Chemopräventive Effekte von *n*-3 mehrfach ungesättigten Fettsäuren und Fisch in humanen Kolonzellen

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Abbreviations

AP-1	activator protein-1
ABC	ATP binding cassette transporter
ACBP	acyl-CoA binding protein
ADP	adenosine diphosphate
AICR	American Institute for Cancer Research
ALA	α-linolenic acid
Apaf-1	apoptotic peptidase activating factor-1
APC	Adenomatous Polyposis Coli
ARA	arachidonic acid
ATP	adenosine triphosphate
Bad	Bcl-2-associated agonist of cell death
Bak	Bcl-2-antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell chronic lymphocytic leukemia/lymphoma 2
BH	Bcl-2 homology domain
Bid	BH3 interacting domain death agonist
Bik	Bcl-2-interacting killer
C	carbon atom, refers to the carbon chain length of the fatty acid
CKI	casein synthase kinase
COX	cyclooxygenase
CpG	cytosine-guanine dinucleoside
CYP	cytochrome p450
d	day
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DCC	Deleted in colorectal carcinoma
DGLA	dihomo-γ-linoleic acid
DHA	docosahexaenoic acid
DISC	death-inducing signal complex
DR	death receptor
DRI	Dietary Reference Intake
DPA	docosapentaenoic acid
EC	Enzyme Commission number, a numerical classification scheme for enzymes
EC_{50}	half maximal effective concentration
EET	epoxyeicosatetraenic acid
e.g.	*exempli gratia* (for example)
EGFR	epidermal growth factor receptor
EPA	eicosapentaenoic acid
EpRE	electrophilic responsive element
et al.	*et alii* (and others)
etc.	*et cetera* (and so forth)
FABS	fatty acid binding proteins
FADD	Fas-associated protein with death domain
FAP	Familial Adenomatous Polyposis
Fas	TNF receptor
FasL	Fas ligand
FITC	fluorescein isothiocyanate
FMO	flavin-monooxygenase
g	gram
g	standard gravity ($9.81 m/s^2$)
GDP	guanosine diphosphate
GKS3β	glycogen synthase kinase 3β

GLA	γ-linolenic acid
GST	glutathione *S*-transferase
GTP	guanosine triphosphate
h-ras	Harvey rat sarcoma
HETE	hydroxyeicosatetraenic acid
HNF-4α	hepatic nuclear factor 4 α
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HpETE	hydroxyperoxyeicosatetraenic acid
HXA4	hipoxilin A4
HXB4	hipoxilin B4
IAP	inhibitor of apoptosis
i.e.	*id est* (that is)
k-ras	Kirsten rat sarcoma
Keap1	Kelch-like ECH-associated protein 1
LA	linoleic acid
LRP	lipoprotein receptor-related protein
LTA4	leukotriene A4
LTB4	leukotriene B4
LTC4	leukotriene C4
LTD4	leukotriene D4
LTE4	leukotriene E4
LTF4	leukotriene F4
LXA4	lipoxin A4
LXR	liver x receptor
MAPK	mitogen-activated protein kinase
MRP	multidrug resistance proteins
MGMT	*O*-6-methylguanine-DNA methyltransferase
MUFA	monounsaturated fatty acids
n-3 / *n*-6 / *n*-9	respectively ω-3 / ω -6/ ω -9, final carbon-carbon double bond at third / sixth / ninth bond from the methyl end of the fatty acid
n-ras	neuroblastoma ras viral oncogene homolog
NAT	*N*-acetyl transferase
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NQO1	NADP(H):quinone oxidoreductase 1
Nrf2	NF-E2-related factor 2
NSAID	non-steroidal anti-inflammatory drug
p53	tumour protein 53
PARP	poly (ADP-ribose) polymerase
PCB	polychlorinated biphenyls
PGG2	prostaglandin G2
PGH2	prostaglandin H2
PGD2	prostaglandin D2
PGE2	prostaglandin E2
PGF2α	prostaglandin F2α
PGI2	prostacycline
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
Smac/Diablo	second mitochondria-derived activator of caspases/direct IAP binding protein with low isoelectric point
SMAD	small mothers against decapentaplectic homolog
SOD	superoxide dismutase
SREBP	sterol regulatory element binding protein
tBid	truncated Bid
TCDD	2,3,4,5-tetrachlorodibenzo-p-dioxin

TEQ	TCDD equivalents
TGFβ	transforming growth factor β
TGFβR	transforming growth factor β receptor
TNF	tumour necrosis factor
TNFSFR1A	tumour necrosis factor receptor superfamily, member 1A
TRADD	TNFRSF1A-associated via death domain
TRAIL	TNF-related apoptosis inducing ligand
TXA2	thromboxane A2
TXA3	thromboxane A3
TXB3	thromboxane B3
UC	ulcerative colitis
UDP	uridine diphosphate
UGT	UDP glucoronosyl-transferase
UK	United Kingdom
USA	United States of America
WCRF	World Cancer Research Fund
Wnt	wingless-type mouse mammary tumour virus integration site family
XIAP	X-linked inhibitor of apoptosis proteins

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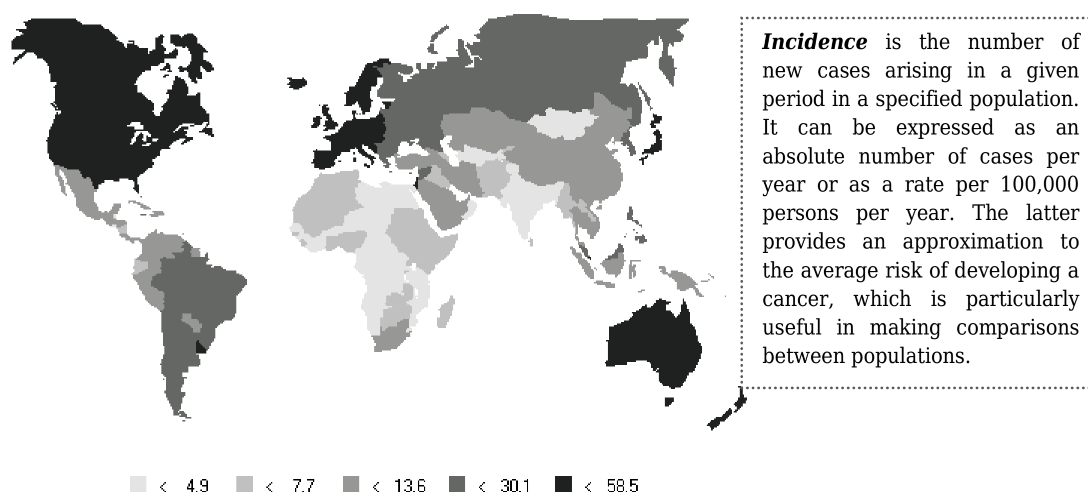
1. PREFACE

1.1 Colorectal cancer

Colorectal cancer includes both types of cancer that form in the tissue of the colon or the tissue of the rectum [National Cancer Institute 2009].

1.1.1 Epidemiology and causes of colorectal cancer

Colorectal cancer is a prevalent disease in the Western World. In Europe, there were an estimated 412,900 cases in 2006 and approximately 207,400 deaths occurred, constituting 12.2% of all cancer deaths [Ferlay *et al.* 2007]. Similar frequencies of occurrence were seen in the U.S. [Jemal *et al.* 2006]. Correspondingly, colorectal cancer is routinely listed as the second most common cause of cancer-related death in Germany, after lung cancer [Robert Koch-Institut und die Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. 2004]. The life-time colorectal cancer risk in the U.S. is around five per cent [Jemal *et al.* 2002]. Incidence rates vary widely between different geographical areas, about 20-fold in the world [Pisani *et al.* 1999], with highest rates seen in the developed countries (*e.g.* USA, Canada, Australia, Western Europe) and lowest in the developing world (Figure 1). Therefore, colorectal cancer is commonly regarded as a Western lifestyle disease.



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Only a small proportion, between 5 and 10%, of colorectal cancer cases are attributable to familial cancer syndromes whereas the majority seems to arise sporadically [Kerber *et al.* 1998]. The two most common inherited syndromes

Table 1. Hereditary colorectal cancer. The table gives a summary of the main hereditary colorectal cancer syndromes [Calva & Howe 2008; da Silva *et al.* 2009; Fearnhead *et al.* 2001; Lindor 2009; Lynch *et al.* 1998; Macrae *et al.* 2009; Sampson & Jones 2009; Sancho *et al.* 2004]**.**

Syndrome	Characteristics	Burden	Alterations
Familial Adenomatous Polyposis **FAP**	Benign neoplasms built from glandular-type elements or adenomas, numerous floric colonic adenomas (100-2500), aggressive (invasiveness & metastasis)	100% CRC at a mean age of 40 years, <1% of CRC cases	CIN, aneuploidy, mutations in *APC, k-ras, p53*
Attenuated FAP **AFAP**	Mutations in 3' and 5' ends of the APC gene and alternatively spliced exon 9	Mean age 45-56	mutations in *APC*
MutYH-associated polyposis **MAP**	Autosomal recessive, higher APC mutation predisposition, adenomatous polyposis ('mild FAP'), also extraintestinal tumours	Mean age 45-56, heterocygote mutation frequency >2%	Mutation in BER (*MutYH*), G→T transversions: *APC* loss, *k-ras* alteration (G12C)
Hereditary Non-Polyposis Colorectal Cancer **HNPCC**	Predisposes to multiple primary cancers without intestinal polyposis, proximal colon	Age of onset 40-45 years, 5-10% of CRC cases	MSI → mutations in MMR genes (*MSH2, MLH1, MSH6, PMS1, PMS2*), as well as *CTNNB1, TGFRB2, Bax, APC*
Juvenile Polyposis Syndrome **JPS**	Juveniles have 50-200 polyps mostly in the rectosigmoid region, Polyps (pedunculated and spherical with a smooth surface) contain chronic inflammatory infiltrate and mucous cysts surrounded by abundant stroma, no muscle fibre	10-50% increased risk of developing GIT polyps and cancer	Mutations in Smad4 and BMPR1A
Peutz-Jegher-Syndrome **PJS**	Pigmentation (hand, lips, feet, buccal mucosa), benign gastrointestinal hamartomatous polyposis, front-like epithelium with cystic dilatations, hypermucinous goblet-cells, spiny smooth muscle bundles	93% increased risk	Germline mutations in *STK11* (80%)
Hyperplastic polyposis **HPP** and serrated pathway syndrome	Large, atypical and dysplastic polyps Serrated polyps	unknown	CpG island methylator phenotype, *B-raf*, *MLH1* promotor mutation
Familial colorectal cancer type X **FCCTX**	Higher adenoma/tumour sequence than HNPCC, distal colon	modest increased CRC risk	no MMR deficiency, no MSI

CRC colorectal cancer, CIN chromosomal instability, APC adenomatous polyposis coli, k-ras Kirsten rat sarcoma, p53 tumour protein 53, MutYH MutY homolog; BER base excision repair, MSI microsatellite instability, MMR mismatch repair, MSH2 mutS homolog 2, MLH1 MutL protein homolog 1, MSH6 mutS homolog 6, PMS1 postmeiotic segregation 1, PMS2 postmeiotic segregation 2, CTNNB1 catenin (cadherin-associated protein) beta 1, TGFRB2 tumour growth factor receptor B2, Bax Bcl2-associated X protein, GIT gastrointestinal, Smad4 small mothers against decapentaplegic homolog 4, BMPR1A bone morphogenetic protein receptor type 1A, STK11 serine/threonine kinase 11, CpG cytosine-guanine dinucleoside, B-raf v-raf murine sarcoma viral oncogene homolog B1

associated with an increased risk of colorectal cancer are Familial Adenomatous Polyposis (FAP) [Macrae *et al.* 2009] and Hereditary Non-Polyposis Colorectal Cancer (HNPCC), also called Lynch Syndrome [Lynch *et al.* 1998]. These syndromes follow an autosomal dominant mode of inheritance. A summary of the main features of these and further principal hereditary colorectal cancer syndromes is given in Table 1.

The sporadic type of colorectal cancer accounts for the majority of diagnosed cases in people carrying no recognised genetic predisposition. This cancer type is common in persons older than 50 years [Neugut *et al.* 1993]. Sporadic colorectal cancer probably arises as a result of dietary and environmental factors as well as ageing [Colditz *et al.* 2006; Doll & Peto 1981; World Cancer Research Fund & American Institute for Cancer Research 2007].

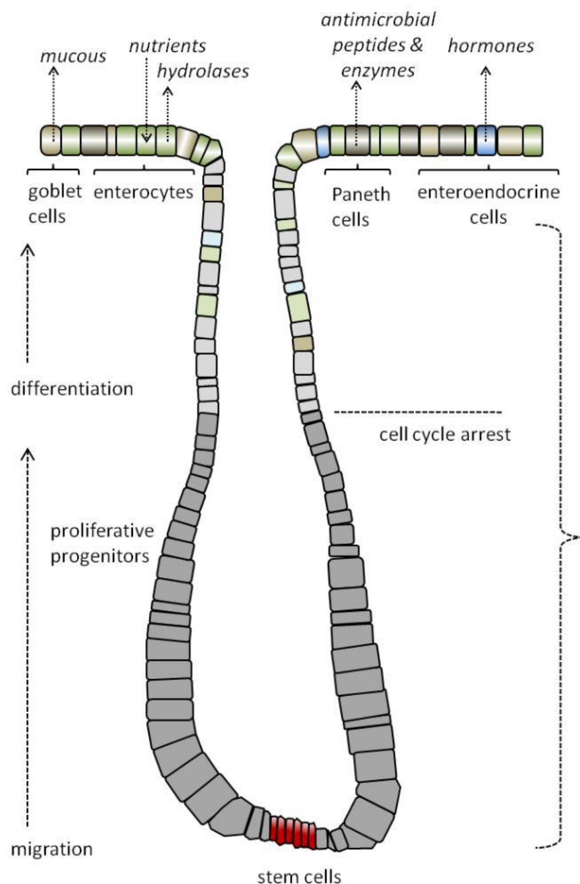
Studies on migrant populations have demonstrated that populations moving from low-risk to high-risk countries rapidly acquire an increased cancer risk, suggesting that local environmental exposures, rather than ethnic differences, influence colorectal cancer susceptibility [McMichael & Giles 1988]. Additionally, a Westernisation of dietary habits in countries formerly known for their low-risk lifestyle, *e.g.* Japan [Tamura *et al.* 1996] and China [Ji *et al.* 1998], have resulted in a rise in incidence and mortality rates [Vainio & Miller 2003].

1.1.2 Organisation of human colonic crypts

This chapter aims to summarise the morphology and cellular structure of the human colon. The intestinal tract consists of the small intestine (duodenum, jejunum, ileum) and the large intestine (caecum, colon, rectum). The length of the human colon is in the region of 1-1.5m. The principal functions are recovery of water and nutrients from the faeces and propulsion of increasingly solid faeces before defaecation in addition to serving as a barrier against luminal pathogens. The colon is inhabited by a variety of commensal bacteria which further degrade food residues [Marchesi & Shanahan 2007].

The colonic mucosa is characterised by the presence of thousands of small invaginations of the surface epithelium, termed crypts. The crypts themselves are dynamic structures which are constantly self-renewing. Intestinal crypts are clonal populations, ultimately derived from a single cell during development, and they house tissue-specific stem cells in the niche at the base of the crypt (Figure 2). In contrast to the surface enlarging structures of the small intestine, which are called villi, the colonic mucosa provides a flat surface epithelium [Sancho *et al.* 2004]. Stem cells at the bottom of the crypt divide about once per day and allow the crypt to be replaced approximately every two days [Potten 1998].

Stem cells were defined as relatively undifferentiated, proliferative cells that maintain their number, while at the same time producing a range of undifferentiated progeny that may continue to divide [Potten & Loeffler 1990].
How colon cancer arises and which molecular defects play a role is the topic of the next chapter.



epithelium
There are four colonic epithelial cell lineages into which progenitor cells mainly mature: mucus-secreting goblet-cells, absorptive enterocytes (colonocytes), enteroendocrine cells (peptide-hormone secreting, less abundant), and Paneth cells (in the ascending colon and at certain disease states).

crypts
The columnar epithelium's folding of the luminar surface forms thousands of crypts. Crypts are supported by the lamina propria and the *muscularis mucosae* (smooth muscle cells). Underneath this, the submucosa containing neural plexus (*Meissner's plexus*) that innervates the epithelium is situated. Beneath lies the *muscularis externa* that is formed by two bands of smooth muscles. An outer serous coat invests the colon.

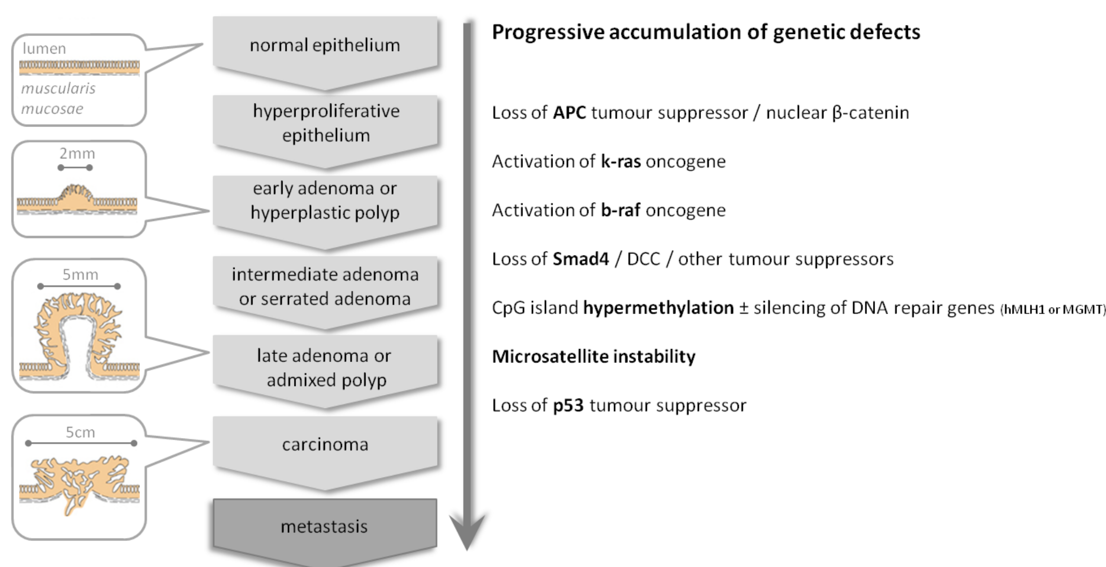
stem cells
The lower cryptal region harbours the multipotent stem cells (3-6 per crypt). They are relatively undifferentiated cells, capable of proliferation and the self-maintained balance of the offspring enabling differentiation into various functionally competent cell types.

Figure 2. Scheme of a healthy human colon crypt [Sancho *et al.* 2004; Willis *et al.* 2008]. The figure shows a profile characterising main epithelial cell types and their basic functions. On the right hand a short outline on colon crypts and their features is given.

1.1.3 Molecular changes during colon carcinogenesis

The evolution of colorectal cancer is a multistep process whereby progression stages range from normal epithelium to aberrant crypt foci, to the development of benign adenomatous polyps, and finally to invasive cancer and metastasis (see Figure 3). This progression is believed to occur over several decades. The colorectal cancer progression sequence is associated with the accumulation of a series of genetic alterations. These genetic alterations and epigenetic changes provide a growth advantage and lead to clonal expansion of altered cells.
The original model by Fearon and Vogelstein [Fearon & Vogelstein 1990] described key genetic alterations. Since then, the number of acquired genetic mutations in colorectal cancer that have been identified has grown and new pathways have been elucidated (see Figure 3). The advancement of this model

can be summarised in the features that (a) colorectal tumours occur as a result of the mutational activation of oncogenes coupled with the inactivation of tumour suppressor genes; (b) mutations in several genes are required for malignant tumours to develop; (c) genetic alterations occur in preferred sequences. It is estimated that at least four distinct genetic changes need to occur to ensure colorectal carcinogenesis. Three tumour suppressor genes (Adenomatous Polyposis Coli, APC; small mothers against decapentaplegic homolog 4, Smad4; and tumour protein 53, p53) and one oncogene (Kirsten Rat Sarcoma, k-ras) are the main targets of these sequential changes [Fodde *et al.* 2001].



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DCC deleted in colorectal carcinoma, CpG cytosine-guanine dinucleotide, MGMT O-6-methylguanine-DNA methyltransferase

Mutations in *APC* are the earliest genetic alterations and seem to be a prerequisite for colorectal carcinogenesis. APC mutation/inactivation is found in 80% of sporadic colorectal cancer cases [Strimpakos *et al.* 2009]. In the case of colorectal cancer, each allele of the gene is mutated, lost (“loss of heterocygocity”), or silenced (by hypermethylation of the gene promotor, see below). APC is an important molecule in the wingless-type MMTV integration site family (Wnt) pathway, which is the primary driving force behind proliferation of epithelial cells in the intestinal crypts [Reya & Clevers 2005]. The central player in the canonical Wnt pathway is β-catenin. In the absence of a Wnt signal, β-catenin is targeted for proteasomal degradation through sequential phosphorylations occurring at its N-terminus. A degradation complex, consisting of the tumour suppressor protein APC, the constitutively active kinases glycogen synthase kinase 3β (GKS3β) and casein kinase I (CKI), regulates β-catenin

phosphorylation in the cell. When Wnt ligands signal through their frizzled and low-density lipoprotein receptor-related protein (LRP) receptors, the destruction complex is inactivated. APC mutations lead to a dissociation of the APC-β-catenin complex, thus resulting in an excess of β-catenin in the nucleus and hence overactive Wnt signalling triggering uncontrolled cell growth [Peifer & Polakis 2000].

Mutations in the oncogene k-ras are found in 50% of sporadic tumours and act synergistically together with mutated APC allowing clonal tumour expansion. However, 50% of the tumours do not comprise mutated *k-ras* indicating other unknown oncogenes are involved. The k-ras protein is involved in the mitogen-activated protein kinase (MAPK) cascade-signalling pathway. Oncogenic mutation in k-ras results in constitutive activation of the MAPK-signalling pathway independent from epidermal growth factor-receptor (EGFR) activation by binding of its ligand. The MAPK pathway is significant in growth-promoting signal transduction from the cell surface receptors to the nucleus. The proto-oncogenes belong to the ras family (h-ras, k-ras, and n-ras) and are located in the inner plasma membrane, binding guanosine diphosphate (GDP) and guanosine triphosphate (GTP) and possess an intrinsic GTPase activity. The k-ras protein is active and transmits signals by binding to GTP and it is inactivated by GTPase driven conversion of GTP to GDP. Most k-ras mutations result in depressed GTPase activity and thus k-ras remains active and this favours cell proliferation and inhibits apoptosis [Ellis & Clark 2000]. Mutations of k-ras are rather a late event in colorectal carcinogenesis, since it is found in only 10% of early adenomas but in 40-50% of carcinomas [Kruzelock & Short 2007; Vogelstein *et al.* 1988].

Loss of Smad4 function is likely to drive malignant progression [Miyaki & Kuroki 2003], an event occurring late in colorectal carcinogenesis [Maitra *et al.* 2000]. Smad4 is also suggested to be causative for the Juvenile Polyposis Syndrome, where often Smad4 loss of heterocygocity accompanies Smad4 germline mutation. Smad4 acts as an intracellular effector of the transforming growth factor receptor β (TGF-β) superfamily of secreted polypeptides. TGF-β signals are transduced by two kinds of receptors (TGF-β receptor I and II, TGFβRI and II) each of them harbouring serine/threonine kinase activity. After TGF-β binding to TGFβRII this receptor phosphorylates TGFβRI and in turn Smads (Smad2 and 3) form complexes with Smad4 in the cytosol after dissociation from the receptor. Regulation of gene transcription by Smad complexes is transmitted by their translocation to the nucleus. Here, they are able to bind to ligand-responsive

promotor sequences. TGF-β-signalling represses epithelial cell growth in normal cells and thus acts in a tumour suppressive manner [Pardali & Moustakas 2007].
Two forms of genetic instability have been described in colorectal cancers which contribute to tumour initiation and progression: chromosomal instability and microsatellite instability. The latter is caused by defects in the DNA mismatch repair machinery resulting in a mutator phenotype at the nucleotide level and consequently in instability of repetitive, non-encoding sequences such as microsatellites. It is found in 15% of sporadic colorectal cancer cases, however, it is the main genetic abnormality (>95%) of HNPCC [Grady & Carethers 2008]. Chromosomal instability causes defects in chromosome segregation and is associated with gross chromosomal abnormalities, such as gene deletions/insertions, activation of proto-oncogenes, inactivation of tumour suppressor genes, as well of aneuploidy or polyploidy of chromosomes [Lengauer *et al.* 1997].
Methylation of cytosine is the only known physiological modification of DNA in normal cells and is regarded as an important mechanism of epigenetic regulation [Jones & Takai 2001]. Abnormal hypermethylation of DNA promotor sequences occurs frequently in colorectal cancer, leading to inhibition of transcription factor binding and hence to the silencing of tumour suppressor genes and DNA repair genes [Goel *et al.* 2007]. If gene promotor cytosine-guanine dinucleotide (CpG) island hypermethylation occurs this leads to the binding of methylated CpG binding proteins and transcription repressors such as histone deacetylases and hence to a block of transcription initiation [Jones & Takai 2001]. Normally, CpG methylation appears throughout the genome but during carcinogenesis it affects mainly unmethylated gene promotor CpG islands resulting in silencing of normally expressed genes [Rashid & Issa 2004].
Furthermore, a contribution to carcinogenesis and cancer cell immortality is associated with telomerase activity and telomere length [Saleh *et al.* 2008; Valls *et al.* 2009]. Telomeres are structures at the end of chromosomes of eukaryotic cells. They shorten progressively in somatic cells during each cell division. This mechanism is important for cellular ageing. Telomerase is an enzyme mainly found in germ cells, which ensures the further transmission of full-length telomeres. An increased expression of telomerase is suggested in the frame of the adenoma-carcinoma sequence of colorectal cancer [Valls *et al.* 2009].
The progression of colorectal cancer can be understood in terms of the Darwinian evolution theory. Cells are thus altered to comprise a growth advantage compared to unaltered cells leading to their clonal expansion. Whereas Fearon and Vogelstein [Fearon & Vogelstein 1990] provided a linear

model representing the main road to colorectal cancer, now a picture emerges in which alternative pathways to that described above may exist though data suggest a rather scarce linear occurrence of mutations in the involved genes [Jass *et al.* 2002; Jass 2007; Smith *et al.* 2002].

1.2 Dietary factors modulating colorectal cancer risk

The contribution of diet to cancer risk has been considered to be about 30% in the developed world, making it second to tobacco as a preventable cause for cancer [Key *et al.* 2004]. However, in the case of colorectal cancer the contribution may be considerably higher [Doll & Peto 1981].

1.2.1 General considerations and recommendations

Western style diets are characterised by high intakes of energy, fat, meat, refined grains, and sugar combined with low intake of calcium, fibre, fruits, and vegetables. The implication of an involvement of nutrition on the development of colorectal cancer is not only plausible but was also recently reviewed by the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR)-Panel resulting in the judgement that “food and nutrition have a highly important role in the prevention and causation of cancer of the colon and rectum” [World Cancer Research Fund & American Institute for Cancer Research 2007]. More precisely, there is convincing evidence, that physical activity decreases the risk for colorectal cancer and red and processed meat as well as body fatness/abdominal fatness and greater adult attained height increase the risk. Foods containing dietary fibre, garlic, milk, and calcium probably protect from this type of cancer. Furthermore, there is limited evidence favouring that non-starchy vegetables, fruits, foods containing folate, vitamin D, or selenium protect against colorectal cancer. Also, support that foods containing iron, cheese, foods containing animal fats or sugars are causes of this cancer is limited (for in detail review see [World Cancer Research Fund & American Institute for Cancer Research 2007]).

In addition to these dietary factors which influence the colorectal cancer risk, lifestyle factors such as cigarette smoking and excessive alcohol consumption [Hermann *et al.* 2009; Huxley *et al.* 2009] or certain types of dietary mutagens such as aflatoxin B1, aristolochic acid and benzo(*a*)pyren are known to increase the risk for cancer [Ferguson 2009].

Fat, *n*-3 polyunsaturated fatty acids, and colorectal cancer

The question arises as to whether a general overconsumption or a certain type of fat is aetiologically important for colon cancer. Fat is the most energy-dense constituent of the diet. In general, dietary recommendations are given for a reduction of fat intake from the approximately 40% of the calories typically found in Western countries to 30% [Food and Agriculture Organization of the United Nations and the World Health Organization 1997]. Based on epidemiological data showing a strong inverse correlation of colon cancer rates and the *per capita* consumption of animal fat and meat, the hypothesis was developed that dietary fat increases excretion of bile acids which can be converted to carcinogens or tumour promoters [Nagengast *et al.* 1995]. Some studies have shown an association between the dietary intake of fat and colorectal carcinomas [Kimura *et al.* 2007; Willett *et al.* 1990] but in a recent meta-analysis no association was found between the highest animal fat intake category and risk of colorectal cancer [Alexander *et al.* 2009].

However, it is still under debate whether specific type of fatty acids might even exert protective effects against certain diseases associated with the Western lifestyle. Especially monounsaturated fatty acids (MUFA), conjugated linoleic acids, and fish-derived *n*-3 fatty acids are discussed in the context of cancer chemoprevention. The WCRF/AICR-Panel quoted, that there is “limited evidence that […] eating fish protects against colorectal cancer” [World Cancer Research Fund & American Institute for Cancer Research 2007].

In a recent meta-analysis by Geelen et al. [Geelen *et al.* 2007] the authors found a borderline significant 12% lower risk in the group with the highest fish consumption compared to the lowest fish consumption. This effect was even more pronounced when the differences between the consumption categories were more than seven-fold. In addition to the epidemiological proof it was also shown that serum eicosapentaenoic acid (EPA, C20:5*n*-3) and docosahexaenoic acid (DHA, C24:6*n*-3) levels are inversely associated with colorectal adenoma risk [Pot *et al.* 2008]. So, of all fatty acids *n*-3 PUFAs seem likely to be involved in protection of cancer.

1.2.1.1 Digestion and bioavailability of fat

In the human diet, around 95% of dietary lipids are triacylglycerols, mainly composed of long chain fatty acids (carbon chain length >16), the remaining being sterols and phospholipids. For metabolic use, triacylglycerols need to be hydrolysed prior to uptake into the cells lining the gastrointestinal tract. Gastric lipase partially digests triacylglycerols to form diacylglycerols and free fatty

acids in the stomach followed by the action of pancreatic lipase in the small intestine which leads to the release of 2-monoacylglycerol and long chain fatty acids [Mattson & Volpenhein 1964].

Long chain fatty acids are only poorly soluble in aqueous solutions and exhibit detergent properties. To overcome these limitations, long chain fatty acids are successively dispersed into mixed micelles within the intestinal lumen, bound to soluble lipid binding proteins in intestinal absorptive cells and, after re-esterification, are secreted into the lymph as triacylglycerol-rich lipoproteins [Black 2007].

Long chain fatty acids are hydrophobic molecules but digestion and absorption must be highly efficient to ensure correct supply to the body as they are key sources of energy and act as substrates for a range of lipid signalling molecules such as steroid hormones and prostaglandins [Russo 2009]. However, the complex mechanisms of absorption into the body remain poorly understood. It was thought for a long time that uptake of long chain fatty acids into the cells only takes place by diffusion by reason of their physicochemical properties. The requirement of both a spontaneous and a facilitated transfer seems to be likely and so the contribution of a protein-mediated transfer was suggested during the last decades [Mu & Hoy 2004]. Once absorbed into the small intestinal enterocyte, the long chain fatty acids are reversibly bound to fatty acid-binding proteins (FABPs) [Chmurzynska 2006] and acyl-CoA binding proteins (ACBP) [Kragelund *et al.* 1999] facilitating their intracellular transfer [Knudsen 1990].

1.2.1.2 Nomenclature, and dietary sources of polyunsaturated fatty acids

Fatty acids can be distinguished by their chain length and degree of unsaturation. The fatty acid with the shortest chain is acetate (C2:0), fatty acids with one double bond are called MUFA (*e.g.* oleic acid (C18:1*n*-9) from virgin olive oil). Fatty acids with two or more methylene-interrupted double bonds in their carbon chain are termed *n*-6 and *n*-3 polyunsaturated fatty acids (PUFAs) depending on the position of the first double bond being at the sixth or third carbon atom from the methyl end of the fatty acid molecule, respectively.

Mammalian cells do not contain enzymes capable of adding double bonds (desaturases) after the ninth carbon atom from the carboxyl end of the molecule. Furthermore, fatty acids of the *n*-6 and *n*-3 families are not metabolically interconvertible [Arterburn *et al.* 2006]. As such, *n*-3 and *n*-6 fatty acids cannot be synthesised and must be provided in the diet. The two fatty acids linoleic acid (LA, C18:2*n*-6) and α-linolenic acid (ALA, C18:3*n*-3) are the major parent

compounds of the family of *n*-6 and *n*-3 fatty acids and hence are considered to be essential. The main *n*-6 fatty acids are LA and arachidonic acid (ARA, C20:4*n*-6) with the former being a mayor fatty acid in both the Western countries and those parts of the developing world with substantial consumptions of seed oils. The intake of LA increased dramatically due to the use of soybean oil, as well as sunflower and safflower. In contrast, the intake of *n*-3 fatty acid remained relatively constant during the past decades, though it has risen where canola oil has been introduced to the diet in the last two decades [Russo 2009].
The predominant sources of *n*-3 fatty acids are fish, and vegetable oils being the main sources of ALA. Fish is the major source of EPA and DHA [Benatti *et al.* 2004]. Noteworthy, the PUFA content in fish varies *e.g.* by feed, season, and species and especially oil-rich coldwater fish are known to provide a good supply of EPA and DHA *e.g.* high contents are found in mackerel (2.5g EPA+DHA / 100g fish), herring (1.7g EPA +DHA / 100g fish), or salmon (1.2g EPA +DHA / 100g fish) whereas lean fish as cod contains as little as 0.3g EPA +DHA / 100g fish [Holub 2002; Schmidt *et al.* 2001].

1.2.1.3 Metabolism of polyunsaturated fatty acids

Studies on fatty acid supplementation and conversion have led to the current belief, that intake of ALA is effective in increasing plasma EPA and docosapentaenoic acid (DPA, C22:5*n*-3) content but has only little effect on the DHA content [Brenna *et al.* 2009]. In other words, the extent of this conversion of ALA to DHA in human appears to be minimal. Utilising stable isotopes, the conversion of ALA to DHA in omnivores was estimated to be below one per cent. Similarly, when evaluating the changes in plasma phospholipids' DHA levels following a supplementation of ALA, it does not increase plasma or erythrocyte DHA phospholipid levels [Whelan & Rust 2006].
Humans have a functional desaturation-chain elongation pathway (Figure 4) predominantly in the liver but also the brain that can convert LA or ALA to the respective long chain PUFA [Barcelo-Coblijn & Murphy 2009]. Thus, EPA and DHA should strictly not be considered as essential fatty acids. The relative health effects of various *n*-3 PUFA do not suffer as a result of any lack of existence of desaturation-chain elongation activity but rather on its poor capacity [Brenna *et al.* 2009]. Two research approaches where used to prove the conversion of the C18 PUFA to their C20 and C22 products: tracer studies and dietary supplementation studies. It is broadly agreed that the conversion of ALA to EPA is low (five per cent) and to DHA even lower, not greater than ~0.5% [Arterburn

et al. 2006; Burdge *et al.* 2002; Burdge & Wootton 2002]. In fact, up to 85% of dietary ALA appears to be oxidised for energy [Barcelo-Coblijn & Murphy 2009].

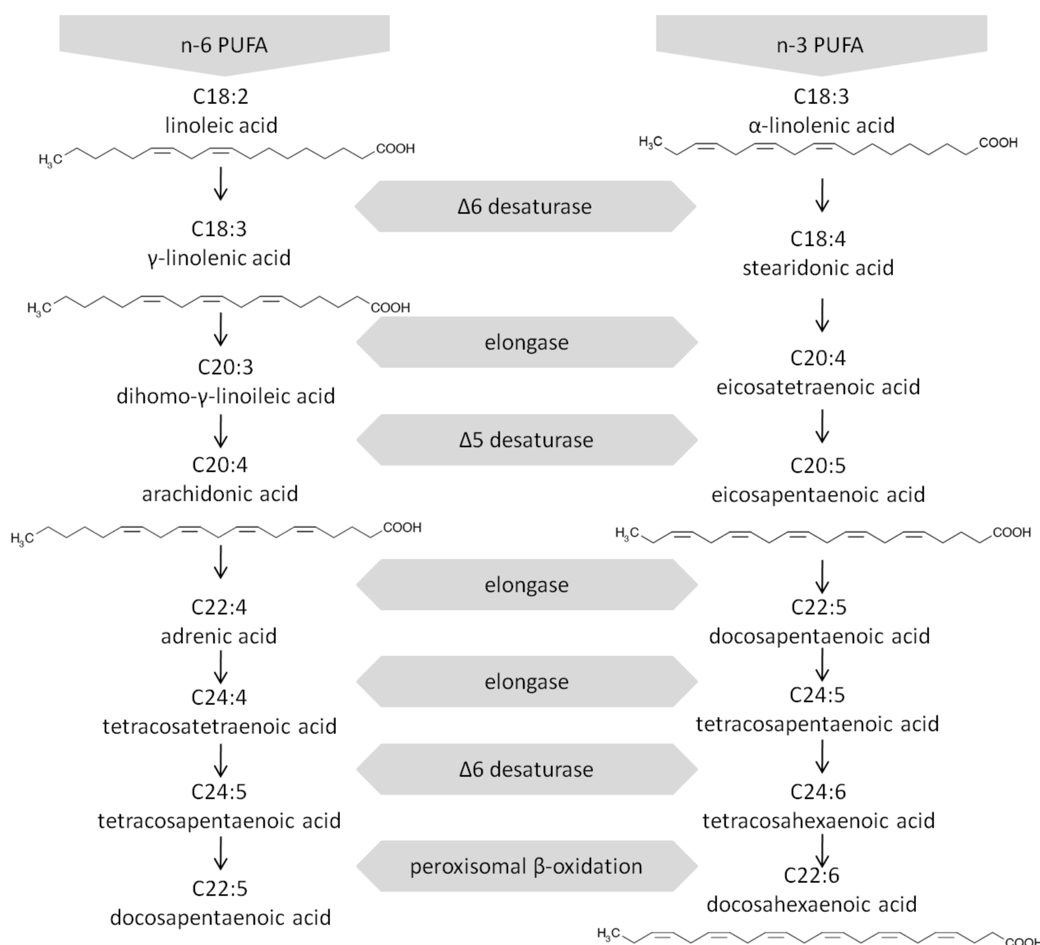

Figure 4. Conversion of long chain *n*-6 and *n*-3 PUFA to their respective very long chain products [Arterburn *et al.* 2006; Nakamura & Nara 2004].
The action of elongase allows the insertion of 2 carbon atoms to the fatty acid backbone and desaturases insert double bonds to the molecule. These steps occur in the endoplasmic reticulum of primarily the human liver, except the last step, the peroxisomal β-oxidation. Additionally, the structures of the fatty acids used within this work are given.

In addition, supplementation with EPA-ethyl esters results in an increase of both EPA and DPA but no changes for DHA in blood and tissues [Mehta *et al.* 2008]. Additionally, one other possible fatty acid has been considered as precursor for long chain *n*-3 fatty acids in humans, namely stearidonic acid (C18:4*n*-3). Consistent with the previous studies, supplementation resulted also in altered EPA and DPA but unchanged DHA levels [Harris *et al.* 2008; James *et al.* 2003]. To note, the body's DHA pool is greater than that of EPA [Arterburn *et al.* 2006], therefore it seems possible that it takes longer until a small contribution of precursor fatty acids' conversion to the plasma DHA pool might be detected.

The only way to enrich tissue phospholipids with DHA is apparently to consume the same. It was shown, that in volunteers given DHA supplements, this fatty acid was rapidly incorporated into their blood stream. Plasma EPA and DHA

phospholipids increased in a dose-dependent, but for DHA in a saturable manner in response to dietary fatty acids [Arterburn *et al.* 2006; Vidgren *et al.* 1997]. Recently, the need to establish dietary reference intakes for individual long chain fatty acids was claimed [Harris *et al.* 2009; Kris-Etherton *et al.* 2009] rather than recommendations on the total intake of *e.g.* EPA+DHA available from numerous health authorities. A summary of a subset of recommendations for fish and EPA+DHA intake from health officials is given in Table 2. Some of them recommend foods (*i.e.* oil-rich fish) whereas others deal with nutrients. However, the resulting EPA+DHA values typically suggested are between 200 and 600mg/d.

Table 2. Recommendations for fish and/or EPA+DHA intakes for healthy adults from governmental and health organisations worldwide (modified according to [Harris *et al.* 2009]).

recommendation	Country	organisation	year
Fish twice/week, one serving of which should be oily, minimum intake 450mg/d	UK	UK scientific advisory committee on nutrition[1]	2004
442mg/d for men, 318 mg/d for women	Australia & New Zealand	Australia Department for Health and Aging[2]	2005
2 servings /week of (preferably oily) fish	USA	American Heart Association[3]	2006
Fish twice/week, one serving of which should be oily to achieve DRI of 450mg/d	The Netherlands	Health Council of The Netherlands[4]	2006
Minimum of 0.3% energy for adults (~667 mg/d)	Belgium	Superior Health Council of Belgium[5]	2006
2 servings/week, both servings oily fish or 500mg/d	USA, Canada	American Dietetic Association/ Dieticians of Canada[6]	2007

[1] [United Kingdom Scientific Advisory Committee on Nutrition (SACN) 2004], [2] [Australian Department of Health and Ageing 2005], [3] [Lichtenstein *et al.* 2006], [4] [Health Council of the Netherlands 2006], [5] [Superior Health Council Belgium 2004] ; [6] [Kris-Etherton *et al.* 2007]; DRI dietary reference intake

1.2.1.4 Eicosanoid synthesis

The eicosanoids are very crucial metabolites of PUFAs. The term eicosanoids, from Greek “eicosa” standing for “twenty”, summarises biologically active signalling molecules which are oxygenated derivatives from three different kinds of PUFAs, namely EPA, ARA and dihomo-γ-linoleic acid (DGLA, C20:3*n*-6), being all of 20 carbon atoms length. In its true sense, the term is used for leukotrienes and the different prostanoides (prostaglandins, prostacyclines, and thromboxanes) but other well known metabolites such as lipoxines, hipoxilins, and epoxy-derivatives are widely included [Hyde & Missailidis 2009].

The signalling pathways of ARA are governed by three classes of enzymes, cyclooxygenases (COX), lipoxygenases, and cytochrome P450 (Figure 5). The

enzymatic products originating from different biological activities in terms of inserting oxygen at different positions of ARA have the advantage of exerting various molecular properties [Cabral 2005].

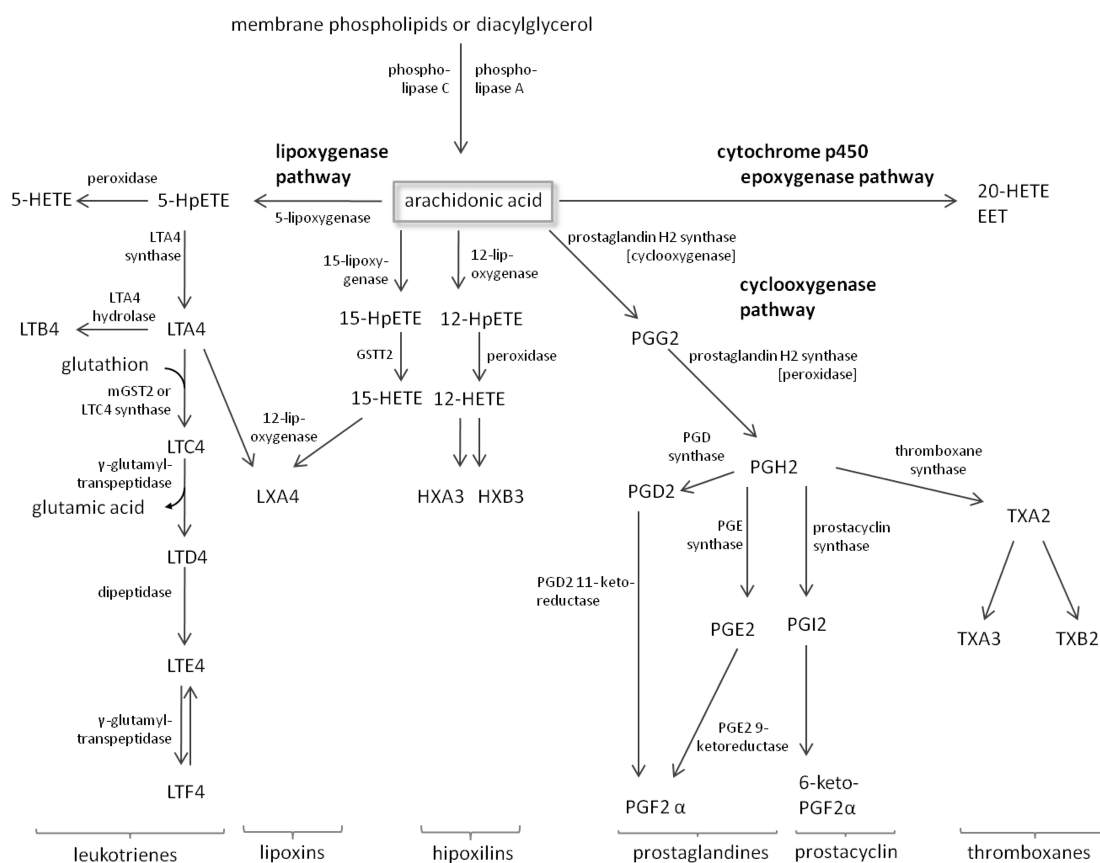

Figure 5. Arachidonic acid metabolism, summarised from [Hyde & Missailidis 2009] with additional remarks [Brash 1999; Fu *et al.* 1988; Hiratsuka *et al.* 1997; Jakobsson *et al.* 1997; Kuhn & Thiele 1999; Mandal *et al.* 2008; Soberman & Christmas 2003].

The first step in the cyclooxygenase metabolic pathway is oxygenation of ARA by its cyclooxygenase activity to form prostaglandin G2 followed by a rapid conversion due to its peroxidase activity to prostaglandin H2. Prostaglandin H2 itself is an unstable intermediate and serves as precursor for a variety of prostaglandins, prostacyclins and thromboxanes. Prostaglandins are exported from their cells of origin through the mediation of putative transporters and can exert effects in both an autocrine and paracrine fashion by signalling through specific G protein-coupled receptors.

Four different types of lipoxygenases have been identified in human cells, 5-, 12-, and 15-lipoxygenases-1 and 2. Generally, they catalyse the dioxygenation of ARA into hydroxyperoxyeicosatetraenoic acid finally resulting in the formation of leukotriens, lipoxines and hipoxilins.

The cytochrome P450 metabolic pathway is the least well described for the ARA metabolism. Several isoforms catalyse the NADPH-dependent conversion of ARA which causes a rise to reactive oxygen species called hydroxyperoxyeicosatetraenoic acids although the epoxy- and hydroxyl-derivatives formed are the major products.

HETE hydroxyeicosatetraenoic acid, HpETE hydroxyperoxyeicosatetraenoic acid, EET epoxyeicosatetraenic acid, LTB4 leukotriene B4, LTA4 leukotriene A4, mGST2 microsomal glutathione *S*-transferase 2, LTC4 leukotriene C4, LTD4 leukotriene D4, LTE4 leukotriene E4, LTF4 leukotriene F4, LXA4 lipoxin A4, HXA4 hipoxilin A4, HXB4 hipoxilin B4, PGG2 prostaglandin G2, PGH2 prostaglandin H2, PGD2 prostaglandin D2, PGE2 prostaglandin E2, PGF2α prostaglandin F2α, PGI2 prostacycline, TXA2 thromboxane A2, TXA3 thromboxane A3, TXB3 thromboxane B3

Eicosanoids are involved in the modulation of intensity and duration of inflammatory responses, have cell- and stimulus-specific sources, and frequently exert opposing effects [Cabral 2005]. The key link between PUFAs and inflammation is that eicosanoids are generated from C20 PUFAs. The incorporation of EPA into human inflammatory cells occurs in a dose-response fashion and is partly at the expense of ARA. EPA can also act as a substrate for lipoxygenases and COX, resulting in a production of eicosanoids with a slightly different structure and functional less potency than those arising from ARA [Calder 2008]. Additionally, EPA was found to act as a substrate for the generation of alternative eicosanoids, termed resolvins. E-series resolvins formed by the action of COX-2 appear to exert anti-inflammatory actions [Serhan *et al.* 2008]. Furthermore, DHA was also described as a substrate for COX-2 yielding in D-series of resolvins and docosatrienes, with apparent anti-inflammatory properties and cellular protective actions [Serhan 2005]. Thus, this has led to the idea that fish oil or fish fatty acids as EPA and DHA may act anti-inflammatory.

1.3 Molecular mechanisms of colon cancer chemoprevention

Reduced mortality and morbidity is associated with an early detection of invasive lesions and precursor adenomatous polyps in the colon. However, most colorectal cancers are diagnosed at an advanced stage. Therefore, a lot of attention has focused on screening for targets to prevent initiation and progression of colorectal cancer in order to reduce the number of patients.

This kind of prevention is termed chemoprevention and is defined as the use of pharmacological agents (including nutrients) to impede, arrest, or reverse carcinogenesis. Based on this idea, the efficacy of numerous nutrients and other dietary factors has been tested [Knasmüller *et al.* 2009; Martinez *et al.* 2008; Sporn 1991].

In chemoprevention, three main phases are distinguished [Wattenberg 1985]. Primary prevention describes the inhibition of initiation, the first step of tumourigenesis by reduction of toxification or induction of detoxification. This can be accomplished by preventing the formation of the ultimate carcinogen or reactive oxygen species as well as by antioxidative effects and is thus called blocking activity [Morse & Stoner 1993]. The promotion and progression of initiated cells to become preneoplastic is inhibited by secondary prevention, *e.g.* by reduction of cell growth or enhancement of differentiation and apoptosis in initiated cells. Agents that effect secondary prevention are suppressing agents. Blockage of progression of carcinogenic devolution is termed tertiary chemoprevention and includes therapeutic approaches.

1.3.1 Modification of biotransformation enzymes and antioxidative defence

Biotransformation is the process by which both endogenous and exogenous compounds are modified facilitating their excretion. Often, lipophilic compounds are converted into more hydrophilic ones. Hence, in the so called phase I the molecule is activated by adding a functional group which is then used for a conjugation step in the so called phase II.

Typical phase I reactions include oxidation, reduction, and hydrolysis, those of phase II glucuronidation, sulfatation, and conjugation with glutathione. Typical enzymes of both reactions are summarised in Table 3. Reactions of phase III represent mechanisms which facilitate the removal of metabolites from the cells. This is accomplished by multidrug resistance proteins (MRP) or ATP-binding cassette (ABC)-transporters [Jones & George 2004].

Table 3. Major phase I and phase II biotransformation enzymes.

phase I enzymes	phase II enzymes
Cytochrome p450 superfamily (CYP)	UDP-glucoronosyl-transferases superfamily (UGT)
Flavin-monooxygensases (FMO)	Glutathione *S*-transferase superfamily (GST)
Cyclooxygenases (COX)	Sulfotransferases (SULT)
estherases	*N*-acetyl-transferases (NAT)
alcohol dehydrogenases	
reductases	

1.3.1.1 Modification of glutathione *S*-transferase T2 expression

A well-studied family of phase II enzymes are the glutathione *S*-transferases (GSTs, EC 2.5.1.18). GSTs catalyse the nucleophilic attack by reduced glutathione on non-polar compounds that contain electrophilic carbon, nitrogen, or sulphur atoms. The result is the formation of (usually) less-reactive, more hydrophilic glutathione-conjugates. Substrates include a wide variety of toxic or carcinogenic electrophiles which are mostly either xenobiotics or products of oxidative stress, *e.g.* benzo(*a*)pyren, but also endogenous molecules such as prostaglandins [Hayes *et al.* 2005]. Three major families of proteins exhibit glutathione transferase activity [Pool-Zobel *et al.* 2005]. Two of them, the cytosolic and mitochondrial GSTs, comprise soluble enzymes that are only distantly related. A third family comprises microsomal GSTs and is now referred to as membrane-associated proteins in eicosanoid and glutathione (MAPEG) metabolism [Hayes *et al.* 2005].

Theta class GSTs are highly conserved and consist of two cytosolic family members, GSTT1 and T2 [Meyer *et al.* 1991]. GSTT2 expression is lower in the human colon compared to GSTT1 or GSTP1, *i.e.* the major GST of the colon

[Ebert *et al.* 2003]. Notwithstanding, GSTT2 has already been shown to be upregulated by apple polyphenols and this may be one mechanism for colon cancer chemoprevention by polyphenols [Veeriah *et al.* 2008]. Conjugation with the endogenous tripeptide glutathione allows lipophilic xenobiotic substances to become more hydrophilic and hence easier to remove. GSTT2 shows affinity to organic hydroperoxides such as DNA-hydroperoxides and lipid-hydroperoxides [Ketterer *et al.* 1988; Tan & Board 1996]. Accordingly, potentially harmful xenobiotics are excreted from the organism. Additionally to the glutathione transferase activity, GSTT2 also exerts peroxidase [Hurst *et al.* 1998] and sulfatase activity [Rossjohn *et al.* 1998].

1.3.1.2 Modification of superoxide dismutase 2 expression

Oxidative stress has been defined as an imbalance between oxidants and antioxidants in favour of the former, resulting in an overall increase in cellular levels of reactive oxygen species. This might for example be one source for DNA damage. One of the well studied oxidative DNA adducts is 8-hydroxydeoxyguanosine in which site-specific mutations result in G→T transversions [Shibutani *et al.* 1991] that are widely found in mutated oncogenes and tumour suppressor genes [Hussain & Harris 1998]. Thus, oxidised DNA bases appear to be mutagenic and capable of inducing mutations commonly found in neoplasia. Hydrogen peroxide is less reactive than either the hydroxyl radical or superoxide anion radicals and more readily diffusible and thus more likely to be involved in the formation of oxidised bases [Guyton & Kensler 1993].

Superoxide dismutases (SODs, EC 1.15.1.1) are one of the most important enzymes in the antioxidant defence against reactive oxygen species, particularly superoxide anion radicals. They catalyse the dismutation of superoxide into hydrogen peroxide and oxygen. At present, three isoforms are described in mammals. Two of them carry copper and zinc at their catalytic centre and are either localised in the intracellular compartment (CuZnSOD or SOD1) or the extracellular space (EC-SOD or SOD3). The third isoform’s cofactor is manganese and is localised in the mitochondria (MnSOD or SOD2) [Zelko *et al.* 2002]. Among all three isoforms SOD2 is shown to be crucial for the survival of aerobic organisms, *e.g.* the very short life-span of $SOD2^{-/-}$ mice. Furthermore $SOD2^{+/-}$ mice feature higher concentrations of 8-hydroxydeoxyguanosine and suffer higher incidences of cancer [Van *et al.* 2003].

Besides the effects on the cellular antioxidant system, SOD2 has been recently discussed as a new type of tumour suppressor [Oberley 2005]. An overexpression of SOD2 correlates with decreased cell growth [Kim *et al.* 2004]. Additionally,

SOD2 overexpression leads to an alteration of different signal transduction pathways in different cell types via mechanisms such as inhibition of transcription factor activator protein-1 (AP-1) and nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) and hence their effects might also affect downstream targets [Li *et al.* 1998b].

1.3.1.3 Modification of cyclooxygenase-2 expression

COX (EC 1.14.99.1), also referred to as prostaglandin endoperoxide synthase, catalyses the conversion of ARA into prostaglandins (see chapter 1.2.1.4 on page 13). COXs are bifunctional enzymes which exist as homodimers of 70kDa subunits, each of which contains one molecule of Fe^{3+}-protoporphyrin IX. They contain an epidermal growth factor binding domain, a membrane binding domain, and two enzymatic activities with distinct active sites. To date, two isoforms have been identified: COX-1 and COX-2 [Chandrasekharan *et al.* 2002; Dou *et al.* 2004; Smith *et al.* 2000]. Although they differ in their pattern of expression and tissue distribution in human cells, they are collectively responsible for the stepwise conversion of ARA to the three classes of prostanoids. COX-1 and COX-2 are encoded by two different genes on two different chromosomes. The COX-1 gene is approximately 22kb long and contains 11 exons. Typical for developmental regulated "housekeeping" genes, the COX-1 gene lacks a TATA box. Recently, different COX-1 splicing variants have been detected on mRNA and protein level. Besides the appearance of only partial COX-1 mRNAs (PCOX-1a and PCOX-1b), mainly one variant, COX-1b which was earlier also referred to as COX-3 is under debate. Its mRNA was found in canines to be of 2.6kb length and its prostaglandin E2 production activity is 20% compared to COX-1 [Chandrasekharan *et al.* 2002; Hersh *et al.* 2005]. Unlike in dogs [Chandrasekharan *et al.* 2002], the human isoform contains the 94bp intron 1 which leads to a frame shift [Dinchuk *et al.* 2003] and thus to a premature termination of the protein [Qin *et al.* 2005], hence its biological significance remains to be revealed.

COXs are integral membrane enzymes [Smith *et al.* 1996] bound to the luminal surface of the endoplasmic reticulum and to the outer and inner membrane of the nuclear envelope.

Whilst COX-1 is ubiquitous and constitutively expressed in most mammalian cells to maintain the baseline level of prostaglandins, COX-2 is absent under normal conditions. The COX-2 gene is of 8kb length and can be modulated through multiple signalling pathways, by growth factors or cytokines that enhance their

expression or inhibitors like non-steroidal anti-inflammatory drugs (NSAIDs) which are already used therapeutically [Cuzick *et al.* 2009].

The overexpression of COX-2 has been well documented in a variety of diseases such as inflammation and cancer, of *e.g.* the breast [Visscher *et al.* 2008], the prostate [Hussain *et al.* 2003], head and neck [Gallo *et al.* 2002] and the colon [Kutchera *et al.* 1996]. In particular increased COX-2 expression is connected with tumour metastasis in colon cancer, where aberrant COX-2 expression was shown to correlate with carcinogenesis in 80% of cancers [Eberhart *et al.* 1994]. COX-2 is upregulated in most human cancers and PGE2 is produced in large amounts in colorectal tumours [Hendrickse *et al.* 1994]. A nested case-control study found that long-term NSAID/COX-2 inhibitor usage was associated with a significantly decreased risk for developing colorectal cancer [Vinogradova *et al.* 2007].

1.3.2 Induction of apoptosis

The term apoptosis describes the morphological process of controlled cellular self-destruction and was first introduced by Kerr [Kerr *et al.* 1972]. It is a physiological phenomenon, in contrast to the pathological burden summarised under the term necrosis.

Apoptosis signalling is triggered by two major routes, the intrinsic and the extrinsic pathway [Hector & Prehn 2009a]. Caspases, a family of cystein proteases, are key molecules within both pathways. They are synthesised as inactive zymogens and after proteolytical cleavage at specific aspartic acid residues, they form heterotetrameric active caspases (each of two small and two large subunits) [Chowdhury *et al.* 2008]. Caspase activation is an important regulatory step of apoptosis signalling and can be achieved by two major mechanisms: 1) cleavage in trans by other caspase molecules (*i.e.* caspase cascade), and 2) by binding of adaptor molecules to the prodomain which is thought to facilitate intramolecular cleavage [Thress *et al.* 1999].

In the extrinsic pathway, caspase activation is a result of the activation of death receptors (such as TNF receptor superfamily, *e.g.* Fas, death receptors 4 and 5, DR4 and DR5) on the cell membrane by their corresponding ligands (such as FasL or TNF-related apoptosis inducing ligand, TRAIL, respectively). This binding is followed by a receptor trimerisation and the recruitment of cytosolic adaptor molecules and procaspase 8 forming the death-inducing signal complex (DISC). Subsequently, procaspase 8 is activated by cleavage and hence activation of downstream caspases is triggered. The intrinsic or mitochondrial pathway is characterised by the B-cell chronic lymphocytic leukemia/lymphoma 2

(Bcl-2) family of protein-driven release of cytochrome c from the mitochondria leading to the activation of caspases. The link between the extrinsic pathway and the mitochondria is given by the Bcl-2 family member BH3 interacting domain death agonist (Bid) [Li *et al.* 1998a; Luo *et al.* 1998]. Bid is cleaved by caspase 8 to its truncated form (tBid) which translocates to the mitochondria acting together with Bcl-2-associated X protein (Bax) and Bcl-2-antagonist/killer (Bak) to release cytochrome c to the cytosol. Cellular stress (*e.g.* DNA damage, oxidative stress, cytokine deprivation) promotes the release of cytochrome c in a caspase independent manner. In response, death-promoting members of the Bcl-2 family (*e.g.* Bax; Bcl-2-associated agonist of cell death, Bad; Bak; Bcl-2-interacting killer, Bik) will translocate to the mitochondria or undergo transformational changes with the mitochondria membrane suggested by pore-formation through which cytochrome c might escape [Korsmeyer *et al.* 2000]. This escape can be prevented by apoptosis-inhibitory members of the Bcl-2 family (*e.g.* Bcl-2 and Bcl-xL). Binding of cytochrome c with dATP and Apaf-1 (Apoptotic peptidase activating factor 1) causes a conformational change allowing Apaf-1 to complex with procaspase 9 to a structure known as apoptosome. This so-called “wheel of death” was revealed as a seven-fold symmetric molecule [Acehan *et al.* 2002]. Activation of the apoptosome will in turn autoactivate caspase 9 facilitating activation of caspase 3.

A class of cell-death inhibitors, the inhibitor of apoptosis proteins (IAPs) could also regulate apoptosis downstream of the apoptosome assembly [Deveraux & Reed 1999]. Thus X-linked inhibitor of apoptosis protein (XIAP) may inhibit apoptosome activity by inhibiting caspase 9 activation and thereby restraining the propagation of the caspase cascade. Smac/Diablo (second mitochondria-derived activator of caspases/direct IAP binding protein with low isoelectric point) was found to be a protein released from the mitochondria during apoptosis and appears to neutralise IAP function [Hector & Prehn 2009b].

No matter by which signalling pathway apoptosis is induced, the activation of effector caspases such as caspase 3 results in execution of ATP-dependent processes [Slee *et al.* 2001] characterised by PARP (poly (ADP-ribose) polymerase)-cleavage, enzymatic DNA degradation to 180bp fragments, phosphatidylserine presentation at the outer membrane layer, cellular shrinkage and formation of apoptotic bodies, and finally in phagocytosis of the residual cell remnants [Hengartner 2000]. These processes are summarised in Figure 6.

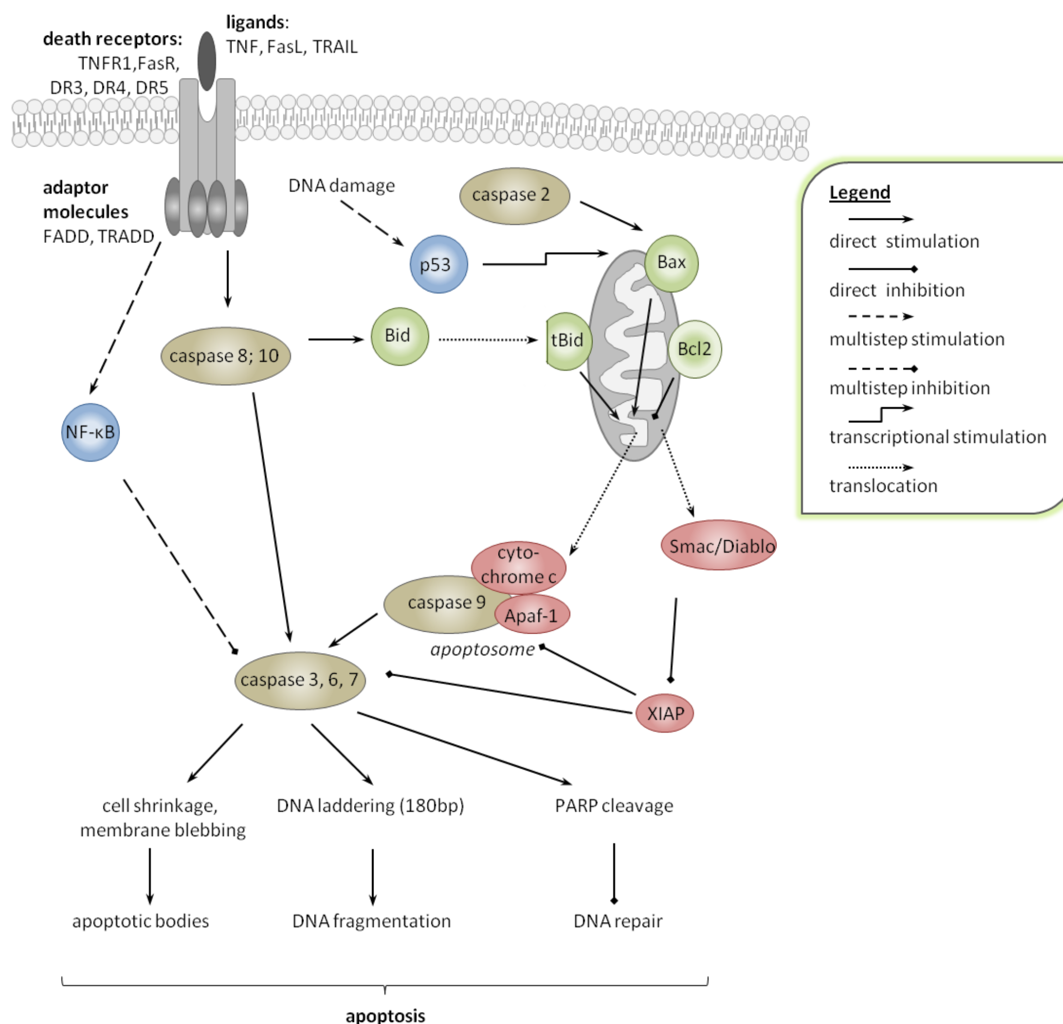

Figure 6. Summary of some major extrinsic and intrinsic apoptotic signal transduction pathways causing the main apoptotic features. The extrinsic apoptotic pathway is a result of receptor activation followed by the caspase cascade. One important step of the intrinsic pathway is the activation of the apoptosome.

TNF tumour necrosis factor, FasL Fas ligand, TRAIL TNF-related apoptosis inducing ligand, TNFR1 TNF receptor 1, FasR Fas receptor, DR3 death receptor 3, DR4 death receptor 4, DR5 death receptor 5, FADD Fas-associated protein with Death Domain, TRADD TNFRSF1A-associated via death domain, p53 tumour protein 53, bid BH3 interacting domain death agonist, tbid truncated bid, bax Bcl-2–associated X protein, bcl2, NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells, Apaf-1 apoptotic peptidase activating factor 1, Smac/Diablo second mitochondria-derived activator of caspases/direct inhibitor of apoptosis protein binding protein with low isoelectric point, XIAP X-linked inhibitor of apoptosis protein

1.4 Use of faecal water as biomarkers for colorectal carcinogenesis

The term biomarker comprises characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [Biomarkers Definitions Working Group 2001]. They can help to understand the relationship between diet and health or disease and offer a useful link between the consumption of specific foods and the biological outcome [Branca *et al.* 2001]. Biomarkers of effect include DNA damage in peripheral lymphocytes [Glei *et al.* 2005], and biomarkers of exposure *e.g.* specific metabolites (*e.g.* 8-hydroxydeoxyguanosine). An example of a susceptibility biomarker for colorectal cancer is the existence of

mutations in tumour suppressor genes (APC-mutation carriers are at higher risk [see FAP, chapter 1.1.1, Table 1 on page 2]), or polymorphisms (*e.g.* single nucleotide polymorphisms [Curtin *et al.* 2009]).

In addition, for studies investigating nutritional effects on chemoprevention a considerable interest arose in the use of the aqueous phase of faeces as a tool to examine mechanisms underlying the aetiology of colorectal cancer. Faeces are a complex mixture and the composition reflects the diet consumed. It does not only contain potential carcinogenic compounds (*e.g.* bile acids, fecapentaens, *N*-nitroso compounds, and heterocyclic amines [de Kok & van Maanen 2000]) but also potential anticarcinogenic substances (*e.g.* the short chain fatty acid butyrate, a gut flora fermentation product of dietary fibre [Scharlau *et al.* 2009]). Therefore, analysing faecal compounds is a promising tool to assess the exposure of the colonic lumen to potential risk and preventive factors. Components of the faecal aqueous fraction are thought to be in direct contact with colonic epithelial cells. Faeces can be obtained easily within human intervention studies and faecal water can be produced by ultracentrifugation [Klinder *et al.* 2007]. It is commonly used *in vitro* to monitor genotoxicity of the luminal environment using the Comet Assay, a technique which allows the screening of DNA-damaging potential of the samples on a single-cell level [Hoelzl *et al.* 2009]. Analysis of the influence of the diet on the genotoxicity of faecal water has been used in several studies to demonstrate that a Western Style-diet can lead to a DNA-damaging luminal environment which is associated with a higher risk for colorectal cancer. Additionally it was shown that a diet high in fat and meat and low in dietary fibre increased faecal water genotoxicity [Rieger *et al.* 1999]. Also, supposedly beneficial nutritional regimens, such as increased intake of high-fibre bread [Glei *et al.* 2005], pre-and probiotics [Burns & Rowland 2004], or polyphenols [Veeriah *et al.* 2008] resulted in decreased faecal water genotoxicity.

1.5 The FISHGASTRO human intervention study

The FISHGASTRO study is a multi-centre parallel randomised controlled intervention trial. The trial is part of the EU-granted project SEAFOODplus and a collaboration of three research centres. It was carried out at the Wageningen University, Wageningen, the Netherlands, and the Institute of Food Research, Norwich, United Kingdom; the Department of Nutritional Toxicology, Friedrich-Schiller-University of Jena, Jena, Germany was included for sample analysis and in study planning. Participants were recruited from out-patient colonoscopy clinic lists in eight clinical centres; six situated in the Netherlands and two in the United Kingdom. Figure 7 provides a summary of the study.

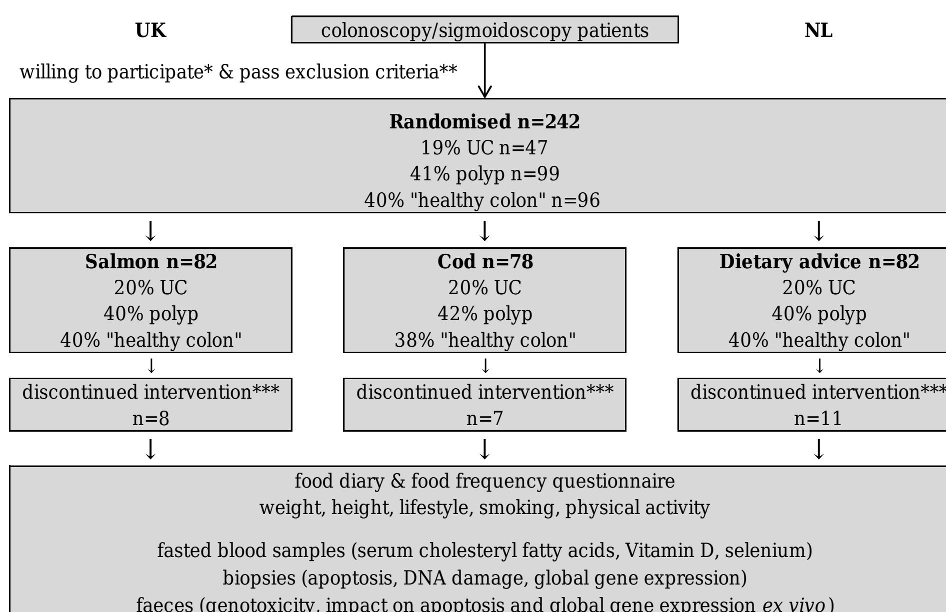

Figure 7. Chart giving an overview of volunteers and main target parameters of the FISHGASTRO study [Pot *et al.* 2009].
UC ulcerative colitis; * main refuse was second colonoscopy/sigmoidoscopy; ** allergic to fish, taking fish oil supplements, increased risk of gut bleeding, pregnant or breastfeeding, organ transplant recipients receiving immuno-suppression therapy, type I diabetics, or at an increased infection risk; *** reasons: fish-related, decline sigmoidoscopy, too busy, pregnancy, UC flare up, health problems, prostate cancer, others.

Between November 2004 and December 2007, 242 male and female volunteers aged 18-80 years were recruited for the study from patients visiting the hospital for a colonoscopy, as part of their regular medical care. They were assigned to three groups of subjects: (i) those with previous colorectal adenomata, (ii) those diagnosed with non-active ulcerative colitis, and (iii) those without any macroscopic signs of disease in the colon. Approximately ten per cent of the invited patients were willing to participate in the trial. After an initial colonoscopy procedure, eligible subjects were randomly allocated to one of three dietary intervention groups: (i) the oil-rich fish group receiving two 150g portions of farmed salmon per week during six months, (ii) the lean fish group receiving two 150g portions of Icelandic cod per week during six months, and (iii) the dietary advice group. The fish was provided to the participants and had to be consumed in addition to their regular fish consumption. All three intervention groups received general dietary advice to achieve a healthy diet.
Of the 242 randomly assigned volunteers, 216 completed the study. The additional consumption of approximately 1.4 portions of salmon/week and 1.3 portions of cod/week provided an additional 0.99g/d and 0.05g/d very long chain *n*-3 PUFA, respectively. The trial is registered at www.clinicaltrials.gov under identifier NCT00145015 and was first reported in the American Journal of Clinical Nutrition [Pot *et al.* 2009].

2. OBJECTIVES

Epidemiological studies and findings from animal work suggest the involvement of fish consumption in colorectal cancer chemoprevention. Fish oil is rich in the *n*-3 PUFAs EPA and DHA and it is likely that above all, these fatty acids contribute to the chemopreventive properties of fish. However, little is known on the exact molecular mechanisms by which fish or fish oil exerts these chemopreventive effects. Thus, the aim of this study was to elucidate the impact of EPA and DHA on certain targets of chemoprevention on colon cells *in vitro*. Furthermore, up to now no intervention study on fish consumption and impairment of colon cancer has been performed. Therefore, for the first time biomarkers of a study intervening with lean and oil-rich fish, the FISHGASTRO trial, were screened *ex vivo*. To address these questions the following work was performed:

First, the uptake of different types of fatty acids (LA, ALA, GLA, ARA, DHA and EPA) into human colon cells (adenoma cell line LT97 and adenocarcinoma cell line HT29) *in vitro* was measured by gas chromatography / flame ionisation detector. Furthermore, their impact on cell growth (DNA staining by 4',6-diamidino-2-phenylindole) and on metabolism (conversion of resazurin to resofurin) was evaluated **(publication I)**. The involvement of EPA and DHA in modulation of gene expression patterns of LT97 human colon adenoma cells was determined in a time course (10 and 24 hours) using a cDNA microarray (PIQOR™, Miltenyi Biotec) containing 306 genes involved in *e.g.* stress response, metabolism, and apoptosis (**publication II**). In addition, the modulation of the biotransformation enzymes SOD2, GSTT2 and COX-2 in LT97 and HT29 cells was determined in a time course (4, 10, 24, and 48 hours) on mRNA level (Real-Time RT-PCR) and protein level (Western Blot) **(publication III)**. The induction of apoptosis as a further mechanism to alter cell number was subject of a further experimental setup. Apoptosis was measured in a time series (10, 24, 24 hours) using a flow cytometer (cell staining with Annexin-V-fluorescein isothiocyanate (FITC) and 7-actinoaminomycin), by Real-Time RT-PCR (expression of the antiapoptotic *bcl-2*), and by Western Blot (PARP cleavage, bid cleavage, pro-caspase 3, pro-caspase 8, and pro-caspase 9) in LA-, EPA- and DHA-treated LT97 and HT29 cells **(publication IV)**. Finally, 89 faecal water samples of a human intervention study giving oil-rich and lean fish in a six-month interval were screened *ex vivo* for alteration of genotoxicity in HT29 cells (Comet Assay) and apoptosis in LT97 cells (flow cytometrical detection of Annexin-V-FITC and 7-actinoaminomycin) (**publication V and IV**, respectively). Additionally, DNA

damage in colonocytes from biopsies was determined by Comet Assay **(publication IV)**. The modulation of global gene expression by GeneChip® (Affymetrix) was determined using a biomarker approach after incubating LT97 adenoma cells with faecal water from a subset of the volunteers (n=39) of the FISHGASTRO human intervention trial **(section 4, additional results)**.

3. PUBLICATIONS

3.1 Publication I: N. Habermann, B. Christian, B. Luckas, B. L. Pool-Zobel, E. K. Lund, M. Glei: „**Effects of fatty acids on metabolism and cell growth of human colon cell lines of different transformation state.**" BioFactors (2009) 35:460-467.

Epidemiological studies suggest that high fish intake is associated with a decreased risk of colorectal cancer which has been linked to the high content of the *n*-3 PUFAs EPA and DHA in some fish. The aim of this study was to compare the uptake of PUFAs (EPA, DHA as well as ALA, GLA, LA, and ARA) into human colon cells, and the resulting growth inhibitory and metabolic effects. Comparatively, these effects were assessed in adenoma and adenocarcinoma cells (LT97 and HT29, respectively) *in vitro*.
All FAs were utilised more efficiently by the human colon adenoma cell line LT97 than by the adenocarcinoma cell line HT29. LT97 were more susceptible than HT29 cells to the growth inhibitory activities of all FAs except for DHA where both were equally sensitive. Inhibition of survival and metabolic activity by EPA and DHA increased with treatment time in both cell lines, but adenoma cells were more susceptible than the adenocarcinoma cells.

Own contribution to the manuscript:

- experimental design
- conduct of cell culture of HT29 and LT97
- practical work on measurement of cell growth and influence of fatty acids on the cells' metabolic activity
- preparation of cells for fatty acid uptake-studies
- data analysis and presentation of results
- compilation of the manuscript

BioFactors

Effects of fatty acids on metabolism and cell growth of human colon cell lines of different transformation state

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Abstract.

Epidemiological studies suggest that high fish intake is associated with a decreased risk of colorectal cancer which has been linked to the high content of the $n-3$ polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in some fish. In this study, two different cell lines are compared in relation to their response to EPA and DHA *versus* the plant derived PUFAs, linoleic acid (LA), γ-linolenic acid (GLA), and α-linolenic acid (ALA) and to the ubiquitous arachidonic acid (ARA). The uptake of 100 μM of each fatty acid (FA) was determined using GC. The 4′,6-diamidino-2-phenylindole assay for DNA quantification and the Cell-Titer-Blue™ assay were used to determine cell survival and metabolic activity at 2–72 h after treatment. All FAs were utilized more efficiently by the human colon adenoma cell line LT97 than by the adenocarcinoma cell line HT29. LT97 were more susceptible than HT29 cells to the growth inhibitory activities of all FAs except for DHA where both were equally sensitive. Inhibition of survival and metabolic activity by EPA and DHA increased with treatment time in both cell lines. ALA or GLA were less growth inhibitory than EPA or DHA and ARA had intermediary activity. The data show that the tested FAs are incorporated into colon cells. Furthermore, adenoma cells are more susceptible than the adenocarcinoma cells.

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Keywords: $n-3$ PUFA, cell growth, fatty acid uptake, EPA, DHA

1. Introduction

Colorectal cancer is one of the most common cancers in western countries. Epidemiological studies have led to the current belief that a significant proportion of colorectal cancer cases may be explained by dietary habits [1]. However, epidemiological studies provide controversial results as to whether or not a diet high in fish is inversely associated with colorectal cancer risk [2]. However, recent findings from the EPIC study in Europe [3] as well as the report of the Physicians Health Study in the US after 22 years of follow-up [4] found a high fish intake to be associated with a decreased risk for colorectal cancer, and in a current meta analysis [5], Geelen et al. argue there is sufficient epidemiological evidence to suggest fish may be protective. Beneficial effects of fish and fish oil, such as protection against cardiovascular diseases [6] and cancer [2], have been attributed to the $n-3$ polyunsaturated fatty acids (PUFA). These include eicosapentaenoic acid (EPA, $20{:}5n-3$) and docosahexaenoic acid (DHA, $22{:}6n-3$). The intake of $n-3$ PUFAs differs in the world and is in direct relation to the relative fish consumption [7–9]. Furthermore, the incidence of colorectal cancer in countries with high fish consumption is reported to be lower [10], but there is relatively little information on how fish or fish specific fatty acids (FAs) could contribute to the process of cancer prevention.

Fish oil has been shown to inhibit chemical-induced carcinogenesis in rats and also to induce apoptosis in the rat colon [11]. In addition, DHA has been shown to reduce the number of azoxymethane (AOM)-induced and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced aberrant crypt foci in rat colon [12,13]. In living cells, linoleic

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acid (LA, 18:2$n-6$) is the substrate for biosynthesis of arachidonic acid (ARA, 20:4$n-6$). α-Linolenic acid (ALA) is a shorter chain $n-3$ PUFA found in some plant oils which is variably converted to EPA and DHA, although the conversion rate in humans is reported to be low [14]. Thus, the conversion of EPA to DHA seems to be insufficient in humans [15] but probably occurs. The PUFA γ-linolenic acid (GLA) is the $n-6$ equivalent of ALA and can be found in small amounts in plant seed oils as well as in human milk [16]. It can be synthesized from LA. The optimal intake of FAs is considered as the ratio of $n-3/n-6$ FAs which is recommended to be 1:5–10 [17], but the ideal intake of fish or fish oil as a supplement is still unclear. Blood level of PUFAs are often used as biomarker in human studies. Recently, it was shown that serum $n-3$ PUFA are inversely and $n-6$ PUFA are positively associated with colorectal adenoma risk in humans [18]. Similarly, levels of fish fatty acids from adipose tissue were inversely and level of ARA and the ratio of ARA to fish oils were both positively associated with colorectal adenoma incidence [19]. Work on human colon biopsies or primary cells has not been widely reported.

The first question to be answered was whether or not cells at different stages of transformation are able to utilize FAs differently. Thus, we examined the uptake of FAs into the cells after PUFA exposure over time. Furthermore, the impact of PUFAs on parameters of cell growth was assessed (cell number and metabolic activity of remaining cells). To relate this to secondary cancer prevention, we determined for the first time biological effects in the preneoplastic human colon adenoma cells LT97 and compared the effects with parameters measured in the highly transformed colon adenocarcinoma cell line HT29. For a comprehensive assessment of the $n-3$ PUFAs EPA and DHA which are currently considered to be the major bioactive compounds in fish [20], their effects were compared with those of other PUFAs. ALA and GLA were included in this comparison, because they presumably would have an intermediate effect on account of the similarities in their chemical structures. ARA was included as its metabolites are well known bioactive compounds. Finally, the plant-derived $n-6$ FA LA, was also included in the comparison and used as negative control, because LA appears to be devoid of similar beneficial activities but is found in abundance in the diet [21].

2. Methods

2.1. Cells and culture

The human colorectal adenoma cell line LT97 represents an early stage of tumor development (kind gift from Professor Marian, Institute for Cancer Research, University of Vienna, Austria) [22,23]. It was established from colon microadenomas of a patient with hereditary familial polyposis. LT97 cells were maintained in a culture medium (MCDB 302) which contained 20% of L15 Leibovitz medium, 0.4 mM glutamine, 2% FCS (fetal calf serum), 0.2 nM triiodo-L-thyronine, 1 μg/mL hydrocortisone supplemented with 10 μg/mL insulin, 2 μg/mL transferrin, 5 nM sodium selenite and 30 ng/mL EGF (epidermal growth factor), 50 μg/mL Gentamicin at 37 °C in a humidified incubator (5% CO_2/95% humidity), as described previously [24]. The cells have a stable karyotype [24,25] are of epithelial nature [23] and have a doubling time of 72–96 h. The LT97 cells used for the experiments presented here were from passages 26–46.

The human colon adenocarcinoma cell line HT29 was isolated in 1964 from a moderately differentiated, grade II adenocarcinoma of the recto-sigmoid colon of a 44-year-old Caucasian woman, and have retained a high level of genomic stability [26,27]. HT29 cells were obtained from the American Tissue Culture Collection (ATCC HTB-38) and maintained as a subconfluent monolayer culture in Dulbecco's Modified Eagle's Medium (DMEM) [Invitrogen GmbH, Karlsruhe, Germany] supplemented with 10% (v/v) FCS at 37 °C in a humidified incubator (5% CO_2/95% humidity). Passages 25–46 of HT29 were used for the experiments in this study.

At regular intervals, a mycoplasm test was performed, that was based on DNA fragment amplification by PCR (VenorGeM, Minerva Biolabs, Berlin, Germany).

2.2. Uptake of FAs by LT97 and HT29 colon cells

Cells were seeded into 25 cm^2 cell culture flasks (density of 4×10^6 cells per flask). After 24 h preincubation, they were treated with a subtoxic concentration (100 μM in EtOH) of the corresponding FAs for 1, 6, 24, 48, and 72 h. FAs were prepared by mixing the purchased stock solutions with ethanol to yield a final concentration of 200 mM and stored in the dark at −20 °C. Before treatment of cells, FAs were further diluted with cell culture medium to yield the final concentration, all with an equal ethanol concentration (0.05%). This ethanol concentration was chosen to be the control. The incubation supernatant was removed and cells were harvested and washed with PBS. The cell number was determined with a haemocytometer using the trypan blue exclusion assay.

The FA content of the incubation supernatant and the cells was determined after extraction with a modified method according to Bligh and Dyer [28]. A mixture of chloroform/methanol (2:1, v:v, 4.5 mL) was added to 1,215 μL of the supernatant. After shaking for 10 sec and ultrasonic treatment (15 min), the sample was heated for 60 min at 60 °C. After cooling, the separation of the phases was performed by addition of 1.5 mL chloroform, shaking and a further addition of 1.5 mL saline followed by centrifugation (700*g*; 3 min) if required. Subsequently, the lower chloroform layer was transferred in a new vial, and additional 1.5 mL chloroform was added to the remaining upper aqueous layer. The mixture of upper aqueous layer and chloroform was shaken (10 sec) for blending. After separation of the phases, the lower chloroform layer was removed, and combined with the first separation and evaporated in a stream of nitrogen at a temperature of 50 °C. The residue was taken up in 900 μL toluene and 100 μL trimethyl sulphonium hydroxide (TMSH) was added [29]. After 30 min of incubation at room temperature, the samples were analyzed by gas chromatography.

Cell pellets were resuspended in 100 μL deionized water. An aliquot containing 4.0×10^6 cells was taken and deionized water was added to a final volume of 1,173 μL before transfer to a 10 mL vial. Lipid extraction and methylation was then carried out as described above using 4.5 mL chloroform/methanol (2:1, v:v). Gas chromatography was performed on a HP 5890 Series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a flame ionization detector (FID), split/splitless injector, automatic liquid sampler HP 7673 (Hewlett-Packard, Waldbronn, Germany), capillary column SP-2380 (60 m, 0.32 mm i.d., 0.2 μm film; Supelco, Bellefonte, PA), and capillary column Stabilwax (30 m, 0.32 mm i.d., 0.5 μm film; Restek, Bad Homburg, Germany), respectively. Helium was used as carrier gas at a pressure of 1.5 bar and 1.2 bar, respectively. The temperature was as follows: The program started at 80 °C (1 min) to reach 180 °C at a rate of 25 °C/min. That temperature was held for 2 min before another raise to 230 °C at 5 °C/min. The final temperature was maintained for 20 min. Temperatures of injector and detector were set to 250 and 260 °C. Injection (5 μL) was performed splitless; after 1 min split was opened and set to a ratio of 1:30. We performed an external calibration for each fatty acid by which the area under the curve per amount fatty acid (mol/L) was determined. The fatty acid content in media and cells where interpolated according to the area under the curve measured in each sample for each fatty acid. For integration of chromatograms, the software package HP 3365 Series II ChemStation version A.03.34. (Hewlett-Packard, Waldbronn, Germany) was used.

2.3. Determination of cell survival/cell growth using the DAPI assay

HT29 cells (8,000/well) were seeded in 96 well microtiter plates and left 24 h before treatment either with LA, ALA, GLA, ARA, DHA [0–1 mM], EPA [0–0.5 mM], or with a solvent control (0.05% ethanol) as described above. The LT97 cells were grown for 2–4 days after seeding before treatment, depending on their confluence. After 2, 4, 6 h (HT29 cells only) 24, 48, or 72 h of incubation, the medium was removed and cells were fixed and lysed with methanol. The amount of DNA per well was measured after adding 100 μL of the DNA dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) [20 μM] (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) for at least 30 min and the resulting fluorescence intensity was measured using a microtiter plate reader (Spectra Fluor Plus, Tecan, Austria; Software: X-Fluor) (excitation at 360 nm/emission at 465 nm). The amount of DNA was considered to reflect the cell number. The data were analyzed by setting the values of the control medium to equal 100%.

2.4. Metabolic activity using the cell titer blue assay

Cells were seeded and incubated in 96 well microtiter plates, as described for the DAPI Assay. CellTiterBlue™ reagent (Promega GmbH, Mannheim, Germany) was added 2 h before the end of the incubation period as described previously [30]. During this time, metabolically active cells convert resarufin into resazorin. The fluorescence intensity was measured (excitation at 520 nm/emission at 595 nm). Results were calculated on the basis of the ethanol controls' resazorin fluorescence which were set to equal 100% and were further related to the number of cells measured afterwards by DAPI staining as mentioned before to calculate the metabolic activity of the remaining cells per well. This assay on its own gives only information on the cell viability per well. But by comparing this value to the final cell number, information on metabolic activity of the remaining cells can be obtained.

2.5. Statistical analysis

Statistical evaluation was performed with the GraphPad Prism Version 4.0 for Windows (GraphPad Software, San Diego California, www.graphpad.com). The statistical analyses used depended on the respective experimental design and are specified in the legends of the figures and tables.

3. Results

3.1. Uptake of $n-3$ and $n-6$ PUFAs into LT97 and HT29 cells

Relative bioavailability of the tested PUFAs is shown in Tables 1 and 2. To assess this, the presence of the FAs was measured in the cell extracts (Table 1) and the medium (Table 2) after 1, 6, 24, 48, and 72 h treatment. It is apparent that all tested FAs were detectable in both cell types and that for both cell lines the greatest increases were seen after 6 h treatment. The accumulation was dependent on time of treatment (two-way ANOVA, $P < 0.001$ for HT29 and LT97 cells; Table 1). The amounts measured differed between FAs (two-way ANOVA, $P < 0.001$ and $P < 0.01$ for HT29 and LT97, respectively) with LT97 cells accumulating generally lower amounts of FAs when compared with HT29 cells. In contrast, it is apparent that there was a rapid loss of FAs from the medium even after 6 h (Table 2), which was not accompanied by an equal accumulation rate especially in LT97 cells.

3.2. Effects of $n-3$ and $n-6$ PUFAs on LT97 and HT29 cell growth and metabolic activity

The influence of the tested PUFAs on metabolic activity was analyzed in HT29 cells for a short time of exposure (2, 4, and 6 h). All concentrations up to 200 μM were noncytotoxic as determined by the DAPI assay. It is apparent from Fig. 1 that the metabolic activity of surviving cells increased slightly after 4 and 6 h in concentration-dependent manner, but there were no significant differences between the tested FAs (two-way ANOVA).

Figure 2 compares the EC_{50} values obtained after treating LT97 and HT29 cells with fish $n-3$ PUFAs (EPA and DHA) as well as the plant-derived PUFAs (GLA, ALA) and ARA

Table 1
Uptake of FAs (nmol) into HT29 and LT97 cells ($n = 3$), respectively

		Uptake of fatty acids into cells (nmol/10^6 cells)					
Cells	*h*	Linoleic acid	α-Linolenic acid	γ- Linolenic acid	Arachidonic acid	Eicosapentaenoic acid	Docosahexaenoic acid
HT29	0	6 (3)	2 (2)	4 (5)	1 (0)	1 (0)	5 (1)
	1	11 (7)	13 (3)	16 (6)	3 (0)	14 (2)	25 (10)
	6	44 (14)	74 (48)*	43 (29)*	7 (2)**	47 (12)***	55 (23)*
	24	60 (47)	25 (6)	31 (1)	8 (1)**	15 (12)	32 (21)
	48	57 (63)	17 (7)	14 (1)	5 (1)	7 (2)	17 (8)
	72	57 (42)	20 (7)	16 (3)	5 (2)	13 (1)	14 (3)
LT97	0	2 (1)	1 (0)	1 (0)	2 (2)	2 (2)	1 (1)
	1	2 (2)	2 (1)	2 (1)	3 (1)	3 (2)	3 (1)
	6	7 (3)	9 (6)	4 (1)	8 (1)	7 (2)	11 (5)
	24	5 (1)	7 (3)	3 (1)	6 (3)	6 (1)	11 (6)
	48	5 (3)	6 (5)	2 (0)	6 (8)	5 (1)	12 (8)
	72	7 (5)	6 (6)	2 (2)	12 (2)	7 (5)	12 (10)

Data represent values per 10^6 cells [mean (SD)]. Asterisks indicate significant increase of the FA when compared with the amount of FA at time 0 h, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA, Bonferroni post test). Each column shows data relating only to the fatty acid added to the cell culture media which is indicated at the top of the column.

for 24, 48, and 72 h. EC_{50} values were not reached within 6 h of treatment in HT29 cells and also not after supplementation with LA, thus, these data are not presented here. It is apparent that the EC_{50} values for the fish oil compounds decreased within time of treatment from 24 to 72 h, indicating a tendency of an increased toxicity with time of exposure. The plant oil derivatives, in contrast, retained similar EC_{50} values throughout the duration of the experiment.

Table 2
Amounts of FAs (nmol) per mL cell culture supernatant after incubation of HT29 and LT97 cells ($n = 3$), respectively, for indicated target times

		Fatty acids present in the medium during incubation (nmol/mL)					
Cells	*h*	Linoleic acid	α-Linolenic acid	γ-Linolenic acid	Arachidonic acid	Eicosapentaenoic acid	Docosahexaenoic acid
HT29	0	348 (84)	321 (121)	331 (41)	399 (178)	298 (44)	426 (167)
	1	227 (32)*	285 (44)	104 (14)***	162 (40)*	231 (17)*	319 (101)
	6	95 (6)***	143 (23)**	23 (10)***	59 (21)**	77 (1)***	126 (41)*
	24	37 (3)***	28 (15)***	25 (8)***	26 (2)***	14 (1)***	34 (12)***
	48	38 (9)***	24 (14)***	25 (12)***	21 (2)***	12 (1)***	32 (12)***
	72	49 (6)***	26 (16)***	17 (9)***	22 (2)***	13 (1)***	39 (17)***
LT97	0	273 (7)	347 (30)	335 (14)	351 (0)	352 (81)	333 (34)
	1	179 (13)***	196 (10)***	195 (26)***	249 (19)***	219 (25)***	248 (55)
	6	81 (16)***	68 (17)***	47 (35)***	75 (7)***	72 (32)***	113 (77)***
	24	25 (7)***	14 (4)***	18 (2)***	28 (6)***	22 (2)***	39 (9)***
	48	26 (13)***	14 (4)***	16 (4)***	24 (2)***	21 (4)***	38 (7)***
	72	29 (11)***	18 (3)***	16 (3)***	25 (0)***	22 (2)***	42 (9)***

Data represent background (medium control) subtracted values per ml [mean (SD)]. Asterisks indicate significant reduction of the FA when compared with the amount of FA at time 0 h, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA, Bonferroni post test). Each column shows data relating only to the fatty acid added to the cell culture media which is indicated at the top of the column.

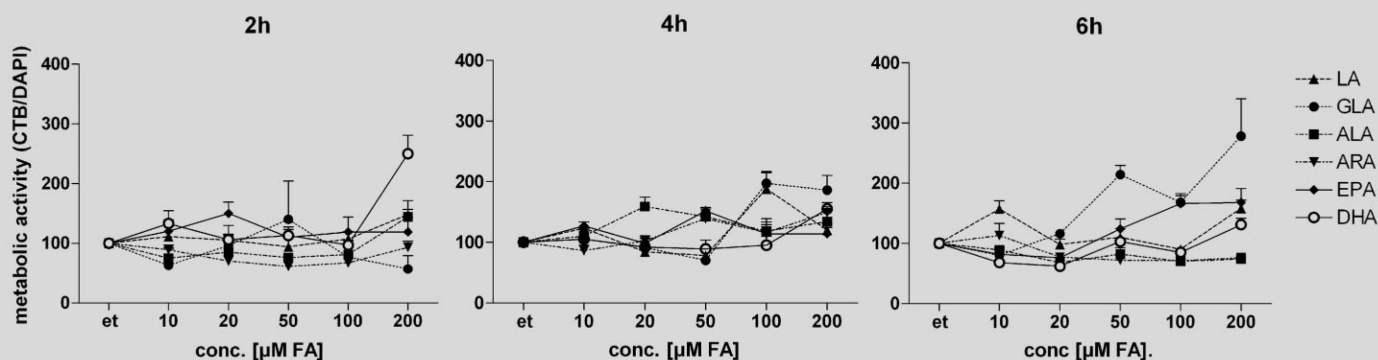

Fig. 1. Metabolic activity (values normalized basing on cell number measured with DAPI) after short time incubation (2, 4, 6 h) in HT29 cells. Dots represent mean ± SD (n = 3) of concentrations up to 200 μM of LA, ALA, GLA, ARA, EPA, and DHA; et = ethanol control.

Overall, LT97 cells were significantly more susceptible to the growth inhibitory actions of the PUFAs than HT29 cells, particularly in relation to treatment with EPA and ARA (two-way ANOVA, $P < 0.01$) (Fig. 3). An exception was DHA which was of equal potency in both cell lines.

Figure 4 compares the growth inhibitory activities, in both cell lines, of EPA and DHA in comparison to ARA and LA over a concentration range where only the influence of the FAs on viable cells is considered; 50 μM for LT97 and 100 μM for HT29 cells. DHA was more effective than EPA and LA for both parameters in HT29 cells. In LT97 cells, DHA and EPA were markedly more effective than LA, but for EPA the effects were more pronounced in LT97 cells than in HT29 cells. Cell growth inhibition by ARA was comparable to that of EPA and DHA in both cell types. Treatment of both cell types with high concentrations of all PUFAs (≥500 μM) resulted in microscopically detectable cytotoxic alterations (not shown).

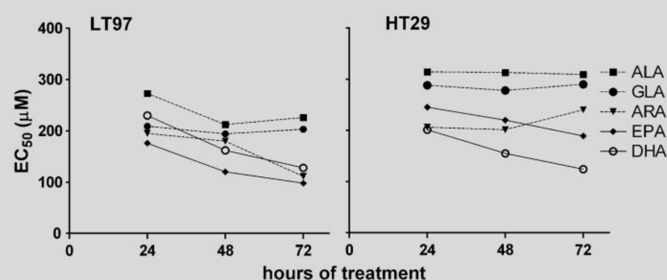

Fig. 2. Survival of LT97 and HT29 cells after treatment with FAs (DAPI assay) (n = 3–6) after 24, 48, and 72 h of incubation. The results are presented as mean concentrations (μM) leading to a reduction of cell growth by 50% (EC_{50}-values). The data were calculated using GraphPad PRISM curve fit with sigmoidal dose-response (variable slope).

4. Discussion

Dietary fat has been implicated as an important factor in the aetiology of colorectal cancer. However, not only the amount of fat ingested but also the pattern of alimentary lipids seems of importance [31]. Fish oil was shown to induce apoptosis *in vivo* in the rat colon [32] as well as *in vitro* in HT29 cells and in CaCo-2 cells [33] as well as in other human colon carcinoma cell lines [34]. Although studies comparing the growth modulatory effect of different $n - 3$ and $n - 6$ FAs in the same colon cell line are rare [21], comparing the effects between colon cell lines reflecting different stages of colon cancer is entirely novel. Here we used LT97 cells which are of adenomatous origin and a model of an earlier stage of colon carcinogenesis than the more common adenocarcinoma cell line HT29. Thus, this work covers an important link between *in vitro* work and animal *in vivo* studies.

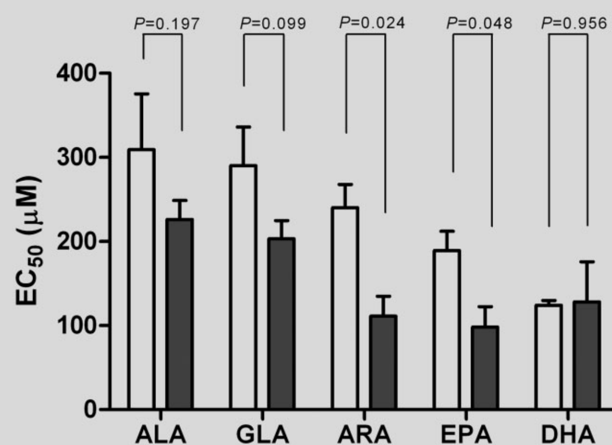

Fig. 3. Comparison of EC_{50} values obtained for LT97 (gray bars) and HT29 cells (white bars) after 72 h FA treatment. P values indicated are obtained by two-tailed unpaired t test. Results are presented as mean ± SD (n = 3–6).

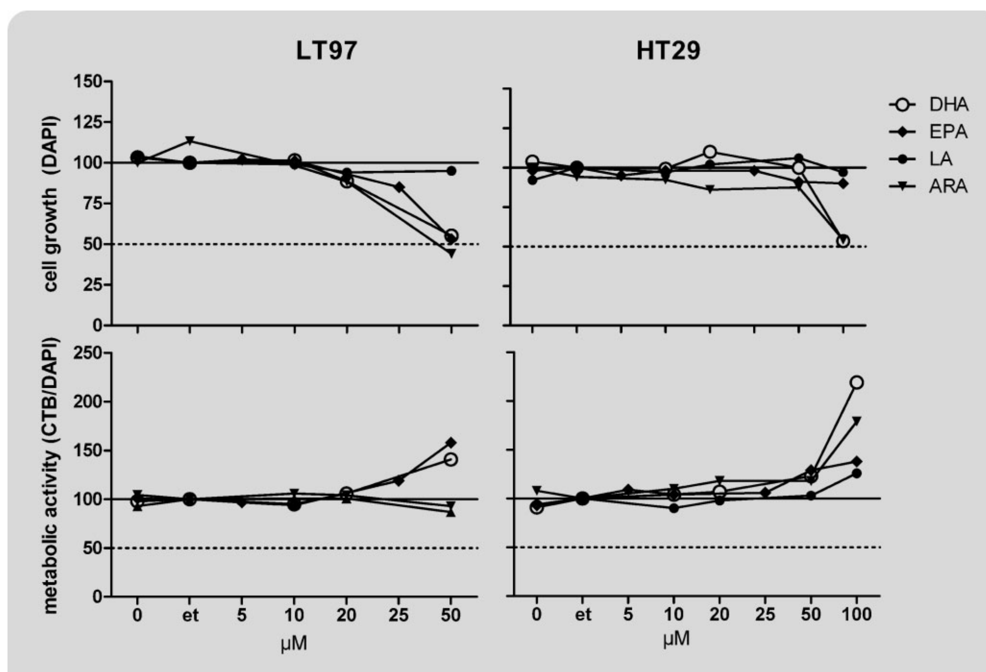

Fig. 4. Comparison of survival curves and metabolic activities of LT97 and HT29 cells after 72 h treatment with the FAs LA, ARA, EPA, and DHA as detected with the method of the DAPI staining and CTB, respectively. The results are presented as background subtracted concentrations (mean, *n* = 3–6).

The first question we addressed was the bioavailability of FAs over a time course of up to 72 h. Efficient absorption of FAs, predominantly in the jejunum and ileum, allows less than 5% of the ingested lipids to reach the colon [35]. Nevertheless, a significant increase of $n-3$ PUFAs in colonic mucosa after alimentary supplementation can be achieved [36,37]. Lipids probably reach the colonocytes primarily *via* the blood, where concentrations may reach as high as 400 µmol/L [38] following supplementation, and, although some might escape absorption in the small intestine this would probably be rapidly metabolized by colonic bacteria. For this study, relevant concentrations of nonesterified FAs dissolved in ethanol as suitable solvent for *in vitro*-studies [39] were used and it was shown, that LT97 and HT29 cells are able to accumulate FAs, as the amount of FAs measurable within the cells increased (Table 1). This is an important finding as the modification of cell FA composition has been shown to affect signaling pathways [40], lipid peroxidation and oxidative stress [41], gene expression [42], and eicosanoid synthesis [43]. The minor differences of measured FAs can be explained by different utilization and metabolism of the FAs in the cells. ARA was found only in small amounts in HT29 cells. It seems likely, that ARA is further metabolized possibly by cyclooxygenase 2 (COX-2), which is over expressed in colon cancer tissue [44] and what may result in higher amount of prostaglandin E2 [45]. Prostaglandin E2 itself was shown to be an important factor for cell proliferation in carcinoma cells [46]. In this study, the amounts of FAs within the adenomatous LT97 cells was much lower than in the HT29 cells even though the loss of FAs from the cell culture supernatant was comparable. This could be observed in different culture conditions between both of the cell lines. Although each cell line was grown to a consistent level of confluence there were differences. LT97 grow as a multilayer and thus 3–4 times more cells were present at the start of incubation compared to the HT29 cells which grow as a monolayer ($14–18 \times 10^6$ *vs*. $4–6 \times 10^6$ cells per flask). Alternatively, it is possible that LT97 cells are able to metabolize FAs better than HT29, a hypothesis which is supported by the increase in metabolic activity of the cells possibly due to FAs providing metabolic fuel especially when providing longer chain PUFAs. This effect was slightly stronger in LT97 than in HT29 cells (Fig. 4). Interestingly, LT97 medium contains lower concentration of glucose compared to HT29 medium (1,442 mg/L *vs*. 4,500 mg/L). Thus, LT97 cells might more readily use added FAs for β-oxidation than HT29 cells.

In contrast to the $n-6$ PUFA LA, both fish $n-3$ PUFAs are potent inhibitors of colon adenocarcinoma cell growth by affecting cell number. EC_{50} values for DHA treatment (Fig. 2) reflect concentrations which can be found in human plasma under physiological conditions following a diet based on fish (168 ± 8 µM) [47]. However, EPA EC_{50} levels were higher than found physiologically (64 ± 5 µM) following a fish-based diet [47].

The location of double bonds apparently had no impact on growth inhibition, because different FAs (ALA, GLA) with the same chain length (C_{18}) and same number of double bonds (18:3) were equally effective. It can be speculated that the number of double bonds might be important in predicting the growth inhibitory action of different FAs in human colon carcinoma cell lines [33]. This is in agreement with our results, as the most unsaturated FA DHA containing six

double bonds had the highest growth inhibitory effect, followed by EPA (five double bonds) and ARA (four double bonds). LA, only containing two double bonds, affected cell growth only at concentrations >500 μM. Therefore, lipid peroxidation can be considered at least in part one reason for the cytotoxic mechanism of PUFAs [48].

Dommels et al. [21] showed a dose-dependent decrease in CaCo-2 cell proliferation by treatment with DHA but not in HT29 cells (48 h incubation, concentration of 0–160 μM). We also only found a significant inhibition of HT29 cell growth after 48 h for DHA at concentrations above 200 μM (Fig. 2).

Comparing a nonmalignant mouse fibroblast cell line (NIH3T3) with its malignant human colon cancer transformants (SIC), Tsai et al. [49] only found inhibition of cell proliferation, following treatment with EPA and DHA, in malignant cells. Similarly, it has been reported that in breast cell lines EPA and DHA selectively inhibited growth of the adenocarcinoma cell line MCF-7 when compared with noncancerous MCF-10A cells [50]. In our study, the growth inhibition in colon cells of adenomatous origin was similar (DHA) or even more pronounced (EPA) when compared with adenocarcinoma cells after treatment with FAs supporting the hypothesis that less transformed cells are more susceptible to the effects of very long chain PUFAs. However, studies in animal models suggest that the pro-apoptotic effects of fish oils are less marked in the healthy colon than in one exposed to carcinogens [11]. To answer the question as to whether healthy and premalignant and malignant colon epithelial cells react differently to the growth inhibitory action of FAs using cell culture models then ideally primary colonocytes should be used. To some extent this question has been addressed by Engelbrecht et al. [40] using the "normal" cell line NCM460 and CaCo-2 cells to compare the effects of palmitic acid (16:0), oleic acid (18:1$n-9$), ARA, and DHA. In their study, the only FA found to be growth inhibitory was DHA and that only in the adenocarcinoma cell line. Thus, combining the data in this study with this data, it would suggest that fish-oil derived PUFAs are growth inhibitory to both malignant and premalignant colonocytes but not to the untransformed cell line NCM460.

This study is novel in that it focuses for the first time on effects of PUFAs on a premalignant colorectal cell line when compared with a colon adenocarcinoma cell line which is important in relation to understanding chemoprevention at the early stages of tumor initiation and progression and may also better reflect normal patterns of FA uptake and metabolism. The data are integrated with an assessment of utilization of a range of PUFAs, highlighting how fast turnover of PUFAs is in this system. These results show that while both colon adenoma and adenocarcinoma cells are able to incorporate the tested FAs this is not the predominant fate of the FAs for either cell line. In fact most FA is lost from the system, presumably utilized as a metabolic fuel. Most interestingly though is the observation that adenoma cells appear to be more susceptible to the growth inhibitory effects of $n-3$ PUFAs than the adenocarcinoma cells; an observation in contrast to the lower susceptibility of "normal" cells reported previously.

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3.2 Publication II: N. Habermann, E.K. Lund, B. L. Pool-Zobel, M. Glei: „**Modulation of gene expression in eicosapentaenoic acid and docosahexaenoic acid treated human colon adenoma cells.**”

Genes and Nutrition (2009) 4:73–76.

The potential colon cancer chemopreventive effect of fish, which has been linked to the high content of the *n*-3 PUFAs EPA and DHA in some fish, is hypothesised to be caused by an alteration of gene expression after application of the PUFAs. The aim of the study was to compare the modulation of gene expression in LT97 colon adenoma cells in response to EPA and DHA treatment. Therefore, we used custom-designed cDNA arrays containing probes for 306 genes related to stress response, apoptosis and carcinogenesis and hybridised them with cDNA from LT97 cells which were treated for 10 or 24 hours with 50µM EPA or DHA. Preneoplastic cells reflecting target cells for chemoprevention were chosen for this study.

There was a marked influence of *n*-3 PUFA on the expression of several gene types, such as detoxification, cell cycle control, signalling pathways, apoptosis, and inflammation. DHA and EPA generally modulated different sets of genes, although a few common effects were noted.

Own contribution to the manuscript:

- experimental design
- co-work on establishment of the PIQOR™ Custom Array
- conduct of cell culture of HT29 and LT97
- isolation of RNA, PIQOR™ Custom Array
- data analysis and presentation and interpretation of the results
- compilation of the manuscript

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RESEARCH PAPER

Modulation of gene expression in eicosapentaenoic acid and docosahexaenoic acid treated human colon adenoma cells

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Abstract Epidemiological studies suggest that high fish intake is associated with a decreased risk of colorectal cancer which has been linked to the high content of the *n*-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acids (EPA) and docosahexaenoic acid (DHA) in some fish. The aim of the study was to compare the modulation of gene expression in LT97 colon adenoma cells in response to EPA and DHA treatment. Therefore, we used custom-designed cDNA arrays containing probes for 306 genes related to stress response, apoptosis and carcinogenesis and hybridised them with cDNA from LT97 cells which were treated for 10 or 24 h with 50 μM EPA or DHA. There was a marked influence of *n*-3 PUFA on the expression of several gene types, such as detoxification, cell cycle control, signaling pathways, apoptosis and inflammation. DHA and EPA generally modulated different sets of genes, although a few common effects were noted. In our approach, we used preneoplastic adenoma cells which are a relevant model for target cells of chemoprevention. If verified with real time PCR, these results identify genes and targets for chemoprevention of colon cancer.

Keywords cDNA array · *n*-3 polyunsaturated fatty acids · Colon cancer · Gene expression

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Short communication

Epidemiological studies suggest that high fish intake is associated with a decreased risk of colorectal cancer (2, 3, 7). Most of the beneficial effects have been linked to the high content of the *n*-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in some fish. EPA and DHA are essential for humans and found most notably in oily fish like salmon or herring. The intake of *n*-3 PUFAs in the world differs and is in direct relation to the relative fish consumption. The incidence of colorectal cancer in countries with high fish consumption such as Greenland or Japan is reported to be lower (9). Also, in vivo studies in rats have shown antitumourigenic effects of a diet rich in fish oil compared to other oils, such as corn oil (5). Evidence suggests that fish-related compounds act post-initiation to reduce transition of adenomas to tumours. However, the molecular mechanisms behind the anticancer effects of a diet rich in fish are not yet clarified.

The aim of the present study was to examine the modulation of expression of 306 genes related to stress response, apoptosis and carcinogenesis by comparing the effects of EPA and DHA. Human colon adenoma cells were chosen since they are a model for target cells for chemoprevention in vivo. Previous in vitro studies in this field have used cancer cell lines such as HT29 (1) or Caco-2 (6) rather than cells derived from adenomas, for example LT97.

Materials and methods

Gene expression studies were performed using LT97 human colon adenoma cells (4, 8) treated with a non-cytotoxic

Table 1 Summary of selected genes that are modulated by treatment of LT97 human colon adenoma cells with 50 µM EPA ($n = 3$) or DHA ($n = 2$)

Accession	Gene name	Gene symbol	FC	SD	*p* value (*t* test)
EPA 10 h					
NM_001228	Caspase 8	CASP8	0.10	0.17	0.17
NM_001074	UDP glucuronosyltransferase 2 family, polypeptide B7, 8, 10, 11	UGT2B7_8_10_11	0.23	0.39	0.39
NM_000410	Hemochromatosis	HFE_3	0.25	0.43	0.37
NM_000578	Solute carrier family 11, member 1	SLC11A1	0.29	0.50	0.92
NM_030753	Wingless-type MMTV integration site family, member 3	WNT3	0.30	0.53	0.95
NM_022467	Carbohydrate sulfotransferase 8	CHST8	2.02	2.66	0.54
NM_000854	Glutathione S-transferase theta 2	GSTT2	4.04	3.71	0.31
EPA 24 h					
NM_000463	UDP glucuronosyltransferase 1 family, polypeptide A1	UGT1A1	2.00	1.06	0.29
NM_004985	v-Ki-ras2 kirsten rat sarcoma viral oncogene homolog	KRAS2A-B	2.02	0.31	0.26
NM_002502	Nuclear factor of kappa light polypeptide gene enhancer in B cells 2	NFKB2	2.12	0.92	0.41
NM_003998	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1	NFKB1	2.16	1.08	0.19
NM_000854	Glutathione S-transferase theta 2	GSTT2	2.41	1.65	0.46
NM_000178	Glutathione synthetase	GSS	2.54	2.31	0.30
NM_001461	Flavin containing monooxygenase 5	FMO5	2.58	0.54	0.15
DHA 10 h					
NM_001228	Caspase 8	CASP8	0.15	0.21	0.80
NM_000617	Solute carrier family 11, member 2	SLC11A2_2	0.27	0.16	0.30
NM_000410	Haemochromatosis	HFE_3	0.28	0.37	0.41
NM_001074	UDP glucuronosyltransferase 2 family, polypeptide B7, 8, 10, 11	UGT2B7_8_10_11	0.29	0.41	0.91
NM_000963	Cyclooxygenase 2	COX2	0.32	0.45	0.98
NM_030753	Wingless-type MMTV integration site family, member 3	WNT3	0.33	0.32	0.34
NM_001880	Activating transcription factor 2	ATF2	3.08	1.88	0.41
DHA 24 h					
NM_000778	Cytochrome P450, family 4, subfamily A, polypeptide 11	CYP4A11	0.07	0.10	0.12
NM_000848	Glutathione S-transferase mu 2	GSTM2	0.12	0.11	0.11
NG_002601	UDP glucuronosyltransferase 1 family, polypeptide A3	UGT1A3_4_5	2.24	0.60	0.09
NM_004324	BCL2-associated X protein	BAX_2	2.25	2.26	0.53
NM_000146	Ferritin	FTL	2.43	0.84	0.04
NM_000765	Cytochrome P450, family 3, subfamily A, polypeptide 7	CYP3A7	3.02	3.20	0.43
NM_014465	Sulfotransferase family 1B, member 1	SULT1B1	3.02	3.60	0.52

Data are presented as fold change (FC) compared to the respective ethanol control for each experiment. A FC $\geq$2 was defined as gene up-regulation whereas a FC $\leq$0.33 was used as cut-off for down-regulated genes. Two-tailed *t* test was performed using Excel software

concentration (50 µM) of EPA ($n = 3$), DHA ($n = 2$) or alternatively 0.1% ethanol. The stock solution of each non-esterified fatty acid was purchased and dissolved in ethanol, thus ethanol was chosen to be the appropriate control. RNA was extracted after 10 and 24 h of incubation with the RNeasy Mini PLUS Kit (Qiagen GmbH, Hilden, Germany). RNA was quantified with the Nanodrop (Peqlab, Erlangen, Germany) and RNA integrity number (RIN) measured by Bioanalyzer (Agilent Technologies Deutschland GmbH, Böblingen, Germany). A control RNA which was labelled with Cy3 for each array was produced using untreated HT29 human colon carcinoma cells. Equal amounts of RNA (1 µg, RIN 8.5–10) were used for synthesising first strand of cDNA by Superscript II reverse transcriptase. A second strand cDNA was synthesized afterwards using Polymerase I, RNaseH and *E.coli* DNA ligase (Invitrogen GmbH, Karlsruhe, Germany). After clean-up of the product the double stranded cDNA was used for amplifying cRNA in order to increase the amount of RNA used for cDNA labelling with Cy3- and Cy5-dCTPs (GE Healthcare, Braunschweig, Germany). The reaction product was purified to yield clean, labelled and single

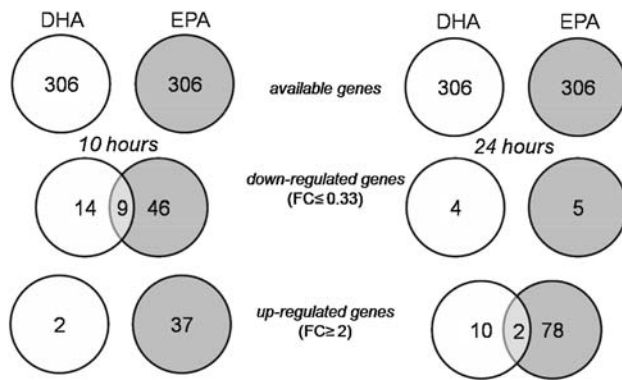

Fig. 1 Venn diagram for cDNA results comparing effects of DHA (*white circles*) and EPA (*grey circles*) in LT97 after 10 (*left panel*) or 24 h (*right panel*) of treatment. The number of genes are shown, which are down- or up-regulated for indicated treatments, overlapping numbers are the result of genes which are regulated by both PUFA in the same manner

stranded cDNA which was hybridised with a customised cDNA array (PIQOR, Miltenyi Biotec, Bergisch-Gladbach, Germany) containing 300 genes of interest and 6 housekeeping genes as described previously (11). Prior to this, the glass slide arrays were preheated and prehybridised. The final hybridisation step was carried out overnight in a water bath (65°C) in a humidified hybridisation chamber. After this, the custom arrays were washed and dried prior to scanning (MArS array reader, Ditabis, Pforzheim, Germany). Fluorescence intensities were analysed using the GeneSpotter software (MicroDiscovery, Berlin, Germany). LOESS-corrected raw data were normalised first in relation to the reference sample and second by setting the value for the housekeeping gene GAPDH equal to one. Data are presented as fold change (FC) compared to the respective ethanol control for each experiment.

Results and discussion

Using a stringent cut-off criteria ($\leq$0.33, $\geq$2) for the obtained FC, 16 genes were differentially expressed in LT97 after treatment with DHA for 10 h (2 up, 14 down) and 14 (10 up, 4 down) after 24 h. Using the same criteria, treatment with EPA resulted in a higher number of differentially expressed genes after both treatment times: 83 (37 up, 46 down) genes were modulated after 10 h, 83 (78 up, 5 down) after 24 h. Examples of modulated genes are given in a summarised form in Table 1. Only a few genes were modulated by both DHA and EPA in the same manner: 9 of the genes were down-regulated by both PUFAs after 10 h and 2 of the genes were up-regulated after 24 h (see Fig. 1).

There was a marked influence of *n*-3 PUFA on the expression of several gene types (Table 1). Of these, some could result in functional effects such as altered biotransformation of phase I [e.g., up-regulation of *CYP3A7* and *FMO5* (24 h)] and phase II [up-regulation, e.g. *GSTT2* (10), *SULT1B1*, *CHST8*, *UGT1A* family, *UGT2B* family down-regulation (10 h)], cell cycle control (e.g. up-regulation of *cyclin dependent kinase 2*), modulation of signalling pathways [e.g. down-regulation of *Wnt3* (10 h), up-regulation of *NFkB* (24 h)], apoptosis [e.g., down-regulation of *casp8* (10 h), *bax* up-regulation (24 h)], and inflammation (e.g. down-regulation of *COX2*). The modulation of gene expression by the *n*-3 PUFAs EPA and DHA has to be verified using real time PCR.

Our data show that *n*-3 PUFAs from fish oils may have a chemoprotective effect by modulating gene expression, which may prevent the development of adenomas to tumours. Our novel approach of studying this specific profile of gene expression modulation in preneoplastic human cells provides a rationale and relevant approach to identify target genes and agents that can contribute to chemoprevention.

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Conflict of interest statement There are no authors' conflict of interest which must be stated here.

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3.3 Publication III: N. Habermann, J. Helmbrecht, M. Glei: "Omega-3 polyunsaturated fatty acids alter SOD2, GSTT2 and COX2 in colorectal cell lines." Manuscript submitted to the British Journal of Nutrition.

EPA and DHA have been shown to affect gene expression using cDNA arrays (publication II). The aim of this study was to validate and compare the modulation of *SOD2, GSTT2* and *COX-2* expression in LT97 colon adenoma and HT29 adenocarcinoma cells in response to EPA and DHA treatment by Real-Time RT-PCR. Additionally, SOD2 and COX2 protein was determined by Western blot. We show effects on oxidative stress reduction by SOD2 induction and probably by enhanced peroxidase activity of GSTT2. A modulation of *GSTT2* though opposing for EPA and DHA was found in HT29 cells. We propose anti-inflammatory action by two mechanisms, first, by a reduction of COX-2 expression which may result in a reduced level of prostaglandin E2, and second, in terms of a potentially reduced level of pro-inflammatory lipid derivatives arising from the fatty acids.

Own contribution to the manuscript:

- experimental design
- experimental set-up of Real-Time RT-PCR analysis and Western Blots
- data analysis
- presentation of results and interpretation
- compilation of the manuscript

1 **Omega-3 polyunsaturated fatty acids alter SOD2, GSTT2 and COX-2 in human**
2 **colorectal cell lines.**

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24 Keywords: gene expression, colon cancer, n-3 PUFA, EPA, DHA
25

26 **Abstract:**

27 Epidemiological studies suggest that high fish intake is associated with a decreased risk of
28 colorectal cancer which has been linked to the high content of the *n*-3 PUFAs EPA and
29 DHA in some fish. The aim of the study was to compare the modulation of superoxide
30 dismutase 2 (SOD2), glutathione *S*-transferase T2 (GSTT2), and cyclooxygenase-2 (COX-2)
31 expression in response to EPA and DHA. Two human colon cell lines of different stage of
32 tumourigenesis, namely LT97 adenoma cells and HT29 adenocarcinoma cells, were treated
33 with 50 µM EPA or DHA for 4, 10, and 24 h. Gene expression analysis was carried out for
34 *SOD2*, *GSTT2* and *COX-2* by real-time RT-PCR using Sybr green. SOD2 and COX-2
35 protein were determined by Western Blot. *SOD2* was found to be up regulated in LT97 cells
36 (24 h). On protein level this effect was less pronounced. *GSTT2* was up regulated by EPA
37 (24 h) and down regulated by DHA (10 h) in HT29 cells. *COX-2* decreased when
38 supplementing the medium of LT97 (10 h) and HT29 cells (10 h and 24 h) with DHA. EPA
39 induced *COX-2* in LT97 cells (24 h). This COX-2 modulation was found on protein level as
40 well. Reduction of oxidative stress by induction of SOD2 and by enhanced peroxidase
41 activity of GSTT2 is hypothesised. Anti-inflammatory action can be proposed by reduction
42 of COX-2 expression which might result in a reduced level of prostaglandins.

43

44 **Introduction:**

45 Epidemiological studies suggest that high fish intake is associated with a decreased risk of
46 colorectal cancer[1,2,3]. Most of the beneficial effects have been linked to the high content of
47 the *n*-3 PUFAs EPA and DHA in some fish. EPA and DHA are essential for humans and
48 found most notably in oil-rich fish like salmon or herring. The intake of *n*-3 PUFAs in the
49 world differs and is in direct relation to the relative fish consumption. The incidence of
50 colorectal cancer in countries with high fish consumption such as Greenland or Japan is
51 reported to be lower[4]. Furthermore, *in vivo* studies in rats have shown antitumourigenic
52 effects of a nutrition rich in fish oil against a carcinogenic burden compared to other oils,
53 such as corn oil[5].

54 Chemoprevention is defined as the use of pharmacological agents (including nutrients) to
55 impede, arrest or reverse carcinogenesis[6,7]. According to Wattenberg[8], blocking agent
56 activity during primary chemoprevention prevents the formation of compounds, which
57 might act as carcinogens. We hypothesise, that fish fatty acids EPA and DHA might
58 influence colon carcinogenesis by different mechanisms, therefore we have chosen to
59 analyse the modification of enzymes involved in oxidative response, biotransformation and
60 inflammation. This can be achieved by the induction of phase II enzymes, such as
61 glutathione *S*-transferase T2 (GSTT2). Generally spoken, GSTs as biotransformation
62 enzymes are capable to detoxify a number of endogenous (e.g. lipidperoxidation products as
63 4-hydroxynonenal[9]) and exogenous (e.g. smoking- or food-borne[10]) carcinogens[11,12].
64 Since their induction is understood to reduce cancer initiation, they can be considered as
65 blocking agents. Additionally, the decrease of reactive oxygen species can be related to
66 chemoprevention, such as the detoxification of superoxide to hydrogen peroxide and
67 diatomic oxygen by superoxide dismutase 2 (SOD2)[13]. Also, it is well known that within an
68 inflammatory environment cells tend to facilitate carcinogenesis[14]. Hence, reduction of a
69 pro-inflammatory environment such as decreased cyclooxygenase-2 (COX-2) activity would
70 counteract[15].

71 To answer the question which genes are altered by the *n*-3 PUFAs EPA and DHA in colon
72 adenoma cells we examined the modulation of expression of 306 genes related to stress
73 response, apoptosis and carcinogenesis by comparing the effects of these fatty acids[16].
74 Human colon adenoma cells (LT97) were chosen since they are an appropriate model for
75 target cells for chemoprevention *in vivo*. There was a marked influence of *n*-3 PUFA on the
76 expression of several gene types. *SOD2, GSTT2* and *COX-2* have been chosen to be verified
77 by real-time RT-PCR in this study. A summary of the fold changes of these genes found by
78 custom array analysis is given in table 1. Additionally to the human adenoma cell line we

79 also examined the effects of both fatty acids on an adenocarcinoma cell model, namely
80 HT29 cells after treatment of the cells for 10 h or 24 h. Finally, Western Blot explorations
81 should reveal the impact of both *n*-3 PUFAs on SOD2 and COX-2 protein.
82

Methods

Cells and Culture

The human colorectal adenoma cell line LT97 (kind gift of Prof. B. Marian, University of Vienna) represents an early stage of tumour development[17]. It was established from colon microadenomas of a patient with hereditary familial polyposis. LT97 cells were maintained in a culture medium (MCDB 302) as described previously[18]. The cells have a stable karyotype[19,20], are of epithelial nature[17] and have a doubling time of 72-96 h. The LT97 cells used for the experiments presented here were from passages 24-38. The human colon adenocarcinoma cell line HT29 was isolated in 1964 from a moderately differentiated, grade II adenocarcinoma of the recto-sigmoid colon of a 44-year-old Caucasian woman, and have retained a high level of genomic stability[21,22]. HT29 cells were obtained from the American Tissue Culture Collection (HTB-38) and maintained as a subconfluent monolayer culture[18] in Dulbecco's Modified Eagle's Medium (DMEM) [Invitrogen GmbH, Karlsruhe, Germany]. Passages 19-34 of HT29 were used for the experiments in this study. At regular intervals, a mycoplasm test was performed (VenorGeM, Minerva Biolabs, Berlin, Germany).

Treatment of cells with fatty acids

LT97 and HT29 (4×10^6) cells were seeded in 25 cm^3 cell culture flasks and incubated until LT97 cells reached 70-80% confluence or in case of HT29 for 24 h. Prior to the experiments, LT97 and HT29 cells were treated with 50 µM EPA or 50 µM DHA. This concentration was shown to affect the expression of genes[16]. For this, the supernatant was removed and fresh medium containing respective PUFA or 0.1% ethanol control was applied. The stock solution of each non-esterified fatty acid was purchased dissolved in ethanol, thus ethanol was chosen to be the appropriate control. All experiments were performed in triplicates.

Isolation of RNA and quality control

RNA was extracted after 4, 10 and 24 h of incubation with the RNeasy Mini PLUS Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. RNA was quantified with the Nanodrop (Peqlab, Erlangen, Germany) and RNA Integrity Number (RIN) was measured by Bioanalyzer (Agilent Technologies Deutschland GmbH, Böblingen, Germany).

Real-Time qRT PCR

The modulation of *SOD2*, *GSTT2* and *COX-2* mRNA was performed by an independent measurement of the mRNA levels. For quantitative RT-PCR-analysis we used the system of iCycler iQ (Bio-Rad GmbH, Munich, Germany), enabling quantitative analysis of the

118 mRNA expression levels. Therefore, aliquots of 2 μg total RNA from each sample were
119 reverse transcribed using reagents from SuperScript II reverse transcriptase (Invitrogen
120 GmbH, Karlsruhe, Germany) according to the manufacturer's protocol. For each reaction 2
121 μl of cDNA was mixed with PCR master mix iQ™ SYBR1 Green Supermix (SYBR Green
122 I, iTaq DNA polymerase, reaction buffer, deoxynucleotide triphosphate mix, 10 mM $MgCl_2$,
123 20 nM fluorescein, and stabilisers; BioRad GmbH, Munich, Germany), and 10 pmol of the
124 gene-specific primers for target *SOD2, COX-2*, and *GSTT2* and for the housekeeping gene
125 *GAPDH* (table 2) in a final volume of 25 μl. All experiments were performed in triplicate
126 and the PCR reaction mixture was set in an iCycler iQ 96-well PCR plate (Bio-Rad GmbH,
127 Munich, Germany). The relative fluorescence signal was captured at the primer nucleotide
128 extension step of each cycle. At the end of the reaction the melting curve analysis was
129 conducted with temperature gradient from 60 to 95°C at 0.108°C/s to differentiate
130 nonspecific primer dimers and specific amplicon. The iCycler iQ1 optical v3.0a software
131 was utilised for obtaining the relative threshold cycle number (CT) and the data
132 normalisation and analysis was carried out as previously described(23). Results were
133 expressed as fold change (FC) compared to the ethanol control.
134 Western Blots targeting SOD2 and COX-2
135 Cells were cultured, incubated and harvested as described above. After a washing step in
136 PBS, cells were chemically lysed in lysis buffer (20 mM Tris/HCl pH 8.0, 150 mM NaCl,
137 10% glycerin, 2 mM EDTA, 1% Nonidet P40) containing a freshly added mixture of 1 mM
138 DTT (reducing agent), 1 mM sodiumorthovanadate (phosphatase inhibitor) and several
139 protease inhibitors (0.5 mM Pefabloc SC, 1 mM PMSF, 1 g/ml pepstatin A, 1 g/ml
140 leupeptin). Total protein concentrations were determined by Bradford assay(24).
141 Equal amounts of total protein (10-20 μg) were diluted with SDS-PAGE loading puffer (125
142 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerin, 0.0005% bromphenol blue, 100 mM DTT)
143 and proteins were separated using discontinuous SDS polyacrylamid gel electrophoresis
144 (stacking gel: 3%, separating gel: 15%). After separation, the proteins were transferred to
145 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using a wet blotting
146 system (Bio-Rad GmbH, Munich, Germany). After Ponceau staining membranes were cut
147 beween the 55 and 60 kDa band and beyond the 35 kDa band of the PageRuler™
148 (Fermentas GmbH, St. Leon-Rot, Germany). Subsequently, unspecific binding sites on the
149 membranes were blocked with 5% nonfat dried milk powder (AppliChem, Darmstadt,
150 Germany) in TBS (100 mM Tris, 150 mM NaCl, pH 7.4). Primary antibody (mouse anti-
151 SOD2, rabbit anti-COX-2, mouse anti-β-actin (Cell Signaling, Frankfurt/M., Germany))
152 were diluted in blocking solution (1:1000 for SOD2, 1:250 for COX-2 and 1:10000 for β-

153 actin) and the respective part of the membrane was incubated with antibody solutions
154 overnight at 4°C. Horseradish peroxidise (HRP)-labeled secondary antibodies (goat anti-
155 rabbit IgG-HRP and rabbit anti-mouse-HRP (Cell Signaling, Frankfurt/M., Germany)), were
156 diluted in blocking solution 1:1000 and were used to visualise primary antibodies. After 1 h
157 incubation with secondary antibodies, membranes were washed twice with TBST and
158 afterwards once with TBS (each 5 min) incubated with ECL Plus Western Detection reagent
159 (Amersham Biosciences Europe GmbH, Freiburg, Germany). Hyperfilm™ ECL (Amersham
160 Biosciences Europe GmbH, Freiburg, Germany) chemiluminescence films were then placed
161 on the membrane to visualise bands. Exposed and developed films were then scanned using
162 Fluor-S documentation system (Bio-Rad GmbH, Munich, Germany).
163 Statistical analysis
164 Statistical evaluation was performed with the GraphPad Prism™ Version 5.0 for Windows
165 (GraphPad Software, San Diego California, USA, www.graphpad.com). Means and SD of 3
166 independent repeats were calculated and afterwards One Way ANOVA with Dunnett's post
167 test was performed.
168

169 **Results:**

170 <u>*SOD2, COX-2, and GSTT2* gene expression</u>

171 Verification of the array data was performed after treating both HT29 (right panel figure 1)
172 and LT97 (left panel figure 1) cells with EPA as well as DHA for 4, 10 and 24 h. Real-time
173 RT-PCR analysis showed enhanced expression of *SOD2* which was significant for LT97
174 cells after 24 h treatment with EPA (FC 2.5±0.6; P<0.05) or DHA (FC 2.0±0.4; P<0.05).
175 Expression of *COX-2* was repressed by DHA after 10 h (LT97: FC 0.4±0.1; P<0.001 and
176 HT29: FC 0.4±0.2; P<0.001) and 24 h in HT29 (FC 0.7±0.1; P<0.05) but not by EPA,
177 which induced *COX-2* gene expression only in LT97 cells after 24 h of treatment (FC
178 1.9±0.5; P<0.05). *GSTT2* was repressed by DHA 10 h after treatment (FC 0.7±0.1; P<0.05)
179 and induced by EPA after 24 h (FC 1.4±0.1; P<0.05) in HT29. *GSTT2* gene expression was
180 not significantly altered in LT97 by any treatment condition.

181 <u>SOD2 and COX-2 protein expression</u>

182 In order to prove whether enhanced mRNA expression is also accompanied by enhanced
183 protein expression, we performed Western Blot analysis over a period up to 48 h specifically
184 for the proteins SOD2 and COX-2. For human GSTT2 no appropriate antibody is available,
185 thus, we had to exclude this issue from our experiments.

186 An alteration of SOD2 protein was hardly to find in our experiments. Neither *n*-3 PUFA nor
187 treatment time seems to impair the expression of this protein (figure 2 above panel) in HT29
188 or LT97 cells.

189 In contrast, we found a down regulation for COX-2 in LT97 (figure 2 left panel) and HT29
190 (figure 2 right panel) cells after treatment with DHA (10, 24, and 48 h). But we did not find
191 a similar effect for EPA. In both cell types COX-2 seems not to be altered by EPA.

192

193 **Discussion:**

194 Colorectal carcinogenesis is a long-standing process which was described in a multistep
195 model to be the result of accumulation of numerous defined mutations[25]. Many of them
196 cause the activation of oncogenes or the inactivation of tumour suppressor genes. There are
197 multiple reasons for these mutations.

198 One crucial factor for mutations is oxidative stress, which has been defined as the imbalance
199 between oxidants and antioxidants in favour of the former, resulting in an overall increase in
200 cellular level of reactive oxygen species which e.g. might be one source for DNA damage.
201 One of the most studied oxidative DNA adducts is 8-hydroxydeoxyguanosine which leads to
202 site-specific mutations and produces G→T transversions[26], that are widely found in
203 mutated oncogenes and tumour suppressor genes[27]. Thus, oxidised DNA bases appear to be
204 mutagenic and capable to induce mutations commonly found in neoplasia. The first step in
205 the removal of superoxide is enabled by the enzymatic action of SOD2. So it is plausible
206 that *SOD2*$^{-/-}$ mice were found to be letal and *SOD2*$^{-/+}$ mice bear higher concentrations of
207 8-hydroxydeoxyguanosine and higher incidences of cancer[28].

208 We found an increase of *SOD2* mRNA which was significant for adenoma LT97 cells after
209 treatment with EPA and DHA for 24 h. This *SOD2* up regulation in cells of a premalignant
210 state of degeneration as seen on our results can be considered as suppressing agent activity
211 since a further transformation is potentially repressed. In our results an up regulation on
212 protein level was only hardly to be found. This can be caused by lacking protein translation.
213 Whether the reason for this effect is an effect of an already high SOD2 protein level in cells
214 *in vitro* or a result of a lacking cofactor, namely manganese, cannot be distinguished. Other
215 reasons for "hidden effects" could be mRNA instability coinciding with unchanged protein
216 synthesis.

217 Besides the effects on the antioxidant system of the cells, *SOD2* had been recently discussed
218 as a new type of tumour suppressor gene[29]. An overexpression of SOD2 decreased cell
219 growth[30]. Additionally, SOD2 overexpression leads to an alteration of different signal
220 transduction pathways in different cell types via mechanisms such as inhibition of
221 transcription factors AP-1 and NF-κB and hence effects might also affect downstream
222 targets[31].

223 GSTT2 is a member of the phase II enzymes superfamily of GSTs which play a role as
224 transferases in the detoxification of a wide variety of toxic or carcinogenic electrophiles.
225 Theta class GSTs are highly conserved and consist of 2 family members, GSTT1 and T2[32].
226 GSTT2 is a minor GST in human colon compared to GSTT1 or the major family member,
227 namely GSTP1[33]. Nevertheless, they have already shown to be up regulated by polyphenols

228 and, however, their up regulation might be one mechanism for colon cancer
229 chemoprevention[34]. Conjugation of the endogenous tripeptide glutathione allows lipophilic
230 xenobiotic substances to become more hydrophilic and hence further easier to be removed.
231 Accordingly, potentially harmful xenobiotics are excreted from the organism. Additionally
232 to the glutathione transferase activity GSTT2 also exerts peroxidase[35] and sulfatase
233 activity[36]. GSTT2 shows affinity to organic hydroperoxides as DNA-hydroperoxides and
234 lipid-hydroperoxides[37,38]. As shown by our results, EPA exerts a GSTT2 mRNA-inducing
235 effect which was significant for HT29 cells after 24h. Interestingly, DHA repressed GSTT2
236 mRNA after 10 h in HT29 cells (figure 1). Since both *n*-3 PUFAs act opposing as GSTT2
237 modulator on gene level, more specific mechanisms than simple lipid peroxidation and
238 resulting oxidative stress must account for these effects. The GST theta class genes lack both
239 TATA and CAAT boxes in their 5' flanking region thus multiple heterogeneous
240 transcription boxes had been described (SP1, PU-1, PEA3, AP-2)[39]. Whether or not EPA
241 and DHA or their derivatives impair gene expression by alteration of these transcription sites
242 and hence alter gene expression via downstream pathways was not yet examined and needs
243 further clarification.
244 Cyclooxygenase (COX), also referred to as prostaglandin endoperoxide synthase, catalyses
245 the conversion of arachidonic acid into prostaglandins. Whilst COX-1 is ubiquitous and
246 constitutively in most mammalian cells to maintain the baseline level of prostaglandins,
247 COX-2 is normally absent[40]. It is well investigated that COX-2 is over expressed in a
248 variety of diseases such as inflammation and cancer, of e.g. the breast[41], the prostate[42], and
249 the colon[43]. Particularly increased COX-2 expression is connected with tumour metastasis
250 in colon cancer, where aberrant COX-2 expression was shown to correlate with
251 carcinogenesis in 80% of the cancers[44]. The effect of COX-2 down regulation by non-
252 steroidal anti-inflammatory drugs such as aspirin is proposed to be a chemopreventive
253 mechanism regarding colon cancer[45]. Our results show a down regulation of COX-2
254 mRNA and COX-2 protein by DHA in both LT97 colon adenoma and HT29
255 adenocarcinoma cells. COX-2 inhibition was already shown in HT29 cells by
256 supplementation with EPA and DHA *in vitro*[46]. Conversely, we found a *COX-2* induction
257 by EPA in LT97 cells. The key link between PUFAs and inflammation is that eicosanoids
258 are generated from C20 PUFAs. Noteworthy, EPA can also act as a substrate for
259 lipoxigenases and COX, resulting in the generation of eicosanoids with a slightly different
260 structure than those from arachidonic acid[47]. The functional consequence is, that those
261 mediators which are formed appear to be less potent then arachidonic acid derivatives.
262 Additionally, EPA and DHA were found to act as a substrate for the generation of

263 alternative eicosanoids, termed resolvins[48]. Resolvins are formed by the action of COX-2
264 and appear to exert antiinflammatory actions and this pathway may be enhanced when
265 supplementing LT97 adenoma cells with EPA. Previously it was shown that COX-2
266 regulation is facilitated in a prostaglandin E2-dependent (via NR4A2) but also in a
267 prostaglandin-independent manner[49]. Taken together, this has led to the idea that fish oil or
268 fish fatty acids as EPA and DHA act anti-inflammatory.
269 To summarise, our data indicate different mechanisms by which the *n*-3 PUFA fish oil
270 components EPA and DHA may exert colon cancer chemopreventive effects. To our
271 knowledge, this is the first time that such effects were described not only in cancer cells but
272 also including an adenoma cell line *in vitro*. First, we showed potential effects on oxidative
273 stress reduction by SOD2 induction and probably by enhanced peroxidase activity of
274 GSTT2 (HT29, EPA 24 h). *SOD2* was significantly induced in LT97 cells (24 h) and in
275 HT29 cells as a trend. This links to a reduction of the harmful potential of superoxide to
276 damage e.g. DNA in premalignant cells. Further, we saw a modulation of *GSTT2* though
277 opposing for EPA (up regulation of GSTT2 mRNA after 24 h) and DHA (down regulation
278 of GSTT2 mRNA after 10 h) in HT29 cells. Whether or not these effects can be translated
279 for protein levels in the cells needs further clarification. And finally, we propose anti-
280 inflammatory action by two mechanisms, first by a reduction of COX-2 expression (by
281 DHA on mRNA and protein level) which may result in reduced level of prostaglandin E2
282 from the ubiquitous arachidonic acid, and second in terms of a induced level of
283 antiinflammatory lipid derivatives arising from EPA and DHA due to COX-2 activity.
284

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292 of the study, co-compilation of the manuscript.

293

294 **Table 1**. Fold changes of *COX-2, GSTT2* and *SOD2* gene expression after treatment of
295 LT97 cells with 50 µM EPA or DHA for 10 or 24 h, respectively. Given are means and
296 standard deviation (SD) of 3 independent repeats, data modified from [16].

		EPA 10h		**EPA 24h**		**DHA 10h**		**DHA 24h**	
locus ID	gene name	mean	SD	mean	SD	mean	SD	mean	SD
NM_000963	COX-2	1.15	0.88	1.62	0.51	0.21	0.37	0.07	0.13
NM_000854	GSTT2	4.04	3.71	2.41	1.65	0.24	0.42	0.33	0.13
NM_000636	SOD2	1.09	0.70	1.78	0.91	0.44	0.42	0.43	0.02

297
298

299 **Table 2.** Sequences of primers used for real-time RT-PCR.

locus ID	**gene name**	sense primer 5’... 3’	antisense primer 5’... 3’	fragment size (bp)
NM_002046	**GAPDH**	acc cac tcc tcc acc ttt gac	tcc acc acc ctg ttg ctg tag	110
NM_000963	**COX-2**	tcc tcc tgt gcc tga tga ttg c	act gat gcg tga agt gct ggg	170
NM_000854	**GSTT2**	tga cac tgg ctg atc tca tgg cc	gcc tcc tgg cat agc tca gca c	142
NM_000636	**SOD2**	gcc ctg gaa cct cac atc aac	caa cgc ctc ctg gta ctt ctc	111

300

301

302

303 **Legends to figures**

304

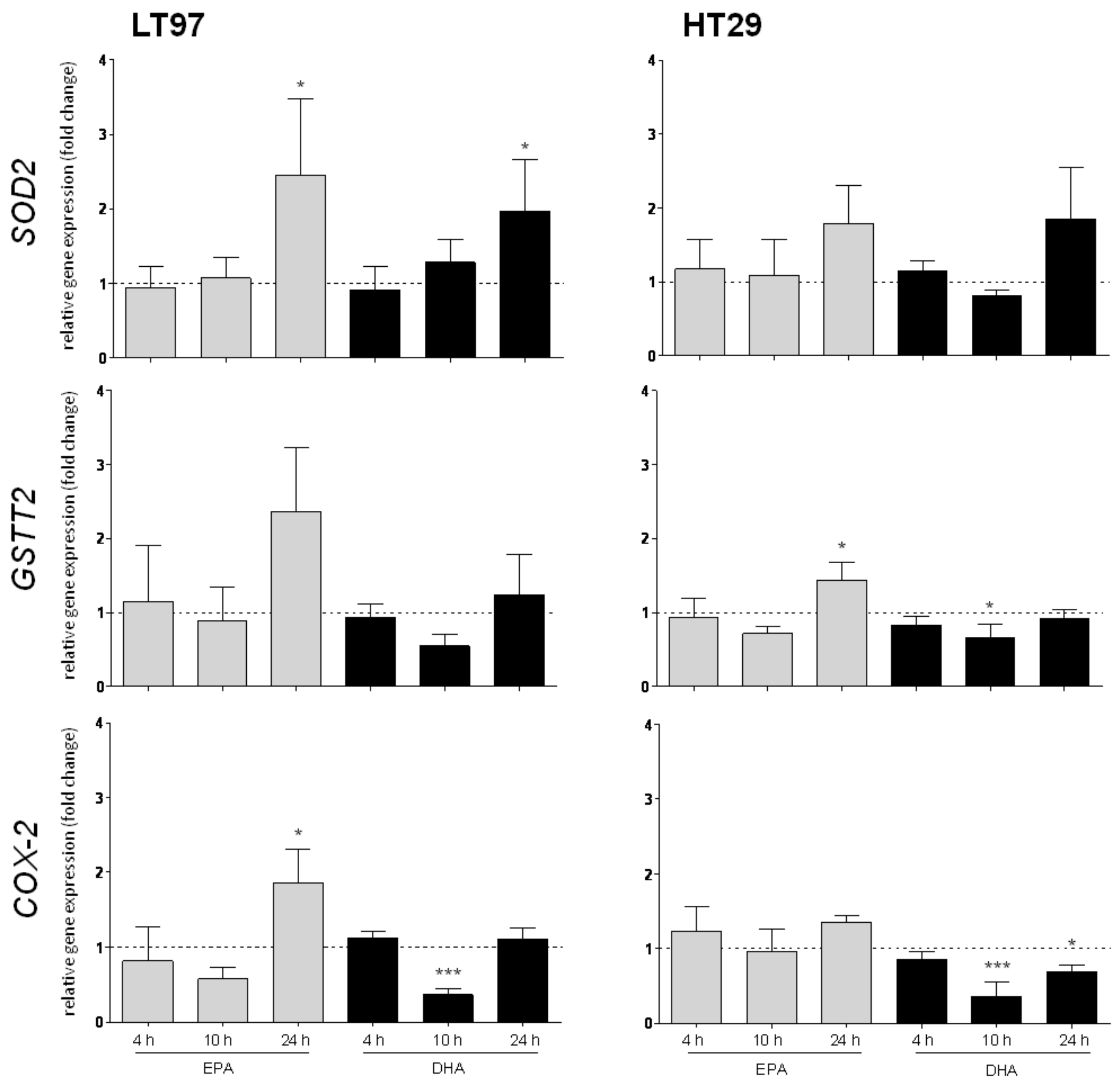
305 **Figure 1.** Gene expression of *SOD2*, *GSTT2* and *COX-2* in LT97 (left panel) and HT29
306 (right panel) after indicated times of treatment by 50 µM EPA and DHA, means + SD (n=3),
307 asterisks indicate differences to the ethanol control (dashed line) by One Way ANOVA
308 followed by Dunnett's multiple comparison test, * $P<0.05$, *** $P<0.05$.

309

310 **Figure 2.** Western Blot analysis for SOD2 and COX-2 protein in LT97 (left panel) and
311 HT29 (right panel) after indicated times of treatment by 50 µM EPA and DHA. The figure
312 shows a representative blot of a series (n=3).

313

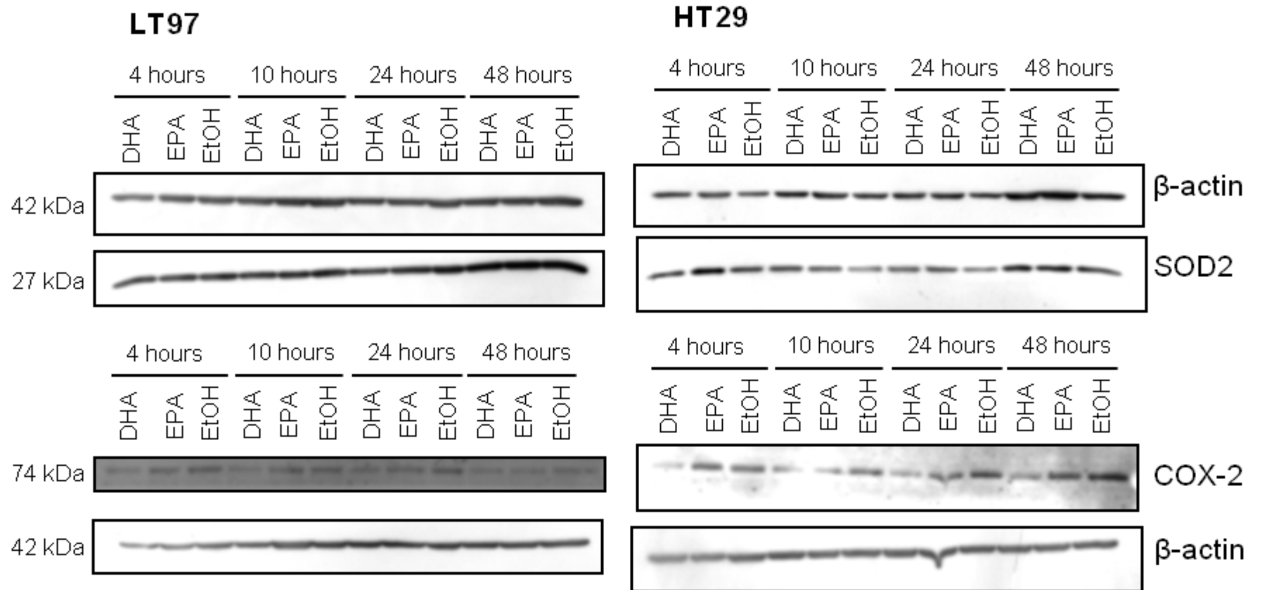
314 **Figure 1.**



315

316

317 **Figure 2.**



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426

3.4 Publication IV: <u>N. Habermann</u>, A. Schön, E. K. Lund, M. Glei: **„Fish fatty acids alter markers of apoptosis in colorectal cell lines but fish consumption has no impact on apoptosis-induction *ex vivo*.**" accepted by Apoptosis, DOI: 10.1007/s10495-010-0459-y.

Studies suggest the *n*-3 PUFAs EPA and DHA, natural food ingredients found in fish, exerting chemopreventive action in colon cancer. One of the involved mechanisms is the facilitation of apoptosis. While pro-apoptotic potential of *n*-3 PUFAs is suggested, it is still unclear whether an additional uptake of fish will also point to comparable results. The aim of this study was to assess EPA- and DHA-mediated effects on different endpoints of apoptosis and to use a novel biomarker-approach to measure modulation of apoptosis by consumption of fish. Apoptosis was detected by flow cytometry after 24 hours treatment of LT97 human colon adenoma cells with 50µM EPA or DHA, by *bcl-2* decrease using Real-Time RT-PCR in LT97 cells after 10 hours treatment, by measuring the decrease of procaspases 3 and 8, PARP cleavage, and truncation of bid in cells treated with 100µM DHA using Western Blot analysis. DHA was more effective in inducing apoptosis compared to EPA. LT97 cells were more prone to the apoptosis-inducing potential of DHA and EPA compared to HT29 cells. Treatment of LT97 cells with faecal water obtained from 89 volunteers of an intervention study giving fish (FISHGASTRO) did not result in changes of apoptosis markers.

Own contribution to the manuscript:

- experimental design
- experimental set-up of Real-Time RT-PCR, Western Blots, and Flow cytometry
- co-work on studies of faecal waters' impact on apoptosis *ex vivo*
- data analysis, presentation and interpretation of the results
- compilation of the manuscript

Apoptosis
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ORIGINAL PAPER

Fish fatty acids alter markers of apoptosis in colorectal adenoma and adenocarcinoma cell lines but fish consumption has no impact on apoptosis-induction ex vivo

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Abstract Previous studies suggest that the *n*-3 polyunsaturated fatty acids (PUFAs) eicosapenteinoic acid (EPA) and docosahexaenoic acid (DHA), constituents of fish oil, exert chemopreventive activity in colon cancer. One of the mechanisms involved is the facilitation of apoptosis. While a pro-apoptotic potential of *n*-3 PUFAs has been suggested, it is still unclear whether additional consumption of fish will also lead to comparable results. The aim of this study was to assess EPA- and DHA-mediated effects on endpoints of apoptosis and to use a novel biomarker-approach to measure modulation of apoptosis by consumption of fish. LT97 human colon adenoma and HT29 human colon adenocarcinoma cells were used to investigate modulation of apoptosis by EPA, DHA or linoleic acid (LA) using a set of endpoints, namely phosphatidylserine staining with Annexin-V (flow cytometry), *Bcl-2* expression (Real-time RT–PCR), and Bid, caspase 3, 8 and 9 expression as well as PARP cleavage (Western Blot). Furthermore, faecal water (FW) of volunteers ($n = 89$) from a human trial intervening with fish was used to investigate changes in apoptosis by flow cytometry. DHA was more effective at inducing apoptosis than EPA. LT97 cells were more prone to DHA and EPA induced apoptosis than HT29 cells. Treatment of LT97 cells with FW from volunteers consuming fish did not result in any changes in apoptosis. Taken together, our results show that adenoma cells are highly susceptible to *n*-3 PUFA-induced apoptosis. By using a biomarker-approach (FW) to measure apoptosis-induction ex vivo no change in apoptosis after additional fish consumption was detectable.

Keywords Apoptosis · Colon cancer · *n*-3 PUFA · Fish consumption · Faecal water

Introduction

Colorectal cancer is the second most common malignancy in the Western world [1, 2]. The sporadic form of colorectal cancer arises over many years as a result of accumulation of genetic errors, many of which affect the control of apoptosis [3]. Effective chemoprevention strategies aim to decrease the accumulation of genetic and epigenetic alterations [4, 5]. Apoptosis is an important chemopreventive mechanism that can be modified by diet e.g. by fish oil [6, 7], resulting in the removal of damaged cells.

Diets high in fish-oil are protective against chemically induced colon cancer in animal models [8–10] and in human populations high fish consumption appears to be chemopreventive [9, 11]. The two major fatty acids eicosapentaenoic (EPA—C20:5) and docosahexaenoic (DHA—C22:6) are naturally occurring *n*-3 polyunsaturated fatty acids (PUFAs) which can only be synthesised in humans from alpha-linolenic acid (C18:3 *n*-3) and then only to a very limited degree [12, 13]. Dietary intake from oil-rich fish such as salmon or mackerel is therefore recommended [14]. In the 1,2-dimethylhydrazine (DMH) rat model of colorectal cancer apoptosis has been shown to be enhanced by fish oil [9] and in human studies consumption of high doses of fish oil has been reported to increase

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apoptosis in the colonic mucosa after 2 years [6]. Recently it has become clear, that PUFAs and some of their derivatives are able to modulate the molecular pathways involved in apoptosis [15]. However, it still needs to be established how apoptosis is induced by *n*-3 PUFAs.

In contrast to studies using high doses of fish oil, an additional intake of salmon (2 portions fish per week giving approximately 1 g EPA + DHA/d over 6 months) failed to show an apoptosis-inducing effect in humans [16]. To our knowledge the question as to whether a high-fish diet in humans can alter the colonic lumen environment in a pro-apoptotic manner has never been assessed. Thus, to test the hypothesis that fish consumption might alter the luminal environment in a pro-apoptotic manner, we have exposed cell lines to faecal water collected during the FISHGASTRO study. Faecal water (FW) is the aqueous phase extracted from stool samples and has been widely used as a non-invasive biomarker for a multitude of tests proving the influence of nutrition on colonic health [17].

The aim of this study was therefore to investigate the impact of *n*-3 PUFAs on cell signalling in apoptosis with a focus on comparing the sensitivities of LT97 and HT29 cells. In addition we wanted to assess whether fish consumption might alter the luminal milieu in a pro-apoptotic manner.

Methods

Cells and culture

The human colorectal adenoma cell line LT97 (kind gift of Prof. Marian, Vienna) represents an early stage of tumour development. It was established from colon microadenomas of a patient with hereditary familial polyposis [18]. LT97 cells were maintained in a culture medium (MCDB 302) as described previously [19]. The cells have a stable karyotype, are of epithelial nature and have a doubling time of 72–96 h [20]. The human colon adenocarcinoma cell line HT29 was isolated in 1964 from a moderately differentiated, grade II adenocarcinoma of the recto-sigmoid colon of a 44-year-old Caucasian woman [21], and have retained a high level of genomic stability [22]. HT29 cells were obtained from the American Tissue Culture Collection (HTB-38) and were maintained as a subconfluent monolayer culture [19] in DMEM [Invitrogen GmbH, Karlsruhe, Germany]. At regular intervals, a mycoplasm test was performed (MycoAlert® Lonza, Cologne, Germany).

Subjects and study design, FW preparation

The design of the intervention study 'FISHGASTRO' has been described in detail elsewhere [16]. The ethical approval was obtained from the Medical Ethical Committee of Nijmegen University Medical Centre St. Radboud (reference 2004/111) and King's Lynn Local Research Ethics Committee (reference 04/Q0105/8). The trial has been registered at www.clinicaltrials.gov under identifier NCT00145015. All subjects gave their written informed consent and a subsample of subjects consented separately for collection of faecal samples.

Three groups of subjects were recruited: (i) those with (previous) colorectal adenomas (ii) those diagnosed with non-active ulcerative colitis (UC), and (iii) those without any macroscopic signs of disease in the colon. The design of the study was a multi-centre parallel randomised controlled intervention trial. After an initial colonoscopy procedure, 242 eligible subjects were randomly allocated to one of three dietary intervention groups: (i) oil-rich fish group receiving two 150 g portions of salmon per week during 6 months (ii) lean fish group receiving two 150 g portions of cod per week during 6 months, and (iii) only dietary advice (DA) group. All three intervention groups received dietary advice to achieve a healthy diet. The fish was provided to the participants at their home and had to be consumed in addition to their regular fish consumption. Salmon and cod provided approximately 1.4 g/d and 0.09 g/d of very long chain *n*-3 PUFA, respectively [16].

A total faeces sample was collected pre- and post-intervention and stored in a cooled container for transport to the laboratory within 4 h. At the laboratory, samples were homogenised, aliquoted and stored at −80°C until further processing. The pre- and post-intervention samples from individual volunteers were analysed in parallel. Samples were defrosted, homogenised by stirring and then transferred to aluminium cap-locked polycarbonate tubes (Beckman Coulter GmbH, Krefeld, Germany), mixed with the same amount (w/v) of ice chilled PBS and centrifuged (Optima LE-80 K Ultracentrifuge, Beckman Coulter GmbH, Krefeld, Germany) at 25,000 *g* for 2 h at 4°C as described previously [17]. The supernatants, representing the FW were aliquoted and stored at −20°C. Faeces of 89 volunteers were used for this study and characteristics of this population is given elsewhere [23].

Treatment of cells with fatty acids, caspase inhibitors, and FW

LT97 and HT29 (1×10^6) cells were seeded in 6-well plates and pre-incubated until LT97 cells reached 70–80% confluence or in case of HT29 24 h (confluency 60–70%). Prior to the experiments ($n = 3$), LT97 and HT29 cells were treated with 50 (only LT97), 100 and 200 μM (only HT29) EPA, DHA or LA, respectively. Controls were exposed to an equal concentration of ethanol to that in the fatty acid exposed samples. For a subset of the experiments using

caspase-inhibitors (Axxora Deutschland GmbH, Grünberg, Germany) 0.002% DMSO was added to the ethanol control. All caspase-inhibitors were dissolved in DMSO and added directly to the DHA-containing cell culture medium. The inhibition of apoptosis caused by 50 μM DHA was tested by co-incubating LT97 cells with 20 μM caspase 3-inhibitor (Ac-DEVD-CHO), caspase 8-inhibitor (Ac-IETD-CHO), caspase 9-inhibitor (AC-LEHD-CHO), or pancaspase-inhibitor (Z-VAD-CHO) for 24 h.

For FW studies, FW was diluted to yield the final concentration of 3.5% FW and afterwards sterile filtered using a 0.45 μm syringe filter before applying to LT97 cells. Supernatant was removed and fresh medium containing PUFAs or FW was applied. Preliminary experiments with FW (3.5, 5 and 10%, $n = 4$) revealed 3.5% as suitable concentration.

Flow cytometry using Annexin-V and 7-aminoactinomycin D

Cells were cultured and incubated as described above. For PUFA experiments, cell culture supernatants were removed and LT97 and HT29 cells were harvested after 24, 48 or 72 h by trypsination for 10 or 5 min, respectively and cells were collected by centrifugation (4°C, 5 min, 400 *g*). For the work with caspase-inhibitors LT97 cells were harvested as described above after 24 h co-incubation with 50 μM DHA and 20 μM of the corresponding caspase-inhibitor. Cell pellets were resuspended in 1 ml cell culture medium for determination of cell number and viability. For staining, 1 x 10^6 cells were resuspended in calcium-buffer (Beckman/Coulter GmbH, Krefeld, Germany) after centrifugation (4°C, 5 min, 400 *g*) and mixed with 10 μl 7-aminoactinomycin D (7-AAD) and 5 μl Annexin-V and incubated on ice for 15 min. Fluorescence was measured using Cell Lab Quanta™ (Beckman Coulter GmbH, Krefeld, Germany) and cells were distinguished by the presence of Annexin-V (early apoptotic), 7-AAD and Annexin-V (late apoptotic/necrotic), and 7-AAD (dead cells). Viable cells do not exert any detectable fluorescence. The distribution by percentage was calculated.

Real-time PCR targeting *Bcl-2*

After 10 or 24 h cells were harvested and RNA extracted using RNeasy Mini PLUS Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified with Nanodrop (Peqlab, Erlangen, Germany) and the RNA integrity number (RIN) was measured with Bioanalyzer (Agilent, Böblingen, Germany). No RNA was used with a RIN below 8.5.

The modulation of *Bcl-2* mRNA was performed by an independent measurement of mRNA levels. For quantitative RT–PCR-analysis we used the system of iCycler iQ (Bio-Rad GmbH, München, Germany), enabling quantitative analysis of the mRNA expression levels. Therefore, aliquots of 1.5 μg total RNA from each sample were reverse transcribed using SuperScript II reverse transcriptase (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's protocol. For each reaction 10 μl of cDNA (1:20 dilution) was mixed with PCR master mix iQ™ SYBR1 Green Supermix (SYBR Green I, iTaq DNA polymerase, reaction buffer, deoxynucleotide triphosphate mix, 10 mM $MgCl_2$, 20 nM fluorescein, and stabilisers; BioRad, Munich, Germany), and 10 pmol of the gene-specific primers for *Bcl-2* and for the reference gene *GAPDH* (Table 1) in a final volume of 25 μl. Primer annealing temperature was 57°C. All samples were analysed in duplicates and the PCR reaction mixture was set in an iCycler iQ 96-well PCR plate (Bio-Rad GmbH, Munich, Germany). The relative fluorescence signal was captured at primer nucleotide extension step of each cycle. At end of the reaction the melting curve analysis was conducted with temperature gradient from 57° to 95°C at 0.108°C/s to differentiate nonspecific primer dimer and specific amplicon. The iCycler iQ1 optical v3.0a software was utilised for obtaining the relative threshold cycle number (C_T) and the data normalisation and analysis was carried out as previously described [24]. Results were expressed as fold-change induction relative to the respective ethanol control.

Western blots targeting PARP, Bid, caspase 3, caspase 8 and caspase 9

Cells were cultured, incubated and harvested as described above. After washing in PBS, cells were chemically lysed in buffer (20 mM Tris/HCl pH 8.0, 150 mM NaCl, 10% glycerin, 2 mM EDTA, 1% nonidet P40) containing a freshly added mixture of 1 mM DTT (reducing agent), 1 mM sodiumorthovanadate (phosphatase inhibitor) and several protease inhibitors (0.5 mM pefabloc SC, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin). Total protein concentrations were determined by Bradford assay [25].

Table 1 Sequences of primers used

Gene	Sense primer 5′-3′	Antisense primer 5′-3′	Fragment size (bp)
GAPDH	acc cac tcc tcc acc ttt gac	tcc acc acc ctg ttg ctg tag	110
Bcl-2	gag gat tgt ggc ctt ctt tg	gcc ggt tca ggt act cag tc	116

Equal amounts of total protein (20–30 μg) were diluted with SDS–PAGE loading buffer (125 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerine, 0.0005% bromphenol blue, 100 mM DTT) and proteins were separated using SDS polyacrylamide gel electrophoresis (stacking gel: 3%, separating gel: 15%). After separation, the proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a wet blotting system (Bio-Rad GmbH, Munich, Germany). Subsequently, unspecific binding sites on the membranes were blocked with 5% non-fat dried milk powder (AppliChem, Darmstadt, Germany) in TBST (100 mM Tris, 150 mM NaCl, pH 7.4). Primary antibody (rabbit anti-PARP, rabbit anti-Bid, rabbit anti-caspase 3, mouse anti-caspase 8, and rabbit anti-caspase 9 (Cell Signaling, Frankfurt, Germany)) was diluted in blocking solution (1:1,000) and the membrane was incubated with antibody solutions overnight at 4°C. HRP-labelled secondary antibodies (goat anti-rabbit IgG-HRP (Cell Signaling, Frankfurt, Germany), rabbit anti-mouse IgG-HRP (Dako, Hamburg, Germany)), were diluted in blocking solution (1:1,000) and were used to visualise primary antibodies. After 1 h incubation with secondary antibodies, membranes were washed twice with TBST and once with TBS (each 5 min) incubated with ECL Plus Western Detection reagent (Amersham Biosciences Europe GmbH, Freiburg, Germany). Hyperfilm™ ECL chemiluminescence films (Amersham Biosciences Europe GmbH, Freiburg, Germany) were then placed on the membrane to visualise bands. To detect the housekeeping-protein the membranes were stripped to wash off the hybridised antibodies and subsequently incubated in the same manner with mouse anti-β-actin (1:1,000, Sigma–Aldrich, Steinheim, Germany) and secondary antibody. Again, Hyperfilm™ was used for detection of chemiluminescence. Exposed and developed films were then scanned using the Fluor-S® documentation system (Bio-Rad, Munich, Germany). Optical density units (OdU) were captured and normalised to the OdU of the β-actin band of the same sample. A fold change of the resulting values to the respective ethanol control value was calculated. Experiments were performed with three independent cell lysates to analyse statistical variance.

Statistical analysis

Statistical evaluation was performed with the GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego California, USA, www.graphpad.com). Means and SD of 3 independent repeats were calculated and afterwards statistical analysis was performed. The statistical analyses used depended on the respective experimental design and are specified in the legends of the figures.

Results

Induction of apoptosis in LT97 cells by DHA

Apoptosis was measured following cell treatment by flow cytometric measurement of stained DNA (7-AAD) together with the presence of flipped phosphatidylserine at the outer membrane layer (Annexin-V). Cells can be distinguished from each other in terms of being viable, dead, early apoptotic or late apoptotic/necrotic, respectively. Figure 1 shows that induction of apoptosis as measured by increase in early apoptosis was more pronounced in LT97 compared to HT29 cells. In LT97 cells 50 μM DHA and EPA increased the percentage of early apoptotic cells whereas higher concentrations of DHA (100 μM) resulted in substantial increases in late apoptosis/necrosis after 24 h treatment. In HT29 cells apoptosis induction was low except at the highest concentration of DHA (200 μM) which led to an increase in late apoptosis/necrosis. LA failed to induce apoptosis in both cell types. After 48 and 72 h effects on early apoptosis were less pronounced whereas late apoptosis/necrosis remained at the same level (data not shown).

The mRNA expression of *Bcl-2* markedly decreased in EPA- and DHA-treated LT97 cells (100 μM, 10 h) with DHA having a stronger effect than EPA; LA had no effect (Fig. 2). After 24 h *Bcl-2* was non-significantly reduced. In HT29 cells no effect was detectable after either 10 or 24 h.

To verify the pro-apoptotic capacity of DHA and EPA a subset of caspase were measured by Western Blot. Procaspase 8, the inactive zymogen which will then be cleaved into the active caspase 8, which is a major initiator caspase of extrinsic apoptosis, was significantly decreased by incubation with 100 μM DHA in LT97 cells (Fig. 3). EPA was less active as were the effects in HT29 cells. Again, LA failed to induce apoptosis. Decline of procaspase 9, the inactive form of the initiator caspase of the intrinsic apoptosis-pathway, was not measureable in any cell line or treatment (Fig. 3). However, decline in the uncleaved form of Bid was detected after treatment with fish-specific fatty acids in both cell types (Fig. 4) and, this loss was particularly apparent for DHA treatment in LT97 cells (50 and 100 μM, $P < 0.05$). As before, the effects were less pronounced in HT29 cells. There was a significant reduction in procaspase 3 in LT97 in response to treatment with the highest concentration of DHA (100 μM, $P < 0.05$) but no such clear effect was seen in HT29 cells (Fig. 4).

Confirmation that apoptosis had occurred was also carried out by measuring PARP cleavage. When PARP is cleaved by the terminal caspase 3, cell death is induced. Figure 5 shows that 100 μM n-3 PUFAs EPA (non-significantly) and DHA ($P < 0.01$) were able to reduce uncleaved PARP in LT97 cells. While the more tumour-like

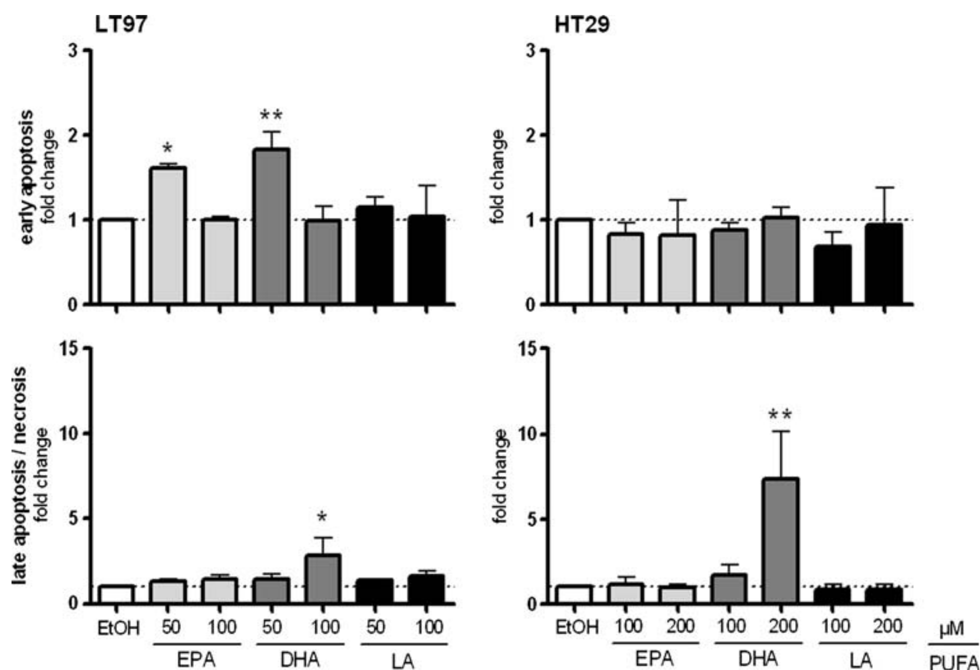

Fig. 1 Fold change of early apoptotic (*above panel*) and late apoptotic/necrotic (*below panel*) LT97 and HT29 cells treated with indicated concentrations of EPA, DHA and LA for 24 h. Relative counts of Annexin-V positive and Annexin V + 7-AAD positive cells were measured using flow cytometry. Data are given as means + SD ($n = 3$). *Asterisks* indicate significant changes relative to the ethanol control (*dashed line*), * $P < 0.05$, ** $P < 0.01$ (One Way ANOVA with Dunnett's post test)

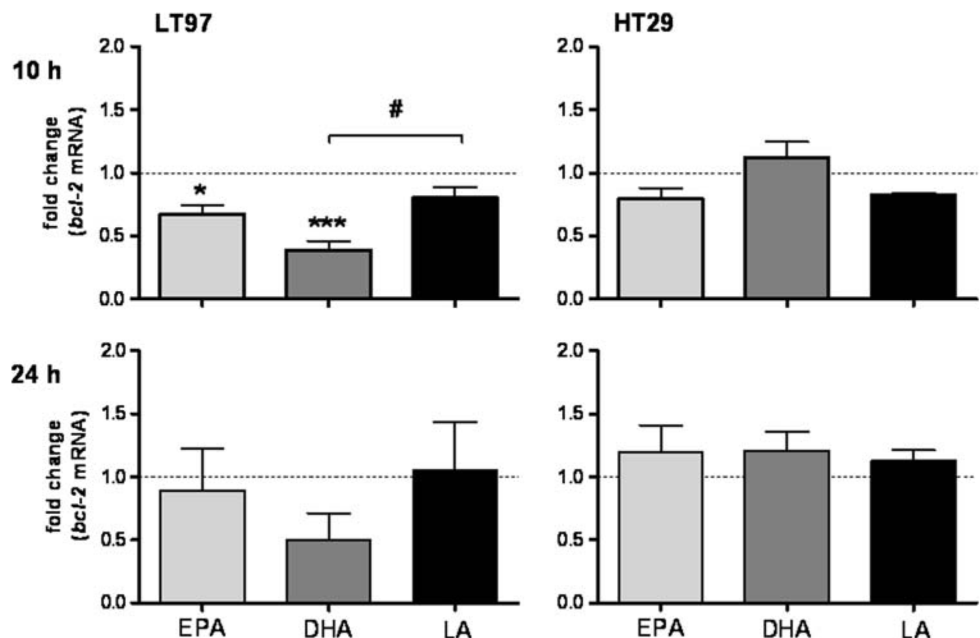

Fig. 2 *Bcl-2* mRNA expression in 100 µM EPA, DHA or LA treated LT97 cells (*left panel*) and HT29 cells (*right panel*) after 10 h and 24 h measured by Real-Time RT–PCR. Data are shown as means + SD ($n = 3$) of the fold changes (ratio treatment/EtOH). *Asterisks* indicate significant changes to the ethanol control (*dashed line*), * $P < 0.05$, *** $P < 0.001$, and the square the difference to LA # $P < 0.05$ (One Way ANOVA with Bonferroni's post test)

HT29 cells only showed induction of apoptosis by DHA at a concentration of 200 µM ($P < 0.001$). LA had no effect on PARP cleavage.

An additional experiment using caspase inhibitors was included to verify the role of the caspases examined in DHA-driven apoptosis (Fig. 6). Analogous to the DHA-caused apoptosis in Fig. 1, LT97 cells were treated with 50 µM DHA for 24 h. The detected level of early apoptosis by flow cytometry was found to decrease when co-incubating the cells with caspase 3-inhibitor and the caspase family-inhibitor. This is in agreement with Fig. 3 which reveals the role of caspase 3 in DHA-driven apoptosis. The addition of inhibitors for caspase 8 and caspase 9 resulted only in a marginal inhibition of apoptosis.

No induction of apoptosis by FW following lean or fatty fish consumption

FW was tested for apoptosis-inducing potential in LT97 cells. Cells were treated for 24 h with 3.5% FW and afterwards apoptosis was measured by staining cells with Annexin-V/7-AAD and detection of resulting dye

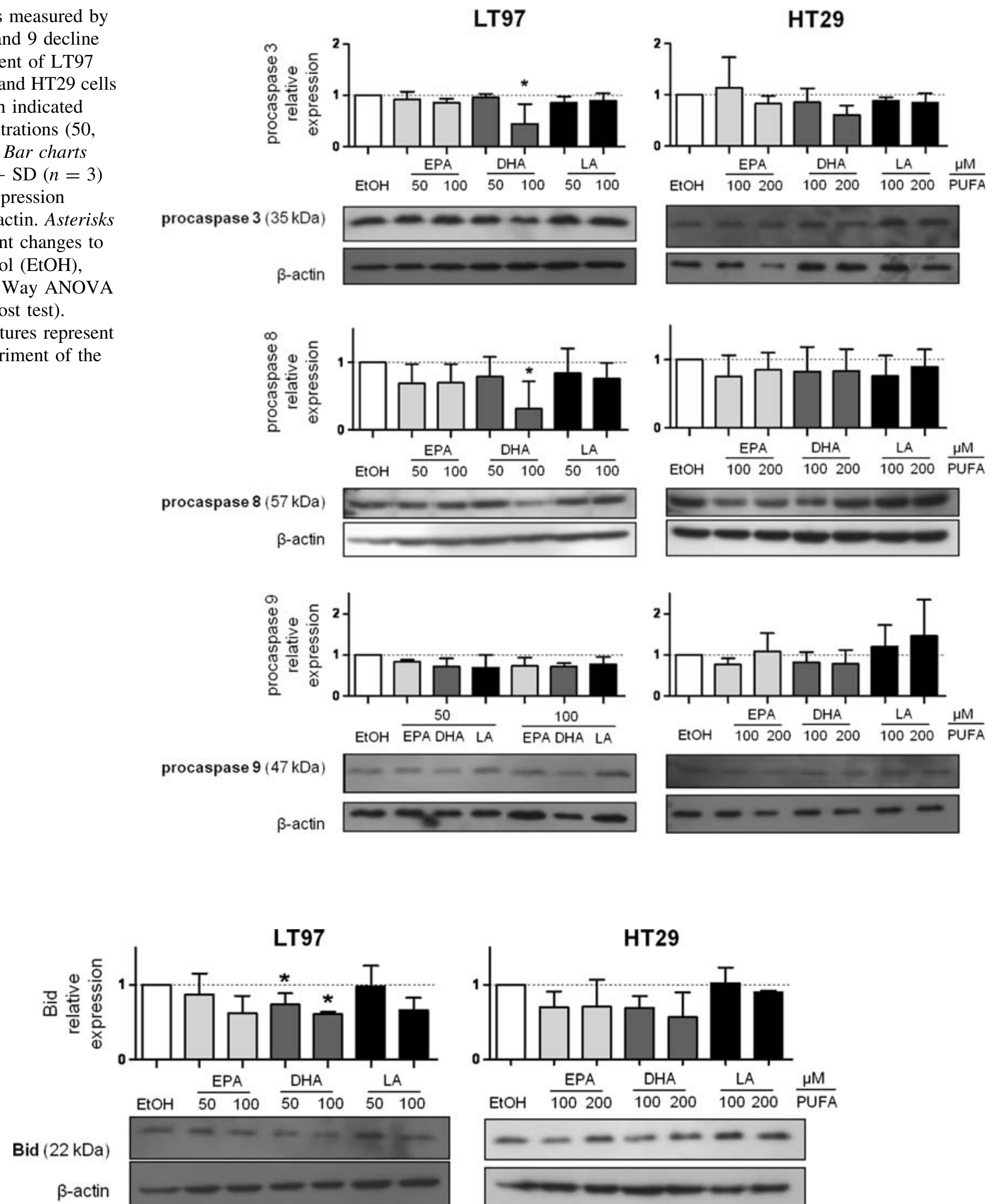

Fig. 3 Apoptosis measured by procaspase 3, 8 and 9 decline after 24 h treatment of LT97 cells (*left panel*) and HT29 cells (*right panel*) with indicated fatty acid concentrations (50, 100 or 200 μM). *Bar charts* represent mean + SD ($n = 3$) of the relative expression normalised to β-actin. *Asterisks* indicate significant changes to the ethanol control (EtOH), * $p < 0.05$ (One Way ANOVA with Dunnett's post test). Western Blot pictures represent an example experiment of the triplicates

Fig. 4 Apoptosis measured by Bid cleavage after 24 h treatment of LT97 cells (*left panel*) and HT29 cells (*right panel*) with indicated fatty acid concentrations (50, 100 or 200 μM). *Bar charts* represent mean + SD ($n = 3$) of the relative expression normalised to β-actin. *Asterisks* indicate significant changes to the ethanol control (EtOH), * $P < 0.05$ (One Way ANOVA with Dunnett's post test). Western Blot pictures represent an example experiment of the triplicates

fluorescence using a flow cytometer. The mean (SD) detected level of early apoptotic cells before intervention was 31.9 (14.4)%. As the participants' health status did not influence the changes in apoptosis (data not shown) Fig. 7 only shows detected changes in apoptosis (after intervention minus before intervention) for the three different intervention groups (salmon, cod and dietary advice). However, there was no detectable modulation of apoptosis-inducing potential of FW after fish-consumption.

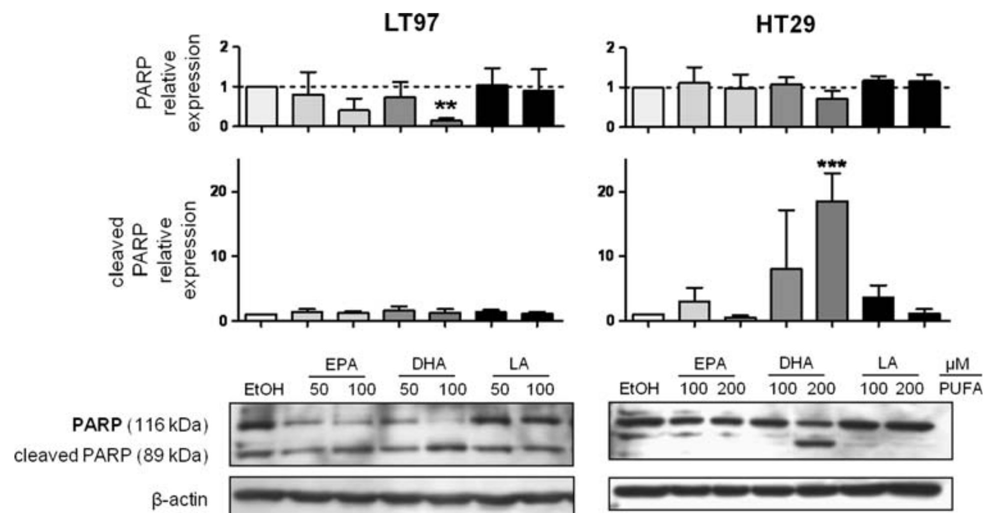

Fig. 5 Apoptosis measured by PARP cleavage after 24 h treatment of LT97 cells (*left panel*) and HT29 cells (*right panel*) with indicated fatty acid concentrations (50, 100 or 200 μM). *Bar charts* represent mean + SD ($n = 3$) of the relative expression normalised to β-actin. *Asterisks* indicate significant changes to the ethanol control (EtOH), ** $P < 0.01$. *** $P < 0.001$ (One Way ANOVA with Dunnett's post test). Western Blot pictures represent an example experiment of the triplicates

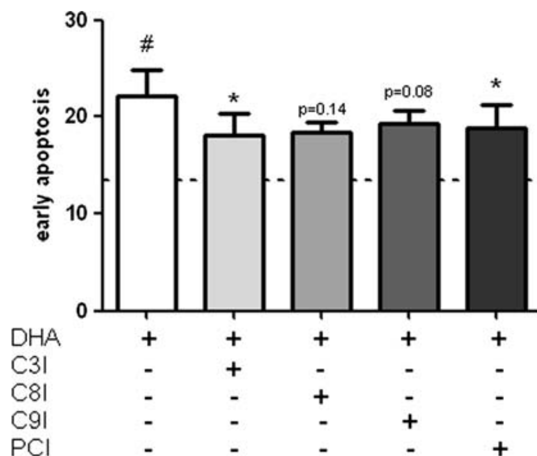

Fig. 6 The percentage of cells in early apoptotis for both LT97 and HT29 cells treated with 50 μM DHA 24 h in combination with either caspase 3-inhibitor (C3I), caspase 8-inhibitor (C8I), caspase 9-inhibitor (C9I), or pancaspase-inhibitor (PCI) as indicated. Relative counts of Annexin-V positive and Annexin V + 7-AAD positive cells were measured using flow cytometry. Data are given as means + SD ($n = 4$). *Asterisks* and *P* values indicate changes relative to DHA, the square indicates a significant change to the ethanol + DMSO control (*dashed line*), # or * $P < 0.05$ (*t* test)

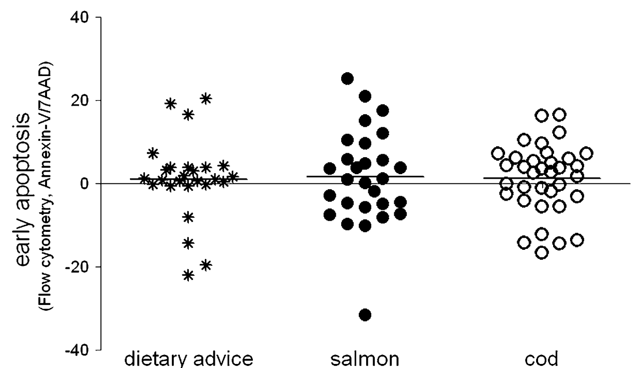

Fig. 7 Early apoptotic LT97 cells caused by FW (3.5%) treatment for 24 h. Annexin-V positive cells were measured using flow cytometry (relative counts). The data show individual results for the change in apoptosis (after intervention minus before intervention) after intervention with salmon ($n = 28$), cod ($n = 35$), and dietary advice ($n = 26$)

Discussion

It has been reported that elevated *n*-3 PUFA intake over 2 years results in markedly increased apoptosis measured using TUNEL assay and immunohistochemical staining of bax, in the colonic mucosa of humans [6]. In this study we have focussed on expanding our understanding of how apoptosis may be mediated and, by comparing two cell lines, one composed of relatively well differentiated adenoma cells (LT97), the other the carcinoma cell line HT29, investigated at what stage in the colorectal carcinoma sequence fish oil is likely to be most protective.

It is hypothesised that PUFA incorporation into cell membranes increases susceptibility towards oxidative damage, and hence cells undergo apoptosis. Furthermore it is probable that an alteration of the membrane composition by *n*-3 PUFA enrichment might also influence downstream signalling pathways and so support apoptosis [26–30]. An increase of *n*-3 PUFAs in cell membranes has previously been demonstrated to occur in humans eating a high-fish diet [31, 32] as well as in rats provided a fish-oil enriched feed [33] and in vitro by supplementing cell cultures with PUFAs [34]. Thus, it is already acknowledged that DHA and EPA in vitro are likely to exert apoptosis-inducing effects in human colon adenocarcinoma cells such as HT29 and Caco-2 [28, 30, 35–37]. In addition fish-oil causes apoptosis in rat models [9, 33]. It was shown that DHA leads to modulation of PI3 kinase and p38 MAPK pathways [28, 30] and a down regulation of Bcl2 and increased caspase 3 activity [35, 37].

Increased apoptosis was detected in the present study using flow cytometry 24 h after treatment (figure 1) in LT97 colon adenoma cells. More cells were found in early apoptosis in response to 50 μM DHA and EPA while higher concentrations (100 μM) caused an enhancement in late apoptosis/necrosis. Analyses at 48 and 72 h did not result in such clear effects (data not shown) and the tumour-like HT29 cells did not show elevated levels of early apoptosis. Our results (Fig. 5) show that both colon adenomatous and adenocarcinoma cells catalyse PARP-cleavage when treated for 24 h with the *n*-3 PUFA DHA, so the effects detected by flow cytometry appear to be late apoptotic events rather than necrosis. Alternatively, since caspase-dependence has previously shown in PUFA-treated HT29 cells [33], the detected PARP cleavage in HT29 cells may reflect an earlier caspase dependent signalling by DHA not detectable in the time frame of this experiment. In LT97 cells the intrinsic apoptosis pathway was triggered by DHA treatment. Our observation in LT97 cells that addition of caspase inhibitors (for all caspases and for caspase 3) decreased the level of early apoptosis supports the hypothesis that caspases, particularly the effector caspase 3, are involved in DHA mediated apoptotic cell death. The flow cytometry data only suggested a trend towards a possible involvement of caspase 8 and caspase 9.

Procaspase 8 was cleaved (Fig. 3), which is likely to have resulted in the elevation of the active form of caspase 8 while there was a marked decrease in the level of Bid for DHA (Fig. 4) and again, the effects on HT29 adenocarcinoma cells were less clear-cut. In the mitochondria, tBid oligomerises with itself and induces the oligomerisation of Bax and Bak which is accompanied by cytochrome c leakage from the mitochondria. It is hypothesised that Bcl-2 suppresses tBid insertion to the mitochondrial membrane which thus results in the preservation of mitochondrial integrity [38]. Therefore our data suggests that DHA > EPA facilitates cytochrome c release in colon adenoma cells first, by downregulation of *Bcl-2* and second, by enhanced proteolytic cleavage of Bid by caspase 8 resulting in tBid production.

We have also shown that DHA is more active than EPA in inducing apoptosis, especially in LT97 cells. Apart from the carbon chain length, the main difference between these two *n*-3 PUFAs is the higher degree of unsaturation of DHA. These double bounds can also serve as a substrate for peroxidation reactions resulting in reactive oxygen species and a pro-oxidant environment [39] which has been shown to induce apoptosis [40]. It has also been shown that increasing the antioxidant potential of cells (e.g. by accumulating ascorbic acid or induction of catalase) can diminish TRAIL- or Fas-induced apoptosis in cancer cells [41, 42] and that lipid soluble antioxidants preloaded into cells block EPA induced apoptosis [33].

Efficient absorption of fatty acids, predominantly in the jejunum and ileum, allows less than 5% of the ingested lipids to reach the colon [43], equivalent to about 15 mmol/l [44]. However this is likely to be rapidly metabolised by the luminal bacteria and the significant increase of *n*-3 PUFAs in colonic mucosa after alimentary supplementation is assumed to be absorbed from the blood where concentrations may reach as high as 400 μmol/l following supplementation [45–47]. We have therefore taken a novel ex vivo biomarker approach to investigate this possibility. To do this we have treated the human colon adenoma cell line LT97 with FW from an intervention study. Human intervention studies measuring apoptosis in vivo are scarce as collection of biopsies is invasive and time consuming on a large scale. Thus, we aimed to use a non-invasive biomarker and, as FW is often used in the context of colon cancer chemoprevention ex vivo, e.g. in Comet Assay studies measuring health beneficial effects of different diets by alteration of DNA-damaging potential [48], we used a similar approach to investigate apoptosis. However, we were not able to detect any additional apoptosis-inducing effects after supplementary consumption of oil-rich or lean fish (salmon or cod, respectively), so one could argue, that this biomarker is not an appropriate one. However, in the same study we also found no effect of fish-supplementation on apoptosis in colonic biopsies from the same volunteers; the reasons as to why no effect was found are discussed by Pot et al. [16]. The validity of this method should be tested in future in a situation where increased apoptosis in the mucosa is established, perhaps using symbiotics [49]. We found no correlation between apoptosis induction after treatment of LT97 cells ex vivo with FW and apoptosis detected in biopsies from the same volunteers [16] (Spearman correlation coefficient was found to be $r = 0.19$; data not shown).

Taken together, our results show pro-apoptotic effects of DHA on LT97 human colon adenoma cells in vitro. The less unsaturated fatty acid EPA is less effective and the more transformed cell line HT29 is less susceptible. Thus, DHA might be the more effective chemopreventive *n*-3 PUFA found in fish oil and fish probably with greatest effect on apoptosis at earlier stages in the adenoma-carcinoma sequence. However, effects of the *n*-3 PUFAs and fish still need to be clarified in humans in vivo.

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3.5 Publication V: G.K. Pot[1] / N. Habermann[1], G. Majsak-Newman, L. J. Harvey, A. Geelen, K. Przybylska-Philips, F. M. Nagengast, B. J. M. Witteman, P. C. van de Meeberg, A. R. Hart, G. Schaafsma, G. Hooiveld, M. Glei, E. K. Lund, B. L. Pool-Zobel, E. Kampman: **"Increasing fish consumption does not affect genotoxicity markers in the colon in an intervention study."** accepted by Carcinogenesis, DOI: 10.1093/carcin/bgp255

Observational studies suggest that fish consumption is associated with a decreased colorectal cancer risk. One mechanism is probably by decreasing luminal genotoxic burden. Therefore, the objective was to investigate the effects of fish on genotoxicity markers in the colon in a randomised controlled parallel intervention study. For a period of six months, subjects were randomly allocated to receive two extra weekly portions of oil-rich fish (salmon), lean fish (cod), or dietary advice. The Comet Assay was used to measure the DNA damage-inducing potential of faecal water (n=89) and DNA damage in colonocytes (n=70) collected pre- and post-intervention as markers of genotoxicity.
Genotoxicity of faecal water was not markedly changed after fish consumption. DNA damage in colonocytes was also not significantly changed after fish consumption compared with the dietary advice group. Measurements of genotoxicity of faecal water and DNA damage in colonocytes did not correlate. In conclusion, increasing consumption of either oil-rich or lean fish did not affect genotoxicity markers in the colon.

Own contribution to the manuscript:

- co-work on experimental design
- establishment of the Comet Assay in both study centres (Wageningen, NL and Norwich, UK)
- data analysis of Comet Assay results
- co-work on statistical evaluation
- co-work on presentation of results and interpretation
- co-compilation of the manuscript

[1] shared first author/equal contribution

Increasing fish consumption does not affect genotoxicity markers in the colon in an intervention study

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35 **Abstract**

36 Observational studies suggest that fish consumption is associated with a decreased colorectal
37 cancer (CRC) risk. A possible mechanism by which fish could reduce CRC risk is by decreasing
38 colonic genotoxicity. However, concerns have also been raised over the levels of toxic
39 compounds found in mainly oil-rich fish, which could increase genotoxicity. Therefore, the
40 objective was to investigate the effects of fish on genotoxicity markers in the colon in a
41 randomized controlled parallel intervention study. For a period of six months, subjects were
42 randomly allocated to receive two extra weekly portions of (i) oil-rich fish (salmon), (ii) lean fish
43 (cod), or (iii) just dietary advice. The Comet Assay was used to measure the DNA damage-
44 inducing potential of fecal water (n=89) and DNA damage in colonocytes (n=70) collected pre-
45 and post-intervention as markers of genotoxicity.
46 Genotoxicity of fecal water was not markedly changed after fish consumption: 1.0% increase in
47 tail intensity (TI) (95% confidence interval (CI) -5.1; 7.0) in the salmon group and 0.4% increase
48 in TI (95% CI -5.3; 6.1) in the cod group compared with the dietary advice group. DNA damage
49 in colonocytes was also not significantly changed after fish consumption, in either the salmon
50 group, (-0.5%TI, 95% CI -6.9; 6.0), or cod group (-3.3%TI, 95% CI -10.8; 4.3) compared with the
51 dietary advice group. Measurements of genotoxicity of fecal water and DNA damage in
52 colonocytes did not correlate (r=0.06, n=34). In conclusion, increasing consumption of either oil-
53 rich or lean fish did not affect genotoxicity markers in the colon.

54

55 ## Introduction

56 Colorectal cancer (CRC) is one of the most commonly occurring cancers worldwide and has
57 been associated with dietary habits [1]. CRC develops over many years as a result of
58 accumulation of DNA damage and mutations, resulting in a loss of control of cell proliferation
59 and failure of damaged cells to undergo apoptosis [1,2]. DNA damage is thought to be caused
60 by genotoxic insults and factors in the diet may modulate genotoxicity in the colon. One of the
61 dietary habits that possibly influences the risk of CRC is consumption of fish. Several
62 observational studies have shown that high intakes of fish could be related to a decreased risk
63 of CRC [1,3-7]. This potential benefit on CRC could be mediated by apoptosis and mitosis,
64 which has been shown in several intervention studies [8-12]. Genotoxicity could be decreased
65 by the intake of fish by modulation of enzymes involved in detoxification of phase I or II
66 enzymes like glutathione S-transferase [13], by a decrease in inflammatory processes via
67 oxidative stress pathways [14,15], or by decreasing the bacterial conversion of bile acids into
68 more genotoxic secondary bile acids [16,17].

69 Whilst the focus has been on the beneficial effects of fish consumption, concerns have been
70 raised as to whether it could also have unfavorable effects, due to the possible presence of
71 toxins. Toxic compounds such as dioxins or polychlorinated biphenyls (PCBs), which can
72 accumulate in the food chain and which are mostly found in oil-rich fish [18,19], could increase
73 colonic genotoxicity. Although mostly associated with beneficial effects, n-3 polyunsaturated
74 fatty acids (PUFA), highly abundant in oil-rich fish, could potentially increase genotoxicity as
75 they are readily oxidized and could enhance lipid peroxidation [20] and oxidative stress [21]
76 leading to an increase in endogenous DNA damage. Thus, oil-rich fish could have differential
77 effects on CRC risk compared with lean fish. To the best of our knowledge, no intervention
78 study has been performed examining the genotoxic effects of consumption of either oil-rich or
79 lean fish in the colon.

80 Colorectal genotoxic effects can be measured indirectly by determining the DNA-damage
81 inducing potential of fecal water in human colon adenocarcinoma cells (e.g. HT29 cells) *in vitro*,
82 or directly by measuring DNA damage in colonocytes extracted from colorectal biopsies *in vivo*.
83 Fecal water represents the aqueous fraction of the feces and diet has been shown to affect
84 fecal water genotoxicity [22-25]. Moreover, it has been demonstrated that fecal water can
85 influence processes related to colorectal carcinogenesis, such as apoptosis [26] and

86 proliferation [27]; patients with colorectal polyps differ in fecal water biochemistry compared with
87 healthy controls [27]. However, it is not clear how genotoxicity of fecal water translates to DNA
88 damage in the colonic epithelium, and ultimately we are interested in the processes in the colon.
89 Therefore, we also included measurements of DNA damage in colonocytes. To sum up, the aim
90 of the current study was to study the effects of fish consumption on markers of genotoxicity in
91 the colon.
92

93 **Subjects and Methods**

94 *Subjects and study design*

95 The design of the study was a multi-centre parallel randomized controlled intervention study and
96 has been described in detail elsewhere (GK Pot et al, in preparation). Three groups of subjects
97 were recruited: those with (previous) colorectal polyps, those diagnosed with non-active
98 ulcerative colitis (UC), and those without any macroscopic signs of disease in the colon. After
99 an initial colonoscopy procedure, 242 eligible subjects were randomly allocated by an
100 independent person to one of three dietary intervention groups: (i) oil-rich fish group receiving
101 two 150g portions of farmed salmon per week for six months, (ii) lean fish group receiving two
102 150g portions of Icelandic cod per week for six months, and (iii) dietary advice (DA) group. All
103 three intervention groups received dietary advice on achieving a healthy diet [28,29]. The fish
104 was delivered to the participants in their home and they were instructed to consume it in
105 addition to their regular fish intake. We provided the participants with fish from the same batch
106 as much as possible.

107 The fatty acid content of the fish provided to the participants was measured using established
108 methods, in the different batches of fish [30]. Salmon provided approximately 3.3g of long chain
109 n-3 PUFA (eicosapentaenoic acid [EPA] + docosahexaenoic acid [DHA]) per 100g fish and for
110 cod this was 0.2g/100g fish. Furthermore, we measured 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
111 (TCDD) equivalents (TEQ) in the fish provided, in a pooled sample of batches provided to the
112 participants [31]. Salmon contained 0.45 TCDD equivalents (TEQ)/g fish and cod contained
113 0.04pg TEQ/g fish. We chose a study duration of six months since this would be sufficient to
114 incorporate n-3 PUFAs into the colonic epithelium [32]. Compliance was checked by food
115 diaries and regular phone calls every two to four weeks, and in the salmon group by serum
116 levels of the long chain n-3 PUFA.

117 Feces was collected by a subgroup (n=128) of the subjects 1-3 weeks prior to the collection of
118 colonic biopsy samples; 89 pairs of pre- and post-intervention fecal samples were randomly
119 selected among those who collected feces and processed for the Comet Assay. Colorectal
120 biopsy samples were collected at baseline during a colonoscopy procedure and post-
121 intervention during a sigmoidoscopy procedure. The preparation of the colonoscopy procedure
122 consisted of Macrogol (Kleanprep, Norgine BV, Amsterdam, NL) in NL, or Picolax (Ferring
123 Pharmaceuticals Limited, Berkshire, UK) in UK; the preparation of sigmoidoscopy procedure

124 consisted of an enema in both NL and UK. Distal colon biopsies were obtained from mucosa of
125 normal-appearance at about 20- 30cm from the anal verge during the colonoscopy or
126 sigmoidoscopy.
127 A subset of complete sets of pre- and post-intervention samples collected in the Netherlands
128 (NL) could be used for the Comet Assay (n=70) based on practical reasons associated with the
129 need to process colorectal biopsies within 24h. A total of 34 subjects had DNA damage
130 measured both in fecal water-treated HT29 cells and in colonocytes at baseline.
131 Fasted blood samples were taken on the day of the colonoscopy or sigmoidoscopy procedure
132 and serum was stored at -80ºC prior to analysis. Serum cholesteryl fatty acids were measured
133 as previously described, in all three intervention groups [30].
134 Life style factors including smoking, weight and height measures were obtained by
135 questionnaire at the start; overall diet and specifically the frequency of fish consumption was
136 assessed pre- and post-intervention by questionnaire.
137 Ethical approval was obtained from the Medical Ethical Committee of Nijmegen University
138 Medical Centre St. Radboud in NL (reference 2004/111) and King's Lynn Local Research Ethics
139 Committee in the United Kingdom (UK) (reference 04/Q0105/8). The trial has been registered at
140 www.clinicaltrials.gov under identifier NCT00145015. All subjects gave their written informed
141 consent and a subset of subjects consented separately for collection of fecal samples.
142
143 *Preparation of fecal water and treatment of HT29 cells*
144 The DNA-damage inducing potential of fecal water in the colonic HT29 cell line was determined
145 as previously described [33]. Cells (passage 14-46) were grown to 70-85% confluence as a
146 monolayer [34] before harvesting. A total fecal sample from one bowel movement was collected
147 and stored in a cooled container for transport to the laboratory within 4h. At the laboratory,
148 samples were homogenized, aliquoted and stored at -80ºC until further processing. Samples
149 were defrosted, homogenised by stirring, mixed with the same amount (w/v) of ice chilled PBS
150 and centrifuged at 25,000xg for 2h at 4°C as described previously [35]. The pre- and post-
151 intervention samples from individual volunteers were analyzed in parallel. After incubation
152 (30min at 37°C) of the cells with fecal water (50% concentration) or control treatments (PBS or
153 75μM H_2O_2) an aliquot was taken and cell viability was assessed by staining the cells with
154 Trypan blue and dead and viable cells were counted using a haemacytometer.

155

156 *Preparation of colonic biopsies*

157 Primary colonocytes were isolated from colonic biopsies within 24h of the endoscopy procedure
158 and kept in Hank's balanced salt solution (HBSS) at 4ºC, as described previously [36]. Briefly,
159 biopsy samples were incubated with 1mg/ml collagenase P and 2mg/ml proteinase K in HBSS,
160 for approximately 90min at 37ºC. The suspension was centrifuged for 5min at 400xg. The pellet
161 was re-suspended in fresh HBSS for further processing. Cell numbers and viability were
162 determined using the Trypan blue exclusion test [35].

163

164 *Alkaline Comet Assay*

165 DNA damage was determined by the Comet Assay as previously described [37] and expressed
166 as % tail intensity (TI), with a higher score indicating more DNA damage. For the Comet Assay
167 in colonocytes, a single batch of HT29 cells which had been treated with or without a known
168 genotoxin (75µM H_2O_2) served as positive and negative control. Colonocytes ($2x10^6$) were
169 mixed with 50µl 0.7% low-melting agarose and distributed onto microscope slides (Trevigen,
170 Gaithersburg, US). Alternatively, fecal water treated HT29 cells ($2x10^6$) were distributed in 90µl
171 0.7% low-melting agarose on agarose pre-covered microscope slides and after 10min covered
172 with another layer of agarose. Slides were then immersed into lysis solution (pH 10) for at least
173 1h. Cells were allowed to unwind in alkaline electrophoresis solution (pH>13) for 20min prior to
174 start of the 20min electrophoresis (pH>13, 300mA and 20V). The slides were removed from the
175 alkaline solution and washed three times with neutralization buffer (pH 7.5). Only slides with
176 colonocytes or controls were treated with 99% ethanol for 10min allowing them to dry prior to
177 storage and shipment. All slides were stained with SYBR Green (2 µg/ml, Trevigen Inc.) and
178 microscopical images were quantified using the image analysis system of Perspective
179 Instruments (Halstead, UK); 50 images were evaluated per slide and the percentage of
180 fluorescence in the tail, %TI was scored. For fecal water genotoxicity mean values of three
181 parallel slides were determined with a coefficient of variation (CV) of 23-26%,and for
182 colonocytes mean values of four replicate slides were determined with a CV of 26-27%. All
183 slides were scored by a single scorer in a blinded manner.

184

185

186 *Statistical analyses*

187 Changes in outcome variables were evaluated using an analysis of covariance (ANCOVA)
188 adjusting for baseline values. The data of the baseline and end measurements were not
189 normally distributed, while the changes in outcome measures were normally distributed. We
190 compared the changes in outcome measures in the salmon and cod group with the changes in
191 the DA group and therefore changes are presented as mean change compared to DA (with a
192 95% confidence interval). We explored if fecal water genotoxicity correlated with measurements
193 of DNA damage in colonocytes using the Spearman correlation coefficient (n=34) and by cross-
194 classification based on the median value. We also explored whether results were different
195 between smokers and non-smokers as it has been shown that smoking could affect genotoxicity
196 [24,38]. We performed analyses using the SAS statistical software program (SAS version 9.1)
197 and considered a p-value <0.05 as significant. The researchers performing the statistical
198 analyses were blinded to the treatment and patient group.

199

200 **Results**

201 Baseline characteristics of subjects whose fecal water was used for the Comet Assay are
202 shown in Table I. The DA group included more women compared with the salmon and cod
203 group. The cod group had a lower percentage of current smokers and was less physically
204 active. Further, subjects in all three intervention groups did not differ in terms of age or BMI.
205 Subjects in whom DNA damage was determined in colonocytes (n=70, data not shown) were
206 comparable in terms of age and BMI. The cod group included somewhat fewer females, no
207 current smokers, and was more physically active. The DA group included fewer polyp patients
208 compared with the salmon or cod group.

209

210 Baseline values of genotoxicity in fecal water was 9.9 ± 7.5% TI in the salmon group, 7.5 ±
211 6.2%TI in the cod group, and 13.9 ± 10.4% TI in the DA group. DNA damage in colonocytes
212 was 17.8 ± 11.1% TI in the salmon group, 17.6 ± 7.0% TI in the cod group, and 15.0 ± 7.5% TI
213 in the DA group at baseline.

214 The Spearman correlation coefficient between fecal water genotoxicity and DNA damage in
215 colonocytes was 0.06 (n=34). The overlap of the cross-classification based on the median
216 values of genotoxicity of fecal water and DNA damage of colonocytes was 19 out of 34 subjects
217 (56%).

218

219 At baseline genotoxicity was higher in smokers than in non-smokers: for fecal water genotoxicity
220 values were 13.6 ± 8.3% TI for smokers (n=15) and 9.3± 8.2% TI for non-smokers (n=74,
221 p=0.07); for colonocytes these values were 18.0 ± 13.9% TI for smokers (n=14) and 16.6 ±
222 8.2% TI for non-smokers (n=55, p=0.63).

223

224 Before the start of the intervention, subjects (n=89) consumed on average 1.1 ± 0.8 portions fish
225 per week. Fish consumption increased by 0.7 ± 0.6 weekly portions in the salmon group, 0.9 ±
226 0.9 in the cod while the DA group changed their fish consumption with 0.2 ± 0.9 weekly portions
227 of fish.

228 Subjects in whom DNA damage was measured in colonocytes (n=70) consumed on average
229 0.8 ± 0.6 portions fish per week at baseline. Fish consumption increased by 0.8 ± 0.7 weekly
230 portions in the salmon group and 0.4 ± 0.7 weekly portions in the cod group while the DA group

231 changed their fish consumption by 0.0 ± 0.4. Overall, diet was not changed in any of the
232 intervention groups (results not shown).
233 Subjects in all three intervention groups had similar levels of serum n-3 VLC-PUFA at baseline,
234 as is shown in **Table II**. Serum n-3 VLC-PUFA levels significantly increased in the salmon group
235 and not in the cod or DA group compared to baseline. This indicated that subjects in the salmon
236 group generally complied with the salmon intervention.
237
238 *Fecal water*
239 At baseline, viabilities of the HT29 cells after 30min incubation with 50% fecal water ranged
240 from 62 to 100%, post-intervention this ranged from 61 to 100%.
241 Changes in genotoxicity of fecal water are presented in Figure 1A. We observed changes of 5.8
242 ± 10.6% TI (mean ± SD) in the salmon group, 5.3 ± 10.4% TI in the cod group, and 4.8 ± 11.9%
243 TI in the DA group. The changes in the salmon and cod group compared with DA were 1.0%TI
244 (95% CI -5.1; 7.0) and 0.4%TI (95% CI -5.3; 6.1), respectively.
245
246 *Colonocytes*
247 At baseline, viabilities of the fresh colonocytes ranged from 70 to 100%, post-intervention this
248 ranged from 84 to 100% indicating that cells were viable for DNA damage measurement.
249 Changes in DNA damage measured in colonocytes are presented in Figure 1B. We observed
250 changes of -0.1 ± 14.3% TI in the salmon group, -2.9 ± 9.8% TI in the cod group, and 0.3 ±
251 8.4% TI in the DA group. After intervention, levels of DNA damage in colonocytes were not
252 changed compared with DA in the salmon group, -0.5%TI (95% CI -6.9; 6.0), or in the cod
253 group, -3.3%TI (95% CI -10.8; 4.3).
254

255 ## Discussion

256 This intervention study showed that increasing fish consumption for six months neither
257 positively nor negatively altered the genotoxicity of fecal water or DNA damage in colonocytes
258 and that results of both measurements did not correlate. To the best of our knowledge, this is
259 the first intervention study that has examined the effects of increasing fish consumption on
260 genotoxic effects in the colon, in both fecal water and colonocytes. We included two types of
261 fish in this trial to study the possible differential effects of oil-rich and lean fish but observed no
262 marked differences between the two types.

263

264 The Comet Assay, which we used in this trial, is considered to be a rapid, simple, economical,
265 and sensitive method to measure DNA damage [39-41]. However, the Comet Assay also has
266 limitations [42]. First, it does not specifically identify the type or site of DNA damage, and
267 second, based on the variation in responses we observed in this study, it appears that a large
268 number of subjects would be necessary to detect subtle effects of for example nutritional
269 interventions. In comparison to previous nutritional intervention studies on DNA damage,
270 however, the number of subjects in this intervention study was comparable [43] or larger
271 [24,44]. In general, there are several aspects that contribute to the internal validity of the Comet
272 Assay [45]. By including positive and negative controls throughout the study, by including a
273 control group (DA group) to compare our results with, and by scoring the slides and analyzing
274 the data in a blinded manner, we have optimized our internal validity.

275

276 In most dietary intervention studies, blood lymphocytes have been used to study the effects of
277 diet on DNA damage. However, DNA damage in lymphocytes does not necessarily represent
278 DNA damage in all cells, tissues, or organs [42,45,46]. Since we are specifically interested in
279 colorectal carcinogenesis, we measured the genotoxicity of fecal water and DNA damage in
280 colonocytes. In previous studies, genotoxicity of fecal water has been considered a suitable
281 marker for the assessment of the rapid changes in genotoxicity in the gut in response to diet
282 [27,37,47]. Previous studies demonstrated that several specific dietary modifications could
283 influence fecal water genotoxicity [23,24,37,48,49], while other studies showed no effect
284 [24,25,37,44,48]. An advantage of using fecal water genotoxicity in intervention studies is that
285 this does not require an invasive procedure to obtain biological material, which decreases the

286 burden on participants. However, the use of fecal water as a marker of genotoxicity also is
287 restricted. For example, for patients with active UC, constipation or other bowel complaints it
288 might not be feasible to collect feces. In our study, we were only able to collect feces from about
289 half of the subjects; a possible reason for this could be that subjects who did not collect feces
290 had more bowel complaints compared with those who did collect feces. It may be possible that
291 more pronounced effects would have been observed in their feces.
292 More importantly, we would like to know the effect of diet specifically in the colon itself. Only one
293 previous dietary intervention study has assessed DNA damage in colonocytes, which
294 demonstrated that levels of colorectal DNA damage were reduced after intervention with a
295 synbiotic preparation containing both pre- and probiotics for 12 weeks [43]. Thus, measuring
296 DNA damage in colonocytes is applicable in intervention studies, though due to the invasive
297 method of obtaining tissue this method is not always preferred or possible. Therefore, we also
298 explored the correlation between measurements of fecal water genotoxicity and DNA damage in
299 colonocytes, but found no agreement between these methods. One reason for this could be that
300 genotoxicity of fecal water and DNA damage in colonocytes do not measure exactly the same
301 endpoint; the fecal water induced DNA-damage represents the genotoxic burden of excreted
302 feces, which is mainly modulated by dietary exposure and processes of the gut flora [50],
303 whereas the DNA damage measured in colonocytes also reflects the effects in the cells which
304 additionally depends on the expression of biotransformation enzymes or the extent of DNA
305 repair mechanisms [51]. However, since the number of subjects in whom both outcomes were
306 measured was low (n=34), this analysis was explorative, and needs to be confirmed in larger
307 studies.
308

309 A limitation of this study was that subjects were all fish consumers at baseline. Subjects in
310 whom fecal water genotoxicity was determined consumed 1.1 ± 0.8 portions of fish week before
311 the start of the intervention. It seems inevitable that in recruiting subjects to an intervention
312 study with fish, mainly fish consumers will volunteer. Another limitation was that whilst the
313 subjects were asked to increase their consumption of salmon or cod by two portions per week,
314 the actual average increase was ranged from 0.7 to 0.9 weekly portions of fish, probably due to
315 the relatively high habitual fish consumption at baseline. This resulted in smaller differences

316 between the fish intervention groups and the DA group than anticipated which could have led to
317 smaller effects of the intervention.
318 Another methodological issue was the use of different bowel preparations pre- and post-
319 intervention. Previous studies have shown that bowel preparation can affect cell proliferation
320 levels in the colon [52], but if this could be expected to affect DNA damage is not known.
321 However, since results are presented as changes in the fish intervention groups as compared
322 with changes in the dietary advice group, where the same protocol of bowel preparation was
323 used, this could not have affected the outcome of the study.
324 An additional limitation was the coincidental imbalance in numbers of smokers between the
325 intervention groups. We observed higher levels of DNA damage in current smokers compared
326 with non-smokers in both fecal water and colonocytes; however, the study lacked power to
327 further investigate the possible effect modification of smoking in our data. It is known that
328 smokers may differ in their enzyme expression of detoxifying enzymes [38] and it could be that
329 smokers respond differently to a possible beneficial diet compared with non-smokers [24].
330 Subjects also differed in baseline levels of physical activity, but since we do not expect that this
331 could have affected our results, we did not further explore this.
332 A strength of this study was that we included two types of fish, salmon and cod, though we did
333 not observe differential effects on genotoxicity. It has been hypothesized that the possible
334 beneficial effects of fish could be outweighed by potential unfavorable effects by toxins,
335 peroxidation, or oxidative stress [53]. We only measured the levels of dioxin equivalents and
336 found that the levels of dioxin equivalents in intervention fish were well below the current
337 maximum tolerable intake of 8 pg TEQ/100g fish [54], and thus the unfavorable effects in this
338 fish intervention due to toxins were considered to be small. However, more studies are needed
339 to further investigate the effects of fish consumption on genotoxicity.
340 In conclusion, increasing consumption of oil-rich and lean fish over six months did not result in
341 genotoxic effects in the colon.
342

343 **Figures and Tables**

344 ***Table I*** Baseline characteristics of the FISHGASTRO population for whom fecal water
345 genotoxicity was determined by the Comet Assay (n=89)

Intervention group:	**Salmon (n=26)**	**Cod (n=34)**	**Dietary advice (DA) (n=29)**
Characteristic			
Age (year, mean ± SD)	57.8 ± 12.6	57.9 ± 8.5	55.6 ± 10.5
Sex (% female)	46	47	58
Smoking (% current)	31	9	15
BMI (kg/m^2, mean ± SD)	25.2 ± 3.7	26.0 ± 4.3	25.9 ± 3.2
Physical activity (% high)	46	24	31
Patient group (% polyp/ UC / healthy)	50/ 19/ 31	50/ 21/ 29	42/ 15/ 42
Country (% NL)	58	56	69

346 Abbreviations: SD (standard deviation), BMI (Body Mass Index), UC (ulcerative colitis), NL (Netherlands)
347
348

349 ***Table II*** Serum measures of very long chain n-3 PUFA (EPA + DHA) per intervention group of
350 participants that completed the intervention, presented as mean ± SD mass% of total fatty acids
351 in cholesteryl esters [1]

Intervention group:	Salmon	Cod	Dietary advice (DA)
Serum very long chain n-3 PUFA	n=26	n=34	n=29
Baseline	3.00 ± 1.18	3.02 ± 1.29	2.63 ± 1.20
End	4.07 ± 1.39	2.80 ± 1.12	2.74 ± 1.27
Change	1.06 ± 1.45	-0.23 ± 0.88	0.03 ± 0.82
Difference in change compared with DA (mean, 95% confidence interval)	1.03 (0.42; 1.64) *	-0.26 (-0.84; 0.32)	

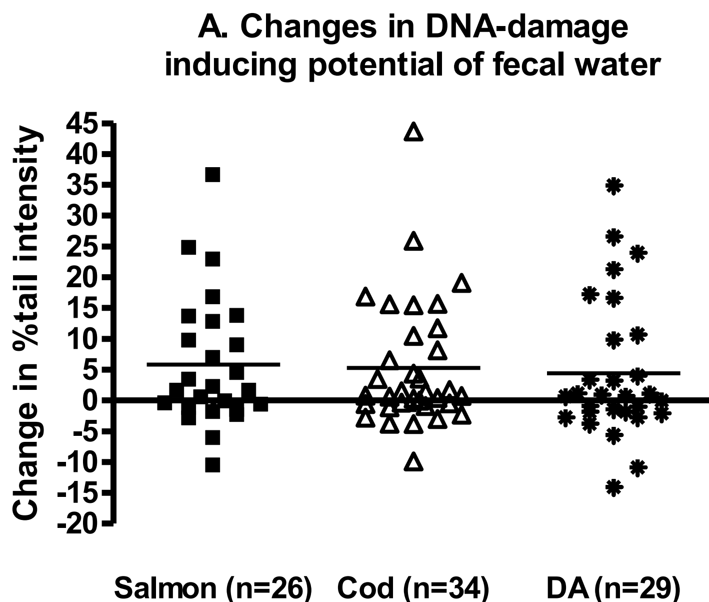
352 [1] missing values due to technical reasons, in salmon group n=2, in cod group n=1, and in DA n=2

353 * Significantly different change compared with DA ($p<0.05$)

354

355

356



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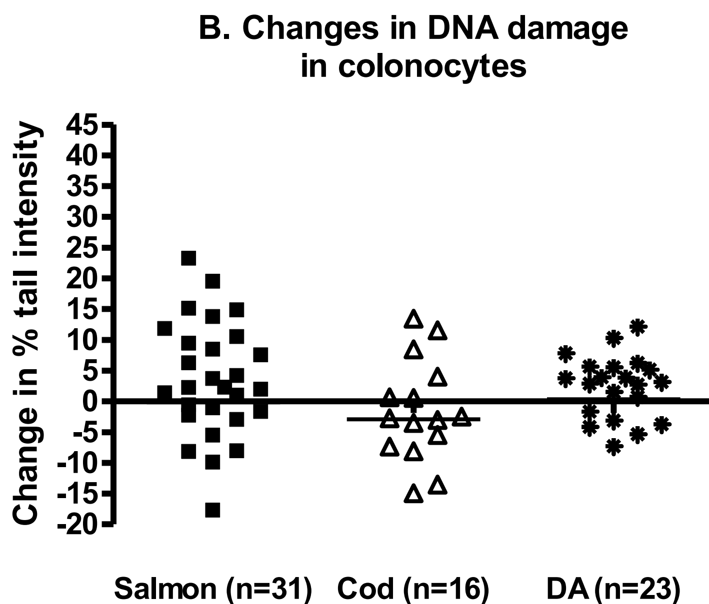

358

359 ***Figure 1*** Results of changes in DNA-damage inducing potential of fecal water (Figure 1A, n=89)

360 and DNA damage of colonocytes (Figure 1B, n=70). The horizontal lines indicate the mean

361 values per intervention group.

362 Abbreviation: DA (dietary advice)

363

364

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388

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391

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531

4. ADDITIONAL RESULTS

4.1 Modulation of LT97 global gene expression by faecal water

We hypothesised, that *ex vivo* faecal water-treated human colon cells are able to reflect nutritional changes of the faeces donors. More specifically, we speculated that these gene expression patterns could show effects of fish consumption/ *n*-3 PUFAs by an additional consumption of oil-rich fish (salmon) compared to a lean fish (cod) in healthy volunteers (n=18) and colon polyp patients (n=21). Thus, the global gene expression pattern was addressed by Affymetrix GeneChip® analysis.

Human colon adenoma LT97 cells were treated with 3.5% faecal water from a subset of the FISHGASTRO study participants. Faecal water was isolated from faeces collected at the beginning of the study and after a six month intervention with either salmon or cod (two 150g portions per week). After 24 hours incubation RNA was isolated from all faecal water-treated LT97 cells using a Qiagen RNeasy Mini Plus kit (Qiagen, Hilden, Germany). The RNA yield was quantified with a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies/Peqlab, Erlangen, Germany), and RNA integrity was measured with an Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, Böblingen, Germany).

Microarray analyses were performed on before intervention and after fish intervention samples of 20 volunteers receiving cod and 19 volunteers receiving salmon. Total RNA from LT97 cells treated with these 78 samples was labeled using a one-cycle cDNA labeling kit (MessageAmp II-Biotin Enhanced Kit; Ambion Inc, Nieuwekerk a/d Ijssel, Netherlands) and hybridised to human whole-genome NuGO GeneChip® arrays encoding 16,554 genes, designed by the European Nutrigenomics Organisation and manufactured by Affymetrix (Affymetrix Inc, Santa Clara, USA). Sample labelling, hybridisation to chips, and image scanning were performed according to the manufacturer's GeneChip® Expression Analysis Technical Manual (Affymetrix).

Microarrays were analysed using the reorganised oligonucleotide probes as described by Dai [Dai *et al.* 2005]. Expression values were calculated using the Robust Multichip Average method and background adjustment by justifying unspecific hybridisation (http://www.bioconductor.org). The Robust Multichip Average signal value estimates are based on a robust average of background-corrected perfect match intensities, and normalisation was performed by using quantile normalisation [Bolstad *et al.* 2003]. Only genes with normalised signals

present on ≥20 arrays were defined as "expressed" and selected for further analysis. Genes were defined as "changed" when comparison of the normalised signal intensities showed a false discovery rate q value [Storey & Tibshirani 2003] <0.25 in a two-tailed paired t test with Bayesian correction (Limma) [Smyth 2004].

Pathway analysis was performed using Gene Ontology Analysis 2.1.17 (http://www.bioinformatics.ubc.ca/ermineJ/) [Lee *et al.* 2005], Gene Set Enrichment Analysis 2.0.4 (http://www.broad.mit.edu/gsea/) [Subramanian *et al.* 2005], and Ingenuity Pathway Analysis 7.5 (www.ingenuity.com). Pathways were defined as significantly changed with a p value <0.05.

From the 16,554 genes present on the microarray, 11,846 were defined as expressed in faecal water-treated LT97 cells. A six month consumption of cod and salmon resulted in differential expression of 88 and 159 genes, respectively, with a fold change of >1.1 (Appendix: Table 5, page 120 and Table 6, page 122). Of these genes, only four were overlapping in both groups, which results in 155 uniquely expressed genes in the salmon group (Figure 8).

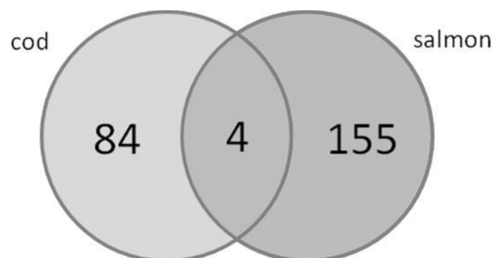

Figure 8. Venn diagram representing number of differentially expressed genes with a fold change >1.1 (p<0.05) after intervention with cod (20 volunteers) and salmon (19 volunteers).

To determine the role of the genes which were found as differentially expressed, we performed pathway analysis. Cod consumption resulted in higher number of upregulated gene sets compared to salmon intervention, only one gene set was found to be downregulated by salmon intervention but this was also downregulated by cod (Figure 9).

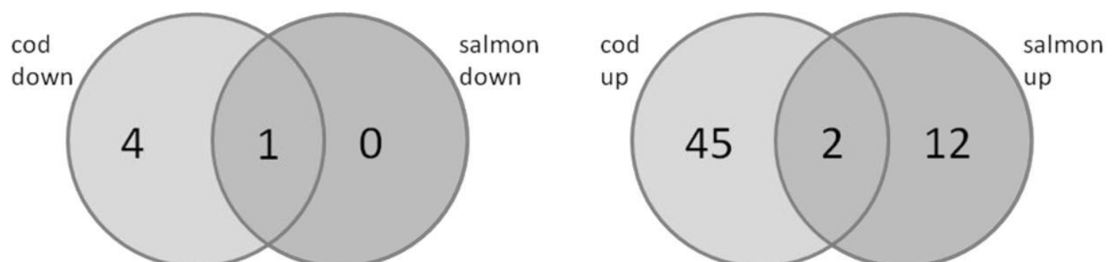

Figure 9. Venn diagram representing number of differentially upregulated ("up") and downregulated ("down") genes sets (false discovery rate <0.25) after intervention with salmon (19 volunteers) and cod (20 volunteers).

The affected pathways determined by Gene Set Enrichment Analysis are presented in Table 4. Intervention with salmon affected *e.g.* pathways involved in glycolysis, fatty acid metabolism and bile acid metabolism. Intervention with cod had an effect on signalling pathways (NF-E2-related factor 2 (Nrf2)-regulated genes, Wnt signalling, EGF signalling, MAPK cascade *etc.*) and xenobiotic metabolising enzymes (as glutathione).

Table 4. Differentially expressed pathways (gene set enrichment analysis) after intervention with salmon (n=19, left hand side) or cod (n=20, right hand side).

salmon up-regulated	NES	p value	FDR	cod up-regulated	NES	p value	FDR
INTEGRIN SIGNALING PATHWAY	2,0668	0,0020	0,0279	NRF2-REGULATED GENES COMBINED	2,2097	0,0000	0,0061
ERK AND PI-3 KINASE (COLLAGEN BINDING)	1,9177	0,0000	0,1169	GLUTATHIONE METABOLISM	2,0585	0,0000	0,0148
GLYCEROLIPID METABOLISM	1,8528	0,0000	0,1584	HS ADIPOGENESIS	1,9770	0,0000	0,0342
ARGININE AND PROLINE METABOLISM	1,6940	0,0021	0,1839	CHOLESTEROL SYNTHESIS/ESTERIFICATION	1,8728	0,0036	0,0672
HS CALCIUM REGULATION IN CARDIAC CELLS	1,6720	0,0000	0,1839	HS IL-1 NETPATH 13	1,8041	0,0000	0,0753
HS ALPHA6-BETA4-INTEGRIN NETPATH 1	1,6726	0,0022	0,1981	METABOLISM OF XENOBIOTICS BY CYTOCHROME P450	1,8773	0,0000	0,0800
LIPOGENESIS	1,6947	0,0077	0,2016	HS EGFR1 NETPATH 4	1,8086	0,0000	0,0808
HS FATTY ACID BETA OXIDATION 1 BIGCAT	1,7535	0,0101	0,2091	TOLL-LIKE RECEPTOR PATHWAY	1,8114	0,0000	0,0894
BILE ACID BIOSYNTHESIS	1,7006	0,0021	0,2143	HS IL-4 NETPATH 16	1,8230	0,0000	0,0924
ROLE OF MAL IN RHO-MEDIATED ACTIVATION OF SRF	1,7122	0,0060	0,2196	REGULATION OF ACTIN CYTOSKELETON	1,7130	0,0017	0,1369
INSULIN SIGNALING PATHWAY KEGG	1,6378	0,0038	0,2211	PYRUVATE METABOLISM	1,7264	0,0055	0,1450
HS FATTY ACID BETA OXIDATION	1,7236	0,0064	0,2301	TGF BETA SIGNALING PATHWAY BIOC	1,7166	0,0112	0,1456
HS GLYCOLYSIS AND GLUCONEOGENESIS	1,7834	0,0000	0,2399	MAPK SIGNALING PATHWAY	1,6771	0,0000	0,1744
RHO CELL MOTILITY SIGNALING PATHWAY	1,7554	0,0077	0,2466	ST ERK1 ERK2 MAPK PATHWAY	1,6522	0,0127	0,2011
				ST GAQ PATHWAY	1,5507	0,0460	0,2069
				NF-KB SIGNALING PATHWAY	1,5548	0,0406	0,2082
				WNT SIGNALING PATHWAY BIOC	1,5657	0,0273	0,2127
				CITRATE CYCLE (TCA CYCLE)	1,5605	0,0294	0,2134
				HS MAPK CASCADE	1,5381	0,0187	0,2148
				HS MONOAMINE GPCRS	1,5548	0,0349	0,2153
				HS INTEGRIN-MEDIATED CELL ADHESION KEGG	1,5856	0,0034	0,2155
				STEROL TRANSPORT/TRAFICKING	1,5333	0,0218	0,2156
				HS INFLAMMATORY RESPONSE PATHWAY	1,6038	0,0237	0,2160
				SIG CHEMOTAXIS	1,5672	0,0271	0,2187
				ST INTEGRIN SIGNALING PATHWAY	1,5275	0,0136	0,2188
				TNF STRESS RELATED SIGNALING	1,5387	0,0331	0,2202
				LIPOGENESIS	1,5713	0,0199	0,2215
				PORPHYRIN AND CHLOROPHYLL METABOLISM	1,6071	0,0231	0,2218
				HS FOCAL ADHESION KEGG	1,5876	0,0000	0,2221
				MTOR SIGNALING PATHWAY KEGG	1,5753	0,0135	0,2231
				AXON GUIDANCE	1,5166	0,0082	0,2235
				ST INTERLEUKIN 4 PATHWAY	1,5204	0,0461	0,2237
				HS INSULIN SIGNALING	1,5905	0,0016	0,2272
				FMLP INDUCED CHEMOKINE GENE EXPRESSION IN HMC-1 CELLS	1,5003	0,0319	0,2334
				GALACTOSE METABOLISM	1,6071	0,0110	0,2339
				HS ALPHA6-BETA4-INTEGRIN NETPATH 1	1,5031	0,0236	0,2339
				PENTOSE PHOSPHATE PATHWAY	1,5054	0,0444	0,2358
				FOCAL ADHESION	1,6202	0,0000	0,2361
				HS STATIN PATHWAY PHARMGKB	1,4754	0,0538	0,2370
				ALK IN CARDIAC MYOCYTES	1,4785	0,0512	0,2371
				HYPOXIA AND P53 IN THE CARDIOVASCULAR SYSTEM	1,4806	0,0464	0,2388
				NFKB ACTIVATION BY NONTYPEABLE HEMOPHILUS INFLUENZAE	1,4837	0,0618	0,2392
				FRUCTOSE AND MANNOSE METABOLISM	1,4869	0,0239	0,2393
				PDGF SIGNALING PATHWAY	1,4930	0,0504	0,2394
				ST GRANULE CELL SURVIVAL PATHWAY	1,6099	0,0248	0,2417
				ST P38 MAPK PATHWAY	1,4881	0,0374	0,2424
				EGF SIGNALING PATHWAY	1,6217	0,0204	0,2477
salmon down-regulated	**NES**	**p value**	**FDR**	**cod down-regulated**	**NES**	**p value**	**FDR**
HS DNA REPLICATION REACTOME	-2,0449	0,0000	0,0130	CELL CYCLE	-1,9536	0,0000	0,0338
				ROLE OF BRCA1, BRCA2 AND ATR IN CANCER SUSCEPTIBILITY	-1,8307	0,0022	0,0681
				HS DNA REPLICATION REACTOME	-1,7456	0,0000	0,0970
				MATURITY ONSET DIABETES OF THE YOUNG	-1,6322	0,0169	0,1477

NES normalised enrichment score, p value normalised p value of the NES, FDR false discovery rate; the estimated probability that the NES represents a false positive finding

Yet, it is not clear whether faecal water can serve as an appropriate *ex vivo* biomarker reflecting the colonic gene expression pattern. Consequently it is of major importance to compare the response of LT97 on faecal water treatment with the gene expression modulation measured in human biopsy samples of the same volunteers (experiments conducted by E.K. Lund, IFR/UK). This comparison study is ongoing.

5. DISCUSSION

Cancer chemoprevention has attracted much attention as one of the most practical and realistic strategies in reducing the global burden of cancer. It has been estimated, that 70% of colon cancer could be avoided by changes in lifestyle. Lifetime non-smoking, physical activity, moderate red-meat consumption, moderate alcohol consumption, a body mass index of < 25, and folic acid supplementation are believed to be of benefit [Willett 2002].

This study focused on colon cancer chemoprevention with a biomarker approach regarding both, *in vitro* effects of *n*-3 PUFA which are abundant in oil-rich fish, and *ex vivo* assessments of samples obtained during a randomised human controlled trial [Pot *et al.* 2009] intervening with lean (cod) and oil-rich (salmon) fish.

5.1 Chemopreventive mechanisms of *n*-3 PUFAs *in vitro*

The first epidemiological evidence on the beneficial effects of dietary fish arose in the 1960/70’s when incidences of cardiovascular diseases in Greenland Inuit were found to be lower compared to those in Europe [Bang *et al.* 1971]. Also other diseases connected with a Western lifestyle had been found to be lower in these populations. During the second half of the 20th century this picture changed and led to a rise in “lifestyle” diseases and now colon cancer is the leading cause of malignancy in the Inuit population. The most likely reason for this is the noticeable nutritional change from a diet based on fish and sea mammals towards a diet more dependent on imported food [Friborg & Melbye 2008].

The outcomes of epidemiological studies on fish consumption and colorectal cancer risk appear to some extent contradictory. But when comparing the studies on the background of the *n*-3 PUFA intake, the situation gains clarity. Of advantage are large-scale studies which address the role of *n*-3 PUFA on colorectal carcinogenesis in populations with a high range of fish consumption and thus a high range of *n*-3 PUFA intake. Examples are the Fukuoko Colon Cancer Study in Japan [Kimura *et al.* 2007], the European Prospective Investigation into Cancer and Nutrition [Norat *et al.* 2005] and the Physician’s Health Study in the U.S. [Hall *et al.* 2008] which all find an inverse association of *n*-3 PUFA/fish intake and colon cancer risk. Also Geelen at al. quoted that the relative protective effect was stronger when differences between highest and lowest fish intake were more pronounced [Geelen *et al.* 2007]. Most studies with fewer participants and investigating smaller differences failed to find an association [Lüchtenborg *et al.* 2005; Tiemersma *et al.* 2002].

A significant increase of EPA and DHA in the colonic mucosa is found in volunteers taking fish oil capsules providing 1.4+1.0g/d EPA+DHA [Gee *et al.* 1999] or 3.2+2.2g/d EPA+DHA [Hillier *et al.* 1991] for up to 12 weeks. For human colonocytes it is likely, that exposure with *n*-3 PUFA is accomplished above all via the lymphatic system (see chapter 5.2.2, page 108). EPA levels in blood respond rapidly (within a week) to dietary changes, whereas DHA changes are much slower [Metherel *et al.* 2009]. Physiological DHA concentrations in plasma following a fish-based diet are found to be 168±8 µM, thus they are higher than EPA concentrations (64±5 µM) [Pawlosky *et al.* 2003]. The concentrations used for the *in vitro* experiments of this study were covering this range.

5.1.1 *n*-3 PUFA impair cell number by affecting cell growth and apoptosis

The first study **(publication I)** demonstrated that PUFAs are incorporated into colon epithelial cells *in vitro*. Of importance, no different effects were elucidated for the tested fatty acids; all fatty acids (EPA and DHA as well as ALA, GLA, LA and ARA) were taken up by the cells in a comparable manner. This finding is not surprising because PUFA incorporation into erythrocyte membranes is an accepted biomarker to reflect rapid changes of the dietary fatty acid profile [Baylin & Campos 2006]. However, it appeared that LT97 cells more readily consumed fatty acids compared to HT29 cells.

If PUFAs reach the cell, it is likely that they exert cellular responses after their incorporation. In contrast to the *n*-6 PUFA LA, both fish *n*-3 PUFAs were potent inhibitors of colon adenocarcinoma cell growth by affecting cell number **(publication I)**. Half maximal effective concentration (EC_{50}) values reached after DHA treatment (HT29 72h: 124±10µM, LT97 72h: 128±117µM) reflect levels which can be found in humans under physiological conditions in plasma following a diet based on fish [Pawlosky *et al.* 2003]. In HT29 cells, DHA was a more potent inhibitor of cell growth. This effect was also seen in other studies treating carcinoma cells [Chamras *et al.* 2002; Schley *et al.* 2005; van Beelen *et al.* 2006].

EPA and DHA are already known to have growth inhibitory or anti-proliferative potential *in vitro* and *in vivo*. In cell-culture models similar effects were described *e.g.* in human cell lines of the breast [Chamras *et al.* 2002; Noguchi *et al.* 1995; Schley *et al.* 2005; Senzaki *et al.* 1998], the pancreas [Shirota *et al.* 2005], the prostate [Pandalai *et al.* 1996], lymphocytes [Verlengia *et al.* 2004b; Verlengia *et al.* 2004a], and the colon [Clarke *et al.* 1999; Tsai *et al.* 1998; van

Beelen *et al.* 2006]. EPA and DHA also reduced cell growth of tumours obtained by inoculating HT29 cells in nude mice [Calviello *et al.* 2004] or by implanting Walker 256 tumours in Wistar rats [Colquhoun *et al.* 2001]. Other rodent animal models revealed reduced tumour growth after fish oil-feed in chemically induced colon cancer models, *e.g.* following azoxymethane [Dommels *et al.* 2003] or 1,2-dimethylhydrazine [Latham *et al.* 1999] treatments in rats.

Additionally, in our study it was shown, that cell growth inhibition was more readily achieved in the adenomatous cell line LT97 compared to the adenocarcinoma cell line HT29 (significant for EPA and ARA). Most often in literature the consensus is quoted, that carcinoma cells are more sensitive to growth inhibitory/anti-proliferative effects of PUFAs than normal cells [Grammatikos *et al.* 1994; Tsai *et al.* 1998]. But *in vitro* comparisons between human cancer cells and human non-cancer cells of the same tissue are rare, due to the unavailability of a “normal” cell line from for example the colon. To the best of my knowledge, there is no comparison of human normal and carcinoma cells from the same organ. For such comparisons mostly fibroblasts [Tsai *et al.* 1998] or non-human cells [Griffiths *et al.* 1997] were used to represent the behaviour of normal cells. Hence, tissue and species specific effects must be taken into consideration when interpreting these results. As no normal cell line exists some researchers take the approach to compare cancer cell lines with non-cancerous cell lines of the same tissue, but the results from these were rather inconsistent. Some found non-cancer cell lines to be more susceptible to PUFAs (*e.g.* non-neoplastic canine prostate epithelial (CAPE) cells vs. adenocarcinoma canine prostatic (CPA) cells [Griffiths *et al.* 1997]), while others found the cancer cell more sensitive (*e.g.* epithelial breast cancer (MCF-7) vs. non-cancerous mammary epithelial (MCF-10A) cell line [Grammatikos *et al.* 1994]). For colon cancer, normal colon mucosa cells (NCM460) were not affected whereas the growth of colon adenocarcinoma epithelial cells (CaCo2) was reduced [Toit-Kohn *et al.* 2009]. Our results showed, what appears to be the opposite in that adenomatous cells were generally more susceptible. Other factors should be kept in mind when evaluating the response of diverse cells *in vitro* to PUFAs. Not only the growth rate is important (doubling time ~24 hours and ~72 hours, for HT29 and LT97 cells, respectively), but the density of the cells at the beginning of treatment (LT97 cells grow as a multilayer, thus, even at the same confluency, a larger number of cells are incubated), but also final density, confluency, and cell number. Additionally, different media (DMEM or MCDB) substituted with different foetal calf serum concentrations (ten or two per cent) are used for HT29 and LT97, respectively. All this means that HT29 are difficult to compare

with LT97 cells as they show a different growth pattern. Altogether, the above mentioned cell culture criteria bias all data published so far. Ideal cell models do not exist and hence PUFA-independent parameters may also alter the effects of the treatment.

It is possible, that a different enzyme system in malignant and non-malignant cells led to a different pattern of PUFA utilisation. In addition, intracellular lipid peroxidation may differ between colon tumour and colon non-tumour cells [Das 1999]. For a characterisation of both *in vitro* cell lines used, an estimation of these markers would be useful for future studies.

A mechanism to control the number of cells and to counteract an excessive cell number is by facilitation of apoptosis **(publication IV)**. The effect of EPA and DHA on triggering apoptosis in cancer cells has been described in a range of *in vitro* models such as the breast [Chamras *et al.* 2002; Sun *et al.* 2008] and the colon [Hofmanova *et al.* 2005; Narayanan *et al.* 2001], but still the nature of any underlying mechanism in the colon remains speculative.

Previously, it has been found that *n*-3 PUFAs exert only marginally apoptotic effects on HT29 cells. DHA was seen to have no effect on PARP cleavage and caspase 3 or 8 expression [Hofmanova *et al.* 2005; Vaculova *et al.* 2005]. This is in concordance with the findings of this study since LT97 cells were found to be much more susceptible to *n*-3 PUFA-induced apoptosis than HT29 cells in terms of the effective concentration needed. Furthermore, the involvement of caspase 8, indicating the contribution of death receptor activation, and bid-cleavage as well as *Bcl-2* decrease, indicating mitochondria-mediated apoptosis has been proven for the first time in LT97 cells. The link between caspase 8 and the mitochondrion is provided by tBid which is formed from Bid. In the mitochondria, tBid oligomerises with itself and induces oligomerisation of Bak and Bax wich is accompanied by cytochrome c leakage from the mitochondria. It is hypothesised that Bcl-2 suppresses tBid insertion to the mitochondrial membrane which thus results in the preservation of mitochondrial integrity [Yi *et al.* 2003]. This work provides support that *n*-3 PUFAs facilitate cytochrome c release first, by downregulation of *Bcl-2* and second, by enhanced proteolytic cleavage of Bid by caspase 8 resulting in tBid. All the described effects were particularly apparent after treatment with DHA in LT97 cells. Downstream effects such as caspase 3 release and PARP cleavage were also detected in HT29 cells but using higher concentrations compared to concentrations needed to affect LT97 cells (200 vs. 100µM).

One finding of our study is that DHA is more active compared to EPA in inducing apoptosis especially considering LT97 cells. Ignoring the carbon chain length,

the main difference between both *n*-3 PUFAs is the higher degree of unsaturation of DHA. These double bonds can also serve as substrate for peroxidation reactions hence resulting in reactive oxygen species [Vaculova *et al.* 2005]. It has previously been shown that reactive oxygen species lead into apoptosis [Simon *et al.* 2000]. Fish oil-supplementation results in higher oxidative burst of peripheral mononuclear cells of healthy young men [Bartelt *et al.* 2008] and this increase in oxidative burst, especially by DHA in LT97 cells, might have led to apoptosis-favouring conditions. It was also revealed that an increasing antioxidative potential of cells (*e.g.* by accumulating ascorbic acid or induction of catalase) might diminish TRAIL- or Fas-induced apoptosis in cancer cells [Perez-Cruz *et al.* 2003; Perez-Cruz *et al.* 2007]. Such findings support the evidence of animal models showing that the chemopreventive effects of *n*-3 PUFAs involved increased oxidative stress [Latham *et al.* 2001].

Concluding remarks:

Following EPA and DHA incorporation both fatty acids affect cellular mechanisms commonly attributed to secondary chemoprevention. Initiated cells, in this *in vitro* work modelled by LT97 cells, may be reduced as a result of on the one hand inhibition of cell growth **(publication I)** and on the other hand by induction of apoptosis **(publication IV**). Assessed effects were less strong in HT29 cells which represent highly altered cells.

5.1.2 Gene expression alteration by *n*-3 PUFAs

Since *n*-3 PUFA are known to alter gene expression, they are candidate nutrients to study the gene expression of possible target genes. The regulation of gene transcription by fatty acids seems to be due to changes in the activity or abundance of transcription factor families as *e.g.* peroxisome proliferator-activated receptor (PPAR), liver x receptor (LXR), hepatic nuclear factor 4α (HNF-4α), and sterol regulatory element binding protein (SREBP) [Jump 2002]. These transcription factors play a major role in hepatic carbohydrate, fatty acid, triglyceride, cholesterol, and bile acid metabolism (Figure 10).

In the liver, the principal action of *n*-3 PUFAs involve a shift from lipid synthesis and storage to lipid oxidation [Jump 2002; Wang *et al.* 2009]. These effects are primarily described for hepatic metabolism and may contribute to the regulation of whole body lipid metabolism and the control of blood triglycerides and cholesterol, important risk factors for chronic diseases.

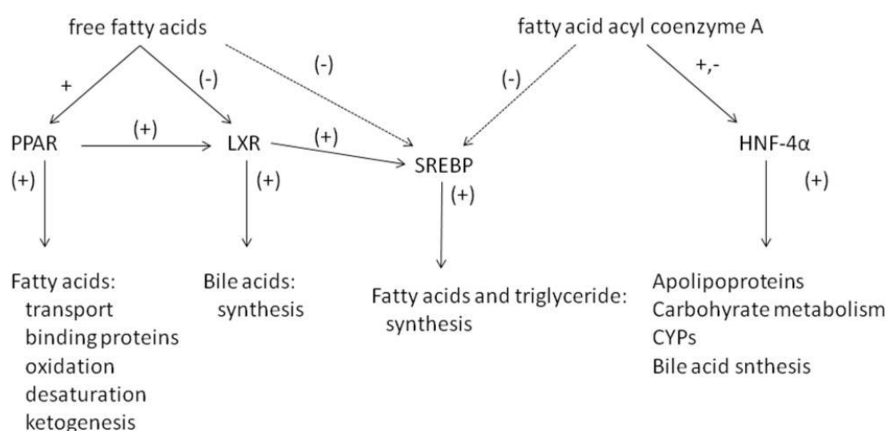

Figure 10. Regulation of transcription factors by non-esterified fatty acids and fatty acid acyl coenzyme A thioesters (modified according to Jump [Jump 2002]). Free fatty acids and fatty acid acyl coenzyme A thioesters serve as potential regulators of nuclear receptors. It is not clear whether free fatty acids or fatty acid acyl coenzyme A thioesters control the turnover of SREBP mRNA.

PPAR peroxisome proliferator-activated receptor, LXR liver x receptor, HNF-4α hepatic nuclear factor 4α, SREBP sterol regulatory element binding protein, + positive association, - negative association, mechanisms in brackets are proposed ones.

To examine direct whole-genome transcriptional changes by *n*-3 PUFA, microarrays are highly valuable as they allow the simultaneous analysis of thousands of genes. Previous studies showed that *n*-3 PUFAs are effective modulators of gene expression *in vitro*. These include whole-genome array analysis of DHA and EPA treated breast cancer cells [Hammamieh *et al.* 2007], and DHA incubated hepatic [Wang *et al.* 2009], and prostate cancer cell lines [Shaikh *et al.* 2008]. The first study examining the influence of DHA on human colon adenocarcinoma cells (CaCo2, 15µM DHA, 48h) using cDNA microarrays showed an inhibition of cell proliferation, induction of apoptosis, regulation of cell cycle, and alteration of COX-2 target genes [Narayanan *et al.* 2001]. In humans, the supplementation of 0.4 or 1.8g EPA+DHA per day (26 weeks) resulted in a shift of the gene expression profile of peripheral blood mononuclear cells to a more anti-inflammatory and anti-atherogenic status [Bouwens *et al.* 2009].

For this study, a custom-designed dual colour cDNA array on a glass slide (PIQOR™, Miltenyi Biotec) targeting 306 genes was performed. Noteworthy, **publication II** is the first to date which examined the gene expression alteration in an adenomatous type of colon cells *in vitro* by EPA and DHA. It was shown that EPA and DHA affect gene expression in partially different ways **(publications II and III)**. Affected genes include those relating to biotransformation (EPA: GSTT2 upregulation), inflammation (DHA 10 hours: COX-2 downregulation), apoptosis (EPA and DHA 10 hours: caspase 8 downregulation), and signalling pathways (EPA and DHA 10 hours: Wnt3

downregulation). Furthermore, effects after 10 and 24 hours were found to be partially different.

In nature EPA and DHA mostly appear concomitantly. Therefore, future studies on expression patterns after simultaneous treatment would be of interest to elucidate probable additive, synergistic, or inhibitory effects.

Concluding remarks:

There are two main conclusions from these results. Firstly, for *in vitro* analyses there is a time-effect of exposure. As time-course studies are rare [Hammamieh *et al.* 2007], the effects of genome wide studies must be understood in terms of reflecting a small sample in the range of physiological effects on gene expression. For gene expression analysis of specific genes, a suitable treatment time must be considered. Secondly, the verification of the array analysis is indispensable. The results of this study were shown to vary in a wide range, thus the p values after t test are comparably high. Verification can be accomplished on mRNA level (*e.g.* Real-Time RT-PCR) or targeting protein (*e.g.* Western Blot, activity assay, or enzyme-linked immunosorbant assay).

5.1.3 Modulation of expression of antioxidative and inflammatory enzymes by *n*-3 PUFAs

To examine the effects in more detail and verify the results from arrays, genes which are involved in colorectal carcinogenesis were chosen for examination in a time course in both adenomatous and adenocarcinoma cells **(publication III)**.

One mechanism by which *n*-3 PUFAs can avoid oxidative stress and detoxification of electrophilic compounds and thus prevent against cancer was suggested by the induction of electrophile-responsive element (EpRE)/antioxidant responsive element-mediated phase II detoxification enzymes thereby increasing the defence capacity against potential carcinogens. The major transcription factor involved in EpRE-mediated gene expression is Nrf2. Under normal physiological conditions, Nrf2 forms an inactive complex with the negative regulator Kelch-like ECH-associated protein 1 (Keap1). By conformational change of Keap1, Nrf2 is released and can translocate to the nucleus. Heterodimerisation with other transcription factors allows interaction with EpRE in the promotor region of various antioxidant and phase II enzymes. Downstream target genes include GSTP1 [Rushmore & Pickett 1990] and NADP(H):quinone oxidoreductase 1 (NQO1) [Tanigawa *et al.* 2007]. Peroxidation products of PUFAs have been linked with Nrf2-related modulation of gene expression [van Beelen *et al.* 2006]. Using a luciferase assay a clear response of EpRE after incubation with EPA<DHA was found, which was much higher fold

than that of ARA [van Beelen *et al.* 2006]. *SOD2* has been predicted to be a novel Nrf2 target in an array based assay in mouse lung cells [Taylor *et al.* 2008] and also SOD enzyme activity was found to be altered in human kidney tubulus cells and fibroblasts in an Nrf2-dependent manner [Boesch-Saadatmandi *et al.* 2008; Zhu *et al.* 2005], which suggests an EpRE in the SOD2 promotor.

An upregulation of SOD2 mRNA caused by EPA and DHA was found in both LT97 and HT29 cells (24 hours). The significant *SOD2* upregulation in cells of a premalignant state of degeneration as seen in our results can be considered as a suppressing agent activity since a further transformation is potentially repressed. The first step in the removal of superoxide anion radicals is enabled by the enzymatic action of SOD2. Induction of *SOD2* and *GSTT2* **(publication III)** can be explained by the Nrf2-pathway.

Besides the effects on the antioxidant system of the cell, SOD2 has been recently discussed as a new type of tumour suppressor gene [Oberley 2005]. An overexpression of *SOD2* decreased cell growth of NIH/3T3 mouse fibroblasts [Kim *et al.* 2004]. Additionally, *SOD2* overexpression led to an alteration of different signal transduction pathways in diverse cell types via mechanisms such as inhibition of transcription factors AP-1 and NF-κB and hence might also affect downstream targets [Li *et al.* 1998b].

Although *SOD2* mRNA was increased, no induction of SOD2 protein after EPA or DHA exposure was found. Such effects have been described before and were discussed as translational block of SOD2 expression: using *in vitro* rodent models, SOD2 activity did not correspond to the oxygen-related *SOD2* mRNA increase [Gomi & Matsuo 2002]. Furthermore, lipopolysaccharide-induced transcriptional *SOD2* induction did not result in elevated SOD2 activity in rat astrocytes [Niu *et al.* 1998]. However, there is still a lack of clarity, whether *SOD2* induction may be also translated at the protein level *in vivo*.

Generally, GST upregulation is considered to be of benefit by enhancing detoxification via conjugation of potential carcinogens to glutathione. GST catalyses the first of four steps required for the synthesis of mercapturic acids [Keen & Jakoby 1978]. Subsequently, the sequential removal of the γ-glutamyl moiety and glycin from the glutathione conjugate will follow prior to *N*-acetylation of the remaining cysteine conjugate. Once formed, the glutathione conjugates are eliminated from the cell by MRP, a family of ABC transporters, more precisely MRP1 and MRP2 [Morrow *et al.* 2000; Paumi *et al.* 2001]. Conjugation of exogenous compounds to glutathione almost always results in the formation of less reactive products that are readily excreted. However, in a few instances the resulting glutathione conjugate is more reactive than its parent

compound [Hayes *et al.* 2005]. For example those compounds with two functional groups are still able to modify DNA by the remaining electrophilic centre after glutathione conjugation (*e.g.* dichloromethane [Guengerich *et al.* 2003]). Mercapturic acid derivatives are excreted in the urine. However, some glutathione conjugates are also found to be eliminated via bile duct and thus found in the colon (*e.g.* simvastatin- [Subramanian *et al.* 2002] or 4,4′-methylenedianiline-conjugates [Chen *et al.* 2008]), where they also may be deconjugated by enzymatic activity of the gut flora implying the result of potential harmful and reactive intermediates in the gut.

As shown by our results, EPA exerts a *GSTT2* mRNA-inducing effect which was significant for HT29 cells after 24 hours. Interestingly, DHA repressed *GSTT2* mRNA after 10 hours in HT29 cells. Since these two *n*-3 PUFAs act in opposite ways on *GSTT2* modulation at the gene level, more specific mechanisms than simple lipid peroxidation and resulting oxidative stress must account for these effects. The GST theta class genes lack both TATA and CAAT boxes in their 5' flanking region thus multiple heterogeneous transcription boxes have been described (SP1 transcription factor, SP1; spleen focus forming virus proviral integration oncogene, spi1; polyomavirus enhancer activator-3, PEA3; activating enhancer binding protein 2 alpha, AP-2) [Ogura *et al.* 1994]. Also, EpRE was first described in the rat *GSTP1* gene [Okuda *et al.* 1989] and later other rat GST family members were also characterised as EpRE harbouring genes [Rushmore *et al.* 1991; Rushmore & Pickett 1990]. Similar, GST activity was found to be increased by DHA in human fibroblasts [Arab *et al.* 2006].

The functional consequence of altered GSTT2 protein still remains to be elucidated; this is due to missing appropriate tests, *e.g.* human-specific antibodies for Western Blot or specific substrates for activity assays. Whether or not the alteration of GSTT2 beneficially affects the cellular defence is not only dependent on the exposition to potential carcinogens, as *e.g.* benzo(*a*)pyren, aflatoxin, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, but also the activity of glutathione supply (catalysed by glutamate cystein ligase and glutathione synthase) and glutathione conjugate removal (enabled by MRP1 and 2) [Hayes *et al.* 2005].

Inflammation is an important tumour-promotor [Erlinger *et al.* 2004]. A well-known hypothesised mechanism for the chemopreventive action of *n*-3 PUFAs is their suppressive potential on the production of ARA-derived prostanoids [Rees *et al.* 2006]. Eicosanoids from EPA and ARA have very similar structures but markedly different effects. Generally, EPA-derived eicosanoids are much less potent inducers of inflammation than ARA-derived eicosanoids [Shapiro *et al.*

1993]. The predominance of *n*-6 fatty acids will result in a pro-inflammatory status with prostaglandins of the 2-series and leukotrienes of the 4-series. As the relative amount of *n*-3 PUFAs increases, the production of prostaglandins of the 3-series and leukotrienes of the 5-series will do in turn [Lee *et al.* 1985]. Some studies suggest a downregulation of COX-2 by EPA and/or DHA by affecting nuclear transcription factors and altering signal transduction and cell signalling. Findings from **publication III** support this hypothesis as they show a downregulation of *COX-2* mRNA and COX-2 protein by DHA in both LT97 colon adenoma and HT29 adenocarcinoma cells. COX-2 inhibition has previously been shown in cancer cell lines by supplementation with 30µM EPA or DHA (24 hours) in HT29 cells *in vitro* [Calviello *et al.* 2004]. However, we found a *COX-2* induction by 50µM EPA in LT97 cells after 24 hours. The key link between PUFAs and inflammation is that eicosanoids are generated from C20 PUFAs. EPA can also act as a substrate for lipoxigenases and COX, resulting in a production of eicosanoids with a slightly different structure than those from ARA. The functional consequence is that the formed mediators appear to be less potent than ARA-derivatives. Additionally, EPA and DHA have been found to act as a substrate for the generation of alternative eicosanoids, termed resolvins [Serhan *et al.* 2008]. Resolvins are formed by the action of COX-2 and appear to exert anti-inflammatory actions [Serhan *et al.* 2008] and this pathway may be enhanced when supplementing LT97 adenoma cells with EPA. Previously it was shown that COX-2 regulation is facilitated in a prostaglandin E2-dependent (via NR4A2) but also in a prostaglandin-independent manner [Holla *et al.* 2006].

Concluding remarks:

Taken together, these results show that EPA and DHA exert colon cancer chemopreventive effects by altering gene expression, more precisely they upregulate *SOD2*, they alter *GSTT2*, and downregulate COX-2 mRNA and protein in LT97 adenomatous cells. This is the first time that such effects have been described not only in carcinoma cell models but also including adenoma cells *in vitro*.

5.2 Alteration of markers of chemoprevention after fish consumption by faecal water *ex vivo*

The FISHGASTRO study was performed as a randomised controlled trial aiming to test the effects of fish-consumption on colorectal carcinogenesis in humans [Pot *et al.* 2009]. To test the health-promoting effects of *n*-3 PUFAs, two types of fish were used within the study, a lean fish (cod) and an oil-rich fish (salmon) compared to no extra fish. All participants were encouraged to eat a healthy diet.

Besides influencing membrane fluidity [Lund *et al.* 1999] *n*-3 PUFA are involved in many physiological processes and with regard to colon carcinogenesis systemic effects of *n*-3 PUFAs were proposed. Thus, fish oil decreased the concentration of secondary bile acids in the colon as compared to a diet high in *n*-6 PUFAs and saturated fats [Bartram *et al.* 1995; Bartram *et al.* 1998]. It has therefore been hypothesised that the secondary bile acids deoxycholic acid and lithocholic acid which are derived from the bacterial degradation of the primary bile acids, cholic acid and chenodeoxycholic acid, respectively, may be involved in the aetiology of colon cancer. Secondary bile acids are known to increase proliferation and act as colon tumour promotors [Nagengast *et al.* 1995]. Furthermore, secondary bile acids are known to be genotoxic [Rosignoli *et al.* 2008] and apoptosis decreases with higher hydrophobicity of bile acids (chenodeoxycholic acid < deoxycholic acid) [Katona *et al.* 2009]. This provided a rationale to use faecal water as a source of human derived material for the identification of biomarkers of local effects of fish consumption on colonocytes; for example by detecting DNA damage inducing potential (Comet Assay) and induction of apoptosis (flow cytometrical detection of Annexin-V-FITC/7-actinoaminomycin) on cultured cells *in vitro*.

Increase of fish consumption is also recommended on the basis that it also contains other beneficial compounds such as selenium [Jackson & Combs, Jr. 2008] and 25-hydoxy vitamin D [Garland *et al.* 2009]. The absorption of selenium from fish is high (~95%) regardless the processing of the fish [Fox *et al.* 2004]. Selenium is an essential part of selenoproteins which include glutathione peroxidases [Allan *et al.* 1999], and additionally it is hypothesised to be an anti-cancer agent because of its role as antioxidant [Rayman 2000]. Dietary supplementation of selenium inversely affected colon cancer incidence (by 58%) in persons with low selenium level [Clark *et al.* 1996], whereas an additional intake of selenium in people with adequate levels is not beneficial [Lippman *et al.* 2009]. There was a profound inverse association of serum 25-hydroxy vitamin D and colon cancer [Freedman *et al.* 2007]. Thus, there was a line of evidence to suggest beneficial effects of vitamin D and underlying mechanisms include upregulation of adherence and signalling between epithelial cells, differentiation, promotion of apoptosis, and downregulation of the Wnt-pathway.

And finally, the alteration of blood lipids is discussed for experimental diets with fish protein [Shukla *et al.* 2006].

Fish may exert health benefits but besides, it may also contain contaminants which resulted in confusion about the role of fish consumption as part of a healthy diet. However, it was concluded, that the benefits of a modest fish

consumption (up to 2 portions per week) outweigh the risk among adults [Mozaffarian & Rimm 2006]. Mercury is a heavy metal and in form of the organic methylmercury it can be readily absorbed and actively transported into tissue. Thus, methylmercury bioaccumulates in aquatic food chains and concentrations depend on level of environmental contamination. Toxicity appears to be related to binding of methylmercury to sulfhydryl groups of enzymes, ion channels, and receptors inhibiting antioxidant systems and leading to oxidative stress [Mergler *et al.* 2007; Mozaffarian & Rimm 2006]. Fish also tends to accumulate halogenated organics including polychlorinated biphenyls (PCBs) and dioxins which had been previously used in industrial processes (paper bleaching, pesticide production, production of polyvinylchloride plastics). Due to the long persistence of these types of compounds, they continue to be present in low concentrations in many foods, also in particularly oil-rich fish [European Commission 2000]. It is suggested that PCBs and dioxins are carcinogenic possibly related to effects on the aryl hydrocarbon receptor, a transcription factor affecting gene expression [Bock & Kohle 2006]. In the FISHGASTRO study only 2,3,4,5-tetrachlorodibenzo-p-dioxin (TCDD) equivalents (TEQ) in salmon (0.45pg TEQ/g) and cod (0.04pg TEQ/g) was measured and it was found to be well below the current maximum tolerable intake of 8pg TEQ/g fish [European Union 2006] and thus unfavourable effects in the FISHGASTRO study due to toxins in salmon or cod were considered to be small (**publication V**).

5.2.1 *Ex vivo* effects of faecal water on the modulation of DNA-damaging effects, apoptosis-inducing potential, and global gene expression

One marker for colon cancer risk is thought to be the genotoxic burden exposing the gut, commonly measured by treatment of *in vitro* cultured cells with faecal water or specific fractions of that (*e.g.* lipophilic faecal water fraction) *ex vivo*. Several previous studies revealed the rapid modification of faecal water genotoxicity by changes in dietary treatments [Glinghammar *et al.* 1997; Rieger *et al.* 1999] while others have shown no effects [Haza *et al.* 2000; Hughes *et al.* 2002].

In a rodent model of chronic inflammation and oxidative stress, fish oil feeding resulted in lower level of 8-hydroxydeoxyguanosine [Bancroft *et al.* 2003; Hong *et al.* 2005]. It is hypothesised that protective effects of *n*-3 PUFAs are partially attributed to the reduction of oxidative DNA damage. Oxidised pyrimidine and purine DNA bases can be detected with a modified version of the Comet Assay using endonuclease III and formamidopyrimide DNA glycosylase which are

bacterial repair enzymes detecting oxidised base alterations [Collins *et al.* 1996]. The alkaline Comet Assay which was used in **publication V** detects single and double strand breaks, and alkali labile sites of the DNA, so oxidised DNA bases were not investigated. It is feasible, that a fish diet in humans also alters the oxidative status in the gut resulting in an impaired level of oxidised DNA bases. However, both rat studies used a dextran sodium sulphate treatment to mimic a chronic inflammatory state featuring a highly oxidative environment which may not reflect the situation of apparently healthy humans. After this inflammatory burst the authors found a decrease in oxidative DNA damage in rats receiving fish oil [Bancroft *et al.* 2003; Hong *et al.* 2005]. In humans, parameters of oxidative stress in urine were not altered by giving EPA + DHA supplements [Dawczynski *et al.* 2009]. It remains elusive as to what extent *n*-3 PUFAs contribute to a decrease of DNA base oxidation in healthy colon and perhaps should be the focus of future studies.

As affective concentration 50% of the faecal water was chosen for a 30min (37°C) exposition of HT29 cells. Prior to the screening of the faecal water samples a dilution series of three randomly chosen faecal waters was conducted. By exposing the cells with 50% faecal water a medium level of genotoxicity was mediated without affecting viability of the cells (see appendix, Figure 13 on page 125).

It is feasible that fatty acids modulate faecal water genotoxicity by decreasing the luminal concentration of bile acids in the colon. Though bile acids were not measured in the FISHGASTRO samples it remains speculative whether or not salmon consumption resulted in decreased levels of especially secondary bile acids. Yet, an estimation of the necessary dose of fish to achieve a reduction of mainly secondary bile acids by fish consumption is not known.

Additionally, probable DNA damage preventing effects exerted by faecal water were measured in analogy by the Comet Assay (30min treatment, 37°C, so-called "antigenotoxicity"). As DNA damage-inducing agent hydrogen peroxide (75µM) was used. Also with this, no beneficial effects of additional fish consumption were detected (appendix, Figure 14 on page 126). In literature, antigenotoxicity against hydrogen peroxide-mediated genotoxicity by ileostomy samples from volunteers receiving cloudy apple juice [Veeriah *et al.* 2008], and also by gut-flora mediated fermentation supernatants of wheat bran arabinoxylanes were described [Glei *et al.* 2006]. For faecal water following fish consumption or fish oil no such test was performed before.

However, since *ex vivo* DNA damage in cultured cells mediated by faecal water does not necessarily reflect the effects found for colonocytes *in vivo*

(publication V), the picture is far from clear. One reason for this could be that genotoxicity of faecal water and DNA damage in colonocytes are different endpoints; the faecal water-induced DNA damage represents the genotoxic burden of excreted faeces, which is mainly modulated by dietary exposure and processes of the gut flora [Burns & Rowland 2004], whereas the DNA damage measured in colonocytes also reflects the effects in the cells which additionally depend on the expression of biotransformation enzymes or the extent of DNA repair mechanisms [Rigas *et al.* 2001] and systemic burden.

An induction of apoptosis was examined in LT97 cells treated with DHA>EPA **(publication IV)**. However, the modulation of the luminal environment by eating additional portions of lean or oil-rich fish (1.3-1.4 portions/week, respectively) has not been examined so far. Results from **publication IV** do not indicate a pro-apoptotic environment in faecal water of the FISHGASTRO study. There is only one study to date examining the effect of faecal water on apoptosis *ex vivo* in HT29 cells [Haza *et al.* 2000]. In this preliminary study a markedly individual difference in effects was observed, apoptosis induction was attributed to the presence or absence of bile acids and butyrate. Bile acids are observed to alter apoptosis [Bernstein *et al.* 2005] and also the gut-fermentation product butyrate can induce apoptosis [Scharlau *et al.* 2009]. Changes in faecal water as a result of consuming fish are probably not related to elevated DHA/EPA concentrations directly but rather to changes in bile metabolism. To further clarify this issue, the analysis of bile acids and short chain fatty acids as butyrate in the faecal water samples of the FISHGASTRO study would be useful.

Additionally, faecal water was screened for its apoptosis inducing potential. By consuming cod or salmon, no additional apoptotic events were examined (**publication IV**). Preliminary tests aiming to find an effective dilution of six different faecal water samples are shown in appendix, Figure 12 on page 124.

Finally, the impact of faecal water on global gene expression of LT97 cells was examined **(additional results)**. One could argue that the used 3.5% faecal water concentration is too low to exert effects in the cells. This concentration was used basing on preliminary tests on cell growth inhibition of LT97 cells which are summarised and further explained at the appendix, Figure 11 on page 123. Only one prior study examining the impact of faecal water on modulation of gene expression has been reported to date [Zeng & Davis 2003] which found an alteration of cell cycle genes. These authors used a concentration of 5% faecal water to treat HT29 cells, thus the tested concentration is comparable to the one applied for the Affymetrix analysis. It is noteworthy, that the study by Zeng *et al.* is preliminary and it does not use the parameter of gene expression alteration to

detect its modification during an intervention trial but rather only the general impact of faecal water. Also, probable reactive compounds found in faecal water (*e.g.* bile acids, short chain fatty acids, or polyphenolic compounds) were not analysed.

Concluding remarks:

An additional consumption of cod or salmon did neither result in alteration of the genotoxic impact of the tested faecal water **(publication V)** nor in potential beneficial effects by preventing hydrogen peroxide-mediated DNA damage. For the first time, impact of fish consumption on alteration of the luminal contents towards apoptosis-induction was examined but no changes by cod or salmon consumption were detected **(publication IV)**. The approach of using faecal water-induced gene modulation to evaluate the impact of diet is new in this study **(additional results)**. However, only marginal changes following the fish-diets were found. Unexpectedly (personal communication with Dr. Guido Hooiveld, Wageningen University, The Netherlands), only a few genes were altered and additionally, the extent of mRNA expressional changes were small (Table 5 and Table 6). Furthermore, abundant compounds being always present in faeces might overwhelm probable changes by the fish-diet.

5.2.2 Evaluation of faecal water as a biomarker of colon cancer risk within a fish-intervention trial

An early diagnosis of colorectal cancer is needed to intervene the further progression of the disease, as treatment is most successful when no metastasis is present [Eisenberg *et al.* 1982]. Sigmoidoscopy and colonoscopy are highly specific and sensitive tests for colorectal neoplasia, but they are limited by patients' compliance and physician's availability and costs [Frazier *et al.* 2000]. The faecal occult blood test is a non-invasive and simple technique which was shown to reduce incidence and mortality of colorectal cancer. The limitation is that the faecal occult blood test is an indirect one, patients with positive results are referred to colonoscopy to confirm the presence of polyps or cancer (for review see [Burt 2000]). For this reason, the development of surrogate tissues or biomarkers for colorectal cancer risk is ongoing.

In most human dietary intervention studies focusing colon carcinogenesis so far, a surrogate tissue, *e.g.* peripheral lymphocytes was used to assess effects of changes in nutrition [Bouwens *et al.* 2009]. When the primary interest of an intervention study emphasises the impact on the gut the situation is complicated. On the one hand, effects in lymphocytes do not necessarily reflect the effects in the target tissue colon, but on the other hand they provide an easily obtainable

and non-invasive biomarker which is what is needed for dietary intervention studies.

The parameter of faecal water-induced genotoxicity is often used and has been shown to be influenced by nutrition. Gut luminal genotoxicity can be regarded as a biomarker of exposure of the colon to potential beneficial/harmful compounds. Though the concentrations of these compounds are thought to be influenced by diet, a modulation of faecal water-genotoxicity can be referred to as biomarker of effect of dietary changes.

In this work, a new type of exploration was the use of faecal water to analyse potential chemopreventive effects *ex vivo*, but no beneficial effect of fish consumption was apparent: there was no change of faecal water genotoxicity **(publication V)** and apoptosis-inducing potential **(publication IV),** furthermore there was only a small effect on the global gene expression **(additional results)**. It is difficult to argue whether these null-results are an effect of lacking benefit of a high-fish diet or whether these are due to a methodological problem suggesting that faecal water may not be an appropriate surrogate for biomarker identification.

If *n*-3 PUFAs are the compounds accountable for beneficial effects of oil-rich fish a lack of impact of faecal water after high oil-rich fish consumption is not surprising. Alimentary fatty acids are expected in faeces only in small amounts. The digestion of fat takes place in the stomach and the intestine with several enzymes contributing to their degradation. Gastric predigestion facilitates fat digestion in the duodenum and affects 10-30% of the dietary fat. Preduodenal lipases preferentially hydrolyse the sn3-ester-bond resulting in the formation of sn1,2 diacylglycerol and free fatty acids [Hamosh & Scow 1973]. The major digestion results from pancreatic lipase in the duodenum. Pancreatic lipase activity results in the formation of sn-2 monoacylglycerol and free fatty acids [Lowe 1997]. The fats are emulsified by bile acids, which are strong detergents, and the available surface is thus increased. This implies a general conservation of 75% of all fatty acids in sn-2 position [Mu & Hoy 2004]. Pancreatic lipase activity towards *n*-3 fatty acids is lower and moreover the lymphatic recovery of EPA and DHA were found to be lower compared to other fatty acids [Ikeda *et al.* 1995]. Products of triacylglycerol digestion may cross the enterocyte membrane by diffusion or by a carrier-mediated process. The chain length of a fatty acid affects its transport and metabolism. Medium chain fatty acids with up to ten carbon atoms can be solubilised in the aqueous phase of intestinal contents, and bound to albumin. They are transported in the *vena portae* to the liver. Longer-chain fatty acids are transported by the lymphatic system [Hunter 2001]. Once

they enter the enterocyte monoacylglycerol and free fatty acids are used to resynthesise first diacylglycerol and than triacylglycerol in the smooth endoplasmic reticulum resulting in the deposition of fat droplets in its lumen [Mu & Hoy 2004]. Lipids are then packed into lipoproteins, predominantly chylomicrons and very low density lipoproteins, which are stable for transport in the aqueous environment. Chylomicrons are light particles (diameters <1.000 g/ml) which are heterogeneous in size (diameters 80–1000 nm) and consist of 90% neutral lipid, predominantly triacylglycerol with some cholesteryl ester, stabilised by a shell of amphipathic lipids, phospholipid and cholesterol, and protein. Intestinal lipoproteins do not enter the blood stream directly. Instead, they are secreted into lymph vessels to be found inside the intestinal villi, and then they enter the circulation in the *vena subclavia* via *ductus thoracicus.* After reaching the liver they are distributed throughout the body. In extrahepatic tissue, the chylomicrons' triglycerides are rapidly hydrolysed by the activities of lipoprotein lipase to form chylomicron remnants. The liver recognises these remnants and they are assimilated by active transport. Triacylglycerides are secreted in form of very low density lipoproteins by the liver, hydrolytic activity of lipoprotein lipase form intermediary density lipoproteins and finally hepatic triacylglycerol lipase forms low density lipoproteins, *i.e.* the major cholesterol transporting lipoprotein [Mu & Hoy 2004]. Fatty acids and as such also *n*-3 PUFAs circulate in blood either in free form bound to albumin or esterified in triacylglycerol in the lipoprotein fraction.

Efficient absorption of fatty acids, predominantly in the jejunum and ileum, allows less than 5% of the ingested lipids to reach the colon [Carey *et al.* 1983]. From ileostomy studies was estimated that 5-8g/d of dietary residual fat (monoglyceride and fatty acids) may reach the caecum [Hill 1998]. Of impact for the fatty acid content of the faeces are gut fermentation products. It was estimated that 20% of ingested carbohydrates pass into the colon and are then fermented by colonic bacteria to form short chain fatty acids (acetate, propionate, and butyrate). Short chain fatty acids are absorbed via passive diffusion in their protonated form across epithelial cell membranes [Cummings *et al.* 1995]. They serve as energy source for non-transformed colonocytes or, alternatively, they are transported to the liver after entering portal bloodstream.

Although it is generally assumed that beneficial effects of fish consumption are mediated through increased concentrations of *n*-3 PUFAs in the blood it is also feasible that effects are exerted luminally. Studies on patients with ileostomies suggest that up to 2% of dietary fatty acids may escape absorption in the small intestine [Normen *et al.* 2006]. A meal containing a 300g portion of the study

salmon would contain 2.13g EPA and 4.11g DHA [personal communication with Gerda Pot]. If 2% escapes absorption that means 42.6mg EPA and 82.2mg DHA would arrive in the colon. If we estimate the volume of the colon to be in the range of 100-500ml[2] then the concentration of EPA and DHA might reach about 0.28-1.4mM EPA and 0.5-2.5mM DHA which in turn may suggest the lipid could have direct effect on the cells. However, the colonic bacteria will rapidly metabolise the fatty acids and so these high concentrations may only be present in the most proximal region of the large intestine.

There might also be study-related reasons which need to be considered when judging the impact of fish consumption on health markers. Firstly, volunteers of the FISHGASTRO study had a higher baseline fish-consumption compared with subjects of other fish oil intervention studies. Perhaps a more pronounced effect would be observed in a population of non-fish consumers. Secondly, the fish which was provided to the volunteers was intended to serve as an additional source of 2 portions fish/week. But it appears that the subjects partially substituted the fish they normally consume by the study fish. This resulted in a lower level of ingested *n*-3 PUFAs than intended (0.99g long chain PUFAs/1.3 portions salmon/week vs. 1.4g long chain PUFAs/2 portions salmon/week). However, there were no correlations between: (i) DNA damage of colonocytes *in vivo* and genotoxicity of faecal water of the same volunteer measured in LT97 cells *ex vivo*, and (ii) apoptosis measured in colon crypts *in vivo* and after treatment of LT97 cells with faecal water *ex vivo* of the same volunteers.

Since the number of subjects for comparative analysis of biomarkers, faecal water and colonocytes/biopsies is low, the results of both studies (apoptosis and genotoxicity) are explorative, and need to be confirmed in larger studies.

This is the first human study to date that has examined health-promoting effects of fish-consumption within an intervention trial. Previously, studies using fish-oil capsules have been performed [Bartelt *et al.* 2008; Bouwens *et al.* 2009; Cummings *et al.* 1995; Lund *et al.* 1999; Rees *et al.* 2006]. It has also been argued as to whether the relative bioavailability of fatty acids from oil-capsules is the same as for food-items. It is noteworthy that the relative bioavailability of fatty acids from oil-rich fish is higher than that of fish-oil capsules [Visioli *et al.* 2003]. So it was calculated, that more than a double EPA dose administered by capsules and almost a nine-fold dose of DHA would be required to reach the same increment in plasma as after a fish meal [Visioli *et al.* 2003].

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[2] Basing on the volume of chymus which reaches the colon (500ml/d) [Elmadfa & Leitzmann 1990] and the volume of the faeces (150-200g/d) [Kasper 1996].

Concluding remarks:

The results of the faecal water experiments do not support the hypothesis that the biomarker of *ex vivo* analysis of faecal water genotoxicity, apoptosis induction and alteration of gene expression reflects nutritional changes of an oil-rich fish intervention. First of all, fish intervention had no or only marginal impact on the above mentioned parameters. Secondly, the *ex vivo* faecal water experiments do not reflect *in vivo* findings regarding DNA damage in colonocytes **(publication V)** and apoptosis in colonic crypts [Pot *et al.* 2009] of the same study.

6. SUMMARY AND FUTURE PERSPECTIVES

The objective of this study was to elucidate the impact of fish-consumption on chemoprevention of colorectal cancer. Of all constituents, *n*-3 PUFA abundant in fish fat, namely EPA and DHA were proposed to be the compounds which act beneficially. Thus, examinations in human colon cells *in vitro* were the subject of the investigations to the chemopreventive effects of fish *n*-3 PUFA in this work:

Uptake of fatty acids into LT97 and HT29 cells and impact of fatty acids on LT97 and HT29 cell growth **(publication I)**

All examined fatty acids (LA, ALA, GLA, ARA, EPA, and DHA) were taken up by LT97 and HT29 cells in the same manner being a prerequisite for fatty acids to exert cellular effects. Furthermore, cell growth was reduced in a concentration- and time-dependent manner. Effects were more pronounced in adenomatous LT97 compared to adenocarcinoma HT29 cells. To elucidate effects of EPA and DHA on human colon cells of different transformation state, further in depth experiments are needed.

Modulation of LT97 gene expression patterns by EPA and DHA **(publication II)**

EPA and DHA are known to alter gene expression and the experiments with adenomatous LT97 cells revealed partially different effects by the tested fatty acids EPA and DHA. Also, a time effect in the gene expression response was observed (10 vs. 24 hours). These preliminary results show probable regulated genes which need to be verified in the future and may then serve as target genes for further studies in this field of research. Additionally, the results also show the importance of treatment times for *in vitro* testings.

Modulation of SOD2, *GSTT2*, and COX-2 expression in LT97 and HT29 cells **(publication III)**

EPA and DHA may reduce oxidative stress by inducing *SOD2*. Effects on *GSTT2* were opposite for EPA (downregulation) and DHA (upregulation) in HT29 cells. Furthermore, anti-inflammatory effects may follow the DHA-caused reduction of *COX-2* in HT29 cells (10 and 24 hours) and LT97 cells (10 hours). In contrast, EPA induced LT97's *COX-2* (24 hours) potentially resulting in anti-inflammatory EPA-derivatives by enzymatic COX-2 activity which needs further clarification.

Induction of apoptosis by EPA and DHA in LT97 and HT29 cells **(publication IV)**

A mechanism to remove altered cells is the induction of apoptosis. EPA and DHA induced apoptosis (measured by flow cytometry) and both, extrinsic (cleavage of procaspase 8 and truncation of bid) and intrinsic pathway molecules (reduction

of *Bcl-2*) were altered. Furthermore, downstream targets such as cleavage of procaspase 3 to become an executioner caspase and PARP cleavage were detected. LT97 cells were more prone to apoptosis inducing potential of DHA and EPA compared to HT29 cells and DHA was more effective in inducing apoptosis than EPA.

In further studies, a biomarker approach was chosen to test the impact of fish-consumption on colonic health. Faecal water of a subset of the volunteers of the FISHGASTRO study was used to test effects *ex vivo:*

Modulation of chemoprevention markers by faecal water of 89 volunteers *ex vivo* **(publication IV and publication V)**

Results from this study do not support the hypothesis of a chemopreventive action by an additional consumption of oil-rich fish regarding the modulation of genotoxicity (measured in HT29 cells by the Comet Assay), and mediation of apoptosis (measured in LT97 by flow cytometry). Additionally, DNA damage in colonocytes from biopsy samples was not altered by fish-consumption. Furthermore, *in vivo* findings (DNA damage in colonocytes, apoptosis in colonic crypts [Pot *et al.* 2009]) did not correlate with *ex vivo* results (genotoxicity of faecal water, apoptotic impact of faecal water, respectively). Whether or not this is related to the lack of impact of a fish consumption on colon cancer still needs to be further elucidated.

Modulation of global gene expression by faecal water of 39 volunteers *ex vivo* **(additional results)**

Ex vivo global genome modulation by faecal water treated LT97 cells was conducted in a subset of the FISHGASTRO volunteers (n=39). Results showed differential expression of several pathways for the cod and the salmon group, though the effect size was rather low compared to other studies. The accordance with global gene expression alteration in colonic biopsies *in vivo* will be the subject of future examinations.

Evaluation of faecal water as biomarker to monitor health beneficial effects of fish

It was not possible to affirm the hypothesis that faecal water-associated biomarkers are suitable to test the impact of fish consumption *ex vivo*. Nevertheless, as faecal water contains a variety of compounds able to impair the gut health and modifiable by nutrition (*e.g.* bile acids by fat, butyrate by dietary fibre, polyphenolic compounds by vegetables and fruits), the analysis of the concentrations of these compounds in the analysed samples would be of interest. A further assessment of the analysed data regarding the influence of probable

confounding factors (*e.g.* bile acids) would be useful. There is promising evidence that future intervention studies may use faecal water as a biomarker. But a prerequisite is that potentially beneficial compounds reach the colon, are significantly altered by the dietary intervention, remain unaltered from bacterial degradation or result from it, and are present in faecal water, *e.g.* an intervention with dietary fibre results in fermentation by commensal bacteria and give rise in short chain fatty acids as butyrate which is discussed as serving beneficial during the development of colorectal cancer.

Overall future perspectives

To further investigate chemopreventive mechanisms of *n*-3 PUFAs *in vitro*, the inclusion of tests on primary human colon cells in comparison to adenoma and carcinoma cell culture is of interest. Since the culture of primary human cells *e.g.* from colon resection samples is not yet well established, this issue remains crucial. Cell culture models such as the used LT97 and HT29 or others like the human colon epithelial cells FHC which are of foetal origin and primary-like could be used to further clarify the influence of EPA and DHA *in vitro* on advanced markers of apoptosis. As it was shown, apoptosis is triggered by both, intrinsic and extrinsic pathway. However, apoptosis-mediation by a specific receptor pathway-activation remains unclear. Also investigations regarding mechanisms of intrinsic apoptosis (cytochrome c, oxidative stress *etc.*) are feasible.

As the FISHGASTRO study was to date the first human intervention trial giving fish, future *in vivo* studies to prove the epidemiological evidence of fish being beneficial for colon cancer prevention would be useful. To strengthen the effect of fish, some alterations compared to the FISHGASTRO study should be made. As it is probably not possible to increase the additional consumption of fish to more than two extra-portions per week, it would be promising to compare fish consumers vs. non-fish consumers allowing the investigation of a broad range of fish intake.

7. ABSTRACT

Of all constituents, *n*-3 polyunsaturated fatty acids are thought to be the crucial cancer chemopreventive factors to which anti-cancer properties of oil-rich fish may be attributed. However, the molecular mechanisms of these effects are not well understood. Thus, the cancer chemopreventive properties of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were the core subject of *in vitro* studies. Furthermore, *ex vivo* studies examining samples of the first randomised controlled human trial intervening with lean and oil-rich fish, the FISHGASTRO trial, were screened for markers of chemoprevention.

For this, the impact of EPA and DHA on growth (DNA staining by 4',6-diamidino-2-phenylindole) and on metabolism (conversion of resazurin to resofurin) of HT29 human colon adenocarcinoma and LT97 human colon adenoma cells were elucidated. Furthermore, different endpoints of apoptosis were analysed [flow cytometrical detection of Annexin-V-FITC and 7-actinoaminomycin; Real-Time RT-PCR analysis of *Bcl-2* (B-cell chronic lymphocytic leukemia/lymphoma 2)-expression, Western Blot detection of caspases 3, 8 and 9, Bid (Bcl-2 Interacting Domain) and poly (ADP-ribose) polymerase (PARP)] in HT29 and LT97 cells. Additionally, the modulation of gene expression patterns in general (PIQOR™ Custom Array, only LT97), and specifically the modulation of antioxidative and inflammatory enzymes [Real-Time RT-PCR analysis of *glutathione S-transferase T2* (*GSTT2), superoxide dismutase 2 (SOD2)*, and *cyclooxygenase-2 (COX-2)* mRNA, Western Blot for SOD2 and COX-2 protein] were determined. For the evaluation of fish consumption in humans, a biomarker approach using faecal water was chosen. Faecal water was collected from a subset (89 volunteers) of the FISHGASTRO participants which additionally consumed lean (cod) or oil-rich (salmon) fish twice a week for half a year compared to a dietary advice. Using faecal water, the modification of genotoxicity (Comet Assay) and induction of apoptosis (flow cytometrical detection of Annexin-V-FITC and 7-actinoaminomycin) as well as the alteration of global gene expression (Affymetrix GeneChip®) were examined.

It was shown that EPA and DHA impair cell growth (LT97 cells were more susceptible compared to HT29 cells) and induce apoptosis (DHA was more active compared to EPA and effects in HT29 cells were weaker compared to LT97 cells). Gene expression was affected in a time- (10 vs. 24 hours) and substance-specific (EPA vs. DHA) manner. Overall, genes connected with biotransformation, cell cycle control, signalling pathways, apoptosis, and

inflammation were altered. More specifically, SOD2 induction and probably an enhanced peroxidase activity due to GSTT2 induction indicate reduction of oxidative stress. Whereas *GSTT2* was induced by EPA it was reduced by DHA in HT29 cells. Anti-inflammatory activity can be attributed to COX-2 decrease by DHA in both cell types. In contrast, EPA induced *COX-2* in LT97 cells.

It was not possible to prove that faecal water-incubated LT97 cells were an appropriate source for biomarker identification to test the hypothesis that additional consumption of fish is beneficial for gut health. There was no marked alteration of genotoxicity or apoptosis by the fish-intervention. The evidence of the comparably small impact of faecal water on the modulation of global gene expression in LT97 cells and the influence of an additional consumption of fish still require final assessment. The extent to which these findings may be related to a lack of impact of oil-rich fish needs further clarification.

In conclusion it was shown, that EPA and DHA exert chemopreventive properties in cell culture models. As the results from the human intervention study were not appropriate to judge the *in vivo* situation, there is still a need to prove the hypothesis that oil-rich fish acts in a colon cancer chemopreventive manner.

8. ZUSAMMENFASSUNG

Die chemopräventiven Effekte des Verzehrs von fettreichem Fisch sind vermutlich auf die *n*-3 mehrfach ungesättigten Fettsäuren zurückzuführen. Zugrundeliegende molekularbiologische Mechanismen innerhalb der Kolonkarzinogenese bedürfen allerdings noch der genaueren Aufklärung. Gegenstand der vorliegenden Arbeit waren daher *in vitro* Analysen zur Rolle von Eicosapentaensäure (EPA) und Docosahexaensäure (DHA) in der Chemoprävention von Kolonkrebs. Darüber hinaus erfolgten *ex vivo* Untersuchungen zum Einfluss von Proben aus der ersten randomisierten, kontrollierten Humanstudie, der FISHGASTRO Studie, in der mit magerem und fettreichem Fisch interveniert wurde, auf verschiedene Marker der Chemoprävention.

Es wurde der Einfluss von EPA und DHA auf das Zellwachstum (DNA Färbung mittels 4',6-Diamidino-2-Phenylindol) und den Metabolismus (Konversion von Resazurin zu Resorufin) von humanen HT29 Kolontumor- und LT97 Kolonadenomzellen bestimmt. Des Weiteren wurden verschiedene Endpunkte der Apoptose [durchflusszytometrische Messung von Annexin-V-FITC und 7-Aminoactinomycin; *Bcl-2* (B-cell chronic lymphocytic leukemia/lymphoma 2)-Expression mittels Real-Time RT-PCR, Western Blot-Untersuchung der Caspasen 3, 8 und 9, sowie Bid (Bcl-2 Interacting Domain)-Abnahme und Spaltung der Poly (ADP-Ribose) Polymerase (PARP)] in HT29 und LT97 Zellen analysiert. Darüber hinaus galt es die Modulation des Genexpressionsmusters (PIQOR™ Custom Array, nur LT97 Zellen) und speziell die Modulation antioxidativer und inflammatorischer Enzyme *(Superoxiddismutase 2 (SOD2), Cyclooxygenase-2 (COX-2)* und der *Glutathion S-Transferase T 2 (GSTT2)* mittels Real-Time RT-PCR zu untersuchen, sowie SOD2- und COX-2-spezifische Western Blot-Analysen durchzuführen. Für die Einschätzung der Wirkung des Fischverzehrs im Menschen sollte ein Biomarker-Ansatz dienen. Dafür wurden Fäzeswässer eines Teils der Probanden der FISHGASTRO Humanstudie, bei der zusätzlich zwei Portionen magerer (Kabeljau) oder fettreicher (Lachs) Fisch pro Woche über einen Zeitraum von sechs Monaten im Vergleich zu einer Ernährungsempfehlung verzehrt wurde, gewonnen. Die Fäzeswässer wurden im Hinblick auf ihr Potential die Genotoxizität (Comet Assay), die Apoptose (durchfluss-zytometrische Bestimmung von Annexin-V-FITC und 7-Aminoactinomycin) sowie die globale Genexpression (Affymetrix GeneChip®) zu modifizieren untersucht.

Eine Verminderung des Zellwachstums durch EPA und DHA (LT97 Zellen waren empfindlicher im Vergleich zu HT29 Zellen) sowie die Induktion der Apoptose (DHA war aktiver im Vergleich zu EPA und HT29 Zellen reagierten schwächer als LT97 Zellen) konnten gezeigt werden. Die Genexpression wurde sowohl zeit- (10 vs. 24 Stunden) als auch substanz- (EPA vs. DHA) abhängig beeinflusst. Dabei wurden vor allem Gene der Biotransformation, des Zellzyklus, von Signaltransduktionswegen, der Apoptose und der Inflammation moduliert. Es könnte zu einer Veränderung des oxidativen Stresses speziell durch die Induktion von SOD2 und einer möglichen Peroxidaseaktivität von GSTT2 führen, wobei *GSTT2* in HT29 Zellen durch EPA gesteigert und durch DHA vermindert wurde. Darüber hinaus können aus der durch DHA-bedingten und in beiden Zelllinien gefundenen Abnahme der COX-2 anti-inflammatorische Wirkungen resultieren. EPA hingegen induzierte *COX-2* in LT97 Zellen (24 Stunden).

Fäzeswasser erwies sich nicht als geeigneter Biomarker zur Bestätigung der Hypothese, dass ein zusätzlicher Verzehr von Fisch zuträglich für die Darmgesundheit ist. Durch die Inkubation von HT29 bzw. LT97 Zellen mit Fäzeswässern unterschiedlicher Spender konnten weder die Parameter Genotoxizität noch die Induktion von Apoptose signifikant moduliert werden. Die genaue Bedeutung des gefundenen vergleichsweise kleinen Einflusses von Fäzeswässern auf die globale Genexpression von LT97 und die Bedeutung eines zusätzlichen Fischverzehrs benötigt abschließender Analysen. Inwieweit diese Ergebnisse einem fehlenden Einfluss durch den Verzehr von fettreichem Fisch zuzuschreiben ist, bleibt weiteren Untersuchungen vorbehalten.

Insgesamt haben die vorliegenden *in vitro* Ergebnisse chemopräventive Eigenschaften von EPA und DHA in Zellkulturmodellen gezeigt. Für die Beurteilung der *in vivo* Situation sind die Ergebnisse der FISHGASTRO Humanstudie allerdings nicht ausreichend. Um zu überprüfen, ob sich fettreicher Fisch chemopräventiv auf die Entwicklung von Dickdarmkrebs auswirkt, sind weitere Untersuchungen notwendig.

9. APPENDIX

9.1 Affymetrix data on LT97 global gene expression modulation

Table 5. Differentially expressed genes of 19 volunteers after salmon intervention for six month.

FC	p value	gene name	Entrez ID	description
1,3229	0,0197	RP4-747L4.3	84832	hypothetical protein MGC12538
1,3097	0,0311	SELM	140606	selenoprotein M
1,2845	0,0028	ADAM21	8747	ADAM metallopeptidase domain 21
1,2652	0,0016	IGFBP6	3489	insulin-like growth factor binding protein 6
1,2570	0,0077	TNNT1	7138	troponin T type 1 (skeletal, slow)
1,2538	0,0279	CTSZ	1522	cathepsin Z
1,2475	0,0088	ABCG2	9429	ATP-binding cassette, sub-family G (WHITE), member 2
1,2365	0,0009	FADS3	3995	fatty acid desaturase 3
1,2361	0,0045	PDE9A	5152	phosphodiesterase 9A
1,2355	0,0382	FOS	2353	v-fos FBJ murine osteosarcoma viral oncogene homolog
1,2348	0,0440	SSFA2	6744	sperm specific antigen 2
1,2296	0,0203	LRSAM1	90678	leucine rich repeat and sterile alpha motif containing 1
1,2271	0,0056	C17orf73	55018	chromosome 17 open reading frame 73
1,2187	0,0186	LY6G6D	58530	lymphocyte antigen 6 complex, locus G6D
1,2144	0,0004	GPR55	9290	G protein-coupled receptor 55
1,2074	0,0066	ALDH3A1	218	aldehyde dehydrogenase 3 family, memberA1
1,2063	0,0161	LRRC58	116064	leucine rich repeat containing 58
1,1983	0,0039	SULT1A1	6817	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1
1,1963	0,0433	ZDHHC20	253832	zinc finger, DHHC-type containing 20
1,1951	0,0179	CSRP2	1466	cysteine and glycine-rich protein 2
1,1909	0,0308	DHRS2	10202	dehydrogenase/reductase (SDR family) member 2
1,1908	0,0058	GSDMA	284110	gasdermin A
1,1905	0,0186	C7orf10	79783	chromosome 7 open reading frame 10
1,1870	0,0246	IQGAP1	8826	IQ motif containing GTPase activating protein 1
1,1850	0,0281	PPP2R2C	5522	protein phosphatase 2 (formerly 2A), regulatory subunit B, gamma isoform
1,1842	0,0179	NUMBL	9253	numb homolog (Drosophila)-like
1,1816	0,0166	CA11	770	carbonic anhydrase XI
1,1815	0,0398	UGT1A8	54576	UDP glucuronosyltransferase 1 family, polypeptide A8
1,1808	0,0411	LRP8	7804	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor
1,1791	0,0459	SCPEP1	59342	serine carboxypeptidase 1
1,1768	0,0146	MMP1	4312	matrix metallopeptidase 1 (interstitial collagenase)
1,1755	0,0033	TM7SF2	7108	transmembrane 7 superfamily member 2
1,1712	0,0027	DMAP1	55929	DNA methyltransferase 1 associated protein 1
1,1711	0,0009	C14orf149	112849	chromosome 14 open reading frame 149
1,1702	0,0062	RTN2	6253	reticulon 2
1,1682	0,0006	ZMYND8	23613	zinc finger, MYND-type containing 8
1,1672	0,0040	CAGE1	285782	cancer antigen 1
1,1663	0,0390	IFNA16	3449	interferon, alpha 16
1,1657	0,0140	FNIP1	96459	folliculin interacting protein 1
1,1649	0,0161	MT1B	4490	metallothionein 1B
1,1644	0,0153	TUBA3C	7278	tubulin, alpha 3c
1,1637	0,0228	RBM14	10432	RNA binding motif protein 14
1,1607	0,0198	TBL1X	6907	transducin (beta)-like 1X-linked
1,1572	0,0026	LOC100129034	100129034	hypothetical protein LOC100129034
1,1566	0,0003	PLEKHH2	130271	pleckstrin homology domain containing, family H (with MyTH4 domain) member 2
1,1565	0,0063	LY6E	4061	lymphocyte antigen 6 complex, locus E
1,1529	0,0344	DAPK3	1613	death-associated protein kinase 3
1,1525	0,0184	IL6ST	3572	interleukin 6 signal transducer (gp130, oncostatin M receptor)
1,1525	0,0463	POLDIP3	84271	polymerase (DNA-directed), delta interacting protein 3
1,1519	0,0051	OR51B4	79339	olfactory receptor, family 51, subfamily B, member 4
1,1518	0,0116	LOC100133362	653325	similar to heterogeneous nuclear ribonucleoprotein A1
1,1498	0,0042	C7orf13	129790	chromosome 7 open reading frame 13
1,1442	0,0031	ACOT8	10005	acyl-CoA thioesterase 8
1,1434	0,0365	NELF	26012	nasal embryonic LHRH factor
1,1427	0,0137	DBNDD1	79007	dysbindin (dystrobrevin binding protein 1) domain containing 1
1,1427	0,0194	SPIRE1	56907	spire homolog 1 (Drosophila)
1,1420	0,0089	CDKN2A	1029	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
1,1419	0,0034	ANXA9	8416	annexin A9
1,1416	0,0309	MAP2K2	5605	mitogen-activated protein kinase kinase 2
1,1415	0,0139	PPM1J	333926	protein phosphatase 1J (PP2C domain containing)
1,1410	0,0144	ADCY7	113	adenylate cyclase 7
1,1402	0,0341	C11orf65	160140	chromosome 11 open reading frame 65
1,1396	0,0174	ZFPL1	7542	zinc finger protein-like 1
1,1395	0,0013	SLC6A20	54716	solute carrier family 6 (proline IMINO transporter), member 20
1,1376	0,0007	BAIAP3	8938	BAI1-associated protein 3
1,1363	0,0092	MOCS3	27304	molybdenum cofactor synthesis 3
1,1362	0,0017	TMEM132A	54972	transmembrane protein 132A
1,1361	0,0109	RNF146	81847	ring finger protein 146
1,1354	0,0111	KLRA1	10748	killer cell lectin-like receptor subfamily A, member 1
1,1354	0,0100	AIM1L	55057	absent in melanoma 1-like
1,1344	0,0393	MPI	4351	mannose phosphate isomerase
1,1343	0,0500	ANXA6	309	annexin A6
1,1341	0,0068	GCHFR	2644	GTP cyclohydrolase I feedback regulator
1,1338	0,0467	RAB6A	5870	RAB6A, member RAS oncogene family
1,1337	0,0120	C15orf17	57184	chromosome 15 open reading frame 17
1,1326	0,0329	CLIP2	7461	CAP-GLY domain containing linker protein 2

FC fold change (after salmon intervention/before salmon intervention), continued on the next page.

Table 5 continued.

FC	p value	gene name	Entrez ID	description
1,1320	0,0470	PYCARD	29108	PYD and CARD domain containing
1,1318	0,0218	PDAP1	11333	PDGFA associated protein 1
1,1314	0,0021	ARL4C	10123	ADP-ribosylation factor-like 4C
1,1310	0,0128	MICAL3	57553	microtubule associated monoxygenase, calponin and LIM domain containing 3
1,1306	0,0145	TNFRSF12A	51330	tumor necrosis factor receptor superfamily, member 12A
1,1305	0,0082	CEACAM1	634	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)
1,1301	0,0114	LOC100128919	100128919	similar to HSPC157
1,1299	0,0026	EML2	24139	echinoderm microtubule associated protein like 2
1,1281	0,0064	HIC2	23119	hypermethylated in cancer 2
1,1277	0,0059	UAP1L1	91373	UDP-N-acteylglucosamine pyrophosphorylase 1-like 1
1,1273	0,0403	KISS1R	84634	KISS1 receptor
1,1273	0,0079	PARP12	64761	poly (ADP-ribose) polymerase family, member 12
1,1260	0,0144	WDR46	9277	WD repeat domain 46
1,1255	0,0405	MME	4311	membrane metallo-endopeptidase
1,1247	0,0348	VCAN	1462	versican
1,1242	0,0018	TRIM2	23321	tripartite motif-containing 2
1,1241	0,0151	PAPSS2	9060	3'-phosphoadenosine 5'-phosphosulfate synthase 2
1,1238	0,0188	SH3GLB2	56904	SH3-domain GRB2-like endophilin B2
1,1224	0,0008	FLJ20712	55025	hypothetical protein FLJ20712
1,1215	0,0187	BTN2A2	10385	butyrophilin, subfamily 2, member A2
1,1213	0,0022	COL6A6	131873	collagen type VI alpha 6
1,1211	0,0134	ZNF121	7675	zinc finger protein 121
1,1210	0,0336	CPEB4	80315	cytoplasmic polyadenylation element binding protein 4
1,1189	0,0162	BCL7A	605	B-cell CLL/lymphoma 7A
1,1187	0,0209	FBXW12	285231	F-box and WD repeat domain containing 12
1,1175	0,0056	RPS6KA4	8986	ribosomal protein S6 kinase, 90kDa, polypeptide 4
1,1174	0,0269	PLCD3	113026	phospholipase C, delta 3
1,1171	0,0120	LOC652968	652968	hypothetical protein LOC652968
1,1169	0,0067	P2RX5	5026	purinergic receptor P2X, ligand-gated ion channel, 5
1,1164	0,0117	DKFZp686O24166	374383	hypothetical protein DKFZp686O24166
1,1160	0,0390	C6orf124	653483	chromosome 6 open reading frame 124
1,1157	0,0176	CCDC18	343099	coiled-coil domain containing 18
1,1155	0,0032	EIF5A	1984	eukaryotic translation initiation factor 5A
1,1148	0,0211	SULF2	55959	sulfatase 2
1,1146	0,0087	EIF2C2	27161	eukaryotic translation initiation factor 2C, 2
1,1143	0,0365	OTUB2	78990	OTU domain, ubiquitin aldehyde binding 2
1,1137	0,0296	ZNF620	253639	zinc finger protein 620
1,1134	0,0079	FBXL17	64839	F-box and leucine-rich repeat protein 17
1,1116	0,0252	SHF	90525	Src homology 2 domain containing F
1,1114	0,0089	ALOX15	246	arachidonate 15-lipoxygenase
1,1114	0,0362	FLJ40142	400073	FLJ40142 protein
1,1109	0,0006	ZFR2	23217	zinc finger RNA binding protein 2
1,1106	0,0233	HIGD1C	613227	HIG1 domain family, member 1C
1,1105	0,0033	GPATCH3	63906	G patch domain containing 3
1,1105	0,0056	SARM1	23098	sterile alpha and TIR motif containing 1
1,1103	0,0401	TSC2	7249	tuberous sclerosis 2
1,1102	0,0174	CCL24	6369	chemokine (C-C motif) ligand 24
1,1101	0,0034	CCL19	6363	chemokine (C-C motif) ligand 19
1,1100	0,0080	PNMT	5409	phenylethanolamine N-methyltransferase
1,1100	0,0353	IL33	90865	interleukin 33
1,1096	0,0193	GPR172A	79581	G protein-coupled receptor 172A
1,1094	0,0247	THSD4	79875	thrombospondin, type I, domain containing 4
1,1092	0,0307	TAGLN	6876	transgelin
1,1082	0,0051	THEM5	284486	thioesterase superfamily member 5
1,1081	0,0153	HMGA1	3159	high mobility group AT-hook 1
1,1080	0,0019	ATP1A3	478	ATPase, Na+/K+ transporting, alpha 3 polypeptide
1,1077	0,0190	C6orf118	168090	chromosome 6 open reading frame 118
1,1074	0,0257	WDR1	9948	WD repeat domain 1
1,1073	0,0429	HDX	139324	highly divergent homeobox
1,1068	0,0065	KLK12	43849	kallikrein-related peptidase 12
1,1063	0,0109	GP6	51206	glycoprotein VI (platelet)
1,1063	0,0208	ATP6AP1	537	ATPase, H+ transporting, lysosomal accessory protein 1
1,1057	0,0220	FKBP8	23770	FK506 binding protein 8, 38kDa
1,1051	0,0348	KIAA1407	57577	KIAA1407
1,1039	0,0154	RP11-278E11.2	442454	ubiquinol-cytochrome c reductase binding protein pseudogene
1,1038	0,0206	RRAS	6237	related RAS viral (r-ras) oncogene homolog
1,1035	0,0143	MRM1	79922	mitochondrial rRNA methyltransferase 1 homolog (S. cerevisiae)
1,1032	0,0277	ENTPD4	9583	ectonucleoside triphosphate diphosphohydrolase 4
1,1031	0,0267	CDYL	9425	chromodomain protein, Y-like
1,1026	0,0437	FGF2	2247	fibroblast growth factor 2 (basic)
1,1025	0,0253	LDLR	3949	low density lipoprotein receptor
1,1021	0,0144	FBXL19	54620	F-box and leucine-rich repeat protein 19
1,1021	0,0397	ZNF341	84905	zinc finger protein 341
1,1019	0,0227	ASZ1	136991	ankyrin repeat, SAM and basic leucine zipper domain containing 1
1,1018	0,0125	LMBR1	64327	limb region 1 homolog (mouse)
1,1014	0,0029	DALRD3	55152	DALR anticodon binding domain containing 3
1,1012	0,0188	IER3	8870	immediate early response 3
1,1012	0,0036	LOC645314	645314	similar to hCG1642995
1,1006	0,0030	PRCC	5546	papillary renal cell carcinoma (translocation-associated)
1,1004	0,0237	CHD4	1108	chromodomain helicase DNA binding protein 4
1,1003	0,0244	SECISBP2	79048	SECIS binding protein 2
1,1003	0,0402	ARTN	9048	artemin
1,1001	0,0036	CXCL17	284340	chemokine (C-X-C motif) ligand 17

FC fold change.

Table 6. Differentially expressed genes of 20 volunteers after cod intervention for six month.

FC	p value	gene name	Entrez ID	description
1,2912	0,0008	AKR1B10	57016	aldo-keto reductase family 1, member B10 (aldose reductase)
1,2816	0,0005	SLC7A11	23657	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11
1,2763	0,0005	ALDH3A1	218	aldehyde dehydrogenase 3 family, memberA1
1,2488	0,0060	UGT1A8	54576	UDP glucuronosyltransferase 1 family, polypeptide A8
1,2436	0,0004	SEMA6A	57556	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A
1,2205	0,0005	STC2	8614	stanniocalcin 2
1,2028	0,0064	MBD6	114785	methyl-CpG binding domain protein 6
1,2015	0,0028	EGF	1950	epidermal growth factor (beta-urogastrone)
1,1832	0,0181	FLT3LG	2323	fms-related tyrosine kinase 3 ligand
1,1770	0,0047	MME	4311	membrane metallo-endopeptidase
1,1761	0,0138	CHAC1	79094	ChaC, cation transport regulator homolog 1 (E. coli)
1,1757	0,0009	TXNRD1	7296	thioredoxin reductase 1
1,1752	0,0003	EPHX1	2052	epoxide hydrolase 1, microsomal (xenobiotic)
1,1751	0,0005	ZNF557	79230	zinc finger protein 557
1,1737	0,0013	SRXN1	140809	sulfiredoxin 1 homolog (S. cerevisiae)
1,1633	0,0492	PIWIL4	143689	piwi-like 4 (Drosophila)
1,1623	0,0215	PLEC1	5339	plectin 1, intermediate filament binding protein 500kDa
1,1620	0,0089	RICH2	9912	Rho-type GTPase-activating protein RICH2
1,1541	0,0003	CBFA2T3	863	core-binding factor, runt domain, alpha subunit 2 /// translocated to, 3
1,1540	0,0023	CBR1	873	carbonyl reductase 1
1,1507	0,0054	CYP4F11	57834	cytochrome P450, family 4, subfamily F, polypeptide 11
1,1505	0,0388	FLYWCH1	84256	FLYWCH-type zinc finger 1
1,1498	0,0002	CPA4	51200	carboxypeptidase A4
1,1493	0,0020	TCP11L2	255394	t-complex 11 (mouse)-like 2
1,1443	0,0138	CPEB2	132864	cytoplasmic polyadenylation element binding protein 2
1,1435	0,0074	EPB41L4A	64097	erythrocyte membrane protein band 4.1 like 4A
1,1432	0,0053	SMURF1	57154	SMAD specific E3 ubiquitin protein ligase 1
1,1425	0,0004	PIR	8544	pirin (iron-binding nuclear protein)
1,1409	0,0211	UCHL1	7345	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)
1,1391	0,0245	MCOLN3	55283	mucolipin 3
1,1358	0,0068	LTBP3	4054	latent transforming growth factor beta binding protein 3
1,1358	0,0043	SBF1	6305	SET binding factor 1
1,1347	0,0250	ARL6	84100	ADP-ribosylation factor-like 6
1,1346	0,0389	TTTY13	83868	testis-specific transcript, Y-linked 13
1,1342	0,0029	TBC1D2	55357	TBC1 domain family, member 2
1,1333	0,0153	ZNF711	7552	zinc finger protein 711
1,1326	0,0155	LOC1001296:	100129624	hypothetical LOC100129624
1,1320	0,0410	KIF21B	23046	kinesin family member 21B
1,1307	0,0160	FLJ10357	55701	hypothetical protein FLJ10357
1,1305	0,0227	APC	324	adenomatous polyposis coli
1,1296	0,0141	WIZ	58525	widely interspaced zinc finger motifs
1,1280	0,0224	PRKAR1B	5575	protein kinase, cAMP-dependent, regulatory, type I, beta
1,1276	0,0113	PARVB	29780	parvin, beta
1,1258	0,0049	C5orf36	285600	chromosome 5 open reading frame 36
1,1241	0,0050	LHFPL1	340596	lipoma HMGIC fusion partner-like 1
1,1240	0,0105	PEX10	5192	peroxisomal biogenesis factor 10
1,1215	0,0020	PGD	5226	phosphogluconate dehydrogenase
1,1213	0,0119	C8orf62	137133	chromosome 8 open reading frame 62
1,1209	0,0088	PRKCD	5580	protein kinase C, delta
1,1207	0,0367	FBXL6	26233	F-box and leucine-rich repeat protein 6
1,1207	0,0139	ATG9A	79065	ATG9 autophagy related 9 homolog A (S. cerevisiae)
1,1201	0,0282	NFKBID	84807	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta
1,1198	0,0049	PLEKHH1	57475	pleckstrin homology domain containing, family H (with MyTH4 domain) member 1
1,1198	0,0011	TRPC5	7224	transient receptor potential cation channel, subfamily C, member 5
1,1182	0,0238	RNF123	63891	ring finger protein 123
1,1181	0,0416	EPB41L5	57669	erythrocyte membrane protein band 4.1 like 5
1,1167	0,0062	OR2F1	26211	olfactory receptor, family 2, subfamily F, member 1
1,1166	0,0214	PLXDC1	57125	plexin domain containing 1
1,1161	0,0084	HOXC4	3221	homeobox C4
1,1159	0,0258	GLTSCR1	29998	glioma tumor suppressor candidate region gene 1
1,1158	0,0212	CLGN	1047	calmegin
1,1152	0,0219	PLCG2	5336	phospholipase C, gamma 2 (phosphatidylinositol-specific)
1,1140	0,0422	RAB27B	5874	RAB27B, member RAS oncogene family
1,1138	0,0193	HSD11B1L	374875	hydroxysteroid (11-beta) dehydrogenase 1-like
1,1130	0,0385	SHD	56961	Src homology 2 domain containing transforming protein D
1,1129	0,0424	LRP5	4041	low density lipoprotein receptor-related protein 5
1,1127	0,0196	L2HGDH	79944	L-2-hydroxyglutarate dehydrogenase
1,1122	0,0078	CDC42BPA	8476	CDC42 binding protein kinase alpha (DMPK-like)
1,1118	0,0467	RORA	6095	RAR-related orphan receptor A
1,1113	0,0064	ZNF296	162979	zinc finger protein 296
1,1104	0,0199	C14orf149	112849	chromosome 14 open reading frame 149
1,1099	0,0205	TFPI2	7980	tissue factor pathway inhibitor 2
1,1089	0,0032	PPP1R12B	4660	protein phosphatase 1, regulatory (inhibitor) subunit 12B
1,1080	0,0035	MMP27	64066	matrix metallopeptidase 27
1,1080	0,0094	NDP	4693	Norrie disease (pseudoglioma)
1,1080	0,0023	RARG	5916	retinoic acid receptor, gamma
1,1063	0,0015	SLCO1C1	53919	solute carrier organic anion transporter family, member 1C1
1,1057	0,0402	SCARB1	949	scavenger receptor class B, member 1
1,1055	0,0138	HTR1D	3352	5-hydroxytryptamine (serotonin) receptor 1D
1,1053	0,0134	ZNF595	152687	zinc finger protein 595
1,1052	0,0084	CAMKK1	84254	calcium/calmodulin-dependent protein kinase kinase 1, alpha
1,1023	0,0287	SOLH	6650	small optic lobes homolog (Drosophila)
1,1022	0,0227	CCNL1	57018	cyclin L1
1,1018	0,0287	C17orf68	80169	chromosome 17 open reading frame 68
1,1017	0,0298	ZNF772	400720	zinc finger protein 772
1,1016	0,0442	JMJD4	65094	jumonji domain containing 4
1,1008	0,0055	GOLGB1	2804	golgin B1, golgi integral membrane protein
1,1001	0,0031	C6orf142	90523	chromosome 6 open reading frame 142

FC fold change.

9.2 Preliminary experiments on faecal water

9.2.1 Modulation of LT97 cell growth

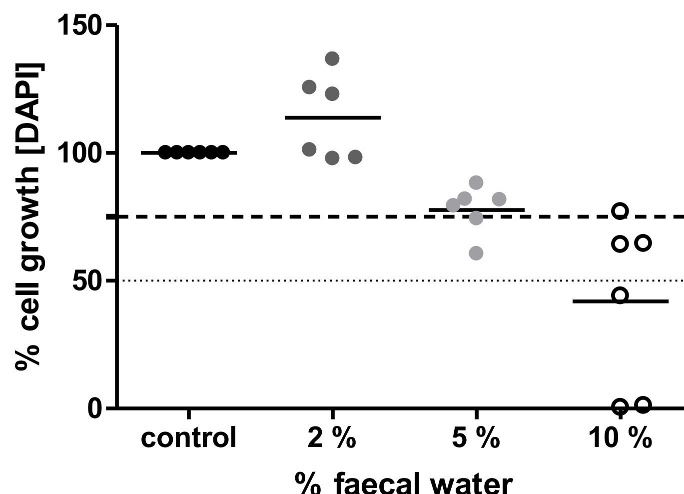

Figure 11. Impact of a 24 hour faecal water-treatment (n=6) with indicated concentrations on cell growth of LT97 cells. Data are presented as normalised fluorescence intensities setting the medium control to equal 100%. Dashed line indicates 75% cell growth, dotted line 50% cell growth.

Total faeces from the FISHGASTRO study was collected and stored at -80°C until further processing. Samples were defrosted, homogenised by stirring, transferred to aluminium cab-locked polycarbonate tubes (Beckman/Coulter GmbH, Krefeld, Germany), mixed with the same amount of ice-chilled PBS and centrifuged (Optima LE-80K Ultracentrifuge, Beckman/Coulter, Krefeld, Germany) at 25.000*g* for 2h at 4°C. The supernatant, representing the faecal water were aliquoted ant stored at -20°C.
LT97 cells were seeded into 96-well plates (Greiner Bio One GmbH, Frickenhausen, Germany), incubated in culture medium until they reached a confluency of ~80% prior to exposure with the indicated faecal water concentrations. Faecal water was diluted in culture medium, the amount of PBS was adjusted, and this dilution was sterile filtered using a 0.45µM syringe filter. The cells were cultured in an incubator (37°C, 95% humidity, 5% CO_2) for 24 hours and afterwards cell culture supernatant was discarded. Prior to staining with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, diluted in PBS, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) cells were fixed and lysed with methanol. After a 30min minimum incubation of the cells with DAPI, the resulting fluorescence intensity was measured using a microtiter plate reader (Spectra Fluor Plus, Tecan, Austria; Software: X-Fluor) (excitation at 360 nm / emission at 465 nm). The amount of DNA was considered to reflect the cell number. The data were analysed by setting the values of the control medium to equal 100%.
This assay was conducted to consider the toxicity of the faecal water samples and to choose a concentration of the Affymetrix global genome analysis (see chapter 4.1, page 91). A concentration of 3.5% was chosen as a marked influence on the cells without necessarily impairing cell growth was expected. Cytotoxic concentrations should be avoided.

9.2.2 Modulation of apoptosis in LT97 cells

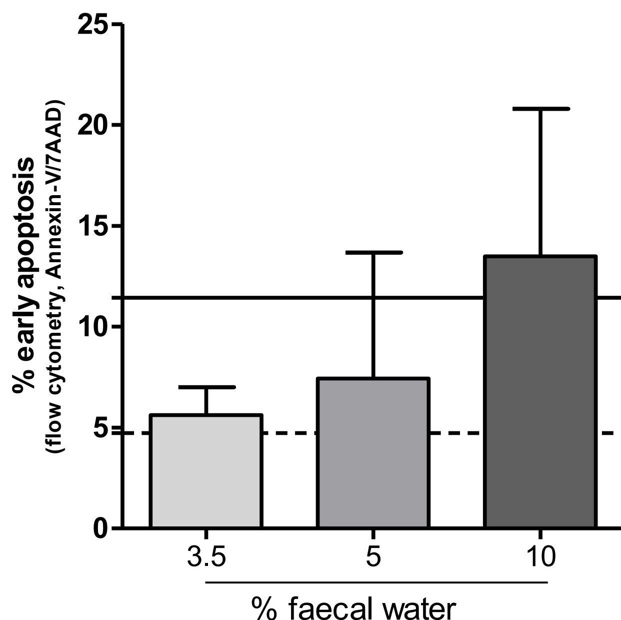

Figure 12. Level of early apoptotic LT97 cells caused by faecal water of indicated concentrations after 24h treatment. Annexin-V-FITC positive cells were measured using flow cytometry (relative counts). Dashed line indicates level of early apoptosis in medium treated cells, solid line indicates level of early apoptosis of cells exposed to 10mM butyrate (positive control).

Faecal water was extracted and applied as described in chapter 9.2.1, page 123.
LT97 cells were seeded in 6-well plates and cultured in medium until they reached a confluency of ~80%. Respective concentrations were applied and apoptosis was measured using a flow cytometer as described in **publication IV**, pages 60ff.
Aim of this preliminary test was the choice of a concentration for apoptosis assay applying faecal water to LT97 cells. Finally, a concentration of 3.5% was taken. Apoptosis by faecal water was already detected at this concentration compared to the medium control (not significant). It was hypothesised that by consuming oil-rich fish the apoptotic effects of the faecal water would increase. At 3.5% faecal water such a raise in apoptosis would be detectable. A second advantage was that the same concentration was also taken for detection of changes in gene expression patterns using Affymetrix Arrays. Exposing the cells with equal concentrations would allow a direct comparison of both parameters.

9.2.3 Genotoxicity of faecal water (concentration series)

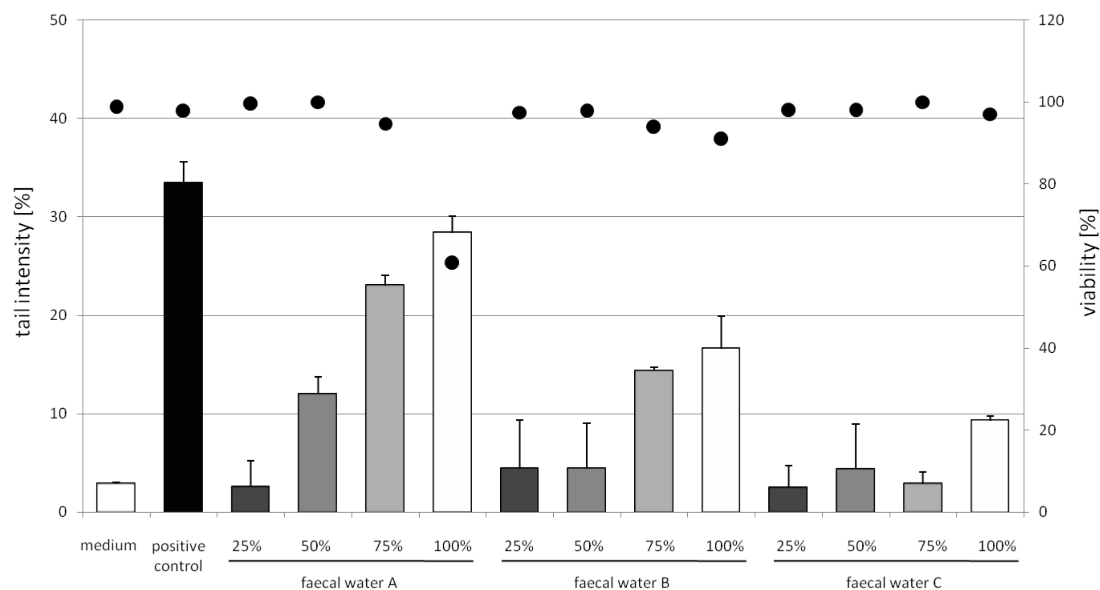

Figure 13. Genotoxicity of of three randomly chosen faecal water samples from the FISHGASTRO Study measured using the Comet Assay. Dilution series (indicated concentrations) in order to find a concentration for screening genotoxicity and antigenotoxicity of the FISHGASTRO faecal water samples. Medium control was PBS adjusted, positive control were H_2O_2-treated HT29 cells (75µM), genotoxicity was measured in HT29 cells (30min incubation, 37°C), tail intensity measured as triplicates, on the right hand side cell viability after indicated treatment is shown which are individually represented by the dots.

Faecal water was extracted and applied to HT29 cells and Comet Assay was conducted as described in publication V, page 71. Faecal water dilutions were adjusted by PBS.
Aim of this study was to find a suitable concentration for genotoxicity and H_2O_2-antigenotoxicity screening of the FISHGASTRO faecal water samples. In conclusion, a concentration of 50% faecal water was taken for the screening assays. At this concentration a trend of genotoxicity was measured without affecting viability in the treated cells, which was the case *e.g.* after incubation with 100% of faecal water from sample A. Detected tail intensities at 50% faecal water incubations allow a simultaneous incubation with 75µM hydrogen peroxide in order to measure antigenotoxicity.

9.3 Antigenotoxicity against hydrogen peroxide induced DNA damage of faecal water from the FISHGASTRO study

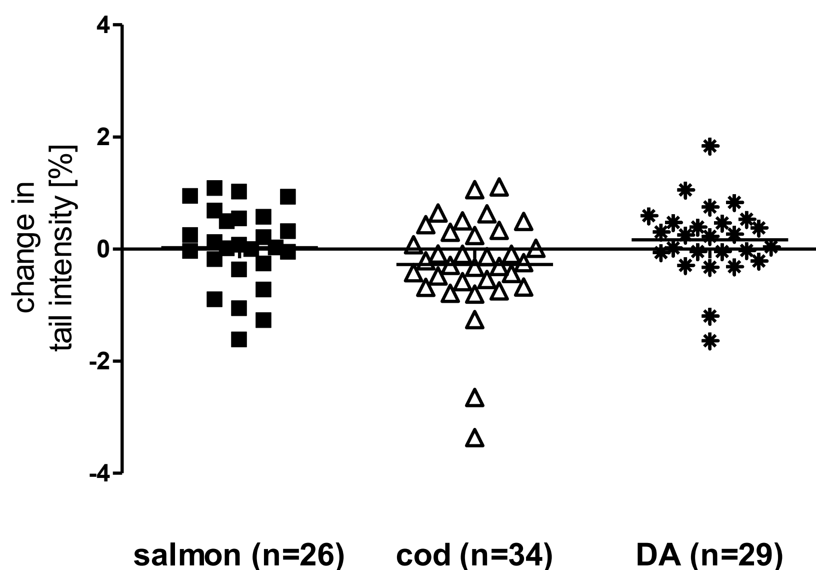

Figure 14. Results of changes in antigenotoxicity of faecal water (after minus before intervention). The horizontal lines indicate the mean values per intervention group.
DA dietary advice.

Faecal water was extracted and applied to HT29 cells and Comet Assay was conducted as described in publication V, page 71. Faecal water dilutions were adjusted by PBS. DNA damage was induced using 75µM hydrogen peroxide and prevention of DNA damage by co-incubation with faecal water was measured (30 min incubation at 37°C).

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-við munum gera betur næst / þetta er ágætis byrjun-

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LIST OF POSTERS AND PUBLICATIONS

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CERTIFICATION OF ORIGINALITY

To the best of my knowledge and belief, this thesis does not contain any material previously submitted for a degree at any university or any material previously written or published by any other person, except where acknowledgment in this regard is made in the text.

Jena, in February 2010 *Nina Habermann*