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The Effect of Omega-3 Polyunsaturated Fatty Acids on Human Cancer Cells

– Molecular Mechanisms Involved

Thesis for the degree of Philosophiae Doctor

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Children's and Women's Health

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Effekten av omega-3 flerumettede fettsyrer på humane kreftceller - involverte molekylære mekanismer

Fet fisk og fiskeolje (tran) har i århundrer vært vanlige bestanddeler i norsk kost. På siste halvdel av 1800-tallet produserte apotekeren Møller ren tran til medisinsk bruk. Den norske professoren Notevarp fant på 1950- og 1960-tallet at fiskeolje inneholdt omega-3 fettsyren (n-3 PUFA) dokosaheksaensyre (DHA). Han var også den første som beskrev en gunstig effekt av n-3 PUFAs på hjerte- og karsykdommer. På 1970-tallet fant legene Bang og Dyerberg at Inuitter fra Grønnland hadde lav forekomst av hjerte- og karsykdommer og relaterte det til økt innhold av n-3 PUFAs i blodet. Noen epidemiologiske observasjonsstudier har vist at n-3 PUFAs kan redusere forekomsten av noen krefttyper, blant annet tarmkreft, men resultatene har ikke vært entydige. Allikevel har noen kliniske studier, samt mange dyre- og cellekultureksperimenter vist at n-3 PUFAs har en vekstinhiberende effekt på noen typer kreftceller, men mekanismene bak dette er uklare. Målet med studiene i denne avhandlingen har vært å se nærmere på hvordan n-3 PUFAs påvirker veksten av kreftceller og studere hvilke mekanismer i cellene som endres ved tilsats av slike fettsyrer i vekstmediet.

Tilførsel av DHA til humane tarmkreftceller (SW620) i kultur viste at veksten ble kraftig redusert i fysiologisk relevante konsentrasjoner, og genekspresjonsanalyser indikerte endringer i uttrykket til flere tusen gener involvert i flere ulike biologiske spor. Tilførsel av n-3 PUFA medførte stress i endoplasmatisk retikulum (ER) og induksjon av ufoldet protein respons (UPR). Noen av hovedfunksjonene til ER er protein folding, regulering av kalsiumnivå og syntese av lipider og steroler. Forstyrrelse av disse mekanismene, som opphopning av ufoldede/feilfoldede proteiner, kan gi ER stress og induksjon av UPR. Stressresponsen som kjennetegnes ved økt fosforylering av eukaryot translasjons initieringsfaktor 2 α (eIF2α-P) ble indusert så tidlig som 3 timer etter DHA-tilførsel. Økt eIF2α-P fører til stopp i protein translasjon, redusert nivå av cellesyklusregulatoren cyclin D og cellesyklusarrest, slik at cellene får tid til reparere skader/gjenopprette homeostasen i ER. Dersom skadene blir for store, vil imidlertid cellenes dødsmaskineri (apoptose) aktiveres.

Genekspresjonsesultatene tydet også på endringer i kalsium- og kolesterolhomeostase. Tilførsel av DHA til SW620 cellene økte nivået av intracellulært kalsium, sannsynligvis som følge av frigjøring av kalsium fra ER og opptak av eksogent kalsium. Den cellulære kolesterolsyntesen reguleres av blant annet sterolregulerende elementbindende protein 2 (SREBP2). Til tross for aktivert SREBP2, ble bare noen av dens målgener oppregulert etter DHA-tilførsel. Nysyntese av kolesterol og inkorporering av nysyntetisert kolesterol til kolesterylestere ble nedregulert, mens det totale kolesterolnivået var lite påvirket.

Endringer i kalsiumhomeostase ble ytterligere undersøkt ved å benytte en human leukemicellelinje (HL-60) og en mutert klon av HL-60 (E2R2) som er resistent mot det kalsiumhomeostase-forstyrrende kjemikalet econazole. Veksten av HL-60 cellene ble kraftig redusert ved tilførsel av eikosapentaensyre (EPA), mens veksten av E2R2 cellene bare ble moderat påvirket. Genekspresjonsanalyser og induksjon av eIF2α-P i EPA-behandlede HL-60 celler indikerte endringer i kalsiumhomeostase og induksjon av UPR. Det ble imidlertid ikke påvist ER stress og UPR i E2R2 cellene. Dette tyder på at EPA-responsen i morcellelinjen var assosiert med endringer i kalsiumhomeostase og induksjon av UPR.

Tilførsel av DHA til SW620 cellene indikerte også endringer i uttrykket av flere gener og proteiner involvert i regulering av cellesyklus og celledød. Proteinnivået av survivin, livin og nukleær faktor kappa B (NFκB) ble redusert, noe som er interessant ettersom disse er indusert i ulike krefttyper og er kjente målproteiner for cellegiftbehandling. Hvorvidt n-3 PUFAs har en plass som supplement til klinisk kreftbehandling beror imidlertid på ytterligere forskning innen dette feltet.

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List of papers

Paper I:

Jakobsen CH*, Størvold GL*, Bremseth H*, Follestad T, Sand K, Mack M, Olsen KS, Lundemo AG, Iversen JG, Krokan HE and Schønberg SA. DHA induces ER stress and growth arrest in human colon cancer cells: associations with cholesterol and calcium homeostasis. *J Lipid Res*. 2008;49:2089-2100.

* Authors contributed equally to this work

Paper II:

Slagsvold JE, **Pettersen CHH**, Follestad T, Krokan HE and Schønberg SA. The antiproliferative effect of EPA in HL-60 cells is mediated by alterations in calcium homeostasis. *Lipids*. 2009;44:103-113.

Paper III:

Slagsvold JE*, **Pettersen CHH***, Størvold GL, Follestad T, Krokan HE and Schønberg SA. DHA alters expression of target proteins of cancer therapy in chemotherapy resistant SW620 colon cancer cells. *Nutr Cancer*. 2010;62(5):611-621.

* Authors contributed equally to this work

Abbreviations

AA	arachidonic acid (20:4 n-6)
ACAT	acyl-CoA:cholesterol acyltransferase
Akt	v-Akt murine thymoma viral oncogene homolog 1
ALA	α-linolenic acid (18:3 n-3)
ATF	activating transcription factor
Atg	autophagy gene
ATP	adenosine triphosphate
BAD	BCl-2 antagonist of cell death
BAK	BCl-2 antagonist killer
BAX	BCl-2-associated X protein
BCl-2	B-cell leukemia/lymphoma 2
BID	BH3-interacting domain death agonist
BIM	BCl-2-interacting protein
BiP/GRP78	heavy chain-binding protein/glucose-regulated protein of 78 kDa
BNIP3L	BCl-2/adenovirus E1B 19 kDa interacting protein 3-like
$[Ca^{2+}]_i$	intracellular calcium concentration
CAK	CDK-activating kinase
CDC25	cell division cycle 25
CDK	cyclin-dependent kinase
CE	cholesteryl ester
C/EBPβ	CCAAT/enhancer-binding protein beta
CHOP/GADD153	C/EBP homologous protein
ChREBP	carbohydrate response element-binding protein
CIP/KIP	CDK interacting protein/kinase inhibitory protein
CKI	CDK-inhibitor
CL	cardiolipin
c-myc	v-myc myelocytomatosis viral oncogene homolog
CO	corn oil
CoA	coenzyme A
CoAS	CoA synthetase
COX	cyclooxygenase
CYP	cytochrome P450 monooxygenase
DAG	diacylglycerol
DGLA	dihomo-γ-LA (20:3 n-6)
DHA	docosahexaenoic acid (22:6 n-3)
DIHETE	di hydroxyeicosatrienoic acid
Ec	econazole
EET	epoxyeicosatrienoic acid
EFA	essential FAs
eIF2α	eukaryotic translation initiation factor 2, α subunit
eIF2α-P	phosphorylated eIF2α
Elovl	elongation of very long-chain fatty acid
EPA	eicosapentanoic acid (20:5 n-3)
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FA	fatty acid
FABP	FA binding protein

FAO	Food and Agriculture Organization of the United Nations
FAP	familial adenomatous polyposis
FFA	free FA
FO	fish oil
G1	gap 1
G2	gap 2
GADD34	growth arrest and DNA damage gene 34
GRP94	glucose-regulated protein 94 kDa
HEPE	hydroxy-EPA
HPEPE	hydroxyperoxy-EPA
HETE	hydroxyeicosatrienoic acid
HMG-CoAR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HMOX1	heme oxygenase 1
HSP	heat shock protein
IAP	inhibitors of apoptosis
IC_{50}	half maximal inhibitory concentration
IP_3	inositol 1,4,5-triphosphate
IP3R	IP_3 receptor
IRE1	inositol-requiring enzyme 1
JNK	cJUN NH2-terminal kinase
LA	linoleic acid (18:2 n-6)
LC3	microtubule-associated protein 1 light chain 3/Atg8
LDL	low density lipoprotein
LIP	liver inhibitory protein
LOX	lipoxygenase
LPO	lipid peroxidation
LT	leukotriene
LX	lipoxin
LXR	liver X receptor
M	mitosis
MAG	monoacylglycerol
MAPK	mitogen-activated protein kinase
MO	menhaden oil
mTORC1	mammalian target of of rapamycin complex 1
MUFA	monounsaturated FA
N-3	omega-3
N-6	omega-6
N-6/n-3 ratio	the ratio between n6 and n-3 PUFAs
NCEH1	neutral cholesterol ester hydrolase 1
NFκB	nuclear factor kappa B
NO	nitric oxide
NPC1	niemann-pick disease type C1
NR	nuclear receptor
Nrf-2	nuclear factor erythroid-2-related factor 2
OA	oleic acid (18:1 n-9)
ORAI/CRACM1	Ca^{2+} release-activated Ca^{2+} modulator 1
P38 MAPK	p38 mitogen-activated protein kinase
$P58^{IPK}$	58 kDa inhibitor of PKR
PA	palmitatic acid (16:0)
PCNA	proliferating cell nuclear antigen

PERK	double-stranded RNA-activated protein kinase (PKR)-like ER kinase
PG	prostaglandin
PKC	protein kinase C
PLA_2	phospholipase A2
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated FA
Rb	retinoblastoma protein
ROS	reactive oxygen species
RXR	retinoid X receptor
RvE	resolvine E
RyR	ryanodine receptor
S	synthesis
S1P	site-1 protease
S2P	site-2 protease
SA	stearic acid (18:0)
SERCA	sarcoplasmic/ER-Ca^{2+}-ATPase
SFA	saturated FA
SFN/14-3-3σ	stratifin/14-3-3 sigma
SQSTM1	sequestosome-1
SREPB	sterol regulatory element binding protein
STIM	stromal interaction molecule
TAG	triacylglycerol
TE	total energy
TG	thapsigargin
TRIB3	tribbles-related protein 3
TX	thromboxanes
UFA	unsaturated FA
UPR	unfolded protein response
VCP	valosin-containing protein
WHO	World Health Organization
XBP-1	x-box binding protein-1

Abstract

Fatty fish and cod liver oil have been common ingredients in the Norwegian diet for centuries. In the latter half of the 1800s the apothecary Møller produced pure cod liver oil for medicinal use. In the 1950s and 1960s, the Norwegian Professor Notevarp found that fish oil contained the omega-3 polyunsaturated fatty acid (n-3 PUFA) docosahexaenoic acid (DHA). He was also the first to describe a beneficial effect of n-3 PUFAs on cardiovascular disease. In the 1970s, the doctors Bang and Dyerberg found that Greenland Inuits had low incidence of cardiovascular disease, and related it to increased n-3 PUFA content in the blood. Some epidemiological observational studies have found that n-3 PUFAs may reduce the incidence of some cancer types like colon cancer; however, the results have not been consistent. Yet, some clinical studies, as well as several animal and cell culture experiments have shown that n-3 PUFAs have a growth inhibiting effect on some types of cancers, but the mechanisms behind this effect are unclear. The aim of the studies has been to explore the molecular mechanisms behind the growth reducing effect of n-3 PUFAs on cancer cells *in vitro*.

Supplementation of DHA to human colon cancer cells (SW620) in culture strongly reduced cell growth in physiological relevant concentrations, and gene expression analysis indicated changed expression of thousands of genes involved in different biological pathways. The DHA treatment resulted in stress in the endoplasmic reticulum (ER) and induction of unfolded protein response (UPR). Some of the main functions of the ER are protein folding, regulation of calcium levels, as well as synthesis of lipids and sterols. Disruption of any of these mechanisms such as accumulation of unfolded/misfolded proteins may result in ER stress and induction of UPR. This stress response is characterized by phosphorylation of the eukaryotic translation initiation factor 2 α (eIF2α-P) which was induced as early as 3 hours after DHA administration. Increased eIF2α-P arrests protein translation, reduces the cell cycle regulator cyclin D and induces cell cycle arrest, allowing the cells to repair the damage/restore ER homeostasis. However, if damage is too severe, the cell death machinery (apoptosis) will be activated.

The gene expression results also indicated changes in calcium and cholesterol homeostasis. Addition of DHA to SW620 cells increased the intracellular calcium level, probably as a result of calcium release from the ER and uptake of exogenous calcium. Cellular cholesterol synthesis is regulated by e.g. sterol regulatory element binding protein 2 (SREBP2). Despite active SREBP2, only some of its target genes were up-regulated by DHA supplementation. New synthesis of cholesterol and incorporation of newly synthesized

cholesterol into cholesteryl esters were down-regulated, while total cholesterol levels were not markedly affected. Changes in calcium homeostasis were further investigated using a human leukemia cell line (HL-60) and a mutant HL-60 clone (E2R2) which is resistant towards the calcium homeostasis-disturbing chemical econazole. The HL-60 cells were strongly growth inhibited by addition of eicosapentaenoic acid (EPA), while the growth of E2R2 cells was only moderately affected. Gene expression analysis and induction of eIF2α-P in EPA-treated HL-60 cells indicated changes in calcium homeostasis and induction of UPR in the mother cell line, while there was no evidence of ER stress and UPR in E2R2 cells.

Administration of DHA to SW620 cells also resulted in changed expression of several genes and proteins involved in regulation of cell cycle and cell death. Protein levels of survivin, livin and nuclear factor κ B (NFκB) were reduced, which is interesting since they are induced in different tumors and are known targetproteins of chemotherapy. Whether n-3 PUFAs have a place as supplements to clinical cancer treatment depends, however, on further research within this field.

1. Introduction

1.1 Fatty acids – structure and function

Fatty acids (FAs) are important macronutrients. They consist of a hydrocarbon chain of variable length, with a carboxyl group (COOH) at one end and a methyl (CH_3) group at the other end (n- or ω-end) (Fig.1). FAs are classified according to the number of carbons in the chain and the type of bond between the carbons. Saturated FAs (SFAs) only have single bonds fully saturated with hydrogen atoms, while mono- (MUFA) and polyunsaturated FAs (PUFAs) have one or more double bonds, respectively, which are not saturated with hydrogen atoms (reviewed in (1-3)). The n-9 MUFA oleic acid (OA, 18:1) is the most common MUFA in human diet. Olive oil is rich in OA, but OA is also synthesized in the human body (reviewed in (3-5)). The most common PUFAs in nature are those of the n-3 and n-6 families, in which the first double bond is positioned either 3 or 6 carbons from the n-end, respectively. The n-3 PUFA α-linoleic acid (ALA, 18:3) and the n-6 PUFA linoleic acid (LA, 18:2) are considered essential fatty acids (EFA) because mammals lack the desaturase enzyme which inserts the double bond into the n-3 and n-6 position of PUFAs; hence these PUFAs have to be provided by the diet. Plants and vegetables have this desaturase and are therefore considered the main source of EFAs in mammals (reviewed in (2, 6)). Dietary sources of n-3 PUFAs are vegetable oils rich in ALA; canola and flaxseed, leafy green vegetables, walnuts, and seafood, fatty fish and fish oil (FO) rich in docosahexaenoic acid (DHA, 22:6) and eicosapentaenoic acid (EPA, 20:5) (Fig. 1). N-6 PUFAs mostly originate from plant oils rich in LA; corn, safflower and soybean (reviewed in (6)).

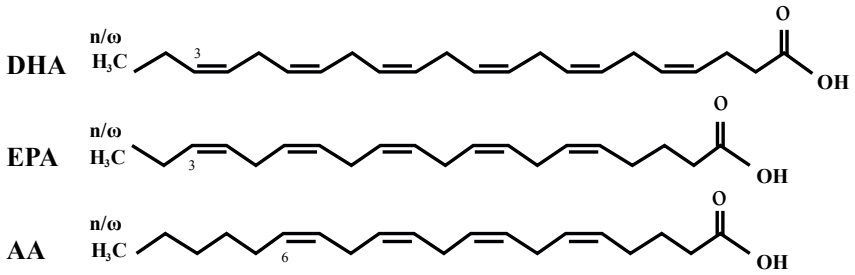

Figure 1. Structure and nomenclature of DHA, EPA and AA.

ALA and LA can be lengthened (elongated) and further desaturated through a series of steps involving elongases and delta-5 (Δ5)- and Δ6 desaturases (Fig. 2). ALA can be converted to EPA and further to DHA, while LA can be converted to e.g. arachidonic acid

(AA, 20:4 n-6). The last step in the conversion of both n-3 and n-6 PUFAs is a cycle of β-oxidation in the peroxisomes. However, n-3 and n-6 PUFAs are not convertible between the two classes. These two PUFA classes compete for the same metabolic enzymes. ALA has higher affinity for the Δ6-desaturase compared to LA, but since LA is considered the most common dietary PUFA source and is presented in higher amounts than ALA in the Western diet, LA will be the more common substrate for the Δ6-desaturase. Consequently, it is important to ensure high enough n-3 PUFA intake, especially through FO, in order to reduce LA desaturation and hence the production of AA and eicosanoids derived from this FA (reviewed in (4, 6, 7)). However, the activities of Δ5- and Δ6 desaturases are known to be slow in humans and different factors may influence their activities. Saturated fat and cholesterol inhibit their actions, insulin activates the Δ6 desaturase and the activity of this desaturase is reduced with age (reviewed in (8)).

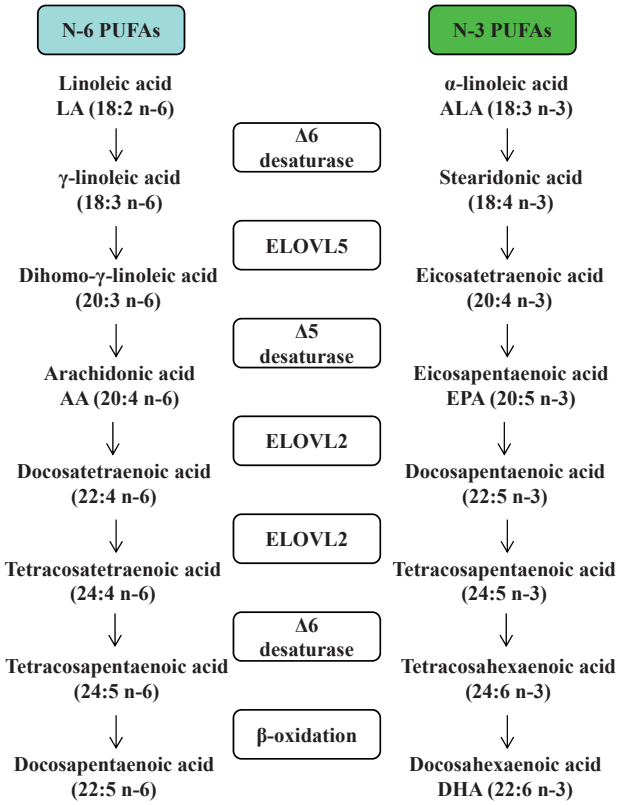

Figure 2. Desaturation, elongation and β-oxidation of n-3 and n-6 essential FAs. Full names are stated in the abbreviations list.

PUFAs are distributed to different degrees in different tissues and organs. The main n-6 and n-3 PUFAs in cellular membranes are AA and DHA, respectively (reviewed in (9, 10)). Of the n-3 PUFAs, DHA is present in all organs, but especially in brain and retina, while ALA and EPA are much less present in tissues. Of the n-6 PUFAs, AA is present in most tissues and LA is the most common PUFA stored in triacylglycerols (TAG) in adipose tissue (reviewed in (9)). The long chain FAs play different roles in cellular homeostasis. They are incorporated into membrane phospholipids, act as substrates for energy production through β-oxidation and serve as energy stores as part of TAGs. They are also involved in modification of proteins i.e. through acetylation in addition to being important in modification of gene expression (reviewed in (11-13)). The functions of FAs will be further dealt with in chapter 1.3.

1.2 Recommendations regarding PUFA intake and bioavailability

The Western diet today shows signs of increased consumption of fat. In the European populations, FAs count for as much as 28-42 % of total energy (TE) consumed, compared to approximately 20-30 % of TE in the diet of our ancestors (reviewed in (14)). The latest recommendations regarding dietary intake of fat and FAs state that the total fat intake should be within 20-35 % of TE, as concluded in the report "Fats and fatty acids in human nutrition" from the 2008 expert consultation held by the World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) (15). This recommendation is fulfilled in Norway (Table 1) (16). The type of fat consumed has changed towards an increase in saturated fat (especially animal fat) and n-6 PUFA rich oils (reviewed in (14)) and in Norway the consumed level of SFAs is higher than recommended (16). There has also been a rapid (from an evolutionary point of view) increase in consumption of foods rich in n-6 PUFAs and a decrease in the intake of n-3 PUFAs in Western societies during the past 150 years. This results in an increased n-6 to n-3 ratio (n-6/n-3), ranging from 10-20/1 compared to 1/1 in the ancestral diet (reviewed in (17)) which contained as much as ~5-6 g/day of n-3 PUFAs with a high EPA and DHA proportion (reviewed in (14)). PUFA consumption by Norwegians is just below the lower recommended intake of total PUFAs (n-3 and n-6) (Table 1). The consumed amount of n-6 PUFAs is above the recommended, however, the intake of n-3 PUFAs is sufficient according to recommendations (15, 16). An n-3 PUFA intake of 0.5 g/day correlates with a minimum of recommended fish intake of 2 fish meals per week (30-40 g/day), one of them oily fish (reviewed in (14)). However, in contrast to the values presented,

a national dietary survey in Norway found that mean daily fish intake was as high as 67 g/day and intake of long chain n-3 PUFAs was 0.9 g/day, counting for as much as 0.4 % of TE (18). Japanese also fulfil the n-3 PUFA recommendations, while Americans consume far too little DHA and EPA (Table 1) (16).

	Total fat		**PUFA**		**LA**		**ALA**		**EPA**		**DHA**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Con Norway	31.5% 97.4g	30.6% 67.0g	5.7%	5.3%	13.5g	8.8g	1.8g	1.2g	0.41g	0.27g	0.59g	0.40g
Con Japan	49.2g	46.2g			12.9g	10.7g	2.0g	1.6g	0.36g	0.31g	0.63g	0.57g
Con USA	98.1g	69.4g			17.1g	12.8g	1.7g	1.3g	0.05g	0.04g	0.09g	0.07g
Rec intake	20-35%		Tot 6-11%[a] n-3 0.5-2%[b]		2.5-9%		1-2 g $\geq$ 0.5%		0.25-2g			

Table 1. Recommended intake of dietary fat and FAs, and intake consumed in Norway, Japan and USA. Con = consumed, Rec = recommended, % = Percent of total energy intake, g = g/day, M = male, F = female. Data collected from (15, 16, 19). [a]Tot = LA + ALA + EPA + DHA, [b]N-3 = ALA + n-3 long chain PUFAs.

An increase in n-3 PUFA intake could be achieved by promoting fish consumption and increasing the use of ALA rich oils instead of dressing oils like safflower oil which is rich in n-6 PUFAs. Both wild fish and wild plants tend to have higher amounts of n-3 PUFAs than their farmed counterparts, hence intake of these should be promoted (reviewed in (14)). Industrial food could be prepared using n-3 PUFA rich oils. Feeding farm animals with n-3 FA-enriched food has resulted in production of eggs and milk with increased n-3 FA content and animals and plants can be genetically manipulated to contain more n-3 PUFAs (reviewed in (20)). FO supplements such as n-3 PUFA capsules are also an alternative way to increase consumption of these FAs. Intake of one 1 g standard FO capsule provides about 300 mg EPA and DHA (reviewed in (21)). Cooking methods also needs to be considered since n-3 PUFAs are highly oxidable resulting in harmful free radicals. The n-3 PUFA content in cooked fish might be reduced by as much as 50% (reviewed in (14)).

One way to assess the bioavailability of n-3 PUFAs is to measure their concentrations in blood serum. The basal n-3 PUFA concentration in healthy humans was in one study found

to be 136 µM EPA and 261 µM DHA when measured in serum. The concentrations increased by 129 % and 45 %, to 312 µM and 379 µM, respectively, after ingesting 57 g cooked salmon containing 0,7 g DHA and 0.5 g EPA/day for 8 weeks (22). Others have also shown that the EPA and DHA levels measured as free fatty acids (FFA) or phospholipids can be increased upon supplementation with n-3 PUFAs (9, 23, 24). Blonk *et al* found that 12 weeks of supplementation with 12 FO capsules (300 mg EPA and 200 mg DHA per capsule, in the form of ethyl esters) led to as much as 362 % and 69 % increase in EPA and DHA in plasma phospholipids, respectively (24). However, there tend to be a saturation level for n-3 PUFAs in blood (9, 24). Harris *et al* found that the DHA and EPA concentrations correlated well between plasma and red blood cells, the latter called the "Omega-3 index". This index is used as an n-3 PUFA bioavailability marker and reflects the n-3 PUFA intake during several weeks. It is expressed as n-3 PUFA % of total FAs in red blood cells, with 8 % as an optimal target level, and 4 % as undesirable, when it comes to cardioprotection (25).

1.3 Lipid metabolism

1.3.1 Lipogenesis

The liver and adipose tissue are the major sites of *de novo* FA biosynthesis, also called lipogenesis. Carbons from glucose (or acetate) are incorporated into FAs through a series of enzymatic reactions starting with the formation of malonyl-CoA from acetyl-coenzyme A (CoA) by acetyl CoA carboxylase (ACC). The multifunctional enzyme FA synthase (FAS) then uses malonyl-CoA as a carbon donor, adding two-carbon units to synthesize mainly the SFA palmitatic acid (PA, 16:0) (reviewed in (26, 27)). PA can be further lengthened by elongation of very long-chain fatty acid (Elovl) enzymes like Elovl6 in the endoplasmic reticulum (ER), or desaturated by the Δ9 stearoyl-CoA desaturaes (SCD), mainly SCD-1, thereby achieving the double bond characteristic of MUFAs (reviewed in (26)).

1.3.2 FA uptake, transport, storage and mobilization by lipolysis

Dietary TAGs are hydrolysed by lingual and pancreatic lipases into monoacylglycerol (MAG) and FAs before uptake by enterocytes, reesterification into TAGs and incorporation into chylomicrons. In the liver, FAs are incorporated into very low density lipoprotein (VLDL). Hence, FAs are transported in the blood as chylomicrons (exogenous FAs), VLDL

(endogenous FAs) as well as FFAs bound to albumin. The enzyme lipoprotein lipase (LPL) hydrolyzes FAs from chylomicrons and lipoproteins, which are then free to be transported into adipocytes for TAG synthesis (reviewed in (28)). However, the mechanisms by which FAs are taken up by adipocytes are not fully understood and it is debated whether the uptake is diffusion- or protein mediated. The diffusion theory is based on the observation that FAs can flip/flop through membranes, while the protein theory considers the action of FA translocase (FAT/CD36), FA transport proteins (FATPs) and plasma membrane bound FA binding protein ($FABP_{pm}$) (reviewed in (29)).

After entering the cell, FAs are bound to FABPs and transported to the ER where acyl-CoA synthetases (CoAS) activate FAs into fatty acyl-CoA thioesters (reviewed in (30)). These are esterified into TAGs by two different pathways. The MAG pathway esterifies FAs with MAG to form first diacylglycerol (DAG) and then TAG. This pathway counts for 75-85 % of synthesized TAG. The glycerol-3-phosphate pathway stepwise acetylates glycerol-3-phosphate and/or dihydroxyacetone (from glycolysis) to phosphatidic acid which is hydrolysed to DAG which is acetylated to TAG (reviewed in (31)). TAGs are stored in lipid droplets in the adipocytes, controlled by perilipins. During basal conditions perilipin-1 protects TAG from cytosolic lipases and promotes TAG storage, while upon increased energetic demands it controls mobilization of FAs from TAGs. This process called lipolysis yields FFAs and glycerol, and is controlled by the three lipases hormone-sensitive lipase (HSL), adipose triglyceride lipase and MAG lipase. The cAMP-dependent protein kinase A pathway is the main pathway known to activate lipolysis and HSL (reviewed in (32)). The FFAs bind to adipocyte FABP and are transported to the plasma membrane (reviewed in (29)). TAG is the main dietary source of FAs and through lipolysis the yield is ~95 g FAs from 100 g TAG (reviewed in (31)), counting for as much as 90 % of the fuel reserves in adults (reviewed in (33)).

1.3.3 β-oxidation of FAs

FAs are degraded to produce energy by a multistep process called β-oxidation. This process takes place mainly in the mitochondria, but also in peroxisomes. To be β-oxidized in the mitochondria, FFAs have to be taken up by the cell and converted to their fatty acyl-CoA thioesters as described above. This can be performed by an acyl-CoAS at the outer mitochondrial membrane. The inner mitochondrial membrane is impermeable to long chain CoA derivatives and therefore carnitine shuttle proteins have to carry these fatty acyl residues

across the membrane. In the mitochondrial matrix, the acyl residues are transferred from carnitine to CoA, thereby regenerating acyl-CoA thioesters which are used for β-oxidation. FAs up to 10 carbons can enter mitochondria independent of carnitine and are activated by acyl-CoASs in the mitochondrial matrix (reviewed in (30)).

There are four enzymatic steps in the β-oxidation spiral. The third step produces 3-ketoacyl-CoA that in the fourth step is cleaved between the α- and β carbons (hence it is called β-oxidation) to give acetyl-CoA and fatty acyl-CoA shortened by two carbons that can then enter the spiral again. The β-oxidation of unsaturated FAs (UFA) in mitochondria requires additional enzymes, in order to handle their double bonds (reviewed in (30)). Acetyl-CoA can be oxidized by the tricarboxylic acid/Krebs cycle to yield energy, used for the formation of ketone bodies through ketogenesis, or take part in cholesterol synthesis (reviewed in (27)). Both β-oxidation and lipolysis are regulated by the ratio of the hormones [glucagon]/[insulin] which again depend on the nutritional state of the animal (reviewed in (30)). During mitochondrial β-oxidation, electrons are transferred to, and hence reduce, flavin-adenine dinucleotide and nicotinamide-adenine dinucleotide (NAD^+) which drives adenosine triphosphate (ATP) synthesis by adding electrons to the electron transport chain. High energy level results in a high level of malonyl-CoA which inhibits the carnitine shuttle protein carnitine palmitoyltransferase I. Hence, β-oxidation increases when the energy and malonyl-CoA level is low and the adenosine monophosphate-activated kinase (AMPK) sensor of energy level is high. Mitochondrial β-oxidation oxidizes short- to long chain FAs, while very long chain FAs are β-oxidized in peroxisomes and ω-oxidized by the cytochrome P450 system (reviewed in (27)).

1.3.4 Incorporation of FAs into phospholipids of cellular membranes

FAs are part of the structural backbone of cellular membranes as they are incorporated into membrane phospholipids. The levels of SFAs and MUFAs in membranes are relatively constant, while the level of n-3 and n-6 PUFAs are influenced by the dietary intake of these FAs. This may be a consequence of the inability of higher animals to synthesize these PUFA classes *de novo*. The type of FAs in membrane lipids is important for membrane function. The length and number of double bonds in the chain of FAs are important for the fluidity of the lipids, with UFAs increasing the fluidity and therefore always occupying the sn-2 position of membrane lipids in order to achieve the correct physical properties of membranes (reviewed in (34)).

Membranes also contain lipid micro domains such as lipid rafts and caveolae which are membrane domains rich in cholesterol, sphingolipids and phospholipids with saturated fatty acyl chains. Several membrane receptors, signaling proteins and lipids are found within such lipid rafts, making these microdomains important in signal transduction. The caveolae, are enriched in the caveolin-1 protein and are, in addition to signal transduction, known to be important in endocytosis and cholesterol transport (reviewed in (35)).

1.3.5 Release of membrane bound FAs and eicosanoid synthesis

PUFAs are released from phospholipids through cleavage by the phospholipase A2 (PLA_2) enzyme family (Fig. 3). The C20 PUFAs dihomo-γ-LA (DGLA, 20:3 n-6), AA and EPA are then further metabolized to eicosanoids which are biologically potent, short-lived, local hormone-like lipids (autacoids) that affect inflammatory and immune responses, and are important in platelet aggregation, cellular growth and cell differentiation (reviewed in (10)). EPA and AA are metabolized by the same enzymes in the three eicosanid synthesis pathways (Fig.3); the cyclo-oxygenase (COX), lipoxygenase (LOX) and cytochrome P450 monooxygenase (CYP) pathways. The COX pathway produces prostaglandins (PGs) and thromboxanes (TXs), the LOX pathway gives leukotrienes (LTs), hydroxy FAs (HETEs, HEPEs) and hydroperoxy FAs (HPETEs and HPEPEs) and lipoxins (LXs), and the CYP pathway yields HETEs, diHETEs and epoxy FAs (EETs) (reviewed in (10, 36)). Eicosanoids derived from the n-3 PUFA EPA include the 3-series of PGs and TXs and the 5-series of LTs, HEPE and LX. Recently, other EPA- and DHA-derived autacoids have also been identified, named E series of resolvins (RvE, EPA-derived through aspirin-modified COX-2 and LOX), D series of resolvins (RvD, through DHA-derived aspirin-modified COX-2 or LOX), protectins (DHA-derived through leucocyte-mediated pathways) and maresins (DHA-derived through macrophage-mediated pathways) which are all anti-inflammatory (reviewed in (36, 37)). N-6 PUFA AA-derived eicosanoids include the 2-series of PGs and TXs, the 4-series of LTs, LXs, EETs, HETEs, diHETEs and aspirin-triggered lipoxin (ATL, through aspirin-modified COX-2 and LOX) (reviewed in (36)). DGLA-derived eicosanoids include the 1-series of PGs. The AA-derived eicosanoids are generally considered pro-inflammatory and some have been linked to carcinogenesis, while EPA-derived eicosanoids are anti-inflammatory and may have anti-cancer properties. Therefore the competition between AA and EPA for being incorporated to phospholipids will affect the inflammation status. As mentioned above, AA is the major PUFA in cellular membranes, but high intake of n-3

PUFAs will result in partial replacement of AA in the membrane phospholipids, less AA-derived eicosanoids and hence reduced inflammation (reviewed in (10)). The dietary n-6/n-3 ratio may also be important in modulation of the AA-derived eicosanoid synthesis, since the capacity of the n-3 PUFAs to suppress the LA to AA conversion depend on the amount of both n-3 and n-6 PUFAs in the diet (reviewed in (4, 38)).

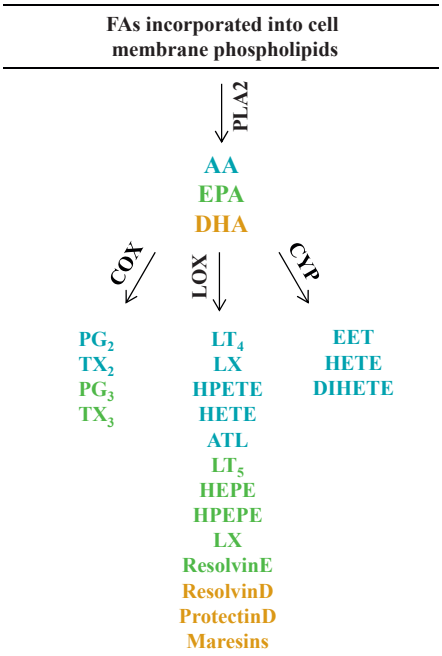

Figure 3. Biosynthesis of AA- and EPA-derived eicosanoids, including EPA- and DHA-derived autacoids. Full names are stated in the abbreviations list.

1.3.6 Regulation of lipid metabolism by PUFAs

Lipidomics is the "omics" research field of lipids, which includes the use of several different techniques for characterization of cellular lipids and the mechanisms by which they affect cellular functions (reviewed in (39)). PUFAs and their metabolites are known to affect gene expression through direct binding to transcription factor members of the nuclear receptor (NR) superfamily, and indirectly through affecting transcription factors like sterol regulatory element binding proteins (SREBP) and carbohydrate response element-binding protein (ChREBP). FAs are known to bind to and activate the peroxisome proliferator-activated

receptors (PPAR) family; PPARα, PPARβ and PPARγ. PPARs form heterodimers with the PUFA-binding retinoid X receptor (RXR) and bind to a PPAR/RXR consensus sequence in the promoter region of target genes, especially genes involved in lipid metabolism (reviewed in (40)). PPARα is a FA sensor regulating FA mobilization and catabolism, all three oxidation-systems described above as well as ketogenesis through its regulation of mitochondrial 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoAS) (reviewed in (27)).

The liver X receptors (LXRα and LXRβ) are also targets for FA regulation. LXRs bind oxysterols, form heterodimers with RXR, and regulate genes involved in hepatic bile acid synthesis. However, UFAs have been shown to antagonize oxysterol activation of LXR (reviewed in (13)). LXRs are also important in regulation of cholesterol homeostasis as well as lipogenesis through regulation of SREBP1c. PUFAs are known to supress lipogenic gene expression and suppress the gene expression, protein maturation and target gene expression of SREBP1C thereby reducing FA and TAG synthesis (reviewed in (27)). PUFAs also supress lipogenesis through interfering with the nuclear translocation of ChREBP (reviewed in (40)). The hepatic nuclear factor 4 (HNF-4α and HNF-4β) class of NRs binds fatty acyl-CoA and is important in regulation of several hepatic genes encoding proteins involved in e.g. lipoprotein metabolism and bile acid synthesis. Saturated acyl-CoA stimulates its transcriptional activity, while polyunsaturated fatty acyl-CoA inhibits the effects of HNF-4 on gene expression. The nuclear factor farnesoid X receptor (FXR) is activated by bile acids; however this activation is antagonized by PUFAs. The last known PUFA-binding NR is the retinoic acid-related orphan receptor beta, also known to regulate lipid metabolism (reviewed in (40)).

1.3.7 Cholesterol homeostasis

Cholesterol is an important lipid in e.g. maintenance of membrane fluidity and permeability, lipid raft formation and transcriptional regulation. It can be synthesized *de novo* or taken up via plasma lipoproteins like low density lipoprotein (LDL). Cholesterol synthesis, uptake, storage, transport and catabolism are tightly regulated by NRs like LXR and SREBPs which senses the cholesterol level (reviewed in (41)). The synthesis can be induced by SREBP1a and SREBP2, the latter known to regulate genes involved in cholesterol synthesis like HMG-CoA reductase (HMG-CoAR), the rate limiting enzyme in *de novo* cholesterol synthesis. In case of low cholesterol level, the SREBP cleavage-activating protein (SCAP) senses the low sterol level and escorts SREBP from its position in the ER membrane to the Golgi where the site-1

and site-2 proteases (S1P and S2P) cleave and activate SREBP. The nuclear SREBP fragment translocates to the nucleus and activates transcription of target genes with sterol response elements. In case of high cholesterol level, SCAP retains SREBP in the ER membrane (reviewed in (42)), hence SREBP-induced expression of genes involved in cholesterol synthesis and uptake is suppressed. NRs, especially LXRs, regulate gene expression involved in the sterol homeostasis towards storage, transport and catabolism. High cholesterol level also facilitates SREBP1c-induced transcription of genes involved in FA synthesis, thereby producing oleyl-CoA that is utilized by acyl-CoA:cholesterol acyltransferase (ACAT) in the esterification of cholesterol into cholesteryl esters (CE). CEs can be stored in lipid droplets or lipoproteins (reviewed in (41)).

1.4 N-3 PUFAs and disease

Pioneering work exploring the beneficial health effects of n-3 PUFAs was performed by the Norwegian professor Notevarp during the 1950s-1960s. A study published by his research group as early as in 1961 described the effect of n-3 PUFAs on cardiovascular disease (43). In the 1970s, Bang and Dyerberg revealed a connection between high consumption of sea food rich in n-3 PUFAs and low cholesterol- and TAG levels among the Greenland Inuit populations. They also speculated that these results could explain the low incidence of coronary heart disease in this population (44). Later, research has suggested a disease preventive effect of n-3 PUFAs, as outlined below.

N-3 PUFAs may improve dyslipidaemias and lower the plasma levels of TAGs. Individuals with established cardiac pathologies have been shown to benefit from n-3 PUFAs as they may slightly decrease blood pressure and inhibit the formation of atherosclerotic plaques, as well as reduce the risk of sudden death, cardiac arrhythmias and stroke (reviewed in (6, 21, 45, 46)). Even if the n-3 PUFA effect on cardiovascular disease has been studied extensively, a meta-analysis by Hooper *et al* found that results considering n-3 fats and cardiovascular disease and total mortality were inconsistent (47). However, their report had several drawbacks, as reflected by an expert panel report requested from the International Society for the Study of Fatty Acids and Lipids (ISSFAL). The authors concluded that the evidence regarding the risk-reducing effect of DHA and EPA on cardiovascular disease were sufficient at that time (2006) (48). Also, the experts attending the FAO/WHO consultation in 2008 concluded that there was convincing evidence for decreased risk of coronary heart disease when SFAs are replaced by PUFAs (15).

Increased consumption of n-3 PUFAs may also be cardio protective in persons with diabetes type 2 (reviewed in (49)), and patients with the multifactor disease metabolic syndrome (MS) (reviewed in (14)). Observational studies have suggested that higher maternal intake of DHA during pregnancy may have positive influence on some foetus developmental outcomes (reviewed in (50)). N-3 PUFAs may also possibly prevent different neurodegenerative diseases, like modify the risk and progression of Alzheimer's disease. In addition, they may have positive effects on inflammatory diseases like rheumatoid arthritis and inflammatory bowel disease (reviewed in (6, 21, 45, 46)). However, the reports on n-3 PUFAs and the effect on different diseases are not fully conclusive, which is reflected in the FAO/WHO report stating that there is a need for further investigation on e.g. the effect of n-3 PUFAs on diabetes, Alzheimer's disease and MS (15).

N-3 PUFAs have been shown to interfere with the catabolic signal transduction pathways involved in cancer cachexia, and may possibly have a positive influence on the loss of weight and lean body mass in advanced cancer patients (reviewed in (51)). However, there is inconsistency between studies reporting on cachexia and n-3 PUFAs, as reviewed in (52). Extensive research has also found that n-3 PUFAs may possibly work as chemopreventive agents. Some studies have found an increased intake of n-3 PUFAs to reduce the risk and incidence of cancers of e.g. colon and breast. However, these results are not consistent; several studies have not found such an association (reviewed in (4)) and the FAO/WHO report encourage more research to be performed also in this field (15). The possible "anti-tumor" effect of PUFAs will be further outlined below.

1.4.1 N-3 PUFAs and cancer

Dietary fats have for a long time been ascribed a role in cancer development (reviewed in (53, 54)). Early epidemiological studies reported a positive correlation between high dietary fat content and e.g. colon cancer risk. However, some of these studies did not consider the different types of fats or their FA content, which later epidemiological observational studies found to be important when assessing the relationship between dietary fats and cancers, and not the total fat *per se* (reviewed in (4, 55)). Diets rich in FO and n-3 PUFAs have been inversely correlated with the incidence of colorectal and breast cancer in some epidemiological observational studies, while diets high in animal fat and n-6 PUFAs correlated with an increased risk of these cancers (reviewed in (54, 56)). However, results from epidemiological studies exploring the relation between PUFAs and cancers have not

been consistent. Even so, cell culture and animal experiments have shown that n-3 PUFAs display a growth inhibitory effect on cancer cells originating from e.g. colon, breast and leukaemia (reviewed in (2, 4, 10, 57-59)). Interestingly, some clinical intervention studies have reported increased effect of conventional chemotherapy and normalization of abnormal colorectal tissue proliferation upon n-3 PUFA supplementation (60-64).

Epidemiological observational studies

Epidemiological observational studies have shown divergent results regarding the anti-tumor effects of n-3 PUFAs. Some studies have reported an association between increased n-3 PUFAs or fish intake and reduced risk for cancers of colon, breast and prostate (reviewed in (10, 59, 65)). There are several factors that may have influenced the epidemiological data, and thereby complicated the interpretation of them, as outlined below.

The level of n-3 PUFA intake varies between different populations and a high level of fish consumption has been correlated with reduced incidence of some cancers. This has been reflected in studies from e.g. Japan, a country with relatively low breast cancer risk and dietary fat intake, but high consumption of n-3 PUFAs (reviewed in (4, 65)). However, the incidence of breast, colon and prostate cancers is now increasing in Japan natives, reflecting a more Westernized diet with decreased fish intake and increased dietary n-6 FAs. The breast cancer incidence in Japanese women has been shown to increase within one generation when migrating to the USA, a country with relatively high breast cancer risk and dietary fat intake, as well as low n-3 PUFA consumption (reviewed in (4, 66)). In Alaskan Eskimos and Aleuts lower rates of breast, endometrium and prostate cancers were reported. They have a diet with a high fat content; however this comes mainly from fish and marine mammals, giving them a high intake of n-3 PUFAs. As in Japan, later studies have shown increasing incidence of both breast and colon cancers in this population as well, possibly due to urbanization and changed dietary habits (reviewed in (4)).

It may also be difficult to reveal the real daily intake of n-3 PUFAs in population studies where the participants are having fish included in the diet as the n-3 PUFA source. This may be due to the differences in n-3 PUFA content between different fish species. Also, marine and farmed fish may be contaminated with carcinogenic organochlorine pesticides. This should be considered in order to avoid introduction of a carcinogenic agent (the pesticides) in addition to antineoplastic agents (n-3 PUFAs), since this could possibly interfere with the n-3 PUFA effect in a negative way. This is especially important for breast and prostate cancer since the incidence of these cancers has been associated with exposure of

such pesticides (reviewed in (57)). How to increase n-3 consumption was outlined in chapter 1.2. It is also important to mention that several epidemiological observation studies have analyzed the intake of n-3 PUFAs independently of the intake of n-6 PUFAs. However, the n-6/n-3 ratio has been found to be associated with breast cancer risk (reviewed in (10, 65, 66)).

Clinical and intervention studies

Several epidemiological observation studies, but not that many clinical studies, exploring the effect of n-3 PUFAs in human cancers have been performed. However, some clinical studies have been published on colon, breast and prostate. Early clinical studies were performed in the 1990s by Anti *et al* (60, 62). In the first study they supplemented high levels of EPA and DHA, 4.1 g and 3.6 g respectively, for periods of 2 weeks to 3 months to persons at high risk of having colon cancer because of sporadic polyposis of the colon. The intervention resulted in increased levels of DHA and EPA, as well as lower level of LA and AA, in the colonic mucosa and plasma. Also, the changed proliferation in colonic mucosa reverted to normal (62). The same outcome was also found in the second study, using the same type of patients, but supplementing with a lower amount of EPA and DHA (2.5 g/day) for 30 days or 6 months (60). Even if 2.5 g/day is somewhat higher than the recommendations from the FAO/WHO report (15), it is important to note that n-3 PUFA doses lower than 3.0 g/day are considered safe (reviewed in (67)).

In the nutritional intervention phase II trial by Read *et al*, a supplement with 0.92 g DHA and 2.18 g EPA/day for up to 9 weeks was given advanced colorectal cancer patients receiving chemotherapy. They found an increase in body weight and energy level. Plasma phospholipid EPA and DHA levels increased, while AA decreased upon the first three weeks of the intervention and were then stable until 9 weeks. Even if not significant, there was a trend towards improvement of the quality of life measurement for overall well-being, reduced diarrhea and fatigue (68).

An interesting randomized, double-blind, placebo-controlled clinical trial carried out by West *et al* enrolled patient with familial adenomatous polyposis (FAP) that had underwent colectomy and were undergoing endoscopy surveillance. Patients taking enteric-coated EPA as FFA 2 g/day for 6 months had reduced polyp number (>20 %) and size (~30 %), as well as decreased polyp burden, compared to the placebo group. EPA supplementation also led to a significant increase in mucosal EPA content. The EPA treatment was safe and well tolerated (44), and the dose was within the FAO/WHO recommendations (Table 1).

Bougnoux *et al* found that breast cancer patients with a higher n-3 PUFA level in breast adipose tissue responded better to chemotherapy (higher degree of tumor regression) and that DHA level was associated with tumor response (63). In a pilot phase II clinical trial, Bougnoux *et al* reported that DHA improved the outcome of women with breast cancer metastasis treated with an anthracycline-based chemotherapy. The dose used was 1.8 g DHA/day (which is within the FAO/WHO recommendations (Table 1)). They found the combination of chemotherapy and DHA to be safe without adverse effects. Both time to progression and overall survival was higher in patients which had a high incorporation of DHA into plasma phospholipids. DHA was found to act as a chemosensitiser, increasing the effect of chemotherapy (64).

Aronson *et al* reported the findings from a randomized prospective phase II trial in which prostate cancer patients undergoing prostatectomy were given either a low-fat or Western diet for 4-6 weeks. The low-fat diet contained 15 % calories from fat and an n-6/n-3 ratio of 2:1, while the Western diet had 40 % of calories from fat and an n-6/n-3 ratio of 15:1. Results showed that the prostate cancer proliferation decreased upon intervention in the low-fat diet group. Also, the n-6 PUFA, TAG and cholesterol levels decreased, while the n-3 PUFA level increased in the prostate tissue membranes after invention (61).

Animal studies

Animal studies using mice or rats bearing human cancer xenografts have shown that an n-3 PUFA-containing diet can slow down growth of different cancers, e.g. colon, breast, prostate and lung, as well as suppress the development and growth of carcinogen-induced cancers in animals (reviewed in (1, 4, 37)). In one study, Fini *et al* supplemented the diet of $APC^{Min/+}$ mice (a model for FAP) with 2 different doses of EPA in the form of FFA for 12 weeks. The lowest dose contained EPA (2.5 % of diet in g) and corn oil (CO, 4.5 %), while the highest contained EPA (5 %) and CO (2.0 %), and the control diet CO (7.0 %). The EPA diets both suppressed the number of polyps with over 70 % and the polyp load with over 80%, however, EPA 5 % was most effective (69). Bathen *et al* showed that supplementing the diet of athymic mice (implanted with human colon cancer cells) with FO (DHA and EPA 12 % of calories) compared to CO (12 % of calories) resulted in tumor growth reduction (70). N-3 PUFAs have also been shown to increase the efficacy of radiation therapy and different cancer chemotherapy drugs in vivo (reviewed in (57, 71). Hardman *et al* implanted lung cancer cells into mice and co-treated them with doxorubicin chemotherapy and FO (19 % of diet in g) and CO (1 %) or CO (20%). They found that FO in combination with chemotherapy significantly

reduced growth of cancer xenografts compared to chemotherapy in combination with CO (72). Reddy *et al* introduced rats to the azoxy-methane carcinogen and supplemented their diet with different levels of Menhaden oil (MO) containing n-3 FAs and CO. Introduction of MO to the diet reduced the incidence of having colon adenocarcinomas, however, only the diet with the highest content of MO (17.6 %) reduced the number of tumors/rat (73).

Studies showing an anti-tumor effect of n-3 PUFAs may be promising in the search for nontoxic alternative cancer therapies or co-treatments, such as combining conventional chemotherapy with n-3 PUFAs in order to sensitize cancer cells to lower concentrations of the antineoplastic drugs and reduce harmful side effects (reviewed in (57, 74)).

Cell culture studies

Several cell culture studies have gathered substantial evidence showing that n-3 PUFAs do have a growth-inhibiting effect on e.g. colon, breast and prostate cancer cells *in vitro.* The anti-tumor effect of these PUFAs occur through multiple mechanisms (reviewed in (2, 4, 10, 58, 65, 75)), as described in chapter 1.5. In vitro cell culture studies also show that n-3 PUFAs increase the sensitivity of several types of cancer cells to different chemotherapies, affecting different molecular mechanisms that inhibit cell growth (reviewed in (74)).

N-3 PUFAs and colon cancer

Among both sexes in Western populations in 2008, colorectal cancer was the 3rd most frequent type of cancer and cause of cancer deaths. Worldwide, the incidence of colorectal cancer in 2008 was ~1,235,000 (9.8 % of all cancers) and number of colorectal cancer deaths ~609,000 (8.1 % of all cancer deaths). Among the new incidences in 2008 about 60 % occurred in developed regions (76). In Norway in 2009, 27,520 new cancer incidents were reported, and colorectal cancer (2,405 new incidents) was the second and third most frequent cause of cancer deaths among women and men, respectively. The cumulative risk of developing colon cancer by the age of 75 in the period of 2005-2009 in Norway ,was 3 % among males and 2.7 % among females (this means that about 1 in 33 Norwegian men may develop this type of cancer before the age of 75). However, there is a trend towards a stabilization of the colon cancer incidence in Norway. More people survive and the mortality is declining for both men and women in regard to both colon and rectal cancer. This may be due to introduction of a new surgery type and preoperative radiation (77). The high incidence and mortality of colon cancer, implies the need for more scientific research, concerning the prevention and treatment of colon cancer.

Some epidemiological case-control and cohort studies have demonstrated an inverse association between n-3 PUFAs or fish intake and the risk of developing colorectal cancer. Yet, the results are inconsistent, since several studies find no such association. However, promising results are emerging from animal and cell culture studies, showing reduced incidence and growth of colorectal cancer after n-3 PUFA treatment. Also, n-3 PUFAs have been shown to increase sensitivity towards different colon cancer therapies (reviewed in (67, 78)). Some human intervention studies have also been performed, like the studies by Anti and West *et al* described above. Recently, Cockbain *et al* published a comprehensive review on n-3 PUFAs and their role in treatment and prevention of colorectal cancer (37).

1.5 Main mechanisms and biological pathways involved in the anti-tumor effect of n-3 PUFAs

Several different biological mechanisms and pathways have been proposed to explain the anti-tumor effects of n-3 PUFAs, as summarized in Fig. 4. Recently it has been suggested that certain PUFAs also are capable of enhancing the uptake of anti-cancer drugs and reducing the drug efflux in drug-resistant cells, thereby increasing their anti-tumor action (79). Before the n-3 PUFAs can be used as chemo preventive agents or as a supplement to existing cancer therapies, clarification of the mechanisms involved is needed.

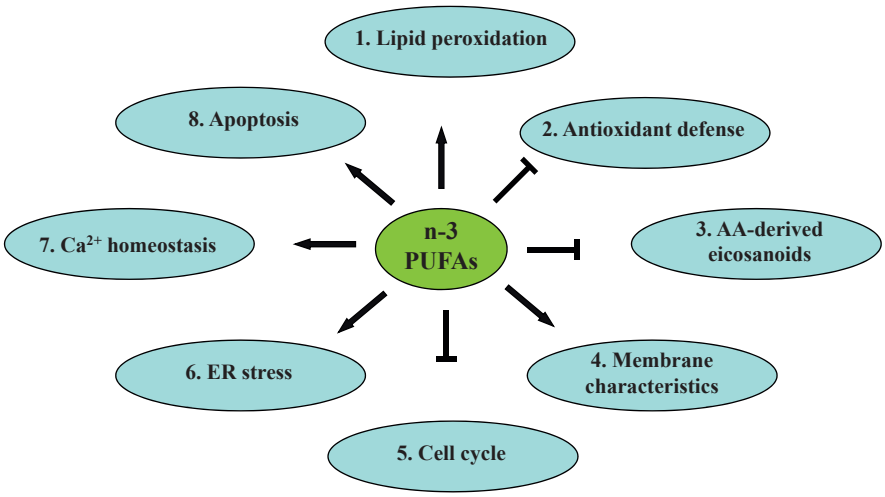

Figure 4. Summary of main mechanisms and biological pathways involved in the anti-tumor effect of n-3 PUFAs

1.5.1 Lipid peroxidation and antioxidant defense mechanisms

Long chain n-3 PUFAs, are highly susceptible to lipid peroxidation (LPO) because of their double bonds. Hence, their incorporation into phospholipids of cellular membranes may sensitize cells to reactive oxygen species (ROS) and thereby induce oxidative stress (reviewed in (80)). LPO is known to degrade phospholipids in cellular membranes, thereby changing their permeability and fluidity, as well as producing a range of reactive LPO products that drives the reaction further (reviewed in (81)).

The level of antioxidant enzymes may be altered in cancer cells; lower levels of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase affect in a negative way their ability to handle oxidative stress (reviewed in (82)). LPO products derived from n-3 PUFAs are involved in the antitumor effects of these PUFAs on some cancer cells, but the mechanisms behind this effect is not yet clear (reviewed in (80)). Antioxidants like vitamin E and SOD may be able to prevent this effect. Free radicals and LPO can lead to cell death by damaging several enzymes, proteins, DNA and depleting ATP levels in the cells. PUFAs have the ability of suppressing the antioxidant content of cancer cells, like inhibiting the expression of the antioxidant and anti-apoptotic B-cell leukemia/lymphoma 2 (BCl-2), thereby rendering the cells even more susceptible to LPO and activation of apoptosis (reviewed in (83)). DHA may decrease the intracellular glutathione level in cancer cells (reviewed in (82)). Also, GPX was reduced in breast cancer xenografts of mice supplemented with a FO concentrate containing n-3 PUFAs. This FO concentrate also potentiated the peroxidizing effect of the chemotherapeutic drug doxorubicin which is known for its oxidative stress-inducing effect (84). LPO was also involved in the growth reduction of DHA-treated colon cancer cells (85). However, a recent review concluded that supplementation of n-3 PUFAs within 0.5-1 g/day or slightly higher doses, do not seem to induce a high grade of cytotoxic or pro-carcinogenic oxidative stress in normal tissues (86).

DHA also incorporates into phospholipids in the mitochondrial membrane of colon cancer cells, preferentially cardiolipin (CL), which is important for the integrity of the mitochondrial membrane. N-3 PUFA rich CL is easily peroxidized, resulting in altered membrane composition and integrity, which together with the resulting CL hydroperoxides initiate apoptosis by triggering the release of pro-apoptotic factors, like cytochrome C from mitochondria (reviewed in (80, 82)).

1.5.2 Eicosanoid production and angiogenesis

The COX enzyme has two isozymes: COX-1, which is constitutively expressed in several cell types, and COX-2, which is induced during inflammation, but not in most normal, non-inflamed tissues. COX-2 is increased in several cancers, including breast and colon cancer (reviewed in (66)). However, n-3 PUFAs may inhibit the induction (87) and reduce the expression of COX-2 (69, 88-91). Since EPA competes with AA for COX activity, n-3 PUFAs will change the type of COX-2-produced products towards less inflammatory and less proliferative. This competition also results in reduction of e.g. the pro-tumorigenic AA-derived PGE_2 in favor of the anti-tumorigenic EPA-derived PGE_3 (reviewed in (37)). Jia *et al* showed that n-3 FA desaturase-transgenic mice producing n-3 PUFAs from n-6 PUFAs with carcinogen-induced colitis-associated colon adenocarcinoma, expressed more PGE_3 and less PGE_2 compared to wild type mice (92). It is important to note that COX inhibitors, like celecoxib, may suppress growth of colon cancer in both mice and humans (reviewed in (66, 80)). N-3 PUFAs can also reduce COX-2 expression by inhibiting nuclear factor kappa B (NFκB) resulting in both reduced COX-2 and reduction of NFκB-induced growth promoting targets (reviewed in (66)). Even if it is not yet known if RvEs has anti-tumor activity, RvE1 inhibits NFκB which influences the regulation of colorectal carcinogenesis at an early stage (reviewed in (37)).

There are links between the eicosanoids and angiogenesis, the development of new blood vessels, which is critical for tumor growth (reviewed in (93, 94)). DHA and EPA reduced growth of HT-29 colon cancer cells *in vitro* and reduced expression of COX-2, vascular endothelial growth factor (VEGF) and reduced PGE2 level. DHA and EPA also inhibited phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK 1 and ERK2) and overexpression of hypoxia-inducible-factor 1α (HIF-1α). Both DHA and EPA inhibited growth of HT-29 xenografts in mice, reduced mircovessel formation and the levels of COX-2, PGE2 and VEGF (91). Nitric oxide (NO) is known to increase the activity of prostaglandin syntethase and the production of PGE2 (reviewed in (95)). NO is produced by the inducible form of NO synthase (iNOS), which have increased activity and/or expression in colon cancer in both animal- and human studies and may increase tumor invasiveness, metastatic potential and angiogenesis (reviewed in (96, 97)). DHA may decrease iNOS expression, at mRNA and protein level, and NO production in colon cancer cells (89, 97). In addition, n-3 PUFAs also inhibit angiogenesis by down-regulating angiogenic mediators, such as platelet-derived growth factor, NFκB, β-catenin and matrix metalloproteinases (reviewed in (98)).

1.5.3 Changes in membrane characteristics and cholesterol synthesis

Cancer cell membranes are different in composition from noncancerous cell membranes. Colonic adenocarcinomas were found to contain more of the SFA stearic acid (SA, 18:0) than normal colon mucosa from the same patients, as well as have increased ratios of SA to OA and LA. This may result in alterations in lipid rafts structure and protein composition; thereby enhancing cancer cell growth and preventing apoptosis ((99) and reviewed in (100)).

N-3 PUFAs like DHA and EPA may affect the formation, composition, structure and function of lipid rafts such as caveolae, which may affect the physiology of the cell and thereby the development of different diseases like cancer. DHA is known to alter the structure of cell membranes as well as changing membrane characteristics like permeability and fluidity, resulting in changes in membrane based signaling (reviewed in (100)).

The cholesterol content of cancer cells may be enriched. Some cancer cell types have lost the feedback regulation of cholesterol synthesis and absorption; hence they have up-regulated cholesterol synthesis. The synthesis of SFAs and cholesterol is regulated by FAS and HMG-CoAR, respectively and the activity of these enzymes are up-regulated in some tumors (reviewed in (100)). EPA may down-regulate the gene expression of FAS (101), and EPA and DHA reduced the expression of HMG-CoAR in liver cancer cells (101, 102). N-3 PUFAs have been found to down-regulate the transcript (102, 103) and nuclear protein level of SREBP1 (104) and SREBP2 in cancer cells (105). Also, a combination of DHA and EPA was incorporated into lipid rafts from breast cancer cells *in vitro* and reduced growth, lipid raft sphingomyelin, cholesterol and DAG levels, and interfered with epidermal growth factor (EGF) signaling which takes place in lipid rafts (106).

1.5.4 Cell cycle regulation

The cell cycle ensures proper chromosome duplication and cell division. This happens through four different cell cycle phases (Fig. 5); gap 1 (G1), synthesis (S), gap 2 (G2) and mitosis (M). Upon cell proliferation signals, cells enter the G1 phase to prepare for DNA synthesis/chromosome duplication. During the S phase cells duplicate their chromosomes before entering the G2 phase in which they prepare for cell division. During the M phase, cells distribute the chromosomes equally to daughter cells. However, most cells in the body enters a resting stage called G0 where they are quiescent and do not divide. Deregulation of the cell cycle usually leads to activation of apoptosis followed by elimination of the deregulated cells from the body. However, if cells overcome their built-in control

mechanisms, they may experience a so called “cell cycle disease”, also called cancer, through which they continue to divide (reviewed in (107)).

In order to maintain correct regulation of the cell cycle, cells have several different classes of proteins involved in this regulation. Especially important are cyclin dependent kinases (CDKs) which are activated in a cell-cycle stage-specific manner through their association with cyclins. CDK activity is also regulated by CDK-inhibitors (CKIs) as well as different kinases and phosphatases. Proper progression through the cell cycle is maintained through “cell cycle checkpoints,” regulatory pathways ensuring correct order and timing of the cell cycle events. If errors like DNA damage occur, checkpoints will delay the cycle until the damage is repaired or if not able to repair the damage, lead the cells into apoptosis (reviewed in (107)).

G1/S phases and checkpoint

During G0- and early G1 phases, the cyclin levels are low, CKI levels are high and hence the CDK activity is low. Upon extracellular proliferative stimulation through G0 or G1, cells enter a new round of cell division. They are committed to perform this cycle when they pass the “G1/S transition point”/“restriction (R) point.” Proliferation signals lead to accumulation of D-type cyclins which bind CDK4/6, and activation of the CDK4/6-cyclin D complex by the CDK-activating kinase (CAK). Active CDK4/6-cyclin D and CDK2-cyclin E complexes phosphorylate the retinoblastoma protein (Rb), leaving members of the E2F protein family free and active to stimulate the transcription of genes whose products are important for S-phase entry. The CDK2-Cylin E complex also induces degradation of inhibitory factors like the CDK interacting protein/ kinase inhibitory protein (CIP/KIP) family member p27. In case of defective DNA, absence of appropriate mitogenic signals or presence of anti-proliferative signals, the G1/S checkpoint is activated. The CKI families inhibitor of CDK4 (INK4; p15, p16, p18 and p19) and CIP/KIP (p21, p27 and p57) serve as effectors at this checkpoint by inhibiting CDKs and preventing cell cycle progression. When the level of proteins required for S phase entry is adequately high, tightly controlled chromosome replication is initiated. Progression through the S phase is regulated by the CDK2-cyclinA complex, which phosphorylates components of the DNA replication machinery and thereby initiating DNA replication (reviewed in (107)).

G2/M phases and checkpoint

After completed chromosome duplication, cells enter the G2 phase in which the newly divided genetic material is controlled. If DNA damage has occurred, checkpoint pathways initiating G2 phase arrest will be activated, leading to CDK1 (CDC2)-inhibition. Potential genotoxic stress may activate phosphorylation of human checkpoint kinases 1 and 2 (Chk1 and Chk2) leading to phosphorylation and thereby inhibition of cell division cycle 25 (CDC25), a process enhanced by the binding of the CDC25 inhibitor stratifin (SFN/14-3-3σ). This blocks CDC25 from dephoshorylating and hence activating CDK1, rendering CDK1 inactive and preventing mitosis entry. Also, SFN and p21 may bind to the CDK1/cyclin B complex, enhancing the G2 arrest. The rate limiting step of mitosis entry is dephosphorylation of CDK1/cyclin B by CDC25 homolog B (CDC25B) and CDC25C thereby increasing its activity. The M phase tightly controls the division into two daughter cells (reviewed in (107)).

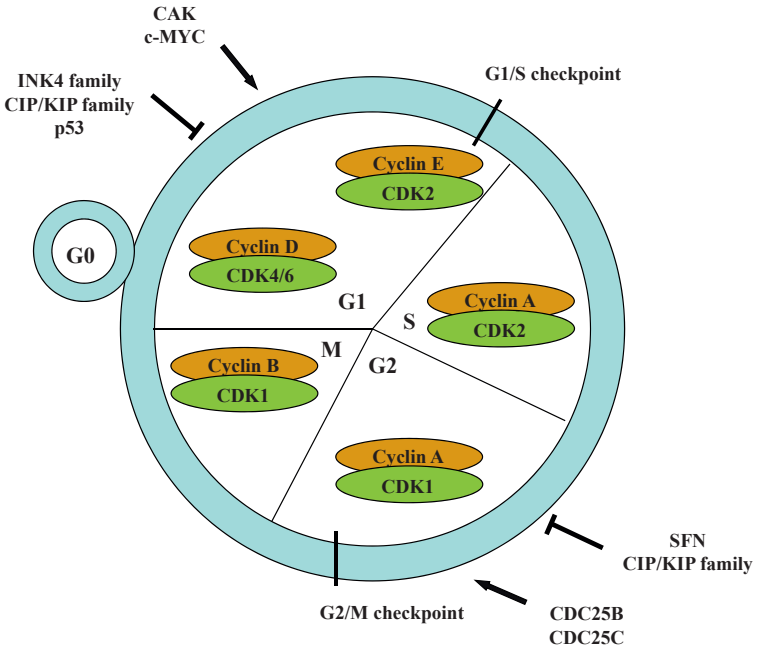

Figure 5. Overview of cell cycle regulation and the G1/S and G2/M checkpoints. Full names are stated in the abbreviations list.

Cell cycle, cancer and PUFAs

Cancer is recognized by loss of normal control of cellular proliferation. This is often correlated to mutations in genes whose proteins are involved in cell cycle regulation.

Mutations, overexpression or gene amplification of positive cell cycle regulators (potent proto-oncogenes), such as v-myc myelocytomatosis viral oncogene homolog (c-myc), may render their activity continuously on and thereby promoting cell proliferation. Overexpression of cyclin A, D1 and E, as well as CDK4 and 6 has been found in some human tumors. The mutation or deletion and hence inactivation of negative cell cycle regulators (potent tumor suppressor genes), such as p53, may destroy their ability to delay/arrest cell cycle progression, and hence leave the cell cycle active. Mutations in the *p53* gene is found in over 50% of human cancers (reviewed in (107)).

Several studies have found n-3 PUFAs capable of interfering with cell cycle progression in cancer cells, inducing cell cycle arrest in either the G1/S or the G2/M phases (88, 104, 108-112) and changing the expression of several genes involved in cell cycle regulation at mRNA and/or protein level in different cancer cell types. DHA and/or EPA may reduce the protein level of cyclin A (88, 112), cyclin D (89, 113), cyclin E (88, 113), CDK2 (88, 112), phosphorylated Rb (110, 112) and proliferating cell nuclear antigen (PCNA) (114), and increase the protein level of p21 (112). Narayanan *et al* showed that DHA induced extensive changes in gene expression of transcripts involved in e.g. cell cycle regulation, including up-regulation of the CKIs p18, p19, p21, p27 and p57 and down-regulation of CDK3 in a colon cancer cell line (97, 115).

PPAR transcription factors are, in addition to their role in regulation of lipid metabolism, also involved in cell proliferation. N-3 PUFAs are known to be PPAR ligands which bind to and thereby enhance their transcriptional activity (reviewed in (116)). PPARγ expression may be up-regulated at mRNA level by DHA and EPA (115, 117), and at protein level by DHA (118). In order to activate PPARγ, the DHA concentration has to be >10-30 μM (reviewed in (116)). Several studies have reported PPARγ to have anti-tumor effects through cell cycle regulation, like inducing expression of the tumor-suppressor gene p53 and thereby the expression of p21, and induction of apoptosis by inducing expression of the Fas ligand and Syndecan 1 among others (reviewed in (116)). Also, the PPARα and PPARγ anti-inflammatory properties may contribute to suppression of tumor growth (reviewed in (10, 94, 119)).

Studies have shown that n-3 PUFAs suppress the expression of protein kinase C (PKC), which is known to be mitosis-stimulating (reviewed in (66, 80)), as well as decrease the activity of the mitosis-stimulators ras and activator protein 1. The latter ones are oncogenic transcription factors known to be frequently activated in cancers (reviewed in (1, 66)). EPA and DHA may also reduce the level of the pro-proliferative transcription factor β-catenin by

increasing its degradation (89, 90, 120) and reducing its translocation into the nucleus (69, 120). Decreased level of β-catenin was followed by down-regulation of some of its tumor-growth involved target genes like c-Met (90) and the inhibitor of apoptosis (IAP) survivin (120).

1.5.5 Endoplasmic reticulum homeostasis

The endoplasmic reticulum (ER) is a metabolic compartment within cells. It has four main functions; maintenance of Ca^{2+} homeostasis, biosynthesis of lipids and sterols, membrane and secretory protein synthesis and protein folding. Perturbation of any of these functions causes ER stress (reviewed in (121)).

ER is an important Ca^{2+} store in eukaryote cells. Within the ER the free [Ca^{2+}] is much higher (~1-2 mM) than in the cytosol (0.1 μM). However, the total [Ca^{2+}] in the ER can be much higher due to the presence of Ca^{2+}-binding proteins. Ca^{2+} in- and efflux into/from the ER are controlled by the ryanodine receptor (RyR), inositol 1,4,5-triphosphate (IP_3) receptor (IP3R) and the sarcoplasmic/ER Ca^{2+} ATPases (SERCA) 1-3. IP3R and RyR are responsible for most of the Ca^{2+} efflux from and SERCA for most of the Ca^{2+} influx to ER. Ca^{2+} depletion of the ER will lead to inhibition of protein folding and target unfolded proteins for ER associated degradation (ERAD), as well as interfering with chaperone function and ER to golgi trafficking of proteins (reviewed in (121)).

The ER membrane is the site for elongation and desaturation of FAs, cholesterol metabolism and phospholipid biosynthesis. Depletion of cholesterol or SFAs may increase the membrane fluidity, resulting in activation of the SREBP transcription factors that induces expression of genes involved in e.g. cholesterol biosynthesis. As mentioned in chapter 1.3.7; if the cholesterol level is too high, cholesterol will be esterified with PUFAs by ACAT and stored as CEs in lipid droplets in the cell. Sterols may also stimulate degradation of HMG-CoAR and thereby inhibit cholesterol biosynthesis (reviewed in (121)).

Within the ER there must be a balance between influx of unfolded proteins, efflux of proper folded proteins and targeting of unfolded proteins for proteasomal degradation. This retains the protein folding homeostasis in the ER and is added to by a range of chaperones like glucose-regulated protein 94 kDa (GRP94), valosin-containing protein (VCP) and several different heat shock proteins (Hsps) which bind the hydrophobic surface patches of unfolded proteins thereby promoting protein folding and prevent them from binding and aggregating with each other. Unfolded or misfolded proteins may be degraded by the proteasomes during

ERAD or autophagy (a degradation pathway described below) (reviewed in (121)). Proper protein folding and maturation requires a highly oxidizing and Ca^{2+}-rich environment in the ER (reviewed in (122)).

Disturbances in any of the ER functions lead to accumulation of unfolded proteins in the ER lumen and thereby induction of ER stress and the common signaling pathway named the unfolded protein response (UPR). Different models exist to describe how ER stress is sensed. The "competition model" describes how the ER luminal domains of the three ER stress sensors double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring 1 (IRE1) compete with unfolded proteins for binding to the heavy chain binding protein/glucose-regulated protein of 78 kDa (BiP/GRP78) (Fig. 6). BiP is normally bound to the luminal domains of these sensor proteins, thereby keeping them in an inactive state. Upon accumulation of unfolded proteins, BiP preferentially associates with the unfolded proteins and is thereby sequestered from the sensors which then can be activated (reviewed in (121)). During the recent years increased knowledge about the sensing of ER stress has emerged, especially on the regulation of IRE1. However, much of this work was performed in yeast, but is currently being explored also in humans (reviewed in (123)).

PERK

Dissociation of BiP from PERK leads to its dimerization and autophosphorylation of PERK's cytosolic kinase domain that phosphorylates eukaryotic translation initiation factor 2 alpha (eIF2α-P) (Fig. 6). This leads to inhibition of translation/protein synthesis to relieve the protein burden of the ER. However, it induces the transcription of specific genes like activating transcription factor 4 (ATF4) (reviewed in (124, 125)). ATF4 regulates genes involved in amino acid import, glutathione biosynthesis and resistance to oxidative stress (reviewed in (126)). ATF4 induces transcription of genes involved in restoring ER homeostasis, a pro-adaptive effect. However, it may also induce a switch from a pro-adaptive to a pro-apoptotic effect (reviewed in (127)). Jiang *et al* found that ATF4 was induced as early as 1 h after thapsigargin (TG)-induced ER stress, followed by induction of activating transcription factor 3 (ATF3) after 1-3 h, CAAT/enhancer binding protein (C/EBP) homologous protein/ growth arrest and DNA damage-153 (CHOP/GADD153) and growth arrest and DNA damage-34 (GADD34) after 6 h. ATF4 was found to be responsible for the induction of ATF3 and CHOP, while ATF3 induced GADD34 (128).

PERK also phosphorylates the nuclear factor erythroid-2-related factor-2 (Nrf2) leading to its dissociation from the inhibitor Kelch-like ECH-associated protein 1 (Keap1). Nrf2 translocates to the nucleus where it stimulates gene transcription of genes with antioxidant response elements (reviewed in (124)) and thereby plays a pro-survival role (reviewed in (126)).

ATF6

BiP dissociates from ATF6, resulting in the transportation of ATF6 to the Golgi where it is cleaved by S1P and S2P (Fig. 6). ATF6 then translocates to the nucleus where it binds to and activates target genes with ATF/cAMP response element and ER stress responsive element, including several ER chaperones (reviewed in (124)). ATF6 is known to induce the transcription of BiP, CHOP and 58 kDa inhibitor of PKR ($P58^{IPK}$) (reviewed in (126)). X-box binding protein 1 (XBP-1) is also induced by ATF6 and their target genes are involved in protein folding and degradation in the ER (reviewed in (127)).

IRE1

Thirdly, the release of BiP from IRE1 leads to dimerization, autophosphorylation and activation of its RNAse activity (Fig. 6). IRE1 removes a nucleotide fragment from the XBP-1 mRNA leading to a spliced mature XBP-1. The precursor and the mature form are both translated, but they have different functions. The mature XBP-1 translocates to the nucleus and activates UPR target genes (reviewed in (129)), such as proteins involved in protein folding and ERAD (reviewed in (127)). The XBP-1 precursor acts as an inhibitor of XBP-1 signaling, especially important during the recovery phase when ER stress declines and IRE1 is no longer active (reviewed in (129)). The IRE1 pathway is known to regulate expansion of the ER during ER stress (reviewed in (126)).

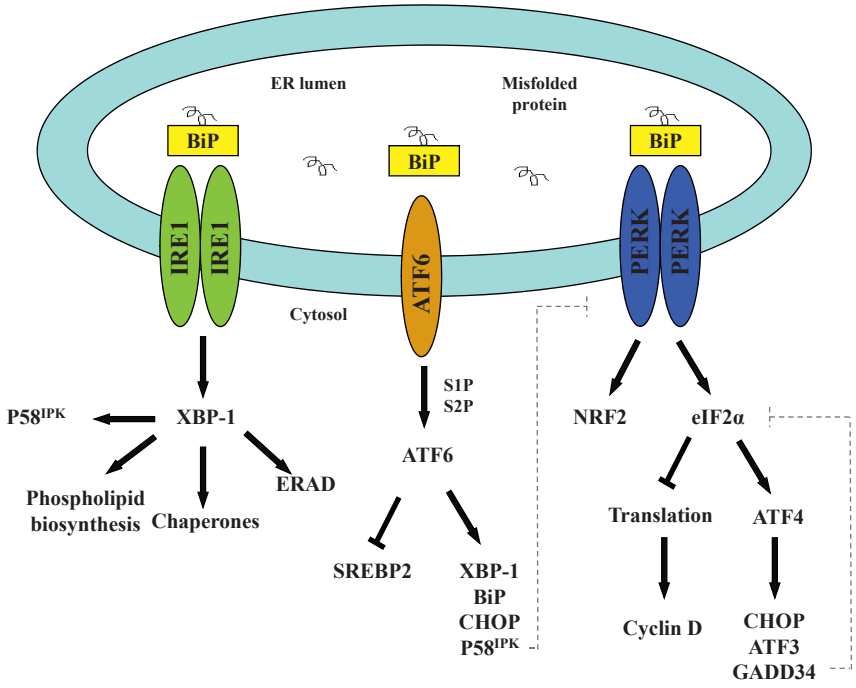

Figure 6. Overview of the UPR and key regulators. Full names are stated in the abbreviations list.

Crosstalk between UPR sensor pathways

There is crosstalk between the three branches of UPR, through XBP-1, which is induced by ATF6, activated by IRE1 and induces the expression of the PERK-interacting protein $P58^{IPK}$, the latter one is discussed below (reviewed in (125)). Crosstalk is also reflected through CHOP which is induced by ATF4, ATF6 and XBP-1 (reviewed in (130)), and in the temporal control of the activation of these three pathways. PERK-dependent eIF2α-P leading to inhibition of protein synthesis, and thereby reducing the protein load on the ER, is the first pathway to be activated. This is followed by activation of ATF6 which requires cleavage and nuclear translocation in order to induce transcription of ER chaperones. The IRE1 pathway is believed to be activated later in UPR. This is because the level of its target XBP-1 is normally low, but is induced by ATF6. Therefore, the time schedule could be; translational shutdown, chaperone synthesis and at last protein degradation (reviewed in (126)).

ER stress-induced cell cycle arrest, apoptosis and autophagy

Activation of UPR is known to trigger cell cycle arrest in the G1 phase, as a consequence of inhibition of cyclin D translation by eIF2α-P. This leads to deactivation of cyclin D-dependent

CDKs. The cell cycle arrest provides time for restoring ER homeostasis and prevents cells from dividing when protein folding is not optimal (reviewed in (131)).

The adaptive responses induced by UPR are pro-survival. However, prolonged ER stress may induce a switch towards cell death (apoptosis is described in chapter 1.5.7). CHOP is known to induce the transcription of GADD34 which acts as a feedback loop on the PERK pathway in stressed cells, by dephosphorylating eIF2α and thereby blocking this protecting pathway. Also, the induction of $P58^{IPK}$ during UPR is known to repress PERK activity and play a role during late UPR. CHOP is also known to induce apoptosis by up-regulation of pro-apoptotic members like BCl-2 antagonist killer (BAK) and BCl-2 antagonist of cell death (BAD), and down-regulation of anti-apoptotic members (BCl-2) of the BCl-2 family. This leads to cytochrome c release from mitochondria to cytosol and activation of downstream caspase-9 and caspase-3 cascades (reviewed in (127)). CHOP suppresses BCl-2 transcription through an interaction with the C/EBP beta (C/EBPβ) isoform liver inhibitory protein (LIP) (reviewed in (130)). Another molecular switch between pro-survival and pro-apoptotic function of the PERK pathway is the CHOP-induced tribbles-related protein 3 (TRIB3). TRIB3 is proposed to exert negative feedback on CHOP so that cells may adapt to mild ER stress. However, TRIB3 inhibits v-Akt murine thymoma viral oncogene homolog 1 (Akt) and thereby leads to apoptosis during persistent ER stress (reviewed in (122)).

IRE1 may recruit tumor-necrosis factor receptor associated factor 2 (TRAF2), thereby activating a MAPK cascade through signal-regulating kinase 1 (ASK1), resulting in activation of cJUN NH2-terminal kinase (JNK) and p38 MAPK. Activation of JNK can either induce autophagy in order to let cells adapt to ER stress, or induce apoptosis when ER stress is persistent (reviewed in (122)). Induction of apoptosis requires that JNK activates the pro-apoptotic BCl-2-interacting protein BIM (BIM) and inhibits BCl-2, thereby leading to cytochrome C release and caspase activation. IRE1-signaling is positively regulated by interaction with the pro-apoptotic BCl-2 members BAK and BCl-2-associated X protein (BAX), and negatively by the anti-apoptotic BAX inhibitor 1 (reviewed in (122, 125)).

Prolonged ER stress can lead to hyperoxidized ER lumen resulting in H_2O_2 leakage to cytoplasm and ROS production. This hyperoxidation has been linked to the CHOP target ER oxidase 1 alpha (ERO1α). CHOP-induced apoptosis has been shown to involve ERO1α-activation of IP3R1, Ca^{2+} signaling in the cytoplasm and activation of the Ca^{2+}-sensing kinase Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (reviewed in (130))

Different conditions that induce ER stress can lead to leakage of Ca^{2+} from the ER to the cytosol. This may activate calpain which induces cleavage of procaspase-12, leading to

activation of the caspase-9 cascade (reviewed in (127)). Increased concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) may lead to its uptake by the mitochondria. This may activate caspase-8 which cleaves B-cell receptor-associated protein 31 (BAP31), an ER membrane-bound protein, creating a truncated BAP20 product that may promote Ca^{2+} release from the ER. Ca^{2+} uptake by the mitochondria leads to increased cytochrome C efflux (reviewed in (125)).

If UPR is not able to restore ER homeostasis, autophagy can be induced to protect against the consequences of misfolded/aggregated proteins and damaged organelles. Autophagy is a mechanism through which damaged DNA, proteins and organelles are sequestered in autophagic vesicles before degradation by lysosomes in order to recycle building stones of important macromolecules. It is known as a survival response to growth factor or nutrient deprivation, but has also been suggested as a second form of programmed cell death (reviewed in (122, 132)). ER stress may induce expansion of the ER membrane as well as ER-phagy; the formation of vesicles containing parts of ER, also called ER-containing autophagosomes or ERAs. The ERAs may contain damaged ER or ER with protein aggregates that have to be degraded. It may also be that ER is degraded in this way in order reduce the ER volume upon declined ER stress (133, 134). In ER-stressed cells, autophagy can be initiated by PERK, IRE1 and Ca^{2+}-release. PERK-induced eIF2α-P leads to up-regulation of autophagy gene 12 (Atg12) which is important in autophagosome formation (reviewed in (122)). Upon hypoxia, PERK may also induce the expression of Atg8/microtubule-associated protein 1 light chain 3 (LC3) and Atg5 through ATF4 and CHOP, respectively (135). Hence, ER stress and autophagy are interlinked.

N-3 PUFAs, ER stress and cancer

ER stress and UPR are known to be induced in different types of cancers. UPR is important in cancer cells in order to induce pro-survival factors such as NFκB, which helps them survive and maintain malignancy. UPR factors linked to tumorigenesis include XBP-1, ATF6, eIF2α-P, ATF4, CHOP and BiP, as well as Hsps that may be implicated in the adaption of cancer cells to different stresses by repairing or degrading damaged proteins. In most normal cells UPR is in a quiescent stage. Activation of UPR during early tumorigenesis may induce cell cycle arrest in the G1 phase as well as activating p38 mitogen-activated protein kinase (MAPK). If UPR induces apoptotic signals at this stage in cancer cells, cells with mutations in apoptotic genes may have increased survival. In cancer therapy this can be utilized by inducing an overload of misfolded proteins or inhibit the adaptive and anti-apoptotic

pathways of UPR. Different cancer drugs targeting the UPR are under development and some are being tested in the clinic e.g. different HSP90- and proteasome inhibitors and an active component of marijuana called delta (9)-tetrahydrocannabiol (THC) (reviewed in (127)). THC was reported to induce cell death in human glioma cells. This was linked to induction of autophagy by increased eIF2α-P and up-regulation of the transcription factors p8, ATF4, CHOP and TRIB3, and subsequent TRIB3-dependent inhibition of the AKT/mammalian target of rapamycin complex 1 (mTORC1) pathway (136). EPA and/or DHA may decrease the phosphorylation and activity of AKT in tumor cells. The AKT/ Phosphoinositide 3-kinase (PI3K) signaling pathway is known to be important in the regulation of the cell cycle and autophagy, and has been suggested to be constitutively activated in different types of cancers (reviewed in (71)). Phosphorylation and hence inactivation of AKT is also associated with induction of apoptosis; it hinders it from phosphorylating and hence inhibiting the pro-apoptotic BAD (137).

N-3 PUFAs may decrease the biosynthetic rate of cholesterol (reviewed in (100), as outlined in chapter 1.5.3. Changes in cholesterol homeostasis in the ER can induce ER stress (reviewed in (121)), and cholesterol depleted cancer cells are known to be prone to induction of apoptosis (reviewed in (100)). ER stress may activate SREBP2 (138). However, Zeng *et al* found that ER stress-induced activation of ATF6 may affect cholesterol synthesis by interfering with the transcription of SREBP2 target genes (139). Some studies have also reported that n-3 PUFAs interfere with Ca^{2+} homeostasis leading to inhibition of translation initiation/eIF2α-P (113), as well as induction of BiP and CHOP (140). Mechanisms for how n-3 PUFAs may increase $[Ca^{2+}]_i$ will be outlined below.

1.5.6 Intracellular calcium homeostasis

Changes in $[Ca^{2+}]_i$ are known to interfere with several different signaling pathways and the Ca^{2+} level within cells must therefore be strictly regulated. This regulation includes Ca^{2+} efflux from the ER to the cytosol (commonly through the IP3R), providing a signal for Ca^{2+} influx via the plasma membrane, a process called capacitative or store-operated Ca^{2+} entry. Hence, the Ca^{2+} level of intracellular Ca^{2+} stores regulates Ca^{2+} influx via the plasma membrane (reviewed in (141, 142)). However, the mechanism for capacitative influx has been largely unknown and under constant research. It is currently believed that it is regulated through the ER Ca^{2+}-sensors stromal interaction molecule 1 (STIM1) and STIM2 which may lead to the activation of the pore-forming subunit of store-operated Ca^{2+} release-activated

Ca^{2+} channels (CRAC); Ca^{2+} release-activated Ca^{2+} modulator 1 (CRACM1/ORAI) (reviewed in (142)). N-3 PUFAs have been reported to release Ca^{2+} from intracellular Ca^{2+} stores; thereby increasing $[Ca^{2+}]_i$ in cancer cells, while the results regarding the ability of n-3 PUFAs to stimulate capacitative influx have not been consistent (113, 143).

TG increases $[Ca^{2+}]_i$ by inhibiting SERCA causing ER Ca^{2+} depletion and capacitative Ca^{2+} influx (144). The antifungal drug econazole (Ec) also increases the $[Ca^{2+}]_i$ in cancer cells by inhibiting SERCA and thereby depleting Ca^{2+} from ER. However, Ec inhibits the TG-induced capacitative influx in some cancer cells (145, 146), while it may induce capacitative Ca^{2+} influx in other cells (147). Studies have shown that also PUFAs like DHA and EPA may decrease TG-induced increase in $[Ca^{2+}]_i$ in cancer cells by inhibiting TG-induced capacitative influx (113, 148, 149). Mutant cell lines resistant to both Ec and TG have been isolated from the human leukemic HL-60 cells. These cells like the E2R2 cells are resistant to ER Ca^{2+} depletion by Ec and have increased capacitative influx. When treated with Ec/TG they maintain protein synthesis, probably due to increased content of ribosomal proteins (150).

1.5.7 Apoptosis in cancer cells

Apoptosis, also called programmed cell death, is tightly regulated by pro- and anti-apoptotic factors. Dysregulation may promote cancer. Apoptotic hallmarks include cell shrinkage, membrane blebbing and bursting, followed by phagocytosis by surrounding cells or packaging into apoptotic bodies. There are two major apoptotic pathways; extrinsic and intrinsic. Upon a death signal, the extrinsic pathway is initiated by Fas ligand binding to a Fas death receptor. This complex further recruits death domain-containing protein (FADD) and procaspase-8 resulting in a protein complex called death inducing signaling complex (DISC) in which procaspase-8 is cleaved and activated before it cleaves and activates the effector procaspase 3 which drives the apoptotic pathway further (reviewed in (132, 151)). The intrinsic pathway is initiated by permeabilization of the mitochondrial membrane thereby causing leakage of cytochrome C to the cytosplasm where it binds apoptosis protease-activating factor 1 (Apaf-1) and procaspase-9 forming an “apoptosome”. Procaspase-9 is activated before caspase-3 activation. The last apoptotic steps, packaging into apoptotic bodies and phagocytosis by surrounding cells, are the same for the extrinsic and intrinsic pathways (reviewed in (151)).

Apoptosis and n-3 PUFAs

Induction of apoptosis is of huge importance in cancer treatment. Several *in vitro* cell culture studies and animal studies have demonstrated that DHA and EPA are able to promote apoptosis in cancer cells (reviewed in (75)). The apoptosis inducing effect includes activation of different pro-apoptotic factors and down-regulation of anti-apoptotic factors. DHA and/or EPA have been found to up-regulate the mRNA level of the procaspases 5, -8, -9, -10 and -13 (115), as well as increase the cleavage and hence activation of procaspase 3 (137, 152, 153) and caspase 8 (152-154). DHA and/or EPA may also induce apoptosis in cancer cells via activation of BAD (137) and BH3-interacting domain death agonist (BID) (152) which are capable of promoting cytochrome C release, or suppression of the anti-apoptotic BCl-2 family members BCl-2 (85, 153-155) and BCl-2 like 1 (BCl2L1/BCl-Xl) (155). DHA also increases the expression of the cell proliferator regulator and pro-apoptotic transcription factor c-MYC (155), induces cytochrome C release from mitochondria and causes mitochondrial membrane depolarization (152). The pro-apoptotic effect of n-3 PUFAs on cancer cells may also involve increased activity of neutral sphingomyelinase (156) and formation of ceramide (112, 156).

NFκB is an important transcription factor in apoptosis regulation. It is usually inhibited by inhibitor of κB (IκB) in mammalian cells, but is released and activated upon pro-apoptotic stimuli. NFκB is constitutively activated in some cancers where it adds to tumor growth and cancer cell survival (reviewed in (66, 132)). N-3 PUFAs have been found to decrease the expression of NFκB (89, 97), decrease its activity (114) and block the degradation of IκB, thereby rendering NFκB inactive (reviewed in (10, 157)).

There is also a strong connection between ER stress, autophagy and apoptosis, as outlined above (chapter 1.5.5). The effect of ER stress and autophagy may depend on the ability of cancer cells to induce apoptosis according to the genetic changes displayed (reviewed in (122)).

1.6 Gene expression analysis

The field of molecular diagnostics includes the elucidation of disease mechanisms to find gene-based biomarkers. An important tool in this field, and in molecular biology research, is global gene expression analysis using mircroarrays, which allows simultaneously examination of the expression of thousands of genes. This tool offers the opportunity to obtain gene expression signatures of diseased cells and patient samples, which can be used in the exploration of biomarkers and thereby improving early diagnosis and individual treatment. In

cancer research this tool can be used for studying changes in gene expression patterns of cancer cells compared to normal cells, or in response to drugs, as well as investigating tumor classification and cancer progression.

Different microarray technologies exist in which the probes can be made of cDNA or oligonucleotides and are either spotted or synthesized on the microarray surface (reviewed in (158)). Different suppliers offer whole genome microarrays, covering the entire transcriptome (all the RNA) of different organisms. The Affymetrix GeneChip® System offers microarrays, chemicals, instruments and data analysis programs for gene expression analysis. Arrays are available for several different organisms, including human. The arrays are produced either as focus arrays, with a specific selection of probes, or whole genome arrays, presenting transcripts of gene sequences with both known and unknown functions (159). The human full genome array from Affymetrix analyzes the expression of about 47,000 transcripts and variants. The array fabrication and handling is well standardized which is reflected by low technical variability. Variation is although introduced due to e.g. differences in RNA quality (reviewed in (160)), which is a critical factor in microarray experiments.

Gene expression analysis by microarrays is a valuable tool in the field of nutritional transcriptomics; the studying of how nutrients may regulate gene expression. Different bioactive food components have been reported to affect gene expression in cells (reviewed in (161)). Microarrays have also added to the understanding of the possible anti-tumor mechanisms of n-3 PUFAs. Scientists have used this technology to outline n-3 PUFA induced changes at mRNA level in cell culture and animal experiments, revealing several different molecular pathways involved (97, 102, 115). Hence, it may be that the anti-tumor effect of n-3 PUFAs involves a combination of different mechanisms, and the elucidation of these mechanisms can be aided by the use of microarrays. The requirement for publishing of raw data from microarray experiments into public databases also adds to the revealing of molecular mechanisms, since the data can be re-analyzed by new approaches in order to find additional pathways involved.

2. Aims of study

N-3 PUFAs in the form of FO have been a common nutritional supplement in Norway for decades. These PUFAs have been associated with positive effects on different diseases like cardiovascular disease, some inflammatory conditions and possibly cancer cachexia. Several epidemiological observational studies have found that high n-3 PUFA intake reduces the risk of some cancers like colon cancer, even if the results are not consistent. These studies are supported by an increasing number of clinical studies, as well as animal- and cell culture experiments reporting a possible anti-tumor activity of n-3 PUFAs. Several different mechanisms have been suggested to be involved in the anti-tumor effect of n-3 PUFAs, but the mechanisms have not yet been fully established. N-3 PUFAs may also have a role as chemotherapy supplements, but in order to use these PUFAs clinically, the molecular mechanisms involved have to be elucidated.

This study aimed at revealing how n-3 PUFAs affect cancer cell growth and in particular assessing the molecular mechanisms and cellular pathways contributing to their anti-tumor effects by using microarrays for global gene expression profiling. The identification of n-3 PUFA-induced early responses in cancer cells and the influence of n-3 PUFAs on known cancer therapy targets were of special interest.

3. Summary of papers

Paper I

DHA induces ER stress and growth arrest in human colon cancer cells: associations with cholesterol and calcium homeostasis

In order to reveal molecular mechanisms behind DHA-induced growth inhibition of cancer cells, gene expression profiling was performed using the Affymetrix GeneChip system and the Affymetrix Human Genome Focus array. Gene expression results revealed extensive changes at transcription level after DHA (70 μM)-supplementation of SW620 colon cancer cells for 12, 24 and 48 h. At 12 h of DHA treatment 839 transcripts were up-regulated and 1066 down-regulated. At 24 h these numbers increased (1157 up-regulated and 1222 down-regulated), while a markedly fewer genes were differentially expressed at 48 h (288 up-regulated and 267 down-regulated). Affected transcripts belonged to numerous different molecular pathways. Interestingly, several transcripts indicative of ER stress and induction of UPR were up-regulated. These included factors from all three UPR branches (ATF6, PERK, XBP-1), especially transcripts downstream of PERK; ATF4, ATF3, GADD34, TRIB3 and heme oxygenase 1 (HMOX1). This was confirmed by phosphorylation of eIF2α (eIF2α –P, 3 h) and induction ATF4 (6 h) at protein level; indicating that ER stress and UPR were early responses to DHA treatment. Tranlational shutdown by eIF2α –P was suggested by reduced cyclin D1. EPA (70 μM) also induced ER stress in SW620 cells, while OA (70 μM) did not. Several chaperones and Hsps like HSP70 were highly up-regulated after 12 h, indicating presence of unfolded proteins and increased need for correct protein folding. Also, several proteasomal subunits were up-regulated at mRNA level and one representative at protein level. Together with up-regulation of GRP94, VCP and sequestosome 1 (SQSTM1) this indicated induction of ERAD. Transcripts involved in the regulation of ER Ca^{2+} homeostasis like IP3R1 and IP3R3 were up-regulated. Further, measurement of Ca^{2+} levels indicated that DHA treatment for 12, 24 and 48 h increased the $[Ca^{2+}]_i$ via Ca^{2+} release from the ER. DHA also enhanced the second phase of ATP-induced increase in $[Ca^{2+}]_i$, probably due to capacitative Ca^{2+} influx. These results imply that DHA treatment interferes with Ca^{2+} homeostasis in SW620 cells. Some pro-apoptotic transcripts like caspase 4 and caspase 7 suggested a link between ER stress and induction of cell death. However, caspase 7 was not induced at protein level. The PERK target Nrf-2 and some of its downstream targets were up-regulated at mRNA level, pointing towards a DHA-induced antioxidant response. The

cholesterol synthesis regulators HMG-CoAR, and the mature form of SREBP2 (mSREBP2) were induced at protein level upon DHA treatment. However, some transcripts encoding proteins involved in cholesterol biosynthesis were down-regulated and d*e novo* cholesterol biosynthesis was found to decrease upon DHA treatment. Transcripts for some proteins involved in cholesterol uptake and transport like LDL receptor (LDLR) and niemann-pick disease type C1 (NPC1) were up-regulated, the latter also at protein level. This may reflect an increased need for cholesterol, but impaired ability to induce cholesterol synthesis. Total cholesterol level did not change at the earliest time points, but increased slightly after 48 h, while there was a reduction in synthesis of CEs from newly synthesized cholesterol. Taken together, these data imply that the growth inhibiting effect of DHA on the SW620 cells involves induction of ER stress, UPR and changes in Ca^{2+}- and cholesterol homeostasis.

Paper II

The antiproliferative effect of EPA in HL-60 cells is mediated by alterations in calcium homeostasis

Based on the results from paper I indicative of DHA-induced changes in Ca^{2+} homeostasis, the influence of such changes on n-3 PUFA sensitivity in cancer cells was further investigated. We used the human leukemia cancer cell lines HL-60 and E2R2 which represent an interesting cell model for studying the influence of changes in Ca^{2+} homeostasis on drug sensitivity and resistance, reflected by their different responses towards Ec and TG treatment. Incubation of HL-60 cells with EPA (35 μM) resulted in a strong growth inhibition, but had no effect on the growth of E2R2 cells. However, doubling the EPA concentration halved the growth of E2R2 cells while abolishing further proliferation of HL-60 cells, indicating a large difference between these two cell lines in the ability of handling the presence of EPA. Gene expression profiling was performed using the Affymetrix Human Genome U133 2.0 plus array. Gene expression results from EPA (35 μM) treatment of HL-60 cells after 12, 24 and 48 h revealed changed expression of a high number of genes, representative for several different molecular pathways. Numbers of differentially expressed genes were highest after 12 h (2974 up-regulated and 2247 down-regulated), decreased at 24 h (908 up-regulated and 138 down-regulated) and was lowest after 48 h of EPA treatment (90 up-regulated and 11 down-regulated). The transcription level of PERK and the ATF4 downstream targets ATF3 and HMOX1 were up-regulated; indicating ER stress and activation of UPR. Induction of eIF2α-P (3 h) and ATF4 (12 h), at protein level, revealed ER stress and UPR as early

responses during EPA treatment. Transcription and protein level of cyclin D1 was down-regulated; suggestive of translational shutdown by eIF2α-P. Transcription of several factors involved in protein folding like molecular chaperones such as Hsp90 and GRP94, and protein degradation like VCP, SQSTM1 (also at protein level) and proteasomal subunits were up-regulated which points towards induction of ERAD. Transcription of Ca^{2+} homeostasis regulators like IP3R1 and CAMK1, and apoptosis like caspase 4 and caspase 7 were up-regulated. Interestingly, in the E2R2 cells the protein levels of eIF2α-P, ATF4, SQSTM1 and cyclin D1 remained unchanged upon EPA-treatment. These results suggest that the anti-tumor effect of EPA on HL-60 cells is mediated by changes in Ca^{2+} homeostasis and induction of ER stress and UPR.

Paper III

DHA alters expression of target proteins of cancer therapy in chemotherapy resistant SW620 colon cancer cells

Gene expression results from paper I indicated changes in different cell signaling pathways upon DHA (70 μM)-treatment of SW620 cells for different time points. The work presented in this paper focused on changes in the expression of transcripts encoding important cell cycle- and apoptosis regulators; some of which are known chemotherapy targets. Changes in gene expression of cell cycle regulators acting at the G1/S checkpoint included up-regulation of p21 and down-regulation of CDK2, CDK4, cyclin D1, cyclin D3 and PCNA. Changes in expression of G2/M checkpoint regulators included up-regulation of stratifin and down-regulation of CDK1, CDC25B, CDC25C, cyclin A2 and cyclin B2. Changes in expression of CDC25C, CDK1, p21 and stratifin were also confirmed at protein level. This indicates that both G1/S and G2/M checkpoints were affected by DHA. The ER stress-related pro-apoptotic transcripts GADD34, TRIB3, caspase 4 and caspase 7 were up-regulated, and CHOP increased at protein level, indicating sustained ER stress. Decreased total protein level of the cancer therapy target NFκB p65 and increased level of phosphorylated p38 MAPK also pointed towards induction of apoptosis. The malignancy-associated IAPs survivin (mRNA down-regulated) and livin (both α- and β isoforms) were reduced at protein level, while the apoptotic cleaved livin (tLivin) increased. These findings may add to the explanation of how DHA may enhance cancer chemotherapy treatments *in vivo*.

4. Discussion

There is common concern about the reduced n-3 PUFA and increased n-6 PUFA intake, and hence increased n-6/n-3 ratio, in the Western diet today. This change in PUFA balance over the last century is reflected by a reduction of DHA in membrane phospholipids, which may lead to "life-style" diseases like cardiovascular disease and neurological disorders (reviewed in (38, 56)). The populations in Norway and Japan have a relatively high fish consumption level and they have a higher intake of n-3 PUFAs compared to USA which has a very low n-3 PUFA intake, as outlined in the introduction (chapter 1.2, table 1). However, the n-3 PUFA intake is declining in Norway as well, as shown by the statistics department of FAO (FAOSTAT) in their food balance sheets for Norway; the fat supply quantity (g/capita/day) consumed from fish or seafood decreased from 6.6 g in 1977 to 4.9 g in 2007 (162). Colorectal cancer has been associated with the type of food we eat (reviewed in (163)), and this cancer form is very common in the Western world (164). Reduced n-6/n-3 ratio has been suggested to have a preventive effect on colorectal cancer (165) and be associated with reduced breast (166) and prostate (167) cancer risk. However, the results from studies reporting on n-6/n-3 ratio in relation to cancer are not consistent (reviewed in (168)). Reduced n-6/n-3 ratio also correlates with reduced death rate from cardiovascular disease (reviewed in (38)). A reduced n-6/n-3 ratio could be achieved by consuming more fatty fish or n-3 FO supplements, less plant oil containing n-6 PUFAs and use optimal cooking methods which preserves the n-3 PUFAs, as outlined in the introduction (chapter 1.2).

Even though results from epidemiological observation studies reporting on fish, n-3 PUFA intake and cancer are inconsistent, the meta-analysis of prospective cohort studies by Geelen *et al* revealed a 4 % and 3 % reduction in colorectal cancer risk per each extra fish meal or 100 g fish consumed per week, respectively (169). One of the studies included in this meta-analysis was the large European Prospective Investigation into Cancer and Nutrition Study, following over 470 000 people from 10 European countries. They found increased fish consumption to have a protective effect on colorectal cancer incidence (170). Few clinical studies on n-3 PUFAs and cancer have been performed, but the positive results from the intervention studies by Anti (60, 62), West (44), Aronson (61) and others, as outlined in the introduction (chapter 1.4.1), are convincing. However, the report from the FAO/WHO expert consultation in 2008 concluded that the evidence reporting on a possible relationship between n-3 PUFA consumption and cancer was insufficient at that time point (15). Even so, the report stated that there is a "probable" decrease in colorectal cancer risk correlated to intake of DHA

and EPA (500 mg/day) or ingestion of 2-3 portions of fish per week. The report also commented on some of the reasons why epidemiological observational studies have been unable to give concluding results on an effect of fat intake like n-3 PUFAs on cancers. Especially two factors were highlighted; the fact that fish also contains vitamin D and selenium which possibly have an effect against some cancers, and that food frequency questionnaires (FFQ) used to asses exposure levels may be error prone since they are not able to quantify the FAs accurately (15). This was also reflected in a report by Dennis *et al* where they reviewed the inconsistency in methods used to measure and report dietary fat and fatty acids in epidemiological studies of prostate cancer; differences in FFQs for dietary measurements, along with other factors, were reported to contribute to large heterogeneity between the reviewed studies (171).

4.1 N-3 PUFAs changes the gene expression profiles of human cancer cells

In the search for mechanisms involved in the anti-tumor effect of n-3 PUFAs, special attention has been given to lipid peroxidation and antioxidant defense, eicosanoid formation, changes in membrane characteristics and gene expression, as well as regulation of cell cycle and apoptosis (reviewed in (4, 10, 172)). Many studies have reported that n-3 PUFAs induce changes in expression of specific genes and proteins. However, the microarray technology brings the opportunity to study changes in gene expression in a new and genome wide manner. The technology is highly hypothesis-creating and exceptionally valuable in screening for drug-induced changes in gene expression and in nutritional transcriptomics, as mentioned in the introduction (chapter 1.6). Anyway, it is important to be aware of the fact that transcriptional changes cannot be directly related to changes at protein level. Gene expression can be regulated at different levels like mRNA synthesis, mRNA stability and regulation of translation; hence, biological interpretation of gene expression results requires further study like verification at protein level (reviewed in (173)). Some studies have presented microarray results from n-3 PUFA treated human cancer cells (97, 102, 115) and colon adenoma cells (174), all reporting changes in the expression of genes involved in regulation of cell growth. However, these studies have used microarrays covering only parts of the human genome. The gene expression profiling results presented in this thesis are based on two microarray types; one covering a selection of ~8500 well characterized human genes (**paper I and III**) and the second covering the whole human genome (**paper II**). Results indicated that n-3 PUFAs induced extensive changes in the expression of several genes belonging to several different

molecular pathways, in cancer cells (**paper I, II** and **III**). Some transcripts were highly up or down-regulated, whereas most differentially expressed transcripts showed moderate changes. However, the level of change in gene expression did not necessary correlate with the importance of specific transcripts when it comes to anti-tumor action of n-3 PUFAs. Differentially expressed genes were representative for e.g. induction of ER stress, UPR, changes in Ca^{2+}- and cholesterol homeostasis, as well as changes in regulation of cell cycle and apoptosis (**paper I, II** and **III**).

4.2 Induction of ER stress and UPR by n-3 PUFAs in human cancer cells

The finding that n-3 PUFAs induced ER stress and UPR in human cancer cells is especially interesting and had to our knowledge not been shown before, at least not to the extent presented here. Even if all three branches of UPR were affected in DHA-treated SW620 cells, the n-3 PUFA-induced expression of PERK and targets downstream of ATF4 were striking in both SW620 (**paper I**, table 1) and HL-60 cells (**paper II**, table 2). Phosphorylation of eIF2α (eIF2α-P) is an important ER stress and UPR marker (reviewed in (126)), and was induced already 3 h after n-3 PUFA supplementation in both cell lines (**paper I**, fig. 2/3 and **paper II**, fig. 3). Together with induction of ATF4 (**paper I**, fig. 2/3 and **paper II**, fig. 3), this indicated that ER stress and UPR were early responses to n-3 PUFA treatment in these cancer cell lines. The reduction of eIF2α-P over time in SW620 cells may be due to up-regulated GADD34 (**paper I**, table 1), which dephosphorylates eIF2α, as outlined in the introduction (chapter1.5.5). OA did not change eIF2α-P or ATF4 protein levels; hence, it did not induce ER stress in SW620 cells (**paper I**, fig. 3). Previously, even if not referring to their observations as "ER stress" or "UPR", Aktas *et al* found that EPA induced eIF2α-P and expression of factors downstream of ATF4 in NIH3T3 cells (140). In line with our results, other FAs have also been found to induce ER stress in different cancer cells (175-178), indicating that not only n-3 PUFAs are capable of inducing ER stress.

Upon ER stress, eIF2α-P leads to translational shutdown and reduction of cyclin D1 which is an important G1 phase regulator (179, 180). Correlating with the induction of eIF2α-P, reduced cyclin D1 at both mRNA and protein level was observed after n-3 PUFA treatment in both SW620 (**paper I**, table 1, fig. 2/3) and HL-60 cells (**paper II**, table 2, fig. 3). Even OA reduced the cyclin D1 level slightly in SW620 cells, although to a much lesser extent and did not affect cell growth (**paper I**, fig. 3). Others have shown that OA did not affect the growth of HL-60 cells (181). Consistent with our results, Palakurthi *et al* showed that EPA

induced eIF2α-P and reduced cyclin D1, leading to G1 cell cycle arrest in NIH3T3 cells. However, they ascribed eIF2α-P to another eIF2α kinase; protein kinase R (PKR) (113), while our results indicate that this happens through the PERK pathway in the cancer cells studied (**paper I** and **II**).

Protein folding in the ER requires an oxidative milieu and disturbances in the oxidative status of the ER may lead to ER stress and an increased production of ROS (reviewed in (126)). ROS may give oxidative damage on DNA and proteins as well as LPO and hence an increased need for antioxidants (reviewed in (182)). Gene expression profiling showed that DHA affected the antioxidant response of SW620 cells, as indicated by up-regulated expression of Nrf-2 and its target HMOX1 (**paper I**, table 1) which was one of the highest up-regulated transcripts. However, short interference RNA (siRNA) knockout of HMOX1 did not affect cell growth during DHA-treatment (**paper I**, results not shown). Nrf-2 may be activated by PERK in a ROS- and eIF2α-P independent manner (183) and has an important role in the redox balance in cells. Nrf-2 induces glutamate-cysteine ligase (GCL) which is the rate limiting enzyme in biosynthesis of the cellular redox buffer glutathione (reviewed in (182)); hence, up-regulated GCL catalytic (GCLC) and modifier (GCLM) subunits point towards an up-leveling of the antioxidant defense in DHA-treated SW620 cells (**paper I**, table 1). Further, we recently found DHA-induced increase in ROS production at early time points (1-4 h), correlating with induction and nuclear translocation of Nrf-2 in SW620 cells (Overland *et al*, submitted). However, the antioxidant vitamin E did not relieve DHA-induced growth inhibition (104); hence, LPO is probably not the main cause of the anti-tumor effect of DHA in the SW620 cells. EPA-treatment of HL-60 cells was previously shown to induce ROS production; hence oxidative stress was suggested to be involved in triggering cell death in these cells (152). However, Finstad *et al* showed that vitamin E did not reduce the effect of EPA on HL-60 cells (184).

Upon accumulation of misfolded proteins in the ER, the ERAD process is activated (reviewed in (121)). The up-regulated expression of several n-3 PUFA-induced proteasomal subunits and Hsps/chaperones in cancer cells (**paper I**, table 1, supplementary table 1 and **paper II**, table 2), like induction of 26S proteasome regulatory subunit RPN2 (RPN2/PSMD1) and Hsp70 in DHA-treated SW620 cells (**paper I**, table 1, fig. 2), may indicate presence of unfolded proteins in the ER lumen and that cells are signaling an increased need for ERAD components. This is also reflected by the up-regulated expression of GRP94/HSP90B1, which may be considered a hallmark of ER stress responses, in both SW620 (**paper I**, supplementary table 1) and HL-60 cells (**paper II**, table 2). It has several

functions during ER stress such as chaperone-induced protein folding, Ca^{2+}-binding and targeting of proteins for ERAD (reviewed in (185)). This ubiquitin selective chaperone VCP and SQSMT1 are also important in ERAD. VCP disassembles protein complexes and translocates proteins from ER to the proteasomes in cytosol, but is also important in cell cycle regulation (reviewed in (186)). SQSTM1 is induced upon accumulation of unfolded proteins (reviewed in (187)); hence, the up-regulated expression of these ERAD components by n-3 PUFA treatment in both of the profiled cancer cell lines (**paper I**, table 1 and **paper II**, table 2, fig. 3) strengthens the suggestion of ERAD induction.

Increased expression of ER stress-induced pro-apoptotic transcripts points towards sustained ER stress. The induction of CHOP during DHA-induced ER stress in SW620 cells (**paper III**, fig. 2) is especially interesting and consistent with previous studies (140, 188). CHOP may be induced by all three UPR branches and regulates the balance between pro- and anti-apoptotic BCl-2 family proteins (as outlined in chapter1.5.5), thereby affecting the balance between protective UPR and induction of cell death (reviewed in (121)). The BID transcript was up-regulated in DHA-treated SW620 cells **(paper I**, supplementary table 1) and others have found activation of BID to be involved in EPA-induced apoptosis of HL-60 cells (152). Also, the BCl-2/adenovirus E1B 19 kDa interacting protein 3-like (BNIP3L) transcript was up-regulated by n-3 PUFA treatment in both SW620 and HL-60 cells (**paper I**, re-examination of gene list and **paper II**, table 2). BNIP3L may interact with BCL-2 (189), and BNIP3L overexpression reduced the growth of SW480 colon cancer cells (190). Taken together, this may illustrate why we find ER stress to be induced early after n-3 PUFA exposure, while changes in cell growth and DNA synthesis appear later. Recently, we used the xCELLigence RTCA DP instrument (Roche) to monitor cell growth in real time during DHA treatment of SW620 cells. Cell index declined after 10-15 h of DHA (but not OA) treatment in these cells (Overland *et al*, submitted).

Sustained ER stress is also indicated by up-regulated XBP-1 (**paper I**, table 1) and its target C/EBPβ (191) in both n-3 PUFA-treated SW620 and HL-60 cells (**paper I** and **II**, re-examination of gene lists). The C/EBPβ LIP isomer has been found to increase during prolonged ER stress, attenuate ATF4-dependent transcription and probably be pro-apoptotic (192). Recently, we also found LIP and the LIP/liver activator protein (LAP) ratio to be increased upon tetradecylthioacetic acid (TTA)-treatment of SW620 cells (193). Increased LIP has been found important for CHOP-induced apoptosis (194), and C/EBPβ/CHOP may be important for the up-regulation of TRIB3 (195). TRIB3 was up-regulated by n-3 PUFA treatment in both cell lines tested (**paper I**, table 1 and **paper II**, table 2) and may promote

apoptosis through inhibiting AKT (196) and NFκB (197). In addition, up-regulated GADD34 (**paper I**, table 1) and caspases 4 and 7 (**paper I**, table 1 and **paper II**, table 2, reviewed in (198)), as well as calpains (**paper I**, table 1, (199)) have been implicated in ER stress-induced apoptosis. However, we were not able to detect activated caspase 7 (**paper I**, results not shown) or observe apoptosis in DHA-treated SW620 cells (104), while others have found EPA-induced apoptosis in HL-60 cells, as discussed below (chapter 4.5).

Extensive ER stress may also induce the expression of genes involved in autophagy (reviewed in (122)). Further examination of differentially expressed genes (**paper I** and **II**) revealed that the already mentioned up-regulated transcripts: SQSTM1/p62, VCP (200), BNIP3L (201) and C/EBPβ (202), in addition to LC3B (reviewed in (187), re-examination of gene lists **paper I**) were also implicated in autophagy. This may indicate induction of autophagy upon n-3 PUFA treatment of SW620 and HL-60 cells. Also, we recently showed that DHA treatment up-regulated several important UPR and autophagy factors as early as after 3 and 6 h in SW620 cells, however, this did not result in increased autophagy level in these cells (Overland *et al*, submitted). Even so, Jing *et al* newly found that DHA induced autophagy and apoptosis in cervical cancer that was p53 dependent (203).

ER stress and UPR are known to play a role in some human diseases. Even if induction of UPR correlates with survival of some tumors, as discussed in chapter 1.5.5, UPR may be detrimental in other diseases like retinitis pigmentosa in which misfolded mutant rhodopsin protein leads to apoptosis, degeneration of the retina and hence results in blindness (reviewed in (204)). ER stress and UPR are also implicated in the pathogenesis of obesity and transcriptional regulation of lipogenesis through all three arms of UPR (reviewed in (205, 206)). Our results show that DHA- and EPA-induced ER stress and UPR in human cancer cells correlated with reduction of cell growth (**paper I** and **II**). However, in search for relevant biomarkers for prediction of PUFA-sensitivity or targeted therapies, there is a need for more research on the molecular mechanisms involved. Currently, different UPR-based "anti-tumor" drugs are being explored and tested, both in the laboratory and the clinic. Given the fact that UPR can be both protective and death-inducing, such drugs may be directed at changing the balance of UPR towards induction of cell death. This can be achieved e.g. by inhibiting proteasomal degradation and thereby ERAD; resulting in extensive ER stress and apoptosis (reviewed in (207)).

4.3 N-3 PUFAs disturb Ca^{2+} homeostasis in human cancer cells

Proper regulation of the Ca^{2+} homeostasis in the ER is important in maintaining overall ER homeostasis (reviewed in (121)). Gene expression results indicated that DHA interfered with the expression of some genes like IP3Rs involved in regulation of Ca^{2+} homeostasis in the SW620 cells (**paper I**, table 1, supplemental table 1). Measurement of cellular Ca^{2+} levels showed that DHA increased the $[Ca^{2+}]_i$, probably by triggering Ca^{2+} release from the IP_3 sensitive ER Ca^{2+} store and increasing capacitative Ca^{2+} flux into cells (**paper I**, fig. 4). Consistent with our results, Aires *et al* previously found that DHA increased $[Ca^{2+}]_i$ via IP_3-dependent release from intracellular Ca^{2+} stores and capacitative Ca^{2+} entry in leukemia cells (143). Chow *et* al also demonstrated an n-3 PUFA-induced two phasic increase in $[Ca^{2+}]_i$ in a leukemic cell line, however, the increase in $[Ca^{2+}]_i$ mobilized from the ER Ca^{2+} pool did not depend on IP_3 and the second phase lower Ca^{2+} level was not due to Ca^{2+} influx (208). Some studies have found that DHA and/or EPA reduced the TG-induced rise in $[Ca^{2+}]_i$ by inhibiting TG-induced capacitative Ca^{2+} influx (113, 148). This is contradictory to our observation in DHA-treated SW620 cells where we found DHA supplementation to increase $[Ca^{2+}]_i$ to an even higher level than TG alone (**paper I**, fig. 4). Hence, results on the capability of n-3 PUFAs to affect capacitative influx are not consistent for unknown reasons. Interestingly, capacitative Ca^{2+} influx is currently believed to include STIM and ORAI proteins (reviewed in (142)). Reentry of the gene expression results (**paper I**) showed that STIM1 was up-regulated at 24 and 48 h upon DHA-stimulation in SW620 cells. This may be to increase capacitative Ca^{2+} flux into cells, in order to refill the ER Ca^{2+} store. The up-regulated calcium modulating ligand (CAMLG) (**paper I**, table 1) is also worth mentioning, since it is known to induce Ca^{2+} flux in to cells (209). OA has been shown to inhibit TG-induced capacitative Ca^{2+} influx (149, 210). OA was not included in the Ca^{2+} measurements, however, we may speculate that the influence of OA on Ca^{2+} transport is different from DHA, and hence OA did not induce ER stress in the SW620 cells (**paper I**).

It is possible that the observed disturbance in Ca^{2+} homeostasis is linked to induction of ER stress in SW620 cells. The measurements of the effect of DHA on Ca^{2+} levels in SW620 cells were performed at late time points. Considering the early induction of eIF2α-P (3 h) and ATF4 (6 h) the observed changes in Ca^{2+} homeostasis could be a result of, rather than the cause of, ER stress induction. These experiments should be repeated at earlier treatment time points in order to explore whether there could be an initial transient Ca^{2+}-

release prior to the observed ER stress or not. Some previous studies have reported that n-3 PUFAs induces a rapid increase in $[Ca^{2+}]_i$ in some cancer cells (113, 143, 208).

The findings of ER stress and UPR induction, as well as changes in the $[Ca^{2+}]_i$ led us to suggest that regulation of Ca^{2+} homeostasis may be important in the "anti-tumor" effect of n-3 PUFAs. In order to further investigate this hypothesis, we took advantage of the already described HL-60/E2R2 cancer cell model in which the two cell lines respond differentially to the Ca^{2+} homeostasis disturbing chemicals Ec and TG (150). Zhang *et al* showed that in contrast to HL-60 cells, E2R2 cells displayed sustained protein synthesis levels and did not induce eIF2α-P upon Ec/TG treatment, which may reflect decreased stress in the E2R2 cells possibly due to increased capacitative influx and increased refilling of the ER (150). We hypothesized that these two cell lines would respond differentially to n-3 PUFA supplementation. EPA (35 μM) did indeed induce strong growth inhibition of HL-60 cells, while E2R2 cells were resistant (**paper II**, fig. 2). These results correlated with an early induction of eIF2α-P (3 h) and ATF4 and reduction of cyclin D1 only in HL-60 cells (**paper II**, fig. 3). Hence, induction of ER stress and UPR, and translational shutdown was not observed in the E2R2 cells. Based on this we suggested that the degree of n-3 PUFA sensitivity correlated with the differences in regulation of Ca^{2+} homeostasis between these cell lines. It has been shown that PUFAs and Ec may mobilize Ca^{2+} from the same intracellular Ca^{2+} store (211). We did not measure Ca^{2+} release in this cancer cell model. However, the gene expression profiling of HL-60 cells showed up-regulation of several genes involved in regulation of Ca^{2+} homeostasis like IP3R, which may indicate increased need of such receptors to regulate Ca^{2+} release from the IP3-sensitive ER Ca^{2+} store (**paper II**, table 2). It would be interesting to explore and compare the effect of n-3 PUFAs on $[Ca^{2+}]_i$ in this model.

Recently it was reported that Ec resistance in the E2R2 cells correlated with high overexpression of the ATP-binding cassette, sub-family F (GCN20), member 1 (ABC50/ABCF1) gene which has a role in translation initiation. Overexpression of ABC50 in HL-60 cells decreased sensitivity towards Ec, decreased eIF2α-P, increased ribosomal content and increased protein translation, while ABC50 knockdown in E2R2 cells gave the opposite characteristics; hence increased the Ec sensitivity in these cells (212). Reinspection of gene expression results (**paper II**) revealed that ABC50 was down-regulated upon EPA treatment in HL-60 cells. This may be consistent with EPA-induced growth inhibition, induction of eIF2α-P and translational shut down in HL-60 cells, but not in E2R2 cells (**paper II).** If the expression of ABC50 is affected by EPA in the E2R2 cells remains to be investigated.

In light of our results it is tempting to speculate that the differences in sensitivity to n-3 PUFAs are correlated with differences in capability of cancer cells to handle changes in Ca^{2+} homeostasis. This may be reflected by a study by Palakurthi *et al* reporting that the cancer cell line U118-MG had a low half maximal inhibitory concentration (IC_{50}) value of 36 µM EPA and induced a rapid increase in Ca^{2+} level, while the less sensitive cancer cell line HTB-174 had a high IC_{50} value of 80 µM and did not give such a rapid increase in Ca^{2+} level. Hence, the degree of EPA sensitivity correlated with the ability to increase in $[Ca^{2+}]_i$ in these cancer cells (113).

4.4 DHA deregulates lipid metabolism in cancer cells

The ER homeostasis is sensitive to changes in cholesterol homeostasis (reviewed in (121)). Interestingly, we found induction of the important cholesterol synthesis regulators SREBP2 (mature form) and HMG-CoAR in DHA-treated SW620 cells (**paper I**, fig. 5), in addition to up-regulated expression level of some genes encoding proteins involved in cholesterol synthesis, uptake and transport like LDLR and NPC1 (**paper I**, table 1, supplementary table 1, fig.5). This could indicate an increased need for cholesterol synthesis upon DHA supplementation. Based on these results, we measured *de novo* cholesterol synthesis which turned out to be down-regulated (**paper I**, fig. 6), accompanied by some down-regulated cholesterol synthesis transcripts (**paper I**, table 1). However, the total cholesterol level in these cells did not change markedly (**paper I**, results not shown). Taken together with the previous observations of accumulated DHA-rich CEs and accumulation of lipids in lipid droplets upon DHA treatment of SW620 cells (104), these results suggested a redistribution of cholesterol from the free form into CEs. The reduction in *de novo* cholesterol synthesis and the accumulation of CEs could possibly lead to depletion of free cholesterol in the ER. DHA supplementation of CaCo-2 cells has been shown to reduce cholesterol transport from the plasma membrane to the ER (213). A similar event in SW620 cells may contribute to reduced ER cholesterol level. Cholesterol depletion may induce ER stress and UPR (214), which could be responsible for the activation of SREBP2 (138). Consistent with the observed reduction in cholesterol synthesis, previous studies have reported that n-3 PUFAs reduced HMG-CoAR mRNA level (101, 102, 215) and nuclear SREBP2 (105), as well as induced LDLR in cancer cells (215, 216). Hence to our knowledge, we are the first to report activation of SREBP-2 upon n-3 PUFA supplementation of cancer cells.

A possible explanation for reduced cholesterol synthesis and reduced expression of some cholesterol synthesis genes could be that ER stress-induced ATF6 might interfere with SREBP2-regulated gene expression by binding the active transcription factor and thereby reducing its activity (139). However, we recently showed that down-regulated expression of some SREBP2 target genes (as early as after 6 h) and increased activated SREBP2 level were most likely regulated independently of ER stress and ATF6 during DHA-treatment of SW620 cells. This was also reflected by the induction of SREBP2 by OA (217), which did not induce ER stress (**paper I**, fig. 3) or accumulation of CEs (104) in these cells. The observed deregulation of cholesterol synthesis by DHA supplementation needs to be further examined.

In addition to SREBP2, cholesterol synthesis is also known to be induced by SREBP1a (reviewed in (42)). Consistent with our previous finding of reduced nuclear SREBP1 protein upon DHA-treatment of SW620 and SW480 cells (104), we found SREBP1 expression to be down-regulated (**paper I**, supplementary table 1). Others have also found reduced SREBP1 mRNA and protein level upon n-3 PUFA supplementation (103, 105). SREBP1 also regulates lipogenesis e.g. through induction of FAS, and consistent with the results of others (101), the expression of FAS was also reduced by DHA-treatment of SW620 cells (**paper I**, reinspection of gene lists). Whether the reduction of SREBP1 may cause down-regulation of the expression of some cholesterol biosynthesis genes upon DHA-treatment needs to be further investigated. Interestingly, SREBP nuclear levels were recently shown to be influenced by the mTORC1 pathway; inhibition of mTORC1 resulted in increased nuclear localization of lipin 1 which could reduce nuclear SREBP (218). Reinspection of the gene lists (**paper I**) revealed that Akt1 expression was down-regulated which may lead to reduced mTORC1 activation. The expression of lipin 1 was up-regulated in DHA-treated SW620 cells (complementary unpublished results).

The finding of reduced incorporation of newly synthesized cholesterol into CEs (**paper I**, fig. 6) correlates with the previous observation of reduced ACAT1 mRNA and protein level upon DHA-treatment of SW620 cells. However, reduction of ACAT1 took place after 24 h and beyond (104); hence there is time for accumulation of CEs preceding this reduction and ACAT1 reduction could possibly be a feedback mechanism to this accumulation. Further, accumulation of DHA-rich CEs in lipid droplets could possibly be due to reduced CE turnover. This speculation is supported by the finding of down-regulated gene expression of neutral cholesterol ester hydrolase 1 (NCEH1, complementary unpublished results) in DHA-treated SW620 cells. NCEH1 degrades CEs from cytosolic lipid droplets (reviewed in (219)); hence its reduction could lead to accumulation of CEs in lipid droplets.

Interestingly, knockdown of NCEH1/KIAA1363 has been found to reduce growth of cancer cell xenografts in vivo (220).

Cholesterol depletion has been reported to induce autophagy (221, 222) and induce SREBP2-dependent transcription of several autophagy genes like LC3B, possibly in order to induce autophagy of lipid droplets to access the sterols stored therein (221). This could be consistent with our observations of activated SREBP2, a possible local cholesterol depletion in the ER (**paper I**) and induction of the expression of autophagic genes after DHA treatment of SW620 cells, even though it did not correlate with increased autophagy in these cells (Overland *et al*, submitted). This is also reflected by the accumulation of cytosolic lipid droplets (104) whose degradation has been shown to be regulated by autophagy-dependent lipolysis (macrolipophagy) (223). Interestingly, Velikkakath *et al* recently showed that the autophagy protein Atg2 co-localized with lipid droplets and found that knockout of Atg2A and Atg2B resulted in accumulation and clustering of lipid droplets in HeLa cells (224). The relation between cholesterol, lipid droplets and autophagy was lately reviewed in (225).

As an important component of cellular membranes, cholesterol is essential for cell growth (reviewed in (41)) and cancer cells may have a higher demand for cholesterol in order to keep up membrane synthesis in rapidly growing cells (226). Cholesterol starvation has been shown to decrease CDK1 activity and arrest cancer cells in the G2 phase of the cell cycle (227). This is also reflected in a study by Kaneko *et al* who showed that the HMG-CoAR inhibitor lovastatin down-regulated the IAP survivin that is normally active in the G2/M phase. They also found that siRNA against survivin induced apoptosis in SW480 cells (228). Hence, there seems to be a link between the reduction in cholesterol biosynthesis (**paper I**) and the previously observed G2/M arrest (104) via reduction of CDK1 and survivin (**paper III**, table 1, fig. 2) after DHA-supplementation of SW620 cells. However, we recently showed that cholesterol supplementation in combination with DHA did not effect DHA-induced growth inhibition of these cells (217).

4.5 N-3 PUFAs affect G1/S and G2/M cell cycle checkpoints and regulation of apoptosis

Based on previous reports of cell cycle arrest in the G2/M and G1 phases of DHA-treated SW620 cells (104) and EPA-treated HL-60 cells (184), respectively, the changed expression of cell cycle regulators was expected (**paper II**, table 2 and **paper III**, table 1, fig. 1). Others have also reported n-3 PUFAs to arrest cancer cells in the G1 and G2/M cell cycle phases (88,

108-112). However, in DHA-treated SW620 cells both G1 and G2/M phase regulators were differentially expressed; hence DHA probably affected both of these phases simultaneously. Down-regulated transcription of G1 phase cyclin D and CDKs in DHA-treated SW620 cells (**paper III**, table 1, fig. 2) may indicate a hinder from passing on to the G2/M phase. DHA-induced p21 (**paper III**, table 1, fig. 2) may inhibit cyclin-CDK complexes at both the G1/S and G2/M checkpoints (reviewed in (229)) and down-regulate PCNA (**paper III**, table 1), in order to halt cell cycle progression (reviewed in (230)). Consistently, others have also reported n-3 PUFAs to induce changes in the expression of p21 (97, 112), CDK2 (88, 112), Cyclin A (88, 112) and PCNA (114). Re-examination of the gene lists from paper II revealed up-regulated expression of the G1 cell cycle inhibitors p18 and p27, as well as down-regulated expression of CDK4 and cyclins D1-3 and cyclin E in EPA-treated HL-60 cells (**paper II**, table 2); hence indicating G1 arrest. Changed transcription of p18 and p27 were previously reported in DHA-treated colon cancer cells (115).

The G2/M phase was also clearly affected by DHA treatment of SW620 cells, as shown by down-regulated expression for several different G2/M phase regulators (**paper III**, table 1). The reduction of nuclear CDK1 and CDC25C (**paper III**, fig. 2) may be due to increased cytosolic SFN (**paper III**, fig. 2) which binds and retains these proteins in the cytoplasm (reviewed in (231)). This, in addition to down-regulation of G2/M cyclins (**paper III**, table 1), may be critical for induction of G2/M arrest in SW620 cells. To our knowledge, DHA-induced SFN, as well as reduced CDK1 and CDC25C have not been reported previously.

In a review on PUFAs and induction of apoptosis in cancer cells, Serini *et al* (75) commented on our results from paper I. Given the fact that conjungated-LA (CLA) has been reported to induce ER stress-associated apoptosis in breast cancer cells (175), they suggested looking for apoptosis following PUFA-induced ER stress in colon cancer cells. However, even if DHA induced changes in different biological pathways that may lead to apoptosis (**paper I** and **III**), we have not able to show induction of apoptosis in SW620 cells by TUNEL-assay (104) or activation of caspase 7 (**paper I**, results not shown). Consistent with our results, Chen *et al* could neither demonstrate apoptosis in SW620 cells upon DHA-treatment, even if DHA induced apoptosis and detectable DNA ladder in SW480 cells in the same study (85). It is important to note that Chen *et al* used double the DHA concentration (150 μM) compared to **paper I** and **III** (70 μM). However, both concentrations are physiologically relevant (22). Intriguingly, Huerta *et al* found that SW620 cells were more resistant to apoptosis than SW480 cells, and that SW620 cells expressed less of some

important apoptotic factors, while the survivin level was higher in SW620 cells compared to SW480 cells, and only SW620 cells expressed the NFκB p65 (232). Considering that the fact that SW480 and SW620 cell lines origin from a primary tumor and a lymph node metastasis of a human Duke's stage B colon adenocarcinoma, respectively, and most likely have a monoclonal origin (233), this may indicate that the ability to induce apoptosis is reduced during progression from primary tumor to metastasis (232). Consistent with the growth inhibiting effect of n-3 PUFAs on the SW620 cells *in vitro* (104), we also showed this effect *in vivo* by feeding nude mice a diet containing FO compared to CO in the control diet. Three weeks after initiation of SW620 xenografts, tumor growth was reduced, the n-3 PUFA level increased, while the n-6 PUFA level decreased. The FO diet also reduced the phosphocholine level in these cells (70) which is known to be increased in different cancers (reviewed in (234)).

Several pro-apoptotic transcripts were up-regulated after 12 h of EPA supplementation in HL-60 cells, including five different caspases like the ER stress-implicated caspases 4 and 7 (**paper II**, table 2). Consistent with these results, Arita *et al* found that EPA reduced HL-60 cell growth in a time- and dose dependent manner, induced apoptosis (above 60 μM for more than 6 h), induced cytochrome C, increased mitochondrial membrane potential and cleaved BID (152). Other studies have also shown that DHA and/or EPA may induce growth reduction and/or apoptosis/necrosis in HL-60 cells (181, 184, 235-237) and other human cancer cells (reviewed in (75)).

4.6 Known anti-tumor targets affected by DHA

PUFAs are emerging as a nontoxic supplement to cancer therapy. It seems to be well tolerated by humans and do not give harmful side effects during clinical trials (61, 64, 68). In cell culture and animal experiments, n-3 PUFAs in combination with conventional cancer therapy have shown increased effect of chemotherapeutic drugs such as doxorubicin and radiation of different cancer types, as well as reduction of chemotherapeutic side effects (reviewed in (74)). Gene expression profiling of DHA-treated SW620 cells revealed changes in expression of genes which are known targets for cancer therapy. Reduction of the IAPs survivin and livin (both α- and β isoforms), and induction of the apoptotic cleaved livin (tLlivin) at protein level were especially interesting (**paper III**, fig. 2). Survivin and livin have been associated with cancer malignancy and found to be up-regulated in different cancers (reviewed in (238)) like colorectal cancer in which their expression was inversely correlated with overall survival

(239). Interestingly, Calviello *et al* previously showed that DHA supplementation to SW480 cells reduced survivin in a time- and dose dependent manner (120). Also, knockdown of survivin mRNA by siRNA increased the sensitivity of radiation-induced apoptosis and arrested SW480 cells in the G2/M phase (240).

Since NFκB is constitutionally active in several cancers and facilitates tumorigenesis, the NFκB-pathway is very interesting for therapeutic targeting in cancer. Unfortunately, several chemotherapies are known to activate NFκB, which may lead to resistance towards the apoptosis inducing effect of these agents (reviewed in (71)). Therefore we were exited to find that DHA-treatment of SW620 cells decreased NFκB p65 (Rel A) at both mRNA and protein level (**paper III**, table 1, fig. 2). Others have also found n-3 PUFAs to interfere with the expression and activity of NFκB (reviewed in (71)). Consistent with our results, Narayayan *et al* found that DHA reduced both NFκB p65 and cyclin D1 at protein level in colon cancer cells (89). Interestingly, ER stress may activate NFκB at an early stage through all three UPR branches and increased $[Ca^{2+}]_i$ or ROS, but also inhibit NFκB if the stress is persistent through e.g. C/EBPβ (reviewed in (241)). In addition, NFκB can be activated by AKT (reviewed in (71)), but this may be counteracted and lead to induction of apoptosis through TRIB3 inhibition of AKT (196). NFκB may also inhibit CHOP (242) and interfere with the regulation of the ER Ca^{2+} storage (243). Hence the DHA-induced reduction of NFκB p65 in cancer cells is an important finding.

The DHA-induced up-regulation, phosphorylation and hence activation of p38 MAPK was also an interesting finding (**paper III**, table 1, fig. 2). In addition to its well-known role in proliferation inhibition (reviewed in (244)), p38 MAPK is also known to be activated during ER stress, and may increase the activity of CHOP (reviewed in (198)). Activation of p38 MAPK has also been suggested as a link between induction of ER stress and ER stress-induced apoptosis and autophagy (245). Further, the anticancer drug celecobix may activate p38 MAPK which is implicated in the down-regulation of survivin and induction of growth inhibition and apoptosis in colorectal cancer cells (246). Others have also found activated p38 MAPK to be involved in the anti-tumor effect of n-3 PUFAs (106, 247). However, the role of p38 MAPK in growth regulation is not clear; some studies have reported a pro-survival role of p38 MAPK in cancer cells and assign it a role in metastasis. Therefore caution should be taken when manipulating p38 MAPK for therapeutic purposes (reviewed in (244)).

4.7 Different cancer cells may respond differently to n-3 PUFA treatment

Animal and cell culture experiments have shown that n-3 PUFAs may have anti-tumor effects. However, the degree of n-3 PUFA sensitivity may vary substantially between different cell lines. Both SW620 and HL-60 cells are sensitive towards quite low doses of DHA and EPA (104, 184), as are several other cancer cell lines from various tissues, while some cancer cell lines seem to be very resistant towards these n-3 PUFAs (113, 184, 248). Ding *et al* showed that the IC_{50} values of DHA treatment of different cancer cell lines varied between ~5-300 μM for the most sensitive and the most resistant, respectively (248). The wide spectra in n-3 PUFA sensitivity between different cancer cells was also reflected by the study of Palakurthi *et al* who tested the growth reduction potential of EPA in several different human cancer cell lines (113). Several factors, probably both known and unknown may be considered in this context since different types of cells display different characteristics; they have different gene- and protein expression which is likely to be very important for their response to different types of stimuli. Therefore different cancer cell lines may respond to exposure of n-3 PUFAs through several different pathways and the degree of growth inhibition or cell death may vary substantially. In addition, Diggle reviewed several factors that may influence the outcome of *in vitro* studies reporting on the association between PUFAs and cancer; cell type differences, grade and stage of tumors, differences in cell cultivation conditions (cell density and medium contents), specific PUFA characteristics (number of double bonds and chain length), as well as PUFA concentration and exposure time (249). Hence, some of these factors may possibly explain why our results show that DHA (35 and 70 μM), but not OA (70 μM), induced ER stress at in the SW620 colon cancer cells (**paper I**, fig. 3), while Caviglia *et al* reported that 400-1200 μM OA, but not 200-800 μM DHA, induced ER stress in a hepatoma cell line (176). It is tempting to mention that supplementation of SW620 cells with 105 μM almost completely abolished further growth, as assessed by MTT assay (unpublished complementary results). In addition, how n-3 PUFAs are delivered to cancer cells; as LDL, bound to albumin (250) or FFAs (**paper I**, **II** and **III**), may also be of importance.

5. Conclusion and future perspectives

Extensive research has been performed on the possible anti-tumor effect of n-3 PUFAs. However, in order to use these PUFAs in cancer prevention or as a supplement to already established cancer therapies it is important to elucidate the molecular mechanisms behind this effect. By taking advantage of the microarray technology, we found that n-3 PUFAs induced extensive changes in the gene expression profiles of cancer cells. Early responses were ER stress and induction of the UPR, which correlated with changes in Ca^{2+} and cholesterol homeostasis. These changes may lead to cell cycle arrest and possibly induction of autophagy and cancer cell death. Importantly, DHA supplementation had a favorable effect on known cell cycle regulators and cancer therapy targets. These results may contribute to the understanding of how natural n-3 PUFAs exert their anti-tumor effect on cancer cells and how they may enhance the effect of conventional cancer chemotherapies.

The observed n-3 PUFA-induced ER stress, UPR and changes in Ca^{2+} and cholesterol homeostasis should be further studied in the established and other cancer cell models, as well as normal cells, to explore if these responses are cancer cell specific and common between different cancer cell types. If cancer cell specific, the differences between normal and cancerous cells counting for these differences should be studied. Further studies are also needed to explore the effect of n-3 PUFAs on cancer therapy target genes and proteins. Also, the presented microarray studies have potential for further investigation of different mechanisms involved in the anti-tumor effect of n-3 PUFAs on cancer cells. In addition, it would be interesting to study the relation between lipids and autophagy (lipophagy) upon n-3 PUFA treatment, as well as the n-3 PUFA effect on micro RNAs in cancer cells.

In the context of personalized diagnostics and treatment, it is likely that specific transcriptomic or proteomic profiles could help reveal patients who would benefit more or less from co-treatment with chemotherapy and specific n-3 PUFAs. This could help reveal possible biomarkers related to n-3 PUFA sensitivity in cancer cells. Whether n-3 PUFAs have potential as supplements to clinical cancer treatment needs to be further elucidated. Especially clinical studies are needed, focusing on cancer prevention and cancer therapy co-treatment, as well as the understanding of the molecular mechanisms involved. Such studies should strictly control preparation and intake of n-3 PUFA dietary sources.

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Paper I

✤ *Author's Choice*

DHA induces ER stress and growth arrest in human colon cancer cells: associations with cholesterol and calcium homeostasis[S]

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Abstract Polyunsaturated fatty acids (PUFAs) are normal constituents of the diet, but have properties different from other fatty acids (e.g., through generation of signaling molecules). N-3 PUFAs reduce cancer cell growth, but no unified mechanism has been identified. We show that docosahexaenoic acid (DHA; 22:6 n-3) causes extensive changes in gene expression patterns at mRNA level in the colon cancer cell line SW620. Early changes include unfolded protein response (UPR) and increased levels of phosphorylated eIF2α as verified at protein level. The latter is considered a hallmark of endoplasmic reticulum (ER) stress and is abundantly present already after 3 h. It may coordinate many of the downstream changes observed, including signaling pathways for cell cycle arrest/apoptosis, calcium homeostasis, cholesterol metabolism, ubiquitination, and proteasomal degradation. Also, eicosapentaenoic acid (EPA), but not oleic acid (OA), induced key mediators of ER stress and UPR at protein level. Accumulation of esterified cholesterol was not compensated for by increased total levels of cholesterol, and mRNAs for cholesterol biosynthesis as well as de novo synthesis of cholesterol were reduced. These results suggest that cytotoxic effects of DHA are associated with signaling pathways involving lipid metabolism and ER stress.— Jakobsen, C. H., G. L. Størvold, H. Bremseth, T. Follestad, K. Sand, M. Mack, K. S. Olsen, A. G. Lundemo, J. G. Iversen, H. E. Krokan, and S. A. Schønberg. **DHA induces ER stress and growth arrest in human colon cancer cells: associations with cholesterol and calcium homeostasis. *J. Lipid Res.* 2008.** 49: **2089–2100.**

Supplementary key words gene expression • phosphorylated eIF2α • antioxidant response • heat shock response • cytosolic free Ca^{2+} • cell cycle • total cholesterol level • cholesterol synthesis

Long-chain polyunsaturated fatty acids (PUFAs) of the n-3 type are important dietary components that may prevent or alleviate coronary heart disease and inflammatory conditions (1, 2). Even though epidemiological studies on the association between fish consumption and cancer risk are not consistent, evidence from animal- and cell-culture studies demonstrate that PUFAs inhibit cancer-cell growth, induce apoptosis, and increase the efficiency of chemotherapeutic drugs (3–5). Several mechanisms have been proposed for the antiproliferative effect of n-3 PUFAs; among these are alterations in eicosanoid formation (6), lipid peroxidation initiated by free radicals (7–9), accumulation of cytotoxic lipid droplets (10), and specific changes in gene expression patterns (11, 12). This could be mediated directly by PUFAs as ligands of transcription factors, or indirectly through metabolites of PUFAs or other secondary events. There is evidence indicating that fatty acids may regulate gene expression directly. The activity and

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✤ ***Author's Choice****— Final version full access.*

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abundance of several nuclear transcription factors, like peroxisome proliferator-activated receptors (PPARα/δ/γ), liver X receptors (LXRα/β), and sterol regulatory element-binding proteins (SREBP1/2), have been shown to be regulated by dietary PUFAs and their metabolites (11, 13).

Cellular stress from cytotoxic agents may result in adaptive mechanisms in several cellular compartments, including endoplasmic reticulum (ER). ER has three main functions: *1*) folding, glycosylation, and sorting of proteins to their proper destination; *2*) synthesizing cholesterol and other lipids; and *3*) maintenance of Ca^{2+} homeostasis. Disruption of any of these processes causes ER stress and activates the unfolded protein response (UPR). The UPR up-regulates genes that support adaptation to and recovery from ER stress as well as initiating apoptotic pathways when damage is severe. Three transmembrane proteins mediate the UPR signal across the ER membrane: inositol-requiring enzyme 1 (IRE1), eukaryotic translation initiation factor 2α (eIF2α) kinase 3 (EIF2AK3/PKR-like ER kinase [PERK]), and activating transcription factor 6 (ATF6). PERK belongs to a family of eIF2α kinases that regulates the translational control during the UPR. Phosphorylation of eIF2α by PERK leads to attenuation of global protein synthesis, but promotes translation of certain mRNAs, like activating transcription factor 4 (ATF4) mRNA (14). Downstream targets of ATF4 are *CHOP*, *GADD34*, *ATF3*, and genes involved in amino acid metabolism, glutathione biosynthesis, resistance to oxidative stress, and protein secretion. Loss of cyclin D1 during ER stress leads to G1 arrest and provides the cell with an opportunity to restore cell homeostasis (15). However, prolonged ER stress may cause cell death. ER stress-induced apoptosis may be mediated by caspase-12, caspase-9, and caspase-7 (16).

Several links exist between signaling pathways controlling the UPR and lipogenesis. Activation of the transcription factors ATF6 as well as the SREBPs that control cholesterol and lipid synthesis requires translocation from the ER to the Golgi followed by cleavage by site-1 protease (S1P) and site-2 protease (S2P) (17). Also, ER stress may activate expression of genes involved in cholesterol biosynthesis (18, 19). Both elevated levels of cholesterol as well as depletion have been shown to induce ER stress (20, 21).

We have previously shown that the human colon cancer cell lines SW480 and SW620, derived from a primary and a secondary tumor of the same patient, respectively, were strongly growth inhibited by docosahexaenoic acid (DHA) (5). DHA enhanced lipid peroxidation in both cell lines significantly, measured as accumulation of the end product malondialdehyde. The antioxidant vitamin E (α-tocopherol) completely abolished the increase in malondialdehyde, without restoring cell growth, demonstrating that the cells were resistant to lipid peroxidation products. DHA accumulated mainly as triglyceride and cholesteryl ester-enriched lipid droplets in SW480 and SW620, respectively. The protein level of the nuclear form of SREBP1 (nSREBP1) decreased in both cell lines, indicating a possible relationship between disturbances in lipid homeostasis and cell-cycle arrest. We demonstrate that DHA-treatment of SW620 cells results in extensive changes in gene expression patterns at the mRNA level. Early changes include induction of ER stress, as evident from the abundant presence of phosphorylated eIF2α (eIF2α-P); increase in cytosolic Ca^{2+}; and disturbances in lipid metabolism. Downstream signaling subsequently results in growth arrest and protein degradation. Key mediators of ER stress, eIF2α-P, as well as ATF4 were also induced by eicosapentaenoic acid (EPA), but not by oleic acid (OA) treatment.

MATERIALS AND METHODS

Cell culture and fatty acid treatment

Human colon adenocarcinoma cell line, SW620, was obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in Leibovitz's L-15 medium (Cambrex, BioWhittaker, Walkersville, MD) supplemented with L-glutamine (2 mM), FBS (10%), and gentamicin (45 mg/l) (complete growth medium) and maintained in a humidified atmosphere of 5% CO_2: 95% air at 37°C. Stock solutions of DHA, EPA, and OA in ethanol (Cayman Chemical, Ann Arbor, MI) were stored at −20°C and diluted in complete growth medium before experiments (final concentration of ethanol $< 0.025\%$ v/v).

RNA isolation

Seeded in 75 cm^2 flasks were 1.5×10^6 cells. After 8 h, complete growth medium supplemented with DHA (70 μM) or an equal volume of ethanol (control) was added, and cells were incubated for 12, 24, and 48 h. Cells were harvested by scraping in ice-cold phosphated buffered saline (PBS) and stored at −80°C. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to instruction manual. RNase inhibitor rRNasin (40U/μl, 1 μl) (Promega, Madison, WI) was added, and RNA was up-concentrated on a speed vac and resuspended in RNase free distilled H_2O. RNA concentration and quality were determined using the NanoDrop1000 (NanoDrop Technologies, Wilmington, DE) and agarose gel electrophoresis.

Gene expression profiling

Five micrograms total RNA was used for cDNA and cRNA synthesis according to the eukaryote expression manual (Affymetrix, Santa Clara, CA). Detailed gene expression profiling procedure can be found in supplementary data. cRNA was hybridized to the Human Genome Focus Array (Affymetrix). Washing and staining were performed using the Fluidics Station 400 (Midi-Euk2v3 protocol). The arrays were scanned using an Affymetrix GeneChip GA2500 Scanner, controlled by GeneChip® Operating Software 1.2 (GCOS, Affymetrix). Expression profiling was performed in triplicates at all time points using RNA from independent biological replicates. All experiments have been submitted to ArrayExpress with accession number E-MEXP-1014.

Statistical analysis of gene expression data

Statistical analysis was performed based on summary expression measures for each probe set of the GeneChips, using the raw data (CEL) files and a linear statistical model for background-corrected, quantile normalized, and log-transformed perfect match values, performed by the robust multiarray average (RMA) method (22, 23).

For each transcript, a linear regression model including parameters representing treatment effects and time effects for the treatment group was fitted to the RMA expression measures. Based on the estimated effects, tests for significant differential ex-

pression due to DHA treatment were performed using moderated t-tests, in which gene-specific variance estimates are replaced by variance estimates found by borrowing strength from data on the remaining genes (24).

To account for multiple testing, adjusted *P* values controlling the false discovery rate were calculated (25) by inserting the estimated value of the proportion of nondifferentially expressed genes (26). Differentially expressed genes were selected based on a threshold of 0.05 on the adjusted *P* values.

Time effect in the control groups were considered negligible and omitted from the model. Statistical analysis was performed in R (27), using the packages Limma and affy from Bioconductor (28). Differentially expressed genes were annotated using the NetAffx Analysis Centre (http://www.Affymetrix.com) and NMC Annotation Tool/eGOn V2.0 (http://www.GeneTools.no).

Immunoblot analysis

DHA treatment of cells and preparation of total protein extracts were performed as described previously (5). Nuclear extracts for detection of ATF4 were prepared using a Nuclear extract kit (Active Motif, Belgium) according to manufacturer's instructions. To detect phosphorylated eIF2α, cells were washed and scraped in ice-cold PBS-1 mM EDTA and pelleted by centrifugation. Pellets were lysed in 2 × packed cell volumes of lysis buffer on ice for 10 min. Equal amounts of protein were separated on 10% precast denaturing NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The blots were incubated with the indicated primary and horse radish peroxidase-conjugated secondary antibodies (DAKO, Carpinteria, CA) and detected by chemiluminescense using Super Signal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) and visualized by Kodak Image Station 4000R (Eastman Kodak Co., Rochester, NY). Quantification was performed using Kodak Molecular Imaging Software (version 4.0.1). Details about buffers and primary antibodies can be found in the supplementary data.

Measurement of cytosolic calcium in single cells

Cytosolic free Ca^{2+} in single cells was determined as previously described (29). In short, cells were incubated for 40 min at 37°C with a solution of 5 μM fura-2, 0.25% DMSO, and 0.025% Pluronic F-127 (TefLab, Austin, TX) in HEPES-buffered salt solution (HSS). Cells were then washed once and incubated in 400 μl HSS. Applications to cells were done by injecting 100 μl of agonist into the well. Ca^{2+} imaging and registration software has been developed by Rotnes and Iversen (30). Cytosolic Ca^{2+} concentration was calculated using the equation: $[Ca^{2+}] = K_d\beta(R - R_{min})/(R_{max} - R)$ (31). Fluorescence data were analyzed using the program LICS (32).

Analysis of total cholesterol levels

Seeded in 175 cm^2 flasks were 4×10^6 cells. The following day, medium was replaced with complete growth medium supplemented with DHA (70 μM) or medium with equal volume of ethanol and harvested after 3, 6, 12, 24, and 48 h incubation. Cells were harvested by trypsination and resuspended in PBS together with floating cells collected by centrifugation. The cell suspension was counted using a Coulter Counter (Beckman Coulter, Fullerton, CA) and an aliquot of 4×10^6 cells was collected by centrifugation. Lipids were extracted from the cell pellet (33) using chloroform-methanol-water (1:2:0.8 v/v/v). Total cholesterol levels (cholesteryl ester and free cholesterol) in the lipid extracts were determined using the Amplex Red Cholesterol Assay Kit (Invitrogen) according to the instruction manual. Data were expressed as μg cholesterol/mg protein.

Analysis of cholesterol and cholesteryl ester synthesis

Seeded in 175 cm^2 flasks were 4×10^6 cells. The following day, medium was replaced with complete growth medium supplemented with DHA (70 μM) or with equal volume of ethanol (control) and incubated for 24 h. Cells were then incubated with complete growth medium containing ^{14}C-acetate (1.2 μCi/ml) and DHA (70 μM) or ethanol (control cells) for 4 and 6 h. Cells (12×10^6) were harvested by trypsination, and cellular lipids were extracted with chloroform/methanol according to a method modified after Bligh and Dyer (33). Lipids were separated by thin layer chromatography using hexane/diethyl ether/acetic acid (70:30:1, v/v/v) and lipids were visualized using iodine vapor. Lipid fractions were solubilized in Insta-gel plus (Perkin Elmer) before counting. Data were expressed as incorporation of ^{14}C-acetate into cholesterol/cholesteryl ester (cpm/mg protein).

RESULTS

Growth inhibition by DHA through ER stress and growth arrest signaling

The human colon cancer cell line SW620 is strongly growth-inhibited by DHA. Between 72 h and 144 h, essentially no growth could be observed after treatment with 70 μM DHA (5). Although growth retardation was modest after 24 h, [^{3}H]thymidine incorporation was reduced 30–40% in SW620 cells already by 12 h, while no effect was observed after 6 h (data not shown).

We demonstrate that complex gene networks and cell signaling pathways are affected at the mRNA level after DHA treatment in SW620 cells (**Fig. 1 and Table 1**). The number of transcripts differentially expressed increased from 12 to 24 h of DHA treatment [up-regulated: 839 (12 h) vs. 1157 (24 h); down-regulated: 1066 (12 h) vs. 1222 (24 h)], while the number decreased at 48 h (up-regulated: 288; down-regulated: 267). The fold change of transcript levels after DHA treatment ranged from 1.2 to 24, the majority of which being toward the lower end. Transcripts could be classified into several functional categories after annotation as shown in Table 1 and outlined in later discussion. More comprehensive information on changes in gene expression is found in the supplementary Table I. Early changes include induction of ER stress and UPR.

The protein levels of selected target genes were measured in SW620 cells after DHA treatment. Importantly, we demonstrate that an abundant amount of eIF2α-P is found as early as 3 h after DHA treatment, preceding the activation of ATF4 and HMOX1 (**Fig. 2A**). Phosphorylation of eIF2α is considered a hallmark of the UPR and ER stress and leads to attenuation of global protein synthesis, but promotes translation of certain mRNAs, like ATF4 mRNA (15). In accordance with this, ATF4 is up-regulated here both at the mRNA and protein level (Table 1 and Fig. 2A). Downstream targets of ATF4, *ATF3*, and genes involved in amino acid metabolism (*ASNS*), glutathione biosynthesis, resistance to oxidative stress (*HMOX1*), and protein secretion are up-regulated at the mRNA level and (when examined) at the protein level (Fig. 2A, Table 1 and supplementary Table I). Also, ATF6, PERK, and X-box binding protein 1

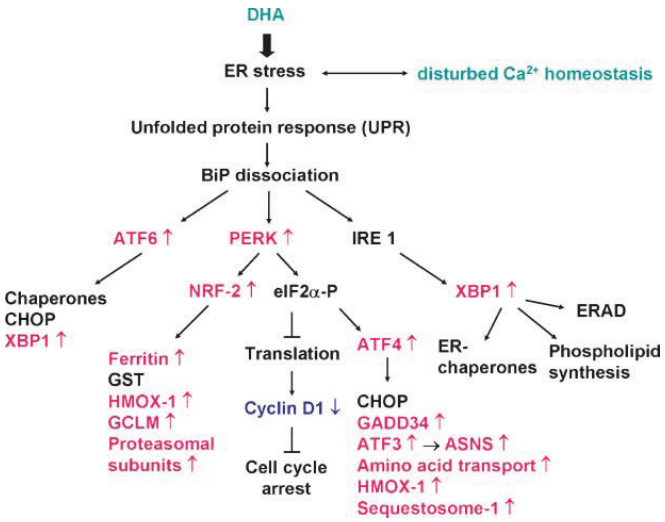

Fig. 1. Docosahexaenoic acid (DHA) induces endoplasmic reticulum (ER) stress in SW620 cells. Diagram showing transcripts found to be affected by DHA treatment in SW620 cells by gene expression analysis (up-regulated, pink; down-regulated, blue) in the main pathways of ER stress signaling. Three transmembrane proteins mediate the unfolded protein response (UPR) across the ER membrane after dissociation from BiP, activating transcription factor 6 (ATF6), PERK, and inositol-requiring enzyme 1 (IRE1). Each of these proteins represents distinct pathways of the ER stress response.

(XBP1), a downstream target of ATF6, are up-regulated at mRNA level in SW620 cells (Table 1). XBP1 regulates a subset of ER-resident chaperones that are essential for protein folding, maturation, and degradation in the ER (34).

Cyclin D1 is significantly down-regulated both at mRNA and protein level (Table 1, Fig. 2B, C), probably mediated by phosphorylated eIF2α that has been shown to attenuate cyclin D1 translation and cause cell-cycle arrest (G1 phase) in response to prolonged ER stress (15). mRNA for GADD34, a subunit in phosphorylated eIF2α phosphatase, is up-regulated in SW620 cells, possibly explaining in part the decrease in phosphorylated eIF2α at 12 h and later (Fig. 2A).

Induction of the UPR is initiated through dissociation of PERK from the ER-resident chaperone BiP/GRP78 that engages in numerous complexes (35). The protein level of BiP remained constant at all time points (data not shown). Treatment of SW620 cells with the ER stress inducers tunicamycin (1 μg/ml) and thapsigargin (0.2 μM) for 6 h, caused a marked down-regulation of cyclin D1 compared with control (results not shown). These results confirm that ER stress in SW620 cells leads to down-regulation of cyclin D1.

Induction of ER stress and UPR is followed by disruption of protein folding and destruction of defective proteins by ER-associated degradation. Several members of the molecular chaperone Hsp40, Hsp70, and Hsp90 families were up-regulated at the mRNA level (Table 1 and supplementary Table I). Hsp70 was also found to be up-regulated at the protein level (Fig. 2A).

Several transcripts belonging to the ubiquitin/proteasome system were up-regulated (Table 1 and supplementary Table I). The proteasome family of proteins is responsible for the degradation of damaged and short-lived proteins. In SW620, mRNA for 27 out of 34 subunits (present on the Human Genome Focus array) of the proteasome 26S were up-regulated. The proteasomal subunit proteasome 26S subunit, non-ATPase, 1 (PSMD1)/Rpn2 was significantly increased relative to control at protein level (Fig. 2A). Also, the mRNA level of sequestosome 1 (SQSTM1), which serves as a storage place for ubiquitinated proteins in the cytoplasm, was up-regulated at all time points in SW620 cells (Table 1). These results support the view that the ER stress response initiated by DHA causes extensive changes in protein homeostasis in these cells.

We have previously shown that EPA has an antiproliferative effect on SW620 cells, although to a lesser extent than DHA, while OA has no effect (5). Key mediators of ER stress and UPR were also induced at protein level by EPA, but not OA, at equal molar concentrations (**Fig. 3**). DHA-treatment (70 μM) of SW620 cells induced phosphorylation of eIF2α already after 3 h (Fig. 2A), while a weaker response is observed after treatment with EPA (70 μM) or half molar concentrations of DHA (35 μM) first at 24 h (Fig. 3). A similar time- and concentration-dependent response is also observed for the induction of ATF4 (Fig. 3). Also, EPA (70 μM) and DHA (35 and 70 μM) reduced the level of cyclin D1 after 24 h (Fig. 2B, C and Fig. 3). After 6 h, only DHA (70 μM) reduced the level of cyclin D1 significantly, whereas DHA (70 μM) and EPA (70 μM) both reduced cyclin D1 substantially and with comparable effects after 24 h. OA did not affect the proliferation of SW620 cells. However, OA reduced the level of cyclin D1 after 24 h, although to a much lesser extent (Fig. 3). This reflects the differences in the antiproliferative effect between the PUFAs observed earlier.

DHA induces antioxidant response

PUFAs are subject to lipid peroxidation, thus causing oxidative stress. Some cell lines that have weak antioxidant defense are highly sensitive to n-3 PUFAs for this reason (36). However, SW480 and SW620 cell lines display little sensitivity to lipid peroxidation products (5). Activated PERK phosphorylates nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (up-regulated in SW620) (Table 1) that

TABLE 1. Functional categories of differentially expressed transcripts affected in SW620 cells treated with docosahexaenoic acid (DHA) (70 μM) at time points indicated

Gene Symbol	Affymetrix ID	Refseq NCBI ID	Description	SW620 Fold Change 12 h	24 h	48 h
ER Stress Response						
ATF3	202672_s_at	NM_001030287 NM_001040619 NM_001674 NM_004024	Activating transcription factor 3	3.7	4.1	3.1
ATF4	200779_at	NM_001675 NM_182810	Activating transcription factor 4	2.1	2.0	1.6
ATF6	203952_at	NM_007348	Activating transcription factor 6	1.3	1.2	—
EIF2S1	201142_at	NM_004094	Eukaryotic translation initiation factor 2-α	NC	NC	NC
GADD34	37028_at	NM_014330	Growth arrest and DNA-damage-inducible 34	6.3	3.9	—
NRF2	201146_at	NM_006164	Nuclear factor E2-related factor	2.0	1.8	—
PERK	218696_at	NM_004836	PKR-like ER kinase	1.4	2.0	—
VCP	208649_s_at	NM_007126	Valocin containing protein	1.9	1.6	—
XBP1	200670_at	NM_001079539 NM_005080	X-box binding protein 1	2.0	1.8	—
Chaperones/Protein Folding/UPR Response						
DNAJB1	200666_s_at	NM_006145	DnaJ homolog, subfamily B, member 1	8.0	4.1	—
HMOX1	203665_at	NM_002133	Heme oxygenase (decycling) 1	24.0	10.6	5.7
HSPA1A/B	200800_s_at	NM_005345 NM_005346	Heat shock 70 kDa protein 1A/B	17.8	9.8	5.1
HSPA1B	202581_at	NM_005346	Heat shock 70 kDa protein 1B	9.8	6.5	3.1
HSP47	207714_s_at	NM_001235	Heat shock protein 47	4.4	1.8	—
Ubiquitine/Proteasome						
PSMD1/RPN2	211198_s_at	NM_002807	Proteasome 26S subunit, non-ATPase, 1	2.2	2.2	—
SQSTM1	213112_s_at	NM_003900	Sequestosome 1	7.7	6.7	5.0
SQSTM1	201471_s_at	NM_003900	Sequestosome 1	6.7	7.3	3.9
$Ca2^+$ Homeostasis						
CAMLG	203538_at	NM_001745	Calcium modulating ligand	1.9	1.9	—
CAPN2	208683_at	NM_001748	Calpain 2, large subunit	1.3	1.8	1.4
CAPN7	203356_at	NM_014296	Calpain 7	—	1.5	—
IP3R1	203710_at	NM_002222	Inositol 1,4,5-triphosphate receptor, type 1	1.5	2.2	1.4
IP3R3	201189_s_at	NM_002224	Inositol 1,4,5-triphosphate receptor, type 3	—	—	1.3
Antioxidants/Oxidative Stress						
CAT	201432_at	NM_001752	Catalase	—	−1.4	—
GCLC	202922_at	NM_001498	Glutamate-cysteine ligase, catalytic subunit	1.6	1.3	—
GCLM	203925_at	NM_002061	Glutamate-cysteine ligase, modifier subunit	3.7	3.5	2.0
HMOX1	203665_at	NM_002133	Heme oxygenase (decycling) 1	24.0	10.6	5.7
SOD1	200642_at	NM_000454	Superoxide dismutase 1	1.5	1.6	—
TXNRD1	201266_at	NM_003330 NM_182729 NM_182742 NM_182743	Thioredoxin reductase 1	3.2	2.9	1.9
Cell Cycle/Apoptosis						
BAG3	217911_s_at	NM_004281	BCL2-associated athanogene 3	9.9	5.4	—
CASP4	209310_s_at	NM_001225 NM_033306 NM_033307	Caspase 4	1.6	2.9	—
CASP7	207181_s_at	NM_001227 NM_033338 NM_033339 NM_033340	Caspase 7	1.6	2.1	—
CCND1	208712_at	NM_053056	Cyclin D1	−1.7	−2.0	—
TRIB3	218145_at	NM_021158	Tribbles homolog 3 (*Drosophila*)	7.4	6.5	3.3
Cholesterol Biosynthesis, Uptake, Metabolism, and Transport						
CAV1	203065_s_at	NM_001753	Caveolin 1, caveolae protein, 22 kDa	−1.5	−1.4	—
DHCR24	200862_at	NM_014762	24-dehydrocholesterol reductase	−1.6	−1.7	—
DHCR7	201791_s_at	NM_001360	7-dehydrocholesterol reductase	−1.6	−1.5	—
FDPS	201275_at	NM_002004	Farnesyl diphosphate synthase	−1.3	−1.2	—
HMGCR	202539_s_at	NM_000859	3-hydroxy-3-methylglutaryl-CoA reductase	NC	NC	NC
LDLR	202068_s_at	NM_000527	Low density lipoprotein receptor	2.4	2.4	—
LSS	202245_at	NM_002340	Lanosterol synthase	−1.3		—
NPC1	202679_at	NM_000271	Niemann-Pick disease, type C1	3.0	4.5	1.9
NPC2	200701_at	NM_006432	Niemann-Pick disease, type C2		1.5	1.5
OSBP	201800_s_at	NM_002556	Oxysterol binding protein	1.4	1.4	—
PMVK	203515_s_at	NM_006556	Phosphomevalonate kinase	-1.3	−1.8	—
SREBP2	201247_at	NM_004599	Sterol regulatory element binding protein 2	NC	NC	NC
TM7SF2	210130_s_at	NM_003273	Transmembrane 7 superfamily member 2	−1.4	−1.9	—
VLDLR	209822_s_at	NM_001018056 NM_003383	Very low density lipoprotein receptor	1.6	1.6	—

NC, no change; UPR, unfolded protein response.

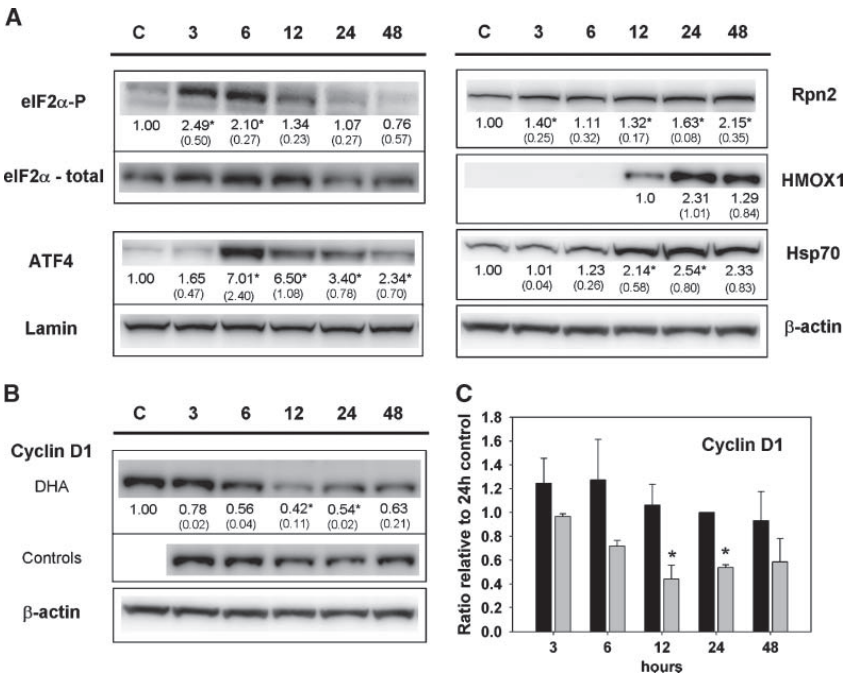

Fig. 2. Analysis of proteins involved in ER stress signaling and UPR. A: Western blot analysis of proteins involved in ER stress signaling and UPR from total cell extracts (except for ATF4: nuclear extracts; eIF2α: cytoplasmic extracts) of SW620 cells treated with DHA for indicated time periods (h). Controls were harvested at all time points; only the 24 h control (C means control) is shown. B: Western blot analysis of cyclin D1 from total extracts of SW620 cells treated or not treated (controls) with DHA for the indicated time periods (h). C: Quantification of cyclin D1 Western blots in B [DHA treated cells (gray bars) compared with controls (black bars)]. Results were verified in three independent experiments; one representative blot is shown. β-actin (total extracts), lamin C (nuclear extacts), or total eIF2α was used as a control for equal protein loading. The blots were quantified and protein band intensities normalized relative to loading control. The adjusted band intensities from the DHA and control membranes were then normalized relative to the 24 h control band, present at all membranes, to adjust for differences in signal intensities between the membranes. The numbers under the blots represent mean fold change (SD) of DHA samples relative to control at the indicted time points for three independent experiments. * Significantly different from control (Student's *t*-test, $P < 0.05$).

promotes transcription of genes involved in redox homeostasis, which contributes to survival of ER stress induced in mammalian cells. Oxidative stress-related genes that were found to be up-regulated in SW620 cells included thioredoxin reductase 1 (TXNRD1), superoxide dismutase 1 (SOD1), HMOX1, glutamate-cysteine ligase modifier (GCLM), and glutamate-cysteine ligase catalytic subunits, (GCLC) indicating a disturbance in the redox balance (Fig. 1 and Table 1). HMOX1, GCLM, and TXNRD1 are downstream targets of Nrf2 (14). HMOX1 displays a 24-fold induction at 12 h and is also strongly induced at the protein level (Fig. 2A and Table 1). HMOX1 catabolizes cellular heme to biliverdin, which is reduced to bilirubin, both being very potent cytoprotective antioxidants (37). However, knockdown of HMOX1 by siRNA did not result in increased DHA-sensitivity in SW620 cells (data not shown), supporting our previous findings that lipid peroxidation is not the key mediator of cytotoxicity.

Effect of DHA treatment on Ca^{2+} homeostasis and genes involved in apoptosis

ER stress-induced depletion of Ca^{2+} stores or dysregulation of Ca^{2+} homeostasis may trigger apoptosis. We found that mRNA for a large number of genes involved in Ca^{2+} homeostasis was changed, mostly up-regulated, after DHA treatment in SW620 cells. Thus, transcripts for the inositol 1,4,5-triphosphate receptors (IP3R1 and 3) were up-regulated, indicating a release of Ca^{2+} regulated by these receptors (Table 1 and supplementary Table I). In agreement with this, treatment with DHA (70 μM) for 12–48 h resulted in an increase in cytosolic Ca^{2+} concentration (**Fig. 4A**). Cytosolic [Ca^{2+}] is mainly regulated by means of transport across cell membranes (e.g., the plasma and ER membranes). Thapsigargin is a specific inhibitor of a Ca^{2+} ATPase, which pumps Ca^{2+} into ER. The rate of [Ca^{2+}] increases in cytosol after addition of thapsigargin thus reflects Ca^{2+} turnover in ER, the main intracellular

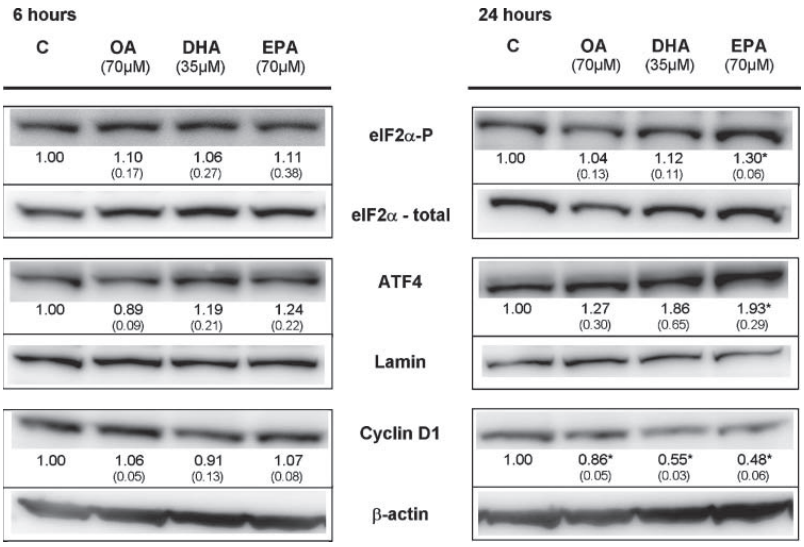

Fig. 3. ER stress signaling and UPR in response to n-3 polyunsaturated fatty acids (PUFAs) and oleic acid (OA). Western blot analysis of proteins involved in ER stress signaling and UPR (ATF4: nuclear extracts; cyclin D1, eIF2α: cytoplasmic extracts) in SW620 cells treated with complete growth medium supplemented with either OA (70 μM), DHA (35 μM), eicosapentaenoic acid (EPA, 70μM), or ethanol (control media, C) for 6 and 24 h. β-actin (cytoplasmic extracts), lamin C (nuclear extracts), or total eIF2α was used as a control for equal protein loading. One representative blot is shown. The blots were quantified and intensities normalized relative to loading control. The numbers under the blots represent mean fold change (SD) relative to control for three independent experiments. * Significantly different from control (Student's *t*-test, $P < 0.05$).

Ca^{2+} store. In SW620 this effect was apparent first after 48 h DHA incubation (average [Ca^{2+}] increase 267nM vs. 187nM in control). The time course of these registrations is shown in Fig. 4B.

A Ca^{2+} signal after agonist-binding to a G protein coupled receptor often displays two phases: an initial peak response, which is due to release from intracellular stores; and a prolonged phase, which is attributable to Ca^{2+} entry through the plasma membrane due to emptying of intracellular Ca^{2+} stores ("capacitative" or store operated Ca^{2+} influx). This Ca^{2+} entry is abolished when free extracellular [Ca^{2+}] is chelated by ethylene glycol tetraacetic acid (EGTA). We find that ATP is an agonist that releases a two-phasic Ca^{2+} signal in most SW620 cells. Incubation with DHA for 12 h and more seemed to accentuate the second phase Ca^{2+} elevation in SW620 cells (Fig. 4C, **Table 2**), whereas the first peak response was virtually unchanged. The time course of the registrations from ATP-stimulated SW620 cells are shown in Fig. 4C. When EGTA was added to SW620 cells treated with DHA for 24 h and then stimulated with ATP, we found that the second phase Ca^{2+} elevation was abolished (Fig. 4D, **Table 3**). The elevation in prolonged Ca^{2+} signal in DHA-treated cells after ATP stimulation can therefore be ascribed to Ca^{2+} entry, probably of the capacitative type.

Disturbances in the Ca^{2+} pool of ER activate calpain in the cytosol, which then converts ER-localized procaspase 12 to caspase 12 (38). Calpain 7 and a large subunit of calpain 2 as well as the ER stress-related caspases (caspase 4 and 7) were up-regulated in SW620 cells (Table 1). The proapoptotic members of the Bcl-2 family, BAD and BIK, were down-regulated, while BID was up-regulated (SW620). BCL2-associated athanogene 3 (BAG3), known to participate in regulation of apoptosis, was up-regulated 9.9-fold in SW620 cells after 12 h incubation with DHA. Also, the proapoptotic factor Tribbles homolog 3 (TRIB3), known to be induced by ER stress through the PERK-ATF4-CHOP pathway, was up-regulated at all time points (Table 1) (39). The protein level of active caspase 7 was found to increase with time in SW480 cells, while not detected in SW620 cells (data not shown).

Effect of DHA on cellular cholesterol and cholesterol metabolism

We have previously shown that treatment of SW480 and SW620 with DHA leads to accumulation of numerous large lipids droplets, mainly containing triglycerides in SW480 and cholesteryl esters in SW620 (5). However, an increase in cholesteryl esters was also seen in SW480. The formation of lipid droplets is probably induced by DHA, since they are highly enriched in this PUFA. To examine whether this accumulation of esterified cholesterol was compensated for by increased total cholesterol levels, we measured cellular cholesterol content after DHA treatment. No significant differences in total cholesterol were found at 3–24 h when comparing control and DHA-treated cells

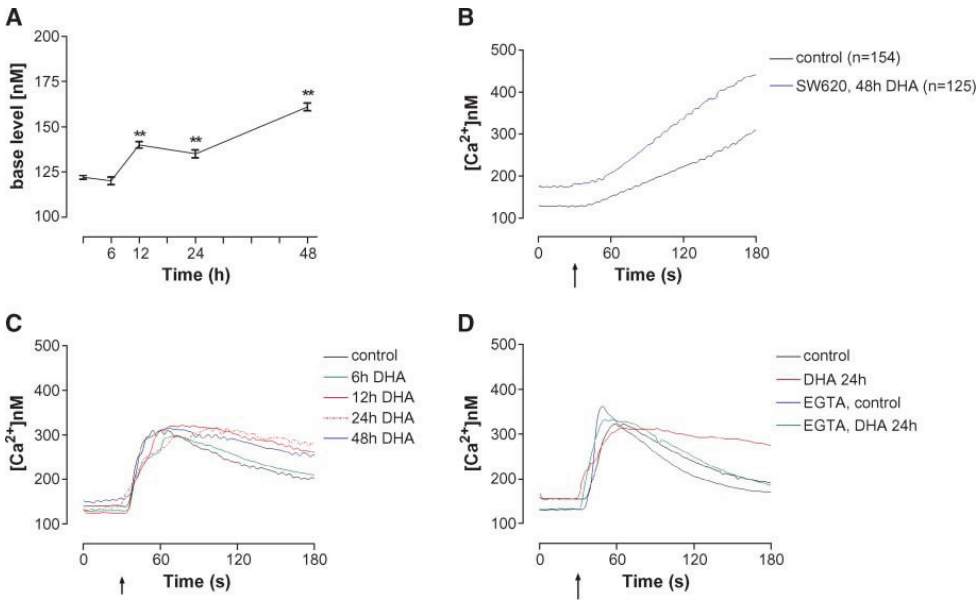

Fig. 4. Cytosolic Ca^{2+} release after DHA treatment. Registrations of cytosolic Ca^{2+} in DHA-treated SW620 cells. A: DHA treatment increases the basic cytosolic Ca^{2+} level in SW620 cells. SW620 cells were incubated with DHA (70 μM) for various time periods as indicated. Average Ca^{2+} concentrations in 196–324 cells are shown. Bars indicate SEM values. The average basic cytosolic Ca^{2+} level from each time period was tested against time 0. Statistically significant difference from control (no treatment): ** $P < 0.01$. B: DHA treatment affects the thapsigargin-inhibited Ca^{2+} transport. SW620 cells were incubated with DHA (70 μM) for 48 h as indicated. After 30 s of [Ca^{2+}] registration thapsigargin (5 μM) (Sigma-Aldrich) or vehicle was added (arrow). Average registrations from all cells are shown since virtually all cells responded. Cytosolic [Ca^{2+}] at the end of the registration (180 s) in DHA-treated cells was statistically significant different from control ($P < 0.05$). C: ATP stimulation causes a prolonged Ca^{2+} signal in DHA-treated cells. SW620 cells were incubated with DHA (70 μM) for various time periods as indicated. After 30 s of [Ca^{2+}] registration ATP (1 μM) was added (arrow). Average registrations from responding cells are shown. D: Removal of extracellular Ca^{2+} with ethylene glycol tetraacetic acid (EGTA) abolishes the prolonged ATP response in DHA-treated cells. SW620 cells were incubated with DHA (70 μM) for 24 h. The cells were incubated in a 10 mM HEPES buffer without Ca^{2+}, but with 0.1 mM EGTA or in a 10 mM HEPES buffer containing 1.2 mM Ca^{2+} for 10 min before registration. After 30 s of [Ca^{2+}] registration, ATP (1 μM) was added (arrow). Average registrations from responding cells are shown.

(data not shown). A slight, but significant increase in total cholesterol levels in DHA-treated cells was observed at 48 h (31.28 ± 1.29 (control) vs. 38.75 ± 3.39 μg cholesterol/mg protein, $P < 0.05$). These results may indicate that cholesterol available for organelles is reduced due to deposition in lipid droplets.

From the gene expression data, it was apparent that several genes encoding proteins involved in cholesterol biosynthesis were down-regulated at the mRNA level in DHA-treated SW620 cells (Table 1). These include 7- and 24-dehydrocholesterol reductase (DHCR7, DHCR24), farnesyl diphosphate synthase (FDPS), phosphomevalonate

TABLE 2. Ca^{2+} registrations in SW620 cells pretreated with DHA (70 μM) at time points indicated: SW620 cells stimulated with ATP, 1 μM

Pretreatment	# Cells (Responding Cells, %)	Maximal [Ca^{2+}] Increase, nM	Decline of the Response
None (control)	120 (56)	190 (± 13.2)	1.64 (± 0.06)
DHA 6 h	110 (53)	196 (± 10.8)	1.66 (± 0.16)
DHA 12 h	107 (55)	226 (± 15.9)[a]	1.39 (± 0.06)[a]
DHA 24 h	94 (51)	201 (± 18.4)	1.27 (± 0.09)[b]
DHA 48 h	97 (45)	198 (± 21.1)	1.23 (± 0.04)[b]

After 30 s of [Ca^{2+}] registration ATP (1μM) (Sigma-Aldrich) or vehicle was added. Maximal [Ca^{2+}] increase is calculated as difference between baseline and peak [Ca^{2+}] in the responding cells. The decline of the response is quantified as the ratio between peak [Ca^{2+}] and [Ca^{2+}] at the end of the registration (180 s). Registrations are depicted in Fig. 4C. The data are presented as means with standard errors (± SEM).

[a] Statistically significant difference from control ($P < 0.05$).

[b] Statistically significant difference from control ($P < 0.01$).

TABLE 3. Ca^{2+} registrations in SW620 cells pretreated with DHA (70 μM) at time points indicated: SW620 cells stimulated with ATP, 1μM

Pretreatment	# Cells	Baseline [Ca^{2+}], nM	Maximal [Ca^{2+}] Increase, nM	Decline of the Response
None (control)	103	123 (± 4.1)	247 (± 10.6)	2.07 (± 0.09)
DHA 24 h	85	141 (± 4.7)[a]	238 (± 14.1)	1.61 (± 0.07)[a]
2. EGTA	81	103 (± 3.7)[a]	259 (± 18.2)	2.51 (± 0.14)[a]
EGTA, DHA 24 h	73	105 (± 3.8)[a,b]	227 (± 17.3)	2.11 (± 0.12)[a,b]

EGTA, ethylene glycol tetraacetic acid. After 30 s of [Ca^{2+}] registration ATP (1μM) (Sigma-Aldrich) or vehicle was added. Maximal [Ca^{2+}] increase is calculated as difference between baseline and peak [Ca^{2+}] in the responding cells. The decline of the response is quantified as the ratio between peak [Ca^{2+}] and [Ca^{2+}] at the end of the registration (180 s). Registrations are depicted in Fig. 4D. The data are presented as means with standard errors (± SEM).

[a] Significantly different from control ($P < 0.05$).

[b] Significantly different from DHA 24 h (÷ EGTA) ($P < 0.001$).

kinase (PMVK), 3β-hydroxysterol Δ14-reductase (TM7SF2) (12, 24 h), and lanosterol synthase (LSS) (12 h). However, some transcripts involved in cholesterol uptake and intracellular cholesterol transport, such as low and very low density lipoprotein receptor (LDLR, VLDLR), the Niemann-Pick C1 protein (NPC1), NPC2, and the oxysterol binding protein (OSBP), were found to be up-regulated in SW620 cells after DHA-treatment (Table 1). NPC1 protein levels were in addition analyzed by Western blot and were increased in DHA-treated cells compared with control at 24 h (**Fig. 5A**).

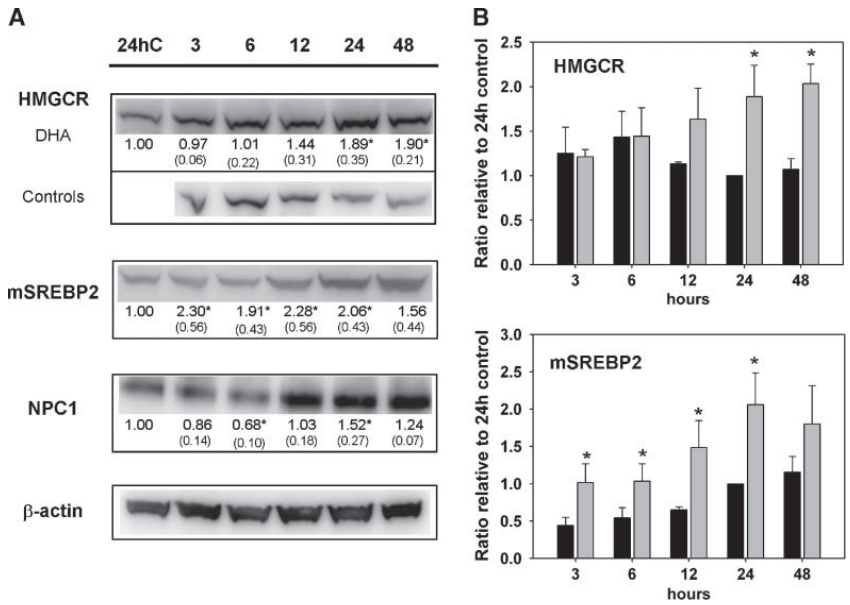

Fig. 5. Changes in cholesterol metabolism induced by DHA. A: Western blot analysis of HMGCR, mSREBP2, and NPC1 protein levels in total protein extracts from SW620 cells treated with DHA for the indicated time periods (h). Controls were harvested at all time points; only 24 h control is shown for mSREBP2 and NPC1. For HMGCR, controls are shown for all time points. β-actin was used as a control for equal protein loading. One blot, representing three independent experiments, is shown. The blots were quantified and protein band intensities normalized relative loading control. The actin adjusted band intensities from the DHA and control membranes were further normalized relative to the 24 h control band, present at all membranes, to adjust for differences in signal intensities between the membranes. The numbers under the blots represent mean fold change (with SD) of DHA samples relative to control at indicted time points for three independent experiments. * Significantly different from control (Student's *t*-test, $P < 0.05$). B: Alterations in HMGCR and mSREBP2 protein levels in control (baseline) and DHA treated cells at the indicated time periods. The plots show the mean value of the actin adjusted band intensitites normalized relative to the 24 h control band for DHA treated cells (gray bars) and control cells (black bars). The data represent the mean and SD of three independent experiments. * Significantly different from control (Student's *t*-test, $P < 0.05$).

Since several of the differentially expressed transcripts listed above are regulated by sterol regulatory element binding protein 2 (SREBP2), the protein levels of mSREBP2 (mature) and pSREBP2 (precursor) were analyzed by Western blot. An increase in mSREPB2 levels was observed in control and DHA-treated cells over the time period assayed, but DHA-treated cells displayed higher levels of mSREBP2 compared with control cells at all time points (Fig. 5A, B). The level of pSREBP2 was unchanged in SW620 control cells at all time points, while a slight decrease was observed after 48 h treatment with DHA (data not shown). We also analyzed the level of the rate-limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) by Western blot after DHA treatment. The protein levels of HMGCR in DHA-treated and control cells were similar at 3 and 6 h. At 12–48 h, the HMGCR protein levels were reduced in controls, while the protein level in DHA-treated cells increased slightly (Fig. 5A, B).

To investigate de novo synthesis of cholesterol in DHA-treated cells, the incorporation of ^{14}C-acetate into cholesterol and cholesteryl esters was measured after treating the cells with DHA for 24 h. The amount of ^{14}C-acetate incorporated into cholesterol in DHA treated cells was slightly, but significantly lower relative to control at 4 h; a similar trend, although not significant was seen after 6 h (**Fig. 6**). The amount of ^{14}C-acetate incorporated into cholesteryl esters in DHA treated cells was reduced by approximately 60% relative to control at both time points (Fig. 6).

DISCUSSION

Exploring how dietary factors interact with and modulate signaling pathways to promote or counteract cancer development and progression constitutes a major challenge. The purpose of the present study was to examine whether n-3 PUFAs like DHA exert their cytotoxicity by changing gene expression patterns and signaling pathways regulating cell growth. We found that ER stress is established already after 3 h treatment with DHA, as demonstrated by increased levels of phosphorylated eIF2α, a hallmark of ER stress. Phosphorylation of eIF2α adapts cells to various conditions of stress by attenuation of protein synthesis. We found that the n-3 PUFAs DHA and EPA, but not OA, cause phosphorylation of eIF2α, thereby generally inhibiting translation initiation. This is in agreement with previous results showing that inhibition of translation initiation mediates the antiproliferative action of EPA in NIH 3T3 cells by decreased levels of cyclin D1 (40). Increased expression of genes downstream of phosphorylated eIF2α is mediated through induction of the transcription factor ATF4 (14). Genes with ATF4 binding sites are involved in restoring ER homeostasis in response to various stresses (41). Several downstream targets of ATF4 are affected at the mRNA and protein level in our study, indicating that ER stress induced by DHA in SW620 cells is mediated through the ER-localized PERK pathway. Induction of the UPR is initiated by dissociation of PERK from the ER-resident chaperone BiP. However, the protein level of BiP remained constant at all time points. Pimpl et al. (42) have reported that transcriptional induction of *BiP* rarely leads to increased protein levels of BiP/GRP78, this being due to increased turnover.

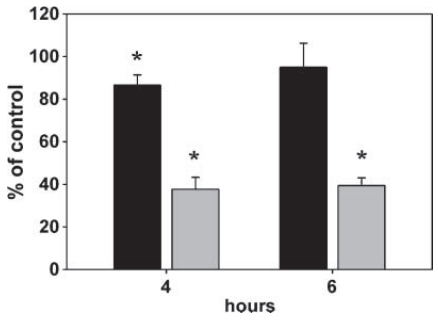

Fig. 6. Effect of DHA on incorporation of ^{14}C- acetate into cholesterol and cholesteryl esters. Amount of ^{14}C-acetate (% of control) incorporated in cholesterol (black bars) and cholesteryl esters (gray bars) in SW620 cells treated with DHA for 24 h, and further coincubated with DHA and ^{14}C-acetate for 4 and 6 h. The mean and ± SD from (4 h, n = 3; 6 h, n = 2) independent experiments is displayed. * Significantly different from control (Student's *t*-test, $P < 0.05$).

UPR is activated to restore cellular homeostasis and induces transcription of genes encoding proteins that mediate ER-associated degradation in response to prolonged ER stress. A large number of 20S and 26S proteasomal subunits were up-regulated in SW620 cells. The proteasome plays a central role in proteolysis of ubiquitinated proteins and are responsible for cleaving many regulatory proteins, like cyclins and members of the NFκB family (43). Prolonged ER stress may cause induction of apoptosis. We show that even though the ER stress-related caspases 4 and 7 are up-regulated in DHA-treated SW620 cells, active caspase 7 is not detectable. On the other hand, active caspase 7 was detected in SW480 cells (data not shown). Chen and Istfan (44) have studied the apoptotic response to DHA in several cell lines, among these SW480 and SW620. A DNA ladder was observed after incubation with DHA (150 μM) for 24 h in SW480, but not in SW620 cells; this is in accordance with our results. Previously, we were not able to detect apoptosis by the TUNEL-assay in either SW480 or SW620 (5). This may indicate that the survival threshold is not exceeded in these cells within the time period assayed and concentration used.

ER is the principal site for protein synthesis and folding, Ca^{2+} storage and signaling, as well as biosynthesis of fatty acids and cholesterol. Any perturbation that interferes with these activities promotes ER stress and initiates the UPR. We found that DHA treatment mobilizes Ca^{2+} from ER into the cytosol, in agreement with previous results investigating the effects of n-3 and n-6 PUFAs (40, 45–47), but the mechanism is not known. Our results indicate that calcium release induced by DHA may be linked to induction of ER stress. A redistribution of cholesterol from intracellular regulatory compartments like ER to DHA-cholesteryl

ester-enriched lipid droplets (5), causing functional depletion of cholesterol in the ER could potentially lead to ER stress and Ca^{2+} mobilization, since total cholesterol is not increased (this work). Harding et al. (21) have shown that compounds that deplete cellular cholesterol stores activate an integrated stress response (ISR) by promoting ER stress.

The observed stabilization of HMGCR, the rate limiting enzyme in cholesterol biosynthesis, and the increased level of mSREBP2 observed in DHA-treated SW620 cells, indicate an increased cellular need for de novo synthesized cholesterol during DHA treatment. Surprisingly, both increased and decreased expression of several SREBP2 target genes is observed in SW620, despite an increase in the active transcription factor. Inhibition of transcription of SREBP2 target genes has previously been associated with ER stress-induced activation and cleavage of ATF6, and is mediated by interaction of the two transcription factors in the nucleus (48). Reduced expression of SREBP2 target genes may result in a decreased ability of the cells to synthesize new cholesterol, in spite of activated SREBP2. In line with this, we show that DHA promotes a reduced de novo synthesis of cholesterol. Surprisingly, we also find a reduced incorporation of newly synthesized cholesterol into cholesteryl esters (this work), despite the previous observed accumulation of cholesteryl esters in SW620 cells treated with DHA (5). This might possibly result from reduced turnover of DHA-enriched cholesteryl esters in droplets resulting in accumulation in spite of reduced synthesis.

Recently, a link between molecular chaperones, heat stress, and cholesterol synthesis was demonstrated (49). In this work, the chaperone DnaJA4 (DnaJ/Hsp40) was identified as a novel SREBP target gene that can be turned on under conditions of low sterol availability and heat shock. They postulated that SREBP-regulated chaperones may function as effectors linking heat-shock response and the maintenance of membrane components. Also, Lee and Ye (18) have shown that both hypotonic conditions and thapsigargin induced ER stress in CHO-7 cells leads to activation of SREBP2, while no increase in cholesterol synthesis was observed.

The reasons why SREBP2 target genes are regulated differently, and consequences thereof, remain to be investigated in our system.

Up-regulation of transcripts involved in cellular uptake and intracellular transport of cholesterol, like LDLR, VLDLR, NPC1/NPC2, and OSBP in SW620 cells treated with DHA, also suggests an increased demand for intracellular cholesterol. A recent report indicates that DHA treatment inhibits transport of exogenous cholesterol from the plasma membrane to the ER by an unknown mechanism in CaCo2 colon cancer cells (50). In addition, a study on a panel of colon carcinoma cell lines revealed a deficiency of the LDLR in SW480 cells, indicating a dependency on endogenous cholesterol biosynthesis (51). This would probably also apply to the SW620 cell line, which is established from a metastasis derived from the primary SW480 tumor. Reduced de novo cholesterol synthesis and inhibition of transport of exogenous cholesterol to the ER pool, in combination with increased cholesterol esterification as observed earlier, may lead to depletion of cholesterol in the ER. This may be an important factor contributing to the observed prolonged ER stress that may cause growth inhibition and eventually cell death.

In vitro studies suggest that pharmacological activation of the UPR can alter the sensitivity of tumor cells to chemotherapeutic agents (52). Understanding how common dietary chemicals like DHA affect gene expression and signaling pathways in tumor cells may reveal possible treatment strategies that may be targeted and possibly enhance the impact of conventional therapy.

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SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Gene expression profiling – detailed method

Synthesis of cDNA and biotinylated cRNA was performed according to the eukaryote expression manual provided by Affymetrix. In brief, 5 µg total RNA from the SW620 cell line was subjected to double-stranded cDNA synthesis using a T7-oligo$(dT)_{24}$-primer (Affymetrix, Santa Clara, CA) and the SuperScript™ Double-Stranded cDNA Labeling Kit (Invitrogen, Carlsbad, CA). After clean up (GeneChip Sample Clean Up Module, Affymetrix), all the prepared cDNA was used for *in vitro* transcription using Enzo BioArray HighYield RNA Transcript Labeling kit (Enzo, Farmingdale, NY). 10 µg fragmented cRNA were hybridized to the Human Genome Focus Array (midi format, Affymetrix) containing probe sets for 8500 of the best characterized human genes. Hybridization and washing/staining were performed using the Hybridization oven 640 and Fluidics Station 400 (Midi-Euk2v3 protocol). Staining was performed using Streptavidin, R-phycoerythrin conjugate (SAPE, Molecular Probes, Eugene, Oregon) and biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA). The arrays were scanned using an Affymetrix GeneChip GA2500 Scanner, controlled by GeneChip® Operating Software 1.2 (GCOS, Affymetrix).

Statistical analysis of gene expression data - comment

First, a model was fitted that included time effects for both the DHA-treated and control groups. However, the time effects in the control group turned out to be negligible, and these effects were omitted from the model.

Antibodies

Primary antibodies detecting the following proteins were used to probe the membranes; HSP70 and HMOX1 (all mouse monoclonal antibodies, Stressgen, Victoria, BC, Canada),

ATF4 (rabbit polyclonal antibody), HMGCR and lamin (goat polyclonal antibodies) (all from Santa Cruz Biotechnology, CA), Cyclin D1 (mouse monoclonal antibody, Cell Signaling Technology, Danvers, MA), RPN2 (mouse monoclonal), active CASP7, NPC1 and SREBP2 (all three rabbit polyclonal antibodies, all four from Abcam, Cambridge, UK). Phosphorylation of eIF2α was assayed using an antibody recognizing phosphorylated eIF2α (Serine 51, Cell Signaling Technology). The membranes were also probed for total eIF2α (Cell Signaling Technology) as a control after stripping the membrane in Restore Western blot Stripping buffer (Pierce, Rockford, IL). All blots were reprobed with β-Actin (Abcam) as a loading control. Membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO, Carpinteria, CA) for one hour at room temperature.

Supplementary Table 1

Functional categories of differentially expressed transcripts affected in SW620 cells treated with DHA (70μM) at timepoints indicated

Gene Symbol	Affymetrix ID	Refseq NCBI ID	Description	SW620 Fold Change		
				12h	24h	48h
ER stress response						
ATF3	202672_s_at	NM_001030287 NM_001040619 NM_001674 NM_004024	Activating transcription factor 3	3.7	4.1	3.1
ATF4	200779_at	NM_001675 NM_182810	Activating transcription factor 4	2.1	2.0	1.6
ATF6	203952_at	NM_007348	Activating transcription factor 6	1.3	1.2	
EIF2S1	201142_at	NM_004094	Eukaryotic translation initiation factor 2-alpha	NC*	NC	NC
GADD34	37028_at	NM_014330	Growth arrest and DNA-damage-inducible 34	6.3	3.9	
GRP94/TRA1	200599_s_at	NM_003299	Glucose regulated protein 94kDa/Tumor rejection antigen 1		1.7	
NRF2/NFE2L2	201146_at	NM_006164	Nuclear factor (erythroid-derived 2)-like 2	2.0	1.8	
PDIA4/ERP70	208658_at	NM_004911	Protein disulfide isomerase related protein		1.4	
PERK/EIF2AK3	218696_at	NM_004836	Eukaryotic translation initiation factor 2-alpha kinase 3	1.4	2.0	
SERP1	200971_s_at	NM_014445	Stress-associated endoplasmic reticulum protein 1	1.3	1.4	
VCP	208649_s_at	NM_007126	Valocin containing protein	1.9	1.6	
XBP1	200670_at	NM_001079539 NM_005080	X-box binding protein 1	2.0	1.8	
Amino acid transport and synthesis						
AARS	201000_at	NM_001605	Alanyl-tRNA synthetase	2.0	2.0	1.6
ASNS	205047_s_at	NM_001673 NM_133436 NM_183356	Asparagine synthetase	6.9	6.0	3.1
CARS	212971_at	NM_001014437	Cysteinyl-tRNA synthetase	2.2	2.0	1.6

		NM_001014438 NM_001751 NM_139273				
EPRS	200843_s_at	NM_004446	Glutamyl-prolyl-tRNA synthetase	1.9	2.0	
GARS	208693_s_at	NM_002047	Glycyl-tRNA synthetase	2.4	2.4	
HARS	202042_at	NM_002109	Histidyl-tRNA synthetase	1.2	1.4	
IARS	204744_s_at	NM_002161 NM_013417	Isoleucine-tRNA synthetase	1.7	1.8	
MARS	213671_s_at	NM_004990	Methionine-tRNA synthetase	2.6	2.5	1.4
NARS	200027_at	NM_004539	Asparaginyl-tRNA synthetase	1.6	1.5	
RARS	201330_at	NM_002887	Arginyl-tRNA synthetase	1.3	1.5	
SARS	200802_at	NM_006513	Seryl-tRNA synthetase	2.4	2.5	1.4
SLC3A2	200924_s_at	NM_001012661 NM_001012662 NM_001012663 NM_001012664 NM_001013251 NM_002394	Solute carrier family 3, member 2	2.6	1.9	2.2
TARS	201263_at	NM_152295	Threonyl-tRNA synthetase	1.5	1.4	
WARS	200629_at	NM_004184 NM_173701 NM_213645 NM_213646	Tryptophanyl-tRNA synthetase	2.1	2.4	1.3
YARS	212048_s_at	NM_003680	Tyrosyl-tRNA synthetase	2.3	2.0	1.4
Chaperones/Protein folding/UPR response						
AHSA1	201491_at	NM_012111	Activator of heat shock 90kDa protein ATPase homolog 1	1.7		
BAG3	217911_s_at	NM_004281	BCL2-associated athanogene 3	9.9	5.4	
C1orf24	217967_s_at	NM_052966	Chromosome 1 open reading frame 24	2.9	6.9	1.7
CANX	200068_s_at	NM_001024649 NM_001746	Calnexin	1.4	1.5	
DNAJA1	200880_at	NM_001539	DnaJ (Hsp40) homolog, subfamily A, member 1	2.2	1.3	
DNAJA3	205963_s_at	NM_005147	DnaJ (Hsp40) homolog, subfamily A, member 3	1.2	1.3	
DNAJB1	200666_s_at	NM_006145	DnaJ (Hsp40) homolog, subfamily B, member B1	8.0	4.1	
DNAJB2	202500_at	NM_001039550 NM_006736	DnaJ (Hsp40) homolog, subfamily B, member B2	2.0	2.0	

DNAJB4	203810_at	NM_007034	DnaJ (Hsp40) homolog, subfamily B, member B4	3.4	2.7	
DNAJB6	208811_s_at	NM_005494 NM_058246	DnaJ (Hsp40) homolog, subfamily B, member B6	1.9	1.3	
DNAJB9	202842_s_at	NM_012328	DnaJ (Hsp40) homolog, subfamily B, member B9	4.4	4.6	1.5
DNAJC1	218409_s_at	NM_022365	DnaJ (Hsp40) homolog, subfamily C, member 1	1.3	1.5	
HMOX1/HSP32	203665_at	NM_002133	Heme oxygenase (decycling) 1	24.0	10.6	5.7
HSF2	209657_s_at	NM_004506	Heat shock transcription factor 2		1.4	
HSP105/HSPH1	206976_s_at	NM_006644	Heat shock 105kDa/110kDa protein 1	4.5	2.7	
HSPA1A/HSPA1B	200800_s_at	NM_005345 NM_005346	Heat shock 70kDa protein 1A/B	17.8	9.8	5.1
HSPA1B	202581_at	NM_005346	Heat shock 70kDa protein 1B	9.8	6.5	3.1
HSPA4L	205543_at	NM_014278	Heat shock 70kDa protein 4-like	3.0	2.0	
HSPA6	117_at	NM_002155	Heat shock 70kDa protein 6	6.7		
HSPA8	210338_s_at	NM_006597 NM_153201	Heat shock 70kDa protein 8	2.2	1.9	
HSPA9B	200691_s_at	NM_004134	Heat shock 70kDa protein 9B	1.7	1.8	
HSPB1	201841_s_at	NM_001540	Heat shock 27kDa protein 1	2.2	1.6	
HSPCA	211969_at	NM_001017963 NM_005348	Heat shock 90kDa protein 1, alpha	1.5	1.5	
HSPCB	214359_s_at	NM_007355	Heat shock 90kDa protein 1, beta	2.2		
HSPD1	200807_s_at	NM_002156 NM_199440	Heat shock 60kDa protein 1	1.4	1.3	
HSPE1	205133_s_at	NM_002157	Heat shock 10kDa protein 1	1.6		
HYOU1	200825_s_at	NM_006389	Hypoxia up-regulated 1	1.6		
PFDN2	218336_at	NM_012394	Prefoldin 2	1.6	1.8	1.4
PFDN4	205361_s_at	NM_002623	Prefoldin 4		1.7	
SEC61B	203133_at	NM_006808	Sec61 beta subunit		1.2	
SEC61G	203484_at	NM_001012456 NM_014302	Sec61 gamma subunit		1.3	
SEC63	201916_s_at	NM_007214	SEC63 homolog (S. cerevisiae)	1.3	1.5	
HSP47/SERPINH1	207714_s_at	NM_001235	Heat shock protein 47	4.4	1.8	
STCH	202557_at	NM_006948	Stress 70 protein chaperone, microsome-associated	2.3	2.3	
STIP1	213330_s_at	NM_006819	Stress-induced-phosphoprotein 1	1.7		
Ubiquitine/Proteasome						

PSMA1	210759_s_at	NM_002786 NM_148976	Proteasome subunit, alpha type, 1	1.5	1.9	1.3
PSMA2	201317_s_at	NM_002787	Proteasome (prosome, macropain) subunit, alpha type, 2		1.4	
PSMA3	201532_at	NM_002788 NM_152132	Proteasome subunit, alpha type, 3	1.3	1.5	
PSMA4	203396_at	NM_002789	Proteasome subunit, alpha type, 4		1.4	
PSMA5	201274_at	NM_002790	Proteasome subunit, alpha type, 5	1.3	1.6	
PSMA7	216088_s_at	NM_002792	Proteasome subunit, alpha type, 7	1.4	1.6	
PSMB1	200876_s_at	NM_002793	Proteasome subunit, beta type, 1	1.3	1.5	
PSMB10	202659_at	NM_002801	Proteasome (prosome, macropain) subunit, beta type, 10	-1.5	-1.5	
PSMB2	200039_s_at	NM_002794	Proteasome subunit, beta type, 2	1.4	1.9	1.3
PSMB3	201400_at	NM_002795	Proteasome (prosome, macropain) subunit, beta type, 3		1.3	
PSMB4	202244_at	NM_002796	Proteasome subunit, beta type, 4	1.5	2.0	1.4
PSMB5	208799_at	NM_002797	Proteasome subunit, beta type, 5	1.4	1.7	
PSMB7	200786_at	NM_002799	Proteasome subunit, beta type, 7	1.5	2.0	1.5
PSMB8	209040_s_at	NM_004159 NM_148919	Proteasome (prosome, macropain) subunit, beta type, 8	-1.6	-2.2	-1.3
PSMB9	204279_at	NM_002800 NM_148954	Proteasome (prosome, macropain) subunit, beta type, 9	-1.4	-1.6	-1.5
PSMC1	204219_s_at	NM_002802	Proteasome 26S subunit, ATPase, 1	1.9	2.1	
PSMC2	201068_s_at	NM_002803	Proteasome 26S subunit, ATPase, 2	1.9	2.2	
PSMC4	201252_at	NM_006503 NM_153001	Proteasome 26S subunit, ATPase, 4	1.8	2.6	1.3
PSMC5	209503_s_at	NM_002805	Proteasome 26S subunit, ATPase, 5	1.2	1.6	
PSMC6	201699_at	NM_002806	Proteasome 26S subunit, ATPase, 6	1.8	2.2	
PSMD1	201198_s_at	NM_002807	Proteasome 26S subunit, non ATPase, 1	2.2	2.2	
PSMD11	208777_s_at	NM_002815	Proteasome 26S subunit, non ATPase, 11	1.9	2.0	
PSMD12	202352_s_at	NM_002816	Proteasome 26S subunit, non ATPase, 12	2.1	2.7	1.3
PSMD13	201232_s_at	NM_002817 NM_175932	Proteasome 26S subunit, non ATPase, 13	1.8	1.7	
PSMD14	212296_at	NM_005805	Proteasome 26S subunit, non ATPase, 14	1.9	2.2	
PSMD2	200830_at	NM_002808	Proteasome 26S subunit, non ATPase, 2	1.7	2.8	1.6
PSMD4	200882_s_at	NM_002810 NM_153822	Proteasome 26S subunit, non ATPase, 4	1.5	1.8	
PSMD7	201705_at	NM_002811	Proteasome 26S subunit, non ATPase, 7	1.7	1.9	

PSMD8	200820_at	NM_002812	Proteasome 26S subunit, non ATPase, 8		1.4	1.4
PSMD9	207805_s_at	NM_002813	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 9		1.2	
PSME1	200814_at	NM_006263 NM_176783	Proteasome (prosome, macropain) activator subunit 1	-1.2	-1.5	-1.3
PSME2	201762_s_at	NM_002818	Proteasome (prosome, macropain) activator subunit 2	-1.3	-1.4	
SQSTM1 (P62)	213112_s_at	NM_003900	Sequestosome 1, ubiquitin-binding protein P62	7.7	6.7	5.0
SQSTM1 (P62)	201471_s_at	NM_003900	Sequestosome 1, ubiquitin-binding protein P62	6.7	7.3	3.9
UBB	200633_at	NM_018955	Ubiquitin B	1.6	1.3	
$Ca2^+$ homeostasis						
ATP2B4/PMCA4	212136_at	NM_001001396 NM_001684	ATPase, Ca++ transporting, plasma membrane 4		1.7	
ATP2C1	212255_s_at	NM_014382 NM_001001486 NM_001001487 NM_014382	ATPase, Ca++ transporting, type 2C, member 1	1.4	1.4	
CALM1	200655_s_at	NM_006888	Calmodulin 1	-2.2	-1.6	
CALM2	207243_s_at	NM_001743	Calmodulin 2 (phosphorylase kinase, delta)	-1.3		
CALM3	200623_s_at	NM_005184	Calmodulin 3	-1.7	-1.6	
CALU	200757_s_at	NM_001219	Calumenin	1.3	1.6	
CAMLG	203538_at	NM_001745	Calcium modulating ligand	1.9	1.9	
CAPN2	208683_at	NM_001748	Calpain 2, large subunit	1.3	1.8	1.4
CAPN7	203356_at	NM_014296	Calpain 7		1.5	
CAPNS1	200001_at	NM_001003962 NM_001749	Calpain, small subunit 1		-1.7	
DGKG	206395_at	NM_001080744 NM_001080745 NM_001346	Diacylglycerol kinase, gamma 90kDa	2.2	1.9	
IP3R1	203710_at	NM_002222	Inositol 1,4,5-triphosphate receptor, type 1	1.5	2.2	1.4
IP3R3	201189_s_at	NM_002224	Inositol 1,4,5-triphosphate receptor, type 3			1.3
PARD3	210094_s_at	NM_019619	Par-3 partitioning defective 3 homolog (C. elegans)	1.6	1.6	1.3
PIK3C3	204297_at	NM_002647	Phosphoinositide-3-kinase, class 3	1.6	1.7	
PIK3CD	203879_at	NM_005026	Phosphoinositide-3-kinase, catalytic, delta polypeptide	1.4	1.7	1.3
PIK4CB	206139_at	NM_002651	Phosphatidylinositol 4-kinase, catalytic, beta polypeptide		1.3	
S100A11	200660_at	NM_005620	S100 calcium binding protein A11 (calgizzarin)		1.4	1.6

S100A11	208540_x_at	NM_021039	S100 calcium binding protein A11 (calgizzarin)		1.4	1.5
S100A14	218677_at	NM_020672	S100 calcium binding protein A14	1.5	1.4	
S100P	204351_at	NM_005980	S100 calcium binding protein P	5.9	6.5	3.4
STC2	203439_s_at	NM_003714	Stanniocalcin 2	2.8	2.1	1.7
Antioxidants/oxidative stress						
ANGPTL4	221009_s_at	NM_001039667 NM_139314	Angiopoietin-like 4		1.4	1.5
BLVRB	202201_at	NM_000713	Biliverdin reductase B (flavin reductase (NADPH))	1.9	3.2	2.4
CAT	201432_at	NM_001752	Catalase		-1.4	
DUSP1	201041_s_at	NM_004417	Dual specificity phosphatase 1	3.4	7.1	
FTH1	200748_s_at	NM_002032	Ferritin, heavy polypeptide 1	1.8	2.0	2.2
GADD45A	203725_at	NM_001924	Growth arrest and DNA-damage-inducible, alpha	1.7	1.9	
GADD45B	207574_s_at	NM_015675	Growth arrest and DNA-damage-inducible, beta	2.0	2.8	2.4
GCLC	202922_at	NM_001498	Glutamate-cysteine ligase, catalytic subunit	1.6	1.3	
GCLM	203925_at	NM_002061	Glutamate-cysteine ligase, modifier subunit	3.7	3.5	2.0
GSTK1	217751_at	NM_015917	Glutathione S-transferase kappa 1	-1.6	-1.7	
HMOX2	218120_s_at	NM_002134	Heme oxygenase (decycling) 2	-1.5	-1.4	
MICA	205904_at	NM_000247	MHC class I polypeptide-related sequence A	1.9	1.8	
MICB	206247_at	NM_005931	MHC class I polypeptide-related sequence B	1.8	1.5	
OXSR1	202696_at	NM_005109	Oxidative-stress responsive 1	1.4	1.4	
SOD1	200642_at	NM_000454	Superoxide dismutase 1	1.5	1.6	
TXNRD1	201266_at	NM_003330 NM_182729 NM_182742 NM_182743	Thioredoxin reductase 1	3.2	2.9	1.9
Cell cycle/Apoptosis						
BAD	1861_at	NM_004322 NM_032989	BCL2-antagonist of cell death		-1.5	
BAG3	217911_s_at	NM_004281	BCL2-associated athanogene 3	9.9	5.4	
BID	204493_at	NM_001196 NM_197966 NM_197967	BH3 interacting domain death agonist	1.3		

BIK	205780_at	NM_001197	BCL2-interacting killer (apoptosis-inducing)	-1.6	-1.8	-1.4
CASP4	209310_s_at	NM_001225 NM_033306 NM_033307	Caspase 4	1.6	2.9	
CASP7	207181_s_at	NM_001227 NM_033338 NM_033339 NM_033340	Caspase 7	1.6	2.1	
CCND1	208712_at	NM_053056	Cyclin D1	-1.7	-2.0	
TRIB3	218145_at	NM_021158	Tribbles homolog 3 (Drosophila)	7.4	6.5	3.3
VEGF	210512_s_at	NM_001025366 NM_001025367 NM_001025368 NM_001025369 NM_001025370 NM_001033756 NM_0033	Vascular endothelial growth factor	5.0	4.2	2.5
Cholesterol biosynthesis, uptake, metabolism and transport						
CAV1	203065_s_at	NM_001753	Caveolin 1, caveolae protein, 22kDa	-1.5	-1.4	
CYB5R3	201885_s_at	NM_000398 NM_007	Cytochrome b5 reductase 3	-1.5	-1.3	
DHCR24	200862_at	NM_014762	24-dehydrocholesterol reductase	-1.6	-1.7	
DHCR7	201791_s_at	NM_001360	7-dehydrocholesterol reductase	-1.6	-1.5	
FDPS	201275_at	NM_002004	Farnesyl diphosphate synthase	-1.3	-1.2	
HMGCR	202539_s_at	NM_000859	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	NC	NC	NC
HMGCS1	221750_at	NM_002130 NM_001098272	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)		1.5	
HSD17B1	205829_at	NM_000413	Hydroxysteroid (17-beta) dehydrogenase 1	-1.3	-1.3	1.3
HSD17B1	204360_s_at	NM_000263	Hydroxysteroid (17-beta) dehydrogenase 1	-1.2	-1.3	
HSD17B4	201413_at	NM_000414	Hydroxysteroid (17-beta) dehydrogenase 4		1.6	
LDLR	202068_s_at	NM_000527	Low density lipoprotein receptor	2.4	2.4	
LSS	202245_at	NM_002340	Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	-1.3		
NPC1	202679_at	NM_000271	Niemann-Pick disease, type C1	3.0	4.5	1.9
NPC2	200701_at	NM_006432	Niemann-Pick disease, type C2		1.5	1.5

OSBP	201800_s_at	NM_002556	Oxysterol binding protein	1.4	1.4	
PMVK	203515_s_at	NM_006556	Phosphomevalonate kinase	-1.3	-1.8	
SC4MOL	209146_at	NM_001017369 NM_006745	Sterol-C4-methyl oxidase-like	2.0	2.3	
SREBP1	202308_at	NM_001005291 NM_004176	Sterol regulatory element binding protein 1		-1.6	-1.4
SREBP2	201247_at	NM_004599	Sterol regulatory element binding protein 2	NC	NC	NC
TM7SF2	210130_s_at	NM_003273	Transmembrane 7 superfamily member 2	-1.4	-1.9	
VLDLR	209822_s_at	NM_001018056 NM_003383	Very low density lipoprotein receptor	1.6	1.6	
WWOX	219077_s_at	NM_016373 NM_130791 NM_130844	WW domain containing oxidoreductase		-1.5	-1.7

* NC means no change

Paper II

Is not included due to copyright

Paper III

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Errata

Errata list

Page 3 (line 5)
Current sentence:
ATF activation transcription factor
Corrected to:
ATF activating transcription factor

Page 5 (line 5)
Current sentence:
PPAR peroxisome proliferators-activated receptor
Corrected to:
PPAR peroxisome proliferator-activated receptor

Page 6 (line 3)
Current sentence:
…the Norwegian professor Notevarp
Corrected to:
…the Norwegian Professor Notevarp

Page 10 (chapter 1.2, line 8)
Current sentence:
The type of fat consumed has changed towards an increase in saturated fat (especially animal fat and n-6 PUFA rich oils)…
Corrected to:
The type of fat consumed has changed towards an increase in saturated fat (especially animal fat) and n-6 PUFA rich oils…

Page 11 (line 9)
Current sentence:
Feeding farm animals with n-3 FA-enriched food have resulted in production of eggs and milk with increased n-3 FA content…
Corrected to:
Feeding farm animals with n-3 FA-enriched food has resulted in production of eggs and milk with increased n-3 FA content…

Page 11 (line 8)
Current sentences:
Cooking methods also needs to be considered since n-3 PUFAs are highly oxidable resulting in harmful free radicals. The n-3 PUFA content in cooked fish might be reduced by as much as 50% (reviewed in 14),
Corrected to:
The sentences have been moved to the end of the paragraph.

Page 11 (line 9)
Current sentence:
The n-3 PUFA content in cooked fish might be reduced by as much as 50% (reviewed in 14), and industrial food should be prepared using n-3 PUFA rich oils.
Corrected to:
Industrial food should be prepared using n-3 PUFA rich oils.

Page 15 (line 1)
Current sentence:

Membrains also contain lipid micro domains such as lipid rafts and caveolae which are membrane domains rich in cholesterol, sphingolipids and phospholipids.
Corrected to:
Membrains also contain lipid micro domains such as lipid rafts and caveolae which are membrane domains rich in cholesterol, sphingolipids and phospholipids with saturated fatty acyl chains.

Page 15 (chapter 1.3.5, line 6)
Current sentence:
EPA and AA are metabolized by the same enzymes in the three eicosanoid syntehesis pathways…
Corrected to:
EPA and AA are metabolized by the same enzymes in the three eicosanoid synthesis pathways…

Page 19 (line 5)
Current sentence:
…(reviewed in (50).
Corrected to:
…(reviewed in (50)).

Page 19 (chapter 1.4.1, line 5)
Current sentence:
…found be important…
Corrected to:
…found to be important

Page 20 (last sentence)
Current sentence:
This is especially important for breast and prostate cancer since the incidence of these cancers has been associated with exposition of such pesticides.
Corrected to:
This is especially important for breast and prostate cancer since the incidence of these cancers has been associated with exposure of such pesticides.

Page 24 (line 7)
Current sentence:
Some human intervention studies have also been performed, like the studies by Anti, West and Aronson *et al* described above.
Corrected to:
Some human intervention studies have also been performed, like the studies by Anti and West *et al* described above.

Page 26 (second paragraph, line 3)
Current sentence:
DHA and EPA reduced micro vessel formation in HT-29 colon cancer cells in vitro, as a result of reduced expression of COX-2 and vascular endothelial growth factor (VEGF), and reduced PGE2 level.
Corrected to:
DHA and EPA reduced the growth of HT-29 colon cancer cells in vitro and reduced expression of COX-2, vascular endothelial growth factor (VEGF) and reduced PGE_2 level.

Page 27 (chapter 1.5.3, last line)
Current sentence:
…(EGF) signaling which to takes place in lipid rafts.
Corrected to:
…(EGF) signaling which takes place in lipid rafts.

Page 32 (PERK chapter, line 3)
Current sentence:
This leads to inhibition of translation/protein synthesis to relive the protein burden of ER.
Corrected to:
This leads to inhibition of translation/protein synthesis to relieve the protein burden of ER.

Page 39 (chapter Apoptosis and n-3 PUFAs, last line)
Current sentence:
…(reviewed in (122).
Corrected to:
…(reviewed in (122)).

Page 41 (line 9)
Current sentence:
…the cause have not yet been fully established.
Corrected to:
…the mechanisms have not yet been fully established.

Page 43 (line 4)
Current sentence:
Transcripts for some proteins involved in cholesterol uptake and transport like LDL receptor (LDLR) and niemann-pick disease type C1 (NPC1), the latter also at protein level.
Corrected to:
Transcripts for some proteins involved in cholesterol uptake and transport like LDL receptor (LDLR) and niemann-pick disease type C1 (NPC1) were up-regulated, the latter also at protein level.

Page 43 (line 9)
Current sentence:
Taken together, these data imply that the growth inhibitiing effect of DHA on the SW620 cells involves induction of ER stress, UPR and changes in Ca^{2+}- and cholesterol homeostasis
Corrected to:
Taken together, these data imply that the growth inhibiting effect of DHA on the SW620 cells involves induction of ER stress, UPR and changes in Ca^{2+}- and cholesterol homeostasis

Page 46 (last line)
Current sentence:
…induced extensive changes in the expression several genes belonging to a several different molecular pathways, in cancer cells.
Corrected to:
…induced extensive changes in the expression of several genes belonging to several different molecular pathways, in cancer cells.

Page 48 (line 24)
Current sentence:
…hence oxidative stress was suggested to be involved in trigging cell death in these cells.
Corrected to:
…hence oxidative stress was suggested to be involved in triggering cell death in these cells.

Page 50 (line 3)
Current sentence:
…have been implicated in ER stressed-induced apoptosis
Corrected to:

…have been implicated in ER stress-induced apoptosis

Page 53 (chapter 4.4, line 5)
Current sentence:
…(paper I, table 1, fig. 5)
Corrected to:
…(paper I, table1, supplementary table 1, fig. 5)

Page 54 (line 15)
Current sentence:
SREBP1 also regulates lipogenesis e.g. trough induction of FAS…
Corrected to:
SREBP1 also regulates lipogenesis e.g. through induction of FAS…

Page 56 (4th line from the bottom)
Current sentence:
It is important to note that Chen et al used the double DHA concentration…
Corrected to:
It is important to note that Chen et al used double the DHA concentration…

Page 58 (line 6)
Current sentence:
Unfortunately, several chemotherapeutics is known to activate NFκB…
Corrected to:
Unfortunately, several chemotherapies are known to activate NFκB…

Page 60 (6th line from the bottom)
Current sentence:
This could help revealing…
Corrected to:
This could help reveal…

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Medicine, NTNU

Dissertations at the Faculty of Medicine, NTNU

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1987

27. Per Martin Kleveland: STUDIES ON GASTRIN.
28. Audun N. Øksendal: THE CALCIUM PARADOX AND THE HEART.
29. Vilhjalmur R. Finsen: HIP FRACTURES

1988

30. Rigmor Austgulen: TUMOR NECROSIS FACTOR: A MONOCYTE-DERIVED REGULATOR OF CELLULAR GROWTH.
31. Tom-Harald Edna: HEAD INJURIES ADMITTED TO HOSPITAL.
32. Joseph D. Borsi: NEW ASPECTS OF THE CLINICAL PHARMACOKINETICS OF METHOTREXATE.
33. Olav F. M. Sellevold: GLUCOCORTICOIDS IN MYOCARDIAL PROTECTION.
34. Terje Skjærpe: NONINVASIVE QUANTITATION OF GLOBAL PARAMETERS ON LEFT VENTRICULAR FUNCTION: THE SYSTOLIC PULMONARY ARTERY PRESSURE AND CARDIAC OUTPUT.
35. Eyvind Rødahl: STUDIES OF IMMUNE COMPLEXES AND RETROVIRUS-LIKE ANTIGENS IN PATIENTS WITH ANKYLOSING SPONDYLITIS.
36. Ketil Thorstensen: STUDIES ON THE MECHANISMS OF CELLULAR UPTAKE OF IRON FROM TRANSFERRIN.
37. Anna Midelfart: STUDIES OF THE MECHANISMS OF ION AND FLUID TRANSPORT IN THE BOVINE CORNEA.
38. Eirik Helseth: GROWTH AND PLASMINOGEN ACTIVATOR ACTIVITY OF HUMAN GLIOMAS AND BRAIN METASTASES - WITH SPECIAL REFERENCE TO TRANSFORMING GROWTH FACTOR BETA AND THE EPIDERMAL GROWTH FACTOR RECEPTOR.
39. Petter C. Borchgrevink: MAGNESIUM AND THE ISCHEMIC HEART.
40. Kjell-Arne Rein: THE EFFECT OF EXTRACORPOREAL CIRCULATION ON SUBCUTANEOUS TRANSCAPILLARY FLUID BALANCE.
41. Arne Kristian Sandvik: RAT GASTRIC HISTAMINE.
42. Carl Bredo Dahl: ANIMAL MODELS IN PSYCHIATRY.

1989

43. Torbjørn A. Fredriksen: CERVICOGENIC HEADACHE.
44. Rolf A. Walstad: CEFTAZIDIME.
45. Rolf Salvesen: THE PUPIL IN CLUSTER HEADACHE.
46. Nils Petter Jørgensen: DRUG EXPOSURE IN EARLY PREGNANCY.
47. Johan C. Ræder: PREMEDICATION AND GENERAL ANAESTHESIA IN OUTPATIENT GYNECOLOGICAL SURGERY.
48. M. R. Shalaby: IMMUNOREGULATORY PROPERTIES OF TNF-α AND THE RELATED CYTOKINES.
49. Anders Waage: THE COMPLEX PATTERN OF CYTOKINES IN SEPTIC SHOCK.
50. Bjarne Christian Eriksen: ELECTROSTIMULATION OF THE PELVIC FLOOR IN FEMALE URINARY INCONTINENCE.
51. Tore B. Halvorsen: PROGNOSTIC FACTORS IN COLORECTAL CANCER.

1990

52. Asbjørn Nordby: CELLULAR TOXICITY OF ROENTGEN CONTRAST MEDIA.
53. Kåre E. Tvedt: X-RAY MICROANALYSIS OF BIOLOGICAL MATERIAL.
54. Tore C. Stiles: COGNITIVE VULNERABILITY FACTORS IN THE DEVELOPMENT AND MAINTENANCE OF DEPRESSION.
55. Eva Hofsli: TUMOR NECROSIS FACTOR AND MULTIDRUG RESISTANCE.
56. Helge S. Haarstad: TROPHIC EFFECTS OF CHOLECYSTOKININ AND SECRETIN ON THE RAT PANCREAS.
57. Lars Engebretsen: TREATMENT OF ACUTE ANTERIOR CRUCIATE LIGAMENT INJURIES.
58. Tarjei Rygnestad: DELIBERATE SELF-POISONING IN TRONDHEIM.
59. Arne Z. Henriksen: STUDIES ON CONSERVED ANTIGENIC DOMAINS ON MAJOR OUTER MEMBRANE PROTEINS FROM ENTEROBACTERIA.
60. Steinar Westin: UNEMPLOYMENT AND HEALTH: Medical and social consequences of a factory closure in a ten-year controlled follow-up study.
61. Ylva Sahlin: INJURY REGISTRATION, a tool for accident preventive work.
62. Helge Bjørnstad Pettersen: BIOSYNTHESIS OF COMPLEMENT BY HUMAN ALVEOLAR MACROPHAGES WITH SPECIAL REFERENCE TO SARCOIDOSIS.
63. Berit Schei: TRAPPED IN PAINFUL LOVE.
64. Lars J. Vatten: PROSPECTIVE STUDIES OF THE RISK OF BREAST CANCER IN A COHORT OF NORWEGIAN WOMAN.

1991

65. Kåre Bergh: APPLICATIONS OF ANTI-C5a SPECIFIC MONOCLONAL ANTIBODIES FOR THE ASSESSMENT OF COMPLEMENT ACTIVATION.
66. Svein Svenningsen: THE CLINICAL SIGNIFICANCE OF INCREASED FEMORAL ANTEVERSION.
67. Olbjørn Klepp: NONSEMINOMATOUS GERM CELL TESTIS CANCER: THERAPEUTIC OUTCOME AND PROGNOSTIC FACTORS.
68. Trond Sand: THE EFFECTS OF CLICK POLARITY ON BRAINSTEM AUDITORY EVOKED POTENTIALS AMPLITUDE, DISPERSION, AND LATENCY VARIABLES.
69. Kjetil B. Åsbakk: STUDIES OF A PROTEIN FROM PSORIATIC SCALE, PSO P27, WITH RESPECT TO ITS POTENTIAL ROLE IN IMMUNE REACTIONS IN PSORIASIS.
70. Arnulf Hestnes: STUDIES ON DOWN´S SYNDROME.
71. Randi Nygaard: LONG-TERM SURVIVAL IN CHILDHOOD LEUKEMIA.
72. Bjørn Hagen: THIO-TEPA.
73. Svein Anda: EVALUATION OF THE HIP JOINT BY COMPUTED TOMOGRAMPHY AND ULTRASONOGRAPHY.

1992

74. Martin Svartberg: AN INVESTIGATION OF PROCESS AND OUTCOME OF SHORT-TERM PSYCHODYNAMIC PSYCHOTHERAPY.
75. Stig Arild Slørdahl: AORTIC REGURGITATION.
76. Harold C Sexton: STUDIES RELATING TO THE TREATMENT OF SYMPTOMATIC NON-PSYCHOTIC PATIENTS.
77. Maurice B. Vincent: VASOACTIVE PEPTIDES IN THE OCULAR/FOREHEAD AREA.
78. Terje Johannessen: CONTROLLED TRIALS IN SINGLE SUBJECTS.
79. Turid Nilsen: PYROPHOSPHATE IN HEPATOCYTE IRON METABOLISM.
80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

1993

82. Gunnar Bovim: CERVICOGENIC HEADACHE.
83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
85. Rune Wiseth: AORTIC VALVE REPLACEMENT.
86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
89. Anne Vik: VASCULAR GAS EMBOLISM DURING AIR INFUSION AND AFTER DECOMPRESSION IN PIGS.
90. Lars Jacob Stovner: THE CHIARI TYPE I MALFORMATION.
91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

1994

92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
97. Bjørn Backe: STUDIES IN ANTENATAL CARE.
98. Gerd Inger Ringdal: QUALITY OF LIFE IN CANCER PATIENTS.
99. Torvid Kiserud: THE DUCTUS VENOSUS IN THE HUMAN FETUS.
100. Hans E. Fjøsne: HORMONAL REGULATION OF PROSTATIC METABOLISM.
101. Eylert Brodtkorb: CLINICAL ASPECTS OF EPILEPSY IN THE MENTALLY RETARDED.
102. Roar Juul: PEPTIDERGIC MECHANISMS IN HUMAN SUBARACHNOID HEMORRHAGE.
103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

1995

104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
107. Finn Egil Skjeldestad: INDUCED ABORTION: Timetrends and Determinants.
108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.
109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

1996

110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANCER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

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124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs

1998

132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.

133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.
134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.
139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

1999

141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

2000

158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
159. xxxxxxxxx (blind number)
160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.

162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.
168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT

192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM
198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.

217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
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