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DISSERTATION

Docosahexaenoic acid suppresses arachidonic acid-induced  
proliferation of LS 174T human colon carcinoma cells

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## Abkürzungen/Abbreviations

|        |  |                |   |
|--------|--|----------------|---|
| 5-FU   | 5-Fluoruracil                          | dNTP           | Deoxynucleotide triphosphate                    |
| AA     | Arachidonic acid                       | EGFR           | Epidermal growth factor receptor                |
| APC    | Adenomatous polyposis coli             | EPA            | Eicosapentaenoic acid                           |
| Apaf-1 | Apoptotic protease-activating factor-1 | FA             | Fatty acid                                      |
| ASS    | Acetylsalicylic acid (Aspirin)         | FAP            | Familial adenomatous polyposis                  |
| ATL    | Aspirin-triggered lipoxin              | FBS            | Fetal bovine serum                              |
| Bak    | Bcl-2 homologous killer protein        | FLIP           | FADD-like inhibitor protein                     |
| Bax    | Bcl-2-associated X protein             | GAPDH          | Glycerinaldehyde-3-phosphate-dehydrogenase      |
| Bcl-2  | B-cell lymphoma-2                      | HIF-1 $\alpha$ | Hypoxia-inducible factor-1 $\alpha$             |
| Bcl-xL | B-cell lymphoma-extra large            | HLTF           | Helicase-like transcription factor              |
| BSA    | Bovine serum albumin                   | HNPCC          | Hereditary nonpolyposis colon cancer            |
| CDK    | Cyclin-dependent kinase                | LOX            | Lipoxygenase                                    |
| CEA    | Carcinoembryonic antigen               | LTs            | Leukotrienes                                    |
| CIMP   | CpG Island Methylator phenotype        | LXs            | Lipoxins  |
| CIN    | Chromosomal instability phenotype      | MGMT           | O <sup>6</sup> -methylguanine-methyltransferase |
| COXIB  | Selective COX-2 inhibitor              | MLH1           | MutL homolog 1                                  |
| COX    | Cyclooxygenase                         | MMP            | Metalloproteinases                              |
| CRC    | Colorectal cancer                      | mRNA           | Messenger RNA                                   |
| CT     | Control                                | MSI            | Microsatellite instability                      |
| DAPI   | 4',6'-diamidino-2-phenylindole         | MSH2           | MutL homolog 2                                  |
| DCC    | Deleted in colorectal cancer           | n-3 PUFA       | Omega-3 polyunsaturated fatty acid              |
| DHA    | Docosahexaenoic acid                   | n-6 PUFA       | Omega-6 polyunsaturated fatty acid              |
| DMEM   | Dulbecco's modified Eagle's medium     |                |   |
| DMSO   | Dimethylsulfoxide                      |                |   |
| DNA    | Deoxyribonucleic acid                  |                |   |

|                     |  |                  |  |
|---------------------|--|------------------|--|
| NFκB                | Nuclear factor κB                          | ERK              | Extracellular-signal regulated kinase                                    |
| NRT                 | Non reverse transcriptase sample           | RNA              | Ribonucleic acid   |
| NSB                 | Non-specific binding controls              | SEM              | Standard error of mean   |
| NTC                 | Non template controls                      | SMAD4            | SMAD family member 4   |
| NSAID               | Nonsteroidal anti-inflammatory drug        | SNP              | Single nucleotide polymorphism   |
| p21 <sup>WAF1</sup> | Cyclin-dependent kinase inhibitor 1A       | TGFβR2           | Transforming growth factor beta receptor 2                               |
| PFA                 | Paraformaldehyde                           | TX               | Thromboxane  |
| PG                  | Prostaglandin                              | TXA <sub>2</sub> | Thromboxane A <sub>2</sub>   |
| PGD <sub>2</sub>    | Prostaglandin D <sub>2</sub>               | VEGF             | Vascular endothelial growth factor                                       |
| PGE <sub>2</sub>    | Prostaglandin E <sub>2</sub>               | XIAP             | X-linked inhibitor of apoptosis protein                                  |
| PGF <sub>2α</sub>   | Prostaglandin F <sub>2α</sub>              | XTT              | 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide |
| PGI <sub>2</sub>    | Prostaglandin I <sub>2</sub>               |                  |  |
| PI                  | propidium iodide                           |                  |  |
| PI3K                | Phosphoinositide 3-kinase                  |                  |  |
| PLA                 | Phospholipase                              |                  |  |
| PPAR                | Peroxisome proliferator activated receptor |                  |  |
| PUFA                | polyunsaturated fatty acids                |                  |  |
| Ras-MAPK            | Ras-mitogen activated protein kinase       |                  |  |

## **1. Einleitung / Introduction**

Worldwide every 3.5 minutes a patient is diagnosed with colorectal cancer (CRC) and every nine minutes someone dies from this disease (Benson 2007).

Various studies have highlighted diet as a very important factor in the context of CRC development, some of which indicating that fish containing high levels of omega-3 polyunsaturated fatty acids (n-3 PUFAs) may be protective against CRC (Hall et al. 2008; Lin 2009; Norat et al. 2005).

However, the exact mechanisms by which n-3 PUFAs influence colorectal carcinogenesis remain unexplained and there has been an ongoing debate about their protective effects in contrast to omega-6 polyunsaturated fatty acids (n-6 PUFAs). In this context we conducted the present *in vitro* study of the effects of n-3 and n-6 PUFAs on a CRC cell line.

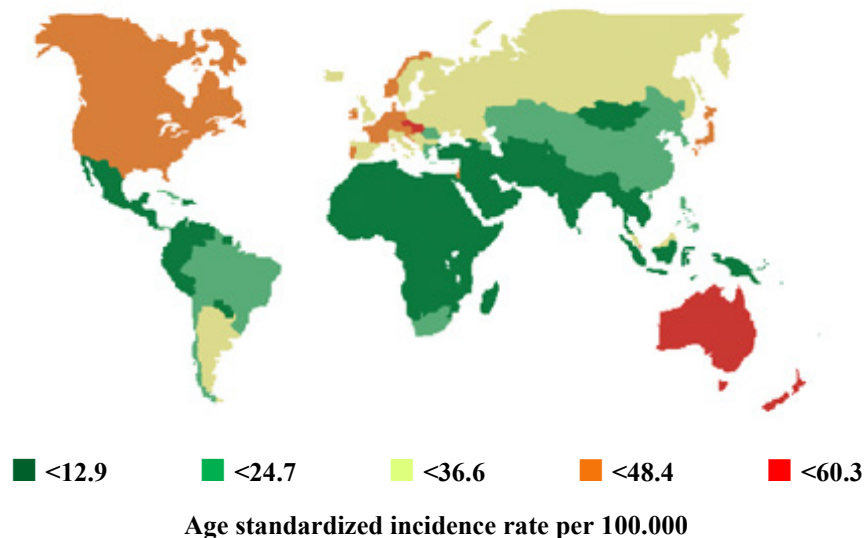
In order to provide the necessary background information, the introduction will first supply general facts regarding CRC, including its epidemiology, its subtypes, as well as the recent understanding of molecular mechanisms involved in its pathogenesis. This is to be followed by a brief review of the environmental risk factors identified for CRC with a particular focus on the impact of polyunsaturated fatty acids (PUFAs). Afterwards, n-3 PUFAs, n-6 PUFAs and lipid mediators derived from these fatty acids (FAs) and their biological functions will be outlined. Finally the current knowledge concerning the most important molecular mechanisms underlying n-3 and n-6 PUFA actions in the context of CRC will be summarized and the specific aim of the present study will be described.

### **1.1 Colorectal cancer - a global burden**

According to the Robert Koch Institute, in 2004 CRC was responsible for almost 28,000 deaths in Germany. Thus, CRC is the second most common cause of cancer-related mortality in both genders, only preceded by breast cancer in women and lung cancer in men. Moreover, approximately 73,200 German men and women were newly diagnosed with CRC in 2004, accounting for about 17% of all diagnosed malignancies. In this context the lifetime risk to be diagnosed with CRC in Germany has been estimated at four to six percent (Batzler et al. 2008; Layer & Riemann 2008).

However, on a global scale, there are approximately one million new cases of CRC and 530,000 CRC-related deaths annually (Parkin et al. 2005). Interestingly, CRC incidence rates strongly

vary (up to 25-fold) among different countries and regions of the world as shown in figure 1.1 (Center et al. 2009a; Center et al. 2009b; Doll et al. 1981; Lamer & Riemann 2008; Parkin 2004).



**Figure 1.1:** World map of colorectal cancer incidence (in males). Adapted from Parkin 2004.

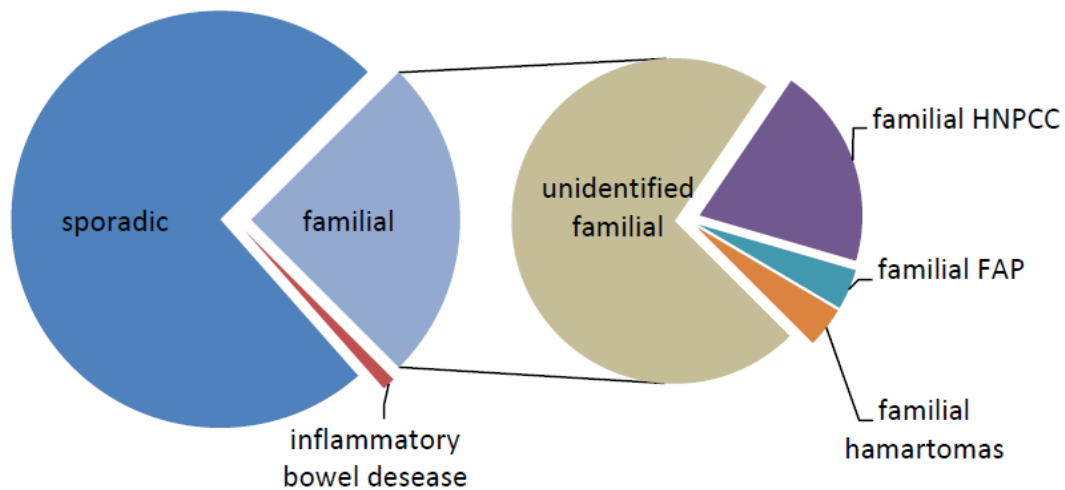
In this context, high incidence rates are reported for the high-income countries of North America, Australia/New Zealand, Western and Eastern Europe and Japan. Here CRC rates are almost four times as high as those found in the middle- to low-income countries of Africa, Asia, and in intermediate and southern parts of Southern America. Yet, CRC incidence rates are reported to increase rather rapidly in countries, which have recently shifted from a low- to a high-income economy and where overall-risk was formerly low (especially Japan, but also elsewhere in Asia). In contrast, the number of CRC cases in high-risk countries is only gradually increasing or stabilizing (North and Western Europe) or even declining (North America) (Benson 2007; Parkin 2001; Parkin et al. 2005; World Cancer Research Fund 2007). On the other hand, five year survival rates of CRC-patients range from approximately 65% in North America and 54-60% in Western Europe to only about 30% in Eastern Europe and in India. This most likely reflects varying levels of treatment and care in different countries and regions of the world (Parkin et al. 2005).

## 1.2 Subtypes of colorectal cancer

Traditionally, CRC is categorized into three subsets: Sporadic, familial and CRC cases associated with chronic inflammatory bowel diseases, such as Crohn's disease or ulcerative colitis (figure 1.2) (Hisamuddin et al. 2004; Kraus et al. 2009).



While chronic inflammatory bowel diseases only account for about one percent of all CRC cases, a positive family history including at least one first- or second-degree relative affected by CRC is found in approximately 25-30% of all CRC patients (Burgart 2005; Hisamuddin & Yang 2004). Nevertheless, only in about one third of these familial CRC cases one of the well-described hereditary CRC syndromes, such as familial adenomatous polyposis (FAP), hereditary nonpolyposis colon cancer (HNPCC - also referred to as Lynch syndrome), or hereditary hamartomatous polyposis syndrome can be identified (Hisamuddin et al. 2006). In the remaining two thirds, underlying molecular mechanisms remain unclear, as these cases often do not follow a Mendelian pattern, presumably due to incomplete penetrance or multi-factorial effects (figure 1.2) (Cheah 2009; Ilyas et al. 1999).



**Figure 1.2:** *Subtypes of CRC:* Sporadic cases ( $\approx 75\%$ ) mostly occur in individuals over the age of 50 years without identifiable predisposing factors. Chronic inflammatory bowel diseases, such as Crohn’s disease or ulcerative colitis account for  $\approx 1\%$  of CRC cases. Familial are cases with a family history of CRC ( $\approx 25\%$ ); unidentified familial CRCs are with a family history of CRC but exclusive of defined CRC syndromes such as FAP ( $\approx 1\%$ ), HNPCC ( $\approx 5\%$ ), and the hamartomatous polyposis syndromes ( $\approx 1\%$ ). Based upon data obtained from Hisamuddin & Yang 2004.

Yet, the vast majority of CRC cases is considered as sporadic in origin, namely with no family history of the disease or other obvious predisposing entities (Cheah 2009; Hisamuddin & Yang 2004; Ilyas et al. 1999). However, although the term “sporadic” may imply that in these cases environmental factors play a more important role than hereditary predispositions, it is noteworthy that even a sporadic case of the illness significantly augments the CRC risk for relatives of the patient in the future. Thus, as in the majority of other cancers, CRC and CRC risk are considered to be strongly influenced by the genetic background (Vogelstein et al. 2004; Wood et al. 2007). In this context it has become evident that genetic variations – be it a single nucleotide polymorphism (SNP) or at the structural level - influence the individual response to

environmental insults and hence determine whether afflicted cells are able to counterbalance the damage or succumb to the insult, which then facilitates the carcinogenic transformation (Cheah 2009).

### **1.3 Colorectal carcinogenesis**

It is widely accepted that colorectal carcinogenesis results from the progressive accumulation of genetic and epigenetic alterations, which subsequently impair key cellular processes that regulate the hallmarks of cancerous behavior (Fearon et al. 1990; Grady et al. 2008; Hanahan et al. 2000). The disruption of these processes, including those responsible for genomic fidelity, signal transduction, cell division, apoptosis, angiogenesis, and the respect of compartmental boundaries, drives the initiation, promotion and progression of cancer (Hanahan & Weinberg 2000; Hawk et al. 2005).

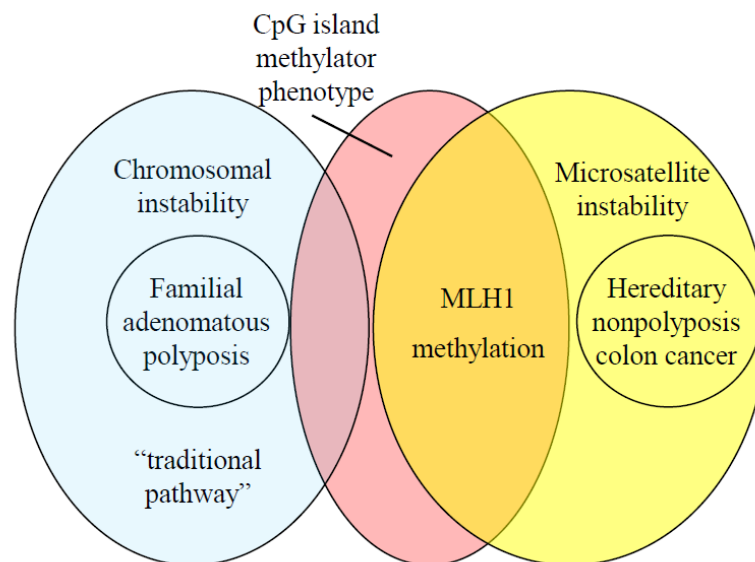
Interestingly, in the colorectum the stepwise acquisition of genetic and epigenetic alterations is usually reflected on a histological level in the transformation of regular epithelium over benign neoplasia (adenoma) into invasive carcinoma and eventually metastatic cancer. This process, taking years or even decades in its development has been molecularly characterized in the seminal classic adenoma to carcinoma progression model proposed by Fearon and Vogelstein in the late 1980s (see also figure 1.4) (Fearon & Vogelstein 1990; Noffsinger 2009; Vogelstein et al. 1988). However, although at its basis this model is still valid, the understanding of colorectal carcinogenesis has evolved considerably over recent decades (Moran et al. 2010).

Based upon the finding that the rate of random mutations alone cannot account for the number of genetic alterations found in most human malignancies, it has been hypothesized that the acquisition of mutations is facilitated by a loss of genomic stability (Lengauer et al. 1998; Loeb et al. 2003; Soreide et al. 2009). In this context, it is noteworthy that approximately 30% of all human genes code for proteins involved in the regulation of DNA fidelity, thus implying that there may be a large variety of different mechanisms that could cause genetic instability (Grady & Carethers 2008; Herrmann et al. 2001). Nevertheless, it is an unresolved question whether genomic instability commonly initiates the neoplastic sequence or whether it occurs early within the process (Grady & Carethers 2008; Michor et al. 2005; Rajagopalan et al. 2003).

Regarding CRC there are at least two chief categories of genomic instability: chromosomal instability (CIN) and microsatellite instability (MSI). While in CIN requisite genetic events occur via the accumulation of structural or numerical chromosomal abnormalities, MSI is characterized

by the disruption of the cellular DNA mismatch repair system (MMR). The mutual exclusivity of the two pathways suggests that genomic instability appears to be a necessity in the context of CRC and that either CIN or MSI are sufficient to drive colorectal carcinogenesis (Noffsinger 2009).

More recently, the impairment of epigenetic stability has been additionally shown to play a key role in particular subsets of CRC. This led to the additional definition of the CpG Island Methylator Phenotype (CIMP), which is also involved in sporadic MSI (Jass 2007; Noffsinger 2009; Soreide et al. 2009; Worthley et al. 2007). This section is intended to provide a short overview of these different pathways of colorectal carcinogenesis and to reveal their differences and interconnections as summarized in figure 1.3.



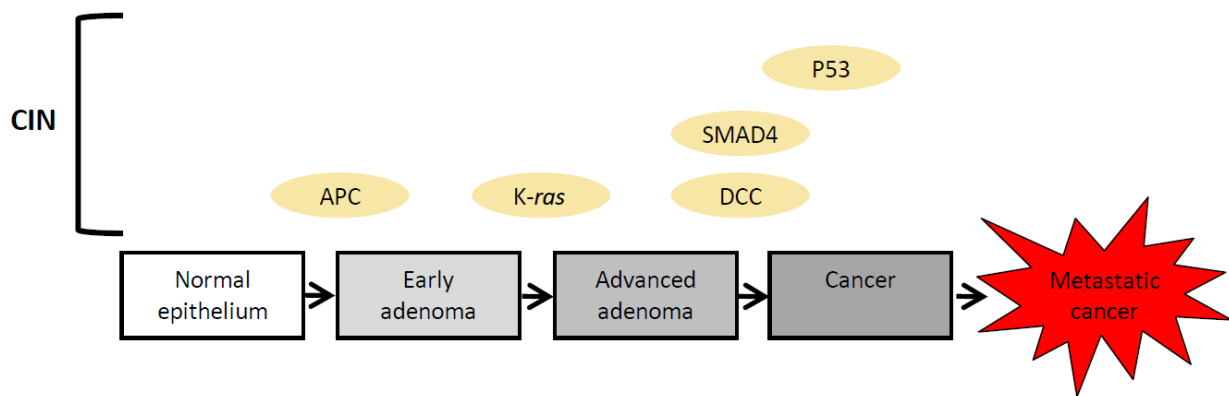
**Figure 1.3:** Genetic instability pathways in the context of colorectal carcinogenesis. Overlapping relationships define the major pathways of genomic and epigenetic instability in CRC. Adapted and in part modified from Markowitz et al. 2009.

### 1.3.1 Chromosomal instability pathway – the traditional model

The majority (approximately 70-85%) of sporadic CRCs develop via the chromosomal instability (CIN) pathway, which is also referred to as “traditional” or “suppressor” pathway and is furthermore exemplified by the hereditary familial adenomatous polyposis CRC syndrome (FAP) (Grady & Carethers 2008; Worthley et al. 2007). As noted above, tumors arising via the CIN pathway are characterized by chromosomal abnormalities including deletions, insertions, and loss of heterozygosity as well as alterations in chromosome number (aneuploidy) (Grady & Carethers 2008; Worthley et al. 2007). Yet, despite its high frequency of occurrence in sporadic

CRC, the mechanisms causing this type of genomic instability remain poorly understood (Pritchard et al. 2011).

Nevertheless, tumors arising via the CIN pathway generally follow the molecular model proposed by Fearon and Vogelstein, as they are characterized by the progressive accumulation of mutations or losses in classical oncogenes and tumor suppressor genes, which are reflected in concurrent histopathological changes (figure 1.4). Genes typically altered in CIN are adenomatous polyposis coli (APC), K-*ras*, deleted in colorectal cancer (DCC), SMAD family member 4 (SMAD4), and p53 (Fearon & Vogelstein 1990; Worthley et al. 2007).



**Figure 1.4:** Simplified progression model of CRC arising via chromosomal instability/traditional pathway (CIN). The hallmarks of the CIN pathway are chromosomal alterations, although the exact underlying mechanisms remain unknown. In this pathway neoplasia is believed to be initiated by disturbance of the Wnt-pathway via mutations in the APC gene, leading to dysfunction in the regulation of growth and apoptosis. Progression is then driven by successive clonal cellular expansion, through which cells acquire enhanced growth characteristics via the accumulation of further mutations in CIN-distinct genes, controlling the hallmarks of cancerous behavior. Adapted in part from Grady & Carethers 2008 and Soreide et al. 2009.

In this context it is noteworthy that the APC protein - via its interaction with  $\beta$ -catenin - not only acts as an essential modulator of the Wnt-pathway, which controls proliferation and apoptosis but also has been implicated to play an important role concerning the perpetuation of chromosomal stability (Behrens et al. 1998; Cadigan et al. 2006; Fodde et al. 2001; Kuhnert et al. 2004; Rubinfeld et al. 1993; Su et al. 1993).

Yet, contrasting sporadic cancers arising via the CIN pathway, patients suffering from the autosomal dominant hereditary disease FAP carry a germline mutation in one of the APC genes (Fodde et al. 2001; Noffsinger 2009; Vogelstein et al. 1988). In these patients the sporadic disruption of the second APC allele leads to the formation of hundreds to thousands of adenomas in the colorectum, some of which subsequently develop into cancer via the pathway outlined above (figure 1.4). Thus, untreated FAP patients face a lifetime CRC incidence of almost 100% (Galiatsatos et al. 2006).

However, although genetic progression outlined above is prototypic, formation of CRC depends on the accumulation of defects rather than on a specific sequence. Hence, only a minority of CRCs characterized by CIN display all of these molecular abnormalities and several of these steps may be bypassed by other genetic events (Hawk & Levin 2005; Uen et al. 2007). In summary, the originally more linear molecular adenoma to carcinoma progression model has evolved into a more complex, comprehensive, and heterogeneous approach (Gatenby et al. 2003; Worthley et al. 2007).

### **1.3.2 Microsatellite instability pathway**

Apart from CIN, the microsatellite instability pathway (MSI) also referred to as the “mutator” pathway is responsible for genetic instability in approximately 15 to 20% of sporadic CRC cases. Unlike CIN, the understanding of mechanisms underlying MSI is more advanced as it is characterized by the disruption of the DNA mismatch repair system (MMR). This results in a subsequent failure of DNA review and repair following cellular replication (Grady & Carethers 2008; Libutti 2008; Pritchard & Grady 2011). Inactivation of the MMR can either be inherited as displayed in the hereditary nonpolyposis colon cancer syndrome (HNPCC) or acquired sporadically.

In most sporadic CRCs following the MSI pathway MMR disruption results from epigenetic silencing, primarily via hypermethylation of the MutL homolog 1 (MLH1) gene coding for an important member of the MMR. Thus, sporadic tumors arising via MSI represent a combination of mutator and epigenetic methylator pathways (figure 1.3 and chapter 1.3.3) (Deng et al. 1999; Grady & Carethers 2008; Kane et al. 1997; Worthley et al. 2007).

In contrast, HNPCC patients carry a germline defect in one of the mismatch repair genes (primarily MLH1 and MutL homolog 2 (MSH2)). The sporadic alteration of the corresponding allele then triggers the accelerated acquisitions of further mutations. Thus, approximately 80% of all HNPCC patients develop CRC with early carcinomas arising before the age of 45 years as well as being prone to other malignancies such as ovarian or endometrial cancer (Higuchi et al. 2005; Kinzler et al. 1996; Salahshor et al. 1999)

Yet, regardless of the underlying etiology due to the fact that the DNA polymerase is highly susceptible to errors in short tandem repeat sequences, also referred to as microsatellites, disruption of the complex MMR results in progressive accumulation of mutations in these microsatellites (figure 1.5) (Hoeijmakers 2001; Thibodeau et al. 1993). Although microsatellites are primarily located in noncoding regions of the genome, some of them reside in genes

implicated to typically play a crucial role in MSI-colorectal carcinogenesis, such as transforming growth factor beta receptor II (TGF $\beta$ R2), Bcl-2-associated X protein (Bax),  $\beta$ -catenin, and insulin-like growth factor 2 receptor (IGF2R) (figure 1.5) (Moran et al. 2010).

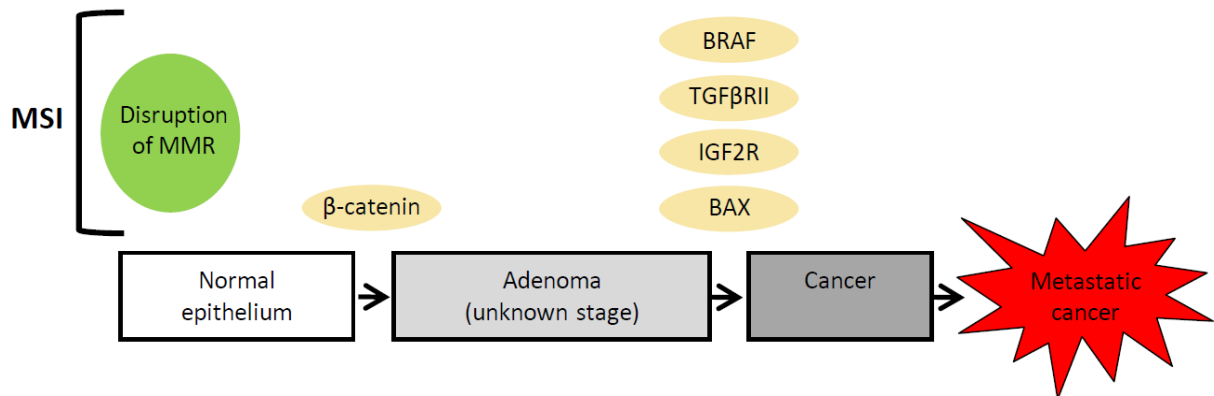


Figure 1.5: *Progression model of CRC arising via microsatellite instability pathway (MSI)*. Disruption of the mismatch repair system (MMR) occurs early within the cancer progression sequence and is caused either by hypermethylation of MLH1 in sporadic cases or inherited defects in one of the MMR genes as found in HNPCC. This leads to the progressive accumulation of mutations in MSI-distinct genes controlling the hallmarks of cancerous behavior, such as TGF $\beta$ R2, Bax,  $\beta$ -catenin, and IGF2R. Mutations of *BRAF* are largely present in sporadic MSI tumors caused by hypermethylation, but usually not in patients suffering from HNPCC. Adapted in part from Grady & Carethers 2008 and Soreide et al. 2009.

Interestingly, alterations typically present in tumors arising via the traditional CIN pathway such as chromosomal abnormalities, allelic loss of or mutations in APC, K-*ras* or p53, are either absent or found in reduced frequencies in sporadic CRC following the MSI pathway (Eshleman et al. 1998; Ionov et al. 1993; Jass et al. 2002; Kinzler & Vogelstein 1996). Yet, a disruption of Wnt-pathway, as typically found in CIN tumors, has also been described in MSI, although deviant underlying mechanisms apart from APC mutations, are implicated, such as mutation of  $\beta$ -catenin (Grady & Carethers 2008).

Molecular differences between cancers following CIN or MSI are also reflected on a histopathological and clinical level: Sporadic MSI-cancers primarily arise in the proximal colon and typically exhibit poor differentiation, a mucinous cell type and a frequent perilymphocytic infiltration (Lynch et al. 2008; Phillips et al. 2004; Young et al. 2001). Furthermore, an association for MSI with older age, female sex and a better over-all prognosis has been suggested (Jass 2007; Lynch et al. 2008). Also, it has been postulated that hyperplastic polyps and other serrated lesions may act as precursors to sporadic MSI-cancers, whereas adenomas are still likely to represent precursors of CRC in HNPCC patients and in CIN tumors (Jass 2007).

### 1.3.3 Epigenetic alterations and epigenetic instability

As already adumbrated in the context of sporadic MSI, epigenetic instability, particularly mediated via aberrant DNA methylation, is also common in CRC (Grady & Carethers 2008). Physiologically, DNA methylation of CpG dinucleotides is an important epigenetic mechanism for the regulation of gene transcription and is widely present and maintained in a relatively stable pattern throughout the human genome (Grady & Carethers 2008; Kondo et al. 2004). However, distinct CpG-rich regions located in the promoters of approximately 50-60% of all genes, which are termed CpG islands, are usually held in an unmethylated state as abnormal methylation within these CpG islands represses gene transcription (Baylin et al. 2000; Grady & Carethers 2008; Jones et al. 1999; Jones et al. 2002). CRC has been reported to frequently display a modest global genomic hypomethylation in combination with aberrant methylation in CpG islands (Bariol et al. 2003; Issa 2004). Besides hypermethylation of MLH1, as found in sporadic CRC following MSI, tumor suppressor genes frequently inactivated by aberrant CpG island promoter methylation include O<sup>6</sup>-methylguanine-methyltransferase (MGMT), p16, p14<sup>ARF</sup>, and helicase-like transcription factor (HLTF) as recently reviewed in great detail by Grady and colleagues (Grady & Carethers 2008).

In this context, several studies suggested that among the loci, which can be aberrantly methylated in CRC, a subgroup undergoes collective methylation of specific genes - a phenomenon referred to as CpG island methylator phenotype (CIMP). Although the distinct underlying mechanism for epigenetic alterations and CIMP in CRC remains uncertain, CIMP has been found in approximately 15-40% of all CRCs (depending upon the altered genes included in the definition of CIMP) and in almost 100% of sporadic cancers arising via the MSI pathway (Issa 2004; Markowitz & Bertagnolli 2009; Toyota et al. 1999; Vandrovcova et al. 2006; Weisenberger et al. 2006; Worthley et al. 2007).

Interestingly, CRCs, which are positive for CIMP but microsatellite stable (intact MMR), share certain clinical and morphological features with sporadic CRCs arising via MSI: Both CRC subtypes show predilections for females, the proximal colon and older age as well as poor and mucinous differentiation and similar precursor lesions. However, overlap between these two subgroups is not exclusive as CRCs, which are positive for CIMP but microsatellite-stable, usually show a more infiltrative growth pattern with discohesive tumor cells, a lack of tumor-infiltrating lymphocytes and are typically diagnosed at an advanced pathological stage. Interestingly, also the better prognosis associated with sporadic tumors displaying MSI does not apply to microsatellite-stable CIMP tumors (Jass 2007).

### 1.4 Environmental risk factors for colorectal cancer and the role of fatty acids

Despite the importance of genetic and epigenetic alterations and predispositions, environmental risk factors play a pivotal role in colorectal carcinogenesis. As mentioned in chapter 1.1, epidemiological studies detected great variations in CRC incidence among different countries and regions around the globe. Although it may be hypothesized that these regional distinctions reflect various genetic predispositions of local populations, migrating populations have been reported to adopt the relative risk of the region they move into within the first generation (Armstrong et al. 1984; Haenszel et al. 1968; McMichael et al. 1980). This implies that the environment constitutes a major component in the development of CRC with diet as one of the most obvious factors (Ilyas et al. 1999).

However, although vast numbers of epidemiological studies investigated environmental risk factors in the context of CRC, clear identifications of these influences remain an issue of ongoing research as available data is heterogeneous and in some respect inconclusive. Nevertheless, a recent meta-analysis, including more than 750 studies, which was conducted by an expert panel of the World Cancer Research Fund suggested environmental factors protective as well as harmful in the context of CRC risk as displayed in table 1.1.

| <i>Panel's judgement</i>    | Decreased Risk  | Increased Risk  |
|-----------------------------|---|---|
| <i>Convincing</i>           | <ul style="list-style-type: none"> <li>- physical activity</li> </ul>   | <ul style="list-style-type: none"> <li>- body fatness</li> <li>- abdominal fatness</li> <li>- red meat (beef, pork, lamb, and goat from domesticated animals)</li> <li>- processed meat (preserved by smoking, curing, or salting, or addition of chemical preservatives)</li> <li>- alcohol &gt;30g/day (men)</li> </ul> |
| <i>Probable</i>             | <ul style="list-style-type: none"> <li>- high fiber diet</li> <li>- garlic</li> <li>- milk</li> <li>- calcium (1200mg/day)</li> </ul>   | <ul style="list-style-type: none"> <li>- alcohol &gt;30g/day (women)</li> </ul>   |
| <i>Limited - suggestive</i> | <ul style="list-style-type: none"> <li>- fish</li> <li>- fruits</li> <li>- non-starchy vegetables</li> <li>- folate</li> <li>- selenium (200µg/day)</li> <li>- vitamin D in fortified foods and animal foods</li> </ul> | <ul style="list-style-type: none"> <li>- foods containing animal fats</li> <li>- high iron diet</li> <li>- cheese</li> <li>- high sugar diet</li> </ul>   |

**Table 1.1:** *Environmental risk factors in the context of CRC.* Influencing factors are graded according to the strength of evidence as judged by experts of the World Cancer Research Fund. Adapted from World Cancer Research Fund 2007.

In addition to body and abdominal fatness, intake of red or processed meat, which were postulated as convincing risk factors by the panel's judgment, a possible role for the intake of fat and FAs in the context of CRC has been discussed for several decades.



As early as 1969, a pioneering case-control study by Wynder et al. suggested an association between high caloric intake from fats and CRC (Wynder et al. 1969). Besides the total amount of fat intake, fat quality appears to be an important factor in this context (McKeown-Eyssen et al. 1985; Nkondjock et al. 2003b; Slattery et al. 1997; Willett et al. 1990).

In this regard, several epidemiological studies reported an inverse correlation between high consumption of fish, a source of omega-3 polyunsaturated fatty acids (n-3 PUFAs), and CRC incidence (Caygill et al. 1996; Kato et al. 1997; Kune et al. 1987; La Vecchia et al. 1988). However, data concerning protective effects of fish consumption and n-3 PUFAs in the context of colorectal carcinogenesis is not fully consistent and has subsequently been challenged in a meta-analysis by MacLean et al. published in 2006 (MacLean et al. 2006). Yet, recent epidemiological studies, not available for or not included in the review by MacLean and colleagues provided additional evidence that high intake of fish, rich in n-3 PUFAs, may significantly diminish the risk for CRC. In particular, the European Prospective Investigation into Cancer and Nutrition Study, including more than 478,000 men from ten European countries and the Prospective Physicians Health Study, including more than 22,000 men in a 22-year follow-up found significant new evidence for the protective effects of diets rich in fish and n-3 PUFAs against CRC (Hall et al. 2008; Norat et al. 2005). Moreover, another recent meta-analysis reported a dose-dependent decrease in CRC risk associated with high fish consumption (Geelen et al. 2007). Highlighting the presumably beneficial effects of n-3 PUFAs in particular, Kojima et al. indicated an inverse correlation between plasma levels of miscellaneous n-3 PUFAs and CRC risk (Kojima et al. 2005).

In contrast to protective effects attributed to n-3 PUFAs, another group of PUFAs, the omega-6 polyunsaturated fatty acids (n-6 PUFAs) have been associated with an elevated risk for CRC (Nkondjock et al. 2003a). Also, colorectal adenoma risk has been reported to be significantly increased by high n-6 PUFA serum levels (Pot et al. 2008). However, epidemiological evidence appears to be not as strong as that concerning the protective effects of n-3 PUFAs.

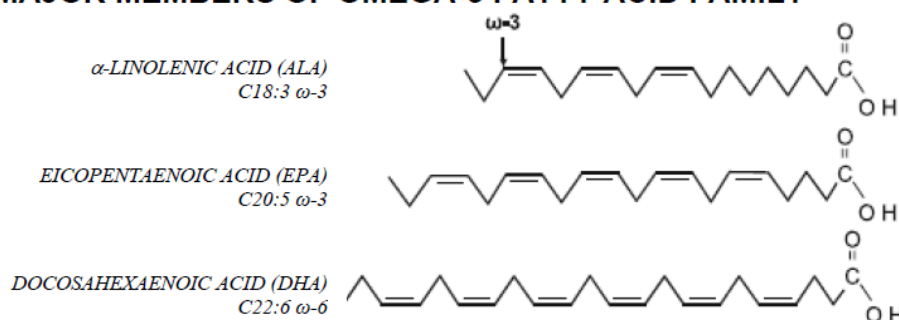
Thus, in summary, quantity, quality as well as configuration of FAs appear to be the critical determinants concerning their role in the context of CRC.

### **1.5 Omega-3 and omega-6 polyunsaturated fatty acids**

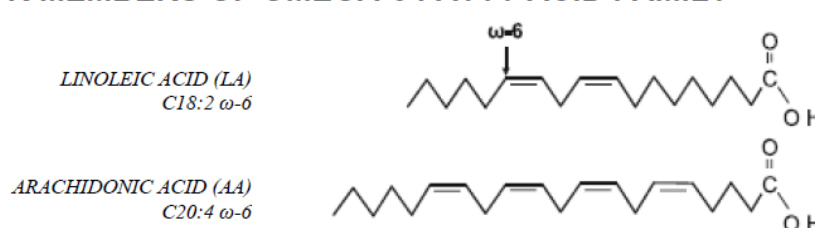
N-3 as well as n-6 PUFAs are both long-chain FAs, which are characterized by the position of their final double bond from the methyl- (or  $\omega$ -) end of the chain. In n-3 PUFAs this double bond is located at the third bond from the methyl end, whereas in n-6 PUFAs it is at the sixth bond

(figure 1.5) (Pauwels et al. 2008). In the designation of FA structure, numerical notation indicates the total number of carbon atoms, followed by the amount of double bonds and the position of the last double bond from the methyl end. Accordingly, the n-3 PUFA docosahexaenoic acid (DHA; 22:6n-3) refers to a 22-carbon FA, containing a total of six double bonds with the first located at the third bond position from the methyl terminus (figure 1.5) (Cunnane 2003; Pauwels & Kostkiewicz 2008). Another typical member of the n-3 PUFA family is eicosapentaenoic acid (EPA; 20:5n-3), whereas arachidonic acid (AA; 20:4n-6) is an important FA in the group of n-6 PUFAs.

### MAJOR MEMBERS OF OMEGA-3 FATTY ACID FAMILY



### MAJOR MEMBERS OF OMEGA-6 FATTY ACID FAMILY



**Figure 1.5:** Structure of n-6 and n-3 polyunsaturated fatty acids. Adapted from Schuchardt et al. 2010.

Interestingly, unlike plants, mammalian organisms do not possess the capacity to endogenously synthesize the characteristic double bonds of n-3 and n-6 PUFAs from scratch. Thus, AA, EPA and DHA have to be either directly provided through diet or may be derived enzymatically from precursors, which likewise have to be supplied via nutrition. Therefore, n-3 as well as n-6 PUFAs are considered *essential* PUFAs (Pauwels & Kostkiewicz 2008; Roynette et al. 2004). Once consumed, PUFA precursors, namely  $\alpha$ -linolenic acid (ALA; 18:3n-3) for n-3 PUFAs or linolenic acid (LA; 18:2n-6) in the context of n-6 PUFAs, are further metabolized via action of various desaturases and elongases through several intermediate stages into EPA or AA. EPA may then be further transformed into DHA. Importantly, in their precursor-related metabolism, n-3 and n-6 PUFAs share an identical set of enzymes thus resulting in a competition between n-3 and

n-6 precursors. Furthermore, mammalian cells are incapable of converting n-6 into n-3 PUFAs or vice versa (Calder 2008; Simopoulos 2009).

Major dietary sources of the n-3 PUFA precursor ALA are green plants, soybeans and rapeseed oils, some nuts, and flaxseed. In contrast, long-chain n-3 PUFAs, such as EPA and DHA, which are synthesized by algae and plankton are found in “oily fish”, such as tuna, salmon, mackerel, herring and sardine. On the other hand n-6 PUFAs are present in vegetable oils, such as corn, soybean or safflower oil as well as in fatty tissues and meat from mammals (Calder 2008; Marszalek et al. 2005; Wendel et al. 2009).

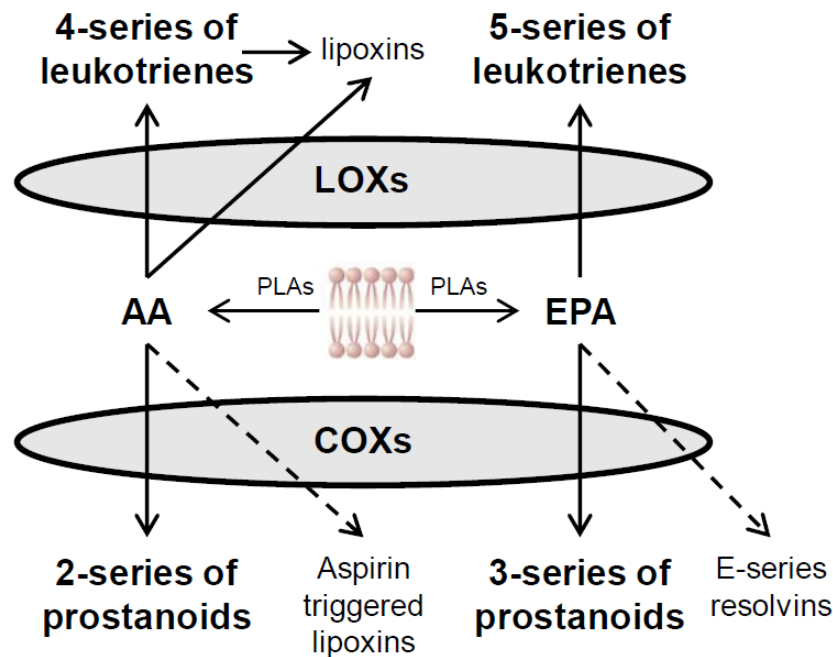
Today n-6 PUFAs represent the major part of PUFAs within diets of industrialized Western countries, whereas n-3 PUFAs only account for much smaller shares, resulting in a n-6 to n-3 ratio of about 15:1 - 20:1 (Simopoulos 2009). However, a high n-6 to n-3 PUFA ratio is reported to be a fairly new development, representing changes in food supply over the past 150 years. In this context studies indicated that for millions of years, while evolution of the *homo sapiens* took its course, n-6 to n-3 PUFA ratio was 1:1, leading to the hypothesis that the human genetic profile was originally established on a balanced n-6 to n-3 PUFA ratio with a much higher intake of n-3 PUFAs than found in today’s Western diets. Based upon this concept, it has been suggested that the recent imbalance of n-6 to n-3 PUFA ratio may significantly contribute to increased risks of several diseases typically found in our modern Western societies, including CRC (Eaton et al. 1985; Eaton et al. 1998; Kang 2005; Leaf et al. 1987; Simopoulos 2009).

### **1.5.1 Polyunsaturated fatty acid metabolism and lipid derived mediators**

The verisimilar importance of changes in PUFA ratio has been further highlighted over recent decades as evidence emerged that FAs are not solely a source of energy for the human body, but that they are also precursors to numerous biologically highly active lipid mediators (Weylandt & Kang 2005).

Once ingested, PUFAs are incorporated into the cellular lipid membrane. In response to various stimuli, such as the binding of growth factors and hormones to membrane receptors, PUFAs are released from the lipid bi-layer through phospholipases (PLAs) and become substrates to miscellaneous enzymes. These enzymes include cyclooxygenases (COXs; consisting of two isoforms COX-1 and COX-2; see also chapter 1.6) and lipoxygenases (LOXs) and subsequently lead to the formation of a large variety of PUFA-derived lipid mediators as recently reviewed in great detail by Stables and Gilroy (Schmitz et al. 2008; Stables & Gilroy 2011). In this context so called eicosanoids, which include prostaglandins (PGs) and thromboxanes (TXs) (collectively

referred to as prostanoids) as well as leukotrienes (LTs) and lipoxins (LXs) are key substances derived from 20-carbon PUFAs such as AA and EPA (figure 1.6) (Schmitz & Ecker 2008; Stables & Gilroy 2011). Given the fact that AA represents the predominant 20-carbon PUFA within cellular membranes compared to n-3 20-carbon PUFA EPA, AA acts as primary substrate of eicosanoid biosynthesis (Pauwels & Kostkiewicz 2008).



**Figure 1.6:** *PUFA metabolism and derived lipid mediators (simplified).* AA and EPA are released from the lipid bi-layer through metabolic activity of phospholipases (PLAs). Via action of cyclooxygenases (COXs) n-6 PUFA AA is metabolized into the 2-series of prostanoids, whereas transformation through lipoxygenases (LOXs) leads to the synthesis of 4-series leukotrienes and lipoxins. In analogy, n-3 PUFA EPA is metabolized to the 3-series of prostanoids via COXs and 5-series of LTs via LOXs. Aspirin-triggered lipoxins as well as E-series resolvins are biosynthesized through complex pathways involving combined actions of Aspirin acetylated COX-2 as well as LOXs. Based upon information obtained from Chan et al. 2010, Stables & Gilroy 2011 and Wendel & Heller 2009.

Physiologically, prostanoids and leukotrienes are involved in the regulation of a plethora of biological functions ranging from inflammatory response, hyperalgesia, and fever to renal function, smooth muscle tone as well as platelet aggregation (DiBona 1986; Eckenfels et al. 1972; Ferreira et al. 1978; Moncada et al. 1973; Moncada et al. 1976; Narumiya et al. 2001; Stables & Gilroy 2011). For both prostanoids and LTs, numerous specific cell-surface receptors have been identified, through which these molecules elicit their various effects. Thus, identical prostaglandins may exert various effects in miscellaneous cell and tissue types, depending upon receptor expression (Hirai et al. 2001; Kanaoka et al. 2004; Lynch et al. 1999; Narumiya & FitzGerald 2001; Weylandt & Kang 2005).

Interestingly, AA-derived eicosanoids have been widely implicated to primarily exert pro-inflammatory actions in a number of conditions and diseases, which include inflammation of the

skin and lung (e.g. asthma) as well as joints (e.g. arthritis) (Kabashima et al. 2007; Le et al. 2009; Matsuoka et al. 2000; McCoy et al. 2002; Peters-Golden et al. 2005; Schmitz & Ecker 2008; Weylandt & Kang 2005). Within the gastrointestinal tract, AA-derived prostaglandins elicit stimulatory as well as protective functions in inflammatory conditions: While sudden dramatic increases of PGs in the mucosa correlate with the illness activity of inflammatory bowel diseases and experimental colitis, baseline expressions of these PGs exert protective functions against gastrointestinal injury and ulcers as well as acute and chronic enterocolitis (Banan et al. 1998; Carty et al. 2000; Kandil et al. 1999; Redfern et al. 1989; Subbaramaiah et al. 2004).

Yet, contrasting the majority of AA-derived eicosanoids, lipoxins (including LXA<sub>4</sub> and LXB<sub>4</sub>) and Aspirin-triggered lipoxins (ATLs), generated from AA either via LOXs or acetylated COX-2, have been found to generate effective anti-inflammatory responses. Hence, these n-6 PUFA-derived substances appear to control the resolution of inflammation by stimulating endogenous anti-inflammatory pathways and thus antagonize pro-inflammatory signals mediated by other COXs- and LOXs-synthesized AA derivatives (Andersson et al. 2004; McMahon et al. 2004).

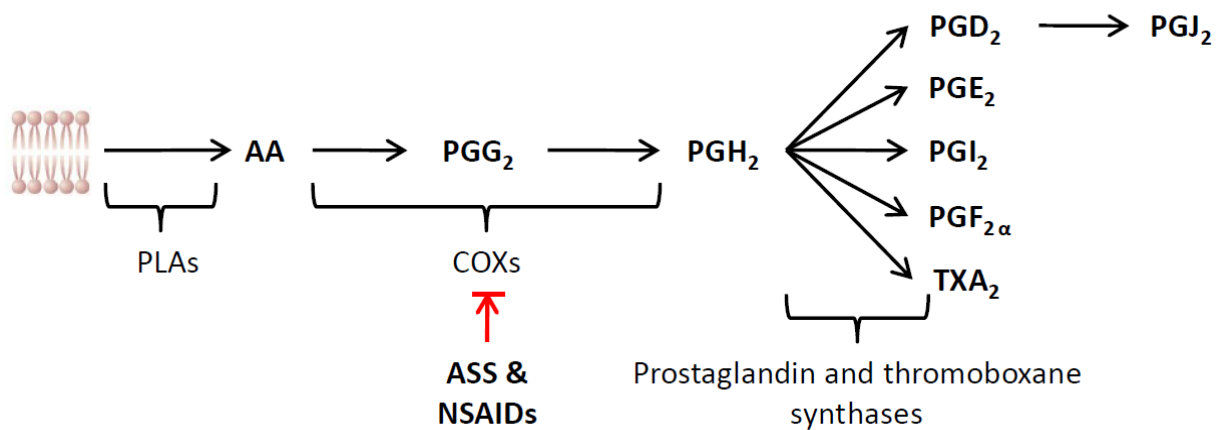
On the other hand n-3 PUFA-derived lipid mediators are believed to be less potent and exert either anti-inflammatory or less pro-inflammatory functions compared to those synthesized from AA (Le et al. 2009; Schmitz & Ecker 2008; Weylandt & Kang 2005). For example, EPA-derived thromboxane A<sub>3</sub> has considerably less pro-aggregatory and vasoconstrictive properties than AA-derived thromboxane A<sub>2</sub>. Moreover, EPA-based 5-series leukotrienes execute partially antagonistic effects compared to their pro-inflammatory AA-derived counterparts (Wendel & Heller 2009).

Moreover, in addition to EPA-derived eicosanoids, recent investigations applying lipidomic analysis identified new classes of n-3 PUFA-derived mediators, namely resolvins, protectins and maresins (Lu et al. 2005; Serhan et al. 2000; Serhan et al. 2002; Serhan et al. 2009). While resolvins are metabolized either from EPA or DHA through acetylated COX-2- and LOX-dependent pathways, resulting in an E-series (resolvin E1 and E2) and a D-series (resolvin D1-D6) respectively, protectins and maresins are both generated from DHA via separate pathways, which have recently been reviewed by Serhan (Serhan 2009). As landmark discoveries in the context of these n-3 PUFA-derived lipid mediators, distinct receptors (the orphan ChemR23 and leukotriene receptor BLT1) have been identified for resolvin E1 (Arita et al. 2005; Arita et al. 2007). Importantly, the newly discovered n-3 PUFA-derived lipid mediators possess potent anti-inflammatory properties and have been found to play an essential role in the physiological resolution of inflammation (Serhan 2009).

Nevertheless, aside from regulatory functions in the context of tissue homeostasis and inflammation as outlined above, COX enzymes as well as (AA-derived) eicosanoids have been implicated to play a pivotal role in the context of CRC, which will be outlined in greater detail in the following paragraphs.

### 1.6 Cyclooxygenases, nonsteroidal anti-inflammatory drugs and AA-derived prostaglandin E<sub>2</sub> in colorectal cancer

As indicated above, COXs, comprised of at least two different isoforms, are key enzymes in the generation of lipid-derived mediators and play a crucial role in the AA-dependent formation of biological active 2-series PGs and TXs, as recently reviewed by Stables and Gilroy and displayed in figure 1.7 (Stables & Gilroy 2011).



**Figure 1.7:** AA-derived 2-series of prostanoids in detail. Following liberation from lipid bi-layer via phospholipases (PLAs), COXs transform AA into prostaglandin G<sub>2</sub> (PGG<sub>2</sub>) and subsequently into prostaglandin H<sub>2</sub> (PGH<sub>2</sub>). PGH<sub>2</sub> is then further transformed into biologically active prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), prostaglandin J<sub>2</sub> (PGJ<sub>2</sub>), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), prostaglandin I<sub>2</sub> (PGI<sub>2</sub>), prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>), and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) through cell- and tissue-specific prostaglandin or thromboxane synthases. Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the enzymatic activity of COXs. Based upon information from Stables & Gilroy 2011.

While COX-1 is constitutively expressed in a large variety of tissues serving as a “housekeeper” responsible for basal levels of prostanoids and thus tissue homeostasis, COX-2 is an immediate early response gene, which is induced in inflammatory and tumorigenic settings but not expressed under regular circumstances by most tissues (Arber 2008; Greenhough et al. 2009; Xie et al. 1991; Yokoyama et al. 1988). Interestingly acetylsalicylic acid (ASS - as in Aspirin) and other nonsteroidal anti-inflammatory drugs (NSAIDs) efficiently inhibit catalytic function of COXs (Arber 2008; Vane 1971).

Hence, first observations concerning an involvement of COXs and their products in colorectal carcinogenesis arose from observations that patients with Gardner’s syndrome (a subset of FAP)

treated with NSAIDs displayed reduced numbers of adenomas (Waddell et al. 1983). Subsequently several studies indicated an association between regular NSAID intake (resulting in a subsequent inhibition of COXs) and lower risk for CRC as well as an improved overall survival in CRC patients (Breuer-Katschinski et al. 2000; Chan et al. 2009; Chan & Giovannucci 2010; La Vecchia et al. 1997; Rothwell et al. 2010; Sandler et al. 1998; Thun et al. 1991).

The pro-tumorigenic effects associated with COXs and n-6 PUFAs in colorectal carcinogenesis are widely attributed to their fundamental roles in PGE<sub>2</sub> biosynthesis (Greenhough et al. 2009; Wang et al. 2008). Elevated levels of PGE<sub>2</sub> have not only been found in the majority of human colorectal adenomas and carcinomas but *in vivo* studies also reported administration of exogenous PGE<sub>2</sub> to strongly increase the adenoma burden in a murine CRC model (APC<sup>Min/+</sup> mice) as well as the incidence and multiplicity of carcinogen-induced CRCs in rats (Backlund et al. 2005; Kawamori et al. 2003; Pugh et al. 1994; Rigas et al. 1993; Wang et al. 2004). Supplementation of PGE<sub>2</sub> also effectively reversed the NSAID-induced regression of small intestinal adenomas in mice, whereas disturbance of PGE<sub>2</sub> degradation via loss of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) promoted the tumor growth in APC<sup>Min/+</sup> mice (Hansen-Petrik et al. 2002; Myung et al. 2006).

Based upon these observations, extensive *in vivo* and *in vitro* research efforts focused on the molecular mechanisms involved in PGE<sub>2</sub>-associated CRC progression. These studies found PGE<sub>2</sub> to exert its cellular effects mainly through distinct cognate receptors (EP1-4), which belong to the family of seven transmembrane G-protein coupled rhodopsin-type receptors. Via activation of these and other receptors, PGE<sub>2</sub> enhances cellular proliferation, promotes angiogenesis, inhibits apoptosis, stimulates cancer cell invasion as well as motility, suppresses immune responses and thus promotes colorectal tumor growth (Greenhough et al. 2009; Wang et al. 2006; Wendel & Heller 2009). Accordingly, homozygous deletions of some of these receptors (EP1 and EP4, but not EP3) have been reported to result in partial resistance to carcinogen-induced CRC precursor lesions in mice (Mutoh et al. 2002; Sonoshita et al. 2001; Watanabe et al. 1999). Numerous molecular pathways are implicated to be altered by, and thus involved in, pro-carcinogenic effects of PGE<sub>2</sub> (Wang & Dubois 2006). For instance, PGE<sub>2</sub> has been found to activate epidermal growth factor receptor (EGFR), which triggers CRC cell growth and mediates PGE<sub>2</sub>-induced cell migration via the subsequent activation of the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway (Buchanan et al. 2003; Pai et al. 2002; Sheng et al. 2001). Furthermore, the PI3K-Akt pathway has been reported to play a substantial role in the PGE<sub>2</sub>-

associated activation of the peroxisome proliferator activated receptor- $\delta$  (PPAR- $\delta$ ), which additionally supports cellular survival (He et al. 1999; Wang et al. 2004).

Moreover, PGE<sub>2</sub> has been identified as activator of the Wnt-pathway, which is a key player in sporadic colorectal carcinogenesis (as indicated in chapter 1.3.1), leading to the accumulation and increased nuclear translocation of  $\beta$ -catenin, which in turn increases CRC cell proliferation (Castellone et al. 2005). Additionally, driving cancer cell proliferation as well as the evasion of apoptosis, PGE<sub>2</sub> also activates the Ras-mitogen activated protein kinase/extracellular signal-regulated kinase (Ras-MAPK/ERK) pathway, which in turn up-regulates expression of COX-2 (Backlund et al. 2006; Wang et al. 2005).

Also PGE<sub>2</sub> is known to induce anti-apoptotic proteins such as B-cell lymphoma-2 (Bcl-2) and nuclear factor  $\kappa$ B (NF $\kappa$ B) as well as to elevate vascular endothelial growth factor (VEGF) expression, hence further supporting evasion of apoptosis and tumor angiogenesis (Fukuda et al. 2003; Poligone et al. 2001; Sheng et al. 1998).

Taking into account the numerous pathways through which PGE<sub>2</sub> promotes colorectal carcinogenesis, beneficial effects of ASS and other NSAIDs, which potently diminish biosynthesis of AA-derived PGE<sub>2</sub>, appear plausible. However, regular use of NSAIDs is associated with primarily gastrointestinal and renal side effects, most importantly gastrointestinal ulcers and bleeds (Chell et al. 2006; Khanapure et al. 2007; Wolfe et al. 1999). In this context adverse effects of ASS and NSAIDs have been reported to be responsible for approximately 260,000 hospitalizations and 26,000 deaths annually all over the world (Larousse et al. 2000). Yet, it has been postulated that the vast majority of adverse effects result from inhibition of COX-1. In contrast, anti-neoplastic effects of NSAIDs are widely attributed to inhibition of COX-2 as supported by several lines of evidence: Approximately 50% of premalignant colorectal adenomas as well as up to 85% of malignant CRCs display an elevated COX-2 expression, which is additionally associated with a worse prognosis (Chell et al. 2006; Eberhart et al. 1994; Elder et al. 1998; Greenhough et al. 2009; Kargman et al. 1995; Ogino et al. 2008; Sano et al. 1995; Sheehan et al. 1999; Wang et al. 2010; Wolfe et al. 1999). Also, animal studies conducted in several murine CRC models additionally deficient for COX-2 showed a reduced colorectal tumor formation (Chulada et al. 2000; Oshima et al. 1996). In contrast, transgene mice over-expressing COX-2 suffered from elevated tumor loads in comparison to their wild-type litter mates in carcinogen-induced CRC (Al-Salihi et al. 2009). However, most convincingly, selective COX-2 inhibitors (COXIBs), which were developed in order to omit the aforementioned adverse effects



of unselective NSAIDs, retain many of the anti-neoplastic properties reported for traditional NSAIDs.

In this context a plethora of evidence suggests that regular use of COX-2 selective COXIBs or NSAIDs reduces the relative risk of CRC development by 40-50% (Chan et al. 2008; Chan & Giovannucci 2010; Flossmann et al. 2007; Greenhough et al. 2009; Rothwell et al. 2010; Rostom et al. 2007; Samoha et al. 2005). Regrettably, several studies indicated that COXIBs significantly elevate the incidence of cardiovascular events by 2.5 to 3-fold.

Thus, primary chemo-prevention of CRC with unselective as well as COX-2 selective inhibitors remains a topic of ongoing debate (Baron et al. 2008; Bresalier et al. 2005; Chan & Giovannucci 2010; Solomon et al. 2005; Solomon et al. 2006).

### **1.7 Experimental findings of n-3 polyunsaturated fatty acids in the context of colorectal cancer**

While many studies aimed to explore the pro-carcinogenic effects associated with n-6 PUFAs and their bioactive metabolites, also large numbers of *in vitro* and *in vivo* studies have been conducted to investigate the protective effects of n-3 PUFAs in the context of CRC. Importantly the majority of these studies confirmed and highlighted the epidemiologically deduced anti-cancerous and protective effects of n-3 PUFAs. For example a study conducted in humans found EPA to reduce colon crypt cell hyperproliferation and to increase mucosal apoptosis (Courtney et al. 2007). Moreover, two investigations applying the *fat-1* mouse, a transgenic model with increased amounts of endogenously synthesized n-3 PUFAs, revealed protective effects of n-3 PUFAs against colon tumor development (Jia et al. 2008; Nowak et al. 2007).

In addition numerous *in vitro* studies conducted in a large variety of CRC cell lines provided further evidence concerning protective properties of n-3 PUFAs in regard to CRC and hinted at some of the underlying mechanisms potentially involved.

In this context, the n-3 PUFA DHA has been reported to exert growth-inhibitory effects *in vitro* via induction of apoptosis in Caco-2 human CRC cells. This was accompanied by a significant alteration of gene transcription and protein levels of a large variety of targets including COX-2, cyclin-dependent kinase inhibitor 1A (p21<sup>WAF1</sup>), inducible nitric oxide synthase (iNOS), cyclin D1,  $\beta$ -catenin and nuclear factor  $\kappa$ B (NF $\kappa$ B), all of which presumably participate in colorectal carcinogenesis (Narayanan et al. 2001; Narayanan et al. 2003; Narayanan et al. 2004). Moreover, Giros and colleagues indicated DHA as well as EPA to elicit pro-apoptotic effects in multiple CRC cell lines, displaying different phenotypes as well as different types of genetic instability.

Further investigating the underlying molecular mechanisms in this study, involvement of the extrinsic (caspase 8) and the intrinsic (caspase 9) pathway in n-3 PUFA-induced apoptosis and altered expression of numerous genes controlling the induction of apoptosis (including Bcl-2, Bid, Bax, Bcl<sub>XL</sub>) as well as a down-regulation of COX-2 expression was revealed (Giros et al. 2009).

As suggested by n-3 PUFA-associated suppression of COX-2 expression reported in the studies mentioned above, beneficial effects of these FAs are, at least in part, attributed to interaction and interference with n-6 PUFA metabolism (Berquin et al. 2008). In this regard, (dietary) supplementation of n-3 PUFAs significantly decreases AA in cellular membranes as it is replaced by DHA and EPA (Calder 2008). As a result the amount of available AA for COXs- and LOXs-dependent metabolism is reduced. Additionally, DHA has been reported to inhibit metabolic activity of COX-2, whereas EPA competes with AA as a substrate for these enzymes, resulting in the formation of less bioactive 3-series of prostaglandins and 4-series of leukotrienes (Ringbom et al. 2001; Wendel & Heller 2009). Hence, the quantity of AA-derived eicosanoids, including pro-inflammatory and pro-proliferative 2-series PGs (such as PGE<sub>2</sub>) and 4-series LTs (also see chapters 1.5.1 and 1.6) is decreased. The importance of n-3 PUFA-related interference with AA metabolism is further highlighted by the EPA- and DHA-induced suppression of PGE<sub>2</sub> formation (and COX-2 down-regulation) in HT-29 CRC cells, resulting in a significant *in vitro* and *in vivo* (when implanted into nude mice) growth inhibition. In the identical study EPA and DHA were furthermore shown to potently inhibit phosphorylation of extracellular signal-regulated kinases (ERK1 and ERK2) and hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) expression, thus decreasing vascular endothelial growth factor (VEGF) levels, which is widely recognized as a potent angiogenic factor in the context of new vessel formation within CRC (Calviello et al. 2004; Warren et al. 1995).

However, n-3 PUFA-derived lipid mediators, which have been mostly investigated in the context of inflammatory resolution, may also directly exert anti-proliferative actions, as for instance, EPA-derived PGE<sub>3</sub> was found to potently exert anti-proliferative effects in human lung cancer cells (Yang et al. 2004).

Nevertheless, several lines of evidence also suggest that n-3 PUFA-related effects do not exclusively rely on COX-related mechanisms: Boudreau et al. demonstrated n-3 PUFAs, including fish oil, as well as separately applied EPA and DHA to inhibit the growth of CRC cells deficient for COXs. These effects were observed *in vitro* as well as in grafted nude mice, and not altered by retroviral COX-1 or COX-2 transfection (Boudreau et al. 2001). Consistent with this,

another study indicated n-3 PUFA DHA to potently induce apoptosis in two CRC cell lines, which lack COX-2 protein but over-express  $\beta$ -catenin. Importantly, in this study DHA significantly reduced  $\beta$ -catenin protein expression via increased proteasomal degradation and decreased translocation of  $\beta$ -catenin into the nucleus. Thus, activation of the aforementioned Wnt-pathway, which regulates transcription and induces cellular proliferation, is decreased (Calviello et al. 2007). In addition several other transcription factors, such as peroxisome proliferator-activated receptor- $\delta$  (PPAR- $\delta$ ), have been shown to be involved in n-3 PUFA-associated gene regulation (Allred et al. 2008; Pauwels & Kostkiewicz 2008).

Moreover, anti-cancerous properties of n-3 PUFAs may also be attributed to their ability to change lipid composition of the plasma membrane, which might affect the membrane fluidity and the way growth factors, cytokines and hormones interact with their receptors, and the resulting signal transduction through secondary messengers as recently reviewed by Berquin and co-workers (Berquin et al. 2008).

In synopsis, numerous mechanisms accounting for the beneficial effects of n-3 PUFA in the context of CRC have been proposed, including the induction of apoptosis, the alteration of gene expression via multiple pathways, the regulation of various cellular signaling pathways as well as the interference with AA-related metabolism. Nevertheless, the exact molecular processes mediating beneficial effects of n-3 PUFAs in the context of CRC are a matter of considerable debate and remain to be fully understood (Chapkin et al. 2008; Slagsvold et al. 2010).

### **1.8 Research aims**

Convincing epidemiological evidence suggests an inverse correlation between the intake of long-chain n-3 PUFAs as found in fish and CRC incidence (as discussed in chapter 1.4). Over the past decades vast numbers of experimental studies have been conducted in order to further investigate effects of n-3 PUFAs and the underlying mechanisms. As outlined above a large body of *in vitro* evidence (chapter 1.7) indicated anti-cancerous effects of n-3 PUFAs in multiple CRC cell lines, thus reflecting and supporting epidemiologically-deduced beneficial effects of n-3 PUFAs (Allred et al. 2008; Baumgartner et al. 2004; Calviello et al. 2004; Calviello et al. 2007; Danbara et al. 2004; Giros et al. 2009; Narayanan et al. 2001; Narayanan et al. 2003; Narayanan et al. 2004; Toit-Kohn et al. 2009).

However, these *in vitro* studies as well as the majority of investigations conducted in the field of n-3 PUFA research did not address effects of n-6 PUFAs, whereas miscellaneous other *in vitro* studies demonstrated n-3 PUFAs and n-6 PUFAs to identically inhibit CRC cell growth and to

induce apoptosis in several CRC cell lines (Dommels et al. 2002; Dommels et al. 2003; Schonberg et al. 2006). Furthermore, additional *in vitro* investigations, which were solely focused on n-6 PUFAs and their impact in CRC, indicated AA as a potent inducer of apoptosis and growth inhibition (Moncada et al. 1973; Oraldi et al. 2009).

In synopsis, these *in vitro* observations have led to some uncertainty concerning a differential role of n-3 and n-6 PUFA in the context of colorectal tumor cell growth. Furthermore, effects of a changed n-6 to n-3 PUFAs ratio on CRC cell proliferation have not yet been assessed. Thus, the present study set out to investigate the effects of n-3 PUFA DHA and n-6 PUFA AA *in vitro*. For this purpose we used the LS 174T CRC cell line, for which a potent PGE<sub>2</sub>-triggered activation of proliferation has been demonstrated previously (Shao et al. 2003; Sheng et al. 2001). Based upon previous epidemiological and experimental findings we hypothesized n-3 PUFA DHA to exert anti-carcinogenic effects, whereas we suspected n-6 PUFA AA to elicit pro-carcinogenic actions in the same experimental model.

In order to test our hypothesis we evaluated the impact of DHA and AA on cancer cell growth, induction of apoptosis, and gene expression as well as the influence on AA-based PGE<sub>2</sub> metabolism. Moreover, taking into account that several lines of evidence suggest that there may be considerable interactions between n-3 and n-6 PUFAs, we strived to assess the effects of DHA co-incubation with AA in regard to cellular proliferation and PGE<sub>2</sub> synthesis.

## **2. Material und Methoden / Materials and methods**

Investigating the influence of n-3 and n-6 PUFAs on CRC cells, we first established a model for the application of FAs to the cells. In order to determine the effects of FAs in the context of cellular viability, cell cycle and apoptosis we applied XXT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assays, flow cytometry experiments, and DAPI stainings. To measure the impact of FAs in the context of cellular mRNA expression real-time PCR (qRT-PCR) experiments were conducted and for the assessment of PGE<sub>2</sub> metabolism a PGE<sub>2</sub>-ELISA was applied.

### **2.1 Cell culture**

The LS 174T CRC cell line was generously provided by Prof. Dr. Liu, Harvard Medical School, Boston, USA. LS 174T cells (also referred to as LS 174 as personally communicated by Dr. Rosemarie Steubing, CLS - Cell Lines Service GmbH, Eppelheim, Germany and Claudia Orhan, LGC Standards GmbH, Wesel, Germany) is a variant of LS 180, which was originally obtained from a 58 years old Caucasian female. LS 174T cells were described as MSI and negative for p53 antigen, but positive for p53 mRNA expression. Furthermore, cells have been reported to synthesize large amounts of carcinoembryonic antigen (CEA) (Abdel-Rahman et al. 2001; American Type Cell Collection 2011; Tom et al. 1976).

In the present study, the LS 174T cell line was grown in Dulbecco's modified Eagle's medium without phenol red (DMEM; Gibco, Carlsbad, CA, USA), which contained 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2µmol/mL glutamine (Sigma Aldrich, St. Louis, MO, USA) as well as 100U/mL penicillin and 100µg/mL streptomycin (both Gibco, Carlsbad, CA, USA).

Cells were maintained in 75cm<sup>2</sup> flasks (BD Biosciences San Jose, CA, USA), split when approximately 80% confluent and kept under a saturated atmosphere of 5% CO<sub>2</sub> and 95% air at 37° Celsius.

### **2.2 Stock solutions of fatty acids**

FAs including docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA) were obtained from NuchekPrep (Elysian, MN, USA) in the quantity of ≥ 100mg. FAs were transferred into glass tubes and the amount was determined by scale. Based upon the amount of FAs obtained and according to the different molecular weight (DHA 328.4g/mol, EPA 302.4g/mol, AA 304.5g/mol), FAs were solved in the appropriate amount of 100% ethanol

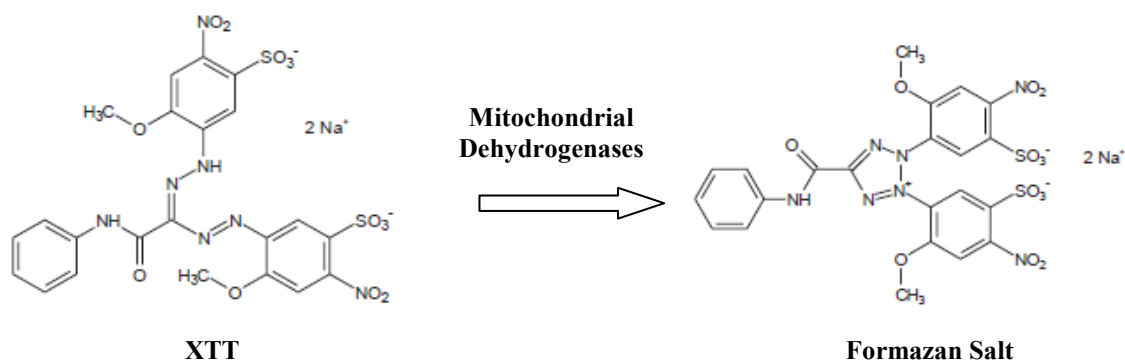
(Sigma Aldrich, St. Louis, MO, USA) to reach a concentration of 100mM for stock solution. Solutions were subdivided into 2mL aliquots in glass tubes, additionally filled with nitrogen gas and stored at -20° Celsius in order to prevent FA oxidation. After 21 days, aliquots in use for experiments were discharged and new glass tubes containing FA were taken from stock.

### 2.3 Preparation of medium containing fatty acids and prostaglandin E<sub>2</sub>

Appropriate amounts of FAs and/or PGE<sub>2</sub> (Cayman Chem, Ann Arbor, MI, USA) or vehicle (ethanol for FAs or DMSO for PGE<sub>2</sub>, respectively) were dissolved in Dulbecco's modified Eagle's medium containing 2% FBS, 2μmol/mL glutamine, 100U/mL penicillin, 100μg/mL streptomycin, and 1mg/mL fatty-acid-free bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO, USA). After addition, tubes containing medium and FAs and/or PGE<sub>2</sub> were vortexed thoroughly for approximately 30 seconds and then incubated at 37° Celsius for another 30 minutes. Instantly prior to addition to cells, tubes were vortexed for another 30 seconds. These measures as well as addition of BSA to medium were undertaken to assure complete dissolution of FAs and PGE<sub>2</sub> in the aqueous medium. In order to avoid unspecific toxic effects of free long-chain FAs, the maximum total FA concentration used in the long-term incubation cell viability experiments did not exceed 100μM.

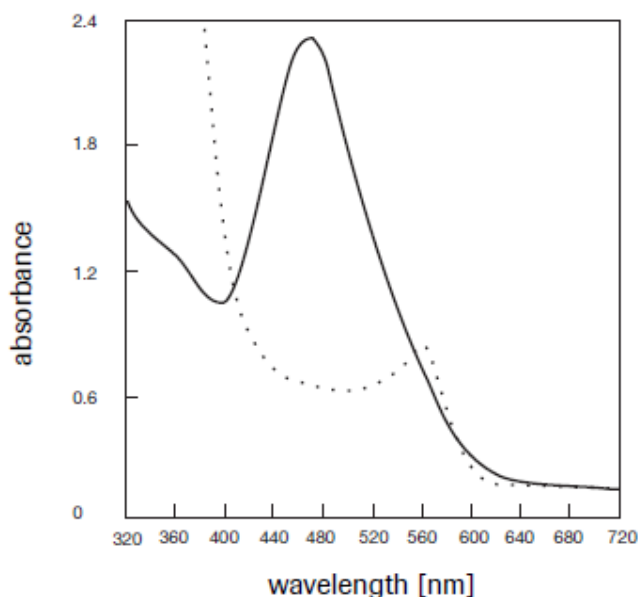
### 2.4 XTT - cell proliferation assay

Cellular viability was determined by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assays according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye in metabolically active cells by mitochondrial dehydrogenases (figure 2.1) (Gerlier et al. 1986).



**Figure 2.1:** Functional principle of XTT assay. Mitochondrial dehydrogenases of viable cells transform XTT into soluble orange formazan salt, which can be assessed photometrically. Roche 2004.

This conversion only occurs in viable cells. Since a higher number of living cells results in an elevated overall activity of mitochondrial dehydrogenase, the amount of orange formazan formed directly correlates with the number of vital cells. Absorbance of orange formazan dye can be accessed photometrically (figure 2.2) (Roche 2004).



**Figure 2.2:** UV-spectra of XTT and formazan dye. Comparison of UV-spectra of XTT labeling mixture (dotted line) and formazan dye (continuous line). Roche 2004.

For the assessment of cellular viability,  $2.5 \times 10^4$  LS 174T cells per well were seeded into a 96-well plate (BD Biosciences, San Jose, CA, USA) in 100 $\mu$ L of regular DMEM without phenol red. After 24 hours, original medium was removed and replaced by 100 $\mu$ L of medium containing the appropriate concentrations of PUFAs and/or PGE<sub>2</sub> or vehicle, which was prepared as outlined in chapter 2.3. Each condition was laid out in four separate wells and incubated for 24 hours, 72 hours or 120 hours.

In order to activate XTT solution prior to addition to the cells, XTT labeling reagent (concentration 1mg/mL dissolved in DMEM without phenol red) was mixed with electron coupling reagent N-methyl dibenzopyrazine methyl sulfate (concentration 0.383mg/mL dissolved in sterile PBS; Invitrogen, Carlsbad, CA, USA). Then 50 $\mu$ L of activated XTT solution was added to each well, resulting in a final XTT-concentration of 0.3mg/mL, and incubated at 37° Celsius. After six hours, formation of orange formazan was assessed photometrically in a plate reader (Victor 1420, Perkin, Elmer, Waltham, MA, USA) at 490nm wavelength. In order to account for possible background absorption, absorbance was measured in a second scan at 620nm.

Absorbance generated at 620nm was subtracted from values measured at 490nm. For graphical layout controls were set as 100%.

### **2.5 Flow cytometry assay**

For cell cycle analysis,  $5 \times 10^5$  cells were plated in 10cm dishes (BD Biosciences, San Jose, CA, USA). After 24 hours, medium was removed and replaced by 10mL DMEM that contained PUFAs (prepared as outlined in chapter 2.3). Cells were harvested for flow cytometry after an additional 24 hours, 72 hours and 120 hours of incubation. For this purpose, medium as well as cells from dishes were collected in pre-chilled 15mL tubes (BD Biosciences San Jose, CA, USA). Tubes were centrifuged for 5 minutes at 1000rpm at 4° Celsius (Centrifuge 5810 R, Eppendorf, Hamburg, Germany), supernatant was removed carefully and the pellet was washed with 5mL of cold PBS. This step was repeated twice and after final removal of PBS, the cellular pellet was dissolved in 1mL of ice-cold ethanol (stored at -20° Celsius) for fixation for 20 minutes. Cells were then washed twice with PBS again.

For detection of the preG1 fraction, as indicator for apoptosis, cells were stained with 0.1mg/mL propidium iodide (PI; Sigma Aldrich, St. Louis, MO, USA), which contained 0.5mg/mL RNase (Sigma Aldrich, St. Louis, MO, USA) and 0.1% NP40 detergent (Sigma Aldrich, St. Louis, MO, USA) for 10 minutes and washed twice with PBS. Afterwards, cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer.

### **2.6 4',6'-diamidino-2-phenylindole (DAPI) staining**

In order to access nuclear morphology after FA incubation, cells were stained with 4',6'-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA). DAPI is a fluorescent stain that strongly binds to deoxyribonucleic acid (DNA). For fluorescence microscopy, DAPI was excited with ultraviolet light. When bound to double-stranded DNA, its absorption maximum is at 358nm and its emission maximum is at 461nm.

For staining,  $1 \times 10^4$  cells were grown on poly-d-lysine (Sigma Aldrich, St. Louis, MO, USA) covered glass slides in 24-well plates (BD Biosciences, San Jose, CA, USA) in 250 $\mu$ L medium. After 24 hours the original medium was replaced by DMEM containing PUFAs (prepared as outlined in chapter 2.3). After 72 hours of incubation, cells were washed twice with PBS and fixed by incubation with 2% paraformaldehyde (PFA; Sigma Aldrich, St. Louis, MO, USA) at 4° Celsius for 20 minutes. Then cells were washed with PBS again and permeabilized with 0.1% Triton X 100 (Sigma Aldrich, St. Louis, MO, USA) for 10 minutes. Cells were stained with



DAPI solution for 5 minutes (concentration 300nM), washed twice and flipped on glass slides using one drop of Immu-Mount (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cells on the slides were assessed for nuclear morphology and apoptotic bodies with an Olympus BX51 Fluorescence Microscope (Olympus, Center Valley, PA, USA) and pictures were taken from representative cells.

## **2.7 Semiquantitative real-time PCR (qRT-PCR)**

For analysis of mRNA gene expression,  $5 \times 10^5$  cells were plated in 10cm dishes. After 24 hours, medium was removed and replaced by 10mL medium containing the appropriate concentration of FAs (prepared as outlined in chapter 2.3).

### **2.7.1 RNA extraction**

After 48 hours of incubation, medium was removed and total RNA was isolated from LS 174T cells using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Briefly, this technology combines the selective binding properties of a silica-based membrane with the microspin technology. A specialized high-salt buffer system allows RNA longer than 200 bases to bind to the RNeasy silica membrane. After complete removal of medium, samples were lysed and homogenized in the cell-culture vessel in the presence of a highly denaturing guanidine-thiocyanate-containing buffer (Buffer RLT), which immediately inactivates RNAses to ensure purification of intact RNA. Then 70% ethanol was added to provide appropriate binding conditions and samples were then applied to an RNeasy mini spin column. Here, in several washing and high speed centrifuging steps at 10,000rpm, the total RNA bound to the membrane, whereas contaminants were efficiently washed away. RNA was then collected from the membrane by addition of RNase-free water to the column followed by an additional spin down. The total RNA was eluded in 30 to 100 $\mu$ L of RNase-free water and stored at -20° Celcius.

RNA concentrations and purity were assessed via spectrophotometrical determination of optical density (OD) at 260nm and 280nm using a GeneQuant pro RNA/DNA analyser (Amersham Bioscience, Piscataway, NJ, USA) in a 1:100 dilution. Purity was judged acceptable above an OD 260nm to OD 280nm ratio of 1.8.

### 2.7.2 Reverse transcription

For synthesis of cDNA from RNA a reverse transcription system (Promega, Madison, WI, USA) applying random primers was used following the manufacturer's instructions. The reverse transcription mastermix was prepared separately and contained for each sample:

- 2 $\mu$ L reverse transcription buffer,
- 4 $\mu$ L 2mM MgCl<sub>2</sub>,
- 2 $\mu$ L dNTP mix,
- 0.5 $\mu$ L recombinant RNasin ribonuclease inhibitor,
- 1 $\mu$ L random primers,
- 0.7 $\mu$ L Avian Myeloblastosis Virus (AMV) reverse transcriptase enzyme.

For each sample, 10.2 $\mu$ L mastermix was added to 1 $\mu$ g of sample RNA diluted in 9.8 $\mu$ L nuclease free water, resulting in a total volume of 20 $\mu$ L per sample. In a next step, the mixture was vortexed and incubated for 10 minutes at room temperature. Reverse transcription was initiated in a thermal cycle controller (PTC-100, MJ Research, Ramsey, MN, USA) for 45 minutes at 42° Celsius. The reaction was stopped by a subsequent heating phase at 95° Celsius for five minutes.

### 2.7.3 Primers

| Target Gene  | Sequences   | Product Length |
|--|---|----------------|
| GAPDH for<br>GAPDH rev                             | GGTGAAGGTCGGAGTCAAC<br>CCATGGGTGGAATCATATTG         | 153bp          |
| COX-2 for<br>COX-2 rev                             | CGCTCAGCCATACAGCAAATCCTT<br>AATCCTGTCCGGGTACAATCGCA | 109bp          |
| p21 <sup>WAF1</sup> for<br>p21 <sup>WAF1</sup> rev | GTGGGGGCATCATCAAAAATT<br>ACCCACCTTCCCCCTGCCTTAC     | 159bp          |
| Bcl-2 for<br>Bcl-2 rev                             | CATGCCAAGGGGGAAACACCAGAA<br>CACGGCCCCAGAGAAAGAAGAGG | 127bp          |

**Table 2.1:** Real-time PCR primer sequences. bp = basepairs.

qRT-PCR primers were developed using Primer Select 5.00 Software (DNASTAR, Madison, WI, USA). At least one primer of the primer pairs was designed to specifically bind to cDNA at Intron-Intron sequences in order to omit amplification of potential contamination with genomic DNA. Dissociation curves were analyzed carefully for primer dimers as well as for additional

DNA-products. Non reverse transcriptase samples (NRT) and non template controls (NTC) were performed in order to assure specific amplification of target cDNA. Designed primer sequences (Table 2.1) were purchased from Invitrogen, Carlsbad, CA, USA.

#### 2.7.4 qRT-PCR

qRT-PCR was carried out applying qRT-PCR SYBR Green Mix (ABgene, Rockford, IL, USA) in an ABI Prism 7000 Sequence detection system (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol.

qRT-PCR mastermix (ABgene, Rockford, IL, USA) for each sample contained:

|                |  |
|----------------|--|
| 2.5 $\mu$ L    | Taq buffer,                              |
| 0.25 $\mu$ L   | Taq polymerase,                          |
| 1 $\mu$ L      | deoxynucleotide triphosphate (dNTP) mix, |
| 2.5 $\mu$ L    | 25mM MgCl <sub>2</sub> ,                 |
| 4 $\mu$ L      | 50% Glycerol,                            |
| 0.75 $\mu$ L   | dimethylsulfoxid (DMSO),                 |
| 0.125 $\mu$ L  | SYBR Green dye,                          |
| 12.875 $\mu$ L | nuclease-free water.                     |

For assessment of mRNA expression, 0.5 $\mu$ L of template cDNA was added to 24 $\mu$ L qRT PCR mastermix. Template DNA and mastermix were transferred to reaction tube stripes and 0.25 $\mu$ L of forward and 0.25 $\mu$ L of backward primer specific for the gene of interest were added, resulting in a final volume of 25 $\mu$ L per well.

Samples were analyzed in the ABI Prism 7000 Sequence detection system applying the following protocol:

|           |  |
|-----------|--|
| 1 cycle   | 15 minutes at 95° Celsius (hot start), |
| 40 cycles | 15 seconds at 95° Celsius,             |
|           | 1 minute at 60° Celsius,               |
| 1 cycle   | dissociation step.                     |

Expression of mRNA for each gene of interest was assessed in triplicates. Means were standardized to expression of house-keeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) of the sample by subtraction of GAPDH cycle values. Semiquantitative mRNA

expression was expressed as fold induction of controls treated with vehicle applying the  $2^{-\Delta\Delta Ct}$  method.

## 2.8 PGE<sub>2</sub>-ELISA

For analysis of PGE<sub>2</sub> formation,  $1 \times 10^5$  cells were plated in 6-well plates (BD Biosciences San Jose, CA, USA) in 3mL regular DMEM medium. After 24 hours medium was replaced by 1mL of medium containing the appropriate amount of FAs (prepared as outlined in chapter 2.3). After 48 hours of incubation, small quantities of medium were removed from the wells, spun down and transferred to a new tube. Then samples were stored at -80° Celsius until further analysis. In order to account for possible changes in cell numbers, the amount of cells per well was assessed by counting. The amount of cells varied only in a range of approximately 5%. Each experimental condition was represented by three independent samples on the plate.

After collection of samples was completed, the PGE<sub>2</sub>-ELISA was performed according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). In brief, after addition of the calibrator solution to the plate, samples, blanks, non-specific binding controls (NSB) and PGE<sub>2</sub> standard were pipetted in duplicates onto the ELISA plate. Next, primary antibody solution was added to each well, excluding NSB wells and the plate was incubated for one hour at room temperature on a Delfia horizontal orbital microplate shaker (PerkinElmer, Waltham, MA, USA) set at 500rpm. Then 50µL of PGE<sub>2</sub> conjugate was applied to each well and the plate was incubated for another two hours at room temperature on the shaker. Next, four washing steps with washing buffer were performed. After the last wash, remaining washing buffer was removed through aspiration and 100µL stop solution was added to each well.

In order to determine PGE<sub>2</sub> synthesis, optical density was measured at 450nm as well as at 570nm (for background subtraction).

Calculation of PGE<sub>2</sub> concentrations was performed according to the manufacturer's protocol: From the average of the duplicate readings for each standard, control, and sample the average NSB optical density was subtracted. PGE<sub>2</sub> standard curve was created using the Prism5 for Windows Software (GraphPad, La Jolla, CA, USA) and PGE<sub>2</sub> concentrations were calculated corresponding to the mean absorbance of the standard.

## 2.9 Statistical analysis

All results are presented as mean  $\pm$  standard error of mean (SEM). For all experiments Student's *t*-test (Gaussian distribution of values tested by Shapiro-Wilk normality test) was used to

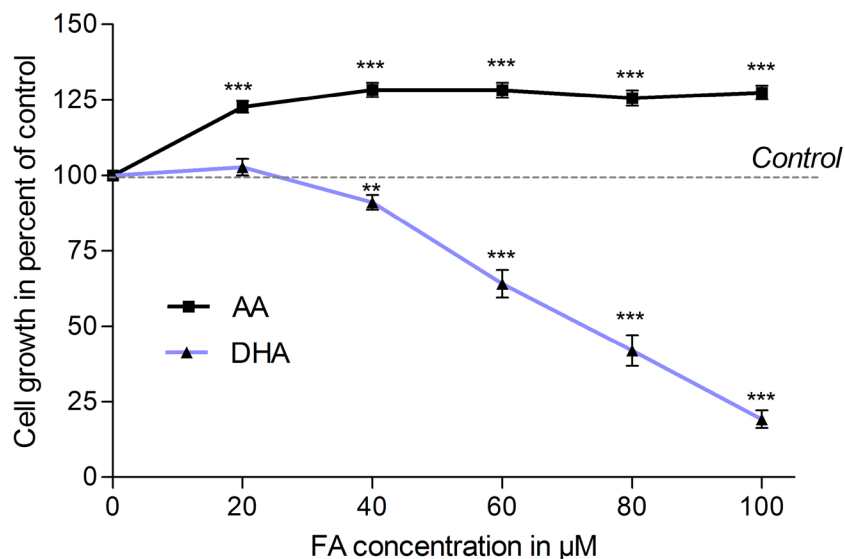
evaluate the difference between two groups unless stated otherwise. In the case of non-Gaussian distribution, the Mann-Whitney test was applied as indicated in figure legends. Statistical significance was accepted at the level of  $P < 0.05$ . XTT results are presented as percentage of control, whereas qRT-PCR results are expressed as fold of control as analyzed by the  $2^{-\Delta\Delta C_t}$  method. GraphPad Prism5 for Windows Software (GraphPad, La Jolla, CA, USA) and Microsoft Excel (Microsoft, Rockwood, WA, USA) were used for calculations.

### 3. Ergebnisse / Results

As several recent studies did not find or did not address differential effects of n-3 PUFA DHA and n-6 PUFA AA in the context of colorectal carcinoma cell growth, it was the intention of the following experiments to investigate the impact of these FAs in the CRC cell line LS 174T. Thus, we assessed cellular viability, apoptosis and cellular mRNA-expression following incubations with miscellaneous concentrations of DHA and AA. In additional co-incubation experiments, we set out to study the interactions between DHA and AA in the context of cellular viability and PGE<sub>2</sub> formation.

#### 3.1 Differential effects of DHA and AA on colorectal cancer cell growth and viability

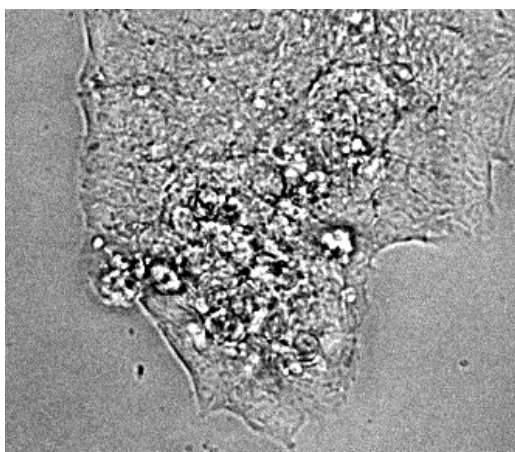
In a first set of experiments we applied XTT assays to investigate the effects of n-3 PUFA DHA and n-6 PUFA AA on cellular viability and proliferation of LS 174T. For this purpose cells were treated with either AA or DHA in medium containing reduced concentration of serum and 1 mg/mL bovine serum albumin (BSA), which was added in order to assure complete dissolution of FAs. After 120h of FA incubation, XTT assays revealed DHA to significantly diminish cellular viability in a concentration-dependent manner, whereas AA increased cellular viability in corresponding concentrations (figure 3.1a).



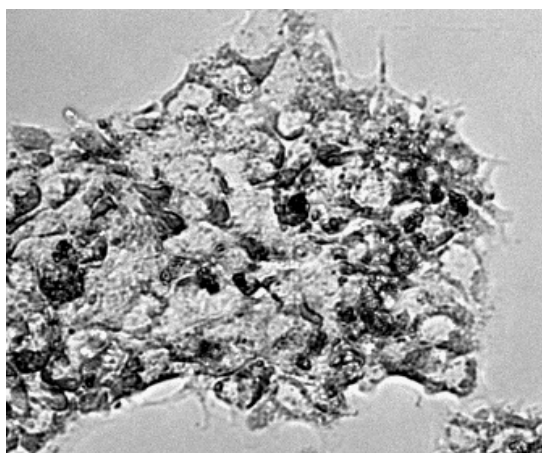
**Figure 3.1a:** Cellular viability of LS 174T assessed via XTT assays after 120 hours of FA incubation. DHA diminished cellular viability in a concentration-dependent manner, whereas AA incubation in equivalent concentrations increased cellular viability. Data points represent at least 13 independent experiments. \*\*P<0.01 versus control, \*\*\*P<0.001 versus control.

Growth promoting effects of AA were evident in concentrations starting as low as 20μM, while a reduction of cellular viability following DHA incubation was observed in concentrations of

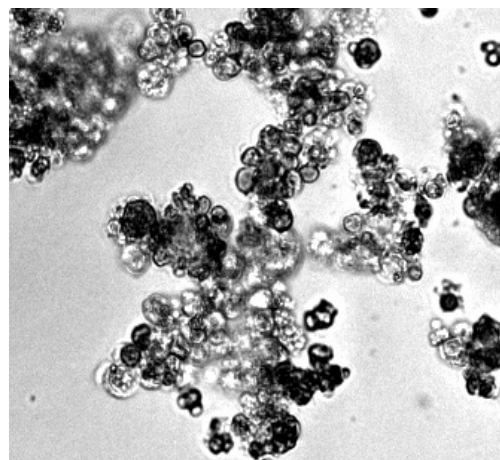
40 $\mu$ M and above. Alterations in cellular viability were also accompanied by changes in cellular morphology as displayed in figure 3.1b. The vast majority of cells incubated with 100 $\mu$ M DHA were found detached from the bottom of cell dish and cellular shape appeared rounded and condensed. In contrast, control cells incubated with vehicle presented unremarkable in an adherent, non-confluent monolayer, while some of the cells incubated with 100 $\mu$ M AA additionally revealed a darker color.



*Control*



*AA 100 $\mu$ M*

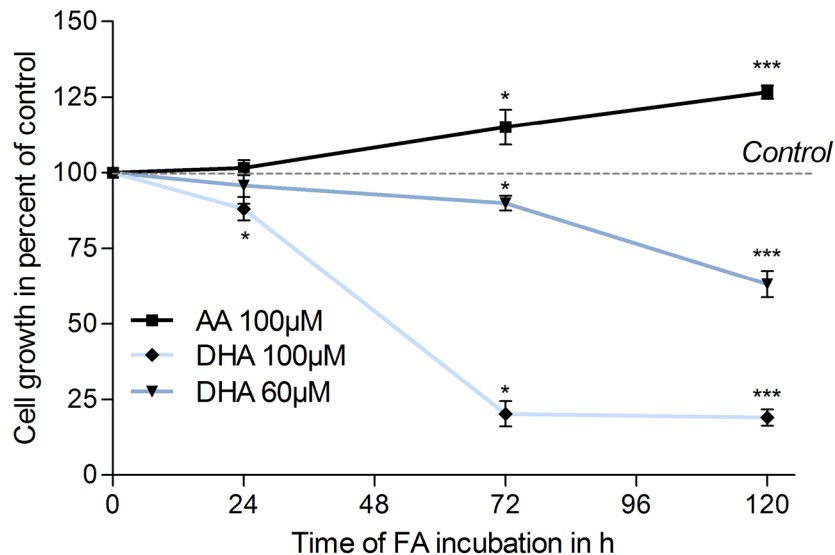


*DHA 100 $\mu$ M*

**Figure 3.1b:** Light-optical microscopic pictures of LS 174T after 120 hours of FA incubation. While control cells appeared healthy, in a confluent cell layer, cells incubated with DHA were detached, rounded and condensed in shape. Cells incubated with AA appeared viable, some of which displaying a darker color.

Additional investigations of the influences of PUFAs after 24 hours and 72 hours of incubation, disclosed that the DHA-triggered decline and the AA-induced elevation of cellular viability were also time-dependent (figure 3.1c). After 24 hours, only high concentrations of 100 $\mu$ M of DHA led to a small but significant decline in cellular viability compared to control cells, while lower concentrations of DHA and AA in general did not result in obvious effects. However, after 72 hours, effects of FA incubation in the context of cellular viability became more evident. At this

time point also DHA at 60 $\mu$ M significantly diminished cellular viability and effects of 100 $\mu$ M DHA became more distinct than after 24 hours. In addition, high concentrations of AA (100 $\mu$ M) significantly augmented cellular viability of LS 174T compared to controls.



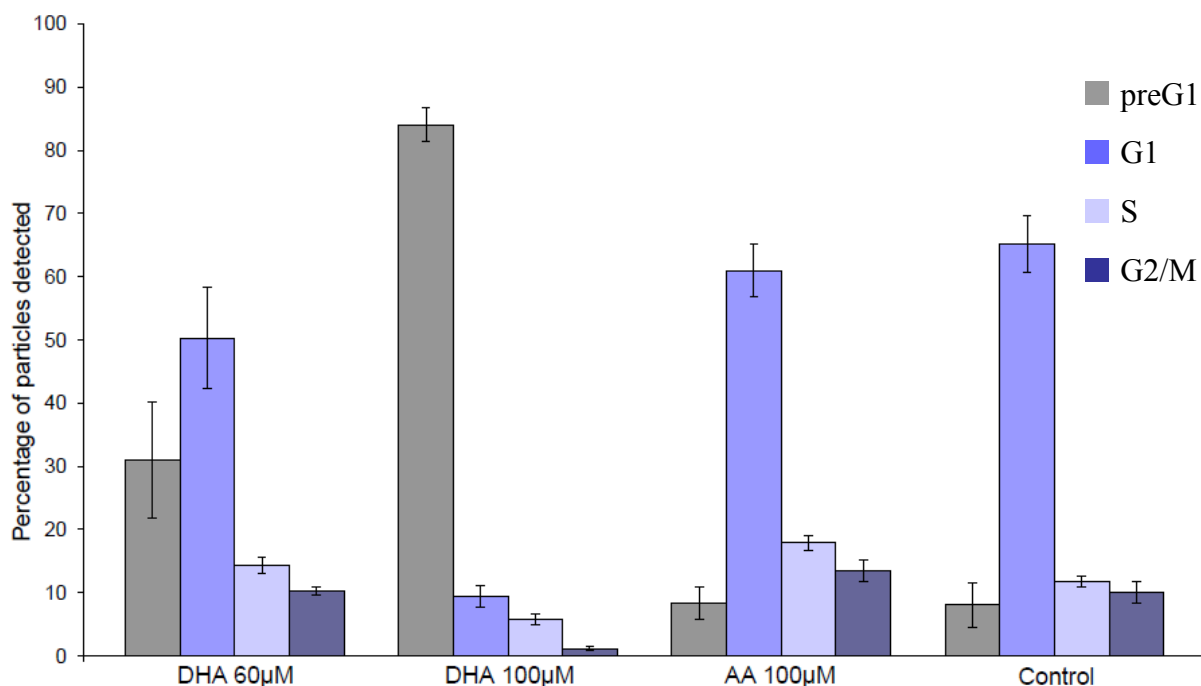
**Figure 3.1c:** Cellular viability of LS 174T assessed via XTT assay following various periods of FA incubation. DHA diminished cellular viability in a time- and dose-dependent manner, whereas AA at 100  $\mu$ M time-dependently increased cellular viability. Data points represent at least five independent experiments. \*P<0.05 versus control, \*\*\*P<0.001 versus control.

### 3.2 DHA induces apoptosis, while AA in identical concentrations fails to do so

The XTT assay represents a valuable tool for assessing cellular viability. Nevertheless, it does not supply information concerning the underlying mechanisms, namely whether decreased viability is due to the inhibition of proliferation, induction of apoptosis, or necrosis. Thus, in order to further investigate the decrease of cellular viability caused by DHA incubation we used flow cytometry analysis as well as DAPI stainings.

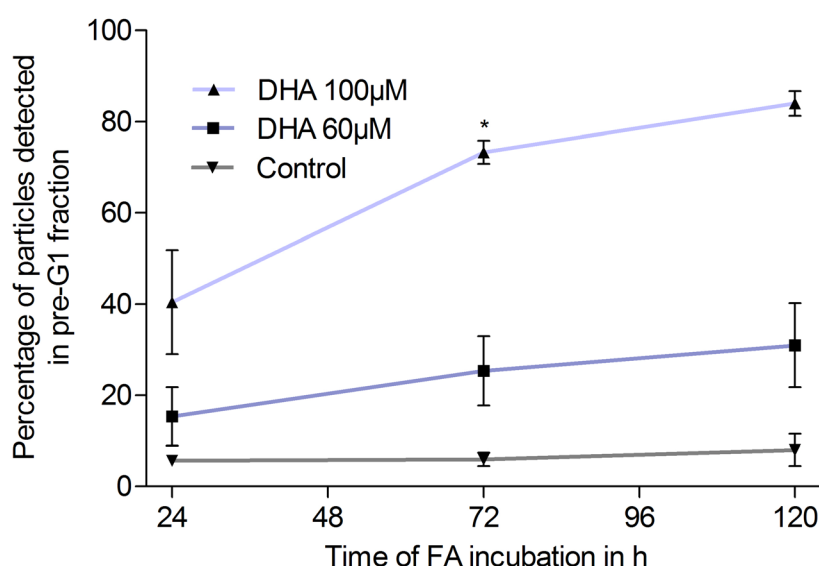
In this context, flow cytometry experiments revealed that 120 hours of DHA incubation resulted in a dose-dependent increase in preG1 fraction (as indicator of apoptosis) in LS 174T cells (figure 3.2a). In contrast AA, in identical concentrations, did not alter the preG1 fraction compared to untreated cells.





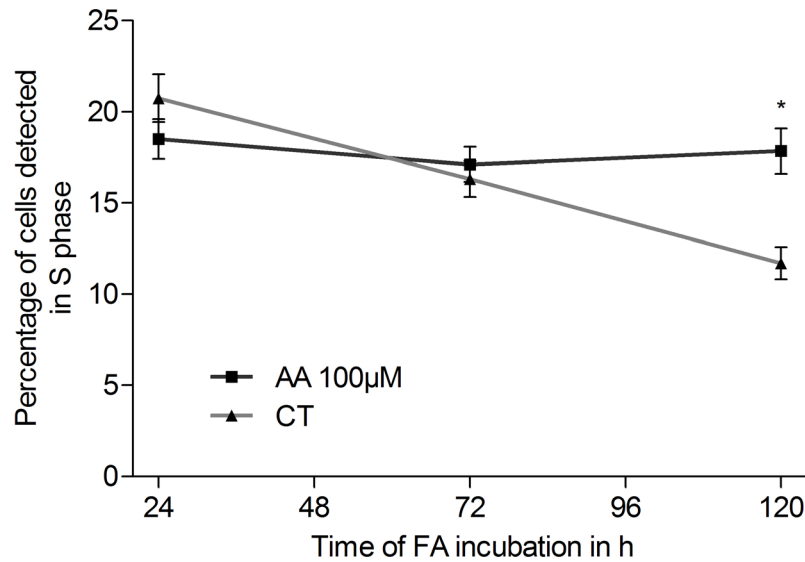
**Figure 3.2a:** Cell cycle analysis of LS 174T by flow cytometry after 120 hours of FA incubation. Induction of apoptosis is indicated by an increased preG1 fraction. DHA increased the preG1 fraction compared to control cells dose-dependently, while incubation with AA did not alter the preG1 fraction. Results represent the mean of four independent experiments.

In analogy to findings in XTT assays, additional flow cytometry experiments conducted after 24 hours and 72 hours of FA incubation indicated the DHA-associated induction of the preG1 fraction to be time-dependent (figure 3.2b).



**Figure 3.2b:** Detection of preG1 fraction of LS 174T by flow cytometry following various periods of FA incubation. DHA incubation induced a dose- and time-dependent increase of the preG1 fraction (as marker of apoptosis) compared to untreated cells. Incubation with AA did not alter preG1 fraction at any given time (data not shown). Each data point represents the mean of at least three independent experiments. Mann-Whitney test was applied for statistical analysis, \*P<0.05 versus control.

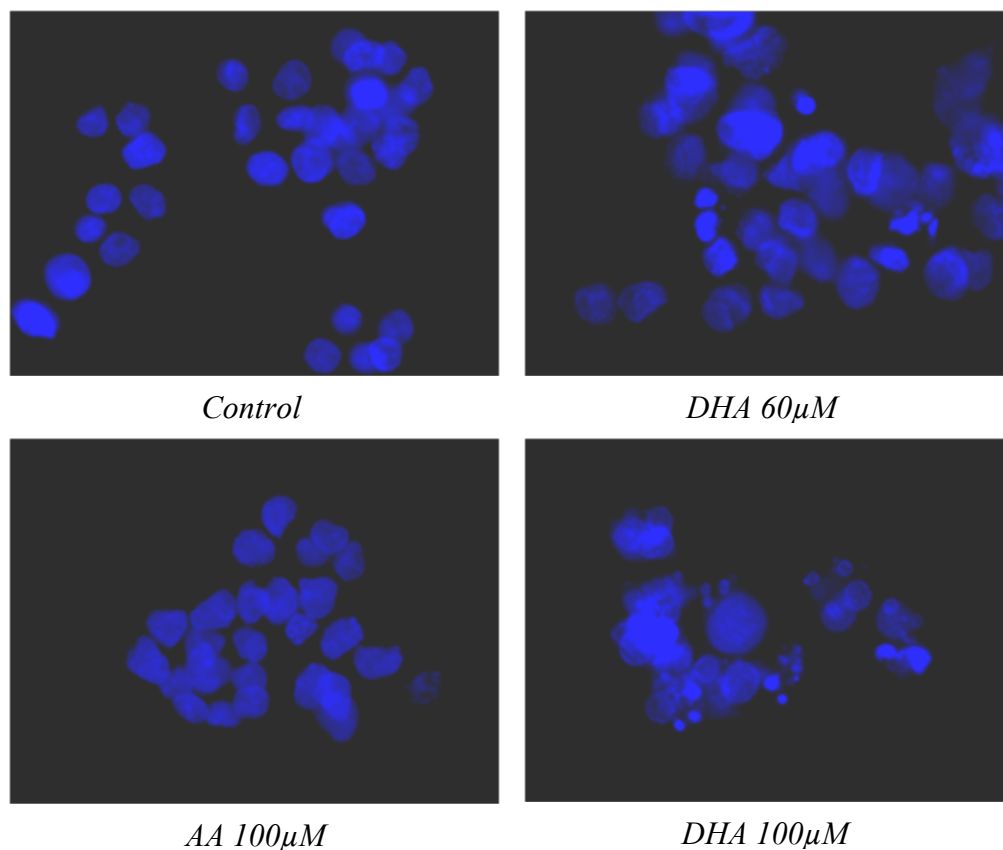
Already after 24 hours, DHA incubation dose-dependently elevated preG1 fraction when compared to cells incubated with vehicle or AA. Pro-apoptotic effects of DHA incubation, especially in high concentrations of 100 $\mu$ M, further progressed after 72 hours of incubation. Apart from the DHA-triggered induction of the preG1 fraction, cell cycle analysis additionally revealed that incubation with 100 $\mu$ M of AA for 120 hours resulted in a significant increase in S-phase, indicating elevated cellular proliferation compared to control cells (figure 3.2c).



**Figure 3.2c:** Detection of S-phase fraction of LS 174T by flow cytometry following various periods of FA incubation. AA incubation induced S-phase fraction after 120 hours as a marker of cellular proliferation. Each data point represents the mean of at least three independent experiments. Mann-Whitney test was applied for statistical analysis, \*P<0.05 versus control.

DHA-induced apoptosis, as indicated by flow cytometry results, was further investigated in DAPI stainings (figure 3.2d). These stainings revealed DHA incubation at 60 $\mu$ M as well as at 100 $\mu$ M to result in hypercondensation and fragmentation of nuclei, which are generally regarded as morphological signs of apoptosis. Thus, flow cytometry results regarding the induction of apoptosis by DHA were confirmed. In contrast, cells incubated with 100 $\mu$ M of AA as well as control cells displayed unremarkable nuclei.

Thus, in summary, these experiments indicate DHA incubation to decrease cellular proliferation and to induce apoptosis in LS 174T CRC cells, whereas AA incubation significantly increases cellular viability and leads to an augmented number of cells detected in the process of proliferation.

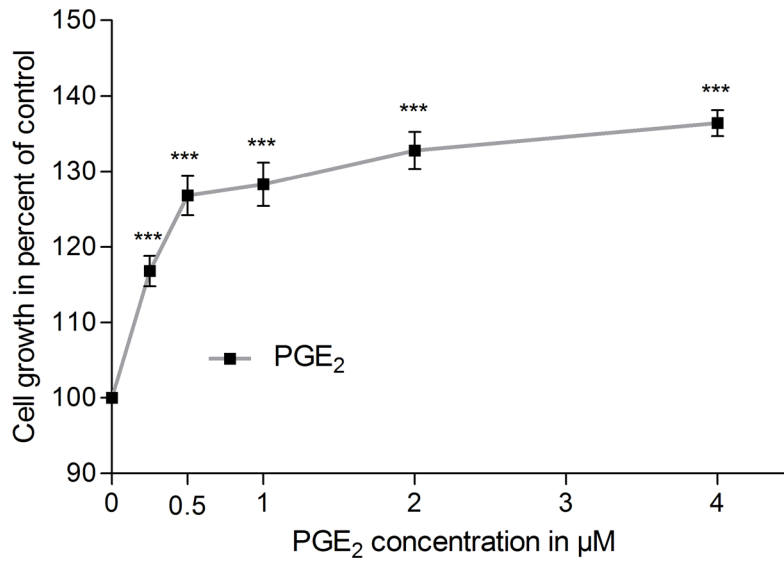


**Figure 3.2d:** DAPI staining of LS 174T cells incubated with different concentrations of FA. Cells incubated with DHA showed an increase of apoptotic bodies (fragmentation and hypercondensation of the nuclei), while cells incubated with AA appeared unaltered compared to control cells.

### **3.3 AA incubation induces COX-2 mRNA expression and synthesis of prostaglandin E<sub>2</sub>, a potent inducer of proliferation in LS 174T**

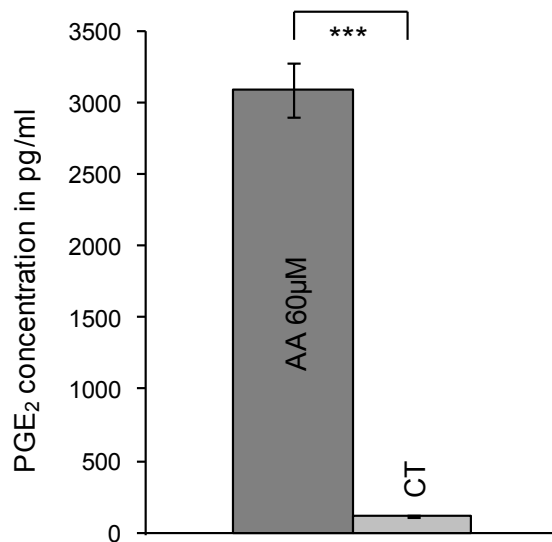
In another set of experiments we strived to further investigate the mechanisms underlying the increased proliferation of LS 174T cells incubated with AA. Recent investigations identified PGE<sub>2</sub> as a potent inducer of proliferation in LS 174T cells (Shao et al. 2003; Sheng et al. 2001). As PGE<sub>2</sub> is derived from AA, catalyzed by COXs (see also chapter 1.6), we hypothesized that AA-dependent PGE<sub>2</sub> formation could at least be partially responsible for the induction of viability found in LS 174T cells incubated with AA.

Indeed, XTT assays confirmed PGE<sub>2</sub> as a dose-dependent promoter of cellular viability and proliferation after 120 hours of incubation in LS 174T cells (figure 3.3a).



**Figure 3.3a:** Cellular viability of LS 174T assessed via XTT assay after 120 hours of PGE<sub>2</sub> incubation. Addition of PGE<sub>2</sub> in micromolar concentrations led to a dose-dependent increase of cellular viability. Data points represent at least 5 independent experiments. \*\*\*P<0.001 versus control.

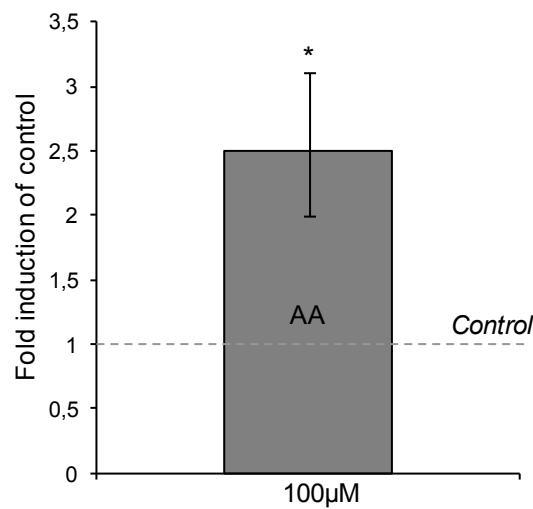
More importantly, ELISA experiments revealed that incubation of LS 174T with AA resulted in a significant formation of PGE<sub>2</sub>, whereas controls incubated with the vehicle only generated little amounts of PGE<sub>2</sub> (figure 3.3b).



**Figure 3.3b:** Detection of levels of PGE<sub>2</sub> formed by LS 174T after 48 hours of AA incubation. AA treatment resulted in significant synthesis of PGE<sub>2</sub>, whereas PGE<sub>2</sub> baseline levels in untreated controls were much lower. Results represent mean of PGE<sub>2</sub> measurements from three independent samples. \*\*\*P<0.001 versus control.

Interestingly, additional semiquantitative real-time PCR (qRT-PCR) investigations showed AA incubation to result in a significant up-regulation of COX-2 mRNA of approximately 2.5-fold when compared to control cells (figure 3.3c). Thereby AA incubation may effect and promote its

own COX-2-related metabolism resulting in elevated synthesis of PGE<sub>2</sub>. In contrast, incubation with DHA significantly diminished COX-2 expression as described in chapter 3.5.

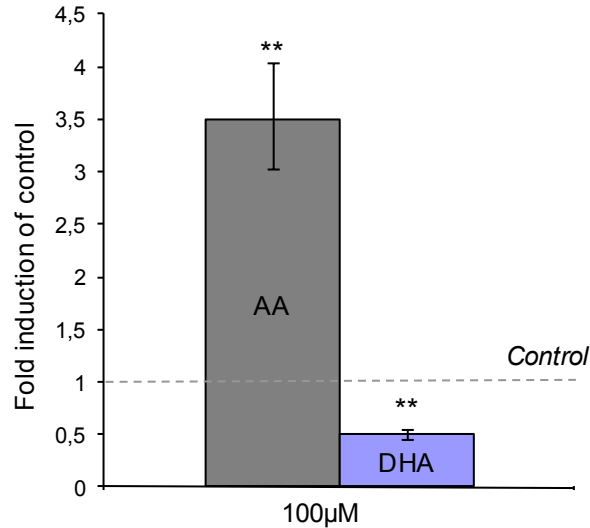


**Figure 3.3c:** *qRT-PCR assessment of COX-2 mRNA expression following 48 hours of FA incubation. AA incubation resulted in elevated amounts of COX-2 mRNA compared to untreated controls. N=3 for each group. \*P<0.05 versus control.*

### **3.4 Differential effects of DHA and AA on the mRNA expression of genes involved in cell cycle regulation and induction of apoptosis**

Further researching the mechanisms involved in the FA-associated regulation of proliferation and apoptosis, we moreover applied qRT-PCR to assess the cellular mRNA expression of genes regulating apoptosis and cell cycle in cells either incubated with DHA or AA.

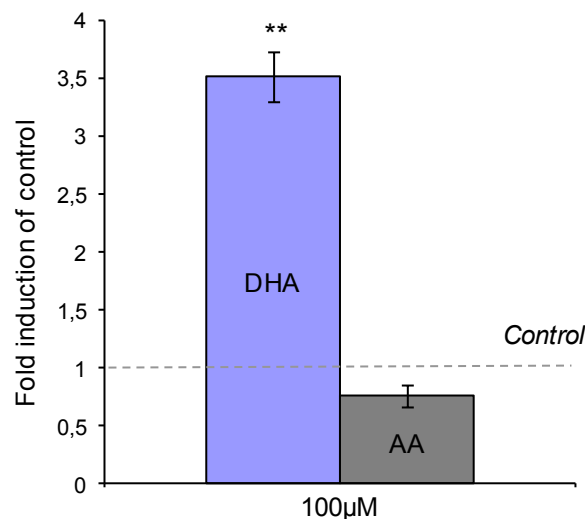
In this context qRT-PCR experiments discovered an opposing regulation of mRNA levels of the anti-apoptotic protein Bcl-2 following n-3 and n-6 PUFA incubation (figure 3.4a). Incubation with n-3 PUFA DHA significantly decreased the amount of detectable Bcl-2 mRNA to approximately 0.5-fold of levels found in controls, which may contribute to the DHA-dependent induction of apoptosis. On the other hand supplementation of n-6 PUFA AA resulted in a significant up-regulation of Bcl-2 mRNA to about 3.5-fold of levels detected in control cells, possibly protecting cells from apoptosis.



**Figure 3.4a:** *qRT-PCR assessment of anti-apoptotic Bcl-2 mRNA expression following 48 hours of FA incubation.* While AA incubation resulted in an elevated expression of Bcl-2 mRNA, DHA incubation led to a decreased amounts of detectable Bcl-2 mRNA compared to the controls. N=3 for each group. \*\*P<0.01 versus control.

Additional investigations concerning the expression of cyclin-dependent kinase inhibitor 1A (also referred to as p21<sup>WAF1</sup>) found DHA to significantly elevate the amount of mRNA of this cell cycle inhibitor to about 3.5-fold of levels measured in control cells. On the other hand, incubation with AA in identical concentrations left the amount of p21<sup>WAF1</sup> mRNA unaltered compared to control cells (figure 3.4b).

Hence, qRT-PCR experiments strongly support the concept of differential effects of n-3 and n-6 PUFAs on a molecular level.

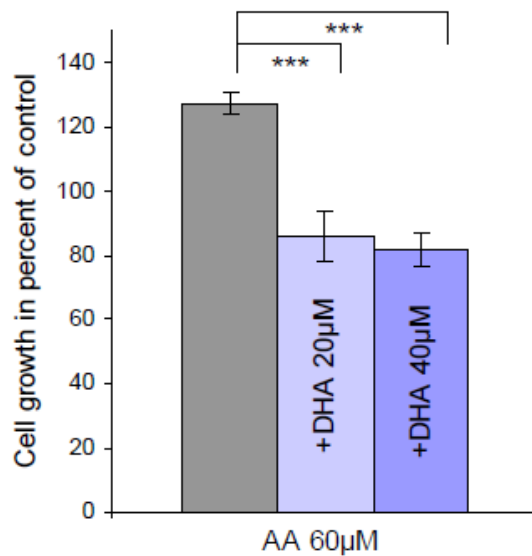


**Figure 3.4b:** *qRT-PCR assessment of cell cycle inhibitor p21<sup>WAF1</sup> mRNA expression following 48 hours of FA incubation.* While DHA incubation led to an elevated expression of p21<sup>WAF1</sup> mRNA, AA incubation resulted in unaltered amounts of detectable p21<sup>WAF1</sup> mRNA compared to controls. N=3 for each group. \*\*P<0.01 versus control.

### 3.5 AA/DHA co-incubation: DHA reverses AA-induced proliferation and reduces AA-induced prostaglandin E<sub>2</sub>-formation

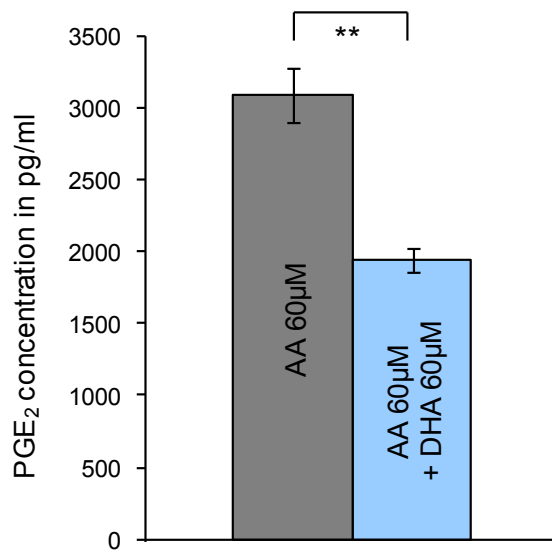
Taking into consideration that within the gastrointestinal tract AA as well as DHA - both supplied via diet - are present, we additionally treated LS 174T cells with combinations of DHA and AA.

Interestingly, XTT assays revealed that co-incubation with DHA was able to reverse AA-induced proliferation as displayed in figure 3.5a. In this context, DHA concentrations of as low as 20 $\mu$ M and 40  $\mu$ M potently counteracted and diminished the pro-proliferative activities of 60 $\mu$ M of AA.



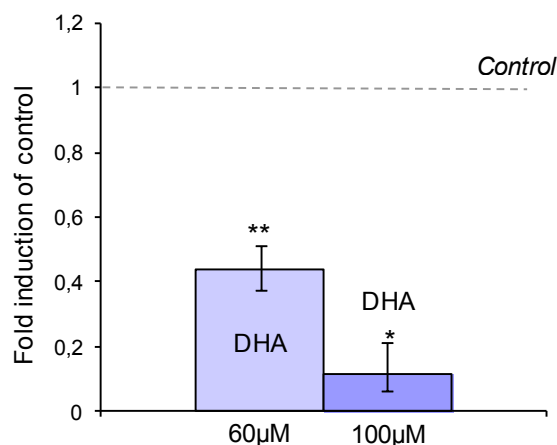
**Figure 3.5a:** Cellular viability of LS 174T assessed via XTT assay after 120 hours of FA incubation. DHA reversed AA-induced proliferation. Data points represent at least six independent experiments. \*\*\*P<0.001 versus AA 60 $\mu$ M.

Moreover, AA/DHA co-incubation significantly reduced the amount of PGE<sub>2</sub> generated from AA (figure 3.5b). In detail, additional supplementation of DHA led to a 30% decrease of AA-induced PGE<sub>2</sub> formation when compared to levels of PGE<sub>2</sub>, which were synthesized by cells incubated with AA alone.



**Figure 3.5b:** Levels of PGE<sub>2</sub> formed by LS 174T after 48 hours of FA incubation. AA-induced synthesis of PGE<sub>2</sub> was suppressed by concomitant DHA incubation. Results represent the mean of PGE<sub>2</sub> measurements from three independent samples. \*\*P<0.01 versus AA 60µM.

In this context, reduced amounts of PGE<sub>2</sub> may be attributed to decreased presence of COX-2, as incubation with DHA significantly reduced COX-2 mRNA expression in a dose-dependent manner (figure 3.5c). While DHA in high concentrations (100µM) diminished COX-2 mRNA levels to approximately 0.1-fold of mRNA levels detected in untreated cells, DHA at 60µM resulted in a decrease to approximately 0.5-fold of the amounts found in untreated cells.

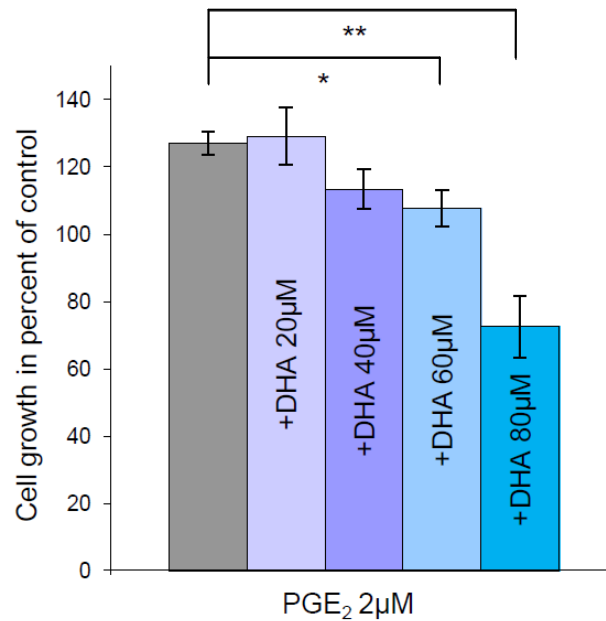


**Figure 3.5c:** qRT-PCR assessment of COX-2 mRNA expression following 48 hours of FA incubation. DHA incubation dose-dependently decreased amounts of COX-2 mRNA compared to untreated control cells. N=3 for each group. \*P<0.05 versus control, \*\*P<0.01 versus control.



### 3.6 Prostaglandin E<sub>2</sub>/DHA co-incubation: DHA also reversed prostaglandin E<sub>2</sub>-induced proliferation

However, PGE<sub>2</sub>/DHA co-incubation experiments, applying PGE<sub>2</sub> in a concentration of 2μM, known from the previous experiments to result in similar growth-induction as in incubation with 60μM AA (as used in the AA/DHA co-incubation setup), revealed that DHA could also directly suppress PGE<sub>2</sub>-induced cellular proliferation (figure 3.6).



**Figure 3.6:** Cellular viability of LS 174T assessed via XTT assay after 120 hours of incubation. Addition of high concentrations of DHA reversed the PGE<sub>2</sub>-induced proliferation. Data points represent at least five independent experiments. \*P<0.05 versus single PGE<sub>2</sub> 2μM, \*\*P<0.01 versus single PGE<sub>2</sub> 2μM.

Yet, in order to suppress PGE<sub>2</sub>-induced growth, higher DHA concentrations than those reversing AA-related proliferation were necessary. Hence, this leads to the conclusion that the effects of DHA-associated growth inhibition are at least in part independent from a pure blocking effect on PGE<sub>2</sub> formation.

#### 4. Diskussion / Discussion

Results of the present work clearly demonstrate opposing effects of n-3 PUFA DHA and n-6 PUFA AA in the context of cellular growth and induction of apoptosis in the CRC cell line LS 174T. While incubation with AA increased cellular proliferation, supplementation of DHA significantly reduced cellular viability and induced apoptosis. Moreover, qRT-PCR experiments revealed differential effects of n-3 and n-6 PUFAs in regard to cellular mRNA expression: DHA incubation significantly decreased mRNA levels of anti-apoptotic Bcl-2 and induced mRNA of cell cycle inhibitor p21<sup>WAF1</sup>, whereas incubation with AA significantly increased expression of Bcl-2 and left amounts of p21<sup>WAF1</sup> mRNA unaltered.

Data also showed that addition of AA increases the amount of COX-2 mRNA and results in a significant biosynthesis of PGE<sub>2</sub> by LS 174T, which was furthermore confirmed as a potent inductor of proliferation in this cell line. In contrast, DHA incubation potently decreased COX-2 mRNA expression and significantly reduced PGE<sub>2</sub> formation from AA in co-incubation experiments. Results obtained in co-incubation experiments moreover indicate DHA to reverse and suppress AA- as well as PGE<sub>2</sub>-induced CRC cell growth.

It is the intention of the following discussion to first consider the results of the present study in the context of other studies, which investigated the impact of n-3 and n-6 PUFAs *in vitro*. Then possible implications of the current data concerning the underlying molecular mechanisms of PUFA action will be discussed and conclusions from co-incubation experiments will be drawn. This is to be followed by a critical analysis of the limitations of the *in vitro* data presented here. In conclusion, the potential impact of our study in the context of CRC prevention will be further outlined.

##### 4.1 Impact of DHA and AA on cellular viability and apoptosis

Data of the present study acquired in XTT assays, DAPI stainings and flow cytometry experiments confirm the results of numerous previous studies, which identified DHA as a potent suppressor of proliferation as well as an inductor of apoptosis in miscellaneous CRC cell lines including Caco-2, HT-29, HCT 116, LoVo, SW420, SW620 and Colo 201 (Baumgartner et al. 2004; Calviello et al. 2004; Danbara et al. 2004; Giros et al. 2009; Narayanan et al. 2001; Narayanan et al. 2003; Narayanan et al. 2004; Toit-Kohn et al. 2009).

However, so far only two studies in this field investigated the effects of DHA in LS 174T CRC cells, which were both aimed at the exploration of synergistic effects of DHA and chemotherapeutic agents (Baumgartner et al. 2004; Calviello et al. 2005). On the one hand,

Baumgartner et al. screened a variety of cancer cell lines for a possible enhancement of arsenic trioxide-mediated cytotoxic effects by addition of DHA and reported DHA alone to dose-dependently reduce cellular viability of LS 174T cells (Baumgartner et al. 2004). As similar concentrations of DHA were applied, these results are almost congruent with XTT-data of the present study. But the effects of DHA were somewhat more potent within our experimental setup. On the other hand, Calviello and colleagues investigated the effects of a combination of DHA with 5-Fluoruracil (5-FU) in LS 174T and also indicated DHA solo incubation to diminish cellular growth and to induce apoptosis in this particular cell line. In accordance with results obtained in flow cytometry experiments of the present study, Calviello et al. also reported DHA to significantly increase the preG1 fraction as indicator of apoptosis. However, the DHA concentration applied by Calviello and coworkers was as low as 10 $\mu$ M, for which neither we nor Baumgartner et al. found a significant effect. This may result from divergent experimental setups. Nevertheless, in summery the finding of the present study that DHA significantly inhibits proliferation of LS 174T and that it potently induces apoptosis is well supported by the available literature.

Moreover, it is noteworthy that the anti-cancerous *in vitro* properties of n-3 PUFAs in regard to CRC appear to be independent from the underlying type of genetic instability (also see chapter 1.3). While LS 174T cells, as used in the present study, have been reported as microsatellite instable (MSI), other microsatellite stable cell lines, such as HT-29 or Caco-2, which follow the chromosomal instability pathway (CIN), were also found susceptible to anti-cancer actions of n-3 PUFAs (Giros et al. 2009). Thus, it may be assumed that n-3 PUFAs, such as DHA, exert their anti-neoplastic effects in a broad variety of CRC cells, which are derived from patients suffering from different types of sporadic CRC.

However, as already pointed out in chapter 1.8, the studies mentioned above - those applying n-3 PUFAs to LS 174T cells as well as those using other CRC cell lines - did not address the impact of n-6 PUFAs. Hence, it may be argued that anti-cancerous properties of n-3 PUFAs reported by these investigations are rather unspecific general effects of PUFAs *in vitro* with n-6 PUFAs exerting the identical effects. In contrast, in the present study, the n-6 PUFA AA, applied under identical experimental conditions as DHA, significantly induced cellular viability and promoted proliferation of LS 174T cells as indicated by XTT assays and flow cytometry experiments. These observations add evidence to the hypothesis that growth-diminishing and pro-apoptotic

effects of DHA are due to specific properties of this particular FA rather than to unspecific effects of PUFAs in general.

Thus, our results additionally help to clarify the aforementioned uncertainty concerning differential effects of n-3 and n-6 PUFAs *in vitro*, which resulted not only from those studies not addressing this issue but also from additional investigations reporting n-3 as well as n-6 PUFAs to equally inhibit cancer cell growth *in vitro* (see chapter 1.8) (Dommels et al. 2002; Dommels et al. 2003; Schonberg et al. 2006).

Moreover the opposing effects of n-3 and n-6 PUFAs found in the present study are in accordance with several studies published recently, which also reported differential effects of these two groups of PUFAs in numerous CRC lines not yet including LS 174T cells (Engelbrecht et al. 2008; Habermann et al. 2009; Hossain et al. 2009; Pan et al. 2009). These studies also found DHA to diminish cellular viability, whereas n-6 PUFA AA reduced cellular viability only slightly or not at all.

#### **4.2 AA-related growth induction and formation of prostaglandin E<sub>2</sub>**

Taking into consideration that AA, via the catalytic activity of COXs, gives rise to PGE<sub>2</sub>, which previously has been identified as a potent inductor of proliferation in LS 174T CRC cells, it may be hypothesized that AA-related growth induction found in the present study is potentially mediated via AA-dependent synthesis of PGE<sub>2</sub>.

Indeed, XTT assays confirmed PGE<sub>2</sub> to increase cellular viability of LS 174T in a dose-dependent manner (Shao et al. 2003; Sheng et al. 2001). In addition, qRT-PCR results indicated COX-2 mRNA to be expressed by LS 174T cells. This is well in agreement with several previous studies that also found COX-2 mRNA as well as COX-2 protein to be present in this particular CRC cell line (Palozza et al. 2005; Shao et al. 2000; Vadlamudi et al. 1999). In contrast, two different *in vitro* studies reported neither COX-2 nor COX-1 to be present in LS 174T cells (Oraldi et al. 2009; Park et al. 2010). The reason for these conflicting results remains unclear.

Nevertheless, further supporting a relevant expression of COXs in LS 174T cells, ELISA experiments of the present study showed LS 174T to potently metabolize exogenous AA into PGE<sub>2</sub> (most likely via the catalytic activity of COXs). At first sight, these findings may appear to be in conflict with results published by Shao and co-workers, who reported LS 174T, despite the presence of COX-2 protein, to only synthesize low amounts of PGE<sub>2</sub> when incubated with exogenous AA (Shao et al. 2000). Yet, the analysis of experimental setups revealed significant differences to the present study: AA concentrations applied by Shao and colleagues were as low

as 15 $\mu$ M, while incubation time was only one hour. In contrast, assessment of PGE<sub>2</sub> synthesis via ELISA in the present study followed 48 hours of incubation with AA at 60 $\mu$ M, thus explaining divergent findings. In this context, qRT-PCR further revealed AA incubation to lead to a significant increase of COX-2 mRNA. Although the direct impact of AA in the context of COX-2 expression in LS 174T CRC cells has not yet been evaluated, PGE<sub>2</sub> has been previously reported to elevate levels of cellular COX-2 expression (Wang & Dubois 2010). Hence, findings of the present study suggest that AA/PGE<sub>2</sub> promote their own metabolism via induction of COX-2 on mRNA level. Furthermore, highlighting differential effects of n-3 and n-6 PUFAs in LS 174T cells, DHA incubation decreased COX-2 expression, directly opposing effects mediated by AA (see chapter 4.4).

In synopsis, experiments of the present study reveal LS 174T cells to potently synthesize PGE<sub>2</sub> from AA. However, it cannot be finally concluded from our data that AA-induced proliferation and impact on mRNA expression is exclusively mediated via synthesis of PGE<sub>2</sub> or that other additional pathways account for AA-related effects. Nevertheless, to our knowledge, the *in vitro* setup established in the present study is the first to display growth promoting effects of a n-6 PUFA in CRC cells, and thus may provide a valuable tool for further investigating n-6 PUFA-related pro-carcinogenic effects in the future.

### **4.3 Differential gene regulation**

Further exploring the effects of DHA and AA in LS 174T CRC cells on the molecular level, we applied qRT-PCR to assess potential regulation of genes involved in apoptosis and cell cycle control. Although representing a powerful tool for the investigation of changes in mRNA expression, in the discussion of qRT-PCR results it is noteworthy that alterations of mRNA do not necessarily correlate with amounts of the corresponding proteins.

#### **4.3.1 Bcl-2 and possible implications for DHA-associated induction of apoptosis**

Semiquantitative real-time PCR experiments revealed opposing regulation of mRNA expression of anti-apoptotic B-cell lymphoma-2 protein (Bcl-2) by n-3 PUFA DHA and n-6 PUFA AA.

Bcl-2 protein was first discovered as an oncogene constitutively over-expressed in B-cell lymphoma with a *t*(14:18) chromosomal translocation and was subsequently identified as a key inhibitor of apoptosis. The discovery of Bcl-2 has subsequently led to the identification of a novel protein group referred to as the Bcl-2 family, which consists of at least 20 members, some

of them exerting pro-, while others executing anti-apoptotic actions (Hector et al. 2009; Hockenbery et al. 1990; Tsujimoto et al. 1984; Vaux et al. 1988).

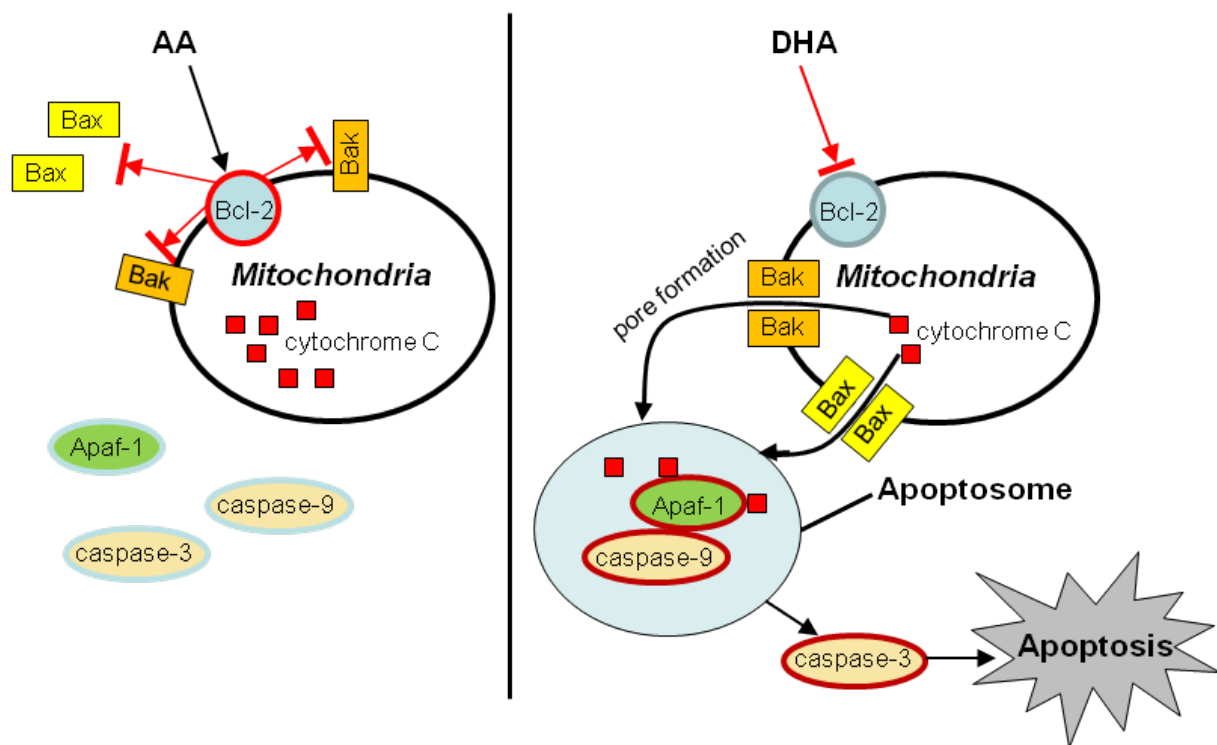
Aberrant expression of Bcl-2 has been reported in a number of solid tumors including CRC. Under healthy conditions in the colorectum, Bcl-2 is solely expressed at the base portion of colonic crypts correlating with very low levels of physiological apoptosis in this area. During colorectal carcinogenesis this specific expression pattern is lost (An et al. 2007; Chan et al. 2000; Liu et al. 1998; Visca et al. 1999; Walker et al. 1995; Watson 2004).

The general explanation of how Bcl-2 protein suppresses apoptosis focuses on the preservation of mitochondrial membrane integrity by this protein. Bcl-2 prevents the oligomerization of the pro-apoptotic proteins Bcl-2 homologous killer protein (Bak) and Bcl-2-associated X protein (Bax), which are both physiologically located in the outer mitochondrial membrane (Borner 2003; Kirkin et al. 2004; Matthews et al. 2006). Decreased amounts as well as modifications of Bcl-2 protein lead to a disinhibition of Bak and Bax, which oligomerize and subsequently form pores. Via these pores several apoptogenic substances are released from the mitochondria into the cytoplasm, including cytochrome C, which associates with apoptotic protease-activating factor-1 (Apaf-1) and caspase-9 forming an apoptosome (Rasola et al. 2007; Seiler et al. 2005; Yang et al. 1997). This activation of caspase-9 culminates in the downstream activation of caspase-3, ultimately resulting in apoptosis (Figure 4.1) (Liu et al. 1998; Matthews et al. 2006).

Results presented here reveal that incubation with DHA leads to a potent down-regulation of Bcl-2 mRNA, which is consistent with several previous studies that indicated DHA-induced apoptosis to be related to decreased amounts of Bcl-2 mRNA and protein in several CRC cell lines including LS 174T (Calviello et al. 2005; Chen et al. 2000; Danbara et al. 2004; Giros et al. 2009; Hossain et al. 2009; Narayanan et al. 2001). Thus it may be hypothesized that the DHA-induced reduction of Bcl-2 mRNA, as found in the present study, unleashes the pro-apoptotic potential of Bak and Bax and thereby contributes to the induction of apoptosis in LS 174T and other CRC cells (Figure 4.1). Indeed, DHA incubation has recently been reported to significantly activate Bax and Bak during the induction of apoptosis in HT-29 and Caco-2 CRC cells, whereas a Bax inhibitor partially reversed DHA-induced apoptosis (Giros et al. 2009).

In the context of LS 174T, Calviello et al. additionally reported DHA to significantly decrease B-cell lymphoma-extra large (Bcl-xL) protein (Calviello et al. 2005). As Bcl-xL also belongs to the anti-apoptotic Bcl-2-like survival factors and inhibits the pro-apoptotic potential of Bax, the

DHA-associated decrease in Bcl-xL protein may further contribute to the induction of apoptosis in LS 174T cells (Willis et al. 2005; Zhang et al. 2000).



**Figure 4.1:** Influence of PUFAs on the regulation of the apoptotic cascade by Bcl-2. Anti-apoptotic Bcl-2, which is up-regulated on the mRNA level by AA incubation, inhibits pro-apoptotic proteins Bax and Bak. In contrast, DHA incubation down-regulated Bcl-2 mRNA, thus possibly preventing Bcl-2-dependent inhibition of Bak and Bax. Following disinhibition, Bax and Bak oligomerize and insert into the mitochondrial membrane. In turn, this results in the formation of pores within the mitochondrial membrane allowing the release of cytochrome C, which associates with Apaf-1 as well as caspase-9, forming an apoptosome. This leads to a downstream activation of caspase-3 and hence induces apoptosis. Back arrows indicate an induction or up-regulation, whereas red arrows display inhibition or down-regulation. Adapted in part from and based upon Borner 2003, Giros et al. 2009 and Matthews et al. 2006.

However, on the other hand, the aforementioned studies did not assess the impact of n-6 PUFAs or other FAs in the context of Bcl-2 expression. Yet, elevated Bcl-2 mRNA levels following AA incubation found in our qRT-PCR experiments could protect LS 174T CRC cells from apoptosis as Bax and Bak oligomerization may be inhibited (Figure 4.1). These results are consistent with previous findings, which revealed PGE<sub>2</sub> to inhibit apoptosis through induction of Bcl-2 in HCA-7 CRC cells (Sheng et al. 1998).

However, contrasting our findings another *in vitro* study recently reported AA incubation to not only down-regulate Bcl-2 mRNA, but also to induce apoptosis in Caco-2 CRC cells (Oraldi et al. 2009). Yet, as these results were obtained in a different cell line and contradict several other investigations, which reported AA in identical concentrations to not induce apoptosis in Caco-2 cells, further investigations are needed to clarify the impact of AA in the context of Bcl-2 expression (Engelbrecht et al. 2008; Hossain et al. 2009).

Moreover, future studies are necessary to elucidate the underlying molecular mechanism through which DHA and AA alter Bcl-2 mRNA expression, as these currently remain elusive. In this context it is noteworthy that a link between COX-2 expression and Bcl-2 regulation has been suggested: Over-expression of COX-2 in intestinal epithelial cells - as found in reaction to AA supplementation in the present study – has been reported to induce expression of Bcl-2, which in turn suppressed apoptosis (Tsujii et al. 1995).

However, putting a possible regulation of Bcl-2 by n-3 and n-6 PUFAs into a broader perspective, over-expression of Bcl-2 has been associated with the resistance to cytotoxic drugs such as 5-FU or cisplatin in several CRC models (An et al. 2007; Violette et al. 2002). Thus, it may be hypothesized that administration of DHA might potentiate anti-cancerous effects of chemotherapeutic agents as suggested by a growing body of *in vivo* and *in vitro* data, recently reviewed by Dupertuis and co-workers (Dupertuis et al. 2007).

#### **4.3.2 Cyclin-dependent kinase inhibitor 1A (P21<sup>WAF1</sup>)**

Similar to the evasion of apoptosis, as mediated by over-expression of Bcl-2, unrestrained cellular proliferation is another characteristic of cancer development. Under healthy conditions, cells of humans and other eukaryotes possess multiple checkpoint mechanisms, which monitor and respond to cellular perturbations by halting cellular progression and proliferation until errors are resolved and the cellular environment becomes permissive again for the adequate transmission of genetic material. Disruption of these checkpoint mechanisms is adverse to genome integrity, promotes carcinogenesis and modifies the efficacy of anti-cancerous therapy (Abbas et al. 2009).

Passage through the cell cycle during cellular proliferation is determined by the function of regulatory (cyclins) and catalytic subunits (kinases) (Jacks et al. 1996; Sherr 1994). In this context cyclin-dependent kinase inhibitor 1A (generally referred to as p21<sup>WAF1</sup>) was originally discovered as a transcriptional target of the tumor suppressor p53, mediating p53-dependent G1-phase cell cycle arrest in response to DNA damage (Chuang et al. 1997; el-Deiry et al. 1993; Harper et al. 1993; Waga et al. 1994).

During proliferation, G1-phase progression is facilitated by the combined activities of cyclin D1 and cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) as well as by activation of the cyclin E and CDK2 complexes (Sherr et al. 1999). p21<sup>WAF1</sup> primarily inhibits CDK2 as well as CDK1 thus disrupting the cell cycle and subsequently leading to cell cycle arrest. Moreover, p21<sup>WAF1</sup>



also suppresses the function of proliferating cell nuclear antigen (PCNA) and thereby interferes with the PCNA-controlled DNA polymerase activity, which under regular circumstances facilitates DNA replication and repair mandatory for S-phase progression (Cayrol et al. 1998; Sherr 1994). Hence, since the original discovery, substantial evidence highlighted p21<sup>WAF1</sup> as a “master effector” of multiple tumor suppressor pathways, some of which independent of the classical p53 route. p21<sup>WAF1</sup> not only controls cell cycle arrest and directly inhibits DNA replication but also regulates fundamental cellular processes, such as gene transcription and apoptosis (figure 4.2) as recently summarized in great detail by Abbas and Dutta (Abbas & Dutta 2009).

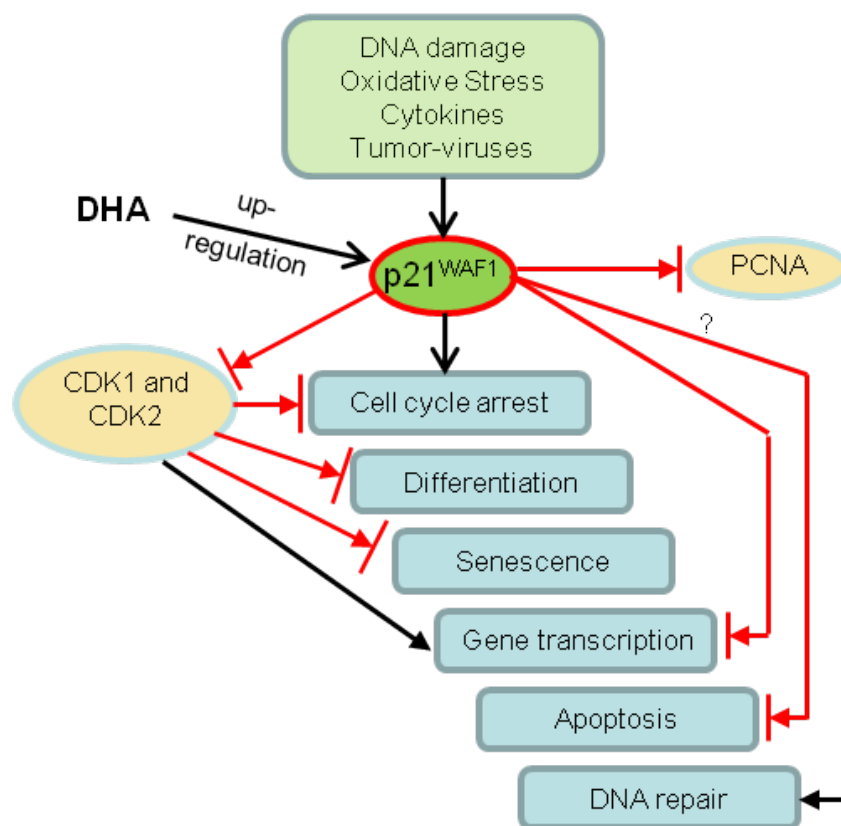


Figure 5.2: *The central cellular function of p21<sup>WAF1</sup>*. p21<sup>WAF1</sup>, which was found to be up-regulated by DHA incubation, responds to a variety of stimuli promoting growth-inhibitory activities and suppressing proliferation. These functions mainly depend on the ability of p21<sup>WAF1</sup> to inhibit the activity of cyclin-dependent kinase 1 and 2 (CDK1 and CDK2) as well as the function of proliferating cell nuclear antigen (PCNA). In addition, some of the anti-proliferative effects of p21<sup>WAF1</sup> rely on its ability to regulate and obstruct gene transcription. Moreover, p21<sup>WAF1</sup> has been suggested to mainly suppress apoptosis, whereas under certain circumstances it may act pro-apoptotically. Back arrows indicate an induction or up-regulation, whereas red arrows denote inhibition or down-regulation. Adapted from Abbas & Dutta 2009.

In the present study, qRT-PCR results revealed DHA to up-regulate p21<sup>WAF1</sup> mRNA levels in LS 174T cells when compared to cells supplemented with AA or control cells. These findings are congruent with results presented by several other investigators, who reported DHA to

significantly elevate p21<sup>WAF1</sup> mRNA expression as well as p21<sup>WAF1</sup> protein levels in Caco-2, SW620, and Colo 201 CRC cells. Furthermore, these studies, which did not investigate LS 174T cells, reported DHA incubation to potentially alter the expression of miscellaneous additional proteins involved in cell cycle regulation such as cyclin E, cyclin D1 and PCNA as well as to significantly induce G1-phase cell cycle arrest (Danbara et al. 2004; Narayanan et al. 2003; Slagsvold et al. 2010). Hence, it may be assumed that DHA potentially disrupts cell cycle progression in CRC cells *in vitro* and may thereby interfere with tumor cell proliferation (Figure 4.2). Moreover, the fact that in the present study AA incubation did not significantly alter amounts of detected p21<sup>WAF1</sup> mRNA suggests that the DHA-related elevation of p21<sup>WAF1</sup> mRNA is not an unspecific cellular reaction to exogenous PUFAs, but is specifically induced by the n-3 PUFA.

However, although best known for the disruption of cell cycle and subsequent growth inhibition, p21<sup>WAF1</sup> has recently been suggested to also act as an oncogene, due to its inhibitory effects in the context of apoptosis mediated via numerous pathways (Abbas & Dutta 2009; Dotto 2000; Gartel 2006). Nonetheless, in the present study, despite elevated levels of p21<sup>WAF1</sup> mRNA, DHA significantly induced apoptosis, while no significant G1-phase cell cycle arrest was detected in flow cytometry experiments. As a possible explanation, pro-apoptotic effects of DHA may superpose and obscure cell cycle arrest, potentially mediated by increased levels of p21<sup>WAF1</sup>. In any case, our data suggest that potential anti-apoptotic properties of p21<sup>WAF1</sup> do not inhibit or reverse DHA-induced apoptosis. In addition, under certain cellular stresses, p21<sup>WAF1</sup> has also been shown to promote apoptosis via p53-dependent as well as p53-independent mechanisms, which yet remain to be fully understood, but may involve Bax protein (see chapter 4.3.1) (Abbas & Dutta 2009; Gartel 2005).

In conclusion, although the present study indicated that DHA significantly increases p21<sup>WAF1</sup> mRNA expression, further studies are needed in order to fully understand the effects of DHA in regard of p21<sup>WAF1</sup>-associated cell cycle regulation and apoptosis in LS 174T CRC cells as well as the underlying molecular mechanisms.

#### **4.4 Co-incubation experiments and DHA interference with AA metabolism**

The most important findings of the present study were obtained in co-incubation experiments: As far as we are aware, results presented here demonstrated for the first time that DHA can directly

suppress and counteract AA-induced growth promotion in a CRC cell line. Hence, these observations provide new evidence concerning the importance of the n-6 to n-3 PUFA ratio in the context of CRC. Although these results are novel in regard of CRC, they are consistent with data obtained in human breast cancer cells, which revealed DHA to significantly diminish AA-induced cancer cell invasiveness *in vitro* (Horia et al. 2007).

As n-3 PUFAs have previously been indicated to significantly interfere with AA-related metabolism (see chapter 1.7) and AA is the precursor to the growth-promoting lipid mediator PGE<sub>2</sub>, it may be hypothesized that the increased DHA content of the cells disrupts AA-dependent PGE<sub>2</sub> biosynthesis, thus inhibiting AA-induced proliferation. Indeed, ELISA experiments revealed DHA to significantly decrease amounts of PGE<sub>2</sub>, derived from exogenous AA in LS 174T cells, by more than one third. Although this matter has not yet been addressed in an explicit AA/DHA co-incubation set up, our data are concordant with results from another *in vitro* study, which reported DHA to significantly inhibit baseline PGE<sub>2</sub> biosynthesis (not triggered by exogenous AA addition) by approximately 50% in HT-29 CRC cells. Highlighting the *in vivo* relevance of these findings in the same study, dietary treatment with DHA significantly inhibited proliferation and reduced PGE<sub>2</sub> levels in inoculated HT-29 cancer cell tumors in athymic mice (Calviello et al. 2004).

In this context it is furthermore noteworthy that qRT-PCR results of the present study indicated DHA to significantly decrease COX-2 mRNA expression in LS 174T cells in a dose-dependent manner. Although not yet investigated in this particular cell line, our data is consistent with numerous previous studies, which also reported decreased COX-2 mRNA and protein levels in response to DHA incubation in several CRC cell lines (Calviello et al. 2004; Giros et al. 2009; Narayanan et al. 2003; Narayanan et al. 2004). Hence, it may be hypothesized that decreased expression of COX-2 contributes to the DHA-associated reduction of synthesis of AA-derived PGE<sub>2</sub>.

In synopsis, these results support the concept that the n-3 PUFA DHA significantly interferes with AA-metabolism. However, DHA does not only potently reverse AA-induced growth, but also possesses the ability to directly inhibit proliferative effects of PGE<sub>2</sub> - although higher concentrations were necessary in this context. Thus, DHA-associated inhibition of AA-induced cellular proliferation is in part independent from a pure blocking effect on PGE<sub>2</sub> formation. This concept is further endorsed by the fact that DHA completely reverses AA-induced proliferation, although levels of PGE<sub>2</sub> are only decreased by approximately one third.

Thus, DHA exerts at least some of its anti-cancerous actions via COX- and AA-independent, yet unknown pathways. This is also implicated by single incubation experiments of the present study, which showed DHA to potentially induce apoptosis and inhibit cancer cell proliferation, although only very low levels of PGE<sub>2</sub> are synthesized in absence of exogenous AA by LS-174T cells. Similarly, other studies have also reported anti-cancerous effects of DHA to be independent of COXs and AA-related metabolism (see also chapter 1.7) (Boudreau et al. 2001; Calviello et al. 2007). In order to further elucidate this matter as well as the underlying mechanisms, additional studies investigating the interaction between DHA, AA and COX-2 in the co-incubation context are needed.

Nevertheless, the *in vitro* finding that n-3 PUFA DHA supplementation suppressed tumor cell growth, even in the presence of high AA and PGE<sub>2</sub> concentrations is important, as it highlights that n-3 PUFAs potentially counteract harmful effects of AA and of AA-derived lipid mediators. Moreover, DHA interference with AA-related metabolism was underlined by the present study, although the exact functional impact still remains to be determined.

#### **4.5 Study limitations**

Although our investigations may provide valuable data concerning the impact of n-3 and n-6 PUFAs in the context of CRC, it has to be taken into consideration that they are deduced from *in vitro* experiments.

As it is impossible to completely reproduce the *in vivo* colorectal microenvironment *in vitro*, transferability of *in vitro* results to a full human organism remains limited. *In vivo*, cells of the colorectal epithelium are exposed to a plethora of influences and stimuli, reaching from luminal stool exposure containing an almost countless number of substances over the influence of bacterial flora to exposition to blood flow transporting oxygen as well as a vast number of hormones and other agents. Furthermore, cellular interactions as found for instance concerning cells of the immune system can hardly be replicated in an *in vitro* setup.

Hence, naturally the microenvironment of a Petri dish in an incubator can only account for a limited number of influencing factors and thus will never fully “live up to” *in vivo* conditions. This may be considered as a general weakness of *in vitro* investigation on the one hand. However, at the same time a limited amount of influencing factors may also be regarded as a particular advantage. Through a reduced number of influences, researchers are enabled to center their focus of attention on the particular matter of interest, while many confounding factors, which exist *in vivo*, may be excluded. In addition accessibility of cells *in vitro* is naturally

superior to *in vivo* conditions. Thus, there are many examples for valuable (mechanistic) data, which were obtained in *in vitro* investigations in the context of many clinical conditions, as for instance in leukemia, rheumatism and also CRC research.

Nevertheless, it also has to be taken into consideration that *in vitro* cells are also exposed to a variety of factors possibly acting as confounders. As one of the most important, cells are maintained in medium supplemented with up to 10% of fetal calf growth serum, containing additional substances, which may influence cellular behavior and thus experimental outcome. However, at least some of these influences may be accounted for via thorough experimental planning and appropriate controls. In the present study all cells, FA-treated as well as controls, were maintained under the identical circumstances.

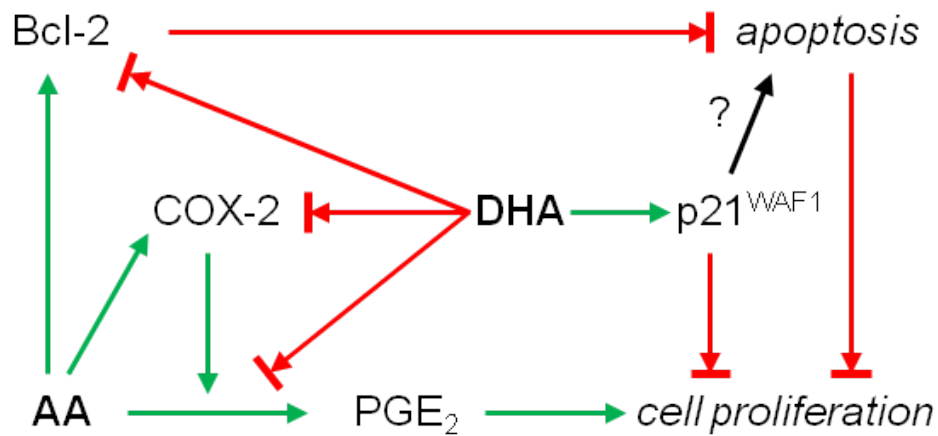
In conclusion *in vitro* studies are a useful tool in order to investigate influences of certain substances in a limited setup, such as PUFAs on cancer cells. Nevertheless, obvious limitations have to be taken into consideration in the discussion of the results obtained.

In regard of the present *in vitro* study, it may furthermore be argued that FA concentrations applied to the cells, reaching up to 100 $\mu$ M in long term incubations, are fairly high. However, the mean daily intake of DHA in North America has been estimated at approximately 100mg, which corresponds to a DHA blood level of approximately 280 $\mu$ M (Arterburn et al. 2006; Chapkin et al. 2008; Hibbeln et al. 2006). Upon DHA supplementation, blood levels have been reported to even exceed 500 $\mu$ M (Conquer et al. 1998). Importantly, these elevated serum levels have been indicated to lead to a corresponding enrichment of FAs in the membranes of colonocytes (Chapkin et al. 2002). Thus, concentrations applied to cells in the current *in vitro* study are well within the physiological range and mimic the microenvironment possibly found in humans. Hence, it is not surprising that the majority of studies conducted in this field of research applied similar FA concentrations with some studies even using considerably higher concentrations of up to 300 $\mu$ M (Chen & Istfan 2000; Habermann et al. 2009; Pan et al. 2009).

#### **4.6 Conclusion and impact of the study**

Although deduced from a limited *in vitro* setup, results obtained in the present study highlight the potential impact of n-3 and n-6 PUFAs as well as the PUFA ratio in the context of CRC. In synopsis, as summarized in figure 4.3, results presented here clearly demonstrate diametrically opposing effects of n-3 and n-6 PUFAs *in vitro*. While n-3 PUFA DHA potently exerted anti-cancerous effects, n-6 PUFA AA promoted cancer cell proliferation. Thus, our data help to

clarify the uncertainty concerning differential effects of these two classes of FAs resulting from previous studies not addressing the effects of n-6 PUFA or reporting similar effects of n-3 and n-6 PUFAs.



**Figure 4.3:** Summary of DHA and AA effects in colorectal cancer cell line LS 174T. Green arrows indicate an induction or up-regulation, whereas red arrows display inhibition or down-regulation.

As a novel aspect not yet investigated by previous studies, we addressed the potential interplay between AA and DHA in the context of CRC cells. As mentioned within the introduction (chapter 1.4), it has been hypothesized that the human genetic profile was originally established on a n-6 to n-3 PUFA ratio of approximately 1:1, as found in diets consumed for millions of years prior to industrialization. In contrast, the n-6 to n-3 PUFA ratio of today’s Western nutrition has been estimated at 15:1 –20:1 and is widely believed to contribute to many serious health issues typically found in our Western societies, including CRC (Eaton et al. 1998; Eaton & Konner 1985; Kang 2005; Leaf & Weber 1987; Simopoulos 2009). In the present study, AA/DHA co-incubations resulted in an (almost) balanced n-6 to n-3 ratio, hence mimicking conditions found in “ancient” diets. Importantly, in this setting, n-3 PUFA DHA potently reversed pro-proliferatory effects of n-6 PUFA AA and of AA-derived PGE<sub>2</sub> towards growth inhibition.

Thus, our data add evidence to the argument that the ratio of n-6 to n-3 PUFA (particularly the ratio of AA/DHA) may be a critical determinant of proliferation and tumor growth in CRC. Based upon our results it may be hypothesized that even the intake of higher amounts of AA, which is an essential precursor to various crucial lipid mediators, does not necessarily have harmful effects in the context of CRC as long as “sufficient” amounts of n-3 PUFA DHA are consumed, which counteract harmful effects of AA and its derived lipid mediators. Taking also

the massive additional *in vivo* and epidemiological evidence into account (chapter 1.4 and 1.7), the present *in vitro* study is clearly in favor of a high intake of n-3 PUFAs as found in oily fish.

Moreover, it is noteworthy that effects of DHA appear to be independent from the type of the underlying type of genetic instability (see chapter 1.3). While LS 174T cells are microsatellite instable (MSI), other microsatellite stable cell lines, such as HT-29 or Caco-2, which follow the chromosomal instability pathway (CIN), have also been reported to be susceptible to anti-cancer actions of DHA in numerous studies (Narayanan et al. 2001; Narayanan et al. 2003; Narayanan et al. 2004; Giros et al. 2009). Thus, it may be assumed that DHA exerts its anti-neoplastic effects in a broad variety of CRC cells. However, additional studies are needed to address the impact of the type of underlying genetic instability in the context of DHA and n-3 PUFA action.

In this context it remains an important question, whether the anti-cancerous effects of DHA observed in the present and in other studies only apply to cancer cells, which already completed the carcinogenetic transformation, or whether they also apply to cells currently within the process of colorectal carcinogenesis as found in adenomas and other precursor lesions (chapter 1.3). The answer to this issue would further explain and elucidate the epidemiologically deduced protective effects of n-3 PUFAs (chapter 1.4). In this regard, it is noteworthy that proteins and pathways found to be altered by n-3 PUFAs in the present and in other studies, including COX-2, Bcl-2, Bax,  $\beta$ -catenine and others have also been implicated as key players in the neoplastic transformation of the colorectal epithelium (Giros et al. 2009; Narayanan et al. 2004). Moreover, several recent *in vivo* and *in vitro* studies provided significant evidence that n-3 PUFAs including DHA may exert at least some of their anti-cancerous actions early within the process of malignant transformation, as for instance total serum n-3 PUFA levels were found to be inversely associated with colorectal adenoma risk and an adenoma cell line was found to be susceptible to DHA-induced cell death (Habermann et al. 2009; Moreira et al. 2009; Pot et al. 2008).

As another important aspect, the present investigation found DHA to potently diminish formation of PGE<sub>2</sub> from exogenous AA and to decrease mRNA expression of COX-2. This may be of special interest in regard of CRC prevention, as protective effects of NSAIDs and selective COX-2 inhibitors in the context of colorectal carcinogenesis have been widely attributed to inhibition of COX-2 and subsequently reduced biosynthesis of PGE<sub>2</sub>.(chapters 1.6 and 1.7). But due to the severe side effects of long term use of NSAIDs and specific COX-2 inhibitors a recommendation as preventive agents against CRC is questionable and still under debate. Thus, based upon the

present data and results obtained in many other investigations, n-3 PUFAs including DHA may be hypothesized as alternative preventive agents interfering with AA-associated metabolism and have consequently recently been referred to as “natural COX inhibitors” (Berquin et al. 2008).

However, besides interferences with the COX-dependent AA metabolism, data presented here suggest that DHA exerts its anti-cancerous actions also via COX- and AA-independent pathways, which yet remain to be fully understood.

Thus, additional research is needed in order to investigate and clarify preventive as well as anti-carcinogenic effects associated with n-3 PUFAs, such as DHA, *in vitro* and *in vivo*.

In conclusion, although deduced from a limited *in vitro* setup, the present study provides significant new evidence for beneficial effects of DHA as well as for the pivotal importance of n-6 to n-3 ratio in the context of CRC (Habbal et al. 2009).



## 5. Abstract

*Background:* Colorectal cancer (CRC) is one of the leading causes of death in Western countries. A potential role of omega-3 and omega-6 polyunsaturated fatty acids (n-3 and n-6 PUFAs) in the context of CRC has been implicated by numerous epidemiological studies, suggesting a protective role of n-3 PUFAs. On the other hand it is well-established that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), generated from n-6 PUFA arachidonic acid (AA), is an important promoting factor in the tumorigenesis of the colorectum.

In this context it has been proposed that the human genetic profile was originally established on a n-6 to n-3 PUFA ratio of approximately 1:1 as found in “ancient” diets, whereas today’s Western diet has been estimated to provide n-6 to n-3 PUFAs in a ratio of 15:1–20:1. It has been hypothesized that this may contribute to many serious health issues typically found in Western societies, including CRC. However, previous *in vitro* observations have led to some uncertainty regarding differential roles of n-3 and n-6 PUFAs in CRC cells. While the majority of investigations conducted in this field addressed neither the effects of n-6 PUFAs nor the impact of a balanced n-6 to n-3 PUFA ratio, several other studies reported n-3 and n-6 PUFAs to exert anti-cancerous effects *in vitro*. Hence, it was the aim of the present study to investigate the impact of n-3 PUFA docosahexaenoic acid (DHA) and n-6 PUFA AA and their combination on CRC cell line LS 174T *in vitro*.

*Methods:* In order to study the effects of n-3 PUFA DHA, n-6 PUFA AA, PGE<sub>2</sub> and several combinations on cellular viability of LS 174T CRC cells, XTT assays were applied. Cell cycle and cell death were assessed via flow cytometry experiments and DAPI stainings. Moreover, mRNA expressions of cyclooxygenase-2 (COX-2), B-cell lymphoma-2 (Bcl-2) and cyclin-dependent kinase inhibitor 1A (p21<sup>WAF1</sup>) in cells incubated with AA, DHA or vehicle were examined by semiquantitative real-time PCR (qRT-PCR). Biosynthesis of PGE<sub>2</sub> in the presence of AA and DHA was measured in a PGE<sub>2</sub>-ELISA.

*Results:* While incubation with n-6 PUFA AA increased cellular proliferation, supplementation of n-3 PUFA DHA significantly reduced cellular viability and induced apoptosis in a time- and dose-dependent manner. Moreover, DHA incubation potently decreased mRNA expression of anti-apoptotic Bcl-2 and induced the expression of cell cycle inhibitor p21<sup>WAF1</sup>. In contrast, incubation with AA resulted in a significantly increased expression of Bcl-2 mRNA, whereas amounts of p21<sup>WAF1</sup> mRNA remained unaltered compared to control cells.

In addition, AA incubation increased mRNA levels of COX-2, the key enzyme in AA-dependent PGE<sub>2</sub> formation, and also resulted in a significant biosynthesis of PGE<sub>2</sub>, an important inductor of cellular proliferation in LS 174T cells. In contrast, DHA incubation decreased COX-2 mRNA expression and significantly diminished AA-induced PGE<sub>2</sub> formation in co-incubation experiments. Importantly, results from co-incubations furthermore indicated DHA to potentially reverse and suppress AA- as well as PGE<sub>2</sub>-induced CRC cell growth.

*Conclusion and impact:* Results presented here clearly demonstrate diametrically opposing effects of n-3 PUFA DHA and n-6 PUFA AA in regard of cellular viability and mRNA regulation, thus helping to clarify the aforementioned uncertainty concerning differential effects of n-3 and n-6 PUFAs *in vitro*.

As novel aspect in this context, the present investigation addresses the interplay between AA and DHA in the context of CRC cells *in vitro*. AA/DHA co-incubations, leading to an (almost) balanced n-6 to n-3 ratio, thus mimicking conditions found in “ancient” diets, showed DHA to potentially reverse pro-proliferatory effects of n-6 PUFA AA and of AA-derived PGE<sub>2</sub> towards growth inhibition. Thus, our data add evidence to the argument that the ratio of n-6/n-3 PUFA (particularly the ratio of AA/DHA) may be a critical determinant of proliferation and tumor growth in CRC. However, further *in vitro* and *in vivo* studies are required in order to fully elucidate this interplay and its clinical impact and relevance.

As another important aspect, DHA potentially diminished PGE<sub>2</sub> formation from exogenous AA and decreased mRNA expression of COX-2. This may be of special interest in regard of CRC prevention as pharmacological inhibition of COXs (via ASS and other nonsteroidal anti-inflammatory drugs), subsequently reducing PGE<sub>2</sub> synthesis, has been found to be protective against CRC development. Nevertheless, long term use of these drugs is associated with severe adverse effects and is a matter of ongoing debate. Thus, based upon the present data and previous studies, n-3 PUFAs including DHA may be hypothesized as an alternative preventive agent against CRC, also interfering with the AA-associated metabolism.

However, besides interaction with the COX-dependent AA metabolism, data of the present study additionally suggest DHA to exert its anti-cancerous actions also via COX- and AA-independent pathways, which yet remain to be fully understood by future investigations.

In conclusion, although deduced from a limited *in vitro* setup, the present study provides significant evidence concerning beneficial effects of DHA as well as for the pivotal importance of n-6 to n-3 ratio in the context of CRC.

## 6. Zusammenfassung

*Hintergrund:* Das kolorektale Karzinom stellt eine der häufigsten Todesursachen in den westlichen Industrienationen dar. Zahlreiche epidemiologische Studien weisen in diesem Zusammenhang auf einen Einfluss mehrfach ungesättigter Omega-3- und Omega-6-Fettsäuren (n-3 PUFAs und n-6 PUFAs) hin, wobei n-3 PUFAs im Allgemeinen eine protektive Wirkung zugeschrieben wird. Andererseits ist bekannt, dass Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), ein aus der n-6 PUFA Arachidonsäure (AA) synthetisierter Lipidmediator, die kolorektale Karzinogenese fördert. In diesem Kontext wurde postuliert, dass sich das menschliche Genprofil ursprünglich auf Basis einer Ernährung mit ausgeglichenem n-6 zu n-3 PUFA-Verhältnis entwickelte, während das n-6 zu n-3 PUFA-Verhältnis heutiger westlicher Nahrung bei etwa 15:1-20:1 liegt. Diese veränderte Nahrungszusammensetzung wird von vielen Experten für die hohe Prävalenz vieler sogenannter „Wohlstandskrankheiten“, zu denen auch das kolorektale Karzinom zählt, mitverantwortlich gemacht. In bisherigen *in vitro*-Untersuchungen zeigte sich jedoch kein einheitliches Bild bezüglich unterschiedlicher Wirkungen von n-3 und n-6 PUFAs auf Kolonkarzinomzellen. Während die meisten Studien in diesem Gebiet weder die Effekte von n-6 PUFAs noch den Einfluss des n-3 zu n-6 PUFA-Verhältnisses untersuchten, zeigten andere Veröffentlichungen antikanzerogene Wirkungen beider Fettsäureklassen. Das Ziel der vorliegenden Arbeit war es daher, die Wirkung der n-3 PUFA Docosahexaensäure (DHA), der n-6 PUFA AA und des AA-basierten Lipidmediators PGE<sub>2</sub> sowie deren Kombination auf die Kolonkarzinom-Zelllinie LS 174T zu untersuchen.

*Methoden:* Der Einfluss von DHA, AA, des Lipidmediators PGE<sub>2</sub> und deren Kombination auf Überleben und Wachstum der Kolonkarzinom-Zelllinie LS 174T wurden mittels XTT Assays untersucht. Mit Hilfe von Durchflusszytometrie und DAPI-Färbungen wurden die Effekte der Fettsäuren auf Zellzyklus und Zelltod evaluiert, während der Einfluss beider Fettsäuren auf die mRNA-Expression von Cyclooxygenase-2 (COX-2), B-cell lymphoma-2 (Bcl-2) und Cyclin-dependent kinase inhibitor 1A (p21<sup>WAF1</sup>) durch semiquantitative real-time PCR ermittelt wurde. Zusätzlich wurde der Einfluss von AA und DHA auf die PGE<sub>2</sub>-Synthese durch ELISA-Untersuchungen quantifiziert.

*Ergebnisse:* Während die Inkubation mit der n-6 PUFA AA die Proliferation der LS 174T Zellen anregte, reduzierte DHA zeit- und dosisabhängig das Überleben der Karzinomzellen und induzierte Apoptose. Weiterhin verminderte die Inkubation mit DHA die mRNA-Expression des antiapoptotischen Proteins Bcl-2 und erhöhte den mRNA-Level des Zellzyklusinhibitors

p21<sup>WAF1</sup>. Im Gegensatz hierzu führte die Zugabe von AA zur vermehrten Expression von Bcl-2-mRNA, während sich die Menge an p21<sup>WAF1</sup>-mRNA unverändert zeigte. Daneben induzierte die Inkubation mit AA die mRNA-Expression von COX-2 (dem Schlüsselenzym der AA-abhängigen PGE<sub>2</sub>-Synthese) und resultierte außerdem in einer gesteigerten PGE<sub>2</sub>-Biosynthese. Demgegenüber verminderte die Inkubation mit DHA sowohl die Expression von COX-2-mRNA als auch die AA-induzierte PGE<sub>2</sub>-Synthese. Weitere Koinkubationsexperimente zeigten, dass DHA dem AA- und PGE<sub>2</sub>-induzierten Wachstum in LS 174T entgegenwirkt.

*Diskussion und Fazit:* Die vorliegende Studie zeigt gegensätzliche Effekte der n-3 PUFA DHA und der n-6 PUFA AA bezüglich Proliferation, Apoptose und mRNA-Regulation in der kolorektalen Karzinomzelllinie LS 174T und trägt somit dazu bei, die Wirkung dieser Fettsäuren weiter zu klären. Als neuer Aspekt wurde die Interaktion zwischen AA und DHA *in vitro* untersucht. AA/DHA-Koinkubationsexperimente mit einem (fast) ausgeglichenen Verhältnis von n-6 zu n-3 PUFAs (entsprechend der Zusammensetzung der Nahrung vor der Industrialisierung) zeigten, dass DHA sowohl dem AA- als auch dem PGE<sub>2</sub>-induzierten Wachstum entgegenwirkt. Diese Ergebnisse unterstreichen den Einfluss des n-6 zu n-3 Verhältnisses (im Besonderen AA zu DHA) im Kontext der kolorektalen Karzinogenese, wobei weitere *in vitro*- und *in vivo*-Studien nötig sind, um die Interaktion der unterschiedlichen Fettsäuren sowie deren klinische Relevanz vollständig zu erfassen. Daneben zeigen die vorliegenden Experimente, dass DHA sowohl die PGE<sub>2</sub>-Synthese aus exogen zugeführter AA als auch die mRNA-Expression des Schlüsselenzyms COX-2 reduziert. Dies ist insofern von besonderem Interesse, als dass die pharmakologische Inhibition der COXs (beispielsweise durch Acetylsalicylsäure oder andere nicht-steroidale Antiphlogistika) mit der hieraus resultierenden Reduktion der PGE<sub>2</sub>-Synthese präventiv gegen das kolorektale Karzinom wirkt, jedoch aufgrund der assoziierten Nebenwirkungen nicht allgemein empfohlen wird. In diesem Zusammenhang legen die vorliegenden Daten in Zusammenschau mit den Ergebnissen anderer Studien nahe, dass alternativ n-3 PUFAs wie DHA als präventive, mit dem AA-Metabolismus interferierende Substanzen eingesetzt werden könnten. Darüber hinaus wird aber aus den vorliegenden Daten zusätzlich deutlich, dass die anti-kanzerogenen Effekte von DHA zum Teil auch auf COX- und AA-unabhängigen Mechanismen basieren, die in zukünftigen Studien weiter erforscht werden sollten. Zusammenfassend liefert die vorliegende Studie neue und signifikante Hinweise bezüglich protektiver Effekte der n-3 PUFA DHA und hebt die Relevanz eines n-6 zu n-3 PUFA-Verhältnisses im Kontext des kolorektalen Karzinoms hervor.

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## **8. Selbstständigkeitserklärung / Declaration of originality**

Ich, Jan-Piet Habel, erkläre an Eides statt, dass ich die vorgelegte Dissertationsschrift mit dem Thema „Docosahexaenoic acid suppresses arachidonic acid-induced proliferation of LS 174T human colon carcinoma cells“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

Datum:

Unterschrift:

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## **10. Lebenslauf / Curriculum Vitae**

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version der Arbeit nicht veröffentlicht.

## 11. Eigene Publikationen / List of Publications

Nowak J\*, Weylandt KH\*, **Habbel P**, Wang J, Dignass A, Glickman JN, Kang JX (\*equally contributed). Colitis-associated colon tumorigenesis is suppressed in transgenic mice rich in endogenous n-3 fatty acids. *Carcinogenesis* 2007 Sep;28(9):1991-5. Epub 2007 Jul 18.

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