* **498**, 178-186 (2016).

134 Minekus, M., Marteau, P., Havenaar, R. & Huis in 't Veld, J. *A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. ATLA Altern Lab Anim*. Vol. 23 (1995).

135 Arhakis, A., Karagiannis, V. & Kalfas, S. Salivary Alpha-Amylase Activity and Salivary Flow Rate in Young Adults. *The Open Dentistry Journal* **7**, 7-15 (2013).

136 Ville, E., Carrière, F., Renou, C. & Laugier, R. Physiological study of pH stability and sensitivity to pepsin of human gastric lipase. *Digestion* **65**, 73-81 (2002).

137 Carrière, F., Renou, C., Lopez, V., de Caro, J., Ferrato, F., Lengsfeld, H., de Caro, A., Laugier, R. & Verger, R. The specific activities of human digestive lipases measured from the *in vivo* and *in vitro* lipolysis of test meals. *Gastroenterology* **119**, 949-960 (2000).

138 Keller, J. & Layer, P. Human pancreatic exocrine response to nutrients in health and disease. *Gut* **54**, 1-28 (2005).

139 Moreno, M. P. d. l. C., Oth, M., Deferme, S., Lammert, F., Tack, J., Dressman, J. & Augustijns, P. Characterization of fasted-state human intestinal fluids collected from duodenum and jejunum. *Journal of Pharmacy and Pharmacology* **58**, 1079-1089 (2006).

140 Larsen, A. T., Sassene, P. & Müllertz, A. *In vitro* lipolysis models as a tool for the characterization of oral lipid and surfactant based drug delivery systems. *International Journal of Pharmaceutics* **417**, 245-255 (2011).

141 Hur, S. J., Lim, B. O., Decker, E. A. & McClements, D. J. In *vitro* human digestion models for food applications. *Food Chemistry* **125**, 1-12 (2011).

142 Fuchs, A. & Dressman, J. B. Composition and physicochemical properties of fasted-state human duodenal and jejunal fluid: a critical evaluation of the available data. *Journal of Pharmaceutical Sciences* **103**, 3398-3411 (2014).

143 Kalantzi, L., Goumas, K., Kalioras, V., Abrahamsson, B., Dressman, J. B. & Reppas, C. Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. *Pharmaceutical research* **23**, 165-176 (2006).

144 World Medical, A. World medical association declaration of Helsinki: ethical principles for medical research involving human subjects. *JAMA* **310**, 2191-2194 (2013).

145 Holm, H., Krogdahl, A. & Hanssen, L. E. High and low inhibitor soybean meals affect human duodenal proteinase activity differently: in vitro comparison of proteinase inhibition. *The Journal of nutrition* **118**, 521-525 (1988).

146 Erlanson, C. & Borgström, B. Tributyrine as a substrate for determination of lipase activity of pancreatic juice and small intestinal content. *Scand J Gastroenterol* **5**, 293-295 (1970).

147 Bernfeld, P. [17] Amylases, α and β. *Methods in Enzymology* **1**, 149-158 (1955).

148 Anson, M. L. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *The Journal of General Physiology* **22**, 79-89 (1938).

149 Walsh, K. A. & Wilcox, P. E. *Methods in enzymology*. Vol. XIX (Academic Press, 1970).

150 Lykkesfeldt, J. Ascorbate and dehydroascorbic acid as reliable biomarkers of oxidative stress: analytical reproducibility and long-term stability of plasma samples subjected to acidic deproteinization. *Cancer Epidemiology Biomarkers & Prevention* **16**, 2513-2516 (2007).

151 Scheers, N. M. & Sandberg, A.-S. Iron regulates the uptake of ascorbic acid and the expression of sodium-dependent vitamin C transporter 1 (SVCT1) in human intestinal Caco-2 cells. *British Journal of Nutrition* **105**, 1734-1740 (2011).

152 Fredrikson, M., Carlsson, N. G., Almgren, A. & Sandberg, A. S. Simultaneous and sensitive analysis of Cu, Ni, Zn, Co, Mn, and Fe in food and biological samples by ion chromatography. *J Agric Food Chem* **50**, 59-65 (2002).

153 Hornsey, H. C. The colour of cooked cured pork. I.—Estimation of the Nitric oxide-Haem Pigments. *Journal of the Science of Food and Agriculture* **7**, 534-540 (1956).

154 Lee, C., Trevino, B. & Chaiyawat, M. A simple and rapid solvent extraction method for determining total lipids in fish tissue. *J AOAC Int* **79**, 487-492 (1996).

155 Bligh, E. G. & Dyer, W. J. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911-917 (1959).

156 Folch, J., Lees, M. & Stanley, G. H. S. A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* **226**, 497-509 (1957).

157 Kaluzny, M. A., Duncan, L. A., Merritt, M. V. & Epps, D. E. Rapid separation of lipid classes in high yield and purity using bonded phase columns. *Journal of Lipid Research* **26**, 135-140 (1985).

158 Cavonius, L. & Carlsson, N. Fatty acid contaminations originating from commercially available solid phase-extraction columns. *Chemical Science Review and Letters* **4**, 1206-1209 (2015).

159 Cavonius, L. R., Carlsson, N.-G. & Undeland, I. Quantification of total fatty acids in microalgae: comparison of extraction and transesterification methods. *Analytical and Bioanalytical Chemistry* **406**, 7313-7322 (2014).

160 Undeland, I., Hultin, H. O. & Richards, M. P. Added triacylglycerols do not hasten hemoglobin-mediated lipid oxidation in washed minced cod muscle. *Journal of Agricultural and Food Chemistry* **50**, 6847-6853 (2002).

161 Shantha, N. & Decker, E. Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. (1994).

162 Guichardant, M., Bacot, S., Moliere, P. & Lagarde, M. Hydroxy-alkenals from the peroxidation of n-3 and n-6 fatty acids and urinary metabolites. *Prostaglandins Leukot Essent Fatty Acids* **75**, 179-182 (2006).

163 Pryor, W. A. & Porter, N. A. Suggested mechanisms for the production of 4-hydroxy-2-nonenal from the autoxidation of polyunsaturated fatty acids. *Free Radical Biology and Medicine* **8**, 541-543 (1990).

164 Tirosh, O., Shpaizer, A. & Kanner, J. Lipid peroxidation in a stomach medium is affected by dietary oils (olive/fish) and antioxidants: the Mediterranean versus Western diet. *Journal of Agricultural and Food Chemistry* (2015).

165 Janero, D. R. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biology and Medicine* **9**, 515-540 (1990).

166 Sakai, T., Matsushita, Y.-i., Sugamoto, K. & Uchida, K. Lipid peroxidation-derived hepatotoxic aldehyde, 4-hydroxy-2-hexenal, in fish. *Bioscience, Biotechnology, and Biochemistry* **61**, 1399-1400 (1997).

167 Barriuso, B., Astiasarán, I. & Ansorena, D. A review of analytical methods measuring lipid oxidation status in foods: a challenging task. *European Food Research and Technology* **236**, 1-15 (2013).

168 Harrysson, H. *Development of an analytical method for measuring the lipid oxidation product 4-hydroxy-trans-2-hexenal from marine fatty acids in digests from a dynamic gastrointestinal mode* Master of Science thesis, Chalmers University of Technology, (2013).

169 Berdyshev, E. V. Mass spectrometry of fatty aldehydes. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1811**, 680-693 (2011).

170 Manini, P., Andreoli, R., Sforza, S., Dall'Asta, C., Galaverna, G., Mutti, A. & Niessen, W. M. A. Evaluation of alternate isotope-coded derivatization assay (AIDA) in the LC–MS/MS analysis of aldehydes in exhaled breath condensate. *Journal of Chromatography B* **878**, 2616-2622 (2010).

171 Baron, C. P., KjÆrsgård, I. V. H., Jessen, F. & Jacobsen, C. Protein and Lipid Oxidation during Frozen Storage of Rainbow Trout (Oncorhynchus mykiss). *Journal of Agricultural and Food Chemistry* **55**, 8118-8125 (2007).

172 Spies-Martin, D., Sommerburg, O., Langhans, C.-D. & Leichsenring, M. Measurement of 4-hydroxynonenal in small volume blood plasma samples: modification of a gas chromatographic–mass spectrometric method for clinical settings. *Journal of Chromatography B* **774**, 231-239 (2002).

173 Pilz, J., Meineke, I. & Gleiter, C. H. Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. *Journal of Chromatography B: Biomedical Sciences and Applications* **742**, 315-325 (2000).

174 Kenmogne-Domguia, H. B., Meynier, A., Viau, M., Llamas, G. & Genot, C. Gastric conditions control both the evolution of the organization of protein-stabilized emulsions and the kinetic of lipolysis during *in vitro* digestion. *Food & Function* **3**, 1302-1309 (2012).

175 Marcin, ák, S., Sokol, J., Bystrický, P., Popelka, P., Turek, P., Bhide, M. & Máté, D. Determination of lipid oxidation level in broiler meat by liquid chromatography. *Journal of AOAC International* **87**, 1148-1152 (2004).

176 Sørensen, G. & Jørgensen, S. S. A critical examination of some experimental variables in the 2-thiobarbituric acid (TBA) test for lipid oxidation in meat products. *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **202**, 205-210 (1996).

177 Grosjean, E., Grosjean, D., Green, P. G. & Hughes, J. M. LC/DAD/MS analysis of carbonyl (2,4-dinitrophenyl)hydrazones. *Agilent Technologies* **No. 5986-8850E** (2000).

178 Deighton, N., Magill, W. J., Bremner, D. H. & Benson, E. E. Malondialdehyde and 4-Hydroxy-2-Nonenal in Plant Tissue Cultures: LC-MS Determination of2, 4-Dinitrophenylhydrazone Derivatives. *Free Radical Research* **27**, 255-265 (1997).

179 Andreoli, R., Manini, P., Corradi, M., Mutti, A. & Niessen, W. M. A. Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **17**, 637-645 (2003).

180 Harris, D. C. *Quantitative chemical analysis*. Vol. 8. (W.H Freeman and Company, 2010).

181 Tiwari, G. & Tiwari, R. Bioanalytical method validation: an updated review. *Pharmaceutical Methods* **1**, 25-38 (2010).

182 Lea, T. in *The Impact of Food Bioactives on Health: in vitro and ex vivo models* (eds Kitty Verhoeckx *et al.*) 103-111 (Springer International Publishing, 2015).

183 Fernández-Blanco, C., Font, G. & Ruiz, M.-J. Oxidative stress of alternariol in Caco-2 cells. *Toxicology Letters* **229**, 458-464 (2014).

184 Wijeratne, S. S. K., Cuppett, S. L. & Schlegel, V. Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells. *Journal of Agricultural and Food Chemistry* **53**, 8768-8774 (2005).

185 Mangas-Sanjuan, V., Gonzalez-Alvarez, M., Gonzalez-Alvarez, I. & Bermejo Sanz, M. *Caco-2 cells for studying intestinal absorption mechanisms: comparison and correlations with rat model*. (2011).

186 Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P. in *Essential Cell Biology* (ed M. Morales) Ch. 1, (Garland Science, 2013).

187 McClements, D. J. & Li, Y. Review of *in vitro* digestion models for rapid screening of emulsion-based systems. *Food & Function* **1**, 32-59 (2010).

188 Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A. & Zucco, F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology* **21**, 1-26 (2005).

189 Gibco & Invitrogen. *Cell culture basics handbook*, www.invitrogen.com/cellculturebasics (2017).

190 Alghazeer, R., Gao, H. & Howell, N. K. Cytotoxicity of oxidised lipids in cultured colonal human intestinal cancer cells (caco-2 cells). *Toxicol Lett* **180**, 202-211 (2008).

191 Patterson, H. B. W. in *Bleaching and Purifying Fats and Oils (Second Edition)* 53-67 (AOCS Press, 2009).

192 Richards, M. P. & Hultin, H. O. Effect of pH on lipid oxidation using trout hemolysate as a catalyst: a possible role for deoxyhemoglobin. *Journal of Agricultural and Food Chemistry* **48**, 3141-3147 (2000).

193 Aarak, K. E., Kirkhus, B., Holm, H., Vogt, G., Jacobsen, M. & Vegarud, G. E. Release of EPA and DHA from salmon oil - a comparison of *in vitro* digestion with human and porcine gastrointestinal enzymes. *The British Journal of Nutrition* **110**, 1402-1410 (2013).

194 Larsson, K., Harrysson, H., Havenaar, R., Alminger, M. & Undeland, I. Formation of malondialdehyde (MDA), 4-hydroxy-2-hexenal (HHE) and 4-hydroxy-2-nonenal (HNE) in fish and fish oil during dynamic gastrointestinal in vitro digestion. *Food Funct* **7**, 1176-1187 (2016).

195 Tullberg, C., Larsson, K., Carlsson, N. G., Comi, I., Scheers, N., Vegarud, G. & Undeland, I. Formation of reactive aldehydes (MDA, HHE, HNE) during the digestion of cod liver oil: comparison of human and porcine *in vitro* digestion models. *Food Funct* **7**, 1401-1412 (2016).

196 Lu, F. S. H., Bruheim, I., Haugsgjerd, B. O. & Jacobsen, C. Effect of temperature towards lipid oxidation and non-enzymatic browning reactions in krill oil upon storage. *Food Chemistry* **157**, 398-407 (2014).

197 Lu, F. S. H., Nielsen, N. S., Baron, C. P. & Jacobsen, C. Oxidative degradation and non-enzymatic browning due to the interaction between oxidised lipids and primary amine groups in different marine PL emulsions. *Food Chemistry* **135**, 2887-2896 (2012).

198 Lu, F. S. H., Nielsen, N. S., Baron, C. P. & Jacobsen, C. Marine phospholipids: the current understanding of their oxidation mechanisms and potential uses for food fortification. *Critical Reviews in Food Science and Nutrition* **57**, 2057-2070 (2017).

199 Labuza, T. D. Kinetics of lipid oxidation in foods. *CRC Critical reviews in Food Technology* **2**, 355-405 (1971).

200 Miyashita, K. & Takagi, T. Study on the oxidative rate and prooxidant activity of free fatty acids. *Journal of the American Oil Chemists' Society* **63**, 1380-1384 (1986).

201 Shewfelt, R. L. Fish muscle lipolysis - a review. *Journal of Food Biochemistry* **5**, 79-100 (1981).

202 Waraho, T., Cardenia, V., Rodriguez-Estrada, M. T., McClements, D. J. & Decker, E. A. Prooxidant mechanisms of free fatty acids in stripped soybean oil-in-water emulsions. *Journal of Agricultural and Food Chemistry* **57**, 7112-7117 (2009).

203 Zhu, X., Ye, A., Verrier, T. & Singh, H. Free fatty acid profiles of emulsified lipids during *in vitro* digestion with pancreatic lipase. *Food Chemistry* **139**, 398-404 (2013).

204 Aarak, K. E., Kirkhus, B., Johansen, S., Vegarud, G. E. & Borge, G. I. A. Effect of broccoli phytochemical extract on release of fatty acids from salmon muscle and salmon oil during *in vitro* digestion. *Food & Function* **5**, 2331-2337 (2014).

205 Carrière, F., Moreau, H., Raphel, V., Laugier, R., Benicourt, C., Junien, J.-L. & Verger, R. Purification and biochemical characterization of dog gastric lipase. *European Journal of Biochemistry* **202**, 75-83 (1991).

206 Richards, M. P. in *Oxidation in Foods and Beverages and Antioxidant Applications* 76-104 (Woodhead Publishing, 2010).

207 Hayashi, T., Uchida, K., Takebe, G. & Takahashi, K. Rapid formation of 4-hydroxy-2-nonenal, malondialdehyde, and phosphatidylcholine aldehyde from phospholipid hydroperoxide by hemoproteins. *Free Radical Biology and Medicine* **36**, 1025-1033 (2004).

208 Kumar Sau, A., Currell, D., Mazumdar, S. & Mitra, S. Interaction of sodium dodecyl sulfate with human native and cross-linked hemoglobins: a transient kinetic study. *Biophysical Chemistry* **98**, 267-273 (2002).

209 Decker, E. A., Huang, C.-H., Osinchak, J. E. & Hultin, H. O. Iron and copper: role in enzymic lipid oxidation of fish sarcoplasmic reticulum at *in situ* concentrations. *Journal of Food Biochemistry* **13**, 179-186 (1989).

210 Maestre, R., Pazos, M. & Medina, I. Involvement of methemoglobin (MetHb) formation and hemin loss in the pro-oxidant activity of fish hemoglobins. *Journal of Agricultural and Food Chemistry* **57**, 7013-7021 (2009).

211 Baron, C. P., Skibsted, L. H. & Andersen, H. J. Concentration effects in myoglobin-catalyzed peroxidation of linoleate. *Journal of Agricultural and Food Chemistry* **50**, 883-888 (2002).

212 Decker, E., Elieas, R. & McClements, J. *Oxidation in foods and beverages and antioxidant applications*. 1 edn, (Woodhead Pub Ltd., 2010).

213 Papuc, C., Goran, G. V., Predescu, C. N. & Nicorescu, V. Mechanisms of oxidative processes in meat and toxicity induced by postprandial degradation products: a review. *Comprehensive Reviews in Food Science and Food Safety* **16**, 96-123 (2017).

214 Sobala, G. M., Schorah, C. J., Sanderson, M., Dixon, M. F., Tompkins, D. S., Godwin, P. & Axon, A. T. R. Ascorbic acid in the human stomach. *Gastroenterology* **97**, 357-363 (1989).

215 Waring, A. J., Drake, I. M., Schorah, C. J., White, K. L. M., Lynch, D. A. F., Axon, A. T. R. & Dixon, M. F. Ascorbic acid and total vitamin C concentrations in plasma, gastric juice, and gastrointestinal mucosa: Effects of gastritis and oral supplementation. *Gut* **38**, 171-176 (1996).

216 Kojo, S. Vitamin C: Basic metabolism and its function as an index of oxidative stress. *Current Medicinal Chemistry* **11**, 1041-1064 (2004).

217 Gorelik, S., Lapidot, T., Shaham, I., Granit, R., Ligumsky, M., Kohen, R. & Kanner, J. Lipid peroxidation and coupled vitamin oxidation in simulated and human gastric fluid inhibited by dietary polyphenols: health implications. *Journal of Agricultural and Food Chemistry* **53**, 3397-3402 (2005).

218 Nieva-Echevarría, B., Goicoechea, E. & Guillén, M. D. Polyunsaturated lipids and vitamin A oxidation during cod liver oil in *vitro* gastrointestinal digestion. Antioxidant effect of added BHT. *Food Chemistry* **232**, 733-743 (2017).

219 Let, M. B., Jacobsen, C. & Meyer, A. S. Ascorbyl palmitate, γ-tocopherol, and EDTA affect lipid oxidation in fish oil enriched salad dressing differently. *Journal of Agricultural and Food Chemistry* **55**, 2369-2375 (2007).

220 Frankel, E. N., Satué-Gracia, T., Meyer, A. S. & German, J. B. Oxidative stability of fish and algae oils containing long-chain polyunsaturated fatty acids in bulk and in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry* **50**, 2094-2099 (2002).

221 Let, M. B., Jacobsen, C., Pham, K. A. & Meyer, A. S. Protection against oxidation of fish-oil-enriched milk emulsions through addition of rapeseed oil or antioxidants. *Journal of Agricultural and Food Chemistry* **53**, 5429-5437 (2005).

222 Alamed, J., McClements, D. J. & Decker, E. A. Influence of heat processing and calcium ions on the ability of EDTA to inhibit lipid oxidation in oil-in-water emulsions containing omega-3 fatty acids. *Food Chemistry* **95**, 585-590 (2006).

223 Yoshida, Y., Niki, E. & Noguchi, N. Comparative study on the action of tocopherols and tocotrienols as antioxidant: chemical and physical effects. *Chemistry and Physics of Lipids* **123**, 63-75 (2003).

224 Kamal-Eldin, A. & Appelqvist, L.-Å. The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* **31**, 671-701 (1996).

225 Telegdy & Berndorfer. On the antioxidative mechanism of tocopherols (α-, β-, γ-, δ-) in Lard. *Nahrung* **12**, 407–414 (1968).

226 Seppanen, C. M., Song, Q. & Saari Csallany, A. The antioxidant functions of tocopherol and tocotrienol homologues in oils, fats, and food systems. *Journal of the American Oil Chemists' Society* **87**, 469-481 (2010).

227 Awada, M., Meynier, A., Soulage, C. O., Hadji, L., Geloen, A., Viau, M., Ribourg, L., Benoit, B., Debard, C., Guichardant, M., Lagarde, M., Genot, C. & Michalski, M. C. n-3 PUFA added to high-fat diets affect differently adiposity and inflammation when carried by phospholipids or triacylglycerols in mice. *Nutr Metab (Lond)* **10**, 23 (2013).

228 Soni, N. K., Ross, A. B., Scheers, N., Savolainen, O. I., Nookaew, I., Gabrielsson, B. G. & Sandberg, A. S. Splenic immune response is down-regulated in C57BL/6J mice fed eicosapentaenoic acid and docosahexaenoic acid enriched high fat diet. *Nutrients* **9**, 50 (2017).

229 Armand, M., Pasquier, B., André, M., Borel, P., Senft, M., Peyrot, J., Salducci, J., Portugal, H., Jaussan, V. & Lairon, D. Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract. *The American Journal of Clinical Nutrition* **70**, 1096-1106 (1999).

230 Leibowitz, G., Ktorza, A. & Cerasi, E. The role of TXNIP in the pathophysiology of diabetes and its vascular complications: a concise review. *Medicographia* **36**, 391-397 (2014).

231 Hall, L. & Martinus, R. D. Hyperglycaemia and oxidative stress upregulate HSP60 & HSP70 expression in HeLa cells. *Springerplus* **2**, 431 (2013).

232 Adarme-Vega, T. C., Thomas-Hall, S. R. & Schenk, P. M. Towards sustainable sources for omega-3 fatty acids production. *Curr Opin Biotechnol* **26**, 14-18 (2014).

233 Kassis, N. M., Gigliotti, J. C., Beamer, S. K., Tou, J. C. & Jaczynski, J. Characterization of lipids and antioxidant capacity of novel nutraceutical egg products developed with omega-3-rich oils. *J Sci Food Agric* **92**, 66-73 (2012).

234 Jayathilake, A. G., Senior, P. V. & Su, X. Q. Krill oil extract suppresses cell growth and induces apoptosis of human colorectal cancer cells. *BMC Complement Altern Med* **16**, 328 (2016).

235 Ishikado, A., Morino, K., Nishio, Y., Nakagawa, F., Mukose, A., Sono, Y., Yoshioka, N., Kondo, K., Sekine, O., Yoshizaki, T., Ugi, S., Uzu, T., Kawai, H., Makino, T., Okamura, T., Yamamoto, M., Kashiwagi, A. & Maegawa, H. 4-Hydroxy hexenal derived from docosahexaenoic acid protects endothelial cells via Nrf2 activation. *PLoS One* **8**, e69415 (2013).

236 Mishra, A., Chaudhary, A. & Sethi, S. Oxidized omega-3 fatty acids inhibit NF-kappaB activation via a PPARalpha-dependent pathway. *Arterioscler Thromb Vasc Biol* **24**, 1621-1627 (2004).

237 Roy, J., Galano, J. M., Durand, T., Le Guennec, J. Y. & Lee, J. C. Physiological role of reactive oxygen species as promoters of natural defenses. *FASEB J* (2017).

238 Naruszewicz, M., Woźny, E., Mirkiewicz, E., Nowicka, G. & Szostak, W. B. The effect of thermally oxidized soya bean oil on metabolism of chylomicrons. Increased uptake and degradation of oxidized chylomicrons in cultured mouse macrophages. *Atherosclerosis* **66**, 45-53 (1987).

239 Surh, J. & Kwon, H. Estimation of daily exposure to 4-hydroxy-2-alkenals in Korean foods containing n-3 and n-6 polyunsaturated fatty acids. *Food additives and contaminants* **22**, 701-708 (2005).

240 Di Domenico, F., Tramutola, A. & Butterfield, D. A. Role of 4-hydroxy-2-nonenal (HNE) in the pathogenesis of alzheimer disease and other selected age-related neurodegenerative disorders. *Free Radical Biology and Medicine* **111**, 253-261 (2017).

241 Bacot, S., Bernoud-Hubac, N., Baddas, N., Chantegrel, B., Deshayes, C., Doutheau, A., Lagarde, M. & Guichardant, M. Covalent binding of hydroxy-alkenals 4-HDDE, 4-HHE, and 4-HNE to ethanolamine phospholipid subclasses. *Journal of Lipid Research* **44**, 917-926 (2003).

242 Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D. & Milzani, A. Biomarkers of oxidative damage in human disease. *Clinical Chemistry* **52**, 601-623 (2006).

243 Uchida, K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Progress in Lipid Research* **42**, 318-343 (2003).

244 Vandemoortele, A., Babat, P., Yakubu, M. & De Meulenaer, B. Reactivity of free malondialdehyde during *in vitro* simulated gastrointestinal digestion. *J Agric Food Chem* **65**, 2198-2204 (2017).

245 Lin, D., Lee, H.-g., Liu, Q., Perry, G., Smith, M. A. & Sayre, L. M. 4-oxo-2-nonenal is both more neurotoxic and more protein reactive than 4-hydroxy-2-nonenal. *Chemical Research in Toxicology* **18**, 1219-1231 (2005).

246 Aw, T. Y. & Williams, M. W. Intestinal absorption and lymphatic transport of peroxidized lipids in rats: effect of exogenous GSH. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **263**, G665-G672 (1992).

247 Ursini, F., Zamburlini, A., Cazzolato, G., Maiorino, M., Bon, G. B. & Sevanian, A. Postprandial plasma lipid hydroperoxides: a possible link between diet and atherosclerosis. *Free Radical Biology and Medicine* **25**, 250-252 (1998).

248 Gorelik, S., Ligumsky, M., Kohen, R. & Kanner, J. A novel function of red wine polyphenols in humans: prevention of absorption of cytotoxic lipid peroxidation products. *The FASEB Journal* **22**, 41-46 (2008).

249 Cerbone, A., Toaldo, C., Laurora, S., Briatore, F., Pizzimenti, S., Dianzani, M. U., Ferretti, C. & Barrera, G. 4-Hydroxynonenal and PPARγ ligands affect proliferation, differentiation, and apoptosis in colon cancer cells. *Free Radical Biology and Medicine* **42**, 1661-1670 (2007).

250 Bae, E. H., Cho, S., Joo, S. Y., Ma, S. K., Kim, S. H., Lee, J. & Kim, S. W. 4-Hydroxy-2-hexenal-induced apoptosis in human renal proximal tubular epithelial cells. *Nephrology Dialysis Transplantation* **26**, 3866-3873 (2011).

251 Anderson, D. R., Duryee, M. J., Shurmur, S. W., Um, J. Y., Bussey, W. D., Hunter, C. D., Garvin, R. P., Sayles, H. R., Mikuls, T. R., Klassen, L. W. & Thiele, G. M. Unique antibody responses to malondialdehyde-acetaldehyde (MAA)-protein adducts predict coronary artery disease. *PLoS One* **9**, e107440 (2014).