Aus der Medizinische Klinik mit Schwerpunkt Hepatologie und Gastroenterologie der Medizinischen Fakultät Charité - Universitätsmedizin Berlin

DISSERTATION

Polyunsaturated fatty acids, colorectal cancer, and inflammation: Effects of three major polyunsaturated fatty acids on the lipid metabolism of colorectal adenocarcinoma HT-29 cells and on the cytokine secretion by peripheral blood mononuclear cells

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von
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Vorwort / Preamble

Parts of the results of this thesis have been published in two recent articles by the author (1, 2):

Wachtel N, Rohwer N, Pietzner A, Loew A, Weylandt KH. Omega-3 fatty acid supplementation – A possible dietary adjunct to enhance immune checkpoint inhibition therapy in cancer? Journal of Cellular Biotechnology. 2018;4:83-8.

Wachtel N, Rohwer N, Pietzner A, Loew A, Weylandt KH. Omega-3 fatty acid supplementation – A possible dietary adjunct to enhance immune therapy in cancer? Preprints. 2018.

All experiments, data analysis, and writing of articles were conducted by the first author, Nikolaus Constantin Wachtel. The co-authors assisted in the interpretation of data. Cited passages are indicated accordingly and previously published figures are identified in the corresponding description of the figure. Moreover, for this thesis the author has been the recipient of the Boehringer Ingelheim Fonds Travel Grant.

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Abbreviations

AA	Arachidonic acid
AICR	American Institute for Cancer Research
APC	Adenomatous polyposis coli
CAC	Colitis-associated cancer
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CM	Conditioned media (of HT-29 cells)
COX	Cyclooxygenase
CRC	Colorectal cancer
CYP	Cytochrome P-450
DHA	Docosahexaenoic acid
DH_20	Distilled water
DT	Docosatriene
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FA	Fatty acid
FBS	Fetal bovine serum
GM	Growth medium
HDHA	Hydroxydocosahexaenoic acid
HEPE	Hydroxyeicosapentaenoic acid
HETE	Hydroxyeicosatetraenoic acid
HPLC	High-performance liquid chromatography
ISTD	Internal standard solution
IBD	Inflammatory bowel disease
LC/MS/MS	Liquid chromatography/tandem mass spectrometry
LO	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
LX	Lipoxin
M	Molar mass (g/mol)
MaR	Maresin
MMR	DNA mismatch repair
MRM	Multiple reaction monitoring mode
MSI	Microsatellite instability
n-3	Omega-3
n-6	Omega-6
NF-κB	Nuclear factor kappa B
NSAID	Nonsteroidal anti-inflammatory drug
OH-FA	Hydroxylated fatty acid
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered Saline
PG	Prostaglandin
PD	Protectin
PD-1	Programmed cell death 1
PUFA	Polyunsaturated fatty acid
RBC	Red Blood Cell
RPM	Rounds per minute
RT	Room temperature (21°C)
Rv	Resolvin
RvD	D-series resolvin
RvE	E-series resolvin
SEM	Standard error of the mean
TX	Thromboxane
WCRF	World Cancer Research Fund

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1. Abstract

Omega-3 and omega-6 polyunsaturated fatty acids (n-3 and n-6 PUFAs) have been shown to modulate inflammation. N-3 PUFAs predominantly seem to suppress inflammatory processes, while n-6 PUFAs have been shown to act as pro-inflammatory agents. Additionally, n-3 and n-6 PUFAs may have an effect on the growth and progression of colorectal cancer (CRC), a tumor in close relationship with immune cells. PUFA-derived lipid mediators play an important role in mediating these effects. The exact role of n-3 and n-6 PUFAs in inflammation and in CRC, however, remains controversial.

To further explore the effect of n-3 and n-6 PUFAs on CRC, in particular with regard to the formation of PUFA-derived lipid mediators**,** we investigated whether incubation with two n-3 PUFAs (eicosapentaenoic acid, EPA; and docosahexaenoic acid, DHA) and one n-6 PUFA (arachidonic acid, AA) affects the lipid metabolism of human colorectal adenocarcinoma HT-29 cells. Additionally, we explored the effect of PUFAs on immune cell function in general, as well as with regard to the crosstalk between immune and CRC cells. For this, we assessed whether incubation with DHA, EPA, or AA would modulate the secretion of TNF-α, IL-6, or IL-10 by human peripheral blood mononuclear cells (PBMCs), stimulated with lipopolysaccharide (LPS) or conditioned media (CM) of HT-29 cells.

Incubation with EPA significantly increased the synthesis of EPA-derived lipid metabolites in HT-29 cells. Incubation of PBMCs with DHA enhanced a pro-inflammatory cytokine profile. Thus, DHA significantly decreased LPS- and CM-induced secretion of IL-10 and increased CM-induced secretion of TNF-α. AA, on the other hand, reduced TNF-α secretion (stimulation with LPS and CM). Analysis of variance revealed that, when compared to incubation with n-3 PUFAs (EPA and DHA), secretion of TNF-α was significantly lower in PBMCs incubated with AA.

The observed increase in EPA-derived lipid metabolites may be a mechanism for how EPA exerts its previously described effects on CRC. Our results further demonstrate that the general paradigm of the role of PUFAs in inflammation represents an oversimplification. Similar to other studies, we were able to show that under certain conditions n-3 PUFAs may have pro-inflammatory and n-6 PUFAs anti-inflammatory

effects. Moreover, with regard to recent studies on tumor immune therapy, as well as prostaglandin-E2-mediated immune dysfunction and tumor growth, changes in CM-induced cytokine secretion upon incubation with DHA raise the possibility of a beneficial effect of n-3 PUFAs in patients with CRC, through a more aggressive immunological response against tumor cells.

2. Zusammenfassung

Mehrere Studien konnten einen Einfluss von mehrfach ungesättigten Omega-3- und Omega-6-Fettsäuren (n-3 und n-6 PUFAs) auf das Immunsystem nachweisen. In diesem Zusammenhang scheinen n-3 PUFAs eine überwiegend antiinflammatorische Wirkung zu haben, während n-6 PUFAs die Immunantwort fördern. Ebenfalls wurde ein möglicher Einfluss dieser Fettsäuren auf das kolorektale Karzinom (CRC) beschrieben, ein Malignom, welches in enger Verbindung mit dem Immunsystem steht. Insbesondere potente Lipidmediatoren, entstanden aus enzymatischer Katalyse von n-3 und n-6 PUFAs, spielen eine wichtige Rolle in diesen beschriebenen Effekten. Der genaue Einfluss von n-3 und n-6 PUFAs auf das Immunsystem und das CRC ist jedoch weiterhin unklar.

Ziel der vorliegenden Studie war daher, die Wirkung von n-3 und n-6 PUFAs auf den Lipidstoffwechsel der Kolonkarzinom-Zelllinie HT-29 sowie auf die Immunantwort, insbesondere mit Hinblick auf das CRC, zu untersuchen. In einem ersten Experiment wurde der Einfluss von zwei n-3 PUFAs (Eicosapentaensäure (EPA) und Docosahexaensäure (DHA)) und einer n-6 PUFA (Arachidonsäure (AA)) auf die Synthese von Lipidmediatoren von HT-29 Zellen untersucht. In einem weiteren Versuch wurde der Effekt von EPA, DHA und AA auf die Zytokin-Sekretion (TNF-α, IL-6 und IL-10) von mononukleären Zellen des peripheren Blutes (PBMCs) untersucht. PBMCs wurden hierfür mit Lipopolysacchariden (LPS) oder konditioniertem Medium von HT-29 Zellen (CM) stimuliert.

Die Inkubation von HT-29 Zellen mit EPA erhöhte den Anteil an EPA-basierten Lipidmetaboliten. Die Inkubation von PBMCs mit DHA führte zu einem proinflammatorischen Zytokinprofil: Es zeigte sich eine reduzierte Sekretion von IL-10 nach Stimulation mit LPS und CM sowie eine erhöhte CM-induzierte Sekretion von TNF-α. Im Gegensatz hierzu, reduzierte AA die LPS- und CM-induzierte Sekretion von TNF-α. Die durchgeführte Varianzanalyse bestätigte diesen Trend: Verglichen mit PBMCs, welche mit n-3 PUFAs (DHA und EPA) inkubiert wurden, verringerte AA die LPS- und CM-induzierte Sekretion von TNF-α.

Eine Zunahme von EPA-basierten Lipidmetaboliten ist ein wahrscheinlicher Wirkmechanismus, über den EPA seine bisher beschriebenen Effekte auf das CRC ausübt. Die Ergebnisse der Versuche mit PBMCs zeigen, dass das bisherige Paradigma von n-3 und n-6 PUFAs in Hinblick auf die Immunantwort eine Vereinfachung darstellt. Ähnlich wie andere Studien, zeigen die vorliegenden Experimente, dass n-3 PUFAs unter bestimmten Bedingungen proinflammatorische und n-6 PUFAs antiinflammatorische Effekte haben können. Diese Ergebnisse sind insbesondere im Hinblick auf aktuelle Studien zur Immuntherapie bei Malignomen sowie zu Tumorwachstum aufgrund von Prostaglandin-E2-vermittelter Immundysfunktion von Interesse: Der demonstrierte Einfluss von DHA auf die CM-induzierte Immunantwort macht deutlich, dass n-3 PUFAs mögliche antikanzerogene Effekte bei Patienten mit CRC über eine verstärkte Immunantwort ausüben könnten.

3. Introduction

The study presented here investigates the impact of omega-3 and omega-6 polyunsaturated fatty acids (n-3 and n-6 PUFAs) on the human immune system and colorectal cancer (CRC). N-3 and n-6 PUFAs have been found to alter the immune response and, as a dietary factor, may additionally influence the development and prognosis of CRC (3-8). Furthermore, it has become evident that the outcome of CRC is strongly influenced by its interaction with immune cells (9-11).

In the context of these findings, we conducted the *in vitro* experiments presented here: We first investigated the impact of n-3 and n-6 PUFAs on the lipid metabolism of CRC cells using liquid chromatography/tandem mass spectrometry (LC/MS/MS). We then established an experimental model which aimed to investigate the effect of n-3 and n-6 PUFAs on immune cells that were activated with inflammatory stimuli. For this, peripheral blood mononuclear cells (PBMCs) were isolated and incubated with three major n-3 or n-6 PUFAs. Subsequently, the PBMCs were stimulated with either a bacterial moiety or CRC cell medium, and cytokine secretion was measured.

With respect to the broad scope and complexity of the topics involved, the introduction aims to provide general information on PUFAs and CRC. Facts regarding PUFAs and their effects, as well as important aspects of the lipid mediator system, will be provided. Subsequently, we will provide an introduction to CRC that presents recent data on the effects of environmental factors, such as PUFAs, on cancer development in the bowel, as well as background information on CRC subtypes and carcinogenesis. In addition, the complex and often unclear relationship between the immune system and CRC will be explored. Finally, we will describe the most recent experimental data that is relevant to this study.

3.1. Polyunsaturated fatty acids (PUFAs)

3.1.1. Overview

PUFAs are fatty acids, which contain multiple carbon-to-carbon double bonds (1, 2, 12, 13). PUFA nomenclature designates the hydrocarbon chain length, number of double bonds, and position of the first double bond (closest to the methyl or omega end), which determines the PUFA family (12). Consequently, "*in omega-3 (n-3) PUFA* [sic!]*, the first double bond is located at the third carbon atom, whereas in n-6 PUFA* [sic!] *it is at the sixth carbon atom*" (Figure 3.1.a) (2, 14, 15). A-Linolenic acid (a-LNA), for example, is an essential member of the n-3 series and is termed as C18:3n-3. It therefore contains 18 carbon atoms with a total of 3 double bonds, the first of which is located at the third carbon atom from the omega end (n-3) (13, 16).

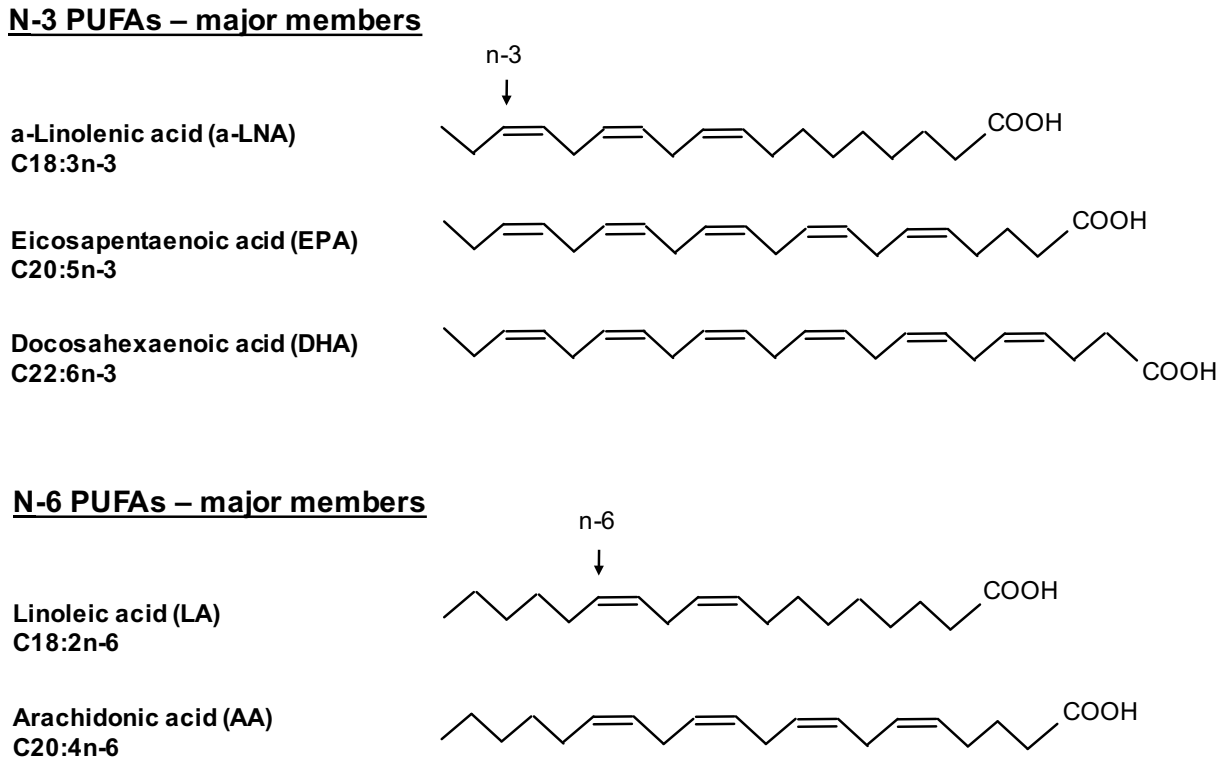

Figure 3.1.a Structure of major n-3 and n-6 PUFAs (14, 17).

As humans and other mammals lack certain desaturases, PUFAs cannot be interconverted from other (non-polyunsaturated) fatty acids and must be provided by direct intake (12, 14, 18, 19). The PUFA-precursors, a-LNA (for the n-3 series) and linoleic acid (LA; C18:2n-6; for the n-6 series), are therefore considered to be essential nutrients.

Eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) for the n-3 series, as well as arachidonic acid (AA; C20:4n-6) for the n-6 series, have been found to be crucial PUFA members for human metabolism (2, 20-23). If their precursors a-LNA and LA are present, non-essential PUFAs, such as EPA, DHA, and AA may be synthesized through further desaturation and elongation (16, 19). It is noteworthy, however, that humans and other mammals are not able to convert n-3 to n-6 PUFAs, or vice versa, and that the precursor-based metabolisms of n-3 and n-6 PUFAs compete for the same enzymes (18, 24). Moreover, tracer and other conversion studies concluded that the precursor-based metabolism of n-3 and n-6 PUFAs lacks efficiency in humans (22, 23, 25). Thus, direct dietary intake of EPA, DHA, and AA serves as an essential contributor for maintaining levels of these fatty acids (14, 26, 27). In acknowledgement of these findings, these three have been termed conditionally essential nutrients (21, 28).

According to the length of their hydrocarbon chain, n-3 PUFAs are found in different dietary products. Major sources for a-LNA are green plants, nuts, and plant seeds (29, 30). Marine n-3 PUFAs, such as EPA and DHA, on the other hand, are synthesized *de novo* primarily by phytoplankton, and accumulate through the food chain in the tissue of marine and freshwater fish (27, 31, 32). The most concentrated source of EPA and DHA are marine, oil-rich or fatty fish, such as sardines, herring and Atlantic salmon (30, 33). The consumption of fish and fish oil therefore serves as the most important source for these fatty acids (23, 34, 35). In contrast, n-6 PUFAs are mainly found in vegetable oils such as safflower, corn or sunflower oil, as well as in egg and meat products (24, 27).

3.1.2. N-3 and n-6 PUFAs in health and disease

N-3 and n-6 PUFAs are not only a source of energy, but have also been found to serve as cell-signaling molecules – in their own right and through their role as precursors to a great number of highly bioactive lipid mediators (21, 26, 36, 37). These are involved in numerous cellular pathways and functions, including inflammation, platelet function, and cellular proliferation (6). Moreover, n-3 and n-6 PUFAs have been found to modulate cell receptor signaling, gene expression, and increase cellular oxidative stress (4, 38, 39).

Interestingly, PUFA intake in Western societies has changed significantly in the last 100 to 150 years; industrialization of agriculture and changes in eating habits, in particular the increased consumption of vegetable oils, resulted in a characteristic imbalance of dietary PUFAs (4, 40). While epidemiological and anthropological evidence suggests that humans evolved on a n-6 to n-3 PUFA ratio of approximately 1, today's Western diet is typically characterized by an excess of n-6 PUFAs and/or low n-3 PUFA intake, resulting in a ratio of around 15 to 1 (4, 41). In this context, it appears that today's Western societies live in a nutritional environment starkly different from the one in which their genetic profile originally evolved (16, 31, 42). This imbalance may be a factor contributing to the development of diseases typical for Western societies (26). Thus, a low n-6 to n-3 PUFA ratio and/or n-3 PUFA supplementation is associated with beneficial effects for patients suffering from conditions with an inflammatory/immune pathogenesis, including inflammatory bowel disease (IBD), rheumatoid arthritis, asthma, and the development of inflammation-related tumors, such as CRC and breast cancer (5, 23, 40, 43-47). Additionally, high intake of marine n-3 PUFAs seems to reduce cardiovascular events, such as myocardial infarction and ventricular arrhythmias as well as neuropsychiatric disease (48-53).

However, as epidemiological and clinical data have proven to be inhomogeneous, controversy about these associations still exists (54-61). Nevertheless, the extensive body of evidence acquired from preclinical studies, as well as both the instrumental and heterogeneous role of PUFA-derived lipid mediators in the modulation of inflammatory processes, make it likely that they not only influence the development and progression of pathologies with an inflammatory component, but also have different effects on inflammation, depending on the type of PUFA (4, 5, 37, 62-65).

3.1.3. N-3 and n-6 PUFAs in the context of inflammation

Inflammation is a vital part of a host's defense against infection and other insults (29). Chronic or uncontrolled inflammation, however, is recognized as a major factor in the development of disease, including autoimmune disorders such as IBD and cancer, in particular CRC (29, 66-68). Research conducted over the last few decades has recognized the ability of n-3 and n-6 PUFAs to modify the reaction of immune cells after exposure to inflammatory stimuli through various pathways. A well-established mechanism is the role of EPA, DHA, and AA in cell signaling through the catalyzation into pro- and anti-inflammatory lipid mediators (3, 26). Further pathways include direct effects on pro- and anti-inflammatory gene expression, as well as on plasma membrane organization of immune cells (3, 69-72). It should be noted that these pathways are all interlinked and likely to affect each other (e.g. the secretion of pro-inflammatory, PUFA-derived lipid mediators also leads to an increased expression of pro-inflammatory genes).

From a simplified point of view, it appears that n-6 PUFAs are predominantly pro-inflammatory modulators of the immune system (2, 4, 6). This effect is largely induced through pro-inflammatory metabolites, derived from n-6 PUFAs, in particular AA. In this context, AA-derived leukotrienes (LTs) and prostaglandins (PGs) have been found to exert potent pro-inflammatory effects on immune cells (1, 2, 6, 73-76). In contrast, n-3 PUFAs seem to reduce inflammatory processes. For one, they inhibit the synthesis of AA-derived, pro-inflammatory mediators (2, 4, 6, 77). Additionally, n-3 PUFA-derived metabolites have been found to induce anti-inflammatory effects. Examples of these include several members of two recently discovered lipid mediator families: resolvins (Rvs) and protectins (PDs) (4, 15, 40, 78) (Figure 3.1.b).

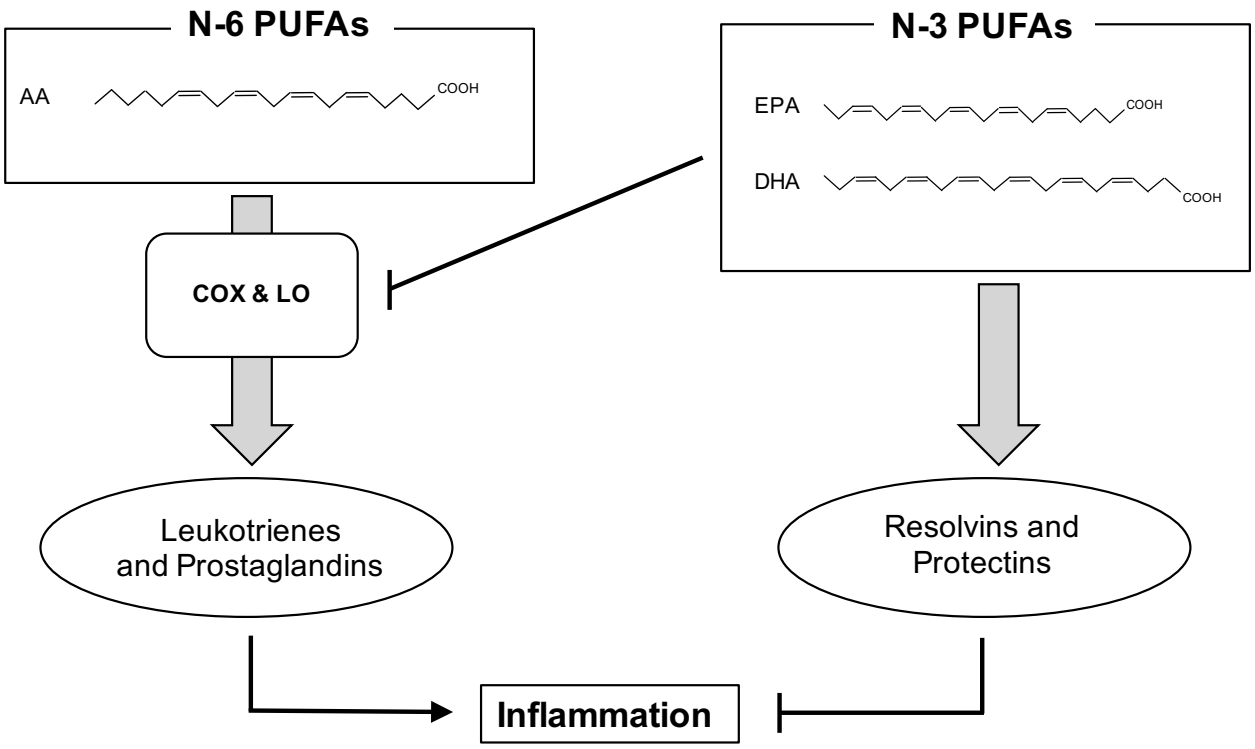

Figure 3.1.b Possible pro- and anti-inflammatory mechanisms of n-3 and n-6 PUFAs. N-3 PUFAs prevent the conversion of AA into pro-inflammatory lipid mediators, such as 2-series prostaglandins and 4-series leukotrienes. In addition, EPA and DHA are precursors to potent anti-inflammatory lipid mediators, most notably resolvins and protectins. COX, cyclooxygenase; LO, lipoxygenase. A similar figure (including title and description) was recently published by the author (2); adapted from: (78) (modified with permission). Additional source: (73).

Despite considerable evidence about these diametrical effects on inflammation, it has become increasingly evident that this paradigm represents an oversimplification (7, 23, 37, 79-85). AA-derived lipoxins (LXs), for example, act as potent anti-inflammatory signaling molecules (37, 86). Moreover, depending on receptor expression on target cells, AA-derived PGs and LTs may also exert anti-inflammatory effects (71, 74, 87-89). Thus, studies were able to demonstrate that n-6 PUFAs, most notably AA and its derivatives, are also capable of inducing a reduction of inflammatory parameters, such as activation of nuclear factor-kappa B (NF-κB) and pro-inflammatory cytokine secretion (37, 59, 83, 90-93). Equally, significant pro-inflammatory effects of n-3 PUFAs have been described in previous studies, including an increase in pro-inflammatory cytokine secretion, such as TNF-α and IL-1 (94-97).

In summary, it seems likely that n-3 and n-6 PUFAs influence immune cell function, however, the exact effects and mechanisms underpinning this influence still have to be determined (4, 37, 70). While n-3 PUFAs seem to have predominantly anti- and n-6 PUFAs largely pro-inflammatory effects, several studies have demonstrated data to the contrary. The discovery of different PUFA-derived lipid mediators, in particular, has provided an explanation for the versatile effects of these fatty acids.

3.2. Fatty acid metabolism and the lipid mediator system

Recent advances in the field of lipidomics (i.e. the structural and functional characterization of lipids and lipid pathways within a living system) have led to the discovery of a vast amount of PUFA-derived bioactive lipid mediators (98, 99). In particular, liquid chromatography coupled to mass spectrometry allows a hitherto unknown insight into PUFA metabolism. The following section describes the LC/MS/MS technology (liquid chromatography/tandem mass spectrometry) that was used in this study, followed by an overview of n-3- and n-6-PUFA-derived lipid mediators.

3.2.1. Liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) – a new era of lipidomics

Mass spectrometry allows the identification and quantification of lipid metabolites. To improve performance in identification, metabolites are usually separated in advance, typically by using high-performance liquid chromatography (98, 100). The resulting LC/MS/MS method is a well-established and sensitive approach, which enables the simultaneous measurement of different metabolites in one sample (101). Usually, samples require a preceding preparation to extract lipid classes of interest (102). Thus, analysis of samples by LC/MS/MS includes several consecutive steps: sample preparation/metabolite extraction, liquid chromatography, mass spectrometry, and data analysis (103).

One of the most commonly used methods for sample preparation is solid-phase extraction (104). Here, a column with a built-in solid sorbent is used to retain metabolites from their biological matrix (36, 102, 104). The extracted metabolites are subsequently eluted from the sorbent by adding a solvent (e.g. hexane:ethyl acetate) (102). To avoid measurement errors, the resulting solution is evaporated under a stream of nitrogen, and reconstituted in an injection-solvent ideal for LC/MS/MS (e.g. acetonitrile) (36).

In the subsequent high-performance liquid chromatography, columns are used to separate the extracted metabolites according to polarity (103, 105). Within this

procedure, reverse-phase chromatography using gradient elution is one of the preferred separation methods for lipid metabolites (36, 103). The chromatographic effluent is coupled to the mass spectrometer and mass spectrometry of metabolites usually directly follows liquid chromatography (98).

Mass spectrometers detect mass-to-charge (m/z) ratio spectra of ionized molecules, and are composed of three main parts: ion source, mass analyzer, and detector (98, 104, 106). The ion source converts metabolites into ions (104). In lipidomic studies, electrospray ionization is the preferred ionization mode; it can generate both positive and negative ion species (i.e. negative and positive mode) (36, 103, 107). Subsequently, the mass analyzer sorts ions according to their m/z ratio, which allows for an accurate measurement by the detector (104). Triple Quadrupole mass spectrometers, like the Agilent 6460 used in this study, are capable of tandem mass spectrometry (MS/MS) (36, 98). MS/MS run in multiple reaction monitoring mode (MRM) greatly improves both sensitivity and specificity of metabolite analysis. It involves multiple rounds of mass spectrometry, allowing mass analyzers to filter two or more sets of ion pairs (precursor and product ions), which are characteristic for the metabolite in question (Figure 3.2.a) (36, 108). Thus, specific precursor ions are selected by a first mass analyzer to be fragmented in a collision cell, resulting in product ions (106). Subsequently, a second mass analyzer filters specific product ions, which are then measured by the detector (98, 108-110).

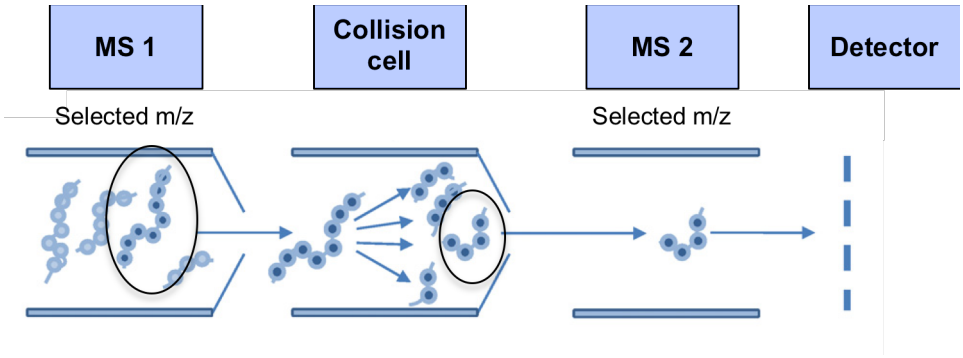

Figure 3.2.a Principle of multiple reaction monitoring mode (MRM) for tandem mass spectrometry (MS/MS). Various modes for MS/MS are available; the depicted MRM has become the method of choice for chemical analysis of metabolites with low molecular weight (109). Circles label precursor and product ion. MS, mass analyzer; m/z, mass-to-charge (ratio). Modified and reproduced permission from: (109). Additional source: (98).

To quantify detected metabolites, an internal standard solution (ISTD) is added to samples before sample preparation. ISTDs contain isotope-labeled metabolites that share almost identical physiochemical properties with their unlabeled analogs, and therefore allow the calculation of calibration curves (36, 105, 111). Additionally, one standard may serve for the quantification of similar metabolite families (36, 111).

3.2.2. PUFA-derived lipid mediators

Advances in LC/MS/MS have led to the identification of a multitude of PUFA-derived cell-signaling molecules (26, 36, 112, 113). Thus, PUFAs are oxygenated into lipid mediators, generally referred to as oxylipins, which are essential for inflammation, platelet function, and cell proliferation (15, 114-118). Mediators, originating from PUFAs with 20 carbon atoms, most notably AA and EPA, are further specified as eicosanoids ("eicosa", Greek for twenty) (36, 71).

N-3- and n-6-derived lipid mediators are "*synthesized through several enzymatic pathways, including cyclooxygenase (COX), lipoxygenase (LOX* [also referred to as LO]), *and cytochrome P-450 (CYP) monooxygenase pathways*" (2, 71, 117, 119). COX enzymes catalyze the oxidative cyclization of their substrates and express two main isoforms, COX-1 and COX-2 (36). In general, COX-1 is responsible for the basal, constitutive synthesis of lipid mediators, while COX-2 is upregulated in response to stress-related situations, such as inflammation (37, 40, 120, 121). Additionally, COX-2 is frequently overexpressed in several cancer cells, including CRC (122-124). Interestingly, COX-2 acetylation by aspirin is known to alter COX function, resulting in the synthesis of alternative (poly)hydroxylated fatty acids, which are further discussed below (15, 125-128). LO enzymes are stereospecific dioxygenases, which are defined by the position at which they oxygenate AA; major mammalian isoforms include 5-, 12-, and 15-LO (112, 121, 129). CYP monooxygenases, well-known for their role in detoxification, also metabolize PUFAs, acting as epoxygenases and hydrolases (112). In addition to COX-, LO-, and CYP-metabolism, similar products may be formed through non-enzymatic reactions (112, 121). Metabolism of AA, EPA, and DHA through these major pathways generates a wide range of oxylipins, often specific to their corresponding precursor PUFA or PUFA family (36, 130).

Hydroxylated fatty acids (OH-FAs) are synthesized through enzymatic as well as non-enzymatic pathways and represent a major group of PUFA-derived oxylipins (118). They are formed during the synthesis of potent lipid mediators, such as Rvs, PDs, LXs, and maresins (MaRs), and are therefore vital for understanding the activity of pathways leading to the formation of these polyhydroxylated metabolites (36, 62, 118). In this context, OH-FAs are more stable than the initially formed hydroperoxide compounds, and each precursor PUFA typically generates a series of specific (poly)hydroxylated metabolite derivatives (36, 118).

Hydroxylation of the n-3 PUFAs EPA and DHA generates EPA-derived hydroxyeicosapentaenoic (HEPEs) and DHA-derived hydroxydocosahexaenoic acids (HDHAs) (118, 130) (Figure 3.2.b). Multiple hydroxylations of EPA and DHA result in the formation of potent mediators, namely Rvs, PDs, and MaRs (15, 62, 73, 131). E-series resolvins (RvEs) derive from EPA, while D-series resolvins (RvDs), PDs, and MaRs originate from DHA (6, 15, 62).

The hydroxylation of AA results in the formation of hydroxyeicosatetraenoic acids (HETEs) (Figure 3.2.b). Multiple hydroxylations of AA generate pro-inflammatory 4-series LTs and anti-inflammatory LXs (76, 120, 121, 126, 130). In this context, 5-HETE is the pathway marker for the formation of LTs (131, 132).

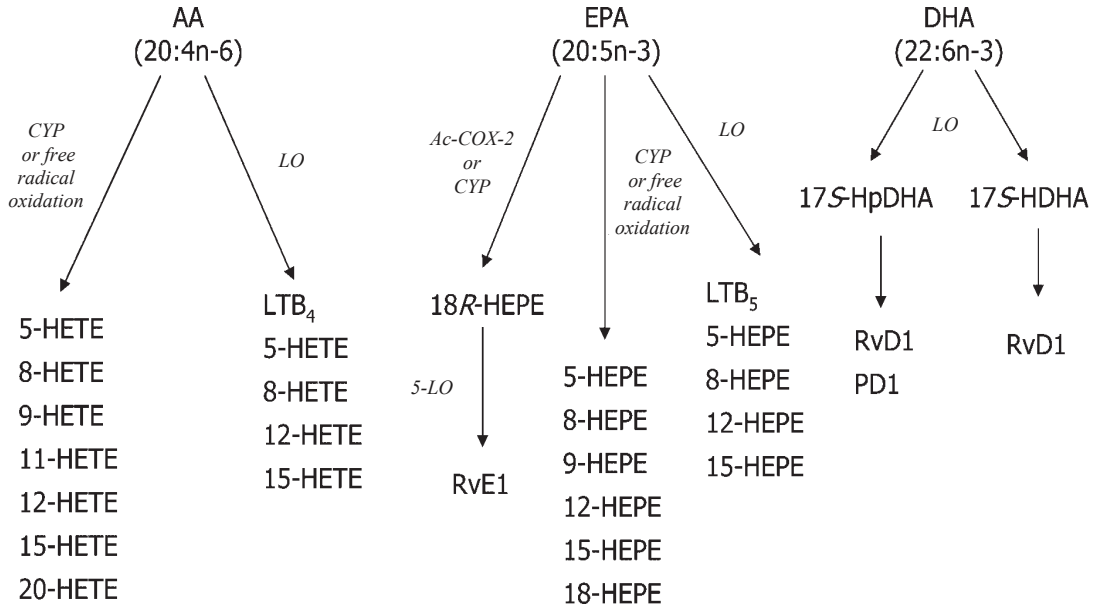

Figure 3.2.b Major hydroxy- and polyhydroxy-PUFA mediators derived from EPA, DHA, and AA. Lipoxygenase (LO), cytochrome P-450 (CYP), acetylated cylcooxygenase-2 (Ac-COX-2), and non-enzymatic free radical-catalyzed pathways are involved in the formation of lipid mediators. HETE, hydroxyeicosatetraenoic acid; HEPE, hydroxyeicosapentaenoic acid; HDHA, hydroxydocosahexaenoic acid; LT, leukotriene; RvE and RvD, E- and D-series resolvins; PD, protectin. Modified and reproduced with permission from: (130).

Interestingly, several OH-FAs additionally act as effector molecules themselves. Thus, OH-FAs, such as 15-HEPE, 17-HDHA, or 15-HETE, have been found to affect behavior of immune and cancer cells (58, 64, 133-137).

Additionally, major lipid mediators are synthesized without OH-FA intermediates. These include a large group of COX-derived eicosanoids (29). Thus, AA and EPA are metabolized into 2- and 3-series PGs and thromboxanes (TXs), respectively (36, 115, 117). Important members include AA-derived PGE_2 and TXA_2 as well as EPA-derived PGE_3 (115, 116, 138, 139).

AA-derived lipid mediators, such as PGs, LTs, LXs, and TXs, play a crucial role in pathologies that are predominantly inflammation-related (as described in chapters 3.1.2 and 3.1.3) (2, 26, 40). Drugs that target pro-inflammatory AA-derived lipid mediators, such as 2-series PGs and 4-series LTs, are therefore used widely and with good efficacy (e.g. nonsteroidal anti-inflammatory drugs (NSAIDs) or leukotriene receptor

antagonists) (6, 26, 115, 120). Until recently, the effects of n-3 PUFAs were predominantly associated with their inhibition of, and competition with AA metabolism, resulting in lower concentrations of AA-derived potent pro-inflammatory eicosanoids, and an increase in n-3 PUFA-derived lipid mediators of the same family with a lower bioactivity (15, 40, 140, 141). The identification of Rvs, PDs, and MaRs by Serhan *et al.* led to an exciting twist in the field of lipid mediator research, possibly establishing the n-3 PUFAs EPA and DHA as precursors to a series of potent, predominantly anti-inflammatory, lipid mediators (15, 119, 130, 142-144). These and other findings led to the characterization of important AA- as well as EPA- and DHA-derived (polyhydroxylated) lipid mediators, taking into account the different, sometimes even diametrical, effects of these two groups (Figure 3.2.c).

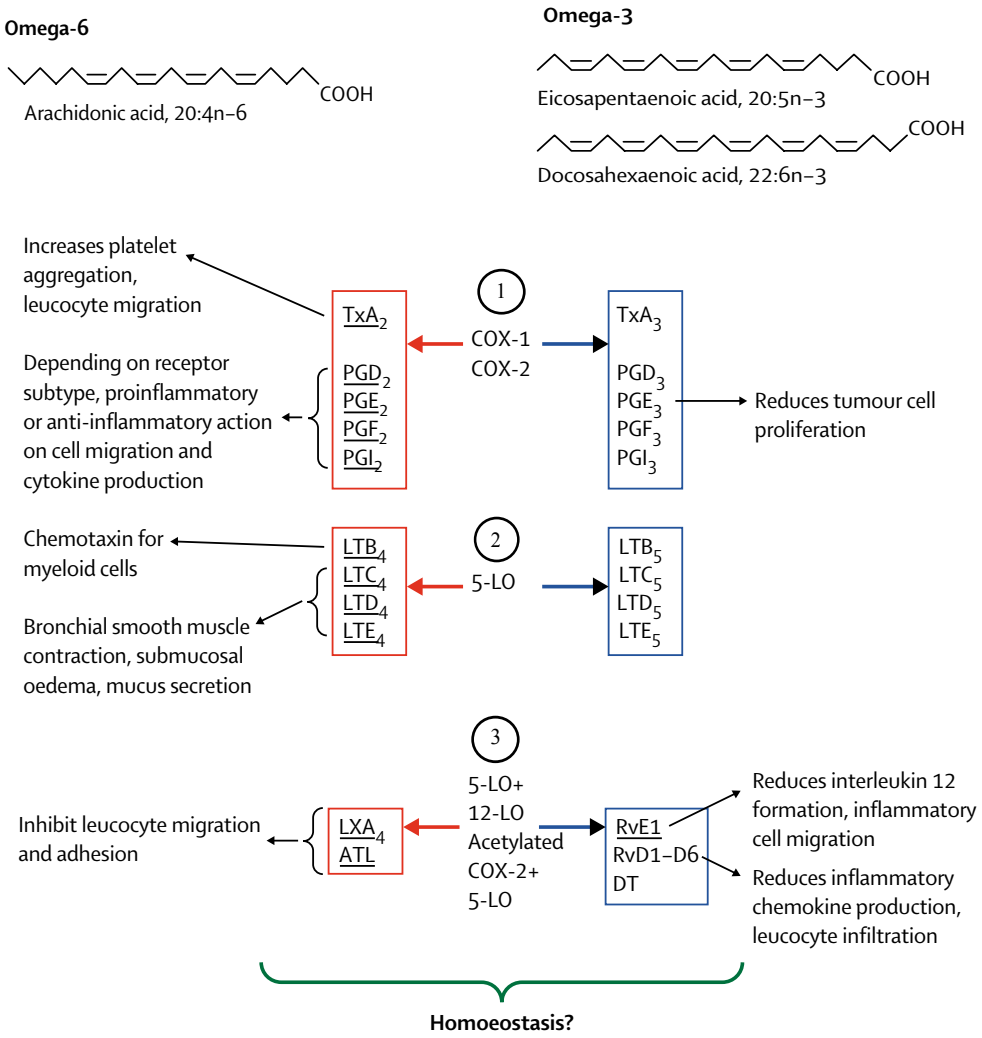

Figure 3.2.c Overview of major lipid mediators derived from EPA, DHA, and AA. ① The formation of n-6-PUFA-derived 2-series prostaglandins (PGs) and thromboxanes (TXs), as well as n-3-PUFA-derived 3-series PGs and TXs is catalyzed by cylcooxygenase-1 and -2 (COX-1 and -2). ② N-6-PUFA-derived 4-series and n-3-PUFA-derived 5-series leukotrienes (LTs) are predominantly generated by 5-lipoxygenase (5-LO). ③ Lipoxins (LXs) are anti-inflammatory mediators, derived from AA via the LO-pathway; aspirin-triggered lipoxins (ATLs) are generated after acetylation of COX-2 by aspirin. Metabolism of EPA and DHA, predominantly through the LO-pathway, gives rise to anti-inflammatory E- and D-series resolvins (RvEs and RvDs). DT, docosatrienes. Mediators with specific receptors are labeled with an underscore. Modified and reproduced with permission from: (40). Additional source: (6).

3.3. Colorectal cancer

The following section portrays the three main pathways in colorectal carcinogenesis. Furthermore, this chapter aims to highlight the importance of environmental factors, with an emphasis on n-3 and n-6 PUFAs, in the development of CRC.

3.3.1. Subtypes of colorectal cancer and colorectal carcinogenesis

From an etiological perspective, CRC can be divided into three subtypes: sporadic, familial, and cases developing due to IBD (145). IBD, such as ulcerative colitis and Crohn's disease, account for a small fraction of CRC cases (approximately 1%), termed colitis-associated cancer (CAC) (146, 147). Approximately 25% of CRC patients are estimated to have a positive family history, and one third of these familial cases are the result of true hereditary CRC-syndromes (148-151). Thus, about 75% of all CRC cases are purely sporadic. Their development is strongly affected by environmental risk factors (see also chapter 3.3.3) (152, 153). So far, three main pathways which result in CRC have been found: chromosomal, microsatellite, and epigenetic instability.

Approximately 65 to 85% of CRCs predominantly develop via the chromosomal instability (CIN) pathway, also referred to as the *"suppressor"* or *"traditional"* pathway (154-157). By contrast, about 15 to 20% of CRCs mainly develop through the microsatellite instability (MSI) pathway, also known as the *"caretaker"* or *"mutator"* pathway (145). These tumors are characterized by defects in the DNA mismatch repair (MMR) system, normally recognizing and correcting mistakes made by DNA polymerases during replication (152). The discovery of epigenetic instability in CRC has led to the definition of a new and third CRC phenotype, the CpG island methylator phenotype (CIMP) (156). Unlike the CIN and MSI pathways, which result in genomic instability, cancerogenesis via the CIMP pathway is based on epigenetic alterations.

The genetic and epigenetic events during CIN, MSI, and CIMP are not mutually exclusive and often show a complex interplay, resulting in the accumulation of genetic and epigenetic alterations of more than one pathway in CRC cells (145, 148, 155). CRCs can therefore be classified according to their genetic and epigenetic status,

resulting in molecular subtypes which are characterized by similar morphology, clinical features, and prognosis (158-161).

3.3.2. Epidemiological aspects

The International Agency for Research on Cancer (IARC) estimates there were about 1.85 million new cases and almost 881,000 CRC-related deaths in 2018 (162). Thus, CRC is one of the most common forms of cancer (1, 2, 163, 164). Interestingly, a significant variation in CRC incidence can be observed worldwide as almost 60% of CRC cases occur in more developed countries (Figure 3.3.a) (162, 164-166). High-income regions like Australia, New Zealand, and Europe show the highest incidence rates of CRC. In these countries, incidence of CRC is up to ten times higher than in the middle- to low-income regions of most parts of Africa and south-central Asia (164).

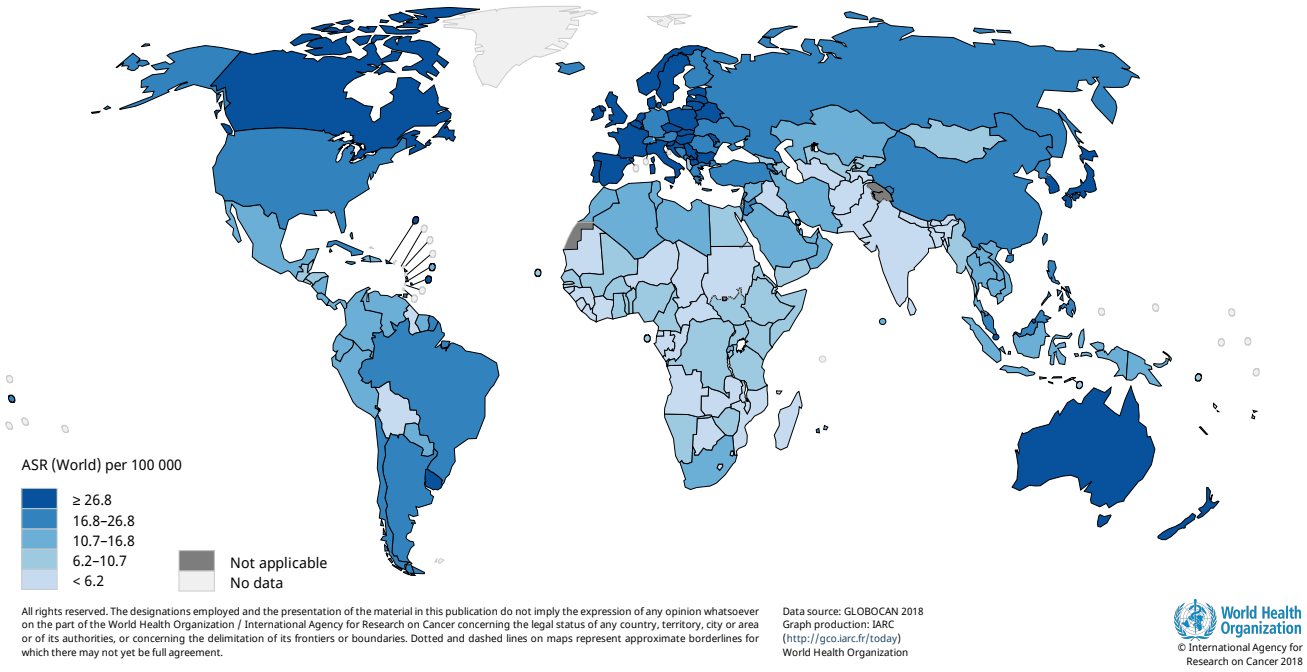

Figure 3.3.a Age-standardized incidence rate (ASR) of colorectal cancer (CRC) in 2018 (both sexes, per 100,000). Reproduced with permission from: (162).

Several studies have demonstrated that development of sporadic CRC is closely related to lifestyle; major risk factors include diet and physical inactivity (see also chapter 3.3.3) (167, 168). The large geographic differences in incidence therefore seem to reflect differing exposures to these environmental risk factors, which are closely linked to economic development and westernization of society (165, 168). In this context, a rapid increase in CRC incidence can be observed in countries that shifted from a low- to a high-income economy during the last century. Japan, in particular, recorded a significant increase in CRC incidence during the last quarter of the 20th century. Here, the age-adjusted incidence rate for CRC increased up to 3.9 times between 1975 and 2000 (162, 168, 169). A similar increase was observed in economically transitioning countries of Eastern Europe, such as the Czech Republic or Slovakia (163, 168). Additionally, studies of migrants further revealed the susceptibility of CRC development to environmental factors. Populations from areas with low CRC incidence that moved to high-risk countries such as Australia or the USA showed a rapid increase of CRC incidence linked with an increasing period of residence (169-172).

3.3.3. Risk factors for colorectal cancer – the unclear role of PUFAs

Several studies and epidemiological observations were able to demonstrate that genetic alterations which result in sporadic CRC are largely dependent on environmental factors (152). Notably, the large-scale meta-analyses by the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) identified several, predominantly dietary, factors modifying CRC risk. Major protective factors include physical activity, food containing dietary fiber and/or garlic, and intake of NSAIDs (in particular aspirin). Red and processed meat, abdominal fatness, and alcohol consumption, on the other hand, are associated with an increased risk for CRC (167, 173-176). In summary, factors influencing sporadic CRC development are therefore related to lifestyle, which led to the conjecture that CRC might be a largely preventable disease (44, 167).

While epidemiological studies on the relationship of n-6 PUFA intake and CRC development failed to show a clear correlation, fish consumption, and thus high intake of marine n-3 PUFAs, was considered as a possible protective factor by the WCRF/AICR expert panel (2, 57, 60, 176-181). Two case-control studies demonstrated a significantly reduced incidence of CRC in patients with high n-3 PUFA blood levels (181, 182). Moreover, Wu *et al.* published a meta-analysis of 19 case-control and 22 prospective cohort studies evaluating the effect of fish consumption on CRC risk in humans. The authors concluded "*that intake of fish* (...) *may significantly reduce the risk of CRC by as much as 12%*" (2, 8). Additionally, several clinical studies demonstrated the beneficial effects of EPA supplementation in patients with colorectal adenomas (183, 184). Although these findings are promising, data concerning the effect of fish or marine n-3 PUFA consumption on CRC still remains controversial; several clinical studies could show no beneficial effect, or even observed a weak positive correlation of n-3 PUFA intake and CRC incidence (60, 61, 185). Heterogeneity of study results may be explained by differences and shortcomings in study design; however, in summary, epidemiological evidence is too limited to attribute a clear beneficial effect of n-3 PUFAs on the development of CRC (8, 38, 44).

Intake of aspirin is associated with the primary prevention of CRC, as well as with higher overall survival in CRC patients (2, 174, 186, 187). Aspirin is believed to counteract the overexpression of COX-2, which is observed in the majority of CRCs and results in high levels of AA-derived, tumor-promoting lipid mediators in the tumor

microenvironment (122-124, 188). Of these, PGE_2 in particular plays a pivotal role in tumor-mediated immune dysfunction, which enables CRC and other tumors to evade the host's immune surveillance (1, 189-192). By reducing PGE_2 levels, aspirin is believed to counteract the immune evasion of tumors (1, 189) (Figure 3.3.b).

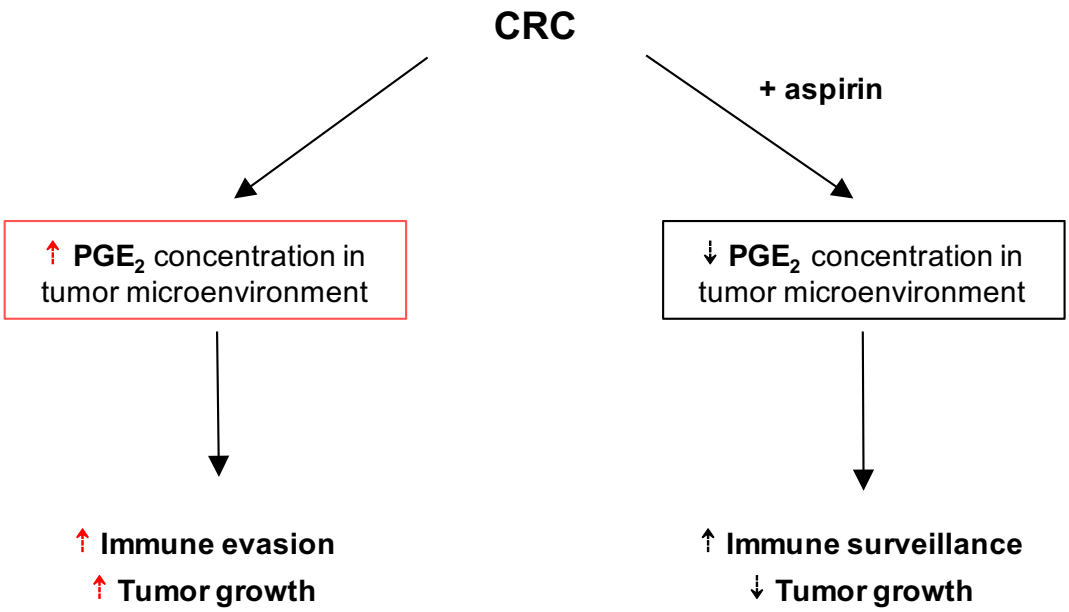

Figure 3.3.b PGE_2-mediated immune dysfunction in colorectal cancer (CRC). PGE_2, prostaglandin E_2. This figure was recently published by the author, reproduced with permission from: (1, 2). Initially adapted from: (189).

These findings, data on the effects of fish and n-3 PUFA intake on CRC incidence, and the current understanding of PUFA-derived lipid mediators, make it biologically plausible that PUFAs, in particular n-3 PUFAs, influence CRC development and proliferation, especially when consumed in high amounts (176, 193, 194).

3.4. Colorectal cancer and the immune system

Carcinogenesis and progression of CRC are affected by the interaction of tumor cells with the host's immune system in the tumor microenvironment (10). Interestingly, data suggest that the immune system is likely to act as a kind of double-edged sword, both promoting and preventing development and progression of cancer in the bowel (10, 195-197). Thus, chronic inflammation has been associated with the development of CRC. Infiltration of CRC with immune cells, on the other hand, correlates with improved survival (9, 11, 198, 199).

In particular, the development of the CRC-subtype CAC is strongly associated with IBD, and serves as a paradigm for the connection between cancer and inflammation (10, 200). The risk of developing CAC correlates both with the severity and duration of inflammation in the bowel (10, 201-205). Moreover, regarding sporadic CRC, several studies demonstrated an inverse correlation between inflammatory markers, such as CRP, IL-6, and plasma fibrinogen, and overall patient survival (206-210).

Conversely, the concept of an anti-tumor immune response is equally well-represented in CRC, a tumor greatly infiltrated by immune cells (1, 10, 195). The inflammatory reaction in and around CRC correlates with a better prognosis, and is believed to be a parameter of the host's tumor immune surveillance (1, 2, 9, 11, 198). In particular, the infiltration of T-cell lymphocytes (CD3, CD45RO, and CD8) has been shown to be a beneficial factor for survival in CRC patients (2, 9, 211-213). In this context, Galon *et al.* demonstrated that "*type, density, and location of immune cells* [T-lymphocytes] *in CRCs had a prognostic value that was superior to and independent of those of the UICC-TNM classification*" (212).

The current understanding of the effects of aspirin (see also chapter 3.3.3), as well as several recent publications on cancer immune therapy in patients with CRC, further demonstrate the importance of immune surveillance in these tumors. By targeting cancer-immune checkpoints in the tumor microenvironment, such as the programmed cell death 1 (PD-1) pathway, cancer immune therapy aims to enhance immune surveillance in patients with malignant tumors (214). The PD-1 pathway suppresses the Th1 cytotoxic immune response and is upregulated in many tumors, thereby enhancing immune evasion of malignant cells. The blockade of this pathway with the PD-1

monoclonal antibody pembrolizumab therefore "*can overcome immune resistance of tumor cells*" (1, 2, 215, 216). Specifically, patients with MSI-high CRC seem to benefit from the blockade of the PD-1 pathway (2, 214, 215, 217, 218). Interestingly, Zelenay *et al.* additionally demonstrated synergistic effects of the blockade of PD-1 and aspirin-mediated anti-tumor mechanisms (1, 189). The authors demonstrated that the inhibition of COX with aspirin or celecoxib synergized with the blockade of the PD-1 pathway, thereby inducing tumor eradication in mice.

While chronic inflammation has been shown to promote development of CRC, more recent studies on tumor microenvironment and cancer immune therapy provide robust evidence for the pivotal role of immune surveillance in preventing further tumor growth and metastasis of existing tumors. Skewing the immune system towards an overall anti-tumorigenic effect therefore seems an essential aim for future studies in this area of research. Considering the previously demonstrated influence of n-3 and n-6 PUFAs on the immune system, it also seems likely that these fatty acids exert an effect on CRC development and progression by influencing the crosstalk between immune and cancer cells in the tumor microenvironment.

3.5. Evidence from preclinical studies in the context of conducted experiments

Animal and *in vitro* studies on n-3 and n-6 PUFAs and their derivatives demonstrated the significant effects of these substances on CRC and immune cells. However, contradicting results have been observed. These are likely to be associated with the heterogeneous, partly diametrical effects of potent lipid mediators that originate from the same PUFA.

3.5.1. Effects of n-3 and n-6 PUFAs on colorectal cancer

Dietary intake of n-3 and n-6 PUFAs possibly influences the development and progression of CRCs. Epidemiological and clinical data concerning this hypothesis remains controversial, as contradicting results or no effect for both n-3 and n-6 PUFAs have been reported previously (also refer to chapter 3.3.3). With regard to n-3 PUFAs, preclinical studies have shown to provide more convincing evidence. For example, numerous *in vitro* experiments demonstrated the anti-proliferative effects of EPA and DHA on various CRC cell lines (2, 193, 219-221). Effects of dietary changes in PUFA intake on CRC incidence have been demonstrated with experiments that used the *fat-1* transgenic mice model. "*These mice carry a transferred gene, which encodes for a fatty-acid desaturase enzyme that converts n-6 to n-3 PUFA* [sic!]*, resulting in a low n-6 to n-3 ratio of almost 1*" (2, 222). In their study, evaluating the rate of CAC in mice, Jia *et al.* showed that *fat-1* mice have a significantly reduced number of colonic adenocarcinomas as well as an elevated rate of colonocyte apoptosis when compared to wild-type littermates (2, 223).

Interestingly, and in agreement with the inconclusive epidemiological and clinical data, AA seems to have pro- as well as anti-tumorigenic properties in experiments with CRC. Several *in vitro* studies reported the anti-proliferative effects of AA on CRC cell lines (193, 220, 221, 224). Conversely, other studies demonstrated that AA and its derivatives possibly stimulate CRC cell growth (225-228). Thus, AA induced proliferation in LS-174T human colon carcinoma cells, and abolished anti-tumorigenic effects induced by EPA in $Apc^{Min/+}$ mice (226, 227).

Several of the effects of EPA, DHA, and AA on CRC are believed to be mediated through the action of the lipid mediators that derive from these fatty acids (58, 71, 129). In this context, EPA-derived 15-HEPE and PGE_3 and DHA-derived 17-HDHA, in particular, reduced cancer cell proliferation in *in vitro* experiments (64, 65, 229, 230). Effects mediated by AA-derived lipid mediators may explain the controversial results of experiments with AA. AA-derived PGE_2, LTB_4, as well as 5- and 12-HETE, have been shown to promote proliferation and/or motility of cancer cells (58, 129, 231-236). 15-HETE, on the other hand, exerts anti-proliferative and pro-apoptotic effects on cancer, which are associated with the activation of peroxisome proliferator-activated receptor gamma (64, 133-135). Considering these findings, it seems vital to further explore the lipid mediator system of n-3 and n-6 PUFAs in the context of CRC. In particular, the controversial findings on AA may be explained by a better understanding of its metabolism in CRC cells.

3.5.2. Immunomodulatory properties of n-3 and n-6 PUFAs

Regarding the effect of n-3 PUFAs on immune cells, the majority of studies have demonstrated the anti-inflammatory properties of these fatty acids. Clinical manifestation and pathology of colitis, for example, is significantly less severe in the *fat-1* transgenic mice model when compared to wild-type littermates (80, 84). Moreover, n-3-PUFAs reduce the secretion of TNF-α, IL-1, IL-2, and IL-6 by immune cells, *in vivo* as well as *in vitro* (2, 47, 237-241). Similar anti-inflammatory effects were demonstrated in studies with n-3 derived lipid mediators, in particular 18-HEPE, 17-HDHA, PDs, Rvs, and MaRs. (1, 2, 58, 62, 131, 136, 137, 242, 243). However, studies have also demonstrated that n-3 PUFAs are capable of augmenting inflammation in certain experimental settings. N-3 PUFA supplementation increased the secretion of TNF-α and IL-1 by immune cells in several *in vitro* experiments (94, 95, 97, 244-247). Similarly, Woodworth *et al* demonstrated that dietary supplementation with high concentrations of fish oil (up to 6%) significantly increased colitis and adenoma formation in a mouse model of inflammatory colitis (248).

Similarly to n-3 PUFAs, a dual role of AA in inflammation has been reported. Research in the last few decades has demonstrated that the assumption that AA and its

derivatives are by definition pro-inflammatory is in fact an oversimplification (63, 85). Despite AA being the precursor molecule for several highly potent pro-inflammatory lipid mediators as well as the clinical efficacy of NSAIDs, several studies have shown that AA may also suppress inflammatory processes (4, 63, 249, 250). This is illustrated by findings showing that dietary supplementation with AA down-regulates inflammatory marker genes in a colitis mice model of IBD (92). Similarly, supplementation with AA and other n-6 PUFAs decreased proliferation of rat and human lymphocytes (251, 252). *In vitro* experiments, using human PBMCs, demonstrated a dose-dependent inhibition of IL-2 by AA (253). Moreover, AA-derived LXA_4 and PGE_2, are known to exert significant anti-inflammatory effects, including the resolution of inflammation and inhibition of TNF-α and IL-2 synthesis (85, 86, 254).

Several theories have been developed aiming to explain the contradictory findings relating PUFAs to inflammation (4, 39, 58, 85). A probable explanation has been provided by the hypothesis that PUFAs exert their effects predominantly through the formation of potent lipid mediators (58, 255). Thus, the role of EPA, DHA, and AA as precursors to a multitude of bioactive derivatives, with partly diametrical functions, is likely to play a significant role in generating paradoxical study results. Additionally, n-3 and n-6 PUFAs have shown to induce alterations in gene expression as well as plasma membrane organization of immune cells. Similarly to the effects mediated by lipid mediators, these may also be pro- as well as anti-inflammatory (3, 69-72). Whatever the exact pathways behind the effects of n-3 and n-6 PUFAs on immune cells are, it seems vital to further assess their role in inflammation as well as in inflammation-related tumors, such as CRC.

3.6. Research aims

Considerable evidence suggests that n-3 and n-6 PUFAs and their derivatives may affect the development and progression of CRC. While numerous studies have described their effects on proliferation and gene expression, surprisingly little evidence is available on the influence of EPA, DHA, and AA on CRC lipid metabolism (118). The identification of lipid mediators, as well as the characterization of pathways involved in PUFA metabolism, are likely to provide explanations for the complex effects of these fatty acids on CRC and other tumors. A recent study quantified EPA-, DHA-, and AA-derived OH-FAs in the supernatant of CRC cell lines, demonstrating that CRCs are capable of OH-FA synthesis, and that OH-FA formation may be modified by incubation with PUFAs (118). Based on these and other findings on the pathways involved in lipid metabolism, the present study set out to investigate effects of EPA, DHA, and AA on the lipid metabolism of human colorectal adenocarcinoma HT-29 cells. Our experiments focused on the quantification of OH-FAs, and of the polyhydroxylated lipid metabolites LTB_4, LTB_5, LXA_4, and RvD_1. We hypothesized that EPA, DHA, and AA would modify the synthesis of their lipid mediators, resulting in an increase in the corresponding HEPEs, HDHAs, HETEs, and polyhydroxylated lipid metabolites. To test our hypothesis, HT-29 cells were incubated with EPA, DHA, or AA, and subsequently the concentration of lipid mediators was quantified using LC/MS/MS.

The second aim of this study was to assess the effects of n-3 and n-6 PUFAs on inflammation, as well as on the inflammatory response of immune cells in the context of CRC. While, as described above, a large body of evidence suggests that n-3 and n-6 PUFAs affect immune cell function, the exact nature of this effect is still in question. We therefore established a method to isolate high numbers of human immune cells (PBMCs) from the leukocyte depletion filters of blood collection systems, and explored the effects of EPA, DHA, and AA on the secretion of TNF-α, IL-6, and IL-10 *in vitro*. First, we assessed whether incubation with PUFAs alone could alter the secretion of cytokines. Here, we did not expect significant changes compared to untreated PBMCs, because evidence suggests that PUFAs affect the reaction of immune cells to inflammation, rather than acting as inflammatory stimuli themselves (4). Subsequently, we aimed to explore the effects of EPA, DHA, and AA on cytokine secretion, induced by

stimulation with lipopolysaccharide (LPS). Given that previous studies demonstrated potent effects of PUFAs on cytokine secretion, we hypothesized that incubation with these PUFAs would alter the secretion of pro- and anti-inflammatory cytokines.

Given the close relationship between CRC and the immune system, as well as the immunomodulatory effects of EPA, DHA, and AA, we also set out to explore the effects of these PUFAs on the interaction between CRC and PBMCs. To test this, we assessed the impact of EPA, DHA, and AA on the secretion of TNF-α, IL-6, and IL-10, which was induced by stimulating PBMCs with conditioned media (CM) of HT-29 cells. Similarly to experiments with LPS-induced cytokine secretion, we hypothesized that incubation with EPA, DHA, and AA would alter CM-induced cytokine secretion.

To summarize, this study encompasses two major research aims: (I) to explore the effects of EPA, DHA, and AA on the lipid metabolism of HT-29 cells and (II) to assess the impact of EPA, DHA, and AA on cytokine secretion by PBMCs induced by LPS or CM.

4. Materials and Methods

4.1. Materials

4.1.1. Consumables

100 x 20 mm cell culture dishes	BD, Franklin Lakes, USA
175 and 75 cm^2 cell culture flasks	BD, Franklin Lakes, USA
6-well cell culture plates	BD, Franklin Lakes, USA
96-well ELISA plates (# 9018)	Corning Inc., Corning, USA
Accu-Jet® tips (5, 10, and 25 ml)	Corning Inc., Corning, USA
BD Plastipak™ syringes (20 and 50 ml)	BD, Franklin Lakes, USA
Cell scrapers	Sarstedt AG&Co., Nürnbrecht, Germany
Cell strainers (70 µm)	Thermo Fisher Scientific Inc., Waltham, USA
Disposable culture tubes 16 x 100 mm	Corning Inc., Corning, USA
Eppendorf vials (1.5 and 2.0 ml)	Sarstedt AG&Co., Nürnbrecht, Germany; Brand, Wertheim, Germany
Falcon tubes (15 and 50 ml)	Corning Inc., Corning, USA
Pipette tips, bioclean (20, 250, and 1000 µl)	Mettler Toledo, Greifensee, Switzerland
Pipette tips (20, 200, and 1000 µl)	Sarstedt AG&Co., Nürnbrecht, Germany

Table 4.1.a Consumables

4.1.2. Buffers and Solutions

AA, EPA, and DHA	Nu-Chek-Prep Inc., Elysian, USA
Absolute ethanol	Merck KGaA, Darmstadt, Germany
Ficoll-Paque™ PLUS	GE Healthcare, Little Chalfont, UK
HPLC grade acetic acid	Thermo Fisher Scientific Inc., Waltham, USA
HPLC grade acetonitrile	Thermo Fisher Scientific Inc., Waltham, USA
HPLC grade formic acid	Thermo Fisher Scientific Inc., Waltham, USA
HPLC grade H_2O	Thermo Fisher Scientific Inc., Waltham, USA
HPLC grade methanol	Thermo Fisher Scientific Inc., Waltham, USA
Laboratory reagent grade ethyl acetate	Thermo Fisher Scientific Inc., Waltham, USA
LPS from E. coli O55:B5 (L6529-1MG)	Sigma-Aldrich, St. Louis, USA
Methanol	Merck KGaA, Darmstadt, Germany; Thermo Fisher Scientific, Waltham, USA
n-Hexane (pure)	Thermo Fisher Scientific Inc., Waltham, USA
PBS without Ca^{2+} & Mg^{2+} (Charge: 0063X)	Merck KGaA, Darmstadt, Germany
Sodium acetate (extra pure)	Thermo Fisher Scientific Inc., Waltham, USA
Sodium hydroxide	Merck KGaA, Darmstadt, Germany
Trypan blue solution (0.4%)	Sigma-Aldrich, St. Louis, USA
Tween®-20	Sigma-Aldrich, St. Louis, USA

Table 4.1.b Buffers and Solutions

4.1.3. Machinery

6460 Triple Quadrupole LC/MS System coupled to an Agilent 1200 HPLC	Agilent Technologies Inc., Santa Clara, USA
Accu-Jet® Pipette Controller	Brand GmbH, Wertheim, Germany
Analytical balance A210P 001 (e = 0.1 mg)	Sartorius AG, Göttingen, Germany
Bond-Elut-Certify-II-Column (200 mg, 3 ml, 50/pk)	Phenomenex Inc., Torrance, USA
ELx800 Universal Microplate Reader	Bio-Tek instruments Inc., Winooski, USA
Fridge - Bosch KGN 33X14	Robert Bosch AG, Gerlingen, Germany
Heating block	Liebisch Labortechnik GmbH, Bielefeld, Germany
Heraeus Biofuge Fresco	Thermo Fisher Scientific Inc., Waltham, USA
Heraeus HERAcell 150 CO_2 Cell Incubator	Thermo Fisher Scientific Inc., Waltham, USA
Heraeus Labofuge 400 centrifuge	Thermo Fisher Scientific Inc., Waltham, USA
Heraeus Laminair HB2448 biological safety cabinet	Thermo Fisher Scientific Inc., Waltham, USA
Kinetex column (150 x 2.1 mm, 2.6 µm)	Phenomenex Inc., Torrance, USA
Lab Dancer Vario, yellow line	IMLAB bvba, Boutersem, Belgium
Laboratory oven (Model UM 100)	Memmert GmbH, Schwabach, Germany
Laboratory water bath	GFL mbH, Burgwedel, Germany
Neubauer counting chamber - Neubauer Improved (0.0025 mm^2)	Marienfeld GmbH, Lauda-Königshofen, Germany
pH meter: CyberScan pH 510 and 765	Eutech Instruments / Thermo Fisher Scientific Inc., Waltham, USA; Knick GmbH, Berlin, Germany
Pipettes (0.5 -10, 10 -100, and 100-1000 µl)	Eppendorf AG, Hamburg, Germany
Rainin Pipet-Lite XLS (0.1-2, 1-10, 10-100, and 100-1000 µl)	Mettler Toledo Inc., Greifensee, Switzerland
RCT magnetic stirrer	IKA®-Werke GmbH, Staufen, Germany
Rotina 35R centrifuge	Hettich Zentrifugen GmbH, Tuttlingen, Germany
Rotina 420R centrifuge	Hettich Zentrifugen GmbH, Tuttlingen, Germany
Transferpette®-8 pipette (30-300 µl)	Brand GmbH, Wertheim, Germany
Vortex-Genie 2 (Model G-560E)	Scientific Industries Inc., Bohemia, USA
Zeiss Axiovert 25 microscope	Carl Zeiss AG, Oberkochen, Germany

Table 4.1.c Machinery

4.1.4. Cells and Cell Culture

0.5% Trypsin-EDTA (10x) (No. 15400-054)	Life Technologies, Carlsbad, USA
Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l D-Glucose and L-Glutamine (PAA E15-843)	PAA Laboratories GmbH, Pasching, Austria
FBS Superior (Lot: 0323W)	Merck KGaA, Darmstadt, Germany
Human colorectal adenocarcinoma HT-29 cells (# HTB38)	ATCC, Manassas, USA Generously provided by AG Christian Fischer, Charité – Universitätsmedizin Berlin, Germany
Macopharma blood collection systems with Leucoflex LST2B leukocyte depletion filter	Maco Pharma International, Langen, Germany
Penicillin / streptomycin (No. 15140-122)	Life Technologies, Carlsbad, USA

Table 4.1.d Cells and Cell Culture

4.1.5. ELISA Kits

Human TNF-α, IL-6, and IL-10 standard	eBioscience Inc., San Diego, USA
"Ready-Set-Go!" ELISA for TNF-α, IL-6, and IL-10	eBioscience Inc., San Diego, USA

Table 4.1.e ELISA Kits

4.1.6. Software

EndNote X7 and X8	Thomson Reuters Corp., NYC, USA
GraphPad Prism 6.0f	GraphPad Software Inc., La Jolla, USA
KC Junior – analysis software for microplate reader	BioTek Instruments Inc., Winooski, USA
MassHunter Workstation software (version b.05.02) – analysis of LC/MS/MS	Agilent Technologies Inc., Santa Clara, USA
Microsoft Office (Office 365 for Mac)	Microsoft Corp., Redmond, USA

Table 4.1.f Software

4.2. Methods

Methods for experiments with PBMCs have been published in the *Supplementary Material* of two recent articles by the author (1, 2). Passages of the following chapter that are similar to methods described previously by the author are italicized.

4.2.1. Cells

The human colorectal adenocarcinoma HT-29 cell line was acquired by Fogh and Trempe from a 44-year-old Caucasian female; cells have shown high culture stability with a comparable karyotype for over 100 passages (256). *PBMCs originate from blood bank donors at the Institute for Transfusion Medicine of the Charité – Universitätsmedizin Berlin, Germany. PBMCs were acquired anonymously, information about age, sex, or other personal data of donors was not collected during the study.*

HT-29 cells and PBMCs were grown in standard conditions (37°C and 5% CO_2). Growth medium (GM), used to maintain cells, consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4.5 g/l D-Glucose, 2 mM L-glutamine,100 µM streptomycin, and 100 IU/ml penicillin. For HT-29 cells, medium changes were performed every 2 to 3 days.

For harvesting (at 80-90% confluence), HT-29 cells were washed twice with 10 ml of PBS. Trypsin-EDTA (10x) was dissolved 1:10 in PBS, resulting in a concentration of 1x Trypsin-EDTA. 5ml of Trypsin-EDTA (1x) was added to HT-29 cells and allowed to settle for 20-30s. Trypsin-EDTA (1x) was removed and cells were incubated for 5 min. 20 ml of GM was added to cell culture flasks. The resulting cell suspension was transferred into a 50 ml Falcon Tube and centrifuged for 5 min at 1,200 RPM, room temperature (RT). Subsequently, the supernatant was removed and the cell pellet was resuspended in 20 ml of GM. The concentration of HT-29 cells was determined using a Neubauer counting chamber and cells were reseeded at a concentration of $1*10^6$ cells/cell culture flask. *CM of HT-29 cells was obtained during medium changes. CM was centrifuged (10 min, 3,000 RPM, RT), pooled, and stored at -20°C.*

4.2.2. Storage and preparation of PUFAs

EPA, DHA, and AA were stored at -20°C while dissolved in 100% ethanol. Before further usage in experiments, ethanol was removed by evaporation to prevent confounding effects on cell cultures. For this, nitrogen gas (preventing PUFA-oxidation) was used. PUFAs were then resuspended in GM.

4.2.3. Lipidomics of colorectal adenocarcinoma HT-29 cells

4.2.3.1. Incubation of cells with EPA, DHA, and AA

HT-29 cells were seeded in 100 x 20 mm cell culture dishes at concentrations of $3*10^6$ cells/well. After 24h, the supernatants were carefully removed and 5 ml of GM supplemented with 20 µM of AA, EPA, or DHA were added to cell cultures. After a subsequent 24h incubation, the supernatants were removed and 500 µl of ice-cold methanol was added to each well. A cell scraper was used to remove cells from the culture plates and the cell-methanol suspension was collected. 1,000 µl of ice-cold PBS was added to each dish to collect the remaining cells, and then transferred to the according cell-methanol suspension. Samples were stored at -20°C until analysis with LC/MS/MS.

4.2.3.2. LC/MS/MS

LC/MS/MS was performed in cooperation with Lipidomix GmbH, Berlin, Germany. 500 µl of water, 500 µl of methanol, and 10µl of internal standard solution (ISTD) were added to HT-29 cell suspension and vortex-mixed. Deuterium labeled metabolites were used for internal standards; these included LTB4-D4, 14,15-DHET-D11, 14,15-EET-D8, 20-HETE-D6, and 5-HETE-D8. Alkaline hydrolysis was performed to increase fatty acid yield of samples. For this**,** 300 µl of 10 M sodium hydroxide was added to samples and the resulting solution was exposed to 60°C for two cycles of 15 min. Each sample was vortex-mixed between the cycles. Samples were allowed to cool down to RT, neutralized with acetic acid (58%), and buffered with 2,000 µl of solid-phase extraction buffer (0.1 mol/l aqueous sodium acetate solution; pH 6) to a pH of 6. Subsequently, solid-phase extraction was performed using a Bond-Elut-Certify-II-Column. 2 ml of n-

hexane:ethyl acetate (4:1) was added to elute oxylipins. The organic phase was evaporated at 40°C under a fine stream of nitrogen, and samples were reconstituted in 100 µl acetonitrile.

The concentration of oxylipins in samples was measured with LC/MS/MS, using an Agilent 6460 Triple Quadrupole mass spectrometer with a Jet Stream ion source, operating in MRM with electrospray ionization (negative mode). The mass spectrometer was coupled to an Agilent 1200 HPLC (degasser, binary pump, well-plate sampler, thermostatted column compartment). The HPLC system used a Phenomenex Kinetex column as stationary phase. Reverse-phase chromatography was performed under gradient conditions with acetonitrile and 0.1% formic acid as mobile phase. The gradient was started at 45% formic acid and decreased to 31% within 14 minutes. The flow rate was 0.3 ml/min during the 20 min run time, with an injection volume of 7.5 µl. Drying gas was adjusted at 250°C/10 l/min, nebulizer at 30 psi, sheath gas at 380°C/10 l/min. The capillary voltage was optimized at 4,500 V (negative), and the nozzle voltage at 1,500 V (negative).

The sample concentrations were determined using linear calibration curves based on the relative peak area dependent on the target compound to ISTD concentration ratios. A complete list of measured target compounds and the corresponding conditions for MRM are shown below (Table 4.2.a).

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision Energy (eV)	Cell Accelerator Voltage (V)	Polarity
15-HEPE	317.2	219.1	120	5	1	Negative
18-HEPE	317.2	259.1	140	3	5	Negative
5-HEPE	317.2	115.1	110	6	1	Negative
12-HEPE	317.2	179.1	120	5	1	Negative
8-HEPE	317.2	155.1	110	7	2	Negative
9-HEPE	317.2	167.1	110	5	1	Negative
12-HETE	319.2	179.1	110	5	1	Negative
15-HETE	319.2	219.1	140	4	8	Negative
5-HETE	319.2	115.1	110	10	1	Negative
11-HETE	319.2	149.1	130	17	1	Negative
8-HETE	319.2	155.1	120	8	1	Negative
9-HETE	319.2	151.1	130	5	2	Negative
LTB_5	333.2	195.1	130	8	1	Negative
LTB_4	335.2	195.1	130	7	1	Negative
17-HDHA	343.2	201.1	110	6	6	Negative
4-HDHA	343.2	101.1	130	7	2	Negative
14-HDHA	343.2	205.1	110	6	2	Negative
10-HDHA	343.2	153.1	110	9	1	Negative
11-HDHA	343.2	149.1	110	5	1	Negative
13-HDHA	343.2	193.1	110	7	1	Negative
16-HDHA	343.2	233.1	130	6	5	Negative
20-HDHA	343.2	241.1	130	5	3	Negative
7-HDHA	343.2	141.1	110	4	1	Negative
8-HDHA	343.2	189.1	110	4	2	Negative
LXA_4	351.2	217.1	110	13	4	Negative
LXA_4	351.2	115.1	110	6	4	Negative
RvD_1	375.2	141.1	110	9	1	Negative

Table 4.2.a Target compounds for multiple reaction monitoring mode (MRM).

4.2.4. Experiments with peripheral blood mononuclear cells

4.2.4.1. Isolation from leukocyte depletion filters

The isolation of PBMCs from leukocyte depletion filters was initiated shortly after the blood donation of patients (within 2.5 hours). Filters were detached from the blood collection systems and one end of the filter was secured with a clip. The other end was flushed with 40 ml of PBS using a syringe (20 ml). To enhance the leukocyte yield, depletion filters were additionally flushed with 20 ml of air. After 30 s of soaking, the clip was removed and the resulting PBS-leukocyte suspension was collected in a cell culture flask. The average yield per depletion filter was approximately 80 ml of PBS-leukocyte suspension.

PBMCs were isolated from the PBS-leukocyte suspension by density gradient centrifugation (2,200 RPM, 20 min, RT, without break). For this, three Falcon tubes (50 ml) were filled with Ficoll-Paque™ PLUS (20 ml). Subsequently, 3 x 25 ml of the PBS-leukocyte suspension were overlaid onto the Ficoll-Paque™ PLUS. After 20 min of centrifugation, the buffy coats (PBMCs) were carefully collected from the interphase and washed with 30 ml of PBS. After a soaking period of 60 s, the PBMCs were again centrifuged (1,500 RPM, 5 min, 4°C) and the supernatant was discarded. 3 ml of Red Blood Cell Lysis Buffer (4.15 g NH_4Cl, 0.5 g $KHCO_3$, 0.02 g EDTA, 500 ml dH_2O; pH 7.2 - 7.4) was then added to cells to minimize the number of residual erythrocytes. 20 ml of GM was added to cells after 8 min and 30 s and the resulting suspension was then filtered, using a cell strainer. Subsequently, 15 ml of PBS were used to rinse the cell strainer. PBMCs were then centrifuged and washed twice with 25 ml of PBS (1,500 RPM, 5 min, 4°C for each centrifugation step). Finally, the PBMCs were assessed for viability (trypan blue exclusion) and counted, using a Neubauer counting chamber. Cells were then resuspended in GM at a concentration of $5*10^6$ PBMCs/ml.

4.2.4.2. Incubation with PUFAs and subsequent stimulation with LPS and CM

Day 1 – first 24 h incubation

*500µl of PBMCs, suspended in GM with a concentration of 5*10^6 PBMCs/ml, were seeded in 6-well cell culture plates. Subsequently, 500 µl of untreated GM (for controls) or GM supplemented with PUFAs (50 µM of EPA, DHA, or AA) was added to each well.*

Day 2 – second 24 h incubation

To evaluate the effect of incubation of PBMCs with PUFAs without subsequent stimulation, PBMCs from day 1 were supplemented with 500 µl of untreated GM. Supernatants of these cell cultures were termed PUFA-only (i.e. EPA-, DHA-, and AA-only).

Alternatively, PBMCs were stimulated with either 500 µl of CM or LPS, dissolved in 500 µl of GM. LPS was dissolved in GM so that the final concentration would be 1 ng/ml/well.

Day 3 – harvesting

Supernatants of wells were transferred into 2.0 ml Eppendorf vials and centrifuged (3,000 RPM, 5 min, 4°C) to remove cells and debris. 1,300 µl of the supernatant was then transferred to new 2.0 ml Eppendorf vials.

Supernatants of untreated PBMCs (500 µl of GM was added on day 1 and 2) and PBMCs stimulated with LPS or CM on day 2, with no prior PUFA incubation on day 1 (termed LPS and CM controls), were used as controls. Experiments evaluating the effect of incubation of PBMCs with PUFAs without subsequent stimulation (termed PUFA-only, i.e. EPA-, DHA-, and AA-only) were set up in single cell cultures. All other experimental and control groups were set up in triplicates. All supernatants were stored at -20°C until subsequent cytokine analysis.

4.2.4.3. Cytokine analysis (ELISA)

Cytokine levels (TNF-α, IL-6, and IL-10) in supernatants of cell cultures were measured in duplicates by using ELISA. All measurements were carried out as recommended by the manufacturer. For each ELISA plate, a standard curve was created by using known concentrations of the cytokine in question. The standard curve ranges were 4 - 500 pg/ml for TNF-α, 2 - 200 pg/ml for IL-6, and 2 - 300 pg/ml for IL-10. *Samples were diluted with GM to lie within these ranges. The absorption of the wells was quantified at a wavelength of 450 nm.*

4.2.5. Statistical analysis

For statistical analysis, GraphPad Prism 6.0f Software was used. The analysis of data was confirmed by the Department of Statistics at the Ludwig Maximilians University, Munich, Germany. A p-value of < 0.05 was considered to be statistically significant.

4.2.5.1. Lipidomics of colorectal adenocarcinoma HT-29 cells

Data from experiments with LC/MS/MS is presented as mean metabolite concentration ± standard error of the mean (SEM). Statistical analysis was conducted with analysis of variance (ANOVA), followed by the Tukey-Kramer test for multiple comparisons. The results represent data obtained from 4 independent experiments.

4.2.5.2. Experiments with peripheral blood mononuclear cells

Results for experiments with PBMCs showed considerable variations in absolute cytokine levels of different donors after stimulation. The results are therefore standardized to be expressed as ratios compared to LPS- and CM-controls. These ratios show the relative mean ± SEM of cytokine levels with respect to the LPS- or CM-controls of each donor. The Student's t-test was used to test for significant differences between experimental groups and controls. ANOVA, followed by the Tukey-Kramer test for multiple comparisons, was used to compare mean stimulated cytokine ratios of experimental groups. A total of 5 PBMC donors were included in the study.

5. Results

Parts of the results of experiments with PBMCs have been published in two recent articles by the author (1, 2). Passages of the following chapter that are similar to results described previously by the author are italicized. Previously published figures are identified in the corresponding description of the figure.

5.1. Lipidomics of colorectal adenocarcinoma HT-29 cells

In a first series of experiments, we tested HT-29 cell cultures for their OH-FA content. LC/MS/MS revealed that HT-29 cell cultures, incubated with EPA, DHA, or AA, are capable of HEPE, HDHA, and HETE formation. These OH-FAs were detected in all samples. Representative chromatograms of HEPE, HDHA, and HETE compounds are shown in Figure 5.1.a to Figure 5.1.d. LC/MS/MS did not measure detectable levels of the polyhydroxylated lipid metabolites LTB_4, LTB_5, LXA_4, and RvD_1 (0 ng/g in all samples).

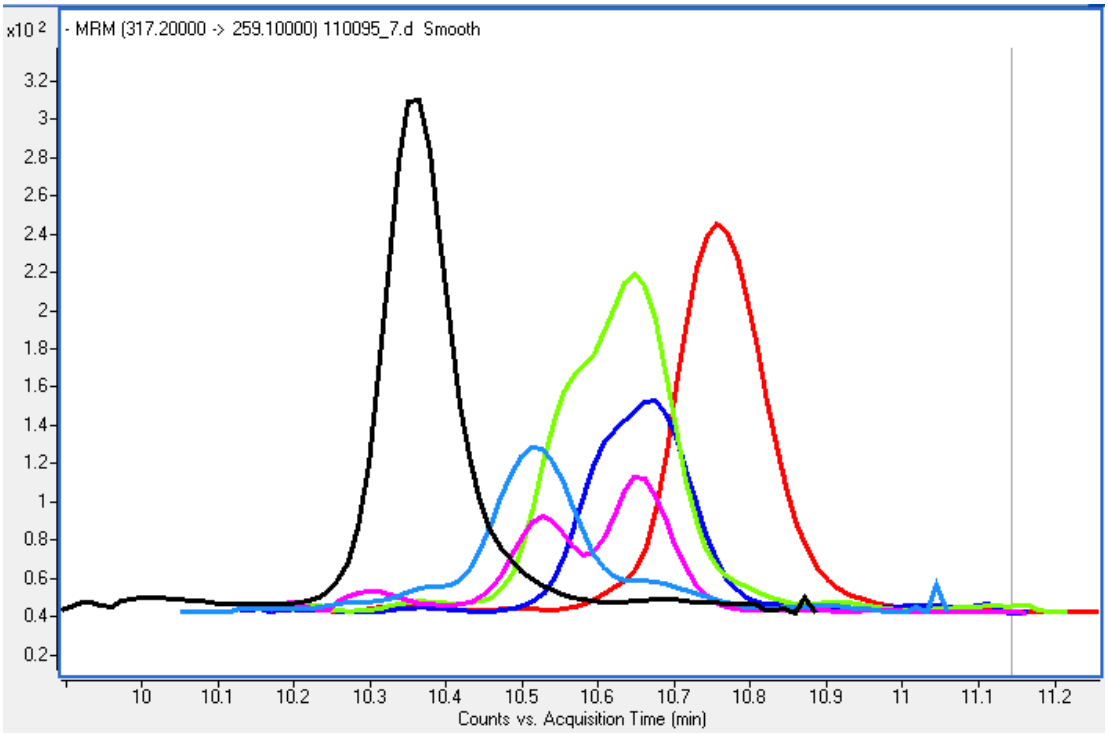

Figure 5.1.a Representative chromatogram of HEPE compounds. 18-HEPE (black), 15-HEPE (light blue), 12-HEPE (pink), 9-HEPE (green), 8-HEPE (dark blue), 5-HEPE (red).

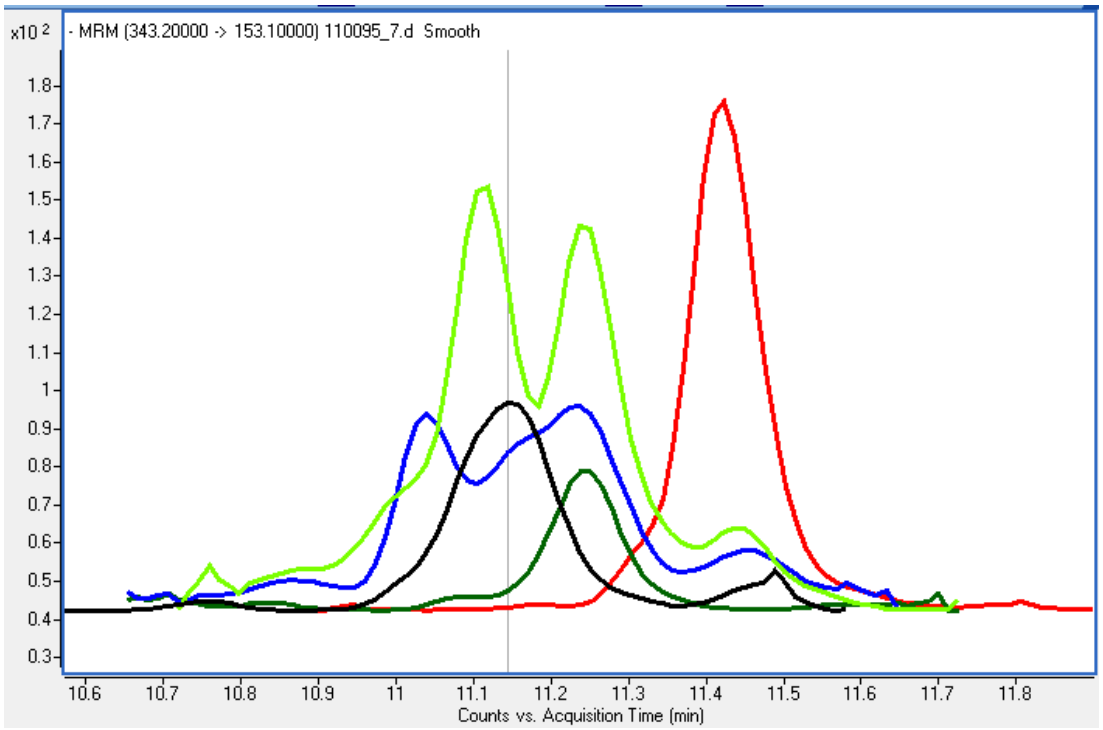

Figure 5.1.b Representative chromatogram of HDHA compounds (I). 11-HDHA (blue), 10-HDHA (black), 8-HDHA (light green), 7-HDHA (dark green), 4-HDHA (red).

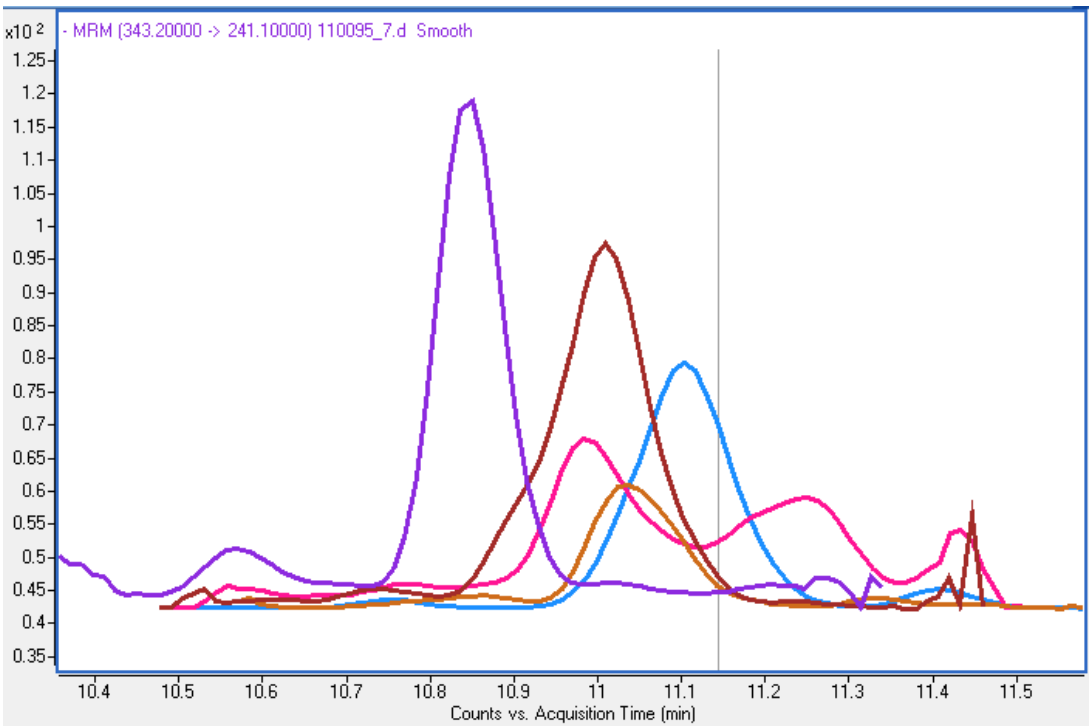

Figure 5.1.c Representative chromatogram of HDHA compounds (II). 20-HDHA (purple), 17-HDHA (pink), 16-HDHA (brown), 14-HDHA (ocher), 13-HDHA (light blue).

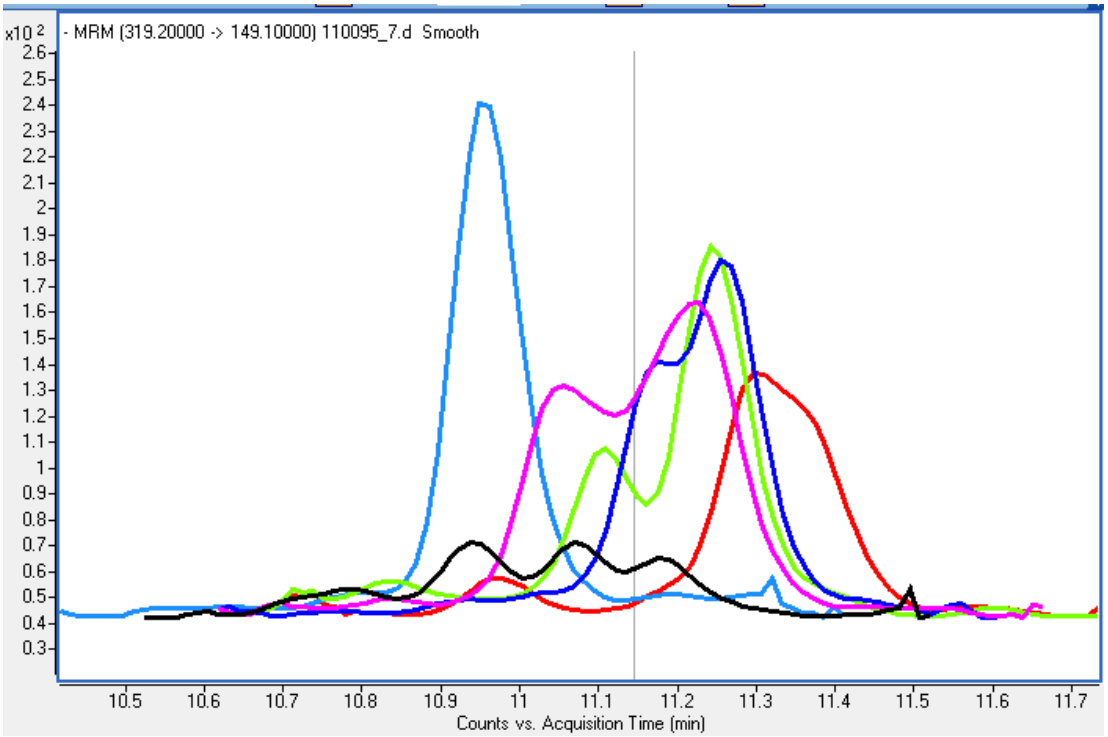

Figure 5.1.d Representative chromatogram of HETE compounds.15-HETE (light blue), 12-HETE (pink), 11-HETE (black), 9-HETE (green), 8-HETE (dark blue), 5-HETE (red).

5.1.1. The effect of incubation with different PUFAs on HEPE, HDHA, and HETE synthesis

ANOVA of HEPE levels after incubation with different PUFAs indicated significantly different concentrations for 5-HEPE ($F(2, 9) = 11.61$; $p = 0.0032$), 8-HEPE ($F(2, 9) = 5.19$; $p = 0.032$), and 15-HEPE ($F(2, 9) = 9.10$; $p = 0.0069$). As discussed in chapter 3.2.2, the production of HEPEs is dependent on their precursor EPA. Consequently, the Tukey-Kramer post-hoc test for multiple comparisons revealed that 5-HEPE, 8-HEPE, and 15-HEPE were significantly increased in HT-29 cell cultures that were incubated with EPA (p of at least < 0.05) (Figure 5.1.e). Furthermore, total HEPE concentration was also significantly different between the three groups ($F(2, 9) = 6.01$; $p = 0.022$), with elevated levels in cell cultures that were incubated with EPA ($p < 0.05$).

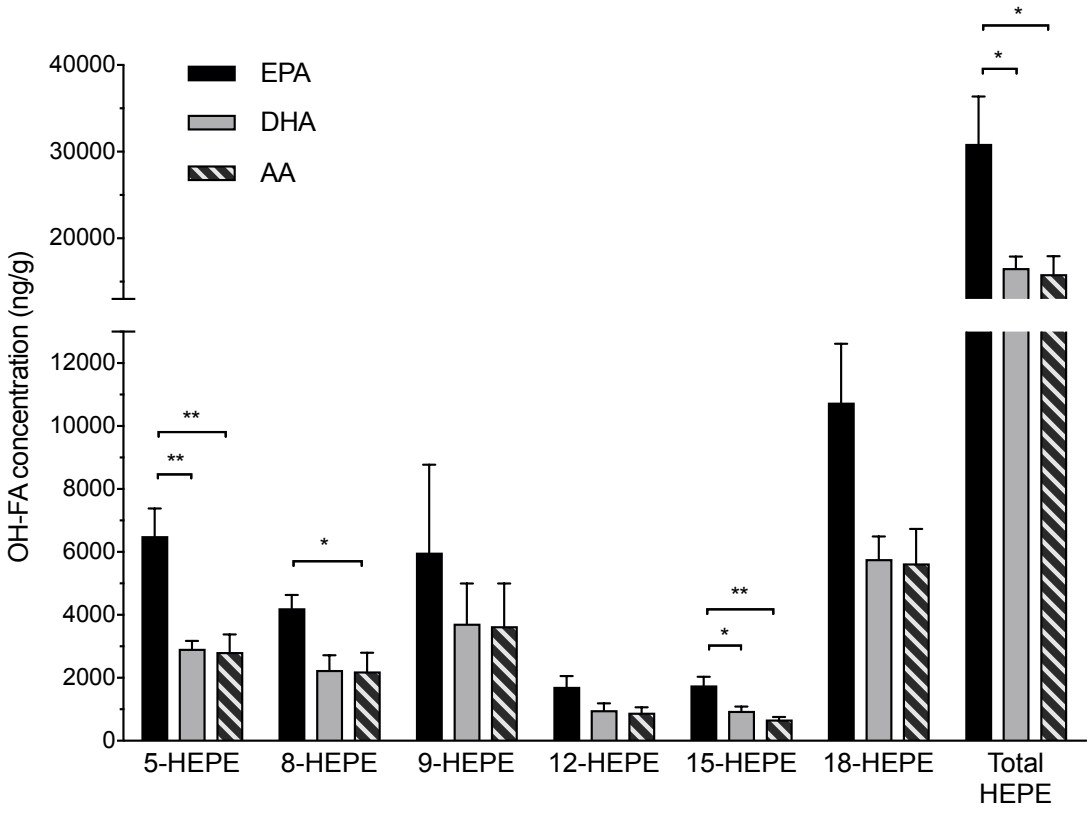

Figure 5.1.e Concentration of HEPE compounds in HT-29 cell cultures after incubation with EPA, DHA, or AA. Data is expressed as the mean hydroxylated fatty acid (OH-FA) concentration of 4 independent experiments. Error bars indicate the standard error of the mean. *$p < 0.05$, **$p < 0.01$.

Moreover, ANOVA indicated significantly different concentrations of 18-HEPE ($F(2, 9) = 4.87$; $p = 0.037$). However, the Tukey-Kramer post-hoc test for multiple comparisons revealed that the observed EPA-induced increase of 18-HEPE was not significant when compared to other groups ($p = 0.061$ when compared to incubation with DHA and 0.055 when compared to incubation with AA) (Figure 5.1.e).

In contrast to findings with EPA, incubation with DHA, the precursor for HDHA compounds, did not result in an increased formation of individual or total HDHA levels (Figure 5.1.f). Similarly, when compared to treatment with EPA or DHA, incubation with AA failed to significantly increase the concentration of HETE compounds (Figure 5.1.g).

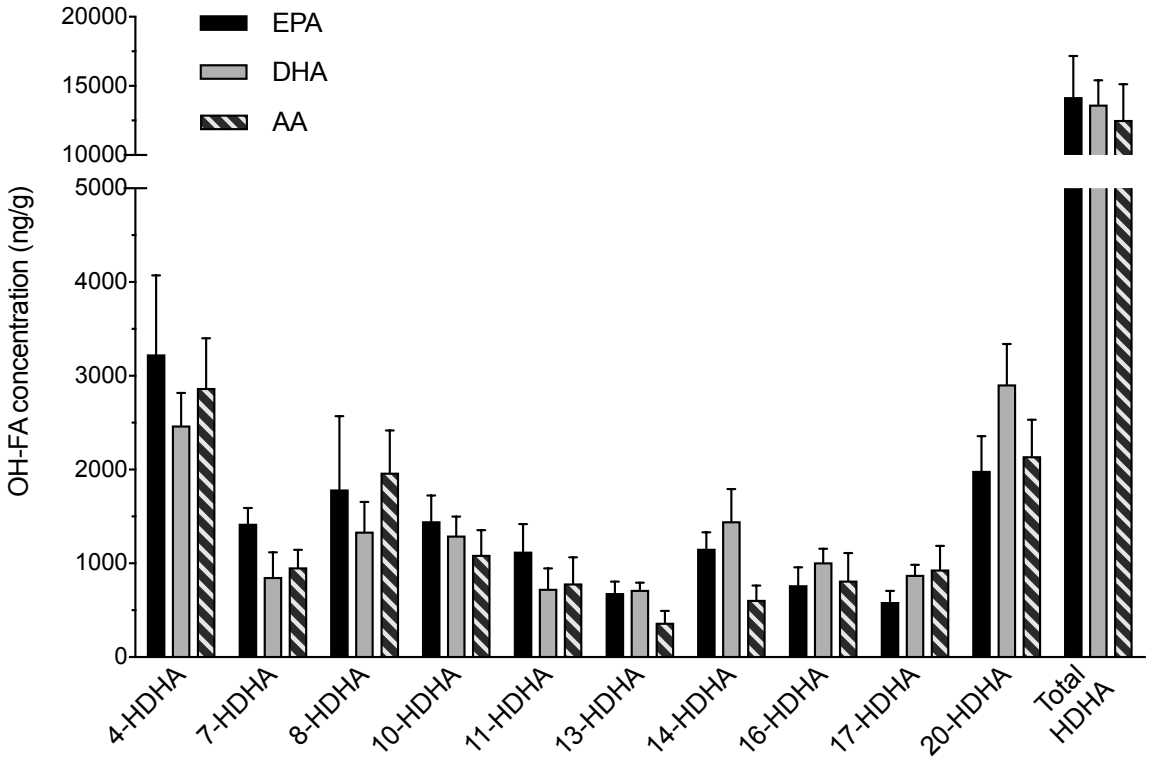

Figure 5.1.f Concentration of HDHA compounds in HT-29 cell cultures after incubation with EPA, DHA, or AA. Data is expressed as the mean hydroxylated fatty acid (OH-FA) concentration of 4 independent experiments. Error bars indicate the standard error of the mean.

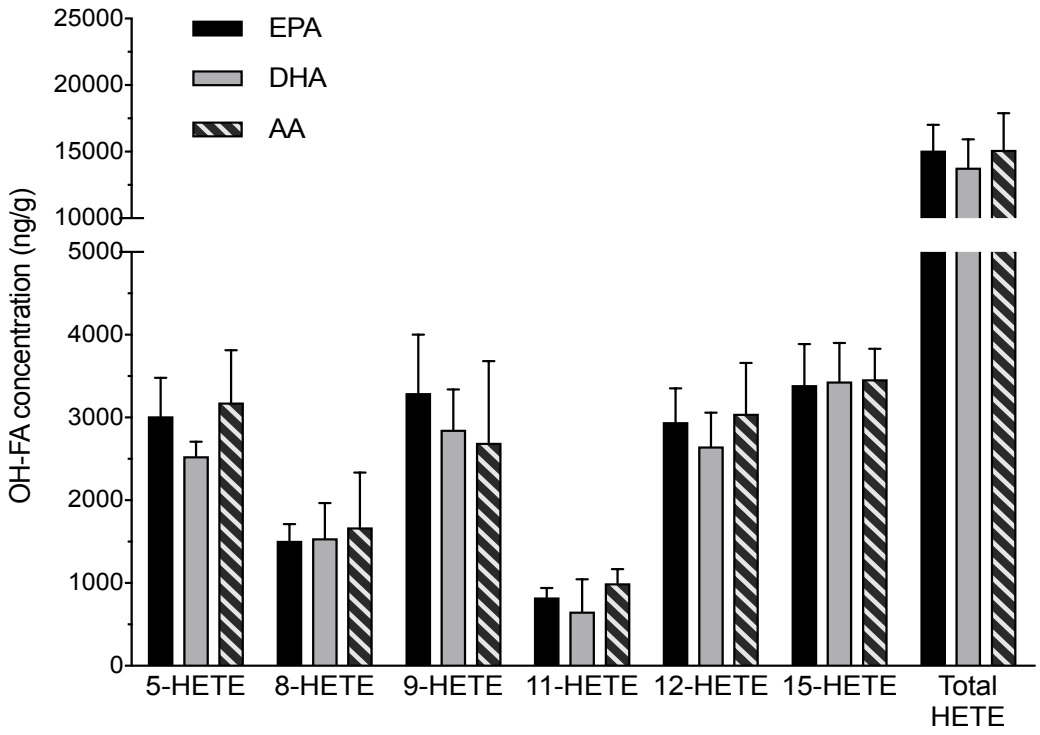

Figure 5.1.g Concentration of HETE compounds in HT-29 cell cultures after incubation with EPA, DHA, or AA. Data is expressed as the mean hydroxylated fatty acid (OH-FA) concentration of 4 independent experiments. Error bars indicate the standard error of the mean.

5.2. Experiments with peripheral blood mononuclear cells

In a second series of experiments, we investigated the effects of EPA, DHA, and AA on the cytokine secretion of human PBMCs. For this, a method for the isolation of PBMCs from leukocyte depletion filters of blood bank donors was established, yielding an average of $1.58 \pm 0.18 * 10^8$ cells per donor, with a cell viability of > 95%. We found that incubation of PBMCs with DHA, and to a lesser extend with EPA (only when compared to AA), promoted a pro-inflammatory reaction in response to stimulation with LPS and CM. Treatment with AA, on the other hand, significantly reduced TNF-α secretion.

5.2.1. Controls and PUFA-only samples

Stimulation of PBMCs with LPS or CM resulted in significantly elevated concentrations of TNF-α, IL-6, and IL-10 ($p < 0.0001$, when compared to untreated PBMCs). *No significant difference was found in cytokine levels of supernatants of PUFA-only samples when compared to each other, or to levels found in untreated PBMCs. When compared to LPS- or CM-controls, TNF-α- and IL-10-levels in supernatants of PUFA-only samples were significantly lower ($p < 0.01$). Interestingly, ELISA of supernatants of PBMCs incubated only with AA (AA-only) measured slightly elevated IL-6 levels. The increase in IL-6 was not statistically significant when compared to levels found in supernatants of other PUFA-only samples or untreated PBMCs*. However, IL-6 levels of LPS controls ceased to be significantly higher than AA-only samples ($p = 0.17$) (Figure 5.2.a.i). Mean IL-6 levels of PBMCs stimulated with CM (CM control), on the other hand, were significantly higher than the ones observed in AA-only samples ($p < 0.01$) (Figure 5.2.a.ii).

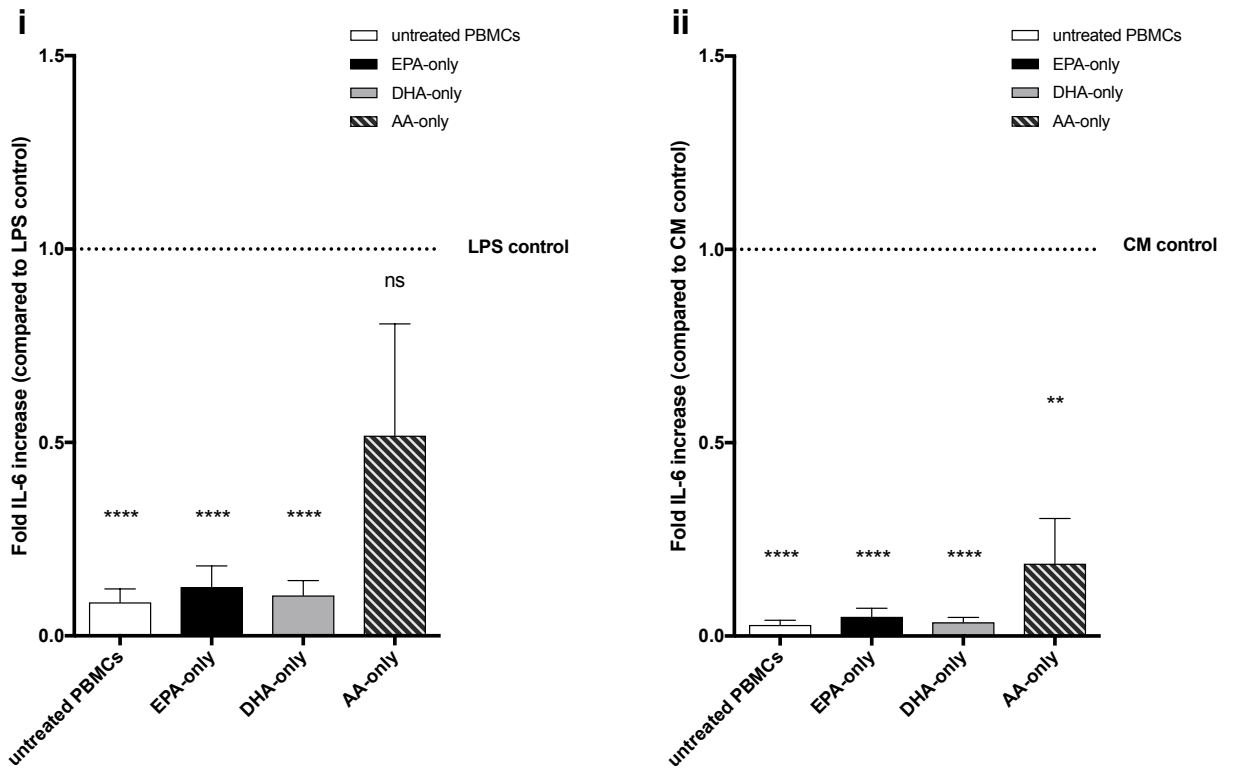

Figure 5.2.a.i and ii IL-6 levels for controls and PUFA-only samples. **i** - Stimulation with LPS: IL-6 concentration in AA-only was similar to levels observed in LPS controls ($p = 0.17$). IL-6 levels in untreated PBMCs and EPA- and DHA-only were significantly lower than in LPS controls. **ii** - Stimulation with CM: IL-6 levels in untreated PBMCs and EPA-, DHA- and AA-only were significantly lower than in CM controls. Data is expressed as the relative mean with respect to LPS or CM controls of 5 PBMC donors. Error bars indicate the relative standard error of the mean. ****$p < 0.0001$; **$p < 0.01$; non-significant p-values are indicated by ns.

5.2.2. The effect of incubation with different PUFAs on LPS- and CM-induced cytokine secretion

Incubation with 50 μM of EPA did not affect the ability of PBMCs to secrete TNF-α, IL-6, or IL-10 after stimulation with LPS (Figure 5.2.b). Similarly, incubation with DHA had no significant effect on TNF-α and IL-6 levels. On the other hand, IL-10 secretion was significantly decreased after incubation with DHA. IL-10 levels were 0.41-fold of levels detected in LPS controls ($p < 0.01$).

While incubation with AA did not affect the LPS-induced secretion of IL-6 and IL-10, TNF-α levels were significantly reduced (Figure 5.2.b). Thus, when compared to LPS controls, TNF-α levels were 0.41-fold lower in supernatants of PBMCs that were incubated with AA ($p < 0.01$).

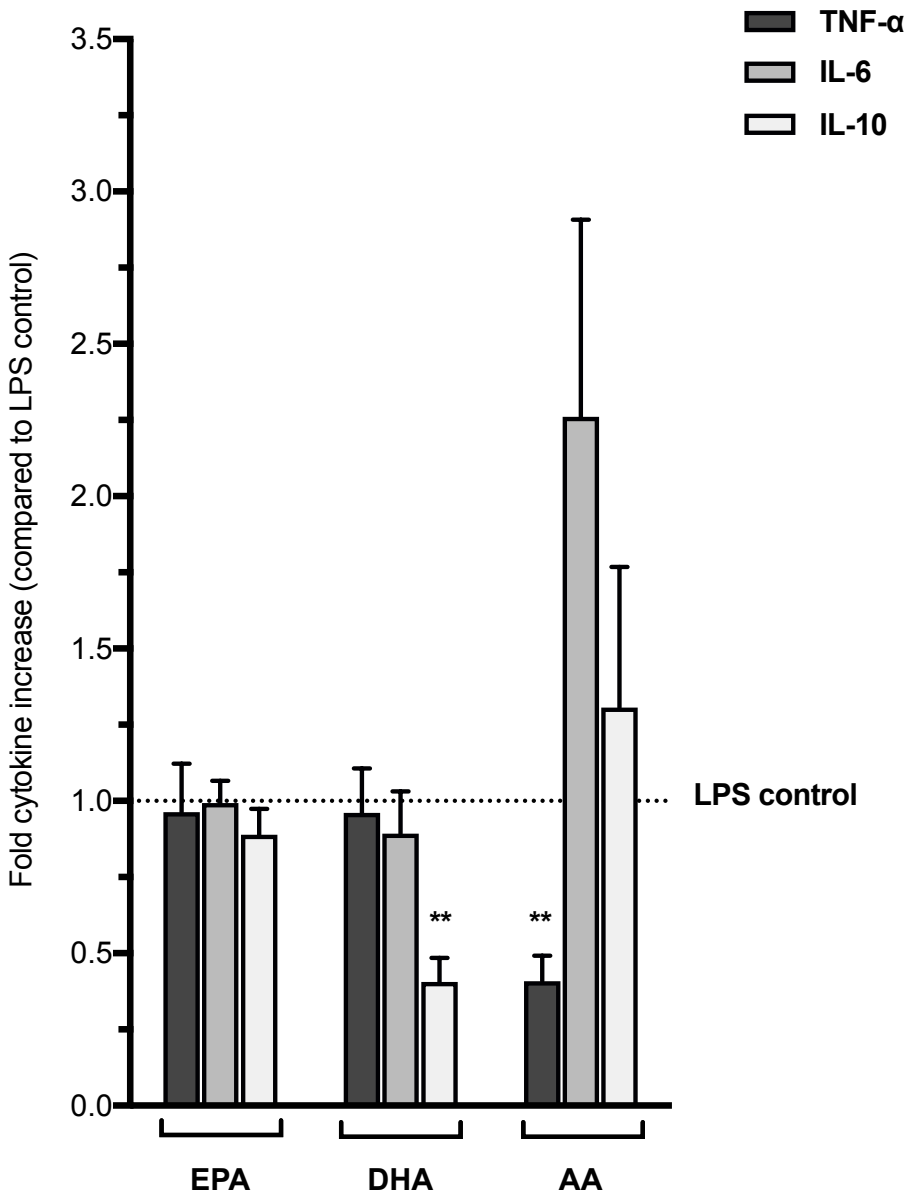

Figure 5.2.b Effect of incubation with n-3 PUFAs (EPA and DHA) and the n-6 PUFA AA on LPS-induced cytokine secretion by PBMCs. Data is expressed as the relative mean with respect to LPS controls of 5 PBMC donors. Error bars indicate the relative standard error of the mean. $^{**}p < 0.01$. This figure was recently published by the author, reproduced with permission from: (1, 2).

Additionally, we evaluated the effects of EPA, DHA, and AA on CM-induced cytokine secretion by PBMCs (Figure 5.2.c). Similar to experiments with LPS, incubation with EPA failed to affect the CM-induced cytokine secretion of PBMCs. TNF-α, IL-6, and IL-10 levels were similar to CM controls.

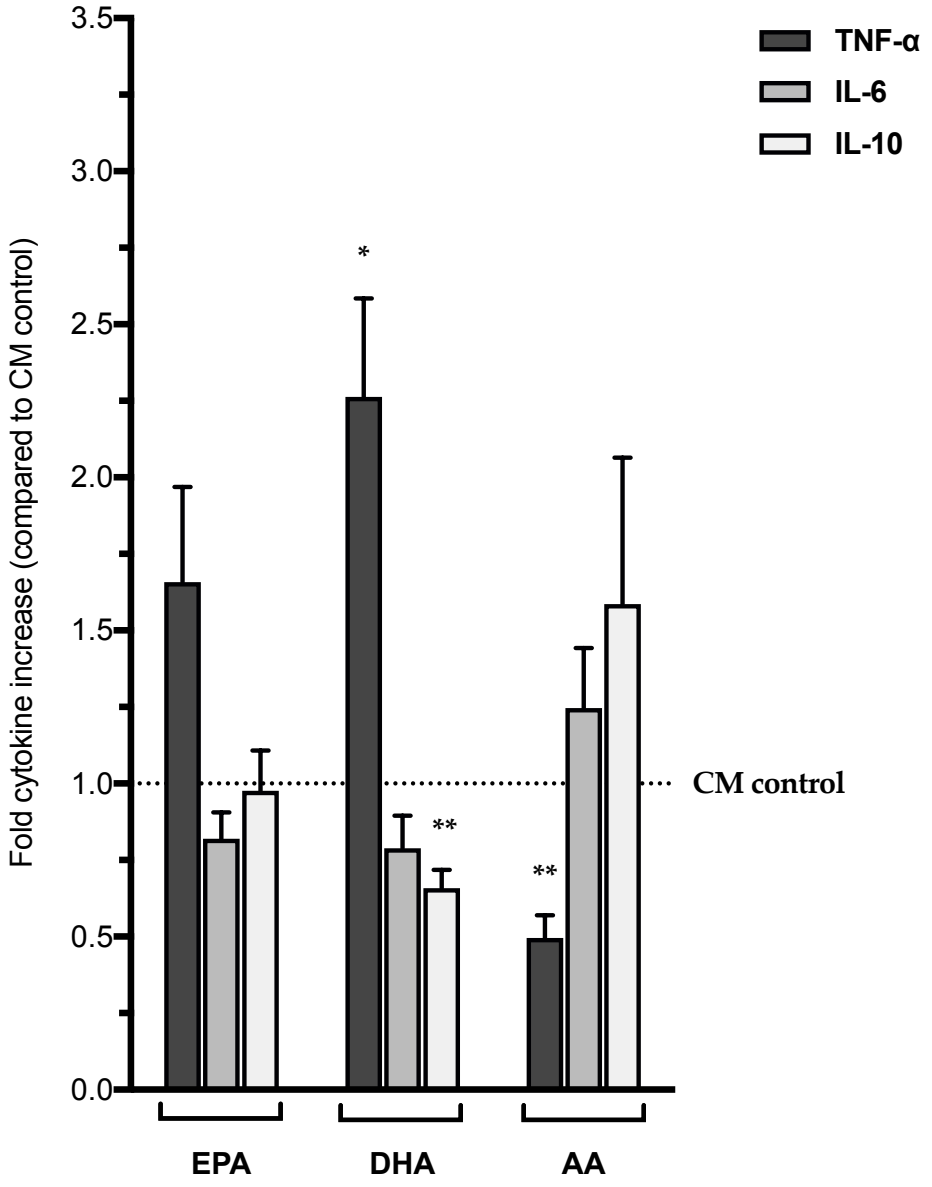

Figure 5.2.c Effect of incubation with n-3 PUFAs (EPA and DHA) and the n-6 PUFA AA on CM-induced cytokine secretion by PBMCs. Data is expressed as the relative mean with respect to CM controls of 5 PBMC donors. Error bars indicate the relative standard error of the mean. *$p < 0.05$; **$p < 0.01$. This figure was recently published by the author, reproduced with permission from: (1, 2).

Incubation of PBMCs with DHA resulted in a significantly altered inflammatory response. While secretion of TNF-α increased to 2.26-fold, secretion of IL-10 was diminished to 0.66-fold as compared to CM controls ($p < 0.05$ and < 0.01, respectively) (Figure 5.2.c).

Incubation with AA did not affect CM-induced IL-6 and IL-10 secretion. Conversely, TNF-α secretion was significantly reduced by AA to 0.49-fold as compared to CM controls ($p < 0.01$) (Figure 5.2.c).

5.2.3. Analysis of variance between experimental groups (EPA, DHA, and AA)

ANOVA revealed a significant difference between the experimental groups in the secretion of TNF-α. We were able to demonstrate this in experiments with LPS- ($F(2, 12) = 5.76$; $p = 0.018$), as well as with CM-induced secretion of TNF-α ($F(2, 12) = 11.80$; $p = 0.0015$). The Tukey-Kramer post-hoc test for multiple comparisons revealed that secretion of TNF-α by PBMCs was significantly reduced after incubation with AA when compared to levels found in PBMCs incubated with n-3 PUFAs (EPA and DHA): In experiments with LPS-induced cytokine secretion, the mean ratios for TNF-α were 0.41 ± 0.08 for AA vs. 0.96 ± 0.16 for EPA and 0.96 ± 0.15 for DHA ($p < 0.05$) (Figure 5.2.d); in experiments with CM-induced cytokine secretion, the mean rations for TNF-α were 0.50 ± 0.07 for AA vs. 1.66 ± 0.31 for EPA and 2.26 ± 0.32 for DHA (p of at least < 0.05) (Figure 5.2.e).

Additionally, ANOVA indicated a significant difference between experimental groups for levels of LPS-induced secretion of IL-6 ($F(2, 12) = 3.93$; $p = 0.049$). The Tukey-Kramer post-hoc test for multiple comparisons, however, revealed, that when compared with each other, no significant difference was found between the three experimental groups. ANOVA for CM-induced secretion of IL-6 and LPS- and CM-induced secretion of IL-10 showed no significant difference when comparing different experimental groups with each other (data not shown).

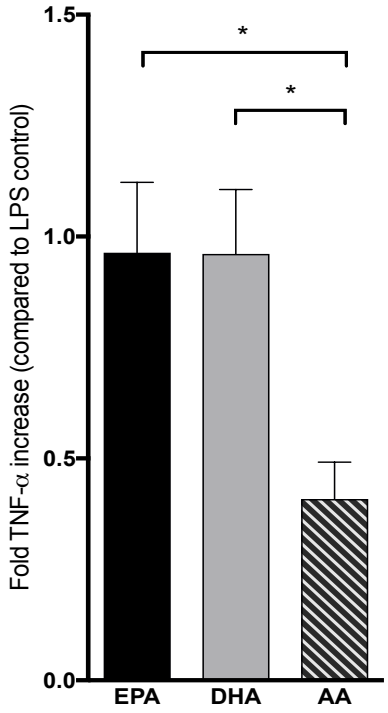

Figure 5.2.d Relative changes in TNF-α secretion between experimental groups after stimulation with LPS. Data is expressed as the relative mean with respect to LPS controls of 5 PBMC donors. Error bars indicate the relative standard error of the mean. *$p < 0.05$.

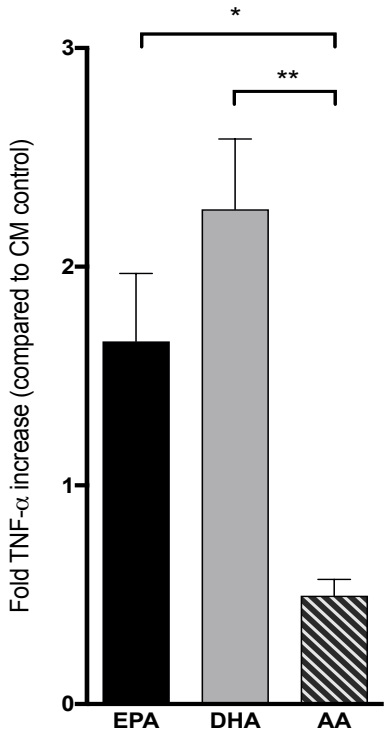

Figure 5.2.e Relative changes in TNF-α secretion between experimental groups after stimulation with CM. Data is expressed as the relative mean with respect to CM controls of 5 PBMC donors. Error bars indicate the relative standard error of the mean. *$p < 0.05$; **$p < 0.01$.

6. Discussion

6.1. Summary of findings

The study presented here investigated the *in vitro* effect of EPA, DHA, and AA on the fatty acid metabolism of colorectal adenocarcinoma HT-29 cells, and on the cytokine secretion of PBMCs. Regarding our hypothesis for experiments on the lipid metabolism of HT-29 cells, we were able to confirm the expected effect of EPA on the synthesis of OH-FAs. Analysis with LC/MS/MS demonstrated that incubation with EPA significantly increased the formation of HEPE compounds in HT-29 cell cultures. Conversely, neither DHA nor AA had an effect on the concentration of OH-FAs when compared to incubation with other PUFAs.

The experiments with human PBMCs allowed for several conclusions. First, LPS and CM markedly increased the TNF-α, IL-6, and IL-10 secretion of PBMCs, while incubation with n-3 PUFAs alone had no significant effect on cytokine secretion. Secondly, the results confirmed our experimental hypothesis by demonstrating that the n-3 PUFA DHA and the n-6 PUFA AA alter the secretion of TNF-α and IL-10 by activated PBMCs. In the established experimental setup, DHA seemed to increase the inflammatory state of PBMCs. Thus, DHA reduced IL-10 (stimulation with LPS and CM) and increased TNF-α secretion (stimulation with CM) (Figure 5.2.b and Figure 5.2.c). Conversely, incubation with AA appears to result in an anti-inflammatory response by reducing TNF-α secretion (stimulation with LPS and CM). These implications are also supported by comparison of LPS- and CM-induced TNF-α levels in supernatants of PBMCs after incubation with n-3 PUFAs versus incubation with AA (Figure 5.2.d and Figure 5.2.e). Our findings are in accordance with the results of several previous studies, which suggest that the paradigm that EPA and DHA exert anti-inflammatory effects, while AA is solely pro-inflammatory, is an oversimplification with several exceptions.

The following section intends to interpret the findings of this study in the context of the role of n-3 and n-6 PUFAs in inflammatory processes as well as in the prevention and treatment of CRC. The results of experiments on the lipid metabolism of HT-29 cells will

be discussed in the context of similar studies that explored the effects of PUFAs on lipid mediator synthesis in CRC (chapter 6.2). The results of experiments with PBMCs will be discussed in chapter 6.3. We will first evaluate the stimulation of PBMCs (through LPS and CM), and assess the role of measured cytokines (TNF-α, IL-6, and IL-10) with regard to inflammation and cancer. Subsequently, the implications of results will be discussed in the context of other studies that demonstrated the complex and often diverse effects of n-3 and n-6 PUFAs on immune cells. Finally, we will analyze the conducted experiments critically, and summarize the implications of our results, as well as future research directions in a concluding chapter.

6.2. Lipidomics of colorectal adenocarcinoma HT-29 cells

In this study we have show that HT-29 cell cultures are capable of forming OH-FAs (Figure 5.1.e, Figure 5.1.f, and Figure 5.1.g). Furthermore, when compared to treatment with DHA or AA, incubation with EPA increased the formation of HEPE compounds in HT-29 cell cultures (Figure 5.1.e). In a recent study by Ostermann and colleagues it was demonstrated that cell culture medium such as the one used in this study contains no relevant concentration of OH-FAs (118). Yet in contrast to the results presented here, the authors found few or no OH-FAs in the supernatants of HT-29 cells after incubation with EPA, DHA, or AA. Moreover, LC/MS/MS of GM supplemented with 50 μM of EPA, DHA, or AA showed that a number of HEPE, HDHA, and HETE compounds also formed through hydrolysis and autoxidation, independent of HT-29 cells. It was concluded that HT-29 cells secreted few OH-FAs into the surrounding GM. However, a crucial difference between the present study and the one by Ostermann *et al*., is that we not only assessed cell supernatants, but whole cell cultures for their OH-FA content (118). It is therefore likely that the method used in this study also detected OH-FAs that were bound inside HT-29 cells. This may explain why, in contrast to Ostermann and colleagues, we detected relevant concentrations of all OH-FAs measured, including the formation of OH-FAs that originate from a PUFA other than the one used for incubation (e.g. formation of HDHAs after incubation with AA). Nevertheless, we cannot rule out the possibility that the observed changes in OH-FA concentration are affected by hydrolysis and autoxidation independent of HT-29 cells.

Keeping this limitation in mind, this study is one of the first to show that HT-29 cell cultures contain OH-FAs. These derivatives may therefore also be formed in other CRC cells, *in vitro* and *in vivo*, thereby possibly affecting tumor development. However, while a relevant formation of OH-FAs was detected in all cell cultures, neither DHA nor AA significantly increased the synthesis of their OH-FA derivatives when compared to incubation with other PUFAs (Figure 5.1.f and Figure 5.1.g). Additionally, none of the polyhydroxylated lipid metabolites which were assessed in this study (LTB_4, LTB_5, LXA_4, and RvD_1) were detected by LC/MS/MS. A possible reason for these findings is the low PUFA to cell ratio that was used in the present study. When compared to similar studies, we used significantly lower PUFA concentrations for incubation with HT-29 cells (118, 220, 257). The PUFA concentration in the present study was 20 μM, while

other studies reported a dosage ranging between 50 and 180 µM (118, 220, 257). This resulted in a relatively low PUFA to cell ratio for our experiments (e.g. 1 µM to 150,000 cells in the present study vs. 1 µM to 15,000 cells used by Ostermann and colleagues), and is a likely reason for the results obtained after incubation with DHA and AA, as well as for the inability to detect LTB_4, LTB_5, LXA_4, and RvD_1. Future studies should therefore consider using higher PUFA concentrations, resulting in an increased PUFA to cell ratio.

Lastly, we were able to demonstrate that incubation with EPA led to a significantly increased formation of 5-, 8-, and 15-HEPE, as well as of total HEPEs in HT-29 cell cultures (Figure 5.1.e). This finding agrees with our hypothesis about the effects of EPA on the OH-FA metabolism. The observed increase in HEPEs after incubation with EPA may be a mechanism for how EPA exerts its previously described effects on CRC (219-221). Indeed, Vang and Ziboh demonstrated that 15-HEPE reduced proliferation of cancer cells (230). Thus, a possible pathway by which EPA exerts its anti-tumorigenic effects on CRC cells may be via their transformation into anti-proliferative and pro-apoptotic OH-FAs, such as 15-HEPE.

6.3. Experiments with peripheral blood mononuclear cells

6.3.1. Isolation and stimulation of peripheral blood mononuclear cells

In the current study, we established a method to isolate high numbers of viable PBMCs from leukocyte depletion filters (average yield of $1.58 \pm 0.18 * 10^8$ cells per donor). Previous studies, which isolated PBMCs from whole blood and/or leukocyte depletion filters using Ficoll-Paque separation, showed that T- and B-lymphocytes make up the majority of PBMCs (up to 75%) (258-262). It is therefore likely that the observed effects of PUFAs on cytokine secretion were mediated by lymphocytes. Additional cell subsets found in PBMCs include monocytes and NK cells, which make up approximately 10 to 15% each (258-261). The proportion of granulocytes is limited (usually < 2%) but increases considerably with prolonged storage times (≥ 24h) (261, 262). In order to minimize this effect, PBMCs were isolated from filters directly after blood donation (within 2.5 hours).

After isolation, PBMCs were stimulated with LPS or CM. In this context, incubation of immune cells with LPS is one of the best-studied methods for stimulating immune cells with a bacterial component (263). LPS are found in the cell wall of gram-negative bacteria, and are recognized by immune cells by binding to cell surface molecules (e.g. Toll-like receptor 4) (264). LPS-induced activation of immune cells results in the upregulation of several intracellular transduction pathways (e.g. NF-κB as well as activator protein-1, AP-1) (264-268). In turn, these induce the secretion of cytokines by PBMCs and their subsets. This effect has been repeatedly observed in numerous studies and was also demonstrated in the current study (265, 266, 269-273).

Conversely, the ability of conditioned media from CRC cells to stimulate PBMCs is a relatively new observation (274, 275). In their *in vitro* studies, Bessler *et al.* hypothesized that soluble factors secreted by CRCs contribute to a reciprocal relationship between cancer and immune cells (274, 276). Thus, while no detectable cytokines were found in the CM of CRC cell lines, such as HT-29 cells, incubation with immune cells led to a partly dose-dependent secretion of pro- and anti-inflammatory cytokines (274, 276, 277). The results of the present study confirm these findings. The CM significantly increased levels of TNF-α, IL-6, and IL-10.

Extracellular vesicles (microvesicles and exosomes) secreted by cancer cells have been proposed as an explanation for the observed cell-to-cell communication. Thus, by secreting microvesicles and exosomes, malignant cells have been found to modulate the behavior of immune cells, including an increased secretion of pro- and anti-inflammatory cytokines (278-282).

6.3.2. Measured cytokines in the context of inflammation and CRC

PBMCs were incubated with EPA, DHA, and AA to observe their effects on the secretion of TNF-α, IL-6, and IL-10. In general, TNF-α and IL-6 are referred to as pro-inflammatory cytokines, which are synthesized by various immune cells, such as lymphocytes and monocytes (209, 283-286). In this context, TNF-α is one of the major mediating cytokines in inflammation (287). After secretion, it activates leukocytes and triggers the production of other pro-inflammatory cytokines (283, 288). Numerous genes, integral for inflammatory processes, have been shown to be regulated by TNF-α-induced activation of NF-κB. These include COX-2, LO-2, and anti-apoptotic proteins (287, 289). Similarly, IL-6 has proven to be essential for the inflammatory response, triggering antibody production and B-lymphocyte differentiation (284, 288, 290, 291). Given its predominantly pro-inflammatory effects, IL-6 plays a pathogenic role in the development of colitis, leading to the formation of CAC (197, 292, 293). In addition, a considerable body of evidence demonstrated that IL-6 promotes the proliferation of CRC cells (210, 277, 286, 294).

The role of TNF-α in the microenvironment of tumors, however, is less clear (1, 2). Barth *et al.* assessed the cytokine expression of tumor-infiltrating lymphocytes in CRC and concluded that expression of TNF-α by even a small proportion of lymphocytes (≥ 3%) correlated with better overall survival (295). Conversely, a more recent study demonstrated that tumor-infiltrating lymphocytes enhance the growth of CRC cells by secreting a combination of cytokines, including TNF-α (296). In this context, *in vitro* and animal studies revealed that TNF-α may exert both pro- and anti-tumorigenic effects on (colorectal) cancer cells (197, 297-302). These opposing activities are most likely the result of different intracellular signaling pathways, initiated by the TNF-dependent trimerization (activation) of TNF-α receptors (2, 297, 302). Thus, NF-κB and AP1

activation via the recruitment of TNFR associated factor 2 (TRAF2) plays an essential role in preventing apoptosis in cancer cells. However, activation of TNF-α receptors may also activate the Fas-associated death domain protein (FADD), initiating the caspase cascade and subsequent cell death (289, 297, 302-305).

IL-10 is a prototypic anti-inflammatory cytokine which is secreted by immune cells, such as T-lymphocytes, B-lymphocytes, and monocytes (306, 307). It decreases the production of pro-inflammatory cytokines (including TNF-α and IL-6) and its main biological function is to limit and terminate the inflammatory response (196, 303, 308-310). Similarly to TNF-α, the role of IL-10 in the context of cancer has proved to be controversial (1, 2). For one, IL-10 reduces antigen-specific T-lymphocyte proliferation. High levels of IL-10 may therefore enhance immune evasion of cancer cells, resulting in tumor progression (1, 2, 192, 310, 311). In contrast, IL-10 has also been shown to prevent tumorigenesis, including the development of CRC. Several studies have demonstrated that IL-10 knockout mice develop chronic enterocolitis and CAC (196, 312, 313). Additionally, the transfer of IL-10 secreting T regulatory lymphocytes (Tregs) has been shown to prevent intestinal adenoma formation in $Apc^{Min/+}$ mice (196, 314).

6.3.3. Implications of results in the context of inflammation

The activation of immune cells with LPS is a well-established method to simulate an inflammatory process *in vitro.* Similarly, IL-10 and TNF-α have been shown to act as essential anti- and pro-inflammatory cytokines, respectively. By taking these previous findings into account, the results of this study indicate that DHA augments the inflammatory reaction of PBMCs, while AA has anti-inflammatory effects. DHA reduced IL-10 secretion after the activation of cells with LPS (Figure 5.2.b). Conversely, incubation with AA had the capacity to scale down inflammation by significantly reducing TNF-α secretion. These implications are further supported by ANOVA of LPS-induced cytokine secretion when comparing TNF-α levels of experimental groups. Here, TNF-α levels were significantly lower when comparing levels found in PBMCs incubated with AA to levels of PBMCs incubated with n-3 PUFAs (EPA and DHA) (Figure 5.2.d).

Surprisingly, PBMCs that were incubated with only AA (without subsequent stimulation) showed a tendency to increase secretion of IL-6 (Figure 5.2.a.i). This finding is in agreement with the complex, sometimes diametrical, effects of AA that were observed in previous studies (see also chapter 3.5.2).

The data presented here, as well as the results of several previous studies, suggest that the paradigm that n-3 PUFAs exert anti-inflammatory effects, while AA is solely pro-inflammatory is an oversimplification (63, 85, 315, 316). It seems that, under certain conditions, n-3 and n-6 PUFAs are also capable of inducing pro- and anti-inflammatory effects, respectively. Moreover, these variations are also reflected in the results of two large-scale clinical studies that assessed the effects of PUFAs on inflammatory markers, the Rotterdam and OmegAD studies (59, 317). The following chapters will discuss previous studies with similar results, and describe the mechanisms how n-3 and n-6 PUFAs may induce a pro- and anti-inflammatory effect, respectively.

6.3.3.1. Impact of n-3 PUFAs on LPS-induced cytokine secretion

In general, n-3 PUFAs are predominantly associated with anti-inflammatory effects. This has been demonstrated by clinical and preclinical trials, reviewed in detail by Harbige and Patterson *et al.* (4, 63). However, these findings are not uncontested, as several studies have also reported contradicting results (63, 97, 318). Moreover, unclear relationships have also been shown in the molecular pathways by which (n-3) PUFAs and their derivatives have been found to alter inflammation. Notably, the varying effects of n-3 PUFAs on the lipid metabolism of cells has been proposed as an explanation for the contradictory nature of previously published results. Additionally, other mechanisms, such as alterations in gene expression and plasma membrane organization of immune cells, have been shown to result in both anti- as well as pro-inflammatory effects (3, 69-72). N-3 PUFAs therefore seem to be able to not only scale down inflammation, but to also act as pro-inflammatory agents via these pathways.

In one of the few studies which assessed the effect of PUFAs on cytokine secretion of human PBMCs, DHA was shown to induce a Th-1-like immune response by increasing the IFNγ/IL-10 production ratio, while AA reduced the secretion of pro-inflammatory cytokines (318). Similarly, Petursdottir and colleagues demonstrated that a diet high in

n-3 PUFAs (i.e. fish oil) results in a lower IL-10 and an increased TNF-α secretion of murine peritoneal macrophages after stimulation with LPS (97). Similar pro-inflammatory effects were also reported by several other studies (94-96, 245). In agreement with these findings, we observed a reduced secretion of IL-10 by PBMCs that were incubated with DHA (Figure 5.2.b).

A limited understanding of the lipid mediator system has been suggested as an explanation for the mixed results obtained with n-3 PUFAs in human trials (58). Indeed, the catalyzation of n-3 PUFAs into either bioactive anti- or pro-inflammatory lipid mediators may be the reason for the contradictory results of previous studies (15, 62, 97, 136, 319, 320). In order to address the mechanisms for pro-inflammatory effects of n-3 PUFAs, Hardardottir *et al.* assessed the concentration of PGE_2 with regard to the n-3 to n-6 PUFA ratio. Their study demonstrated that an increase in the dietary n-3 to n-6 PUFA ratio correlated with a reduced formation of PGE_2, while increasing TNF-α secretion in murine macrophages (96). Moreover, several authors who reported similar results also identified a decrease in AA-derived PGE_2 as a possible mechanism the for the pro-inflammatory effects of n-3 PUFAs (95-97, 246, 321). PGE_2 inhibits the function of various immune cells, suppresses acute inflammatory mediators, and promotes a regulatory T-cell immune response (1, 87, 96, 97, 320, 322-324). A decrease in PGE_2 due to incubation with DHA, therefore seems a plausible mechanism for a reduced secretion of IL-10 by PBMCs (Figure 5.2.b).

However, additional mechanisms are likely to exist. Similarly to our findings, Petursdottir and colleagues demonstrated a reduced secretion of IL-10 by murine peritoneal macrophages due to a diet high in n-3 PUFAs (97). Inhibition of PG-synthesis with indomethacin, however, had only a limited effect on the ability of n-3 PUFAs to reduce IL-10 secretion. Moreover, depending on the mode of inflammatory response, PGE_2 concentration, cell type, and receptor, PGE_2 may also exert potent pro-inflammatory effects (71, 74, 87, 323, 325). Examples of additional pro-inflammatory mechanisms of n-3 PUFAs include direct effects on pro-inflammatory gene expression, and on the plasma membrane organization of PBMCs. Indeed, dietary supplementation with n-3 PUFAs causes a significantly increased genetic expression of TNF-α and pro-inflammatory CD11b in experimental nonalcoholic steatohepatitis in mice, as well as an increased expression of LPS-receptor molecules in murine macrophages (244, 326, 327).

To summarize, numerous studies have demonstrated that n-3 PUFAs affect inflammatory processes. The majority of data indicate that DHA and EPA predominantly attenuate inflammation. However, a considerable number of studies also reported contradicting results, thereby agreeing with our findings. A combination of pathways by which n-3 PUFAs enhance the immune response (most notably a decrease in PGE_2) is likely to be the reason for the reduced secretion of IL-10, which was observed after incubation with DHA (Figure 5.2.b).

6.3.3.2. Impact of AA on unstimulated PBMCs and LPS-induced cytokine secretion

The majority of clinical and animal studies implicate that AA and other n-6 PUFAs enhance autoimmune and inflammatory disorders such as IBD or rheumatoid arthritis (4, 77, 84, 328, 329). However, contradicting results have been reported recently, associating AA with anti-inflammatory effects as well (59, 92, 330, 331). Thus, several studies have demonstrated that AA and other n-6 PUFAs are capable of inhibiting pro-inflammatory NF-kB-mediated gene expression, as well as the secretion of pro-inflammatory cytokines such as TNF-α (6, 93, 97, 318, 332-337).

As with n-3 PUFAs, it seems likely that the anti-inflammatory effects of AA are associated with effects mediated by AA-derived anti-inflammatory lipid mediators. While 4-series LTs (e.g. LTB_4) and some 2-series PGs (depending on cell type and receptor) have been identified as pro-inflammatory lipid mediators, AA-derived lipid mediators may also exert potent anti-inflammatory effects (6, 40, 74-76, 338, 339). For example, Hubbard and colleagues showed that AA significantly inhibited TNF-α secretion by 100%, using a model with LPS-induced cytokine secretion by murine macrophages (333). Interestingly, in a second step the authors added indomethacin, inhibiting PG-synthesis of cells. Here, TNF-α activity was restored to 90% of control values, which suggests that the effect of AA on TNF-α secretion was mediated via increased PG-levels (1, 333). Indeed, several authors suggest that PGE_2 plays a crucial role in mediating the anti-inflammatory effects of AA (74, 323, 324, 340). For example, PGE_2 has shown to reduce TNF-α secretion of immune cells via the induction of IL-1R–associated kinase-M (87). Additionally, AA-derived LXs exert potent anti-inflammatory and pro-resolving effects on immune cells (88, 341). In particular, LXA_4 and 15-epi-LXA_4

attenuate inflammation, and have been shown to reduce the secretion of pro-inflammatory cytokines (88, 93, 342). Therefore, an increase in PGE_2 and LXs seems to be a likely mechanism for the decrease in TNF-α levels that was observed in the present study (Figure 5.2.b and Figure 5.2.d).

As stated before, PGE_2 may also exert pro-inflammatory properties, predominantly depending on cell type and receptor (71, 74, 323, 325, 343). Therefore, the association between a possible increase in PGE_2 and the obtained results remains unclear. Supporting this notion, PGE_2 has been shown to also increase the secretion of pro-inflammatory IL-6 by macrophages (344). Moreover, it was demonstrated that this effect is mediated by a different mechanism than the anti-inflammatory effects of PGE_2 on cytokine secretion (344). In the present study, we observed that unstimulated PBMCs as well as stimulated PBMCs (with LPS), showed a tendency to secrete more IL-6 when incubated with AA (Figure 5.2.a.i and Figure 5.2.b). Consequently, ANOVA between experimental groups also indicated a difference in LPS-induced IL-6 levels (chapter 5.2.3). However, these findings remain only suggestive, as the effects on IL-6 levels were not significant when compared to the corresponding controls (p = 0.18 and 0.12 when compared to untreated PBMCs and LPS control, respectively). Equally, the Tukey-Kramer post-hoc test revealed no significant difference when comparing the LPS-induced IL-6 levels of the three experimental groups. Nevertheless, an increase in PGE_2 might be a cause for PBMCs, which were incubated with AA, to show a tendency to secrete more IL-6.

In conclusion, AA is generally associated with pro-inflammatory effects; however, previous data as well as the present study demonstrate that AA may also reduce inflammatory parameters. An elevated synthesis of PGE_2 and LXs after incubation of PBMCs with AA seems a likely reason for the subsequent decrease in LPS-induced TNF-α, which was observed in the present study.

6.3.4. Implications of results on the interaction between immune cells and CRC

In a subsequent series of experiments, we assessed the CM-induced cytokine secretion by PBMCs in order to explore the impact of PUFAs on the interaction between CRC and immune cells. The effects of EPA, DHA, and AA were similar to the ones observed with LPS-induced cytokine secretion. Thus, incubation with DHA had the capacity to reduce CM-induced IL-10 secretion, while increasing the concentration of TNF-α (Figure 5.2.c). Conversely, AA significantly reduced CM-induced secretion of TNF-α. EPA only had an effect on cytokine levels when compared to AA; ANOVA between experimental groups revealed that after incubation with AA, CM-induced secretion of TNF-α was significantly reduced when compared to levels found in PBMCs incubated with n-3 PUFAs (EPA and DHA) (Figure 5.2.e).

TNF-α is a typical pro-inflammatory cytokine, while IL-10 has been shown to exert potent anti-inflammatory properties (see also chapter 6.3.2). Our results therefore imply that DHA induced a pro-inflammatory and more aggressive response by PBMCs against tumor cells. Conversely, by reducing TNF-α levels, AA had the opposite effect. However, with regard to the pro- or anti-tumorigenic effects of immune cells against CRC, the implications of the observed changes in cytokine secretion are limited for several reasons. For one, the general understanding of the role of immune cells in the tumor microenvironment of CRC is still limited (see also chapter 3.4). Secondly, probably due to the activation of different intracellular pathways (NF-κB and AP1 versus FADD), TNF-α may exert pro- as well as anti-tumorigenic effects (2, 289, 297). Thirdly, similarly to TNF-α, studies on the role of IL-10 in the microenvironment of tumors produced contradicting results (2, 345).

Nevertheless, keeping these limitations in mind, our results may allow several hypotheses on the effect of PUFAs on the microenvironment of CRC. In general, systemic inflammation is inversely correlated with overall survival in patients with CRC (206, 209, 210). However, an increased local inflammatory reaction against tumor cells, such as the one we observed after incubation with DHA, seems to be a positive prognostic factor in CRC (198, 346). This hypothesis is backed by numerous studies on tumor-infiltrating immune cells. Therefore, inflammatory infiltrate in and around CRC correlates with improved survival, suggesting that a local inflammatory reaction induces anti-cancer properties (9, 198). Furthermore, the beneficial effects of NSAID intake, in particular aspirin, are believed to be mediated by a reduced synthesis of PGE_2. In turn,

PGE_2 has been shown to be essential for the immune evasion of cancer cells (1, 122-124, 188, 189). Our results therefore raise the question of whether n-3 PUFAs, such as DHA, may exert some of their previously demonstrated beneficial effects on CRC, through a reduced synthesis of PGE_2 in the tumor microenvironment. Low levels of PGE_2 in immune cells would, in turn, lead to a more aggressive immunological response against tumor cells, such as the one observed in this study (Figure 5.2.c and Figure 5.2.e). This pro-inflammatory effect is likely to be mediated, at least in part, by lymphocytes, as these cells make up the majority of PBMCs. Moreover, tumor-infiltrating lymphocytes, in particular, have been shown to be a positive prognostic predictor in CRC (9, 213). These possible effects are of particular interest with regard to the recently demonstrated synergistic effects of aspirin-mediated anti-tumor mechanisms and immune checkpoint inhibition by PD-1 blockade (see also chapter 3.4) (189, 215, 216). Therefore, similarly to aspirin, n-3 PUFAs (in particular DHA) could work as an adjunct to cancer immune therapy by reducing PGE_2 in the tumor microenvironment (Figure 6.3.a) (1, 2).

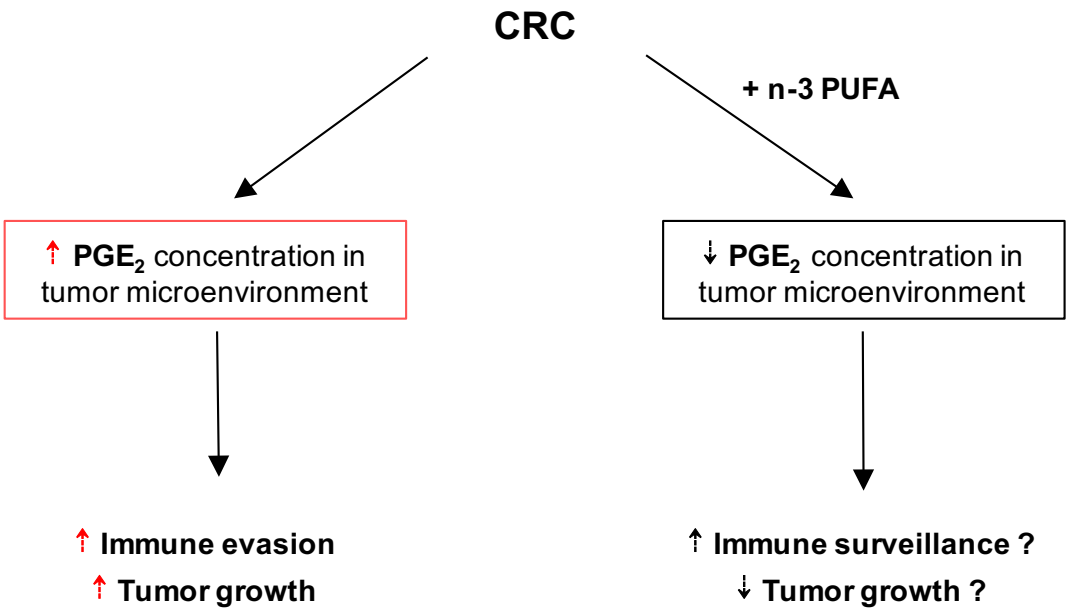

Figure 6.3.a Possible effect of n-3 PUFAs (in particular DHA) on colorectal cancer (CRC). In analogy with the previously proposed anti-tumor immune response induced by aspirin intake (Figure 3.3.b) (189), DHA and other n-3 PUFAs might have similar effects. PGE_2, prostaglandin E2. This figure (including title and description) was recently published by the author, reproduced with permission from: (1, 2).

Incubation of PBMCs with AA resulted in a reduced secretion of TNF-α after stimulation with CM (Figure 5.2.c and Figure 5.2.e), which suggests that AA exerts anti-inflammatory effects on immune cells in the tumor microenvironment. Similarly to observations of n-3 PUFAs, this effect is likely to be mediated by lymphocytes. With regard to findings on tumor-infiltrating immune cells, our results imply that AA has the capacity to induce pro-tumorigenic effects via a reduced local inflammatory reaction against tumor cells. Indeed, a limited number of studies demonstrated pro-tumorigenic effects of AA or its derivatives on CRC (223, 225, 226, 228). However, the validity of these implications is limited as we only observed a significant increase of CM-induced TNF-α levels after incubation of PBMCs with AA. The concentrations of neither IL-6 nor IL-10 were affected by incubation with AA.

In conclusion, the results of CM-induced cytokine secretion imply a more aggressive immunological response of PBMCs against tumor cells, due to incubation with DHA. Conversely, AA seems to exert anti-inflammatory effects. Further deductions on the effect of PUFAs on the interaction between immune and CRC cells would be limited in their validity. Nevertheless, previous findings on the effect of PUFAs on immune cells, as well as on the interaction of the immune system and CRC, agree with the implications of our results. Thus, it is probable that a crosstalk between CRC cells and leukocytes exists. Moreover, at least *in vitro*, this interaction seems to be influenced by n-3 and n-6 PUFAs. Interestingly, the results after incubation with DHA raise the possibility of an immune-enhancing anti-tumorigenic effect of n-3 PUFAs, in particular by DHA, in the tumor microenvironment of CRC (Figure 6.3.a).

6.4. Evaluation of experiments – limitations of the conducted study

Although our results allow multiple conclusions, adding to a better understanding of the role of n-3 and n-6 PUFAs in the context of CRC and the immune system, the present study displays several shortcomings and limitations. It should be noted that all our experiments were conducted *in vitro.* In general, the findings of *in vitro* studies have limited validity when compared to *in vivo* studies. They are simplified approaches to elucidate highly complex mechanisms, such as the effect of n-3 and n-6 PUFAs on CRC and immune cells. Therefore, a plethora of variables, which would be likely to affect the behavior of CRC and immune cells in "real life", were not considered in the present study. Examples of these include enteral bacteria and stool, cell-to-cell interactions (e.g. between PBMCs and CRC, as well as between PBMCs and immune cells not present in PBMCs), and the effect of numerous hormones and other messenger molecules. This is a general weakness of all *in vitro* studies. Nevertheless, a mechanistic approach, like the one used in the present study, allows for the assessment of a specific research question in a controlled experimental setting, while excluding numerous confounding influences, which are normally found *in vivo*. In particular, considering the complex and diverse effects of n-3 and n-6 PUFAs on immune cells, such an approach is likely to allow a more controlled experimental setting.

A possible unspecific confounding factor in this and other *in vitro* studies is the need to complement the GM of cells with 10% FBS. FBS contains several factors that were likely to have an effect on our results. To minimize this effect, the GM used in this study was supplemented with the same lot of FBS. Moreover, we used FBS Superior, which is additionally standardized by the manufacturer (Merck KGaA, Darmstadt, Germany).

6.4.1. Lipidomics of colorectal adenocarcinoma HT-29 cells

Regarding experiments with LC/MS/MS, several major limitations have to be considered. First, while we were able to demonstrate the presence of OH-FAs in HT-29 cell cultures, the implications of our results are significantly undermined by the fact that we did not assess PUFA derivatives in PUFA-supplemented GM without cells present.

Especially in the context of the recently published study by Ostermann *et al.*, it seems crucial to include such a control (see also chapter 6.2) (118). As stated earlier, we cannot rule out completely that the measured compounds were affected by hydrolysis and autoxidation, independent of HT-29 cells. Secondly, the absence of a control group of HT-29 cell cultures in GM without PUFA supplementation further undermines the implications of our results, as we have no data on the baseline levels of OH-FAs. Thirdly, we were not able to demonstrate that DHA or AA affect the synthesis of their OH-FA derivatives, nor did we detect the presence of polyhydroxylated lipid metabolites. This is likely to be caused by a PUFA concentration in cell cultures that was too low (20 µM). Indeed, other studies with a similar setup used considerably higher PUFA to cell ratios (118, 220, 257).

Future research should therefore include control groups that assess the concentration of OH-FAs without cells present to rule out cell-independent hydrolysis and autoxidation and cell cultures without PUFA supplementation to measure baseline levels of OH-FAs. Moreover, similar studies should consider using a higher PUFA to cell ratio when assessing OH-FA formation, as this is likely to result in more pronounced results, and in the detection of polyhydroxylated lipid metabolites.

Zhou and Astarita *et al.* thoroughly summarize the challenges of collecting and preparing samples before LC/MS/MS (36, 104). For this procedure, samples should be harvested in cold solvents to instantly halt their lipid metabolism. Moreover, for the same reason, it is recommended that samples should be stored at low temperatures (preferably -80°C) until analysis with LC/MS/MS. Our samples were stored at -20°C. Therefore, residual metabolism might have taken place in samples, which would have an effect on the PUFA derivatives measured. All HT-29 cell samples, however, were stored under equal conditions before analysis. Therefore, it is likely that any residual metabolism would affect all samples in a similar manner.

6.4.2. Experiments with peripheral blood mononuclear cells

For this study, we established a relatively cheap method to isolate high numbers of PBMCs from leukocyte depletion filters (Leucoflex, LST2B). Interestingly, Meyer *et al.*

demonstrated that buffy coats from Leucoflex filters are *"most similar to standard buffy coats in their relative proportions of leukocyte subpopulations"* (260). Additionally, the authors showed that PBMCs obtained from leukocyte depletion filters express similar levels of the activation markers CD69 and CD25, when compared to PBMCs isolated from whole blood (260). Therefore, the PBMCs used in this study are likely to have similar properties to PBMCs isolated from whole blood.

However, our study design has several limitations. First, while the acquisition of leukocyte depletion filters is an easy method to gain access to an adequate number of human donors, we have no further information about these individuals. Therefore, crucial variables, such as sex, age, disease, medication, and diet are unknown. These limitations are likely to be responsible for the considerable variations in absolute cytokine levels when comparing PBMC cell cultures from different donors. We therefore standardized cytokine levels to be expressed as ratios compared to LPS- or CM-controls.

Secondly, when assessing CM-induced cytokine secretion, it has to be considered that CM was obtained from cells that used to belong to a foreign human host. It is therefore possible that the observed increase of cytokines after contact with CM is of allo-immunogenic nature, thus, limiting the validity of the used model of CM-induced cytokine secretion to mimic an *in vivo* immune reaction against CRC.

Thirdly, by measuring three cytokines at one time point, we only assessed a limited number of variables to determine the complex role of n-3 and n-6 PUFAs on immune cells. The assessment of inflammatory gene expression (e.g. NF-kB-expression), as well as of the lipid metabolism of PBMCs (especially the formation of PGs), would have allowed a more thorough understanding of the effects of PUFAs on these cells. In particular, the lower potency of EPA to induce changes in cytokine secretion (when compared to DHA), which we observed in the present study, may have been explained through assessing changes in lipid metabolism and gene expression of PBMCs.

Finally, it has to be argued that an assessment of the versatile effects of n-3 and n-6 PUFAs on the immune system requires a more complex experimental setup than the one used in this study. The sum of data indicates that n-3 and n-6 PUFAs are capable of inducing both pro- and anti-inflammatory effects. Interestingly, previous studies have suggested major variables which determine the effect of PUFAs on the immune system. In this context, it was demonstrated that n-3 PUFAs may have diametrical effects on the

cytokine secretion of different immune cells of the same animal; n-3 PUFAs decreased TNF-α and IL-10 secretion by murine T-cells, but increased the secretion of these cytokines in macrophages (244). Moreover, Wallace and colleagues showed that the activation state of immune cells significantly alters the overall effect of n-3 and n-6 PUFAs on the inflammatory reaction (347). A diet high in n-3 PUFAs, for example, reduced the secretion of TNF-α and IL-1 by peritoneal macrophages, while resident macrophages showed an increased secretion of these cytokines. Thus, the cell type used, and the activation state of PBMCs, are likely to have affected the results presented here. While we demonstrated that incubation with PUFAs alters the secretion of TNF-α and IL-10, the use of different immune cells, a different activation state of PBMCs, or quantification of cytokines at a different point of time, may have yielded different, even contradicting results. Future studies should therefore modify their experimental setup to consider these variables (e.g. isolation of specific cell-subpopulations for experimental use, and multiple time-dependent quantification of inflammatory markers).

6.5. Concluding remarks and future directions

Although the present study has several limitations, our results demonstrate the potential of n-3 and n-6 PUFAs to influence the lipid metabolism of colorectal adenocarcinoma HT-29 cells, and to alter the cytokine secretion of immune cells (PBMCs). First, by using LC/MS/MS, we were able to verify the presence of OH-FAs in HT-29 cell cultures. Furthermore, incubation with EPA resulted in the expected increase of EPA-derived HEPEs. This may be a mechanism for how EPA exerts its previously described beneficial effects on CRC.

Secondly, we demonstrated that n-3 and n-6 PUFAs have the capacity to alter cytokine secretion by PBMCs. Our results for stimulation with LPS and CM have similar implications; DHA altered the secretion of cytokines to a more pro-inflammatory profile, while AA had an anti-inflammatory effect on cells. In the context of inflammation, n-3 PUFAs are predominantly associated with anti- and AA with pro-inflammatory effects. This contrasts with the findings of the present study. Moreover, it has become increasingly apparent that this paradigm represents an oversimplification (85). As outlined in chapters 3.1.3 and 6.3.3, several studies have reported results that are consistent with our findings. Furthermore, our results may be explained by the mechanisms by which n-3 and n-6 PUFAs have been found to alter inflammation, in particular their transformation into bioactive lipid mediators, as it has been shown that n-3 and n-6 PUFAs may exert anti- as well as pro-inflammatory effects via these pathways.

With regard to the effect of PUFAs on the interaction between immune cells and CRC, the implications of our results are less clear. This is largely due to the fact that the understanding of the tumor microenvironment in general, and also with regard to TNF-α and IL-10, is limited. Therefore, while our findings raise the question of whether n-3 PUFAs, such as DHA, could work as an adjunct to cancer immune therapy via reduced PGE_2 levels, evidence for this hypothesis is still limited. Similarly, our observation that incubation with AA reduced the CM-induced secretion of TNF-α has limited implications, as TNF-α is associated with pro- as well as anti-tumorigenic effects. Nevertheless, our results demonstrate that n-3 and n-6 PUFAs are capable of influencing the crosstalk between PBMCs and CRC *in vitro*.

Interestingly, it remains largely unclear under what circumstances n-3 and n-6 PUFAs have predominantly pro- or anti-inflammatory effects. Knowledge of how the potentially opposing effects of these PUFAs are integrated by immune cells, and the immune system as a whole, is paramount in understanding the role of these fatty acids in the context of inflammation. Therefore, additional trials are warranted, preferably using human immune cells isolated after dietary PUFA supplementation, which may elucidate the mechanisms of how these fatty acids affect the immune system and, cancer immune therapy in particular. Moreover, future studies should consider previously published variables, such as type and activation state of immune cells, that have been shown to alter the influence of n-3 and n-6 PUFAs on inflammation.

LC/MS/MS of immune cells, in particular, seems to be a promising method for future studies, as it would reveal alterations in lipid mediator concentrations after supplementation with fatty acids. Indeed, Wang *et al.* argue that lipid mediators, rather than their parent PUFAs, are likely to be the major effector molecules that alter immune cell function (58). Similarly, using LC/MS/MS to reveal lipid mediators in human CRC tissue is likely to be an important approach for explaining the possible effects of n-3 and n-6 PUFAs on cancer cells, as well as on immune cells, in the cancer microenvironment.

In conclusion, while our results were obtained from a limited *in vitro* setup, we were able to demonstrate that derivatives of n-3 and n-6 PUFAs are present in HT-29 cell cultures, and that their concentration is affected by incubation with EPA. Moreover, the present study provides significant evidence of the capacity of n-3 and n-6 PUFAs to alter the immune cell function of cells being challenged with a bacterial moiety (LPS) or CM. More studies in this research area are warranted. In particular, the assessment of an enhanced effect of cancer immune therapy due to high n-3 PUFA supplementation is of high interest. As the various and complex effects of n-3 and n-6 PUFAs may often be explained by their metabolism into bioactive lipid mediators, the experimental setup of future studies should include the quantification of these mediators via LC/MS/MS.

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8. Statutory Declaration

“I, Nikolaus Constantin Wachtel, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic “*Polyunsaturated fatty acids, colorectal cancer, and inflammation: Effects of three major polyunsaturated fatty acids on the lipid metabolism of colorectal adenocarcinoma HT-29 cells and on the cytokine secretion by peripheral blood mononuclear cells*“ independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I am aware of the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice and that I commit to comply with these regulations.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

9. Author Contribution Statement

I, Nikolaus Constantin Wachtel, contributed the following to the below listed publications:

Publication 1:

Wachtel N, Rohwer N, Pietzner A, Loew A, Weylandt KH. **Omega-3 fatty acid supplementation – A possible dietary adjunct to enhance immune therapy in cancer?** Preprints. 2018.

Contribution:

All experiments, data analysis, and writing of articles were conducted by the first author, Nikolaus Constantin Wachtel. The co-authors assisted in the interpretation of data. The manuscript was proofread by all co-authors.

Publication 2:

Wachtel N, Rohwer N, Pietzner A, Loew A, Weylandt KH. **Omega-3 fatty acid supplementation – A possible dietary adjunct to enhance immune checkpoint inhibition therapy in cancer?** Journal of Cellular Biotechnology. 2018;4:83-8.

Contribution:

All experiments, data analysis, and writing of articles were conducted by the first author, Nikolaus Constantin Wachtel. The co-authors assisted in the interpretation of data. The manuscript was proofread by all co-authors.

Date Signature

10. Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

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11. List of Publications

Wachtel N, Rohwer N, Pietzner A, Loew A, Weylandt KH. Omega-3 fatty acid supplementation – A possible dietary adjunct to enhance immune therapy in cancer? Preprints. 2018.

Wachtel N, Rohwer N, Pietzner A, Loew A, Weylandt KH. Omega-3 fatty acid supplementation – A possible dietary adjunct to enhance immune checkpoint inhibition therapy in cancer? Journal of Cellular Biotechnology. 2018;4:83-8.

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„*Optimism is true moral courage”*

Sir Ernest Henry Shackleton