N-3 FATTY ACIDS, LIPID METABOLISM AND CANCER

N-3 VETZUREN, VETSTOFWISSELING EN KANKER

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Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam op gezag van de
rector magnificus
Prof.dr. P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 23 februari 2000 om 13.45 uur

door

Sonja Donate Zuijdgeest-van Leeuwen

geboren te Delft

Promotiecommissie

Promotor:	Prof. J.H.P. Wilson
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Financial support of this research project by Numico Research B.V. is gratefully acknowledged.

n-3 Fatty acids, lipid metabolism and cancer

Thesis Erasmus University Rotterdam, Rotterdam, The Netherlands
ISBN: 90-73235-18-9
Cover: 'Message from the Sea'
Cover photo: Wim van der Burgh, Delft
Printed by Optima Grafische Communicatie, Rotterdam

We thank Numico Research B.V., Nutricia Nederland B.V. and Croda GmbH for providing financial support for printing this thesis.

Ter nagedachtenis aan mijn opa's

Contents

List of abbreviations

ACTH	adrenocorticotropic hormone
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
BMI	body mass index
BW	body weight
CE	cholesteryl esters
CHD	coronary heart disease
CRP	C-reactive protein
DHA	docosahexaenoic acid (22:6n-3)
EE	ethyl ester
EPA	eicosapentaenoic acid (20:5n-3)
FFA	free fatty acid
FFM	fat-free mass
FM	fat mass
GI	gastrointestinal
HDL	high-density lipoprotein
HSL	hormone-sensitive lipase
IBW	ideal body weight
LDL	low-density lipoprotein
LMF	lipid-mobilizing factor
LPL	lipoprotein lipase
LT	leukotrienes
MTBSTFA	N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide
NIDDM	non-insulin dependent diabetes mellitus
OA	oleic acid (18:1n-9)
PG	prostaglandins
PIF	proteolysis-inducing factor
PL	phospholipids
R_a	rate of appearance
REE	resting energy expenditure
RQ	respiratory quotient
TAG	triacylglycerol
TX	thromboxanes
VLDL	very-low-density lipoprotein

1

INTRODUCTION

Background

Lipid metabolism in healthy subjects

n-3 Fatty acid metabolism

Cancer cachexia: etiology and potential treatments

Aims

Outline of the thesis

Background

Cachexia is a frequent problem in cancer patients which is characterized by weight loss, impaired performance and fatigue.[75,127] In general, administration of nutritional support does not improve the condition[155] nor is any other adequate treatment available.[127] The mechanisms underlying cancer cachexia are poorly understood.[79] Factors that may play a role in the etiology of weight loss include increased lipolysis from adipose tissue,[212,213] and increased proteolysis in muscle.[130] The potential role of elevated lipolysis in the occurrence of weight loss in cancer is supported by the recent isolation of a lipid-mobilizing factor (LMF) from urine of weight-losing cancer patients,[97,219] and by the observation of increased whole-body lipolysis observed in cancer patients.[124,192]

Animal studies have shown that weight loss in tumor-bearing mice was effectively attenuated by n-3 fatty acids derived from fish oil,[214] of which eicosapentaenoic acid (EPA; 20:5n-3) proved to be the active component.[213] Since EPA was found to inhibit lipolysis and proteolysis *in vitro*,[21,212,213] inhibition of lipolysis and/or proteolysis were assumed to play a role in these beneficial effects *in vivo*. Furthermore, EPA inhibited tumor growth in these animals.[21,213] Recent uncontrolled clinical studies suggest that EPA supplementation may reverse weight loss in patients with pancreatic cancer as well.[17,230] It is, however, not yet known whether inhibition of lipolysis and/or proteolysis plays a role in the effects of EPA in cancer. In the present thesis, the results of several studies on the effects of EPA on lipid metabolism in cancer patients and healthy subjects will be presented.

In this introductory chapter, background information is provided. In the first part of this chapter, the nomenclature of lipids is explained, and the types of lipids, the process of intestinal absorption of fat, the transport of lipids throughout the body, and mechanisms regulating lipolysis are reviewed. In the second part, literature findings regarding n-3 fatty acids in epidemiological studies are briefly reviewed, metabolic pathways of n-3 and n-6 fatty acids are explained, and the effects of n-3 fatty acids on lipid metabolism and the inflammatory response are reviewed. In the third and last part of this chapter, different aspects of cancer cachexia are discussed such as changes in body composition, underlying mechanisms of weight loss with special reference to metabolic abnormalities, and potential treatments of cancer cachexia. Finally, the aims of the current studies and the outline of this thesis are presented.

Lipid metabolism in healthy subjects

Lipids[139]

Although lipids fulfill multiple functions in the body, two main functions can be recognized. At first, lipids are an important component of cellular membranes, which are layers with highly selective permeability that regulate the passage of materials into and out of the cell. The second important function is the storage of body lipids in adipose tissue, as an energy source via oxidation pathways. Lipids exist in many different forms, and are characterized by their a-polar character. A subgroup of this large group of compounds is formed by lipids containing fatty acids, often in combination with one or two other molecules such as glycerol or cholesterol. Fatty acids consist of a polar, hydrophilic acid group and a hydrophobic tail which may be saturated, or unsaturated with double bonds. The specific characteristics of fatty acids are determined by the length of the carbon chain, the number of double bonds, and the location of the first double bond within the chain. The nomenclature of fatty acids is based on these characteristics: the number before the colon indicates the total number of carbon atoms, whereas the number after the colon represents the number of double bonds. The number behind the 'n' indicates the position of the first double bond seen from the methyl end of the chain. According to this notation, palmitic acid is indicated as 16:0, with 16 carbon atoms and no double bonds. Eicosapentaenoic acid (EPA) is indicated as 20:5n-3 due to the 20 carbon atoms, 5 double bonds, and the first double bond starting at carbon 3 from the methyl end of the chain (Figure 1.1). Another method to indicate the location of the double bond, is the so-called 'Δ'-notation. In this notation, the location is determined from the carboxyl-end instead of the methyl end of the chain.

Besides the saturated fatty acids which are mainly present in mammals, three main families of unsaturated fatty acids exist: the n-9 fatty acids which are found in mammals as

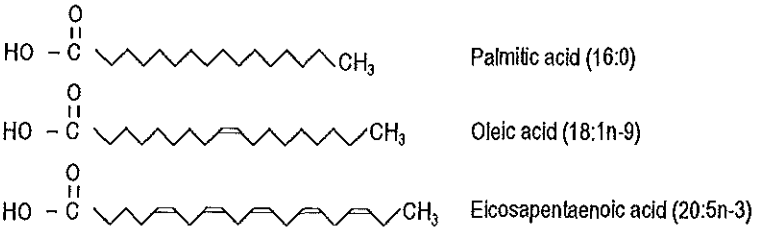

Figure 1.1

Molecular structure of palmitic acid (16:0), oleic acid (18:1n-9) and eicosapentaenoic acid (20:5n-3).

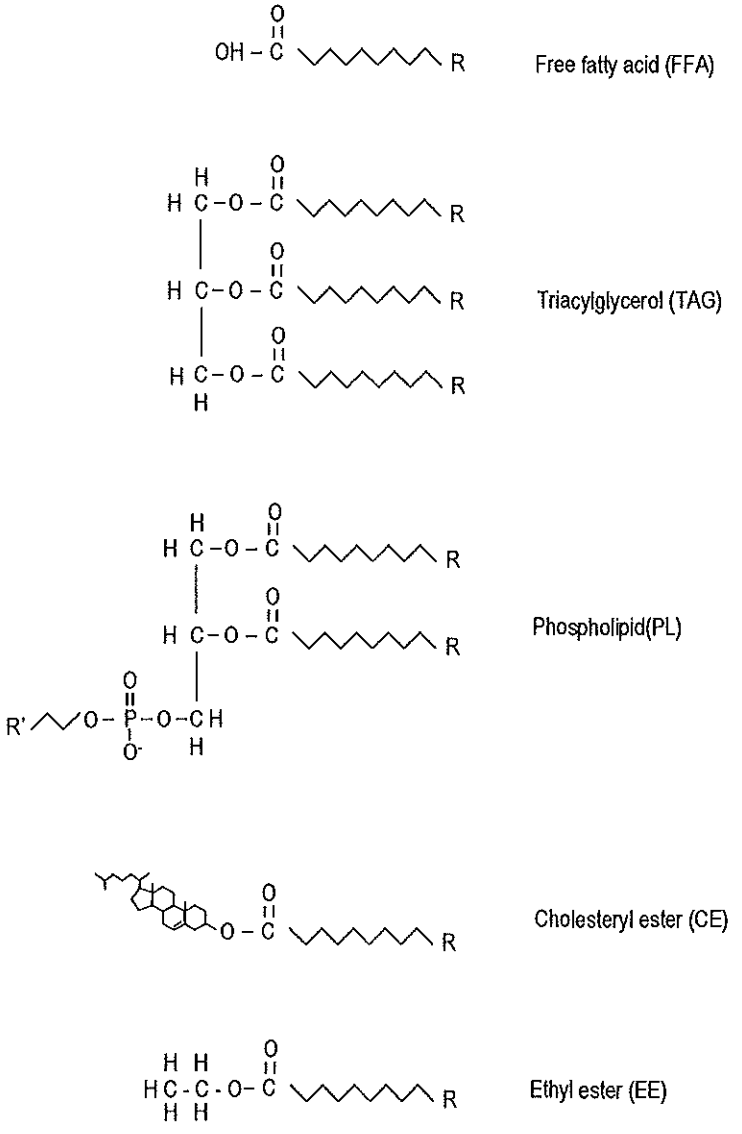

Figure 1.2
Molecular structure of triacylglycerols (TAG), phospholipids (PL), cholesteryl esters (CE), free fatty acids (FFA) and ethyl esters (EE).

well as in plants, the n-6 fatty acids which are mainly found in vegetables and plants, and the n-3 fatty acids which are mainly found in marine oils derived from fatty fish and algae.

During life, fatty acids can be obtained from the diet, or they can be synthesized within the body. Since human enzyme systems are not able to introduce new double bonds beyond Δ9, fatty acids from the n-9, n-6 and n-3 families are not metabolically interconvertible in mammals. Consequently, the fatty acids 18:2n-6 (linoleic acid) and 18:3n-3 (α-linolenic acid) are called essential fatty acids and must therefore be provided by the diet. Within the body, elongation and desaturation of fatty acids takes place to form various long-chain polyunsaturated fatty acids for specific functions.

Different types of lipids[139,187]

Within the human body, different types of lipids have distinct physiological functions (Figure 1.2). Triacylglycerols (TAG) consist of three fatty acids esterified to a glycerol backbone and are mainly used for fat storage within the adipose tissue. After hydrolization of TAG, free fatty acids (FFA) are released into the circulation which can serve as an energy substrate for most cells. Phospholipids (PL), which are also called glycerophospholipids, consist of a glycerol backbone containing two fatty acids and one phosphoric acid group linked to a polar molecule. PL are, together with cholesterol, the major lipid components of cell membranes. Cholesteryl esters (CE) are fatty acids esterified to cholesterol. CE are mainly found in lipoproteins in blood which provide for the transport of cholesterol throughout the body. Whether fatty acids are incorporated into TAG, PL or CE, depends on local lipid concentrations and enzyme activities, but also on chain length and degree of saturation. Cellular membranes are mainly composed of long-chain polyunsaturated fatty acids to ensure membrane fluidity, whereas fat stored in adipose tissue contains relatively high proportions of saturated fatty acids. A type of fatty acid esterification that does not naturally occur but which is used in oral supplements to supply specific fatty acids in relatively higher doses, are the ethyl esters (EE). These synthetic preparations exist of fatty acids esterified to an ethyl group. Some advantages of EE over naturally occurring TAG mixtures are that specific fatty acids can be administered in high concentrations without interference with other components or fatty acids, and that the amount of oil to be taken can be reduced.

Intestinal lipid absorption[2,187]

After ingestion of dietary fat, large lipid droplets within the intestine are emulsified by bile salts, which results in lipid droplets of about 1 μm in diameter. Lipids on the surface of these micelles are hydrolyzed by pancreatic lipase, resulting in free fatty acids, 2-monoacylglycerides, and glycerol. CE are broken down by pancreatic hydrolase into free cholesterol and fatty acids. After formation of so-called mixed micelles (structures of 50 to

100 nm in diameter), the free fatty acids, glycerol and 2-monoacylglycerides diffuse through the cell membranes into the epithelial cells. Within the epithelial cells, TAG, CE and PL are resynthesized and incorporated into droplets called chylomicrons. The core of these chylomicrons consists of TAG, CE and cholesterol, whereas PL and apolipoproteins are found on the outside. Chylomicrons are released from the cell into the extracellular fluid, and are subsequently transported to the systemic venous circulation via the lymph vessels and the main lymphatic duct. Short-chain fatty acids are not reesterified within the enterocyte, but are either transported via the portal vein to be reesterified into TAG within the liver, or directly oxidized. A similar pathway is observed for fatty acids derived from EE, which are also directly transported to the liver as albumin-bound free acids instead of being reesterified within the cell. As a consequence, only a minor part of the fatty acids derived from EE is incorporated into chylomicrons. In contrast with fatty acids supplied as TAG, chain length does not appear to affect the route of absorption for fatty acids from EE.

Transport of lipids[187]

TAG-containing chylomicrons within the circulation are taken up by the liver. After metabolization and reesterification, TAG constitute the basic components of very-low-density lipoproteins (VLDL) together with TAG formed from free acids. VLDL is released into the circulation to deliver TAG to the peripheral tissues. At the surface of peripheral endothelial cells, VLDL-TAG are hydrolyzed by lipoprotein lipase into glycerol and free fatty acids which are taken up by the cells. After fatty acid uptake by adipocytes, reesterification of fatty acids to TAG takes place. Also other lipoproteins such as low-density lipoprotein (LDL) and high-density lipoprotein (HDL) play an important role in the transport of cholesterol and TAG throughout the body.

Regulation of lipolysis

Lipids and carbohydrates are the main substrates for energy production. In the postprandial state, glucose is the most important energy substrate for most organs. In the post-absorptive state, however, energy production from glucose is gradually shifted towards processes that use fatty acids as substrate for oxidation. In order to provide this substrate, lipolysis -the processes in which TAG are hydrolyzed to fatty acids and glycerol- increases, and free fatty acids are released from adipose tissue into the circulation. In this condition, lipolysis is mainly mediated by adipose tissue hormone-sensitive lipase (HSL) (Figure 1.3). HSL is activated by hormones such as glucagon, epinephrine, growth hormone, adrenocorticotropic hormone (ACTH), thyroid hormone and cortisol, whereas insulin is the most important inhibitory hormone. However, lipolysis does not only take place within adipose tissue, but also in the liver and on the surface of peripheral

endothelial cells. During the post-absorptive state, these lipases (*i.e.* lipoprotein lipase and hepatic lipase) play only a relatively minor role in whole-body lipolysis. Within this thesis, the term 'whole-body lipolysis' will refer to the total release of glycerol into the plasma compartment as a result of all lipolytic processes within the body. The term 'palmitic acid release' will refer only to those lipolytic processes where fatty acids are released into the circulation, i.e. HSL-mediated lipolysis within the adipose tissue.

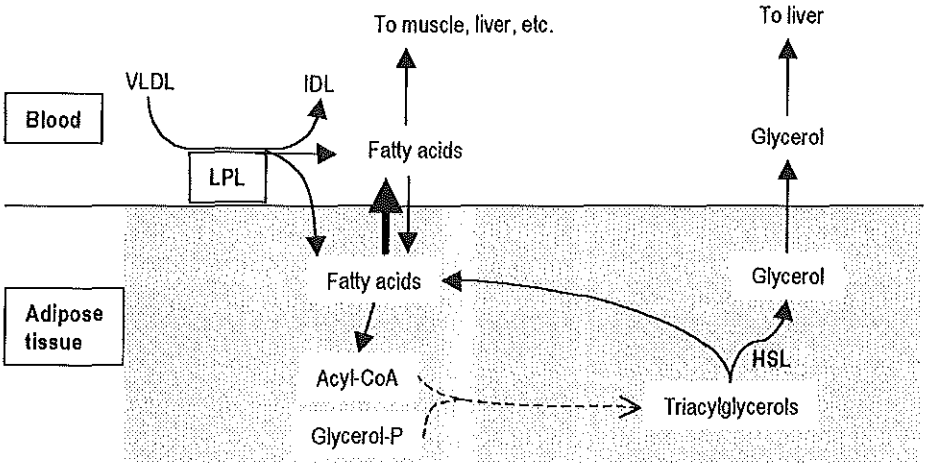

Figure 1.3

After a meal, triacylglycerols from very-low density lipoprotein (VLDL) are hydrolyzed by lipoprotein lipase (LPL), and VLDL is converted into intermediate density lipoprotein (IDL). Fatty acids diffuse into the adipose tissue, and are stored as triacylglycerols after reesterification. During the postabsorptive state, triacylglycerols are degraded into glycerol and fatty acids by hormone-sensitive lipase (HSL). Glycerol diffuses out of the cell and is taken up by the liver. Fatty acids also diffuse into the blood to be oxidized, stored or metabolized in muscle, liver or other tissues, or fatty acids are reesterified within the tissue.

After fatty acids have been taken up by peripheral tissues such as muscle, oxidation via mitochondrial β-oxidation takes place. In each cycle of β-oxidation, a fatty acid is shortened by two carbons, yielding reduction equivalents for adenosine triphosphate (ATP) production via the respiratory chain. Besides β-oxidation, fatty acids can also be degraded by peroxisomal oxidation. This pathway can handle very long chain fatty acids (≥20 carbons) which are poor substrates for mitochondrial β-oxidation. In contrast with the mitochondrial β-oxidation, the peroxisomal oxidation does not generate ATP since it is not coupled to the respiratory chain. The process of fatty acid oxidation is not directly regulated but is proportional to FFA concentrations in plasma and in the specific organ or cell. This implicates that fatty acid oxidation is mainly regulated at the level of adipose tissue HSL which provides the substrate for this this process.

n-3 Fatty acid metabolism

n-3 Fatty acids in epidemiological studies

In the 1970s, it was reported that Eskimos had low rates of coronary heart disease (CHD) and cancer despite their high-fat diet.[11,12,55] EPA appeared to play a role in these effects, since EPA was found to have anti-thrombotic effects by reducing platelet aggregation.[56] In 1985, the 20-year follow-up study from the Dutch town of Zutphen renewed the interest in the 'fish oil hypothesis' by reporting that mortality from CHD was reduced by more than 50% in those subjects who had eaten the equivalent of 30g of fish per day.[121] In the following years, some studies confirmed the potentially beneficial effects of the consumption of fatty fish[31,199] or a Mediterranean alpha-linolenic acid-rich diet[46] on CHD, but other studies did not detect any significant effects of fish consumption on coronary heart disease[8] or of fish oil supplementation on coronary atherosclerosis.[184] Although it is now generally accepted that n-3 fatty acids have beneficial effects in preventing CHD, the potential benefit of n-3 fatty acids in other diseases such as cancer, chronic inflammatory diseases and diabetes is currently under investigation.

Effects of n-3 fatty acids on lipid metabolism

Epidemiological studies have suggested that cardiovascular disease[10,78,111,149,202] and non-insulin-dependent diabetes mellitus (NIDDM)[67,165] are associated with elevated levels of TAG and/or FFA in blood. It may therefore be beneficial to reduce plasma lipid concentrations in subjects at risk.[4] Supplementation of EPA and/or docosahexaenoic acid (DHA; 22:6n-3) was shown to reduce postabsorptive[71,81,109,175] as well as postprandial[226] serum TAG concentrations and FFA[45] levels, but the effects of n-3 fatty acids on serum cholesterol concentrations are inconsistent.[27,71,94,99,224] Despite extensive research, there is still much uncertainty about the mechanism by which n-3 fatty acids reduce plasma lipid concentrations. The decrease in serum TAG concentrations appears to be caused by a reduction in hepatic TAG synthesis.[88,183] This reduction may in part be related to low substrate availability due to decreased plasma FFA concentrations[45,89,109,182,198] as a consequence of either reduced peripheral lipolysis,[213] or enhanced FA oxidation.[1,70,231]

Studies have shown that stimulation of lipolysis by a tumor-derived lipid-mobilizing factor (LMF) *in vitro* is effectively attenuated by EPA.[170,212,213] Hormonal stimulation of adenylate cyclase, and thus the formation of cyclic AMP, is also inhibited by EPA.[170,211] This effect appears to be mediated by an inhibitory effect of a guanine nucleotide-regulatory protein.[170,211] This effect of EPA on lipid metabolism is assumed to play a role in the potentially beneficial effects of EPA in cachectic cancer patients.[230]

Metabolism of n-3 and n-6 fatty acids[139,197]

As already mentioned, the fatty acids of the n-6 and the n-3 series are essential fatty acids, since they are necessary for proper physiological functioning and cannot be synthesized within the human body. After 18:2n-6 (linoleic acid) and 18:3n-3 (α-linolenic acid) have been absorbed from the diet, they are elongated to fatty acids with 20 and 22 carbon atoms. In this pathway, n-6 and n-3 fatty acids compete, since they are desaturated and elongated by the same enzymes (Figure 1.4). Although both Δ4- and Δ6-desaturases prefer n-3 fatty acids over n-6 fatty acids,[197] 20:4n-6 is the major long-chain polyunsaturated fatty acid within the body since the intake of n-6 fatty acids is usually substantially higher than that of n-3 fatty acids. However, when the intake of n-3 fatty acids increases, the contribution of 20:5n-3 and 22:6n-3 will increase at the expense of 20:4n-6.[227] Small amounts of n-3 fatty acids in the diet may therefore substantially affect metabolic pathways within the human body.

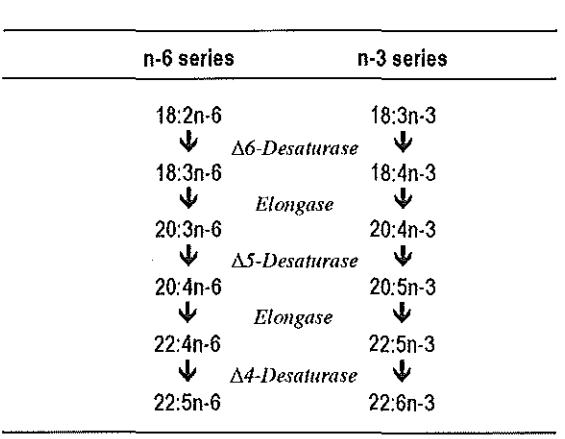

Figure 1.4

Essential fatty acid metabolism: elongation and desaturation of n-6 and n-3 fatty acids.

Effects of n-3 fatty acids on the inflammatory response

Competition between n-6 and n-3 fatty acids also appears to play a role in the regulation of the pathways of eicosanoid synthesis.[227] Eicosanoids are important compounds in the regulation of the inflammatory response and blood clotting.[32] They are synthesized from C_{20} polyunsaturated fatty acids (Figure 1.5) and can be divided into three groups: prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs). PGs and TXs are products from the cyclooxygenase pathway and contain a cyclopentane ring or a six-membered oxygen-containing ring, respectively. LTs are produced via the lipoxygenase pathway.

The major biologically active members of the eicosanoid family are those derived from arachidonic acid (AA; 20:4n-6), i.e. the PGs and TXs of the 2-series (i.e. PGE_2 and TXA_2) and the LTs of the 4-series (LTB_4). These compounds stimulate the inflammatory response as well as platelet aggregation.[32] In contrast, eicosanoids derived from EPA, i.e. the PGs and TXs of the 3-series (i.e. PGE_3 and TXA_3) and the LTs of the 5-series (LTB_5), are biologically less active than the eicosanoids derived from AA, with anti-aggregating and immunosuppressive effects as a result.[52] Most human studies regarding the effects of n-3 fatty acids on cytokine production showed a decrease in the production of one or more proinflammatory cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor-α.[26,44,60,147,148] The inhibitory effects of EPA on the inflammatory response may play an important role in the potentially beneficial effects of EPA in inflammatory bowel disease,[9,24] rheumatoid arthritis,[119,191] psoriasis[80,140] and cancer cachexia.[230]

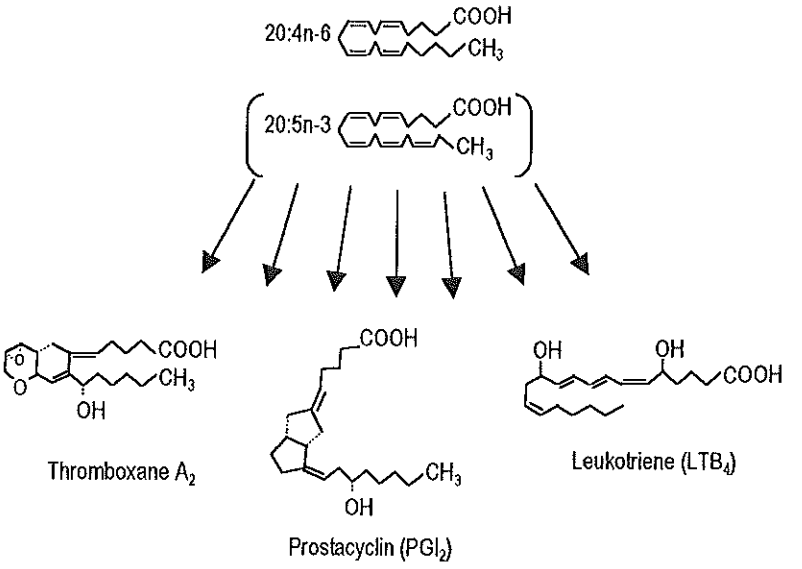

Figure 1.5

Examples of molecular structures of thromboxanes, prostaglandins and leukotrienes synthesized from arachidonic acid (AA; 20:4n-6) or eicosapentaenoic acid (EPA; 20:5n-3).

Cancer cachexia: etiology and potential treatment

Many patients with cancer suffer from involuntary weight loss which is called cancer cachexia. The term 'cachexia' is derived from the Greek words 'κακος' meaning 'bad', and ''ηξις' meaning 'condition'. The occurrence of weight loss is related to the type of tumor and ranges from 30% in patients with favorable non-Hodgkin's lymphoma to nearly 90% in patients with gastric cancer.[50] Patients with cachexia have a decreased survival time[50,164] and an impaired response to chemotherapy,[49,50] and they suffer from fatigue[75] and a reduced quality-of-life.[162] Weight loss in cancer can partly be explained by reduced food intake due to factors such as abdominal fullness, taste change, constipation, mouth dryness, nausea and vomiting,[83] or by impaired food uptake due to intestinal malabsorption.[146,168,225] However, weight loss in cancer patients differs from that during simple starvation. During starvation, fat from adipose tissue is usually used as the main fuel whereas muscle mass is conserved. In contrast, in cancer cachexia, substantial loss of muscle mass is observed in addition to loss of fat mass.[65,96] These changes in body composition suggest that factors other than reduced food intake play a role in the loss of body mass in cancer. This assumption is supported by the observation that increased energy intake in cancer patients does not reverse the cachexia syndrome.[38,161] Nutritional support increased only body fat but not total body nitrogen,[40,193] and total parenteral nutrition did not stimulate overall protein synthesis in malnourished cancer patients.[104]

Metabolic alterations

Although the mechanisms underlying cachexia have not been fully clarified, metabolic alterations appear to play an important role in the occurrence of weight loss in cancer. In healthy subjects, reduced food intake usually results in reduced resting energy expenditure (REE) whereas in many cancer patients, REE is increased despite normal or even reduced energy intake.[5,103,204] This is illustrated by REE data of a patient with progressive pancreatic cancer from our study during long-term follow-up, showing that an increase in REE preceded the decrease in body weight (Figure 1.6). In general, tumor type appears to play an important role in determining whether REE is elevated or not. In lung cancer[69,204] as well as in pancreatic cancer,[62] REE was higher than in healthy subjects, whereas in gastric or colorectal cancer, REE was not altered.[68]

Furthermore, alterations in lipid, protein and carbohydrate metabolism have been reported in cancer. With regard to lipid metabolism, an elevated turnover of glycerol and fatty acids has been observed in cancer patients,[124,192] although one study did not reveal any differences in lipolytic rates between the groups.[108] Cancer patients with weight loss were shown to have an increased turnover of both glycerol and fatty acids when compared with patients without weight loss.[192] In another study, however, it was concluded that the observed increase in lipolysis and triglyceride-fatty acid cycling in cachectic patients with

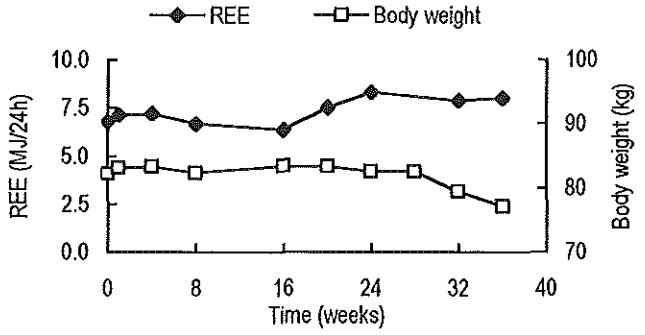

Figure 1.6

Resting energy expenditure (REE) and body weight during long-term follow-up in a patient with progressive pancreatic cancer. REE increased after 20 weeks, followed by a decrease in body weight.

esophageal cancer was due to alterations in their nutritional status rather than the presence of tumor itself.[116] Besides alterations in lipolysis, concentrations of specific fatty acids may also be affected by tumor presence and tumor type: in colorectal cancer,[18] 18:2n-6 and 18:3n-3 were reduced and 18:1n-9 was increased in red blood cells, whereas in platelets of lung cancer patients,[172] 18:2n-6 and the n-3 fatty acids were reduced. In patients with bladder cancer,[142] total n-3 and n-6 fatty acids were reduced in plasma PL.

Alterations in protein metabolism in cancer cachexia are clearly illustrated by the undesired loss of muscle mass.[96] In metabolic studies, muscle protein synthesis was significantly reduced in malnourished patients with advanced gastric carcinoma[54] and in weight-losing cancer patients of different tumor types,[58] whereas protein breakdown was elevated in patients with hepatocellular carcinoma.[159] In muscle biopsy specimens from 43 newly diagnosed cancer patients with weight loss, both reduced protein synthesis and elevated protein degradation were detected.[132] Furthermore, plasma amino acid profiles may be altered in patients with cancer cachexia, as suggested by a low plasma glutamine:cystine ratio,[85] high plasma glutamate levels[86] and reduced serum tryptophan levels.[107]

Changes in glucose metabolism also occur in cancer.[51] The rate of endogenous glucose production is increased, and the magnitude of this increase appears to be related with factors such as tumor stage, tumor histology and cachexia.[51] Increased gluconeogenesis, *i.e.* hepatic production of glucose from lactate, alanine and glycerol, is one of the most important alterations in glucose metabolism in cancer.[51] The rate of Cori cycling, the energy-consuming process in which lactate is released as a result of glycolysis

in peripheral tissues and resynthesized to glucose by the liver, is also increased in cancer cachexia.[51]

Underlying mechanisms

The mechanisms underlying the metabolic alterations in cancer have not yet been clarified. Besides alterations in food intake, many metabolic factors appear to play a role in cancer cachexia, such as the recently identified lipid-mobilizing factor (LMF)[219] and proteolysis-inducing factor (PIF).[130] Recent studies have stressed the important role of the acute-phase response associated with high levels of tumor necrosis factor-α, interleukin-1 and interleukin-6, and interferon-γ.[25,62,64,196,203] Other factors such as hormonal alterations and metabolic competition between tumor and host may also play a role in the metabolic alterations in cancer cachexia.[79]

Potential treatments

An adequate treatment for cancer cachexia is not available. Corticosteroids and progestational drugs have been shown to improve appetite, food intake and the sensation of well-being, but the beneficial effects on muscle mass are minimal.[73] Although hydrazine sulfate has received much attention, this compound was shown to be ineffective in improving the symptoms of the patient with cancer cachexia.[73] Megestrol acetate had beneficial therapeutic effects on appetite, body weight and quality-of-life,[134] but these beneficial effects appeared to be related to conservation of fat mass rather than of muscle mass.[30] Melatonin may be effective in the treatment of cancer cachexia by decreasing TNF blood concentrations,[128] and medroxyprogesterone acetate may contribute to downregulation of the acute-phase response.[134] In conclusion, the n-3 fatty acid EPA may have beneficial effects in cancer cachexia by inhibiting proteolysis[130] and lipolysis,[170] and by modulation of the acute-phase response.[229]

Aims of the study

The main aims of this thesis were the following:

- To determine whether lipid metabolism is altered in cancer patients
- To determine whether supplementation of EPA reduces lipolysis and lipid oxidation in healthy subjects and in weight-losing cancer patients
- To obtain pilot information on the long-term effects of EPA supplementation on body weight in weight-losing cancer patients

Outline of the thesis

Differences in lipolysis and lipid oxidation between weight-losing cancer patients and healthy subjects are presented in *Chapter 2*. Since it was not known whether whole-body lipolysis and lipolytic activity *in vitro* are related, *Chapter 3* describes the measurements of lipolytic activity in the plasma samples of our cancer patients. In *Chapter 4*, we report differences in plasma n-3 fatty acid concentrations between patients with pancreatic cancer, lung cancer and esophageal cancer in comparison with healthy subjects. The level of incorporation of EPA and DHA in different plasma lipid fractions during short-term n-3 fatty acid ethyl ester supplementation in healthy subjects is reported in *Chapter 5*. The effects of short-term EPA ethyl ester supplementation on lipolysis and lipid oxidation in healthy subjects and weight-losing cancer patients are presented in *Chapter 6 and 7*, respectively. In *Chapter 8*, the short-term metabolic effects of different n-3 fatty acid supplements are compared, and *Chapter 9* describes our pilot study on the effects of long-term EPA ethyl ester supplementation on body weight, body composition and biochemical parameters in weight-losing cancer patients. Finally, the main results of this thesis are discussed in *Chapter 10*, and a summary of results is presented in *Chapter 11*.

2

LIPOLYSIS AND LIPID OXIDATION IN WEIGHT-LOSING CANCER PATIENTS AND HEALTHY SUBJECTS

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Abstract

Increased lipolysis has been suggested as one of the possible mechanisms underlying cancer cachexia. The aim of our study was to assess whether lipolysis is increased in weight-losing cancer patients taking differences in food intake and body composition into account.

Sixteen healthy subjects and 18 cancer patients with different tumor types and weight loss of ≥5% in the previous six months were included in the study. Food intake was recorded for four days. After an overnight fast, [1,1,2,3,3-$^{2}H_5$]glycerol was infused to determine the rate of appearance (R_a) of glycerol as a measure of whole-body lipolysis, and [1-^{13}C]palmitic acid was infused to determine the R_a of palmitate as a measure of palmitic acid release. Palmitate oxidation was determined by measuring $^{13}CO_2$ enrichment in breath samples and body composition was measured by bioelectrical impedance analysis.

After adjustment for energy intake, whole-body lipolysis was significantly higher in cancer patients than in healthy subjects (6.46 $\pm$ 0.63 and 4.67 $\pm$ 0.46 μmol/kg.min, respectively; $P < 0.05$). The difference in palmitic acid release did not reach statistical significance. The rate of palmitate oxidation was also significantly higher in patients than in healthy subjects (1.15 $\pm$ 0.10 and 0.93 $\pm$ 0.07 μmol/kg.min, respectively; $P < 0.05$). No differences in body composition were observed between groups.

In conclusion, whole-body lipolysis as measured by the R_a of glycerol, and palmitate oxidation are elevated in weight-losing cancer patients, but palmitic acid release was not significantly different.

Introduction

Cancer cachexia is a syndrome of involuntary weight loss, impaired physical performance and fatigue, and is frequently seen in patients with malignant tumors.[75,127] Different studies have shown that weight loss is associated with increased morbidity, attenuated response to therapy, decreased quality of life[50] and reduced survival.[48] The beneficial effect of dietary supplementation is modest, and pharmacological agents have failed to improve the condition.[38] Therefore, better knowledge of the mechanisms underlying cancer cachexia is important in order to develop new treatment strategies.

One of the factors contributing to the development of weight loss is reduced food intake, which may be caused by decreased appetite or tumor treatment,[146,168] by mechanical obstruction of the gastrointestinal tract,[48] or by intestinal malabsorption.[146] In addition, metabolic aberrations may contribute to cachexia in cancer patients.[103,204] The combination of reduced energy intake and increased energy expenditure in cancer patients will result in substantial weight loss. It has been shown that weight loss in cancer patients comprises both muscle mass and fat mass (FM).[96] One of the mechanisms that may be involved in the reduction of FM is increased lipolysis. In literature, several studies have reported elevated lipolysis in cancer patients,[116,124,192] although one study did not detect any difference in lipolysis between cancer patients and healthy subjects.[108]

The mechanisms responsible for elevated lipolysis in cancer patients are poorly understood. Lipolysis may be stimulated by reduced energy intake, weight loss or by the presence of cancer as such. In urine of weight-losing cancer patients, a probably tumor-derived lipolytic material has been detected[219] which stimulated lipolysis[101] and proteolysis *in vitro*,[130,216] and induced weight loss in animals.[218] Furthermore, inflammation mediated by cytokines such as interleukin-6 may affect lipolysis in cancer patients.[79,84] Studies in healthy subjects have demonstrated that lipolytic rate depends on energy intake[34,114] and the size of fat-free mass (FFM).[34,118] To our knowledge, however, no data have been published on lipolysis in cancer patients after adjustment for energy intake or body composition. Therefore, the aim of the present study was to assess whether whole-body lipolysis, palmitic acid release and palmitate oxidation are increased in weight-losing cancer patients taking differences in food intake and body composition into account.

Methods

Subjects

Eighteen patients with histologically proven cancer and weight loss ≥ 5% in the previous 6 months, and 16 healthy subjects with stable weight were included in this study.

The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam, and written informed consent was obtained from all participants prior to start of the study. The following exclusion criteria were used: treatment with chemotherapy or radiation therapy in the 2 weeks preceding the study, surgery in the previous 2 months, concurrent corticosteroid treatment, insulin-dependent diabetes mellitus, uncontrolled hyper- or hypothyroidism, edema or fever. Clinical characteristics of cancer patients are summarized in Table 2.1.

Table 2.1

Clinical characteristics of weight-losing cancer patients.[a]

Age	Sex	Tumor type	Time since diagnosis (mo)	Metastases	Prior Treatment[b]	Weight loss (% / 6 mo)
		Gastrointestinal tumors:				
50	M	Adenocarc. of the oesophagus	1	None	None	6.9
60	M	Adenocarc. of the oesophagus	48	Liver, lung	S,C	7.3
69	M	Squamous cell carc. of the oesophagus	8	Lymph nodes	C	18.1
59	M	Squamous cell carc. of the oesophagus	22	None	C,R	14.8
49	M	Squamous cell carc. of the oropharynx	14	None	C,R	10.7
59	M	Carc. of the rectum	24	Liver	S,R	18.0
63	M	Pancreatic cancer (locoregional relapse)	19	None	S,R	14.3
75	M	Pancreatic cancer (locoregional relapse)	7	None	S	7.3
59	M	Gall bladder carc.	2	Liver	None	25.0
		Other tumors:				
74	M	Adenocarc. of the lung	12	Liver	S	10.8
55	M	Undifferentiated large cell carc.of the lung	16	Mediastinum	R	5.3
65	M	Mesothelioma of the lung	3	None	None	14.0
73	F	Adenocarc. of the mamma	180	Lung, bone	S,H,R	14.4
74	F	Adenocarc. of the mamma	1	Bone, lymph nodes	H	7.2
65	F	Carc. of cervix (locoregional relapse)	24	None	S,R	5.3
66	F	Carcinoid	96	Liver, omentum, lymph nodes	None	17.1
65	M	Adenocarc. of the kidney	15	Lung	S,I	13.1
65	M	Adenocarc. of unknown primary	1	Liver	None	11.1

[a] Carc.: carcinoma

[b] S = Surgery; R = Radiotherapy; C = chemotherapy; H = Hormonal treatment; I = Interferon treatment

Study protocol

After an overnight fast of ≈12 hours, subjects attended the outpatient department between 8 and 9 a.m. for measurements of whole-body lipolysis and lipid oxidation, resting energy expenditure (REE) and body composition. After fifteen minutes of absolute rest at the department, teflon catheters were inserted into the antecubital vein of one arm for the infusion of isotopes and into the contralateral dorsal hand vein or forearm vein of the other arm for blood sampling. Baseline blood samples were collected in heparinized vacuum tubes. Four baseline breath samples were collected by exhaling through a straw into 10mL Exetainer tubes. Blood samples were centrifuged immediately at 1200g for 10min at 4°C, and plasma was stored at -80°C under nitrogen until analysis.

After baseline sampling, a plasma protein solution (40g protein/L; albumin ≥ 85%; CLB, Amsterdam, The Netherlands) was infused containing the stable isotope labeled tracers [1,1,2,3,3-$^{2}H_5$]glycerol (MassTrace, Woburn, USA) and [1-^{13}C]palmitic acid (MassTrace). Labeled glycerol was infused at a rate of ~0.08μmol/kg.min (prime 1.2μmol/kg) to determine the rate of appearance (R_a) of glycerol as an index of whole-body lipolysis[233] using a Perfusor® Secura pump (B.Braun, Melsungen, Germany). Labeled palmitate was infused at a rate of ~0.04μmol/kg.min to determine the R_a of palmitate as an index of fatty acid release.[233] The plasma bicarbonate pool was primed with $NaH^{13}CO_3$ (~1.7μmol/kg) dissolved in saline. The exact amount of tracers infused during the study was determined afterwards by measuring the isotope concentration in the infusate.

During the isotope infusion, REE was measured for 30 minutes by indirect calorimetry using a ventilated hood system (Deltatrac™ MBM-100, DATEX/Instrumentarium Corp., Helsinki, Finland). The amounts of O_2 consumed and CO_2 produced during the last 20 minutes of the measurement were used to calculate REE and total respiratory quotient (RQ). At 50, 60, 70, 80 and 90 minutes after start of the isotope infusion, arterialized venous blood samples[239] were taken in heparinized tubes to measure [$^{2}H_5$]glycerol and [1-^{13}C]palmitate enrichment. Breath samples were taken to measure $^{13}CO_2$ enrichment in order to calculate [1-^{13}C]palmitate oxidation. All blood samples were placed on ice immediately, and centrifuged and stored at the end of the infusion period. In order to prevent obstruction, the venflon sampling device was flushed with 2 mL saline containing heparin in low concentration. Heparin-released lipoprotein lipase activity was verified to be less than 3% of the measured values for total whole-body lipolysis.

Body composition was determined by bioelectrical impedance analysis (BIA; HUMAN-IM SCAN, Dietosystem, Milan, Italy), using the equation of Deurenberg et al..[47] Body weight (BW) and height were measured. Subjects recorded their dietary intake

for four days preceding the measurements of lipolysis. Dietary intake was calculated using the nutritional package 'Komeet' (B.ware Nutrition Software, Arnhem, the Netherlands).

Analysis of blood samples

To isolate plasma glycerol, 0.2mL plasma was deproteinized by subsequently adding and mixing 0.5mL H_2O, 0.2mL 0.15M $CuSO_4$ and 0.2mL 0.15 M Na_2WO_4. After centrifugation at 15000g and 15°C for 8 minutes, the supernatant was passed through a mixed ionexchange column (AG50W-X8, AG1-X8, 200-400 mesh, 0.2 g each; Biorad, Richmond, USA). The column was washed with 4mL H_2O and the effluent containing glycerol was collected and dried under nitrogen. Derivatives of glycerol were formed during incubation with 0.030mL pyridine and 0.015mL N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA, Pierce, Omnilabo, Breda, The Netherlands) for one hour at 60°C.

To measure [1-^{13}C]palmitate enrichment, lipids were extracted from 250µL plasma using chloroform/methanol (2:1, by vol.; Merck; Darmstadt, Germany) according to Folch et al.[66] in the presence of butylated hydroxytoluene (BHT; 1mg/mL) as an antioxidant. Plasma free fatty acids (FFA) were isolated by thin layer chromatography (silica plates; Merck, 5721) using hexane/diisopropylether/acetic acid (60:40:3, by vol.; Merck) as a developer. Spots were scraped off, extracted using chloroform/methanol (2:1), dried under nitrogen and converted to their derivatives by MTBSTFA.

Plasma enrichment of free [$^2H_5$]glycerol and [1-^{13}C]palmitic acid were analyzed on a Carlo Erba GC8000 gaschromatograph coupled to a Fisons MD800 mass spectrometer (Interscience BV, Breda, The Netherlands) in electron impact ionization mode with an interface temperature of 280°C and a source temperature of 200°C. All measurements of isotopic enrichment were carried out by injecting 1µL with a split ratio of 50:1 on a fused silica capillary column of 25m x 0.22mm, coated with 0.11m HT5 (SGE, Victoria, Australia). Natural glycerol and [^{2}H5]glycerol (mass 387 and 381), and natural palmitic acid and [1-^{13}C]palmitic acid (mass 313 and 314) were measured by selected ion monitoring. For both [$^2H_5$]glycerol and [1-^{13}C]palmitic acid, the coefficient of variation was 0.2Mol% and no concentration effect was observed for the Mol% enrichment. $^{13}CO_2$ in breath samples was measured on an isotope ratio mass spectrometer (ABCA; Europa Scientific, Van Loenen Instruments, Leiden, The Netherlands) with a standard deviation of 0.0002Atom%$^{13}CO_2$.

Blood hemoglobin, albumin, prealbumin and C-reactive protein (CRP) were measured according to standard clinical chemical methods. Thyroid hormones T_3, T_4 and rT_3 were measured according to the method of Bauer et al..[20] Plasma insulin was measured by radioimmunoassay (Biosource, Fleurus, Belgium).

Calculations

Calculations of the rate of appearance (R_a) of glycerol and palmitate were made according to Klein et al.,[116] using the equation described by Steele:[205]

R_a (μmol/kg.min) = (IE_i/IE_p – 1) * F

where F is the isotope infusion rate (μmol/kg.min), IE_i is the isotopic enrichment of the infusate (atom percent excess) and IE_p is the isotopic enrichment in plasma (atom percent excess) during steady state conditions. The R_a of total free fatty acids (R_a FFA) was calculated from the R_a of palmitate assuming that the palmitate concentration was 23% of total plasma FFA concentrations.[110] An index of the relative rates of lipolysis and reesterification was calculated as the ratio of R_a FFA / R_a of glycerol.[233]

Palmitate oxidation was calculated according to the following equation:

Palmitate oxidation (μmol/kg.min) = (IE_{CO2} * VCO_2) / (IE_p * BW * k)

where IE_{CO2} is the isotopic enrichment of the expired CO_2 (atom percent excess), VCO_2 is the CO_2 production (μmol/min), BW is body weight (kg) and k is the bicarbonate correction factor for incomplete recovery of ^{13}C (k = 0.75) according to Wolfe et al..[234]

Statistical analysis

Data are expressed as mean ± SEM. Statistical differences between cancer patients and healthy subjects were assessed using linear regression analysis including as covariates: 1. a dummy variable to indicate tumor presence and 2. energy intake. Pearson correlation coefficients were calculated. Due to the skewed distribution of CRP data, a logtransformation was applied for plasma CRP concentrations. *P*-values less than 0.05 were considered statistically significant. Analyses were performed with SPSS software (SPSS for Windows version 6.1.3, SPSS Inc., Chicago).

Results

The characteristics of cancer patients and healthy subjects are summarized in Table 2.2. The mean age of cancer patients was significantly higher than that of healthy subjects although the age range was comparable, i.e. 49–75 year in patients and 40–75 year in healthy subjects. BW, body mass index (BMI; kg/m^2), percentage of ideal BW (%IBW), arm circumference and sum of four skinfolds were significantly lower in cancer patients than in healthy subjects. Absolute values of FFM and FM were reduced in cancer patients, but the percentage of FFM (%FFM) was not different between cancer patients and healthy subjects. Since %FFM was also comparable in patients with gastrointestinal (GI) cancer and patients with other types of tumors (non-GI), %FFM was not included as a covariate in the statistical analyses.

Table 2.2

Characteristics of study population.[a]

	Cancer patients			Healthy subjects
	Gastrointestinal (n = 9)	Other (n = 9)	Total group (n = 18)	(n = 16)
Age (y)	60 ± 3	67 ± 2[b]	64 ± 2[b]	54 ± 2
Sex	9 M	5 M; 4 F	14 M; 4 F	10 M; 6 F
Weight (kg)	67.2 ± 3.4	63.8 ± 3.1[b]	65.5 ± 2.3[b]	77 ± 3.4
Weight loss (%)	13.6 ± 2.1[b]	10.9 ± 1.4[b]	12.3 ± 1.2[b]	0 ± 0
BMI (kg/m^2)	21.5 ± 1.1[c]	23 ± 1	22.2 ± 0.7[c]	25.3 ± 1
%IBW[d]	97.8 ± 4.9[b]	108 ± 5.1	102.9 ± 3.6[c]	118.2 ± 4.6
Arm circumference (cm)	27.5 ± 1.3[b]	28.5 ± 1.0[c]	28 ± 0.8[b]	31.9 ± 0.8
Sum of four skinfolds (mm)	33.3 ± 4.6[b]	45 ± 7.7[c]	39.1 ± 4.6[b]	69.2 ± 7.4
Fat-free mass (kg)	47.6 ± 2.2	44.3 ± 2.5[c]	45.9 ± 1.7[c]	54.1 ± 2.9
Fat mass (kg)	20.4 ± 2.4	19.5 ± 1.8	19.9 ± 1.4	23.6 ± 2.2
Fat-free mass (%)	71 ± 3	70 ± 2	70 ± 2	70 ± 2

[a] mean ± SEM

[b,c] Significantly different from healthy subjects (t-test): [a] $P < 0.01$, [b] $P < 0.05$,

[d] Percentage of ideal body weight

Total energy intake (kJ/day) was significantly lower in cancer patients than in healthy subjects, especially in patients with non-GI tumors (Table 2.3). When expressed per kg BW, energy intake in patients with non-GI tumors remained 25% lower than in healthy controls ($P = 0.055$). Because of the differences in energy intake between groups, further statistical analyses were adjusted for energy intake. No significant differences in energy percentage of fat, protein or carbohydrate intake were observed between any of the groups.

Blood hemoglobin, albumin and prealbumin were found to be significantly decreased in cancer patients, whereas CRP was significantly increased (Table 2.4). With regard to thyroid hormones, T_3 was significantly lower and rT_3 significantly higher in cancer patients than in healthy subjects, whereas T_4 did not show any significant difference between the groups. Urinary creatinine excretion was significantly lower in cancer patients than in healthy subjects. Again, no significant difference in any biochemical parameter was detected between cancer patients with GI tumors and patients with other types of tumors.

Table 2.3

Dietary intake in weight-losing cancer patients and healthy subjects[a]

	Cancer patients			Healthy subjects
	Gastrointestinal (n = 9)	Other (n = 9)	Total group (n = 18)	(n = 16)
- total (kJ/d)	8348 ± 902	5903 ± 568[b]	7054 ± 588[c]	9081 ± 621
- per kg body weight (kJ/kg.d)	128 ± 15	92 ± 8	109 ± 13	122 ± 10
- Protein (E%)[d]	17.8 ± 1	17.4 ± 1	17.6 ± 0.7	16.9 ± 0.8
- Fat (E%)[d]	34.9 ± 2	33.9 ± 1.1	34.4 ± 1.1	36.2 ± 1.5
- Carbohydrate (E%)[d]	45 ± 3	50 ± 2	47 ± 2	47 ± 1

[a] $\bar{x}$ ± SEM

[b,c] Significantly different from healthy subjects: [b] $P < 0.01$; [c] $P < 0.05$

[d] E%: energy percent

Table 2.4

Biochemical parameters in weight-losing cancer patients and healthy subjects[a]

	Cancer patients			Healthy subjects
	Gastrointestinal (n = 9)	Other (n = 9)	Total group (n = 18)	(n = 16)
Blood:				
Hemoglobin (mmol/L)	7.7 ± 0.4[c]	7.1 ± 0.3[b]	7.4 ± 0.3[b]	8.6 ± 0.2
Albumin (g/L)	39 ± 2[b]	39 ± 1[b]	39 ± 1[b]	48 ± 1
Prealbumin (g/L)	0.19 ± 0.02[b]	0.17 ± 0.01[b]	0.18 ± 0.01[b]	0.28 ± 0.01
T_3 (nmol/L)	1.22 ± 0.15[b]	1.52 ± 0.13[c]	1.39 ± 0.11[b]	1.89 ± 0.1
T_4 (nmol/L)	93 ± 8	112 ± 9	102 ± 6	90 ± 4
rT_3 (nmol/L)[d]	0.54 ± 0.14[c]	0.36 ± 0.11[c]	0.48 ± 0.09[b]	0.25 ± 0.02
C-Reactive Protein (mg/L)[d]	25 ± 13[c]	17 ± 15[c]	21 ± 10[b]	1 ± 1
Urine:				
Creatinine excretion (g/24h)	0.14 ± 0.01[c]	0.13 ± 0.02	0.14 ± 0.01[c]	0.17 ± 0.01

[a] Mean ± SEM unless otherwise stated

[b,c] Significantly different from healthy subjects (t-test): [b] $P < 0.01$; [c] $P < 0.05$

[d] Median ± SEM

Table 2.5

Rate of appearance (R_a) of glycerol and palmitate and the ratio of R_a of total free fatty acids (FFA) / R_a of glycerol in plasma, rate of palmitate oxidation and resting energy expenditure (REE) in weight-losing cancer patients and healthy subjects.[a]

	Cancer patients			Healthy subjects
	Gastrointestinal (n = 9)	Other (n = 9)	Total group (n = 18)	(n = 16)
R_a of glycerol (μmol/kg.min)	5.88 ± 0.6	7.04 ± 1.12[b]	6.46 ± 0.63[c]	4.67 ± 0.46
R_a of palmitate (μmol/kg.min)	4.23 ± 0.41	4.38 ± 0.55	4.3 ± 0.33	3.59 ± 0.23
Ratio R_a FFA / R_a glycerol	3.23 ± 0.25	2.82 ± 0.17	3.03 ± 0.16	3.75 ± 0.37
Palmitate oxidation (μmol/kg.min)	1.14 ± 0.16	1.16 ± 0.14[b]	1.15 ± 0.10[b]	0.93 ± 0.07
REE (kJ/kg.24h)	97 ± 4[d]	96 ± 4[d]	97 ± 3[d]	84 ± 2.40

[a] Mean ± SEM

[b-d] Significantly different from healthy subjects after adjustment for energy intake:
[b] $P = 0.05$; [c] $P < 0.05$; [d] $P < 0.02$

Whole-body lipolysis, as measured by the R_a of glycerol, was 38% higher after correction for energy intake in cancer patients than in healthy subjects (Table 2.5; $P < 0.05$). When the two subgroups of cancer patients were considered separately, a 50% elevation of R_a of glycerol was observed in patients with non-GI cancer ($P < 0.05$), whereas the elevation of R_a of glycerol did not reach statistical significance in the GI patients. Although palmitic acid release, as measured by the R_a of palmitate, also tended to be higher in cancer patients than in healthy subjects, this difference failed to reach statistical significance ($P < 0.10$). Reesterification, as indicated by the ratio R_a FFA / R_a glycerol, did not differ significantly between cancer patients and healthy subjects, although values tended to be lower in cancer patients than in healthy subjects. Palmitate oxidation was significantly elevated in the total group of cancer patients ($P < 0.05$) as well as in the subgroup of non-GI cancer patients ($P < 0.05$). REE per kg BW was significantly elevated in both patients with GI cancer and with other tumor types ($P < 0.01$).

Palmitate oxidation ($R = 0.52$; $P < 0.05$) and REE ($R = 0.65$; $P < 0.01$), but not lipolysis or fatty acid were significantly correlated with the logarithm of plasma CRP concentrations.

Discussion

Increased lipolysis is one of the factors that may contribute to development of weight loss in cancer cachexia.[97] It is not known, however, whether lipolysis is increased in cancer patients when alterations in energy intake and body composition are taken into account. In the present study, we investigated whether lipolysis was higher in weight-losing cancer patients than in healthy subjects taking differences in food intake and body composition into account. Since no differences in %FFM were observed, this factor was not included in the statistical analyses. Energy intake, however, showed substantial differences between groups, and was therefore included in the statistical analyses as a covariate. Whole-body lipolysis was assessed using infusion of $^{2}H_5$-labeled glycerol, whereas palmitic acid release was measured using ^{13}C-labeled palmitate. Whole-body lipolysis and palmitate oxidation were significantly higher in cancer patients than in healthy subjects, but the difference in fatty acid release between the two groups failed to reach statistical significance.

Lipolysis is the hydrolysis of triglycerides into free glycerol and fatty acids. For every triacylglycerol molecule hydrolyzed, one molecule of glycerol is released into the plasma. The R_a of glycerol is therefore a direct reflection of the rate of lipolysis.[233] In contrast, the R_a of palmitate may be affected by other factors such as reesterification within the tissue[233] and differential mobilization of different fatty acids from adipose tissue.[43] The index for reesterification, calculated as the ratio of R_a FFA / R_a glycerol, did not differ significantly between groups in the present study.

Several studies have shown that lipolysis expressed per kg BW does not differ significantly between younger and older subjects.[35,117,221] Based on those observations and the fact that age did not correlate with lipolysis in the present study, we conclude that the slight difference in age between cancer patients and healthy subjects in the present study has not affected study outcome.

Previous studies have shown that REE is higher in cancer patients with an acute-phase response than in patients without this response.[62,203] This finding was confirmed in the present study, where REE was significantly correlated with plasma CRP concentrations. Whole-body lipolysis was, however, not correlated with plasma CRP in cancer patients, although it has been suggested that cytokines and inflammation may mediate changes in lipid metabolism.[84] Rather, the presence of a lipolytic factor[101,219] or lipolytic hormones may have stimulated whole-body lipolysis in cancer patients.

It may be noted that our values of the R_a of glycerol and palmitate in cancer patients as well as in healthy subjects were higher than those reported in comparable studies.[108,116,124,192] Although there is no simple explanation for this, it should be emphasized that the differences between our values and those reported in literature did not

affect the comparison between patients and healthy subjects within our study, since identical methodology was applied for all subjects.

We conclude that lipolysis and palmitate oxidation are elevated in weight-losing cancer patients when energy intake is taken into account. Body composition did not differ between patients and healthy subjects, so that adjustment for this potential confounder was not needed. Differences in palmitic acid release and reesterification between cancer patients and healthy subjects did not reach statistical significance. Prospective studies are needed to assess the role of increased lipolysis and fat oxidation in the etiology of weight loss of cancer patients.

Acknowledgements

This work was supported by Numico Research B.V., Wageningen, The Netherlands. For help i the recruitment of patients, we gratefully acknowledge the physicians of the departments of Oncology, Radiation therapy, Surgery and Internal Medicine of the University Hospital Rotterdam. We also thank M. van Seters for assistance, Dr. P.I.M. Schmitz for performing the randomization, the pharmacists of the University Hospital Rotterdam for preparing the isotope solution, and professor H. Jansen for his help to perform validation tests.

3

PLASMA LIPOLYTIC ACTIVITY AND WHOLE-BODY LIPOLYSIS IN WEIGHT-LOSING CANCER PATIENTS

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Abstract

In urine of cachectic cancer patients, a lipid-mobilizing factor (LMF) has recently been identified which may be responsible for the increased breakdown of adipose tissue in these patients. It is not known whether the presence of this LMF in plasma, which can be measured as plasma lipolytic activity, is associated with increased lipolysis *in vivo*. The aim of the present study was to assess whether whole-body lipolysis and plasma lipolytic activity are correlated in weight-losing cancer patients and healthy control subjects.

In 18 weight-losing cancer patients of different tumor types and in 16 healthy subjects, the rate of appearance of glycerol was determined as a measure of whole-body lipolysis using $^{2}H_5$-labeled glycerol. *In vitro* lipolytic activity of plasma was determined by measuring glycerol release from incubated adipocytes.

In vitro lipolytic activity towards isolated fat cells was significantly higher in plasma from weight-losing cancer patients than in plasma of healthy subjects ($P<0.01$), as was the rate of whole-body lipolysis ($P < 0.05$). However, no significant correlation was detected between lipolytic activity *in vitro* and whole-body lipolysis *in vivo* in the total study group, nor in healthy subjects or cancer patients separately.

Introduction

Many cancer patients suffer from involuntary weight loss which is caused both by reduced food intake and by metabolic alterations.[127] Recent studies have identified a lipid-mobilizing factor (LMF), which shows homology with the plasma protein Zn-α_2-glycoprotein.[97] LMF was shown to induce lipolytic activity in incubated adipocytes[143,219] and to stimulate lipid mobilization and catabolism in mice.[97] Therefore, LMF may play an important role in the excessive breakdown of adipose tissue in cancer. In a recent study in weight-losing cancer patients of different tumor types and healthy subjects, we showed that whole-body lipolysis, as measured by stable isotope steady state kinetics, was increased in cancer patients *(Chapter 2)*. We decided to measure lipolytic activity in the plasma samples of these patients to assess whether whole-body lipolysis *in vivo* and lipolytic activity *in vitro* in weight-losing cancer patients and healthy control subjects are correlated.

Subjects and methods

Eighteen patients with histologically proven cancer of different tumor types and weight loss ≥5% in the previous six months, and 16 healthy subjects with stable weight were included in the study. Whole-body lipolysis was determined by measuring the rate of appearance of glycerol.[116] After baseline blood sampling, [1,1,2,3,3-^{2}H$_5$]glycerol (MassTrace, Woburn, USA) was infused by approximately 0.08μmol/kg.min (priming dose 1.2μmol/kg). After 50, 60, 70, 80 and 90 minutes of infusion, blood samples were taken for determination of plasma [^{2}H$_5$]glycerol enrichment as described previously *(Chapter 2)*.

Plasma lipolytic activity was determined via a modified method of Beck and Tisdale,[22] measuring glycerol release from incubated adipose cells. Adipocytes from recently sacrificed NMRI mice were separated and incubated with plasma for 2 hours. The release of glycerol was determined spectrophotometrically according to the method of Wieland.[228]

Differences between patients and controls were tested using the Mann-Whitney U test. Spearman's rank correlation coefficient was calculated between plasma lipolytic activity and whole-body R_a of glycerol. Differences were considered statistically significant when $P < 0.05$.

Results

Results showed that lipolytic activity *in vitro* was significantly higher in weight-losing cancer patients (median 0.007 μmol/10^5 adipocytes.2h; range 0.000 - 0.195) than in healthy subjects (median 0.000 μmol/10^5 adipocytes.2h; 0.000 - 0.031; $P = 0.006$).

Lipolysis *in vivo* was also higher in cancer patients (6.46±0.63 μmol/kg.min; Mean±SEM) than in healthy subjects (4.67±0.46 μmol/kg.min; $P < 0.05$). No significant correlations were detected between lipolytic activity *in vitro* and lipolysis *in vivo* (Figure 3.1).

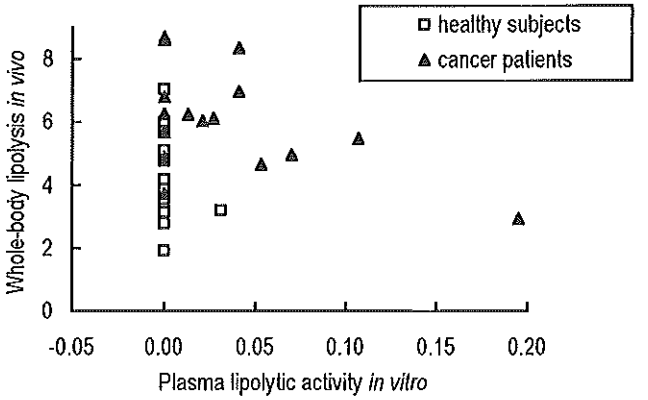

Figure 3.1

No significant correlation between whole-body lipolysis (Rate of appearance of glycerol; μmol/kg.min) and plasma lipolytic activity in vitro (mmoles glycerol / 10^5 adipocytes / 2h) in weight-losing cancer patients and in healthy subjects.

Discussion

The presence of LMF is assumed to be one of the factors that stimulate lipolysis in cancer patients.[97] Our results confirm that both lipolysis *in vivo* and lipolytic activity *in vitro* are increased in cancer patients. However, as there was no correlation between the two measures, factors other than LMF probably play a role in whole-body lipolysis in cancer patients. Such factors may include proinflammatory cytokines such as tumor necrosis factor-alpha, interleukine-1, interleukin-6 and interferon-gamma.[6,84] Also hormonal alterations have been reported in cancer patients, such as decreased plasma insulin, increased glucagon levels[19] and alterations in glucocorticoids.[6] We conclude that both lipolysis *in vivo* and lipolytic activity *in vitro* are elevated in weight-losing cancer patients. No correlation was detected, however, between these two measures. Further studies are needed to study the mechanisms underlying elevated lipolysis in cancer patients.

4

PLASMA FATTY ACID COMPOSITION IN PANCREATIC, LUNG AND ESOPHAGEAL CANCER

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Abstract

Despite the general notion of impaired nutritional status in cancer patients, studies on fatty acid status in cancer patients are limited. Plasma n-3 fatty acid concentrations may be reduced due to reduced uptake or intake, or due to enhanced metabolic demands. The aim of the present study was to investigate whether plasma n-3 fatty acids concentrations are reduced in patients with different tumor types.

We measured fatty acid composition in plasma phospholipids (PLs) and cholesteryl esters (CEs) in 45 healthy subjects and 71 newly diagnosed, untreated cancer patients of three tumor types: esophageal or cardia cancer (n=35), non-small cell lung cancer (n=22) and pancreatic cancer (n=15).

In pancreatic cancer, plasma n-3 fatty acids showed a substantial reduction in both plasma PLs and CEs. Although n-3 fatty acids in lung cancer also tended to be reduced, this difference failed to reach statistical significance. In esophageal cancer, n-3 fatty acid concentrations were comparable to those in healthy subjects. n-3 Fatty acid levels were lower in weight-losing than in weight-stable patients with lung cancer, whereas pancreatic cancer patients with diabetes had significantly higher levels of n-3 fatty acids than those pancreatic cancer patients without diabetes. For all tumor types combined, total n-3 fatty acids were also significantly reduced in patients with plasma C-reactive protein concentrations > 10 mg/L.

We conclude that plasma n-3 fatty acid levels are reduced in pancreatic cancer, possibly reduced in lung cancer, but not altered in esophageal cancer. Further studies are needed to assess the mechanisms underlying the observed changes in n-3 fatty acid concentrations.

Introduction

Many cancer patients suffer from substantial weight loss due to reduced food intake, impaired intestinal absorption, metabolic alterations or a combination of these factors, with consequent increased risk of developing nutritional insufficiencies. The risk of essential fatty acid deficiency, including deficiency of n-3 fatty acids, is especially elevated in conditions of decreased intake, intestinal malabsorption, and inflammation. Little is known, however, on the plasma concentrations of n-3 fatty acids in specific groups of cancer patients. In patients with bladder cancer, plasma n-3 and n-6 fatty acid levels were reduced in plasma phospholipids (PLs).[142] A small study in cancer patients with different tumor types reported reduced concentrations of eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) in total plasma lipids in comparison with healthy subjects.[200] In lung cancer patients, reduced levels of EPA, DHA and linoleic acid (18:2n-6) in platelet phosphatidylcholine have been reported.[172] Mosconi et al.[152] described reduced levels of polyunsaturated fatty acids in malnourished cancer patients, but did not report any specific data regarding n-3 fatty acids. In colorectal cancer patients, no significant alterations in plasma n-3 fatty acid levels were detected.[18] Most studies are small, however, and to our knowledge, direct comparisons between different tumor types have not been made.

Recent studies have reported potentially beneficial effects of n-3 fatty acid supplementation in cancer such as inhibition of weight loss[15,17,230] and a prolonged survival.[76] Although the mechanisms underlying these effects are not known, they may be related with impaired n-3 fatty acid status in cancer patients.

The aim of the present study was to investigate whether plasma n-3 fatty acid concentrations are reduced in patients with pancreatic cancer, lung cancer and esophageal cancer when compared with healthy subjects.

Subjects and Methods

Subjects

The study protocol was approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam. Written informed consent was obtained from every subject before inclusion into this study. Fifteen patients with pancreatic cancer, 22 patients with non-small cell lung carcinoma and 35 patients with esophageal or cardia carcinoma were included in the study. All patients were newly diagnosed and untreated, and were recruited in consultation with clinicians of the Departments of Surgery, Pulmonary Diseases, Oncology and Thoracic Surgery of the University Hospital Rotterdam, The Netherlands. Exclusion criteria were a previous history of malignant disease and previous

anti-tumor treatment. Forty-five healthy subjects were included as a control. Exclusion criteria for the healthy subjects were a history of malignant disease and the presence of diabetes, which was defined as treatment with insulin, oral antidiabetics or a special diet. Mean alcohol consumption, smoking behavior and weight loss over the previous six months were recorded using a questionnaire, as were diseases other than cancer. Age, sex, weight, height and medication were also noted.

Determination of plasma fatty acid composition

A heparinized blood sample was collected from every subject after an overnight fast, and was centrifuged at 1600 g at 4°C for 10 minutes. Plasma samples were frozen under nitrogen at -80 °C until analysis. Fatty acid composition of plasma PLs and cholesteryl esters (CEs) was determined as described in *Chapter 5*. Briefly, plasma samples were extracted using a chloroform-methanol mixture (2:1 by vol.) according to Folch et al..[66] Lipid fractions were separated by thin-layer chromatography, CE and PL fractions were scraped off and fatty acids were transmethylated by an acetylchloride-methanol mixture (1:20 by vol.) according to Lillington et al..[125] Fatty acid compositions were analyzed by Gas Liquid Chromatography (GLC; Carlo Erba HRGC 5160) using a FID detector and a CP-sil 88 column (Chrompack; length 50m, diameter 0.25mm) using 'on column' injection. The carrier gas was helium (pressure: 150 kPa). The temperature program started at 90°C, and then increased to 170°C by 10° per minute, continuing to 220°C with 2° per minute, and to the final temperature of 225°C by 1° per minute, until the end of analysis. In the chromatograms of the PL fractions, the following fatty acids were identified: palmitic acid (16:0), stearic acid (18:0), linoleic acid (18:2n-6), dihomo-γ-linolenic acid (20:3n-6), arachidonic acid (20:4n-6), EPA (20:5n-3), docosapentaenoic acid (22:5n-3), docosahexaenoic acid (22:6n-3) and the total sum of vaccenic acid (18:1n-7) and oleic acid (18:1n-9). Since 18:1n-7 and 18:1n-9 could not be appropriately separated in all chromatograms, the sum of 18:1n-7 and 18:1n-9 was used for data analysis (referred to as 18:1). 18:1n-7 accounted for approximately 16% of total 18:1 in plasma PLs. Total n-3 fatty acids in PLs were calculated as the sum of 20:5n-3, 22:5n-3 and 22:6n-3. In CE, the fatty acids 16:0, palmitoleic acid (16:1n-7), 18:0, 18:1, 18:2n-6, α-linolenic acid (18:3n-3), γ-linolenic acid (18:3n-6), 20:3n-6, 20:4n-6, 20:5n-3 and 22:6n-3 were identified. In plasma CE, 18:1n-7 accounted for about 5% of total 18:1. Total n-3 fatty acids in CEs were calculated as the sum of 18:3n-3, 20:5n-3 and 22:6n-3. The weight percentage of each fatty acid was calculated by defining the sum of all nominated fatty acids as 100 percent. Three desaturation indices were calculated as follows (based on the PL data):

Δ9-desaturation index = 18:1 / 18:0

Δ6-desaturation index = [(20:4n-6 + 20:3n-6 + 18:3n-6) / 18:2n-6]

Δ5-desaturation index = [(20:4n-6 / (20:3n-6 + 18:3n-6 + 18:2n-6)]

Table 4.1

Characteristics of healthy subjects and cancer patients.[a]

	Healthy subjects (n=45)	Pancreatic cancer (n=15)	Lung cancer (n=22)	Esophageal cancer (n=35)
Age (y)[b]	59 (50-83)	64 (45-78)	66 (45-83)	63 (32-79)
Sex	26 M; 19 F	6 M; 9 F	15 M; 7 F	28 M; 7 F
Body weight (kg)	74.3 ± 2.0	67.4 ± 4.1§†	76.6 ± 3.1	75.5 ± 1.8
Body mass index (kg/m^2)	24.0 ± 0.7	24.6 ± 1.1	26.0 ± 1.0	24.5 ± 0.5
Weight loss (kg)[c]	0.1 ± 0.1	7.1 ± 1.2‡	3.8 ± 1.7‡	4.9 ± 0.8‡
Weight loss (%)[c]	0.05 ± 0.13	10.1 ± 1.8‡	4.8 ± 2.0‡	5.7 ± 0.9‡
Alcohol use (units/week)	6.7 ± 1.1	2.1 ± 1.1	8.1 ± 2.6	15.4 ± 2.4$^{\#\S\ddagger}$
Smoking (cig/day)	1.3 ± 0.6	3.7 ± 1.1	7.9 ± 1.6‡	7.8 ± 2.2‡
Prevalence of NIDDM[d]	n=0 (0%)	n=6 (40%)§†‡	n=2 (9.5%)	n=3 (8.6%)

[a] Mean ± SEM

[b] Median (range)

[c] Weight loss in the preceding six months

[d] NIDDM: Non-Insulin Dependent Diabetes Mellitus

‡ Significantly different from healthy subjects ($P < 0.05$)

$^{\#}$ Significantly different from pancreatic cancer ($P < 0.05$)

§ Significantly different from lung cancer ($P < 0.05$)

† Significantly different from esophageal cancer ($P < 0.05$)

C-reactive protein (CRP) was measured in plasma of cancer patients using a standard assay based on the principle of immunological agglutination (Boehringer Mannheim GmbH, Mannheim, Germany).[129]

Statistical analysis

Results are expressed as mean ± SEM unless otherwise stated. Differences between the four groups were assessed using one-way analysis of variance, followed by the post-hoc least-significant difference method. Differences between two groups were assessed using the Student's t-test, and Pearson's correlation coefficients were calculated between fatty acid concentrations and other factors. For statistical analyses on CRP concentrations, analysis of variance including tumor type as a covariate was used and log-transformation was applied because of skewed distribution. An inflammatory response was assumed to be

present when CRP > 10 mg/L.[210] CRP levels were not measured in healthy subjects, since they were assumed to be normal in this group (< 10 mg/L).[3] Statistical analyses were performed by SPSS for Windows 6.1.3 (SPSS Inc., Chicago). Differences were considered significant when $P \leq 0.05$.

Table 4.2

Fatty acid composition of plasma phospholipids (PL) in healthy subjects and in cancer patients. Figures are expressed as weight percentage of total PL.[a,b]

Fatty acids in PL	Healthy subjects (n=45)	Pancreatic cancer (n=15)	Lung cancer (n=22)	Esophageal cancer (n=35)
16:0	29.1 ± 0.2	30.4 ± 0.5 ‡	30.1 ± 0.5 ‡	30.2 ± 0.4 ‡
18:0	14.8 ± 0.2	14.3 ± 0.5	15.0 ± 0.4	14.4 ± 0.3
18:1[c]	10.6 ± 0.2	12.0 ± 0.6 †‡	10.8 ± 0.4	10.8 ± 0.3
18:2n-6	24.7 ± 0.5	22.3 ± 0.9 §‡	24.6 ± 0.8 #†	21.8 ± 0.6 §‡
20:3n-6	3.2 ± 0.1	3.0 ± 0.3	3.4 ± 0.2	3.5 ± 0.2
20:4n-6	11.2 ± 0.4	12.3 ± 0.9 §	10.5 ± 0.7	11.9 ± 0.4
20:5n-3	1.2 ± 0.1	0.7 ± 0.1 †‡	0.9 ± 0.1 †	1.3 ± 0.2
22:5n-3	1.1 ± 0.1	0.9 ± 0.1 †‡	1.0 ± 0.1	1.1 ± 0.1
22:6n-3	4.1 ± 0.2	4.2 ± 0.4	3.9 ± 0.4	5.0 ± 0.2 §‡
Indices:				
total n-3	6.5 ± 0.4	5.7 ± 0.5	5.7 ± 0.5	7.5 ± 0.4 §#
total n-6	39.0 ± 0.5	37.6 ± 0.5	38.5 ± 0.5	37.2 ± 0.5 ‡

[a] Mean ± SEM

[b] Statistical differences are assessed by one-way ANOVA with post-hoc LSD method

[c] 18:1 refers to the sum of 18:1n-7 and 18:1n-9

‡ Significantly different from healthy subjects ($P < 0.05$)

Significantly different from pancreatic cancer ($P < 0.05$)

§ Significantly different from lung cancer ($P < 0.05$)

† Significantly different from esophageal cancer ($P < 0.05$)

Results

Baseline characteristics

Characteristics of the healthy subjects and cancer patients are summarized in Table 4.1. No differences in age were observed between any of the groups. Body weight was significantly reduced in pancreatic cancer patients when compared with all other groups. Mean weight loss in the previous six months was significantly different between all patient groups and healthy subjects, and ranged from 4.8±2.0% in lung cancer to 10.1±1.8% in pancreatic cancer. Alcohol use was significantly higher in esophageal cancer than in other groups, and patients with esophageal and lung cancer smoked significantly more cigarettes per day than healthy subjects. None of the patients had insulin-dependent diabetes, but non-insulin-dependent diabetes mellitus (NIDDM) was observed in 40% of the pancreatic cancer patients which was significantly more than in the other patient groups. Plasma fatty acid composition of the different patient groups is presented in Tables 4.2 and 4.3. Fatty acid ratios and desaturation indices are reported in Table 4.4.

Plasma fatty acid concentrations

Pancreatic cancer: Compared to healthy subjects, patients with pancreatic cancer showed considerable reductions in total n-3 fatty acid concentrations in plasma PLs as well as CEs (Tables 4.2 and 4.3), but this reduction only reached statistical significance in plasma CEs ($P < 0.05$). The fatty acids 20:5n-3 and 22:5n-3 showed significant reductions both in PLs and CEs in pancreatic cancer ($P < 0.05$), as did 18:3n-3, 18:2n-6, 18:3n-6 and total n-6 fatty acids in CEs ($P < 0.05$). Most saturated and monounsaturated fatty acids were higher in pancreatic cancer patients than in healthy subjects. The ratio of total polyunsaturated fatty acids to total saturated fatty acids (P/S ratio) was reduced in pancreatic cancer (Table 4.4; $P < 0.05$), whereas no significant difference in n-3/n-6 ratio was detected between patients and controls. The Δ9- and Δ5-desaturation indices in PLs were elevated in pancreatic cancer ($P < 0.05$). Pancreatic cancer patients with NIDDM showed higher concentrations of 20:5n-3, 22:5n-3, 22:6n-3 as well as total n-3 fatty acids than patients without diabetes, both in plasma PLs and CEs (Table 4.5; $P < 0.05$). Only 18:3n-3 in plasma CEs did not differ between the two groups.

Lung cancer: Although levels of n-3 fatty acid concentrations in plasma PLs and CEs tended to be reduced in lung cancer, these reductions failed to reach statistical significance (Tables 4.2 and 4.3). The observed reductions in total n-3 fatty acids in PLs and CEs were not statistically significant, nor were the changes in 20:5n-3 and 22:6n-3. 18:3n-3 was significantly reduced in plasma CEs ($P < 0.05$), whereas 16:0 and 18:0 were significantly elevated in plasma PLs and CEs ($P < 0.05$). No significant differences in

Table 4.3

Fatty acid composition of plasma cholesteryl esters (CE) in healthy subjects and in cancer patients. Figures are expressed as weight percentage of total CE.[a,b]

Fatty acids in CE	Healthy subjects (n=45)	Pancreatic cancer (n=15)	Lung cancer (n=22)	Esophageal cancer (n=35)
16:0	11.8 ± 0.2	13.4 ± 0.4 §†‡	11.5 ± 0.3	12.3 ± 0.3 §#
16:1n-7	2.8 ± 0.2	3.1 ± 0.2	2.6 ± 0.3	2.8 ± 0.3
18:0	1.6 ± 0.0	2.2 ± 0.3 †‡	2.1 ± 0.1 ‡	1.9 ± 0.1
18:1[c]	18.9 ± 0.4	21.3 ± 0.8 §‡	19.0 ± 0.6	20.4 ± 0.5 ‡
18:2n-6	54.6 ± 0.8	49.6 ± 1.1 §‡	54.9 ± 1.1	51.5 ± 1.1 §‡
18:3n-6	0.8 ± 0.1	0.5 ± 0.1 †‡	0.8 ± 0.1	0.8 ± 0.1
20:3n-6	0.7 ± 0.0	0.9 ± 0.1 ‡	0.8 ± 0.1	0.9 ± 0.1 ‡
20:4n-6	6.9 ± 0.3	7.7 ± 0.8	6.6 ± 0.5	7.3 ± 0.4
18:3n-3	0.5 ± 0.0	0.3 ± 0.0 §†‡	0.4 ± 0.0 ‡	0.4 ± 0.0 ‡
20:5n-3	1.0 ± 0.1	0.5 ± 0.1 †‡	0.8 ± 0.1	1.1 ± 0.1
22:6n-3	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Indices:				
total n-3	2.0 ± 0.1	1.3 ± 0.1 †‡	1.7 ± 0.2	2.2 ± 0.2
total n-6	63.0 ± 0.7	58.7 ± 1.1 §†	63.1 ± 0.9	60.5 ± 0.9 §†

[a] Mean ± SEM

[b] Statistical differences are assessed by one-way ANOVA with post-hoc LSD method

[c] 18:1 refers to the sum of 18:1n-7 and 18:1n-9

‡ Significantly different from healthy subjects ($P < 0.05$)

Significantly different from pancreatic cancer ($P < 0.05$)

§ Significantly different from lung cancer ($P < 0.05$)

† Significantly different from esophageal cancer ($P < 0.05$)

fatty acid ratios or desaturation indices were detected between lung cancer patients and healthy subjects, except for a 6% reduction in P/S ratio (Table 4.4; $P < 0.05$).

Esophageal cancer: Plasma n-3 fatty acid concentrations in PLs and CEs in esophageal cancer showed a tendency for higher values than in healthy subjects, although

Table 4.4

Desaturation indexes in plasma phospholipids in healthy subjects and in cancer patients[a,b]

Index	Healthy subjects (n=45)	Pancreatic cancer (n=15)	Lung cancer (n=22)	Esophageal cancer (n=35)
P/S ratio[c]	1.0 ± 0.0	1.0 ± 0.0 ‡	1.0 ± 0.0 ‡	1.0 ± 0.0 ‡
n-3/n-6 ratio	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0 §#
20:4n-6/18:2n-6	0.5 ± 0.0	0.6 ± 0.1 §	0.5 ± 0.0	0.6 ± 0.0 §‡
Δ9-desaturation index[d]	0.7 ± 0.0	0.9 ± 0.1 ‡	0.7 ± 0.0	0.8 ± 0.0
Δ6-desaturation index[e]	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.0 §‡
Δ5-desaturation index[f]	0.4 ± 0.0	0.5 ± 0.1 §‡	0.4 ± 0.0	0.5 ± 0.0 §‡

[a] Mean ± SEM

[b] Statistical differences are assessed by one-way ANOVA with post-hoc LSD method)

[c] P/S ratio; ratio total polyunsaturated fatty acids / total saturated fatty acids

[d] Δ9-desaturation index: 18:1 / 18:0

[e] Δ6-desaturation index: [(20:4n-6 + 20:3n-6 + 18:3n-6) / 18:2n-6]

[f] Δ5-desaturation index: [20:4n-6 / (20:3n-6 + 18:2n-6 + 18:3n-6)]

‡ Significantly different from healthy subjects ($P < 0.05$)

Significantly different from pancreatic cancer ($P < 0.05$)

§ Significantly different from lung cancer ($P < 0.05$)

‡ Significantly different from esophageal cancer ($P < 0.05$)

the difference did not reach statistical significance (Tables 4.2 and 4.3). The slight increase in n-3 fatty acid levels was mainly the result of elevated concentrations of 22:6n-3, especially in plasma PLs. As in the other patients groups, 18:3n-3 showed a significant reduction in plasma CEs ($P < 0.05$). Total n-6 fatty acid concentrations were significantly reduced in both plasma PLs and CEs ($P < 0.05$). The Δ6- and Δ5-desaturation indices were elevated by 23% and 20%, respectively (Table 4.4; $P < 0.05$).

Relation with weight loss

In PLs and CEs of lung cancer patients with weight loss, concentrations of 20:5n-3 and total n-3 fatty acids were significantly lower than those patients without weight loss (Table 4.6; $P < 0.05$). In esophageal cancer, the n-3 fatty acid content was not different between weight-losing and weight-stable patients. The effect of weight loss could not be assessed in pancreatic cancer since all patients of this tumor type had lost weight in the previous six months.

Table 4.5

n-3 Fatty acids in plasma phospholipids (PL) and cholesteryl esters (CE) in pancreatic cancer patients with and without non-insulin dependent diabetes mellitus (NIDDM).
Figures are expressed as weight percentage of total PL and CE.[a,b,c]

Fraction	Fatty acid	No NIDDM (n=9)	With NIDDM (n=5)	*P*-value
Plasma PL	20:5n-3	0.5 ± 0.1	1.0 ± 0.1	0.001
	22:5n-3	0.8 ± 0.1	1.2 ± 0.1	0.002
	22:6n-3	3.5 ± 0.2	6.0 ± 0.5	0.000
	total n-3	4.8 ± 0.3	8.2 ± 0.5	0.000
Plasma CE	18:3n-3	0.3 ± 0.3	0.2 ± 0.0	0.307
	20:5n-3	0.4 ± 0.1	0.8 ± 0.1	0.003
	22:6n-3	0.5 ± 0.1	0.8 ± 0.1	0.012
	total n-3	1.1 ± 0.1	1.8 ± 0.2	0.004

[a] Mean ± SEM

[b] Statistical differences are assessed by the Student's t-test

[c] Presence or absence of DM unknown in one patient

Relation with inflammation

Plasma CRP concentrations in pancreatic cancer were 20 (1 - 385) mg/L (median and range), in lung cancer 21 (0 - 205) mg/L, and in esophageal cancer 5 (0 - 214) mg/L. Plasma CRP concentrations were higher than 10 mg/L in 14 lung cancer patients (63.6%), in 9 esophageal cancer patients (25.7%) and in 10 pancreatic cancer patients (66.7%). In patients with lung and pancreatic cancer, plasma CRP concentrations were significantly higher than in patients with esophageal cancer ($P < 0.05$). After correction for tumor type, patients with CRP concentrations higher than 10 mg/L showed significantly reduced levels of total n-3 fatty acids ($P = 0.04$) and 22:5n-3 ($P = 0.02$) in PLs, and of 18:3n-3 in CEs ($P = 0.003$), when compared with patients with CRP concentrations < 10 mg/L. Total n-3 fatty acid concentrations also tended to be reduced in plasma CEs of patients with CRP > 10 mg/L, although this difference did not reach statistical significance ($P = 0.06$). The P/S ratio and the n-3/n-6 ratio were also lower in patients with CRP values > 10 mg/L ($P = 0.02$ and $P = 0.07$, respectively).

Table 4.6

n-3 Fatty acids in plasma phospholipids (PL) and cholesteryl esters (CE) in weight-losing and weight-stable lung cancer patients.
Figures are expressed as weight percentage of total PL and CE.[a,b,c,d]

Fraction	Fatty acid	Weight-losing (n=10)	Weight-stable (n=11)	*P*-value
Plasma PL	20:5n-3	0.6 ± 0.1	1.1 ± 0.1	0.01
	22:5n-3	1.0 ± 0.1	1.0 ± 0.1	0.17
	22:6n-3	3.2 ± 0.3	4.4 ± 0.7	0.12
	total n-3	4.8 ± 0.4	6.5 ± 0.8	0.06
Plasma CE	18:3n-3	0.3 ± 0.1	0.4 ± 0.1	0.19
	20:5n-3	0.5 ± 0.1	1.0 ± 0.2	0.02
	22:6n-3	0.6 ± 0.1	0.7 ± 0.1	0.62
	total n-3	1.4 ± 0.1	2.1 ± 0.3	0.03

[a] Weight-losing: when weight loss in previous six months > 5%

[b] Presence or absence of weight loss unknown in one patient

[c] Mean ± SEM

[d] Statistical differences are assessed by the Student's t-test

Relation with other factors

No significant correlations were detected between plasma n-3 fatty acid concentrations and factors such as body weight, BMI, alcohol intake, or smoking behavior.

Discussion

In the present study, we investigated whether plasma n-3 fatty acids were reduced in different types of cancer. Results showed that differences in fatty acid pattern exist between different tumor types. In pancreatic cancer, plasma total n-3 fatty acids were significantly reduced in plasma CEs. In lung cancer, total n-3 fatty acids tended to be reduced as well, but these differences failed to reach statistical significance. In esophageal cancer, the total n-3 fatty acid concentrations in plasma PLs and CEs were comparable to or even higher than those in healthy subjects. Total n-3 fatty acid levels were lower in lung cancer patients with weight loss than in those without weight loss, and pancreatic cancer patients with diabetes showed significantly higher levels of total n-3 fatty acids than those patients without diabetes. For all tumor types combined, total n-3 fatty acid levels were

Table 4.7

Fatty acids in plasma phospholipids (PL) and cholesteryl esters (CE) in cancer patients with and without an inflammatory response, as defined by C-reactive protein (CRP) concentrations > 10 mg/L. Figures are expressed as weight percentage of total PL and CE.[a,b]

Fraction	Fatty acid	CRP ≤ 10 mg/L (n=39)	CRP > 10 mg/L (n=33)	*P*-value
Plasma PL	20:5n-3	1.2 ± 0.1	0.9 ± 0.1	0.14
	22:5n-3	1.1 ± 0.1	0.9 ± 0.1	0.02
	22:6n-3	4.8 ± 0.3	4.1 ± 0.3	0.10
	total n-3	7.1 ± 0.4	6.0 ± 0.4	0.04
	n-3/n-6 ratio	0.19 ± 0.01	0.16 ± 0.01	0.07
	P/S ratio[c]	1.00 ± 0.01	0.97 ± 0.01	0.02
Plasma CE	18:3n-3	0.4 ± 0.0	0.3 ± 0.0	0.00
	20:5n-3	1.0 ± 0.1	0.7 ± 0.1	0.14
	22:6n-3	0.7 ± 0.1	0.6 ± 0.1	0.38
	total n-3	2.0 ± 0.2	1.6 ± 0.1	0.06

[a] Mean ± SEM

[b] Statistical differences are assessed by analysis of variance with correction for tumor type

[c] P/S ratio; ratio total polyunsaturated fatty acids / total saturated fatty acids

reduced in plasma PL and CE in patients with CRP plasma levels > 10 mg/L in comparison with patients with CRP levels < 10 mg/L.

Fatty acids in plasma are present mainly as components of triacylglycerols (TAGs), CEs and PLs. We measured fatty acid composition in plasma PLs and CEs, since these fractions are good indicators of the resultant of dietary fat intake[133] and probably also of metabolic demands, and the day-to-day variation of their fatty acid composition is smaller than in TAGs.[151] Furthermore, fatty acid composition of the plasma PL fraction is closely related to that of PLs in platelet and erythrocyte membranes.[28,169]

The patient groups included in the present study all represent cancer patients at risk to develop nutritional deficiencies. The underlying mechanisms may, however, differ between the different groups, since contributing factors such as decreased food intake, reduced uptake and increased metabolic demands may differ between tumor types. The observed presence of an acute-phase response has previously been reported in patients with lung cancer[209,232] and pancreatic cancer,[13,62,64] and appears to be related with plasma levels of n-3 fatty acids in the present study. Previous studies in non-cancer patients with

an evident inflammatory response reported that plasma n-3 fatty acid levels were inversely correlated with inflammation.[61,163] Plasma n-3 fatty acids were also significantly lower in patients with rheumatoid arthritis than in healthy subjects.[208] An inflammatory response in humans is also attenuated by supplementation of n-3 fatty acids.[26,33] These findings may indicate that reduced plasma n-3 fatty acid levels in patients could contribute to an inflammatory state. Another possible explanation of reduced plasma n-3 fatty acid levels might be an increased metabolic demand for n-3 fatty acids during inflammation, since n-3 and n-6 fatty acids are the precursor molecules for inflammatory substances such as prostaglandins and leukotrienes.

Yet another factor that might have contributed to the difference in fatty acid pattern between lung cancer patients and healthy subjects is smoking, since smoking has been reported to be inversely associated with 22:6n-3 and 20:4n-6 in serum PLs and CEs.[194] However, we were not able to detect a significant correlation between smoking behavior and plasma n-3 fatty acids in the present study.

Plasma n-3 fatty acid levels in pancreatic cancer were even lower than those in lung cancer. Patients with pancreatic cancer often suffer from intestinal malabsorption due to impaired excretion of pancreatic enzymes.[225] Reduced absorption of polyunsaturated fatty acids may subsequently result in essential fatty acid deficiency.[112] The observation that n-3 fatty acid levels in pancreatic cancer patients with NIDDM are not reduced but are instead comparable to those in healthy subjects, is difficult to explain. Some studies in non-cancer NIDDM patients reported reduced plasma levels of polyunsaturated fatty acids in comparison to healthy subjects,[173,176,190] whereas one study in newly diagnosed NIDDM patients reported increased levels of elongated polyunsaturated fatty acids.[167] Further studies are needed to identify the underlying mechanism of the relation between the presence of NIDDM and plasma n-3 fatty acid levels in pancreatic cancer.

In esophageal cancer, alcohol intake was higher than in the other patient groups, which may explain the elevated n-3 fatty acids plasma levels observed in our study. Previous studies showed that plasma n-3 fatty acid levels were higher in moderate drinkers than in nondrinkers.[195] and that 22:6n-3 and 18:3n-3 were elevated in esophageal tissue of rats administered ethanol, especially after tumor induction.[153] Furthermore, weight loss may have played a role in the changes in plasma lipid composition. In contrast with many other tumor types, weight loss in esophageal cancer is thought to be the result of decreased food intake due to inability to swallow, rather than of metabolic alterations.[63] When energy intake is reduced, fatty acids are mobilized from the adipose tissue to meet energy requirements. Since the rate of mobilization is higher for n-3 fatty acids than for saturated fatty acids,[37,41,180] the proportion of n-3 fatty acids in blood may increase. Thus, a study in obese women showed that total n-3 fatty acids increased during weight reduction,[39] and it may partly explain the relatively high concentrations of n-3 fatty acids in esophageal

cancer patients in the present study. However, this is not consistent with the fact that we did not detect any difference in n-3 fatty acids between esophageal cancer patients with and without weight loss.

We conclude that differences in plasma fatty acid composition exist between different tumor types. When compared with healthy subjects, total n-3 fatty acids in plasma CEs are significantly reduced in pancreatic cancer, tend to be reduced in plasma PLs and CEs in lung cancer, but are unchanged in esophageal cancer. Further studies are warranted to clarify the mechanisms underlying the observed differences in n-3 fatty acid concentrations in cancer. This knowledge may contribute to better understanding of potential benefits of n-3 fatty acid supplementation in different types of cancer.

Acknowledgements

We gratefully acknowledge D. Dezentjé for his practical assistance. Furthermore, we thank all physicians and others who assisted us in the recruitment of patients, and we thank all healthy subjects who volunteered in our study.

5

INCORPORATION AND WASHOUT OF ORALLY ADMINISTERED N-3 FATTY ACID ETHYL ESTERS IN DIFFERENT PLASMA LIPID FRACTIONS

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Br J Nutr 1999;82(6):481-8
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Abstract

The aim of our study was to quantify the incorporation of EPA and DHA in plasma lipids after oral administration of n-3 fatty acid ethyl esters, since little is known about the rate and pattern of incorporation into plasma lipid fractions. In addition, we aimed to obtain pilot information regarding EPA half-life, which is needed to establish a optimal dosing schedule.

Five healthy volunteers ingested 2 x 8.5g n-3 fatty acid ethyl esters per day for 7 days, supplying 6.0g EPA and 5.3g DHA per day. The fatty acid compositions of plasma phospholipids (PL), cholesteryl esters (CE) and triacylglycerols (TAG) were determined during supplementation and during a washout period of 7 days. Half-lives of EPA and DHA were calculated.

The proportion of EPA in PL showed a 15-fold increase after 7 days ($P < 0.001$), while DHA showed a smaller increase ($P < 0.01$). In CE, EPA also increased ($P < 0.05$), while DHA did not increase at all. Remarkably, incorporation of DHA into TAG was even higher than that of EPA. Half-life of EPA in PL ranged from 1.63 to 2.31 days (1.97 ± 0.15 days; mean ± SEM), whereas mean half-life of EPA in CE was 3.27 ± 0.56 days. In three subjects, washout of EPA and DHA from TAG seemed to follow a bi-exponential pattern, with a short half-life (< 1 day) in the initial phase and a half-life of several days in the second phase. In conclusion, EPA ethyl esters are rapidly incorporated into plasma lipids, especially into PL. The relatively long half-life of EPA in plasma would permit a dosing schedule with intervals of ≥12 hours in supplementation studies.

Introduction

Beneficial health effects of n-3 fatty acids in fish oil have been described in several diseases. n-3 Fatty acids, especially eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA), are known to reduce plasma triacylglycerol (TAG) concentrations and may be beneficial in atherosclerosis and inflammatory diseases.[52,197]

Since fish oil contains a mixture of TAGs with various fatty acids, the concentration of n-3 fatty acids is relatively low. A higher concentration of EPA and DHA can be achieved by using a supplement of unesterified fatty acids. Ingestion of free fatty acids (FFA), however, causes gastrointestinal complaints and may be toxic.[23]

In contrast to FFA, no side effects are induced by n-3 fatty acid ethyl esters (EE), and EE would therefore be appropriate for supplementation of n-3 fatty acids. However, the absorption of EPA and DHA as EE may be lower than of EPA and DHA as TAG or FFA, since previous studies reported that the plasma incorporation of EPA- and DHA-EE was lower than the incorporation of EPA- and DHA-TAG after a single dose[23,57,122] as well as during prolonged supplementation of n-3 fatty acids as EE.[92] However, other studies did not find any differences in incorporation between supplements.[120,156,177] The diversity of results may partly be explained by the fact that some studies measured EPA and DHA only in plasma TAG and not in plasma phospholipids (PL) or cholesteryl esters (CE). Before using n-3 fatty acid EE in a clinical setting, it is important to determine the actual level of incorporation when using n-3 fatty acid EE.

In addition, information about the kinetics of EPA and DHA is needed to devise proper dosing schedules. However, only limited data are available about the rate of incorporation and the half-life of EPA and DHA in blood, and the data available are mainly restricted to single dose supplementation. A few studies investigating the rate of incorporation of EPA after a single dose of oral EPA-EE reported a maximal incorporation of EPA into TAG after 4-6 hours.[57,122,156] In studies with prolonged supplementation of n-3 fatty acid EE or TAG, EPA was found to reach its maximum level within one week.[92,98] In contrast, the rise in DHA continued for several weeks.[92,98] Although this difference in incorporation between EPA and DHA may be due to a difference in EPA or DHA concentration in the supplement, it may also be caused by a difference in metabolism between EPA and DHA. Some studies have shown that the decrease in EPA after cessation of supplementation is much more rapid than that of DHA,[98] but no information is available about the half-life of EPA and DHA in plasma lipids.

The first aim of our study was to determine the level of incorporation of EPA and DHA in different plasma lipid fractions during supplementation with n-3 fatty acid EE. In addition, we attempted to quantify the half-life of EPA and DHA in the different plasma lipid fractions.

Subjects and methods

Subjects

Study subjects were five healthy volunteers, four females and one male, of normal weight (body mass index 20-25) and ages between 23 and 30 years. None had a history of gastrointestinal complaints or metabolic or other serious diseases. All subjects consumed a normal Dutch diet, containing not more than one meal of fish per week. From one week before start of the protocol until the end of the study, subjects did not ingest any fish or fish products apart from the supplement. This study was approved by the Medical Ethical Committee of the University Hospital Dijkzigt, Rotterdam.

Experimental protocol

Duration of the study was 14 days: 7 days of n-3 fatty acid EE supplementation and 7 days of washout. On day 0, subjects attended the outpatient department after an overnight fast. After 15min of rest, venous blood samples were taken in EDTA tubes. Hereafter, subjects consumed 8.5g of n-3 fatty acid EE as a fluid (Incromega E2573; Croda Oleochemicals, North Humberside, England) twice daily with breakfast and supper respectively, for a period of 7 days. To improve the taste, the n-3 fatty acid EE were mixed with two drops of peppermint oil just before consumption. The supplement contained 35.0 w/w% EPA and 31.2 w/w% DHA according to information from the supplier, resulting in an intake of 6g EPA and 5.3g DHA/d. The fatty acid composition of the supplement is shown in Table 5.1.

Table 5.1

Contents of the relevant fatty acids in the n-3 fatty acid ethyl ester supplement.

Fatty acid	w/w% of total fatty acids
18:1n-9	4.7
18:2n-6	1.5
18:3n-3	0.4
20:3n-6	0.3
20:4n-6	2.2
20:4n-3	1.0
20:5n-3 (EPA)[a]	35.0
22:5n-3	7.5
22:6n-3 (DHA)[b]	31.2
Other fatty acids	19.1

[a] EPA: eicosapentaenoic acid

[b] DHA: docosahexaenoic acid

Subsequently, fasting blood samples were taken during supplementation in the morning of days 1, 2, 3, 4 and 7. To determine the rate of washout of EPA and DHA, blood sampling was continued on days 8, 9, 10, 11 and 14. Blood samples were placed in ice immediately after sampling and centrifuged within 5min. Plasma was stored in -70°C under nitrogen until analysis within 6 weeks.

Fatty acid composition

Plasma lipids were extracted using chloroform/methanol (2:1, by vol.; Merck; Darmstadt, Germany) according to Folch et al.[66] in the presence of butylated hydroxytoluene (1mg/ml) as antioxidant. Prior to extraction, internal standards were added to be able to calculate absolute plasma concentrations of PL, CE and TAG (purity 96%, 74% and 95%, respectively). Plasma lipids were separated into PL, TAG and CE by thin layer chromatography (silica plates; Merck, 5721) using hexane/diisopropylether/acetic acid (60:40:3, by vol.; Merck) as a developer. Fatty acids were converted into their methyl ester using a mixture of acetyl chloride (Merck) and methanol.[125] Fatty acid analyses were performed by Gas Liquid Chromatography (GLC; Carlo Erba HRGC 5160) using a FID detector, a Chrompack CP-sil 88 column (length 50m, diameter 0.25mm), an uncoated methyl deactivated retention gap, helium as a carrier gas and 'on column' injection. Concentrations of individual fatty acids are presented as mole per 100 mole and not as absolute values, because of the statistically significant decreases in total plasma PL, CE and TAG concentrations.

Biochemical analyses

Plasma TAG concentrations were measured on day 0, 2, 7 and 11 using a standard enzymatic assay from Boehringer-Mannheim (no. 917; Mannheim, Germany). Plasma FFA concentrations were measured on day 0, 2, 7 and 11 using an enzymatic colorimetric method (NEFA C test kit; Wako Chemicals, Neuss, Germany). Total plasma PL and CE were determined on all days by adding up the GLC peak areas of all fatty acids for the fraction concerned.

Calculation of EPA and DHA half-life

The Scientist® software package (MicroMath Scientific Software, Utah) was used for least squares fitting of the data during the washout period, using a mono-exponential and a bi-exponential model (Powell, 1970):

Mono-exponential model:

Concentration = a*exp((-b)*time)

Half-life = 0.693/b

Bi-exponential model:

Concentration = a1*exp((-b1)*time) + a2*exp((-b2)*time)

Half-life 1 = 0.693/b1

Half-life 2 = 0.693/b2

The Model Selection Criterion (MSC) was defined by the formula:

$$MSC = \ln\left[\frac{\sum_{i=1}^{n} w_i (Yobs_i - Yobs)^2}{\sum_{i=1}^{n} w_i (Yobs_i - Ycal_i)^2}\right] - \frac{2p}{n}$$

where w_i are the weights applied to the points, Y_{obsi} is the observed value of Y, Y_{cal} is the calculated value of Y and n is the number of data points.

The MSC attempts to represent the 'information content' of a data set, and indicates whether the model is suitable for the data or not. To calculate EPA and DHA half-life, the model with the largest MSC is in principle the best model for the given data, and was used for data analysis. A MSC<0.5 was considered to be inadequate to give a good fit of the data. When one of the calculated parameter values was identical to the upper or lower limits purposely set as model constraints, data were considered invalid and were excluded from statistical analyses.

Statistical analysis

Based on an estimated mean difference of 3 mol/100 mol, a standard deviation of 1.4 mol/100 mol and use of the paired t-tests, power of the study was 0.94. Results are expressed as mean ± SEM. Changes in plasma lipid concentrations were tested by the Student paired t-test, and in the figures by the Proc Mixed procedure for repeated measures of the SAS statistical software package. *P*-values less than 0.05 were considered to be significant.

Results

n-3 Fatty acid EE supplementation was well tolerated by all subjects. However, when belching occurred, a fishy smell or taste was noted. All subjects completed the study, but in one subject the blood sample of day 4 could not be taken. During supplementation, concentrations of individual plasma lipid fractions showed moderate to substantial changes (Table 5.2). Plasma TAG concentrations decreased significantly during supplementation (days 2 and 7; $P < 0.05$), returning to near baseline values after 4 days of wash out (day 11). Plasma free fatty acid (FFA) concentrations also decreased significantly within 2 days of supplementation ($P = 0.01$) and remained low during

supplementation, although the difference from baseline after 7 days failed to reach statistical significance ($P = 0.07$). Both plasma PL and CE concentrations showed a significant decrease during n-3 fatty acid EE supplementation.

Supplementation of n-3 fatty acid EE for one week resulted in considerable changes in plasma fatty acid composition. In plasma PL, a highly significant increase of EPA (20:5n-3) was observed, approaching a plateau level after approximately 4 days (Figure 5.1). During washout, the EPA concentration decreased rapidly, with an almost complete return to baseline values after 7 days of washout. A significant increase was also found for DHA (22:6n-3) concentrations in plasma PL, although this increase was much less pronounced than that of EPA. Arachidonic acid (20:4n-6) also showed a slight increase, which reached significance only on days 2 and 3 when using the paired t-test. In contrast, levels of 18:2n-6, 18:1n-9 and 20:3n-6 in plasma PL decreased significantly during supplementation, and returned to initial levels by day 14. No significant changes in plasma PL were found for 22:5n-3.

Table 5.2

Plasma concentrations (mmol/L) of total phospholipids (PL), cholesteryl esters (CE), triacylglycerols (TAG) and free fatty acids (FFA) before (day 0), during (day 1-7) and after (day 8-14) supplementation of fish oil ethyl esters.[a]

Day	PL[b,c]	CE[b]	TAG	FFA
0	1.26 ± 0.09	2.29 ± 0.21	0.84 ± 0.15	0.29 ± 0.04
1	1.21 ± 0.09	2.01 ± 0.13		
2	1.16 ± 0.08	1.85 ± 0.13	0.50 ± 0.13[d]	0.12 ± 0.02[d]
3	1.10 ± 0.09	1.86 ± 0.16		
4	1.11 ± 0.11	1.75 ± 0.14		
7	1.15 ± 0.08	1.85 ± 0.11	0.51 ± 0.04[d]	0.14 ± 0.05
8	1.19 ± 0.08	1.89 ± 0.10		
9	1.26 ± 0.08	1.95 ± 0.08		
10	1.25 ± 0.10	1.92 ± 0.09		
11	1.31 ± 0.09	1.98 ± 0.07	0.72 ± 0.09	0.27 ± 0.03
14	1.29 ± 0.07	2.04 ± 0.06		

[a] Mean ± SEM; n=5.

[b] Significant decrease during supplementation ($P < 0.05$; repeated measures).

[c] Significant increase during washout period ($P < 0.05$; repeated measures).

[d] Significantly different from day 0 ($P < 0.05$; paired t-test).

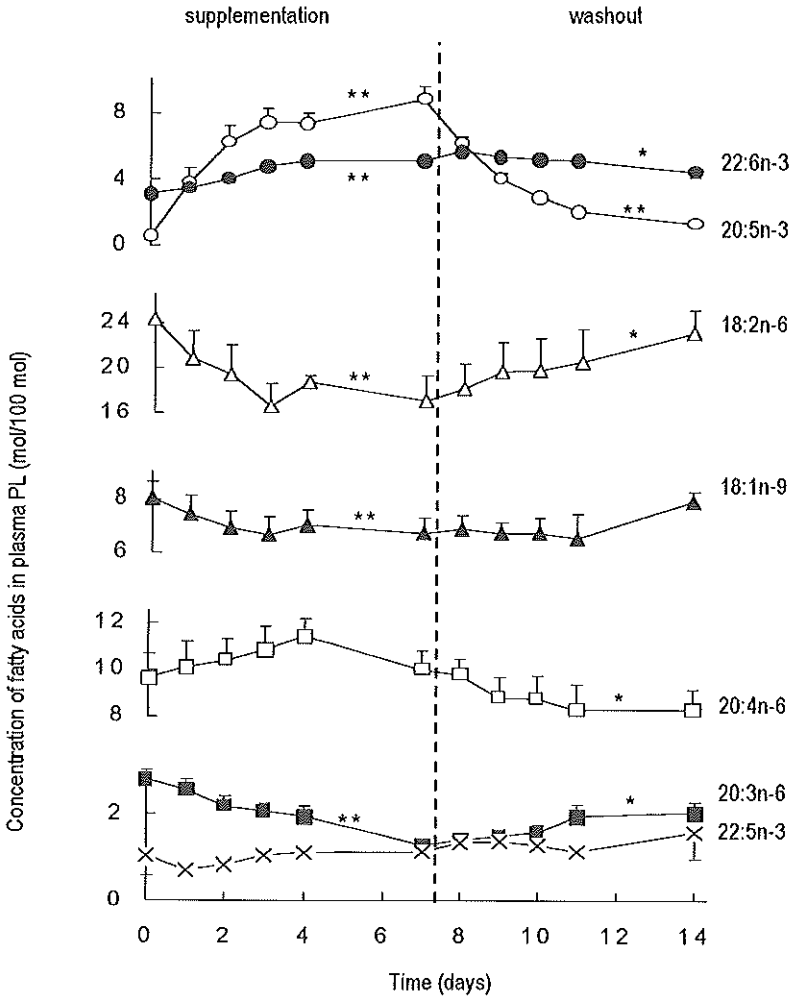

Figure 5.1

The concentration of different fatty acids in plasma phospholipids (PL) during daily intake of n-3 fatty acid ethyl esters (17 g/d) and during the washout period (in mole percent of total fatty acids in the PL fraction).

* $P < 0.05$ (repeated measurements analysis)

↔ $P < 0.0001$ (repeated measurements analysis)

In plasma cholesteryl esters (CE), changes in fatty acid composition were less pronounced than in plasma PL (Figure 5.2). Incorporation of EPA into the CE fraction was less rapid than in the PL fraction, and the EPA concentration did not reach a plateau level within 7 days. Subsequently, the decrease of EPA during washout was less rapid than in PL. DHA incorporation into CE was minimal, while the proportion of 20:4n-6 increased significantly. A small and insignificant increase of 20:3n-6 and 22:5n-3 was found. No changes were detected in the levels of 18:2n-6 and 18:1n-9 in plasma CE during supplementation.

A completely different pattern of incorporation was observed for plasma TAG (Figure 5.3): here, DHA was incorporated to an even higher extent than EPA, in contrast to the incorporation into PL and CE. Both fatty acids showed a rapid decrease during washout. EPA had completely returned to baseline on day 14, while DHA was still significantly increased by this time. While incorporation of 22:5n-3 into PL and CE was minimal, a fivefold increase of 22:5n-3 was detected in TAG, reaching a plateau value within 2 days. This was followed by a rapid return to baseline values during washout. In addition, 20:4n-6 showed a significant increase on days 2 and 3 (paired t-test), while 20:3n-6 was significantly increased at day 1. This was accompanied by a decrease in 18:1n-9, which only reached significance on day 3 (paired t-test). Again, no significant changes in the levels of 18:2n-6 in TAG were found during the study.

Plasma half-life of EPA could be calculated in all plasma lipid fractions, but calculation of half-life of DHA was only possible in plasma TAG, due to the high scatter of data in plasma PL and the small changes of DHA in plasma CE. Individual washout curves are shown in Figure 5.4.

The washout of EPA from plasma PL was well fitted by a mono-exponential model, resulting in an EPA half-life ranging from 1.63 to 2.31days (1.97 ± 0.15 days; Mean ± SEM) (Table 5.3). The high MSC value showed that the model used was suitable for the data. In plasma CE, data were also best fitted mono-exponentially, although the MSC value was lower than in PL due to the higher intra-individual scatter. Half-life of EPA in CE could be calculated in four subjects and ranged from 1.63 to 4.08 days (3.27 ± 0.56 days).

The decrease of EPA in plasma TAG was initially fitted by the mono-exponential model (Table 5.3). Half-life of EPA in TAG could be calculated in four subjects and ranged from 0.47 to 1.63 days (mean 1.12 days). However, in three of them, the MSC value showed an increase when fitting the data with a bi-exponential model, whereas in the fourth subject, the use of a bi-exponential model was not possible. In the three subjects, the initial half-life of EPA ranged from 0.11 to 0.77 days (0.41± 0.19 days), whereas half-life in the second phase was 1.29-4.17 days (2.92 ± 0.86).

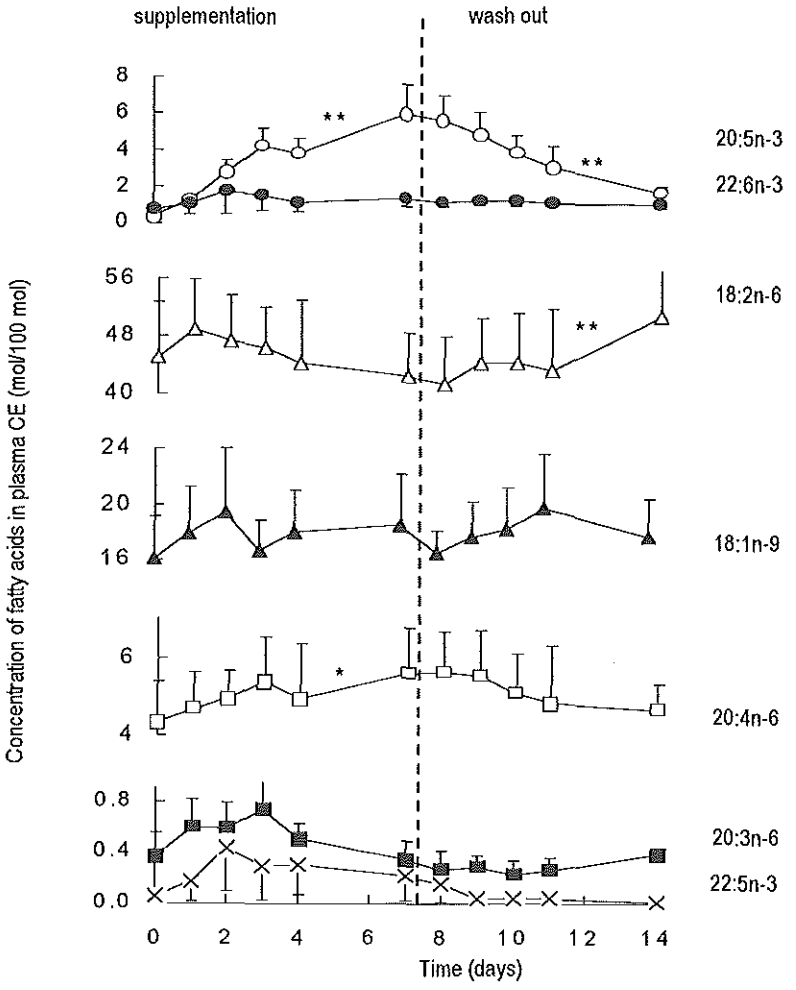

Figure 5.2

The concentration of different fatty acids in plasma cholesteryl esters (CE) during daily intake of n-3 fatty acid ethyl esters (17 g/d) and during the washout period (in mole percent of total fatty acids in the CE fraction).

* $P < 0.05$ (repeated measurements analysis)

** $P < 0.0001$ (repeated measurements analysis)

For the decrease of DHA in plasma TAG, comparable results were found (Table 5.3). A half-life ranging from 0.89 to 2.57 days was found with a mean of 1.63 days when using the mono-exponential model. Use of the bi-exponential model resulted in an increase of the MSC value. The initial half-life of DHA in plasma TAG ranged from 0.18 to 0.38 days, followed by a half-life of 3.81-5.32 days in the second phase (4.75 ± 0.33 days).

Discussion

Our study demonstrates that n-3 fatty acid EE are absorbed from the intestine, as was shown by the significant increase of EPA and DHA in different plasma lipid fractions during supplementation. In general, a relatively consistent pattern of incorporation and washout was shown in most lipid fractions with small deviations between subjects.

EPA was incorporated into PL and CE, as well as in TAG. In contrast, the DHA incorporation pattern was quite different: a very low incorporation into CE, but a high incorporation into TAG which was even higher than that of EPA. Although not all data could be used for the calculation of plasma half-lives, this study provides pilot information regarding washout of EPA and DHA from plasma. During washout, the decrease of EPA in PL and CE was found to be mono-exponential with mean half-lives of 1.97 and 3.27 days, respectively. In plasma TAG, however, the data of EPA and DHA were better fitted by a bi-exponential washout model, with a short initial half-life of less than 1 day, followed by a longer half-life of several days.

The incorporation of EPA into plasma lipids was considerably higher than the incorporation of DHA in our study. This finding is consistent with results from other studies investigating the incorporation of n-3 fatty acid TAG[92,156] as well as studies in which EE were used.[74,123,207] Only Blonk et al.,[27] who used a supplement of n-3 fatty acid ethyl esters, did not find any difference in incorporation between EPA and DHA into plasma PL after 12 weeks of supplementation. In these studies, supplementation ranged from a single dose[74] until one year of supplementation.[123] The pattern of incorporation of different fatty acids appeared to be independent of the different fish oil types and dosages. Sadou et al. investigated whether the intramolecular structure of n-3 fatty acid TAG affects the incorporation of EPA and DHA.[185] They found that DHA (mainly situated at the sn-2 position) was mainly incorporated into plasma TAG, while EPA (predominantly situated at sn-1/3) was mainly incorporated into plasma PL.[185] They concluded that the difference in incorporation was due to the intramolecular structure. However, our study shows that a supplement containing EE instead of TAG resulted in a similar plasma distribution, which would suggest that the incorporation pattern is the result of properties of EPA and DHA rather than of the position of the fatty acid on the glycerol backbone.

Table 5.3

Half-lives of EPA and DHA (days) in different plasma lipid fractions, calculated by a mono-exponential and a bi-exponential model. The higher the value of the Model Selection Criterion (MSC; an indicator for the suitability of the model), the better the model.

		Mono-exponential		Bi-exponential		
	Subject	half-life	(MSC)[a]	half-life 1	half-life 2	(MSC)[a]
EPA in PL[a]	1	2.02	(5.1)	-[b]	-	-
	2	2.31	(3.9)	-	-	-
	3	1.63	(3.6)	-	-	-
	4	2.27	(3.8)	-	-	-
	5	1.65	(3.5)	-	-	-
	Mean	1.97		-	-	
	SE	0.16		-	-	
EPA in CE[a]	1	[c]	-	-[b]	-	-
	2	4.08	(1.9)	-	-	-
	3	3.59	(2.9)	-	-	-
	4	3.80	(4.7)	-	-	-
	5	1.63	(1.5)	-	-	-
	Mean	3.27		-	-	
	SE	0.56		-	-	
EPA in TAG[a]	1	0.47	(3.6)	0.11	1	(5.4)
	2	1.05	(4.0)	[d]	[d]	-
	3	1.37	(2.7)	0.77	4	(2.8)
	4	1.06	(2.0)	0.33	3	(3.6)
	5	1.63	(1.5)	[e]	[e]	-
	Mean	1.12		0.41	3	
	SE	0.19		0.19	0.86	
DHA in TAG[a]	1	0.89	(1.9)	0.35	5	(3.5)
	2	1.18	(2.0)	0.38	4	(4.4)
	3	2.57	(0.9)	0.35	5	(1.1)
	4	1.89	(0.9)	0.18	5	(2.2)
	5	[c]	-	[c]	[c]	-
	Mean	1.63		0.31	5	
	SE	0.38		0.04	0	

[a] PL, phospholipids; CE, cholesteryl esters; TAG, triacylglycerols; MSC, Model Selection Criterion.

[b] No bi-exponential fit possible due to good fit of mono-exponential model.

[c] No suitable fit due to high scatter of data.

[d] No suitable fit because parameter value was equal to estimated upper constraint.

[e] No suitable fit due to negative value of half-life 2.

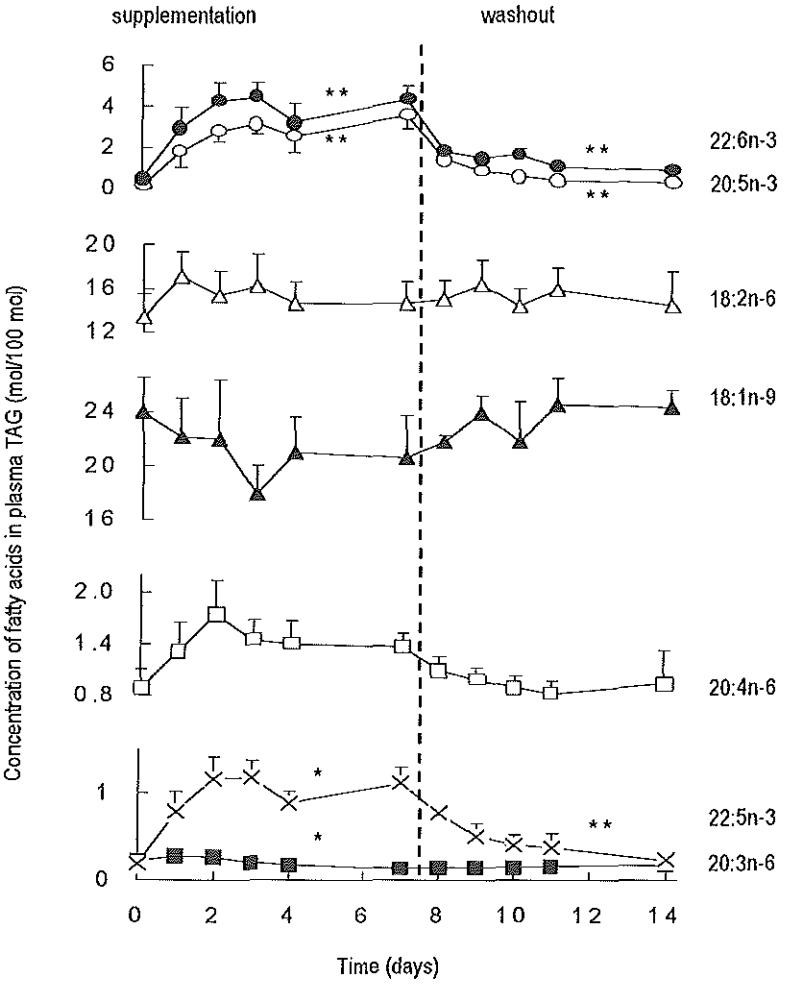

Figure 5.3

The concentration of different fatty acids in plasma triacylglycerols (TAG) during daily intake of n-3 fatty acid ethyl esters (17 g/d) and during the washout period (in mole percent of total fatty acids in the TAG fraction).

* $P < 0.05$ (repeated measurements analysis)

** $P < 0.0001$ (repeated measurements analysis)

A more plausible explanation for the difference in incorporation between EPA and DHA was given by Subbaiah et al..[207] These authors suggested that the difference is caused by the fact that EPA and DHA are competitors for the enzymatic transfer of fatty acids from PL to CE. The affinity of the enzyme lecithin-cholesterol acyltransferase is higher for EPA than for DHA. Thus, the conversion of EPA from PL to CE is higher compared to DHA. This would explain why, in our study, incorporation of EPA into CE is high, while the incorporation of DHA in CE is negligible. It would also explain why DHA incorporation into PL was more rapid than into CE, a finding that has also been described in other studies.[29,185,207]

When the transfer of DHA from PL to CE is reduced, a relative accumulation of DHA in PL would be expected. Indeed, in our study DHA in PL was still increasing after one week of supplementation. However, the increase of DHA in PL was less rapid than the increase of EPA. This could be explained by a relatively high clearance of DHA compared to EPA from the plasma towards the adipose tissue, since DHA has been suggested to be the preferred storage form of n-3 fatty acids.[113]

N-3 fatty acid supplementation was also shown to induce changes in other fatty acids than EPA and DHA in the present study. We observed a small increase in 20:4n-6 in plasma PL on day 2 and 3, followed by a decrease in concentration. It is likely that the observed increase is the result of the small amount of 20:4n-6 in the supplement. However, this increase does not correspond to other studies, which generally report a decrease in 20:4n-6 during n-3 fatty acid supplementation,[92,120,135,171,177] most likely as a consequence of competition between the n-3 and n-6 fatty acids. Yet, when examining literature data in detail, some of these studies show a temporary increase in 20:4n-6 during the first week of supplementation,[92,177,224] which is in accordance with our findings. This observation combined with the significant decrease of 18:2n-6 in PL indicates that incorporation of EPA and DHA into PL in the first days of supplementation mainly occurs at the expense of 18:2n-6 and not of 20:4n-6.

Not only was the incorporation level of EPA higher than that of DHA, but also the incorporation rate. Similarly, the decline during washout was more rapid for EPA than for DHA. The same finding has also been described by others[98,135,136,207] who showed that DHA had not returned to baseline values within 4 weeks of washout. So far, no information has been available about the washout in the separate plasma lipid fractions. In our study, the decrease of EPA in the PL and CE fractions was mono-exponential, implicating that EPA probably originates from one pool, i.e. plasma PL. Unfortunately, it was not possible to calculate half-life of DHA in PL and CE due to the high intraindividual scatter and the slow rate of washout.

As for plasma TAG, our results would indicate a bi-exponential washout of EPA as well as DHA. The slow decline in the second phase of washout may be due to the release

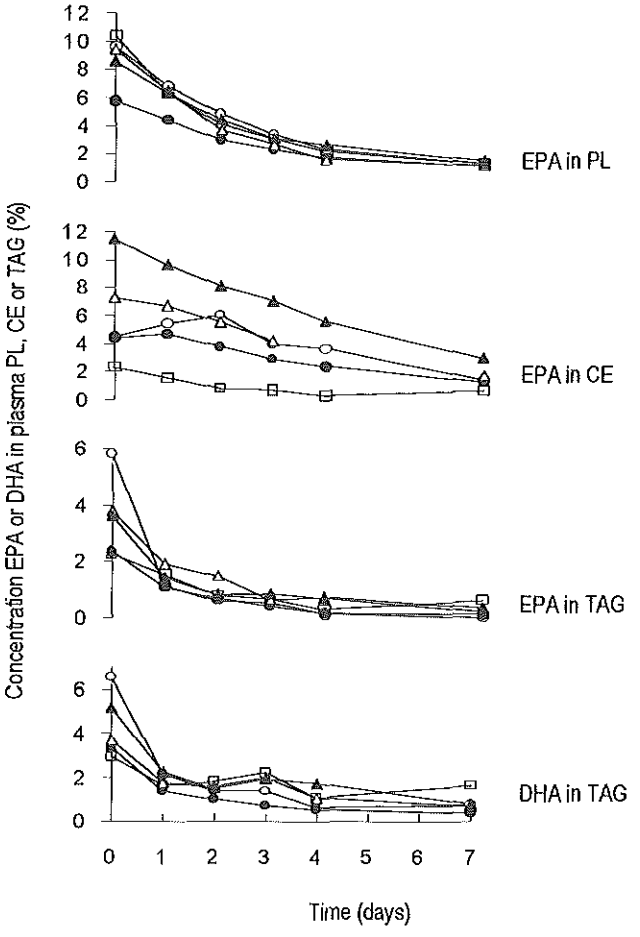

Figure 5.4
Individual washout curves of EPA from plasma phospholipids (PL), cholesteryl esters (CE) and triacylglycerols (TAG), and of DHA from plasma TAG.

of n-3 fatty acids from body stores. EPA and DHA are incorporated into TAG by the liver to be incorporated into VLDL and to re-enter the plasma pool. The half-life in the second phase is longer for DHA than for EPA, which is consistent with the notion that DHA is the main storage form of the n-3 fatty acids.[113]

In summary, we conclude that n-3 fatty acids ethyl esters are sufficiently well incorporated into plasma lipids to allow the use of n-3 fatty acid ethyl esters in clinical practice. Our results suggest that the washout of EPA and DHA from plasma PL and CE is mono-exponential, but that EPA and DHA washout from TAG may follow a bi-exponential pattern. Based on the relatively long half-life of the n-3 fatty acids in plasma, we conclude that supplementation with 6g of EPA in a frequency of once per 24 hours would probably be sufficient to obtain a substantial increase in plasma EPA and DHA levels.

Acknowledgements

This work was supported by Numico Research B.V., Wageningen, The Netherlands.

6

EFFECTS OF EICOSAPENTAENOIC ACID ETHYL ESTER SUPPLEMENTATION ON WHOLE-BODY LIPOLYSIS AND PALMITATE OXIDATION IN HEALTHY SUBJECTS

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Abstract

Supplementation with fish oil may play a role in the prevention and treatment of cardiovascular diseases by reducing serum triacylglycerol (TAG) and free fatty acid (FFA) concentrations. Although the mechanism underlying this effect has not yet been clarified, reduced lipolysis and/or increased lipid oxidation may play a role. The aim of the present study was to assess the effects of short-term eicosapentaenoic acid-ethyl ester (EPA-EE) supplementation on lipolysis and lipid oxidation in healthy subjects.

In sixteen healthy subjects (10M, 6F; age 40- 75y), whole-body lipolysis (using [1,1,2,3,3-$^{2}H_5$]glycerol) and palmitic acid release (using [1-^{13}C]palmitic acid) were measured at baseline, and after 2 and 7 days of EPA-EE (6 g/d) or placebo (oleic acid-EE, OA-EE; 6 g/d) supplementation using a double-blind, randomized study design. Palmitate oxidation was determined by measuring $^{13}CO_2$ enrichment in expired breath.

No significant differences in whole-body lipolysis, palmitate oxidation, or palmitic acid release were detected between the groups receiving EPA-EE and OA-EE supplementation for one week. It was remarkable, however, that both palmitic acid release and palmitate oxidation showed substantial reductions in both treatment groups as compared with baseline values.

Introduction

Epidemiological studies have suggested that cardiovascular disease[10,78,111,149,202] and non-insulin-dependent diabetes mellitus (NIDDM)[67,165] are associated with elevated levels of triacylglycerol (TAG) and/or free fatty acid (FFA) in blood, and that it may be beneficial to reduce plasma lipid concentrations in subjects at risk.[4] Since it has been shown that supplementation of fish oil reduces serum TAG[1,81,166,175] and FFA[45] levels, fish oil may play a role in the prevention and treatment of cardiovascular diseases[42,138,160] and NIDDM.[77]

The main constituents of fish oil are eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Several studies showed that EPA, but not DHA, is responsible for the TAG-lowering effect of fish oil,[71,175,231] although other investigators reported that both EPA and DHA had a TAG-lowering effect.[81] Despite extensive research, there is still much uncertainty about the mechanism by which n-3 fatty acids reduce plasma lipid concentrations. The decrease in serum TAG concentrations appears to be caused by a reduction in hepatic TAG synthesis.[88,183] This reduction may in part be related to low substrate availability due to decreased plasma FFA concentrations.[45,89,109,182,198]

Two different mechanisms have been proposed to explain the reduction in plasma FFA concentrations by n-3 fatty acids. Firstly, reduced peripheral lipolysis may be involved, since EPA was shown to inhibit lipolysis *in vitro*.[213] Secondly, enhanced fatty acid (FA) oxidation may play a role, since mitochondrial and/or peroxisomal β-oxidation appeared to increase during fish oil supplementation.[1,70,231] However, a study in rats showed a reduction rather than an increase in whole-body fat oxidation with fish oil, despite increased hepatic peroxisomal FA oxidation.[181] In view of these paradoxical findings, it remains unclear whether reduced lipolysis or increased fat oxidation is the prevalent mechanism by which fish oil attenuates serum TAG concentrations.

The aim of the present study was to assess the effects of short-term EPA-ethyl ester (EPA-EE) supplementation on lipolysis and lipid oxidation in healthy subjects.

Subjects and methods

Subjects and study design

Sixteen weight-stable, healthy subjects (10 M; 6 F) aged 40 - 75 y were included in the study. The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam, and written informed consent was obtained from all subjects prior to start of the study. Exclusion criteria were: surgery in the last two months, underlying metabolic disease such as liver disease, diabetes, hyper- or hypothyroidism,

hart failure or fever, treatment with anti-epileptic drugs, corticosteroids or xanthine deviates, anorexia, extremely restrictive diets and alcohol consumption > 15 units per week.

After a baseline measurement of whole-body lipolysis and palmitate oxidation on day 0, subjects were randomized over two groups according to a double-blind study design. One group received a supplement of 6 grams of EPA-EE (purity 96.8%, EPA95EE; Pronova, Sandefjord, Norway) per day for one week, and the other group received 6 grams of oleic acid (OA)-EE (purity 79%, Ethyl Olivate; Croda Oleochemicals, North Humberside, England) as a control for one week. Supplements were provided in 0.5g capsules, to be taken as 3x2g with the main meals. On days 2 and 7, measurements of lipolysis and palmitate oxidation were repeated according to the same protocol as on day 0. All participants filled in dietary records from four days before the first measurement until the end of the study period.

Measurement of lipolytic rate and CO_2 production

On day 0, subjects attended the outpatient department between 8 and 9 a.m. after an overnight fast of approximately 12 hours. Fifteen minutes after arrival, teflon catheters were inserted into the antecubital vein of one arm and into the contralateral dorsal hand vein or forearm vein of the other arm. Baseline blood and breath samples were taken, blood was centrifuged immediately and plasma was stored at -80°C under nitrogen until analysis. After baseline sampling, infusion of a pasteurized plasma protein solution (40 g protein/l, albumin ≥85%; CLB, Amsterdam, The Netherlands) containing [1,1,2,3,3-$^2H_5$]glycerol (MassTrace, Woburn, USA) and [1-^{13}C]palmitic acid (MassTrace) was started using a calibrated syringe pump (Perfusor Secura; B.Braun, Melsungen, Germany). Glycerol was administered by primed-constant infusion with a priming dose of 1.2 μmol/kg and an infusion rate of approximately 0.08 μmol/kg.min and palmitic acid was given by a constant infusion rate of approximately 0.04 μmol/kg.min. To prime the plasma bicarbonate pool, a bolus of $NaH^{13}CO_3$ dissolved in saline (1.7 μmol/kg) was administered intravenously. The exact infusion rates were determined afterwards by measuring isotope enrichment in the infusate. During isotope infusion, CO_2 production was measured for 30 minutes by indirect calorimetry using a ventilated hood system (DeltatracTM MBM-100, DATEX/Instrumentarium Corp., Helsinki, Finland), using only data from the last 20 minutes for analysis. Respiratory quotient (RQ) was calculated using CO_2 production and O_2 consumption. After 50, 60, 70, 80 and 90 minutes of infusion, arterialized venous blood samples[239] were taken for determination of plasma [$^2H_5$]glycerol and [1-^{13}C]palmitate enrichment. Blood samples were placed on ice immediately, and centrifuged and stored at the end of the infusion period. Breath samples were taken to measure $^{13}CO_2$ enrichment. Weight and height were measured.

Analysis of blood samples

Plasma [$^2H_5$]glycerol and [1-^{13}C]palmitate enrichment were measured as reported previously (Chapter 2). In brief, for measuring plasma [$^2H_5$]glycerol enrichment, plasma was deproteinized by subsequently adding and mixing water, $CuSO_4$ and Na_2WO_4. After centrifugation, the supernatant was applied to a mixed ionexchange column (AG50W-X8, AG1-X8, 200-400 mesh, 0.2 gram each; Biorad, Richmond, USA) and glycerol was converted to its derivative by MTBSTFA during a one-hour incubation at 60°C. For measuring plasma [1-^{13}C]palmitate enrichment, lipids were extracted from plasma using chloroform/methanol according to Folch et al.,[66] plasma FFA were isolated by thin layer chromatography (silica plates; Merck, 5721) and converted to their derivatives by MTBSTFA (N-methyl-N-(tert-butylmethylsilyl)-trifluoroacetamide; Pierce, Rockford, IL).

Plasma enrichment of free [$^2H_5$]glycerol and [1-^{13}C]palmitic acid was analyzed on a Carlo Erba GC8000 gaschromatograph coupled to a Fisons MD800 mass spectrometer (Interscience BV, Breda, The Netherlands). Natural glycerol and [$^2H_5$]glycerol (fragments m/z 377 and 381) and natural palmitic acid and [1-^{13}C]palmitic acid (fragments m/z 313 and 314) were measured by selected ion monitoring. Breath samples were analyzed on an isotope ratio mass spectrometer (ABCA; Europa Scientific, Van Loenen Instruments, Leiden, The Netherlands).

Serum TAG concentrations were measured using a standard enzymatic assay from Boehringer-Mannheim (no. 917; Mannheim, Germany), FFA were determined using an enzymatic colorimetric method (NEFA C test kit; Wako Chemicals, Neuss, Germany). Plasma glucose was measured by standard techniques, and plasma insulin was measured by radioimmunoassay (Biosource, Fleurus, Belgium).

Calculations

Calculations of whole-body lipolysis (glycerol) and palmitic acid release were made according to Klein et al.,[116] using the equation described by Steele:[205]

Rate of appearance (R_a; μmol/kg.min) = (IE_i/IE_p - 1) * F, where F is the isotope infusion rate (μmol/kg.min), IE_i is the isotopic enrichment of the infusate (atom percent excess) and IE_p is the isotopic enrichment (atom percent excess) in plasma during steady state conditions.

Palmitate oxidation was calculated according to the following equation:

Palmitate oxidation (μmol/kg.min) = (IE_{CO_2}/IE_p) * V_{CO_2}/BW, where IE_{CO_2} is the isotopic enrichment of the expired CO_2 (atom percent excess), V_{CO_2} is the CO_2 production (μmol/min) and BW is body weight (kg).

Table 6.1

Characteristics of study participants.[a]

	EPA group	OA group
Body weight (kg)	75 ± 11	79 ± 17
Body mass index (kg/m^2)	24 ± 3	27 ± 5
Age (yr)[b]	51 (40 - 65)	55 (48 - 75)
Sex	4 M; 4 F	6 M; 2 F

[a] Mean ± SD unless otherwise stated

[b] Median (range)

Statistical analyses

Data are expressed as mean ± SEM or as median (range) (as indicated). As R_a of palmitate, R_a of glycerol and palmitate oxidation showed a skewed distribution, log-transformed data were used for the statistical analyses. To analyze treatment effects, the Proc Mixed procedure for repeated measures of the statistical software package of SAS (SAS Inc., Cary, N.C. USA) was used. This procedure can be used for longitudinal regression analysis in which observations are collected on the same subjects over time.[137] The independent variables in the model were the treatment indicator variable, baseline measurement, time and interaction between time and treatment. Spearman's correlation coefficients were calculated. *P*-values less than 0.05 were considered statistically significant.

Results

Characteristics of subjects are summarized in Table 6.1. No differences in body weight, body mass index, age or sex were observed, although age tended to be lower in the group receiving EPA-EE than in the group receiving OA-EE. All subjects had normal

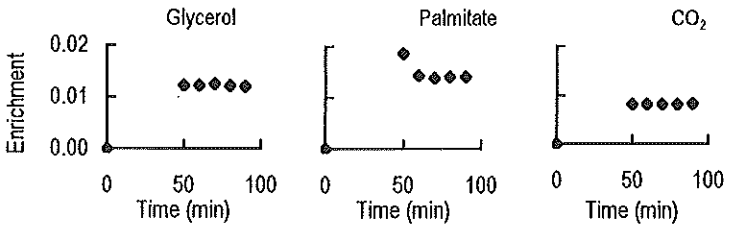

Figure 6.1

Steady state conditions were obtained for $^2H_5$-glycerol, ^{13}C-palmitate and $^{13}CO_2$ within 60 minutes during the turnover studies.

Table 6.2

Dietary intake during the study.[a,b,c]

		EPA	OA
Energy intake (kJ/d)	on day before day 0	8513 ± 2649	8422 ± 2894
	on day before day 2	8288 ± 1626	8880 ± 2763
	on day before day 7	8013 ± 2074	8435 ± 1928
Mean E-intake (kJ/d)	on 4 preceding days	8927 ± 1694	9027 ± 2548
	during study	8919 ± 1556	8956 ± 1474
Mean E%-fat	on 4 preceding days	36 ± 7	36 ± 6
	during study	34 ± 4	36 ± 5
Mean E%-carbohydrate	on 4 preceding days	47 ± 7	47 ± 4
	during study	51 ± 5	47 ± 5
Mean E%-protein	on 4 preceding days	17 ± 3	16 ± 4
	during study	16 ± 3	16 ± 3

[a] Mean ± SD

[b] No significant differences between treatment groups or between days

[c] E%: energy percentage

liver function tests. No significant differences were detected between the two treatment groups with regard to energy intake on the days preceding each measurement, nor in energy intake or energy percentages of fat, carbohydrate or protein (Table 6.2). During the measurements, isotopic steady state (as shown in Figure 6.1) was reached after approximately 60 minutes in all subjects.

No significant differences in whole-body lipolysis, palmitic acid release, palmitate oxidation, or RQ were observed between the groups receiving EPA-EE and OA-EE (Table 6.3). It was remarkable, however, that both palmitic acid release and palmitate oxidation tended to decrease with EPA-EE as well as with OA-EE supplementation.

No significant differences in serum TAG, FFA, glucose or insulin concentrations were detected between the two treatment groups (Table 6.4), despite a substantial reduction in serum TAG concentrations during both EPA-EE and OA-EE supplementation when compared with baseline values. Baseline plasma FFA or TAG concentrations were not significantly correlated with whole-body lipolysis, palmitic acid release or palmitate oxidation ($P > 0.25$). A significant correlation between the changes in whole-body lipolysis and serum TAG concentrations was only detected after two days of EPA-EE supplementation ($R = -0.85$, $P < 0.01$).

Table 6.3

Whole-body lipolysis (as measured by $^{2}H_{5}$-glycerol), palmitate release (as measured by ^{13}C-palmitate) and palmitate oxidation at baseline, and after two and seven days of eicosapentaenoic acid (EPA; n=8) or oleic acid (OA; n=8) ethyl ester supplementation (6 g/d).[a]

		day 0	day 2	day 7	*P*-value[b]
Whole-body lipolysis[c]	EPA[d]	4.05 ± 0.46	3.19 ± 0.54	3.77 ± 0.44	0.23
	OA	5.39 ± 0.73	5.48 ± 0.65	5.57 ± 1.01	0.52
Palmitate release[c]	EPA[d]	2.92 ± 0.47	2.78 ± 0.21	2.62 ± 0.36	0.03
	OA	3.79 ± 0.18	2.89 ± 0.13	3.28 ± 0.10	0.002
Palmitate oxidation[c]	EPA[d]	1.40 ± 0.23	1.11 ± 0.11	1.17 ± 0.17	0.01
	OA	1.54 ± 0.14	1.29 ± 0.08	1.39 ± 0.07	0.002

[a] Median ± SEM

[b] Prox Mixed for repeated measures analysis, comparison with baseline (see Methods)

[c] μmol/kg.min

[d] No significant differences between treatment groups (Proc Mixed for repeated measures)

Discussion

Despite extensive studies on the effects of fish oil on lipid metabolism, the mechanism by which n-3 fatty acids reduce serum TAG concentrations has remained unresolved. In the present study we investigated the effects of EPA-EE on whole-body lipolysis and lipid oxidation in sixteen healthy subjects using stable isotope tracer techniques. Results showed that whole-body lipolysis, palmitic acid release and palmitate oxidation were not significantly different between the groups receiving EPA-EE and OA-EE. It was remarkable, however, that both palmitic acid release and palmitate oxidation tended to decrease in both treatment groups.

In a previous study in healthy subjects, fish oil reduced plasma FFA concentrations by approximately 40 percent within one day of supplementation.[45] The observed effect was not explained by increased hepatic β-oxidation and/or ketogenesis alone, since blood concentrations of ketone bodies did not show any change. It was suggested that reduced lipolysis might be involved in the reduction of serum TAG levels by fish oil.[45] *In vitro* studies supported this suggestion by showing that EPA inhibited lipolysis in incubated adipocytes.[170,213] In the present study, however, no reduction in whole-body lipolysis was detected during EPA-EE supplementation. This may be related to the observation that *in vitro* measurements of lipolysis are not directly related with *in vivo* FFA metabolism.[126]

Table 6.4

Plasma concentrations of free fatty acids (FFA), triacylglycerols (TAG), glucose and insuline at baseline, and after two and seven days of EPA or OA ethyl ester supplementation (6 g/d; n = 8 + 8).[a]

		day 0	day 2	day 7	*P*-value[b]
FFA (µmol/L)	EPA[c]	794 ± 92	645 ± 77	633 ± 83	0.14
	OA	893 ± 97	840 ± 95	880 ± 107	0.20
TAG (mmol/L)	EPA[c]	1.04 ± 0.15	0.81 ± 0.12	0.83 ± 0.11	0.0004
	OA	1.19 ± 0.25	0.99 ± 0.2	0.97 ± 0.15	0.06
Glucose (mmol/L)	EPA[c]	4.40 ± 0.1	4.30 ± 0.1	4.40 ± 0.1	0.97
	OA	4.80 ± 0.2	4.60 ± 0.2	4.40 ± 0.2	0.26
Insulin (mmol/L)	EPA[c]	5.94 ± 0.57	7.11 ± 0.9	6.65 ± 0.84	0.35
	OA	8.44 ± 1.38	7.60 ± 1.30	8.46 ± 1.44	0.20

[a] Mean ± SEM

[b] Prox Mixed for repeated measures analysis, comparison with baseline (see Methods)

[c] No significant differences between treatment groups (Proc Mixed for repeated measures)

Our findings suggest that factors other than reduced lipolysis are involved in the TAG reducing effects of fish oil.

Although increased fat oxidation has been assumed to play a role in the effects of EPA on lipid metabolism,[141] in the present study, palmitate oxidation tended to be reduced instead of increased during EPA-EE supplementation. The generally made assumption that fat oxidation is increased with EPA appears to be based on the observation that peroxisomal β-oxidation, as measured by enzyme activity *in vitro*, was increased in hepatocytes from fish oil-fed rats.[1,82,89] It is, however, questionable whether an increase in peroxisomal β-oxidation would substantially enhance whole-body lipid oxidation since peroxisomal β-oxidation in rat tissue *in vitro* was shown to make up for only 14-46% of total fat oxidation, depending on the tissue and the type of fatty acid.[178] In other words, peroxisomal β-oxidation is probably not representative for overall fatty acid oxidation.

A study in fish oil-fed rats showed that total FA oxidation as measured by indirect calorimetry was reduced whereas whole-body energy expenditure was not affected by fish oil supplementation in these rats, indicating that the reduction in lipid oxidation was compensated for by increased carbohydrate utilization.[181] As far as we know, no studies have been reported on the effects of fish oil supplementation on whole-body lipid oxidation

in humans. Results from the present study suggest that the well-known reduction of serum TAG by EPA is not explained by increased lipid oxidation.

In the present study, no significant changes in plasma FFA levels were detected with EPA-EE which is in contrast with an earlier study in healthy subjects supplying a comparable dosage of EPA as TAG instead of EE for one week.[45] This inconsistency may imply that the esterification of EPA may affect the effects on lipid metabolism. A previous study in tumor-bearing rats showed that EPA-EE and EPA as free acid exerted different effects on weight loss.[100] It is possible that the chemical form and composition of the EPA supplement influences its physiological effects. Clearly, this issue would require further study.

It was remarkable that whole-body lipolysis, palmitic acid release and palmitate oxidation tended to decrease in the OA-EE group as well. Since we were not able to identify factors other than fatty acid supplementation that might have affected study outcome, the possibility cannot be excluded that OA-EE supplementation affected lipolysis as well. OA-EE might therefore not be suitable as a placebo supplement.

We conclude that supplementation of EPA-EE does not affect whole-body lipolysis, palmitate oxidation and palmitic acid release in comparison with OA-EE as a placebo supplement.

Acknowledgements

We gratefully acknowledge all subjects who volunteered in our study. For financial support, we thank Numico Research BV, Wageningen, The Netherlands. We also thank M. van Seters for her practical assistance, L. Hanff for preparing the isotope solutions, and P. Schmitz for his assistance in subject randomization and administration.

7

EICOSAPENTAENOIC ACID ETHYL ESTER SUPPLEMENTATION IN CACHECTIC CANCER PATIENTS: EFFECTS ON LIPOLYSIS AND LIPID OXIDATION

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Abstract

Recent reports suggested that weight loss in cachectic cancer patients may be inhibited by supplementation of the n-3 fatty acid eicosapentaenoic acid (EPA; 20:5n-3), presumably due to inhibition of lipolysis. The aim of the present double-blind randomized trial was to assess whether short-term oral EPA ethyl ester (EE) supplementation inhibits lipolysis and lipid oxidation in weight-losing cancer patients.

Seventeen weight-losing cancer patients of different tumor types and 16 healthy subjects were randomized to receive EPA-EE (6 g/d) or placebo (oleic acid (OA)-EE; 6 g/d) for seven days. At baseline (day 0) and during supplementation (days 2 and 7), whole-body lipolysis and palmitic acid release were measured in the overnight fasting state using [1,1,2,3,3-$^{2}H_5$]glycerol and [1-^{13}C]palmitic acid. Palmitate oxidation was determined by measuring $^{13}CO_2$ enrichment in expired breath.

No significant differences in whole-body lipolysis, palmitate oxidation, or palmitic acid release were detected between the groups receiving EPA-EE and OA-EE supplementation for one week. Also resting energy expenditure and plasma CRP concentrations did not differ significantly between the two treatment groups.

We conclude that EPA-EE do not significantly inhibit lipolysis or lipid oxidation in weight-losing cancer patients during short-term supplementation when using OA-EE as a placebo supplement.

Introduction

Cachexia is a frequent problem in cancer patients which is characterized by weight loss, impaired performance and fatigue.[75,127] In general, administration of nutritional support does not improve the condition,[155] nor has any other adequate treatment become available.[127] The mechanisms underlying cancer cachexia are poorly understood,[79] but it was suggested that increased lipolysis may play a role.[212,213] This is supported by the recent isolation of a lipid-mobilizing factor (LMF) from urine of weight-losing cancer patients[97,219] and by the increased whole-body lipolysis observed in cancer patients.[124,192]

Recent studies showed that the lipid-mobilizing effect of LMF *in vitro* was effectively attenuated by the n-3 fatty acid eicosapentaenoic acid (EPA), due to inhibition of the adenylate cyclase activity.[170] Furthermore, uncontrolled clinical studies in patients with pancreatic cancer suggested that EPA supplementation reversed weight loss for at least three months.[17,230] This was attributed to down-regulation of the acute-phase response by EPA as suggested by the decrease in serum C-reactive protein (CRP) concentrations.[229]

The aim of the present double blind, randomized study was to assess whether EPA ethyl esters (EE) inhibit lipolysis and lipid oxidation in weight-losing cancer patients during short-term supplementation.

Subjects and methods

Study design

In 17 weight-losing cancer patients, whole-body lipolysis, palmitic acid release, palmitate oxidation, and resting energy expenditure (REE) were measured. Thereafter, patients were randomized according to a double-blind study design. Nine patients received a supplement of 6 grams of EPA-EE (purity 96.8%, EPA95EE; Pronova, Sandefjord, Norway) per day for one week. The other patients received 6 grams of oleic acid (OA)-EE (purity 79%, Ethyl Olivate; Croda Oleochemicals, North Humberside, England) as a control for one week. The supplements, provided in 0.5g capsules, were taken as 3x2g with the main meals. Measurements of lipolysis and lipid oxidation were repeated on days 2 and 7, according to the same protocol as on day 0. Indirect calorimetry was only repeated on day 7. From four days before the first measurement until the end of the study period, all patients recorded their dietary intake. The same protocol was performed in 16 healthy subjects, randomized to receive EPA-EE or OA-EE.

Ethical permission was granted by the Medical Ethics Committee of the Erasmus University Medical Center of Rotterdam, and written informed consent was obtained from each subject.

Subjects

Seventeen weight-losing cancer patients were included in the study. Weight loss was defined as loss of body weight of more than 5% in the previous 6 months. Patients treated with chemotherapy or radiation therapy 2 weeks before start of the study, or elective surgery in the last 2 months were excluded from the study, as well as patients with corticosteroid treatment, insulin-dependent diabetes mellitus, uncontrolled hyper- or hypothyroidism, edema or fever.

The patient group receiving EPA-EE consisted of nine patients with the following tumor types: cancer of the upper gastrointestinal tract (4), pancreatic cancer (1), rectal cancer (1), renal cancer (1), breast cancer (1) and non-small cell lung cancer (1). All but one patient had metastatic disease and/or a locoregional relapse. Weight loss ranged from 6.9 to 18.1% in the preceding six months. The patient group receiving OA-EE consisted of eight patients with the following tumor types: esophageal cancer (1), pancreatic cancer (1), breast cancer (1), cervical cancer (1), carcinoid (1), adenocarcinoma of unknown primary (1), non-small cell lung cancer (1) and mesothelioma (1). Seven patients had metastatic disease and/or a locoregional relapse, whereas the patient with mesothelioma had direct invasion of the right liver lobe. Weight loss ranged from 5.3 to 17.1%. Furthermore, sixteen healthy subjects (10 M; 6 F) aged 40 - 75 y (54 ± 10 y; mean ± SD) were included in the study as a reference.

Measurements of lipolytic rate and REE

After an overnight fast of approximately 12 hours, subjects attended the outpatient department between 8 and 9 a.m. on the first day of the study period (day 0). Fifteen minutes after arrival, teflon catheters were inserted into the antecubital vein of one arm and into the contralateral dorsal hand vein or forearm vein of the other arm. Baseline blood and breath samples were taken, blood was centrifuged immediately and plasma was stored at -80°C under nitrogen until analysis. After baseline sampling, infusion of a pasteurized plasma protein solution (40 g protein/l, albumin ≥85%; CLB, Amsterdam, The Netherlands) containing [1-^{13}C]palmitic acid (MassTrace, Woburn, USA) and [1,1,2,3,3-$^{2}H_5$]glycerol (MassTrace) was started using a calibrated syringe pump (Perfusor Secura; B.Braun, Melsungen, Germany). Glycerol was administered by primed-constant infusion with a priming dose of 1.2 μmol/kg and an infusion rate of approximately 0.08 μmol/kg.min and palmitic acid was given by a constant infusion rate of approximately 0.04 μmol/kg.min. To prime the plasma bicarbonate pool, $NaH^{13}CO_3$ dissolved in saline was administered intravenously (1.7 μmol/kg). The exact infusion rates were determined afterwards by measuring isotope enrichment in the infusate.

During isotope infusion, resting energy expenditure (REE) was measured for 30 minutes by indirect calorimetry using a ventilated hood system (DeltatracTM MBM-100, DATEX/Instrumentarium Corp., Helsinki, Finland). REE and respiratory quotient (RQ) were calculated using the amount of O_2 consumed and of CO_2 produced during the last 20 minutes of the measurement. After 50, 60, 70, 80 and 90 minutes of infusion, arterialized venous blood samples[239] were taken for determination of plasma [$^2H_5$]glycerol and [1-^{13}C]palmitate enrichment. Blood samples were placed on ice immediately, centrifuged and stored at the end of the infusion period. Breath samples were taken to measure $^{13}CO_2$ enrichment. Weight and height were measured.

Analysis of blood samples

Plasma [$^2H_5$]glycerol and [1-^{13}C]palmitate enrichment were measured as described in *Chapter 2*. In brief, lipids were extracted from plasma using chloroform/methanol according to Folch et al..[66] Plasma free fatty acids were isolated by thin layer chromatography (silica plates; Merck, 5721) and converted to their derivatives by N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA, Pierce, Omnilabo, Breda, The Netherlands). To isolate plasma glycerol, plasma was deproteinized by subsequently adding H_2O, $CuSO_4$ and Na_2WO_4. After centrifugation, the supernatant was passed through a mixed ionexchange column (AG50W-X8, AG1-X8, 200-400 mesh, 0.2 g each; Biorad, Richmond, USA). Derivatives of glycerol were formed during incubation with pyridine and MTBSTFA for one hour at 60°C.

Plasma enrichments of free [$^2H_5$]glycerol and [1-^{13}C]palmitic acid were analyzed on a Carlo Erba GC8000 gaschromatograph coupled to a Fisons MD800 mass spectrometer (Interscience BV, Breda, The Netherlands). Natural palmitic acid and [1-^{13}C]palmitic acid (fragments m/z 313 and 314) and natural glycerol and [$^2H_5$]glycerol (fragments m/z 377 and 381) were measured by selected ion monitoring. Breath samples were analyzed on an isotope ratio mass spectrometer (ABCA; Europa Scientific, Van Loenen Instruments, Leiden, The Netherlands).

CRP, albumin and prealbumin were measured by standard techniques.

Calculations

Calculations of palmitate and glycerol turnover were made according to Klein et al.,[116] using the equation described by Steele.[205]

The rate of appearance (R_a) of palmitate and glycerol was calculated as follows: R_a (μmol/kg.min) = (IE_i/IE_p – 1) * F, where F is the palmitate isotope infusion rate (μmol/kg.min), IE_i is the isotopic enrichment of the infusate (atom percent excess) and IE_p is the isotopic enrichment (atom percent excess) in plasma during steady state conditions.

Palmitate oxidation was calculated according to the following equation:
Palmitate oxidation (μmol/kg.min) = (IE_{CO_2}/IE_P) * V_{CO_2}/BW, where IE_{CO_2} is the isotopic enrichment of the expired CO_2 (atom percent excess), V_{CO_2} is the CO_2 production (μmol/min) and BW is body weight (kg).

Statistical analyses

Data are expressed as mean ± SEM, unless stated otherwise. To analyze treatment effects, the Proc Mixed procedure for repeated measures of the statistical software package of SAS (SAS Inc., Cary, N.C. USA) was used. This procedure can be used for longitudinal regression analysis in which observations are collected on the same subjects over time.[137] The independent variables in the model were the treatment indicator variable, baseline measurement, time and interaction between time and treatment. In addition, energy intake on the day preceding the measurement was included as a covariate. Spearman's correlation coefficients were calculated. *P*-values less than 0.05 were considered statistically significant.

Table 7.1

Characteristics of weight-losing cancer patients.[a]

	EPA group (n=9)	OA group (n=8)
Age (y)	64 ± 3	64 ± 2
Sex	8 M; 1 F	5 M; 3 F
Weight (kg)	67 ± 3	63 ± 3
BMI (kg/m^2)	22 ± 1	22 ± 1
Weight loss (%)	13 ± 1	10 ± 2
Albumin (g/l)	39 ± 1	40 ± 2
Prealbumin (g/l)	0.17 ± 0.01	0.18 ± 0.01

[a] Mean ± SEM

Results

Characteristics of the patients included in the study are summarized in Table 7.1. No differences in age, sex, weight or biochemical parameters were observed between the treatment groups. In two patients receiving EPA-EE and two patients receiving OA-EE, the second measurement (*i.e.* after two days of supplementation) could not be performed due to physical weakness and no patent venous access, respectively. The measurement on day 7 could not be performed in one patient receiving EPA-EE because of surgery on day 4.

Table 7.2

Mean dietary intake (kJ/d) before and during EPA or OA supplementation (6 g/d), and dietary intake on the days preceding the measurements.[a]

	EPA group (n=9)	OA group (n=8)
Mean energy intake		
- At baseline (week 0)	8249 ± 942	6130 ± 720
- During supplementation (week 1)	8814 ± 1072	6494 ± 594
Energy intake on the day preceding the measurement on:		
- day 0	7701 ± 1113	5157 ± 580
- day 2	9695 ± 1224[b]	6457 ± 819
- day 7	7847 ± 1160	6033 ± 700

[a] Mean ± SEM

[b] $P \leq 0.05$ compared to day 0

Despite randomization, energy intake at baseline was higher in the EPA-EE group than in the OA-EE group. During supplementation, a temporary rise in dietary energy intake on the day preceding the measurement on day 2 was observed in patients receiving EPA-EE ($P < 0.01$; Table 7.2), which had returned to baseline values on the day preceding the third measurement on day 7. Compared to the OA-EE group, the increase in energy intake on day 2 in the EPA-EE group did not reach statistical significance. Since it was previously reported that lipolysis is negatively associated with total daily energy intake,[34,114] it was decided to include energy intake as a covariate in the statistical analyses.

No significant differences in whole-body lipolysis or palmitic acid release were observed between the two treatment groups (Figures 7.1 and 7.2, respectively). Although palmitate oxidation tended to decrease during EPA-EE supplementation, the difference between the groups did not reach statistical significance (Figure 7.3).

Plasma CRP concentrations did not change significantly with EPA-EE (37±15 and 41±17 mg/L, respectively) or with OA-EE (37±14 and 31±8 mg/L, respectively). Also REE did not show any changes during EPA-EE supplementation (93.7±2.9 kJ/kg.24h at baseline, and 94.6±3.7 kJ/kg.24h during supplementation), or during OA-EE supplementation (98.2±4.7 kJ/kg.24h at baseline, and during supplementation 100.0±3.9 kJ/kg.24h).

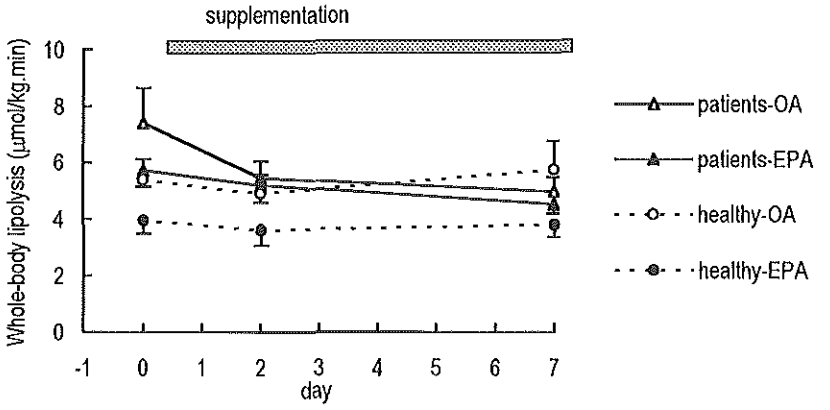

Figure 7.1

Whole-body lipolysis as measured by the rate of appearance of $^{2}H_5$-labeled glycerol in plasma of weight-losing cancer patients during supplementation of EPA (black symbols) or OA (open symbols). In comparison, whole-body lipolysis during EPA or OA supplementation in healthy subjects is shown (dotted lines). Mean ± SEM.

Discussion

In the present study, we investigated whether short-term supplementation with EPA-EE inhibits lipolysis and lipid oxidation in weight-losing cancer patients. Whole-body lipolysis, palmitic acid release, palmitate oxidation and REE were measured at baseline and during EPA-EE or OA-EE supplementation. Results revealed that EPA-EE supplementation does not affect whole-body lipolysis, palmitic acid release, palmitate oxidation or REE when compared with OA-EE as a placebo supplement.

In our study, no change in REE was detected during EPA supplementation, which is in agreement with a previous study reporting stabilization of REE during EPA supplementation.[230] Although we did not detect any significant changes in CRP concentrations, recent studies have reported significant reductions in CRP concentrations and stabilization of body weight after one month of supplementation of EPA as a free acid.[15,17,229,230] It is possible that our study period of one week was too short to detect any significant changes in lipolysis or plasma CRP levels. Findings by Dagnelie et al.[45] suggested, however, that the effect of EPA on lipid metabolism would be rapid, since plasma FFA levels were significantly decreased within one day of fish oil supplementation. Furthermore, a previous study showed that EPA-EE were rapidly incorporated into plasma

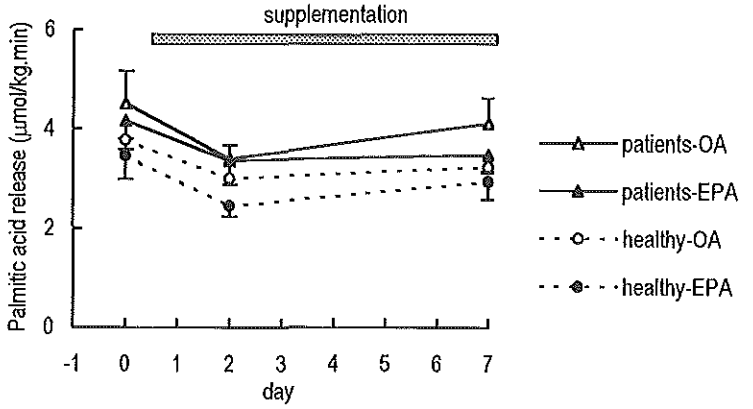

Figure 7.2

Palmitate release of ^{13}C-labeled palmitic acid in plasma in weight-losing cancer patients during supplementation of EPA (black symbols) or OA (open symbols). In comparison, palmitate release during EPA or OA supplementation in healthy subjects is shown. Mean ± SEM.

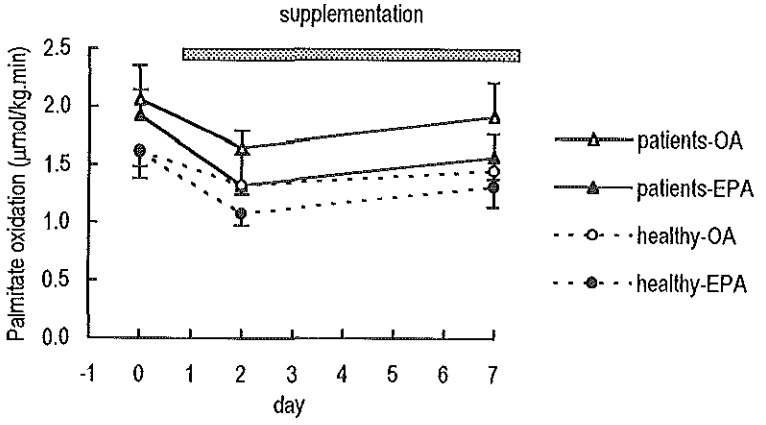

Figure 7.3

^{13}C-Palmitate oxidation rate in weight-losing cancer patients during supplementation of EPA (black symbols) or OA (open symbols). In comparison, the rate of palmitate oxidation during EPA or OA supplementation in healthy subjects is shown (dotted lines). Mean ± SEM.

lipids, especially plasma phospholipids, within four days of supplementation (*Chapter 5*) which suggests that EPA may have rapid metabolic effects within the human body. It is possible
that the effects of EPA differ between EE and free acids, and also that the effects of EPA-EE vary among the different groups of cancer patients.

It is remarkable that whole-body lipolysis, palmitic acid release and palmitate oxidation tended to decrease in the OA-EE group as well. Despite careful standardization of procedures on different study days, the possibility cannot be excluded that the observed changes were in fact caused by factors such as the physical activity level or sleeping pattern. Else, it is possible that the use of OA-EE as a placebo supplement has affected study outcome, since OA-EE might have inhibited lipolysis as well.

In the present study with OA-EE as a placebo supplement, we conclude that lipolysis is not inhibited by EPA-EE during short-term supplementation, and that inhibition of lipolysis is probably not the mechanism by which EPA appears to inhibit weight loss in weight-losing cancer patients.[17]

Acknowledgements

We thank Numico Research BV, Wageningen, The Netherlands, for financial support. For help in the recruitment of patients, we wish to acknowledge the physicians of the Departments of Oncology, Radiation Therapy, Surgery and Internal Medicine from the University Hospital Rotterdam. We also thank M. van Seters for practical assistance, L. Hanff for preparing the isotope solutions, and P. Schmitz for assistance in patient registration and randomization.

8

N-3 FATTY ACIDS ADMINISTERED AS TRIACYLGLYCEROLS OR AS ETHYL ESTERS HAVE DIFFERENT EFFECTS ON SERUM LIPID CONCENTRATIONS IN HEALTHY SUBJECTS

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Abstract

Fish oil supplements of different compositions have been used to study the effects of n-3 fatty acid supplementation on serum lipid concentrations. The aim of the present study was to assess whether n-3 fatty acids administered as triacylglycerols (TAG) or as ethyl esters (EE) have different effects on serum lipid concentrations on short term, and whether the effect of eicosapentaenoic acid (EPA; 20:5n-3) alone is different from that of a mixture of EPA and docosahexaenoic acid (DHA; 22:6n-3).

Forty-nine healthy subjects were randomized over five groups to receive a supplement containing 6g EPA per day (16g EPA/DHA-TAG/d; 12g EPA/DHA-EE/d; or 6g EPA-EE/d), or a supplement containing oleic acid (OA; 16g OA-TAG/d; or 6g OA-EE/d) for seven days. Extra butter was supplied to all groups with supplement dosage < 16 g/d. Overnight-fasted blood samples were taken at baseline and after 2 and 7 days of supplementation to measure serum lipid concentrations.

Serum TAG concentrations decreased significantly during EPA supplementation ($P < 0.05$). Both EPA/DHA-TAG and EPA/DHA-EE reduced total/HDL-cholesterol ratios ($P < 0.05$ and $P < 0.01$, respectively). However, this was caused by a reduction in total cholesterol concentrations in the EPA/DHA-TAG group ($P < 0.05$), but by an increase in HDL-cholesterol in the EPA/DHA-EE group ($P < 0.05$). No effects of EPA-EE on serum total or HDL-cholesterol concentrations were observed.

The effects of n-3 fatty acids on serum lipid concentrations on short term are affected both by the presence of DHA and by the type of esterification, which should be taken into account in future studies using n-3 fatty acids.

Introduction

Early epidemiological studies suggested that fish consumption and fish oil supplementation may be beneficial in the prevention and treatment of coronary heart disease (CHD).[31,56,121] Since high plasma lipid concentrations are considered as an important risk factor for CHD,[4] many studies have assessed the effects of fish oil supplementation on blood cholesterol and triacylglycerol (TAG) concentrations. Results showed that postabsorptive[71,81,109,175] as well as postprandial[226] serum TAG concentrations are markedly reduced by supplementation of eicosapentaenoic acid (EPA; 20:5n-3) and/or docosahexaenoic acid (DHA; 22:6n-3). These effects appear to be related with reduced TAG and VLDL synthesis as well as increased catabolic rate of VLDL.[42] Results of studies on the effects of n-3 fatty acids on serum cholesterol concentrations are inconsistent.[27,71,94,99,224]

Less attention has been paid to the effects of fish oil supplementation on plasma free fatty acid (FFA) concentrations which may also play an important role in the development of cardiovascular disorders.[67] Alterations in FFA concentrations may provide important information on the underlying mechanism of the effects of n-3 fatty acid supplementation on lipid metabolism and may act as an early indicator of changes in lipid metabolism. This is supported by the finding in healthy subjects that the decrease in plasma FFA concentrations was complete within one day of fish oil supplementation, whereas serum TAG concentrations continued to decrease until at least three days of supplementation.[45]

Many investigators used native purified fish oil as TAG as the source of EPA and/or DHA. In recent years, however, an increasing number of studies have used n-3 fatty acid supplements consisting of ethyl esters (EE) instead of TAG,[27,71,81,91,135] since this allows administration of specific fatty acids without interference by other components. Furthermore, the amount of oil required to obtain clinical effects can be reduced because of the high concentrations of specific fatty acids in EE-supplements. Since the intestinal absorption and plasma lipid incorporation of EPA and DHA did not differ between EE and TAG supplements,[90,92,120,136,156] it is generally assumed that the physiological effects of n-3 fatty acid EE are similar to those of n-3 fatty acid TAG. However, since routes of absorption may differ between n-3 fatty acid EE and TAG,[2,105,106,154] it remains possible that n-3 fatty acids administered as TAG or as EE have different effects on serum lipid concentrations. Indeed, two recent studies at our department showed that EPA and DHA administered as TAG significantly reduced plasma FFA,[45] whereas EPA administered as EE did not *(Chapter 6)*.

The aim of the present study was to assess whether n-3 fatty acids administered as TAG or as EE have different effects on serum TAG-, FFA and cholesterol concentrations

on short term, and whether the effect of EPA alone is different from that of a mixture of EPA and DHA.

Subjects and methods

Subjects and study design

Fifty healthy subjects, 18 men and 32 women, with stable weight without any gastrointestinal, metabolic or endocrine diseases were included in the study. Subjects with plasma TAG concentrations >3 mmol/L were excluded from participation, as were pregnant women. The study was approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam, and written informed consent was obtained from all subjects prior to start of the study. After an overnight fast starting at 20.00h the preceding evening, subjects attended the outpatient department between 7.00 and 9.30h. Weight and height were recorded, and baseline blood samples were taken after at least ten minutes of rest. EDTA blood samples were centrifuged immediately at 15000 g for 10 minutes at 4°C and blood samples for determination of serum TAG, cholesterol and HDL-cholesterol were centrifuged in a similar way after clotting. Serum TAG concentrations were determined within 4 hours after sampling, whereas EDTA plasma and serum for cholesterol determination was frozen at –80 °C under nitrogen until analysis within 4 weeks.

Subjects were randomized over five groups to receive different EPA (approximately 6 g/d) or OA (5 or 12 g/d) supplements. Extra butter was added to supplements containing <16g fat to achieve a total fat amount of 16 grams per day in all groups. The following supplements were used:

Group I: EPA plus DHA as TAG (EPA/DHA-TAG; Triomar; Pronova, Lysaker, Norway);

Group II: EPA plus DHA as EE (EPA/DHA-EE; Omacor; Pronova, Lysaker, Norway);

Group III: Purified EPA administered as EE (EPA-EE; purity 96.8%, EPA95EE; Pronova, Sandefjord, Norway);

Group IV: OA-fatty acid-mixture administered as TAG (OA-TAG; Super Refined Olive Oil; SR3912; Croda Oleochemicals, East Yorkshire, England) (serving as a control for EPA/DHA-TAG);

Group V: OA-EE (purity 79%, Ethyl Olivate; Croda Oleochemicals, North Humberside, England) in group V (serving as a control for EPA/DHA-EE and EPA-EE).

Details of the supplementation schedule are summarized in Table 8.1.

Table 8.1

Detailed information about the fatty acid supplementation.

Treatment group[a]	Capsule size (g)	Fatty acid in supplement (g/d (%[b]))			Dosage (g/d)		
		OA	EPA	DHA	Suppl.	Butter	Total fat
EPA/DHA-TAG[c]	1.0	1.1 (7%)	4.9 (31%)	3.2 (20%)	16	0	16
EPA/DHA-EE[d]	1.0	0 (0%)	5.9 (49%)	4.2 (35%)	12	4	16
EPA-EE[e]	0.5	0 (0%)	5.8 (97%)	0 (0%)	6	10	16
OA-TAG[f]	none[g]	12.6 (79%)	0 (0%)	0 (0%)	16	0	16
OA-EE[h]	0.5	4.7 (79%)	0 (0%)	0 (0%)	6	10	16

[a] EPA, eicosapentaenoic acid (20:5n-3); DHA, docosahexaenoic acid (22:6n-3); OA; oleic acid (18:1n-9); TAG, triacylglycerols; EE, ethyl esters

[b] Weight / weight%

[c] Triomar (Pronova, Lysaker, Norway)

[d] Omacor (Pronova, Lysaker, Norway)

[e] EPA95EE (Pronova, Sandefjord, Norway)

[f] SR3912 (Croda Oleochemicals, East Yorkshire, England)

[g] Administration by pre-weighed bottles (5.4g) instead of capsules

[h] Ethyl Olivate (Croda Oleochemicals, North Humberside, England)

After two and seven days of supplementation, blood samples were taken after a similar overnight fast to measure plasma FFA and TAG concentrations, according to the same protocol as on day 0. In addition, a questionnaire regarding side effects was filled in by each subject.

Laboratory analyses

Serum TAG, total cholesterol and HDL-cholesterol concentrations were measured using standard enzymatic colorimetric tests from Boehringer-Mannheim GmbH, Mannheim, Germany. Plasma FFA concentrations were determined using an enzymatic colorimetric method (NEFA C test kit; Wako Chemicals, Neuss, Germany).

Statistical analyses

Data are expressed as mean ± SEM. The Proc Mixed procedure for repeated measures of the statistical software package of SAS (SAS Inc., Cary, N.C. USA) was used, a procedure suitable for longitudinal regression analysis in which observations are collected on the same subjects over time.[137] Gender was included as a covariable due to differences between groups. For pairwise comparisons of treatment groups, the independent variables in the model were: the treatment indicator variable, baseline measurement, gender, time, and interaction between time and treatment. To compare data with their baseline values within treatments, the independent variables in the model were: a value to indicate the absence (day 0) or presence (day 2 and 7) of supplementation, gender, and time. P-values less than 0.05 were considered statistically significant.

Results

Characteristics of healthy subjects included in the study are summarized in Table 8.2. Fifty subjects were randomized over five groups, but one subject in the EPA/DHA-TAG group withdrew from the study for personal reasons within two days after randomization. This resulted in an EPA/DHA-TAG group of nine subjects and a total number of evaluable subjects of 49. The mean age of the groups ranged from 31 to 40 years (individual range 18-69), with all subjects having normal weight for height. Since, despite randomization, groups were different with regard to gender distribution, gender was included as a potential confounder in all subsequent statistical analyses. No apparent side effects of supplementation were reported, except for eructation mainly in the groups receiving EPA/DHA-TAG and EPA-EE.

Table 8.2

Characteristics of healthy subjects.[a]

Treatment group[b]	n	Age (y)	Sex	Weight (kg)	BMI (kg/m^2)
EPA/DHA-TAG	9[c]	35 ± 3	1 M; 8 F	65 ± 4	22 ± 1
EPA/DHA-EE	10	36 ± 3	2 M; 8 F	64 ± 2	22 ± 1
EPA-EE	10	38 ± 4	8 M; 2 F	77 ± 4	23 ± 1
OA-TAG	10	31 ± 4	4 M; 6 F	70 ± 3	24 ± 1
OA-EE	10	40 ± 4	3 M; 7 F	68 ± 4	23 ± 1

[a] Mean ± SEM

[b] EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; OA, oleic acid; TAG, triacylglycerols; EE, ethyl esters

[c] One subject withdrew from the study for personal reasons within two days after randomization.

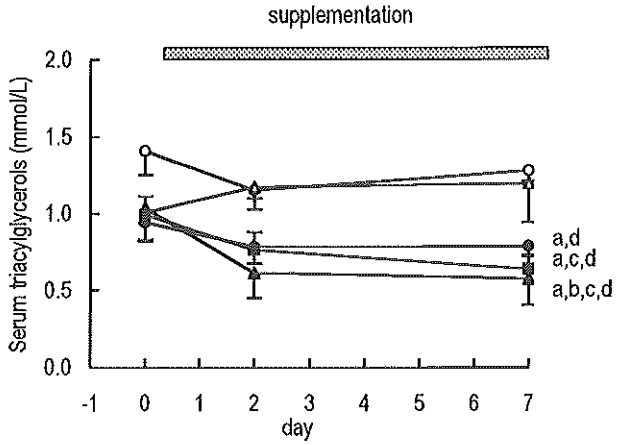

Figure 8.1

Effects of EPA/DHA-TAG (▲), EPA/DHA-EE (■), EPA-EE (●), OA-TAG (△) or OA-EE (○) supplementation on serum triacylglycerol concentrations. Mean ± SEM. Statistical analysis: Repeated measures; $P < 0.05$)

[a] Significantly different from baseline values

[b] Significantly different from EPA-EE

[c] Significantly different from OA-TAG

[d] Significantly different from OA-EE

Significant decreases in serum TAG concentrations were detected in all three groups receiving EPA, whereas serum TAG concentrations in the OA groups did not show any significant change (Figure 8.1). However, the reduction of serum TAG during EPA-EE supplementation (17%) was significantly less than during EPA/DHA-TAG supplementation (42%). Supplementation of EPA/DHA-EE had an intermediate effect on serum TAG concentrations which did not differ significantly from the other two EPA supplements. The change of serum TAG in the EPA/DHA-TAG group differed significantly from the EPA-EE group ($P = 0.013$) as well as from the OA-TAG group ($P = 0.012$).

Plasma FFA concentrations during supplementation showed a strikingly dissimilar pattern in different groups. A significant reduction was observed in the group receiving EPA/DHA-TAG, but not in any of the other groups (Figure 8.2). In this group, plasma FFA concentrations decreased by 23% and 33% after 2 and 7 days of EPA/DHA-TAG supplementation, respectively ($P = 0.03$). Compared to OA-TAG supplementation, the change in plasma FFA concentrations during EPA/DHA-TAG supplementation was highly significant ($P = 0.002$).

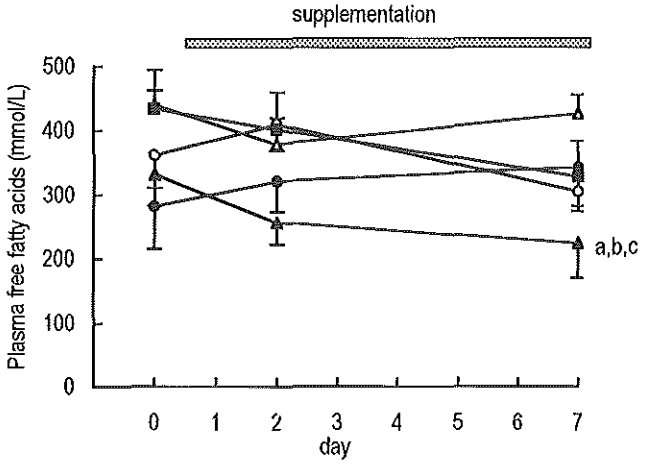

Figure 8.2

Effects of EPA/DHA-TAG (▲), EPA/DHA-EE (■), EPA-EE (●), OA-TAG (△) or OA-EE (○) supplementation on plasma free fatty acid concentrations. Mean ± SEM. Statistical analysis: Repeated measures; $P < 0.05$)

[a] Significantly different from baseline values

[b] Significantly different from EPA-EE

[c] Significantly different from OA-TAG

The total/HDL-cholesterol ratio showed a significant reduction both during EPA/DHA-TAG and EPA/DHA-EE supplementation ($P = 0.02$ and $P = 0.008$, respectively; Figure 8.3A), but not during supplementation of EPA-EE, OA-TAG or OA-EE. In comparison with other treatment groups, EPA-EE supplementation had significantly less effect on the total/HDL-cholesterol ratio than supplementation of EPA/DHA-TAG or EPA/DHA-EE ($P < 0.05$). Supplementation of EPA/DHA-TAG also differed significantly from that of OA-TAG ($P < 0.05$).

Serum total cholesterol concentration was significantly reduced by approximately 14% during EPA/DHA-TAG supplementation ($P = 0.02$), but, remarkably, was not affected in the group receiving EPA/DHA-EE nor in any other treatment group (Figure 8.3B). The change in serum cholesterol concentration in the EPA/DHA-TAG group was significantly different from the groups receiving EPA-EE ($P < 0.01$) and OA-TAG ($P < 0.01$).

In contrast to total cholesterol concentrations, serum HDL-cholesterol concentrations were only affected in the EPA/DHA-EE group where they showed a significant increase of approximately 19% ($P = 0.03$; Figure 8.3C). No significant change in HDL-cholesterol from baseline was observed in any other treatment group. No significant differences were detected between any of the groups.

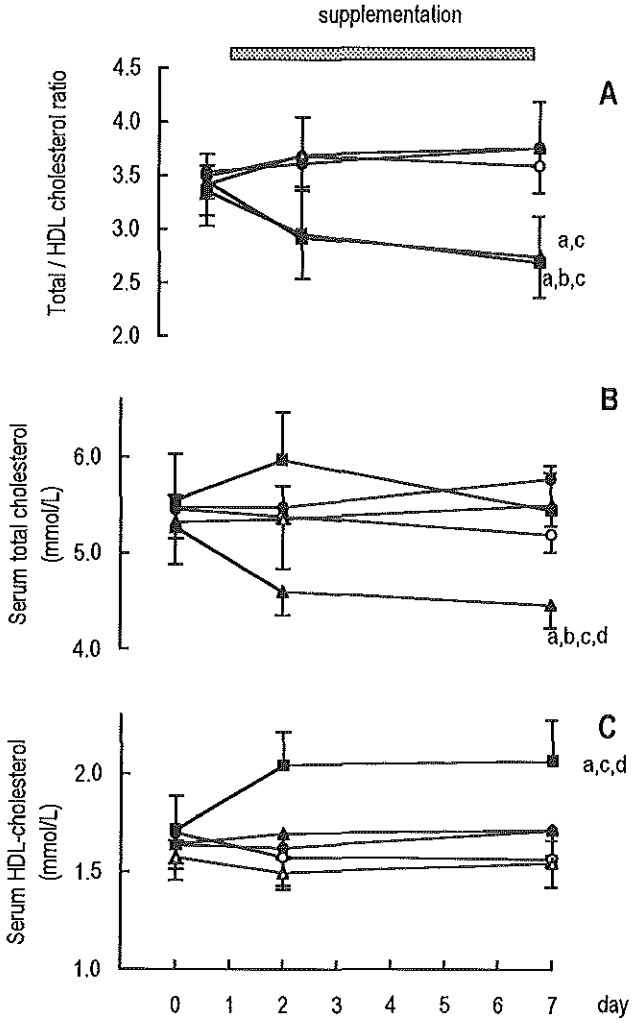

Figure 8.3

Effects of EPA/DHA-TAG (▲), EPA/DHA-EE (■), EPA-EE (●), OA-TAG (△) OA-EE (○) supplementation on serum lipoprotein concentrations. Mean ± SEM. Statistical analysis: Repeated measures; $P < 0.05$)

[a] Significantly different from baseline values

[b] Significantly different from EPA-EE

[c] Significantly different from OA-TAG

[c] Significantly different from OA-EE

Discussion

In the present study we investigated whether n-3 fatty acids administered as either TAG or EE have different effects on serum TAG, FFA and cholesterol concentrations, and whether the effect of pure EPA is different from that of a mixture of EPA and DHA. Results showed that serum TAG concentrations significantly decreased during n-3 fatty acid supplementation, although the decrease was significantly greater in the EPA/DHA-TAG group than in the EPA-EE group. Both EPA/DHA-TAG and EPA/DHA-EE reduced the total/HDL-cholesterol ratio, however this decrease was caused by a decrease in total cholesterol concentrations in the EPA/DHA-TAG group but by an increase in HDL-cholesterol in the EPA/DHA-EE group. No effects of EPA-EE on serum total or HDL-cholesterol concentrations were observed. A statistically significant reduction in plasma FFA concentrations was only observed in the group receiving EPA/DHA-TAG. It should be noted, however, that post-hoc power calculations showed that the study was underpowered for measuring FFA levels because of within-subject variability.

In 1989, Harris showed in an extensive review that the effects of n-3 fatty acid supplementation on plasma lipid concentrations vary considerably between different studies.[93] Systematic differences between studies may be induced by factors such as study population, dosage and duration of the study, and differences have also been reported between animal and human studies.[94] As shown by the present study, the use of different n-3 fatty acid supplements by different authors may give another explanation for the markedly different effects of n-3 fatty acids on lipid metabolism. Very little is known about mechanisms underlying the observed differences in effect between the TAG- and EE-containing supplements, but the route of intestinal absorption of fatty acids may differ between n-3 fatty acid EE and TAG.[2] In general, fatty acids esterified as TAG are hydrolyzed in the intestinal lumen by pancreatic lipase to yield FFA and monoacylglycerols (MAG). FFA and MAG diffuse through the cell membrane and are reesterified into TAG within the enterocyte. Subsequently, the TAG are excreted as chylomicrons into the lymphatic system to enter the circulation via the vena cava. The same may, however, not be true for the absorption of fatty acid EE. Some studies have reported that the rate of hydrolysis of EE by pancreatic lipase *in vitro* is lower than that of TAG,[120,236] although this may not be relevant to the more complex *in vivo* situation. Ackman[2] suggested that hydrolysis of EE may not take place in the intestinal lumen, but rather after absorption in the intestinal wall, followed by transport of FFA to the liver via the portal vein. However, if indeed n-3 fatty acids were directly transported to the liver, one would expect that the effect of n-3 fatty acid EE on liver metabolism would be more pronounced than that of n-3 fatty acid TAG. This is, however, not consistent with our data which show that the effect of n-3 fatty acid EE on serum TAG and cholesterol concentrations is less than that of n-3 fatty acid TAG.

A more plausible explanation for the differential effects of EE and TAG supplements may be a reduced rate of intestinal absorption of n-3 fatty acid EE. In rats, EPA- and DHA-EE were recovered in lymph at a much slower rate than EPA and DHA administered as TAG or as free acid.[105,237] The slower rate of recovery could be due to differences in metabolic pathways between EE and TAG within the enterocyte.[235] Fatty acids administered as EE have been proposed to undergo an additional metabolic step via the phosphatidic acid pathway prior to conversion into TAG.[235,238]

Besides the type of esterification of EPA, the fatty acid composition of the supplement also appears to play a role in the effect of EPA on serum lipid concentrations. Although some authors suggested EPA and not DHA to be responsible for the serum TAG reducing effect of fish oil,[71,72,175] others reported that both EPA and DHA have TAG lowering effects in healthy subjects.[81,92] Results of the present study show that the lipid-reducing effect of a supplement containing both EPA and DHA is markedly different from a supplement containing only EPA. Our observation that the EPA/DHA-EE supplement increased HDL-cholesterol concentrations whereas other supplements did not, is consistent with a previous report of Grimsgaard and coworkers[81] who found that DHA-EE increased HDL-cholesterol concentrations, whereas EPA-EE reduced serum total cholesterol concentrations. It has been suggested that the cholesterol-lowering effect of EPA-EE may be secondary to the serum TAG-reducing effect.[71] If this is true, the present study may have been too short to induce any effect of EPA-EE on serum total cholesterol concentrations. A study in rats suggested that EPA-EE may inhibit intestinal cholesterol absorption and hepatic cholesterol biosynthesis.[150] To our knowledge, the mechanism by which DHA increases HDL-cholesterol is not yet resolved.

It could be argued that the administration of a small amount of extra butter in the EPA-EE receiving group might have counteracted a possible reduction in serum cholesterol concentrations during EPA-EE supplementation. If so, however, one would expect serum cholesterol concentrations to increase in the OA-EE group, since this group received extra butter as well. The fact that we did not observe any significant effects of OA-EE on plasma lipid parameters suggests that the small amount of additional butter did not affect study outcome.

In conclusion, although it is not possible to draw conclusions on underlying mechanisms, it is quite clear from these results that the short-term effect of n-3 fatty acids as EE on plasma lipid concentrations is not equivalent to n-3 fatty acid as TAG. Moreover, DHA may interact with the physiological effects of EPA. Our results strongly suggest that, on short term, use of n-3 fatty acid TAG is superior to that of n-3 fatty acid EE in reducing serum TAG, FFA and total cholesterol concentrations. Future studies will have to show whether these differential effects of the n-3 fatty acid supplements persist during long-term supplementation, and to unravel the underlying mechanisms.

Acknowledgements

We gratefully acknowledge the subjects who volunteered in this study. We thank Numico Research B.V. for financial support, and Pronova, Lysaker, Norway, and Croda GmbH, Nettetal-Kaldenkirchen, Germany, for kindly providing the supplements.

9

LONG-TERM SUPPLEMENTATION OF EICOSAPENTAENOIC ACID IN WEIGHT-LOSING CANCER PATIENTS

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Abstract

Previous uncontrolled studies suggested that supplementation of eicosapentaenoic acid (EPA; 20:5n-3) may inhibit weight loss in cachectic cancer patients. The aim of the present double-blind, randomized study was to obtain pilot information on the effects of EPA supplementation on body weight, body composition and biochemical parameters in weight-losing cancer patients.

Nineteen weight-losing cancer patients of different tumor types were randomized to receive EPA ethyl esters (EE) (6g/d) or oleic acid (OA)-EE (6g/d) as a placebo for a maximum period of 12 weeks. At baseline and after 1, 4, 8 and 12 weeks, antropometric measures and blood samples were taken, and dietary intake was recorded for 4-7 days preceding the measurements.

For reasons of surgery, progressive disease and death, only 11 patients (EPA, n=4; placebo, n=7) were assessable for statistical analyses after four weeks of supplementation.

Repeated measures analysis showed a statistically significant favourable effect of EPA on body weight, fat mass and sum of four skinfolds after correction for baseline values ($P = 0.03$; $P = 0.001$ and $P = 0.002$, respectively). No significant effects of EPA on fat-free mass, mid-arm muscle circumference, or arm, hip or mid circumference were detected. A significant favourable effect of EPA was also detected on serum C-reactive protein (CRP) concentrations, since CRP remained stable in the group receiving EPA and increased in the placebo group ($P = 0.01$).

In conclusion, although selective patient dropout in the EPA group may have affected study outcome, our results are consistent with literature reports suggesting that body weight, fat mass and blood CRP levels appear to improve during long-term EPA supplementation.

Introduction

Many cancer patients suffer from cachexia with involuntary weight loss, impaired condition, reduced quality-of-life and shortened duration of survival.[75,127] In general, cachexia is not effectively reversed by nutritional support,[155] and adequate treatment is not available.[127] Metabolic derangements such as elevated energy metabolism, an ongoing acute-phase response, and excessive breakdown of muscle protein and adipose tissue are common in cachectic cancer patients,[179] but the underlying mechanisms have not yet been clarified. Cytokines as well as tumor-derived proteolytic[131] and lipolytic[219] factors may contribute to weight loss in cancer patients by stimulating catabolism. Recently, n-3 fatty acids from fish have aroused interest as a potential promising tool in the treatment of cancer cachexia.[230] Animal studies showed that n-3 fatty acid supplementation inhibited weight loss in a murine cancer cachexia model.[214] Eicosapentaenoic acid (EPA; 20:5n-3) was suggested to be the active component, since purified EPA was found to inhibit loss of body weight in tumor-bearing mice to the same extent.[21,213] Other studies, however, reported that docosahexaenoic acid (DHA; 22:6n-3) also inhibited weight loss in tumor-bearing mice.[158] *In vitro* studies suggest that inhibition of lipolysis and proteolysis may play a role in the observed effect, since EPA inhibited tumor-induced lipolysis[212,213] as well as protein degradation[21,212] *in vitro*. Furthermore, supplementation of EPA was shown to inhibit tumor growth and to prolong survival in mice.[21]

In a recent double-blind, randomized study, we investigated whether EPA inhibited lipolysis in weight-losing cancer patients after one week of supplementation *(Chapter 7)*. At the end of the week, patients were asked to continue supplementation for another 11 weeks to obtain pilot information on the long-term clinical effects of EPA supplementation. This report presents pilot information on the long-term effects of EPA supplementation on weight loss, body composition, biochemical blood parameters and quality-of-life in these weight-losing cancer patients.

Methods

Subjects

The study was approved by the Research Ethics Committee of the University Hospital of Rotterdam and written informed consent was obtained from all participants prior to the start of the study. Nineteen cancer patients of different tumor types with weight loss ≥5% in the preceding 6 months were included in this study. Patients treated with chemotherapy or radiation therapy in the previous 2 weeks or elective surgery in the previous 2 months were excluded, as were patients with corticosteroid treatment, insulin-dependent diabetes mellitus, uncontrolled hyper- or hypothyroidism, edema or fever.

Study design and methods

At start of the study, body weight, height, circumferences of arm, waist and hip were measured. Skinfold thickness (biceps, triceps, subscapula, suprailiac) was measured using Harpenden calipers (Holtain Ltd, Crymich, UK) and fat mass was calculated according to Durnin and Womersly.[53] Mid-arm muscle circumference (MAMC) was calculated using the following equation:

MAMC = MAC – π * TSF, where MAC = mid-arm circumference and TSF = triceps skinfold.

Blood samples were taken to measure blood C-reactive protein (CRP), albumin, total protein, hemoglobin, leucocytes, thrombocytes, and triacylglycerol (TAG). Baseline nutritional intake was measured by a four-day dietary record preceding start of the study and quality-of-life was determined using the Rotterdam Symptom Checklist (RSCL),[87] a validated questionnaire of quality-of-life in cancer patients.

Patients were randomized in a double-blind fashion to receive capsules containing 6 grams of EPA as ethyl esters (EE) per day (purity 96.8%, EPA95EE; Pronova, Sandefjord, Norway) or 6 grams of oleic acid EE (OA-EE) per day (purity 79%, Ethyl Olivate; Croda Oleochemicals, North Humberside, England) for a period of maximal 12 weeks. Supplements were provided as 500 mg capsules to be taken as 3x4 capsules with the three main meals.

Follow-up measurements of body weight, skinfolds, biochemical parameters, quality-of-life and nutritional intake (using a seven-day dietary record) were performed after 1, 4, 8 and 12 weeks of supplementation.

Statistical analyses

Results are presented as changes from baseline values and expressed as median (range) unless otherwise stated. Differences over time between the two groups were examined by repeated measures analysis of covariance using the linear regression model, a procedure suitable for longitudinal regression analysis in which observations are collected on the same subjects over time.[137] To account for the within-patient correlation in the measurements of the dependent variable, the Generalised Estimating Equations approach was followed. These analyses were performed with the SAS procedure Proc Mixed (version 6.12-Windows; SAS Inc., Cary, NC, USA), using the independence working correlation structure. Independent variables in the model were the treatment indicator variable, baseline measurement, measurement time and interaction between time and treatment. The model assumes a linear relation between measurement and time in both treatment groups. Statistical significance of the treatment effect was assessed by testing the

Table 9.1

Baseline characteristics of weight-losing cancer patients.[a]

	EPA group			OA group
	< 4 wks	≥ 4 wks	Total EPA group	
	(n=5)	(n=4)	(n=9)	(n=10)
Age (y)	59 (49 - 74)	69 (59 - 75)	65 (49 - 75)	65 (55 - 74)
Sex	5 M	3 M; 1 F	8 M; 1 F	5 M; 3 F
Body weight (kg)	66 (50 - 75)	72 (60 - 82)	66 (50 - 82)	63 (49 - 80)
Body mass index (kg/m^2)	22 (14 - 23)	25 (20 - 25)	23 (14 - 25)	23 (18 - 26)
Fat mass (kg)	11 (5 - 17)	19 (12 - 23)	14 (5 - 23)	17 (6 - 27)
Fat-free mass (%)	82 (77 - 90)	76 (61 - 81)	79 (61 - 90)	74 (58 - 88)
Weight loss (%)	8 (5 - 13)	10 (7 - 17)	10 (5 - 17)	7 (3 - 13)
Energy intake (MJ/d)	4.9 (3.3-12.3)	7.9 (7.9-7.9)	6.9 (3.3-12.3)	6.1 (3.5-8.5)
Biochemical parameters:				
C-reactive protein (mg/L)	61 (4 - 128)	7 (4 - 26)	9 (4 - 128)	21 (4 - 126)
Albumin (g/L)	37 (35 - 45)	41 (38 - 42)	38 (35 - 45)	41 (33 - 46)
Hemoglobin (mmol/L)	7.1 (6.2 - 9.6)	6.8 (5.7 - 8.6)	7.1 (5.7 - 9.6)	7.0 (6.0 - 9.0)
Leucocytes (*10.e^9/L)	8.8 (5.0 - 11.8)	5.9 (5.4 - 6.8)	6.8 (5.0 - 11.8)	6.4 (3.4 - 12.5)
Thrombocytes (*10.e^9/L)	300 (216 - 502)	285 (231 - 345)	300 (216 - 502)	305 (255 - 651)

[a] Median (range)

null-hypothesis that the coefficients of the treatment indicator and its interaction with time are simultaneously equal to zero. *P*-values less than 0.05 were considered significant.

Results

The patient group receiving EPA consisted of nine patients with the following tumor types: cancer of the upper gastrointestinal tract (4), pancreatic cancer (1), rectal cancer (1), renal cancer (1), breast cancer (1) and non-small cell lung cancer (1). All but one patient had metastatic disease and/or a locoregional relapse. The patient group receiving OA-EE consisted of ten patients with the following tumor types: oesophageal cancer (1), pancreatic cancer (2), gall bladder (1), breast cancer (1), cervical cancer (1), carcinoid (1), adenocarcinoma of unknown primary (1), non-small cell lung cancer (1) and mesothelioma (1). Seven patients had metastatic disease and/or a locoregional relapse, whereas the patient with mesothelioma had direct invasion of the right liver lobe.

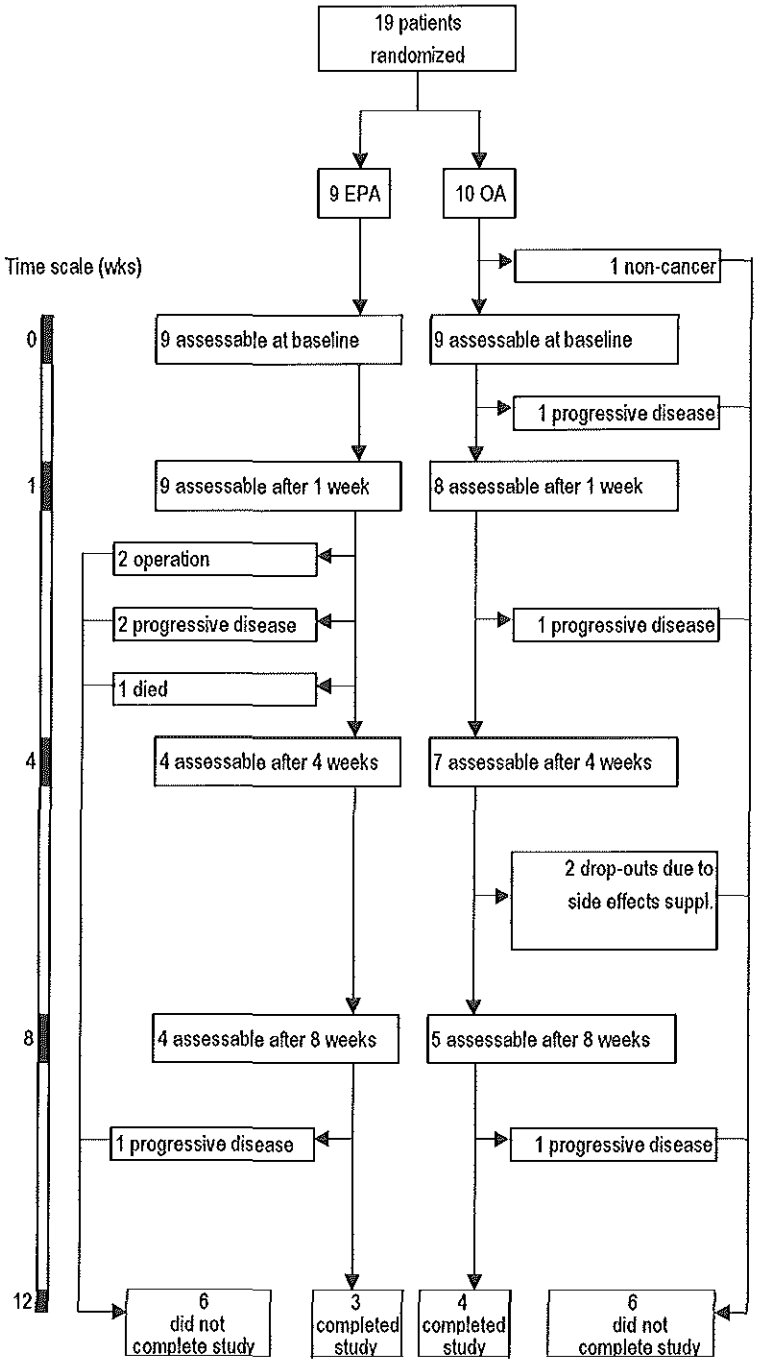

Figure 9.1

Trial profile

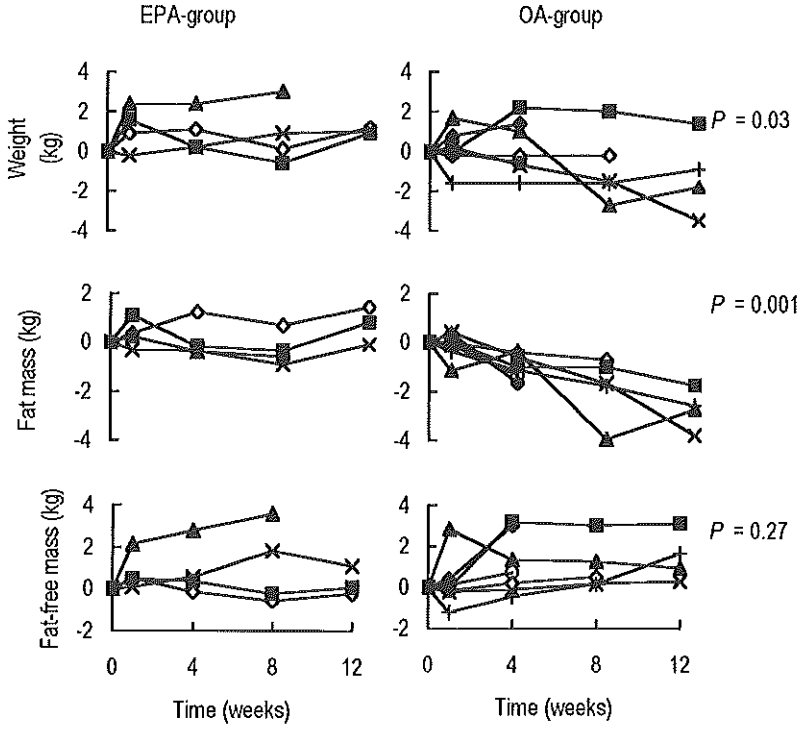

Figure 9.2

Individual changes in body weight, fat mass and fat-free mass during EPA (left) or OA (right) supplementation in weight-losing cancer patients.
The Proc Mixed procedure for Repeated Measures was used for statistical analyses.

No differences in any of the baseline patient characteristics such as age, sex, weight or biochemical parameters were observed between the treatment groups (Table 9.1). Of the randomized patients, one patient from the placebo group was excluded from the analysis because of non-malignant disease, and two patients from the placebo group withdrew from the study within four weeks because of progressive disease. Five patients from the EPA group dropped out before the follow-up measurement at four weeks due to death (1 patient), progressive disease (2 patients), and operations which were already scheduled at the time of inclusion into the study (2 patients) (Figure 9.1). In the placebo group, one patient withdrew within four weeks due to progressive disease. This resulted in a total number of four assessable patients in the EPA group and seven assessable patients in the

placebo group (Figure 9.1). A comparison of baseline characteristics between assessable and non-assessable patients from the EPA group showed that dropouts had a lower body weight, body mass index, fat mass and blood albumin than the assessable patients, wheras blood CRP concentrations, percentage of fat-free mass and leucocyte count were higher in the non-assessable patients. No differences in the percentage of weight loss were observed between the groups.

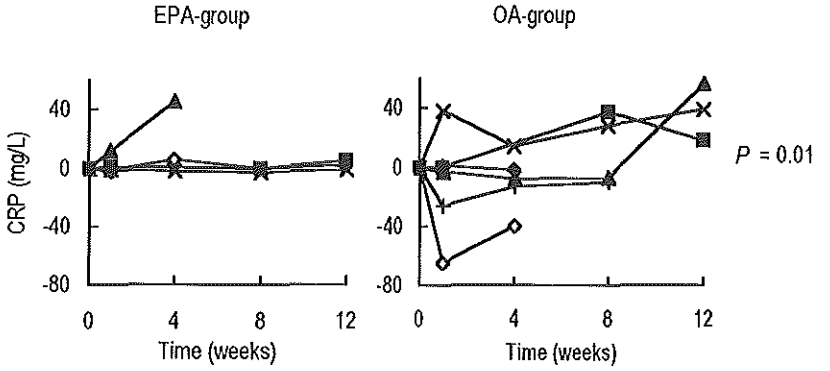

Figure 9.3

Individual changes in blood C-reactive protein (CRP) concentrations during EPA (left) or OA (right) supplementation in weight-losing cancer patients. Significant difference between the treatment groups. The Proc Mixed procedure for Repeated Measures was used for statistical analyses.

Repeated measures analysis showed a statistically significant favourable effect of EPA on body weight, fat mass and sum of four skinfolds after adjustment for baseline values ($P = 0.03$; $P = 0.001$ and $P = 0.002$, respectively; Figure 9.2). Also individual skinfolds were higher with EPA than with placebo supplementation (biceps, $P = 0.003$; triceps, $P = 0.08$; subscapular, $P = 0.04$; suprailiac, $P = 0.06$). No significant effects of EPA on fat-free mass (Figure 9.2), MAMC, or arm, hip or mid circumference were detected. A significant favourable effect of EPA was also detected on serum CRP concentrations after adjustment for baseline values, since CRP remained stable in the group receiving EPA and increased in the placebo group (Figure 9.3; $P = 0.01$). Blood total protein, albumin, prealbumin, vitamin A and vitamin E did not differ significantly between the treatment groups, although both blood hemoglobin, leucocyte count and thrombocyte count tended to improve with EPA (Figure 9.4).

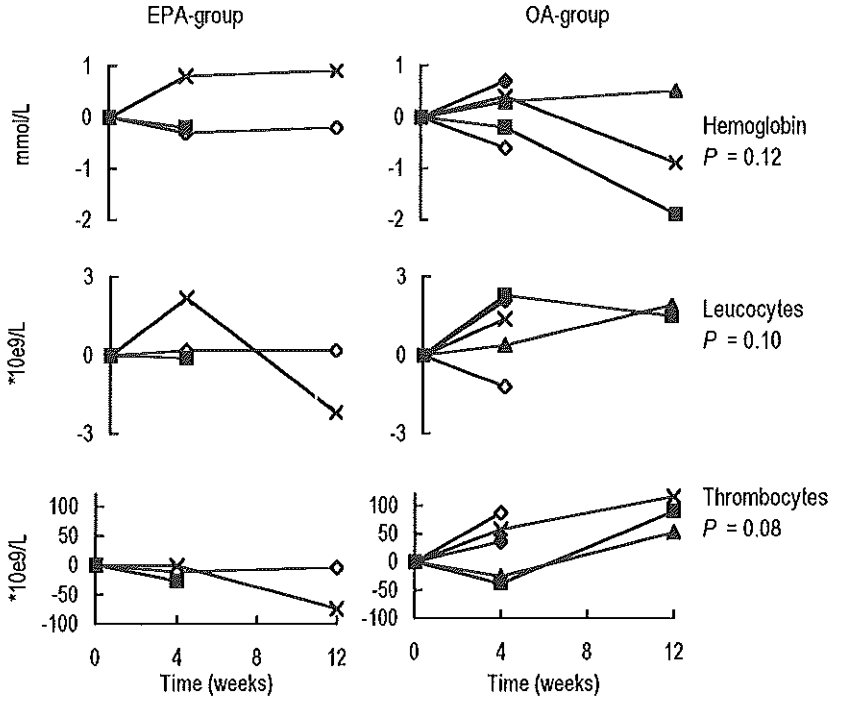

Figure 9.4

Individual changes in blood hemoglobin concentrations, leucocyte count and thrombocyte count during EPA (left) or OA (right) supplementation in weight-losing cancer patients. The Proc Mixed procedure for Repeated Measures was used for statistical analyses.

No significant treatment effect for quality-of-life was observed, since the physical score, psychological score, activity score and overall score of the RSCL did not differ between patients receiving EPA or placebo.

Although energy intake did not differ significantly between the treatment groups, the change in energy intake tended to be higher in the EPA group than in the placebo group (Figure 9.5; $P = 0.08$).

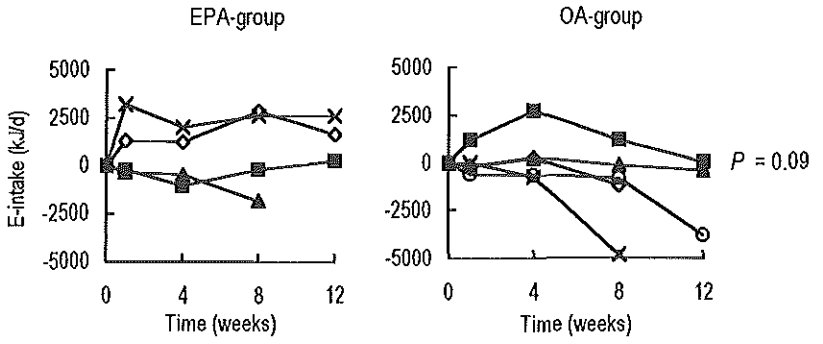

Figure 9.5

Individual changes in energy intake during EPA (left) or OA (right) supplementation in weight-losing cancer patients.
The Proc Mixed procedure for Repeated Measures was used for statistical analyses.

Discussion

The aim of the present study was to obtain pilot information regarding the effects of EPA on weight loss, body composition, biochemical parameters and quality-of-life in weight-losing cancer patients. Repeated measures analysis showed a significant favourable effect of EPA on body weight, fat mass, skinfold thickness and serum CRP concentrations after adjustment for baseline values.

However, results may have been biased by the fact that only a minor part of the patients included in the study were able to complete the study. The relatively high sample attrition in the present study was related to the fact that patients had advanced disease, and that the study was initially designed to investigate the effect of EPA on lipolysis on short-term. Therefore, several patients were included of whom it was suspected at the time of inclusion that they would not be able to complete the period of follow-up. Disease progression in combination with short life-expectancy, forced several patients to withdraw from the study. Since some baseline characteristics differed between assessable and non-assessable patients in the EPA group, the possibility may not be excluded that selective patient dropout has affected study outcome. This should be taken into account when interpreting the results of the study.

Recently, a lipid-mobilizing factor (LMF) has been identified in urine of weight-losing cancer patients,[97,186] which induced lipolysis in white adipocytes by stimulation of cAMP production.[115] This stimulation was inhibited in adipocytes pretreated with EPA.[170] A proteolysis-inducing factor has also recently been identified which stimulated protein

degradation in mice.[36,131] The effect of proteolysis-inducing factor was attenuated by EPA.[102,130] These studies on the effects of EPA on lipolysis and proteolysis suggest that EPA could be useful in the treatment of cancer cachexia. This is supported by recent reports of uncontrolled clinical studies in weight-losing patients with pancreatic cancer which showed that weight loss was inhibited and fat and fat-free mass were preserved by EPA.[16,17,230] Although the present results confirm the effects of EPA on body weight and fat mass, we could not confirm a significant effect of EPA supplementation on fat-free mass or MAMC. This may be related with the small number of patients and the relatively high drop out rate.

Our observation that the change in serum CRP was significantly smaller in the EPA group than in the placebo group is consistent with recent reports that EPA reduces serum CRP concentrations in patients with pancreatic cancer.[15,17,230] This effect may be due to downregulation of the acute-phase response by EPA via suppression of interleukin-6 production.[158,229] Since the presence of an acute-phase response is thought to play an important role in cachexia in pancreatic cancer[64] as well as in other tumor types,[25,145,220] the effect of EPA on the acute-phase response could partly explain the beneficial effects of EPA in cancer cachexia.[179]

Despite the small number of patients and the potential bias by selective patient dropout, the present study does provide pilot information concerning the effects of EPA on preservation of fat mass and stabilization of CRP concentrations. Our findings confirm results reported so far from uncontrolled studies suggesting that supplementation of EPA in cachectic cancer patients may be beneficial. Further double-blind, randomized studies with larger patient groups are therefore warranted to assess the effects of EPA on body weight, body composition, biochemical parameters and quality-of-life.

10

GENERAL DISCUSSION

Whole-body lipolysis in weight-losing cancer patients

Plasma fatty acid composition in cancer

n-3 Fatty acid supplements: Intestinal absorption of ethyl esters

Effects of EPA ethyl esters on lipolysis

Conclusions

The studies in this thesis describe the alterations in lipid metabolism in cancer patients, the effects of short-term eicosapentaenoic acid (EPA; 20:5n-3) supplementation on lipid metabolism in healthy subjects and cancer patients, and the long-term effects of EPA supplementation on weight loss, body composition and inflammation in weight-losing cancer patients.

The studies showed the following results:

- Both whole-body lipolysis and plasma lipolytic activity *in vitro* are higher in weight-losing cancer patients than in healthy subjects *(Chapters 2 & 3)*
- Plasma n-3 fatty acids are significantly reduced in pancreatic cancer and tend to be reduced in lung cancer, but are unchanged or even increased in esophageal cancer when compared with healthy subjects *(Chapter 4)*.
- Supplementation of EPA ethyl esters induces a rapid increase of EPA in plasma phospholipids and cholesteryl esters within four to seven days in healthy subjects *(Chapter 5)*.
- EPA ethyl ester supplementation does not inhibit whole-body lipolysis and lipid oxidation in healthy subjects *(Chapter 6)* nor in weight-losing cancer patients *(Chapter 7)* when compared with oleic acid (18:1n-9) ethyl esters as a placebo supplement.
- The reduction of serum triacylglycerol, free fatty acid and cholesterol concentrations by a mixture of EPA and docosahexaenoic acid (DHA; 22:6n-3) adminstered as triacylglycerols is different from that by pure EPA administered as ethyl esters *(Chapter 8)*.
- Pilot data in a limited group of cancer patients suggest that long-term supplementation of EPA ethyl esters inhibit the loss of body weight and fat mass and the rise in blood C-reactive protein (CRP) concentrations, which is not seen in patients receiving oleic acid ethyl esters as a placebo supplement *(Chapter 9)*.

Whole-body lipolysis in weight-losing cancer patients

Weight loss in cancer patients comprises both loss of muscle and fat mass.[96] One of the mechanisms thought to be involved in the reduction of fat mass is increased lipolysis. Results of the present study show that lipolysis and lipid oxidation were elevated in weight-losing cancer patients in comparison with healthy subjects *(Chapter 2)*, which is consistent with some[116,124,192] but not all[108] studies in literature. Several factors and mechanisms may underlie the observed elevation in lipolysis.

Firstly, measurements of lipolysis may be affected by energy intake. In many cancer patients, food intake is reduced due to decreased appetite[146,168] or mechanical

obstruction of the gastrointestinal tract.[48] The observation in a previous study that lipolysis was not only elevated in weight-losing cancer patients but also in weight-losing patients without cancer,[116] suggests that reduced energy intake could be responsible for the observed elevation in lipolysis. Previous studies in healthy subjects have also shown that energy intake can affect whole-body lipolysis,[34,114] and therefore, energy intake was included as a covariable in the data analyses of the present study. The difference in lipolysis between cancer patients and healthy subjects, however, remained statistically significant after this correction for energy intake, and we therefore conclude that our results are not confounded by differences in food intake between patients and healthy subjects.

Secondly, since lipolytic rate is usually presented per kilogram body weight, measurements of lipolysis may be distorted by changes in body composition. It has been shown that lipolysis is related with the size of the fat-free mass and not of the fat mass,[34,118] implying that the lipolytic rate per kilogram body weight may decrease when the percentage of fat-free mass decreases. Data analysis in our study showed, however, that the percentage of fat-free mass was similar in patients and healthy controls in the present study, and alterations in body composition can therefore not explain the observed differences in lipolysis between cancer patients and healthy subjects.

Since our study showed that study results are not confounded by energy intake or body composition, it follows that the observed elevated lipolysis in cancer patients was induced by other, disease-related factors. Recent reports suggest that the presence of an inflammatory response may affect lipolysis.[79,84] In the present study, however, no significant correlation between whole-body lipolysis and plasma CRP in cancer patients was detected. As another potential mechanism, hormonal alterations such as altered insulin, glucagon and glucocorticoid levels[6,19] may be involved in the observed elevation in lipolysis in cancer. A third factor that may have stimulated whole-body lipolysis in cancer patients is the presence of a lipid-mobilizing factor in blood.[101,215] To determine whether this lipid-mobilizing factor was present in the blood of the patients, plasma lipolytic activity was measured *(Chapter 3)*. The results confirmed that besides whole-body lipolysis *in vivo*, also plasma lipolytic activity *in vitro* was increased in cancer patients, indicating that the lipid-mobilizing factor was present in these patients. However, as there was no correlation between the *in vivo* and *in vitro* measurements, it seems unlikely that the presence of a lipid-mobilizing factor is the major factor for the elevation in lipolysis in cancer patients. Based on current knowledge, it is not likely that one single factor is responsible for the elevation in lipolysis in weight-losing cancer patients. Rather, a combination of the above-mentioned factors may stimulate lipolysis in cancer patients. Clearly, this issue will require further research.

Plasma fatty acid composition in cancer

Plasma total n-3 fatty acids were significantly reduced in pancreatic cancer and tended to be reduced in lung cancer, but were unchanged in esophageal cancer when compared with healthy subjects *(Chapter 4)*. Fatty acid composition was measured in plasma phospholipids and cholesteryl esters because the fatty acid composition of these fractions has a smaller day-to-day variation than plasma triacylglycerols.[151] The fatty acid composition of the plasma phospholipid fraction is a valuable indicator for fatty acid status because of the close relation with the phospholipids in platelet and erythrocyte membranes.[28,169]

The observation that n-3 fatty acid levels were significantly reduced in patients with elevated serum CRP concentrations (> 10 mg/L) suggests that the presence of an acute-phase response may be one of the potential mechanisms underlying the reduction in plasma n-3 fatty acid concentrations in cancer. This is consistent with other studies in non-cancer patients showing inverse correlations between plasma n-3 fatty acid levels and inflammatory state.[61,163,208] The observation that pancreatic cancer patients with non-insulin dependent diabetes mellitus (NIDDM) have higher levels of n-3 fatty acids than those without NIDDM is not consistent with most studies in non-cancer NIDDM patients. These studies showed reduced plasma levels of n-3 and n-6 fatty acids in comparison with healthy subjects,[173,176,190] although one study in newly diagnosed NIDDM patients showed elevated levels of elongated n-3 and n-6 fatty acids in newly diagnosed NIDDM patients.[167]

n-3 Fatty acid supplements: Intestinal absorption of ethyl esters

In previous studies on the effects of EPA on lipolysis and cancer cachexia, EPA was provided as triacylglycerols[45,230] or as free acids[17] but not as ethyl esters. However, the use of triacylglycerols has the disadvantage that the concentration of a specific fatty acid is relatively low, and that only a mixture of fatty acids can be administered. On the other hand, free fatty acids have been reported to induce gastrointestinal complaints.[23] For this reason, it was proposed to use EPA as ethyl esters in the present intervention studies on the effects of EPA on lipolysis. The use of ethyl esters allows administration of specific fatty acids in high concentrations, without interference by other substances. However, a review of the literature revealed some controversy concerning the absorption of ethyl esters: some studies showed that plasma incorporation of EPA and docosahexaenoic acid (DHA; 22:6n-3) was similar between ethyl esters and triacylglycerol supplements,[92,156] but other studies suggested that the absorption of ethyl esters may be impaired.[23,57,122] Furthermore, EPA ethyl ester supplementation was found to be ineffective in reversing

host body weight loss and inhibiting tumour growth in mice, presumably due to the inability to reach effective plasma and tumour concentrations of EPA over the initial time period in this tumour model.

Therefore, in order to assess whether it was justifiable to use EPA ethyl esters instead of EPA triacylglycerols in the intervention studies, we decided to perform a separate study to measure the incorporation pattern of EPA into plasma lipids during administration as ethyl esters in healthy subjects. This study, which is described in *Chapter 5*, showed that EPA ethyl esters were highly incorporated into both plasma phospholipids and cholesteryl esters within one week of supplementation. It was therefore concluded that n-3 fatty acid ethyl esters were sufficiently well incorporated into plasma lipids to allow the use of n-3 fatty acid ethyl esters in further metabolic and clinical studies.

An unexpected finding in this study was the observation that the incorporation patterns of EPA and DHA were quite different: EPA was mainly incorporated into plasma phospholipids, whereas DHA was mainly incorporated into TAG. This finding is consistent with results from other studies on the incorporation of n-3 fatty acids supplied as triacylglycerols[92,156] as well as studies using ethyl esters.[74,123,207] Only Blonk et al.,[27] who used a supplement of n-3 fatty acid ethyl esters, did not find any difference in incorporation between EPA and DHA into plasma phospholipids after 12 weeks of supplementation. The difference in incorporation between EPA and DHA may be related with the fact that EPA and DHA are competitors for the enzymatic transfer of fatty acids from phospholipids to cholesteryl esters.[207] Since the affinity of lecithin-cholesterol acyltransferase is higher for EPA than for DHA, the conversion of EPA from phospholipids to cholesteryl esters may be relatively higher than that of DHA. This would explain why, in this study, incorporation of EPA into cholesteryl esters was high, whereas the incorporation of DHA in cholesteryl esters was negligible.

Effects of EPA ethyl esters on lipolysis

Results in *Chapters 6 and 7* showed that short-term supplementation of EPA does not inhibit whole-body lipolysis in healthy subjects or weight-losing cancer patients when using oleic acid ethyl esters as a placebo supplement. However, several questions remain to be solved in order to integrate this result with findings from previous studies. The first question is whether a study period of one week was long enough to be able to detect any potential effects of EPA ethyl esters on lipolysis. Another question is whether the type of EPA supplement, pure EPA esterified as ethyl esters, could have affected study outcome. A third question is whether the choice of oleic acid ethyl esters for use as a placebo supplement was justified. Below, the potential influence of these factors on study outcome are discussed.

Study period

The first question concerns the duration of the study. The observation of Dagnelie et al.[45] that plasma FFA levels were significantly reduced within one day of fish oil supplementation in healthy subjects, suggested that the effect of EPA on lipid metabolism would be rapid. Furthermore, the study described in *Chapter 5* showed that EPA ethyl esters were rapidly incorporated into plasma lipids, especially into plasma phospholipids, within four days of supplementation. Although, based on these previous findings, it is unlikely that the effect of EPA on lipid metabolism takes more than seven days, the possibility cannot be excluded that a longer study period in the present studies would have resulted in different findings.

Ethyl esters versus triacylglycerols

The second question addresses the use of EPA ethyl esters in the present study. This question was raised by the fact that in our studies mentioned above *(Chapter 6 & 7)*, the rapid decrease of plasma FFA as reported by Dagnelie et al.[45] was not observed. In the latter study, a supplement containing both EPA and DHA esterified as TAG was used, whereas in the present studies, a supplement containing purified EPA esterified as EE was administered. It was suggested that the use of these different n-3 fatty acid supplements might have affected study outcome. Therefore, an additional study was performed in order to determine whether the various n-3 fatty acid supplements have differential effects on plasma lipid concentrations *(Chapter 8)*. Even though all EPA supplements did reduce serum TAG concentrations significantly, the degree of reduction was significantly greater in the group receiving both EPA and DHA as triacylglycerols (EPA/DHA-TAG) than in the group receiving pure EPA as ethyl esters (EPA-EE). In addition, plasma free fatty acid concentrations were only reduced in the EPA/DHA-TAG group but not in the groups receiving EPA/DHA-EE or EPA-EE. Differential effects of the different supplements on plasma lipoprotein concentrations were also detected *(Chapter 8)*.

Although little is known about mechanisms underlying the observed differences in effect between the triacylglycerol- and ethyl ester-containing supplements, the route of intestinal absorption of n-3 fatty acid ethyl esters and triacylglycerols may differ in several aspects.[2] Firstly, the rate of hydrolysis by pancreatic lipase may be lower for ethyl esters than for triacylglycerols.[120,236] Secondly, the rate of absorption may be reduced[105,237] due to an additional metabolic step via the phosphatidic acid pathway.[235,238] Thirdly, fatty acids from ethyl esters may be transported from the enterocyte as free acids bound to albumin instead of being reesterified and released into the circulation as triacylglycerols.[2] Especially the latter situation might induce interactions with receptors and / or hormones that could differ from those of fatty acids esterified as triacylglycerols.

Besides the type of esterification of EPA, the presence of DHA also appears to influence the effect of EPA on serum lipid concentrations *(Chapter 8)*. Although some authors suggested that EPA and not DHA is responsible for the serum triacylglycerol reducing effect of fish oil,[71,72,175] others reported that both EPA and DHA have triacylglycerol-lowering effects in healthy subjects.[81,92]

In retrospect, the results of this study suggest that EPA as ethyl esters may not have been the most appropriate supplement for studying the effects of EPA on lipolysis. When interpreting the results of the current study, it should therefore be taken into account that the metabolic effects of supplements containing EPA ethyl esters as described in this thesis might differ from the effects of a mixture of EPA and DHA as triacylglycerols.

Use of oleic acid ethyl esters as a placebo

The third question addressed the question whether oleic acid ethyl esters are suitable as a placebo supplement. Oleic acid was chosen as a placebo for being one of the most common monounsaturated fatty acids present in daily food, especially in olive oil. Also, olive oil[7,95,201,206,223] and oleic acid[144,188] had been used as a placebo supplement in many other studies. In the study on the effects of EPA on lipolysis, it was concluded that EPA ethyl esters did not affect whole-body lipolysis in comparison with the placebo group which received oleic acid ethyl esters. However, it was remarkable that both whole-body lipolysis, palmitic acid release and palmitate oxidation tended to decrease in the EPA as well as the placebo group. If oleic acid ethyl esters are assumed to be a valid placebo supplement which did not affect lipolysis, the observed changes should have been caused by factors other than fatty acid supplementation. However, it was not possible to identify such factors. This raises the question whether oleic acid ethyl esters could also have affected lipolysis. In the study described in *Chapter 8*, two different oleic acid supplements containing either triacylglycerols or ethyl esters were included. This study showed that neither oleic acid ethyl esters nor oleic acid triacylglycerols affected serum triacylglycerol, free fatty acid or lipoprotein concentrations. These results would suggest that oleic acid ethyl esters do not specifically affect lipid metabolism and are therefore a valid placebo supplement. It should be noted, however, that the effects on absolute serum lipid concentrations do not necessarily reflect lipid fluxes within the body.

Recent findings regarding the effects of EPA in cancer

The research described in this thesis was based on various studies performed in the early 1990's which showed that EPA inhibited both weight loss in tumor-bearing mice and lipolysis *in vitro*.

However, since 1996, several studies have been published on the effects of EPA on body weight and inflammatory response in patients with pancreatic cancer.[14-17,230] These

uncontrolled clinical trials[16,17] suggested that supplementation of EPA attenuates the loss of body weight, especially that of fat-free mass. In our long-term supplementation study in cancer patients *(Chapter 9)*, this beneficial effect of EPA on body weight and CRP was confirmed, but no beneficial effects of EPA on fat-free mass were observed. It should be taken into account, however, that the study was underpowered and that selective dropout of patients in the EPA-group might have affected the results of this long-term study.

The present results do show a preservation of fat mass during long-term supplementation of EPA, which may suggest that EPA does inhibit lipolysis on long term. This is consistent with a recent study reporting that stimulation of lipolysis *in vitro* was effectively attenuated by EPA.[170] Nevertheless, based on the findings reported in *Chapters 6 & 7*, we suppose that inhibition of lipolysis *in vivo* may not be the key mechanism underlying the observed effects of EPA in cancer cachexia.[212-214] Instead, other mechanisms are likely to contribute to the apparent anti-cachectic effects of EPA.

In recent years, it has become apparent that the presence of an inflammatory response is related with the occurrence of weight loss in cancer,[13,64,145,157,189,196,222] and that resting energy expenditure is elevated during inflammation.[203] Furthermore, it is now known that supplementation of EPA can modulate the inflammatory response in cancer patients[15,174,229] and in non-cancer patients.[26,33,59] Our long-term supplementation study *(Chapter 9)* also showed that plasma CRP concentrations were lower in the group receiving EPA than in the placebo group. Based on these findings, it is speculated that the beneficial effects of EPA in cancer cachexia could be related with modulation of the acute-phase response rather than with direct inhibition of lipolysis by EPA.

Another mechanism which may possibly play a role in the anti-cachectic effect of EPA is the inhibition of proteolysis. Recent studies showed that the proteolytic effects of a newly identified,[131,217] tumor-derived proteolysis-inducing factor were effectively attenuated by EPA *in vitro*.[102,130]

Conclusions

Based on the results of this thesis, the following conclusions can be drawn:

- Whole-body lipolysis, palmitate release and palmitate oxidation are significantly elevated in weight-losing cancer patients in comparison with healthy control subjects. These differences remain significant after correction for energy intake, and are not affected by changes in body composition.
- Plasma n-3 fatty acid concentrations are reduced in pancreatic cancer and tend to be reduced in lung cancer, but are unchanged in esophageal cancer when compared

with healthy subjects. Total n-3 fatty acids are lower in patients with an inflammatory response (CRP > 10 mg/L) than in patients without inflammation.

- Whole-body lipolysis and lipid oxidation are not inhibited by supplementation of EPA ethyl esters in weight-losing cancer patients nor in healthy subjects during short term supplementation when compared with oleic acid ethyl esters as a placebo supplement.
- The effects of EPA administered as ethyl esters on lipid metabolism may differ substantially from the effects of a mixture of EPA and DHA administered as triacylglycerols. Therefore, the conclusion that supplementation of EPA ethyl esters do not inhibit lipolysis should not be extrapolated to EPA administration in general, since the metabolic effects of EPA might be different when using another type of esterification or when supplying EPA in combination with DHA.
- Our findings during long-term supplementation of EPA suggest that body weight, fat mass and skinfold thickness are higher in cancer patients receiving EPA than in patients receiving the placebo supplement. Furthermore, EPA supplementation appears to reduce serum CRP concentrations.

Future studies

Future studies are needed to elucidate the mechanisms underlying cancer cachexia. Since recent studies suggest that the inflammatory response may play an important role in cancer cachexia, further research should focus on the clinical effects of inhibition of the acute-phase response in cancer. Furthermore, the recent identification of a lipid-mobilizing factor and a proteolysis-inducing factor suggests that specific stimulation of lipolysis and proteolysis by a tumour-derived factor may also play a role in the occurrence of weight loss. However, the precise role of lipolysis and proteolysis in cancer cachexia remains to be clarified.

Although studies in literature suggest that supplementation of n-3 fatty acids may be beneficial in cancer patients, it is not known whether this potential effect is related with baseline plasma n-3 fatty acid status in these patients. Also, it remains uncertain whether the fatty acid concentrations in plasma reflect the fatty acid status in tissues. The present study has shown that plasma n-3 fatty acids levels are reduced in pancreatic cancer and appear to be reduced in lung cancer, but the mechanism underlying these alterations is not known. Therefore, further studies are needed to clarify whether the fatty acid status in plasma and in tissues are related, and whether any reductions in n-3 fatty acid levels in plasma and/or adipose tissue are related with reduced intake, with reduced availability or with elevated metabolic demands. Clarification of the underlying mechanisms may contribute to the identification of patient groups at risk for developing n-3 fatty acid

deficiencies, which may lead to the designation of specific target groups of patients who may benefit from n-3 fatty acid supplementation.

Furthermore, before performing additional studies on the clinical effects of n-3 fatty acids in cancer or in other diseases, the mechanisms underlying the observed differences in physiological effects of various n-3 fatty acid supplements will have to be clarified. These studies will have to address whether to use triacylglycerols, ethyl esters or supplements with some other type of esterification, and whether pure EPA, pure DHA or a mixture of fatty acids should be used to obtain desired clinical effects. Possibly, the type of supplement will vary with the nature of the effect to be achieved.

Once the problems regarding lipid metabolism in cancer and the use of different n-3 fatty acid supplements have been solved, double-blind, randomized, long-term clinical trials on the effects of EPA should be performed. These studies should include sufficient numbers of patients to detect whether long-term supplementation of EPA does inhibit loss of weight, fat mass and fat-free mass in cancer patients. Such studies should concentrate especially on patients with those types of cancer in which weight loss frequently occurs, and factors such as inflammation and n-3 fatty acid deficiency should be taken into account. Obviously, quality-of-life and survival will have to be important additional outcome measures in such trials.

11

SUMMARY

Cachexia is a frequent problem in cancer patients which is characterized by weight loss, impaired performance and fatigue. In general, administration of nutritional support does not improve the condition nor is any other adequate treatment available. The mechanisms underlying cancer cachexia are poorly understood, but factors such as increased lipolysis and proteolysis may play a role. Weight loss in tumor-bearing mice has been found to be attenuated by n-3 fatty acids derived from fish oil, with eicosapentaenoic acid (EPA; 20:5n-3) being the active component. EPA was shown to inhibit lipolysis and proteolysis *in vitro*, and recent uncontrolled clinical studies suggested that EPA supplementation may reverse weight loss in patients with pancreatic cancer *(Chapter 1)*.

The aims of the studies reported in this thesis were to assess whether lipolysis and plasma lipid composition are altered in cancer patients and whether supplementation of EPA reduces lipolysis and lipid oxidation in healthy subjects and in weight-losing cancer patients. Furthermore, we aimed to obtain pilot information on the long-term effects of EPA supplementation in these cancer patients.

In order to develop new treatment strategies for cancer cachexia, better knowledge of lipid metabolism is needed. Since studies in healthy subjects have demonstrated that lipolytic rate depends on energy intake and the size of fat-free mass, the aim of the first study within this thesis (*Chapter 2*) was to assess whether whole-body lipolysis, palmitic acid release and palmitate oxidation were increased in weight-losing cancer patients when taking differences in food intake and body composition into account. Measurements were performed in 18 weight-losing cancer patients with different tumor types and in sixteen healthy subjects after an overnight fast, and food intake was recorded for four days. Body composition was measured by bioelectrical impedance analysis. After adjustment for energy intake, whole-body lipolysis was significantly higher in cancer patients than in healthy subjects. The difference in palmitic acid release did not reach statistical significance. The rate of palmitate oxidation was also significantly higher in patients than in healthy subjects. No differences in body composition were observed between groups. It is concluded that whole-body lipolysis and palmitate oxidation are elevated in weight-losing cancer patients when differences in food intake and body composition are taken into account.

Recently, a lipid-mobilizing factor has been identified in the urine of weight-losing cancer patients, which was shown to stimulate lipolysis *in vitro* as well as *in vivo*. To assess whether whole-body lipolysis *in vivo* and lipolytic activity *in vitro* are correlated, lipolytic activity was measured in plasma samples of weight-losing cancer patients and healthy control subjects *(Chapter 3)*. *In vitro* lipolytic activity towards isolated fat cells was significantly higher in plasma of weight-losing cancer patients than in plasma of healthy subjects ($P < 0.01$). However, no significant correlation was detected between lipolytic activity *in vitro* and whole-body lipolysis *in vivo* in the total study group, nor in

healthy subjects or cancer patients separately. This suggests that factors other than the presence of the lipid-mobilizing factor probably also contribute to whole-body lipolysis in cancer patients.

The risk of developing essential fatty acid deficiency is increased in cancer patients due to reduced food intake, impaired intestinal absorption or increased metabolic demands. In the study described in *Chapter 4*, we investigated whether plasma n-3 fatty acid concentrations were reduced in different groups of cancer patients in comparison with healthy subjects. In pancreatic cancer, plasma n-3 fatty acids showed a substantial reduction in both plasma phospholipids and cholesteryl esters. Although total n-3 fatty acids in lung cancer also tended to be reduced, this difference failed to reach statistical significance. In esophageal cancer, however, total n-3 fatty acid concentrations were comparable with or even higher than those in healthy subjects. n-3 Fatty acid levels were lower in weight-losing than in weight-stable patients with lung cancer. Pancreatic cancer patients with diabetes had significantly higher levels of n-3 fatty acids than those pancreatic cancer patients without diabetes. For all tumor types combined, total n-3 fatty acids were significantly reduced in patients with plasma C-reactive protein concentrations > 10 mg/L.

Beneficial health effects of n-3 fatty acid supplementation have been described in several diseases such as cancer cachexia, atherosclerosis and inflammatory diseases. Since natural fish oil contains a relatively low concentration of n-3 fatty acids, it might be advantageous to use purified n-3 fatty acid ethyl esters. However, studies investigating the intestinal absorption of eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) ethyl esters are controversial. In *Chapter 5*, the level of incorporation of EPA and DHA in different plasma lipid fractions of healthy subjects during supplementation with n-3 fatty acid ethyl esters is described. Within 7 days of supplementation, the proportion of EPA in plasma phospholipids showed a 15-fold increase ($P < 0.001$). The proportion of DHA only showed a two-fold increase. In cholesteryl esters, EPA also increased significantly ($P < 0.05$), whereas DHA did not increase at all. In contrast, incorporation of DHA into triacylglycerols was even higher than that of EPA. These results showed that EPA is rapidly and substantially incorporated into plasma lipids when provided as ethyl esters.

In *Chapter 6*, the effects of short-term EPA ethyl ester supplementation on whole-body lipolysis and lipid oxidation in healthy subjects are reported. In sixteen healthy subjects, whole-body lipolysis, palmitic acid release and palmitate oxidation were measured using stable isotopes. Subjects were randomized to receive EPA ethyl ester (6 g/d) or placebo (oleic acid ethyl esters; 6 g/d) for seven days in a double-blind design. After 2 and 7 days of supplementation, turnover measurements were repeated. Whole-body lipolysis, palmitate oxidation, or palmitic acid release were not significantly different between the

treatment groups, despite substantial reductions in palmitic acid release and palmitate oxidation in both treatment groups when compared with baseline values.

A similar study on the effects of EPA on lipolysis in weight-losing cancer patients is described in *Chapter 7*. Seventeen weight-losing cancer patients of different tumor types were randomized to receive EPA ethyl esters or placebo for seven days, and whole-body lipolysis, palmitic acid release and palmitate oxidation were measured at baseline, and after 2 and 7 days of supplementation. Again, no significant differences in whole-body lipolysis, palmitate oxidation, or palmitic acid release were detected between the groups receiving EPA ethyl ester and oleic acid ethyl ester supplementation. Furthermore, no significant treatment effects on resting energy expenditure and plasma CRP concentrations were detected. Also in patients, whole-body lipolysis tended to decrease in the placebo group.

Results of both studies reported above *(Chapters 6 and 7)* differed from an earlier study from our group in that a rapid decrease in plasma FFA concentrations was not observed during n-3 fatty acid supplementation. This raised the suspicion that different types of n-3 fatty acid supplements could have varied effects on lipid metabolism. We therefore performed an additional study, described in *Chapter 8*, in which forty-nine healthy subjects were randomized over five treatment groups. Group I received both EPA and DHA as triacylglycerols, group II received EPA and DHA as ethyl esters, and group III received pure EPA as ethyl esters for a period of seven days. Groups IV and V received placebo supplements containing oleic acid. Overnight-fasted blood samples were taken at baseline and after 2 and 7 days of supplementation. Serum triacylglycerol concentrations decreased significantly in all groups receiving EPA ($P < 0.05$), but notably, the triacylglycerol reduction in the group receiving EPA ethyl esters (17%) was significantly less than in the group receiving the mixture of EPA and DHA as triacylglycerols (42%) ($P < 0.05$). Furthermore, although both supplements containing EPA plus DHA reduced the total/HDL-cholesterol ratio, this was caused by a reduction in total cholesterol concentrations in the EPA plus DHA triacylglycerol group, but by an increase in HDL-cholesterol in the EPA plus DHA ethyl ester group ($P < 0.05$). The effects of n-3 fatty acid supplements on serum lipid concentrations are thus affected both by the presence of DHA and by the type of esterification.

Besides measuring the effects of short-term EPA ethyl ester supplementation on lipolysis, we also obtained pilot information on the long-term clinical effects of EPA supplementation. In *Chapter 9*, we explored whether supplementation of EPA influences loss of body weight, body composition and biochemical blood parameters. For this study, cancer patients were asked to continue supplementation for another 11 weeks after completion of the one-week study of metabolic measurements (described in *Chapter 7*). Anthropometric measures and blood samples were taken at baseline and after 1, 4, 8 and 12 weeks, and dietary intake was recorded for 4-7 days preceding the measurements. Due

to death, progressive disease and surgery, only 11 out of 19 randomized patients (EPA, n=4; placebo, n=7) were assessable for statistical analyses after four weeks of supplementation. Body weight, fat mass and sum of four skinfolds were significantly higher in the EPA group than in the placebo group ($P = 0.03$, $P = 0.001$ and $P = 0.002$, respectively). C-reactive protein (CRP) levels were significantly lower in the group receiving EPA than in the placebo group ($P = 0.01$). Although selective patient dropout in the EPA group may have affected study outcome, our results are consistent with literature reports suggesting that body weight, fat mass and blood CRP levels appear to improve during long-term EPA supplementation.

In conclusion, whole-body lipolysis is significantly elevated in weight-losing cancer patients when compared with healthy control subjects. Plasma n-3 fatty acids are reduced in pancreatic cancer and tend to be reduced in lung cancer, but are unchanged in esophageal cancer in comparison with healthy subjects. Short-term supplementation of EPA ethyl esters does not reduce whole-body lipolysis or lipid oxidation in weight-losing cancer patients or in healthy subjects in comparison with oleic acid ethyl esters as a placebo supplement. Furthermore, the metabolic effects of EPA administered as ethyl esters differ substantially from the effects of a mixture of EPA and DHA administered as triacylglycerols. Long-term EPA supplementation may improve body weight, fat mass, skinfold thickness and serum CRP concentrations, although these results may have been affected by selective patient dropout.

12

SAMENVATTING

Veel kankerpatiënten lijden aan gewichtsverlies, wat meestal gepaard gaat met een slechte conditie, vermoeidheid, een slechte weerstand en een kortere levensduur. Over het algemeen verbetert het geven van bijvoeding de situatie niet of nauwelijks. Ook bestaat er geen geneesmiddel tegen het optreden van gewichtsverlies bij kanker. De oorzaak van dit gewichtsverlies is niet bekend. Veel patiënten hebben een verminderde eetlust, maar daarnaast lijkt het er ook op dat vetweefsel en spieren versneld worden afgebroken. Uit dierstudies is gebleken dat het geven van n-3 vetzuren uit visolie het gewichtsverlies zou kunnen tegengaan. Het vetzuur eicosapentaeenzuur (EPA; 20:5n-3) was hierbij het actieve bestanddeel. Ook was EPA in staat om de vet- en eiwitafbraak in geïsoleerde cellen te remmen. Docosahexaeenzuur (DHA; 22:6n-3) had daarentegen geen remmend effect in deze studies. Recent is gebleken dat EPA waarschijnlijk ook bij patiënten met pancreaskanker het gewichtsverlies kan remmen, maar omdat in dat onderzoek geen controlegroep aanwezig was, moeten de resultaten met enige voorzichtigheid worden bekeken *(Hoofdstuk 1)*.
In de studies die beschreven staan in dit proefschrift, werd onderzocht:

1. of de vetafbraak bij kankerpatiënten met gewichtsverlies hoger is dan bij gezonde mensen;
2. of de vetzuursamenstelling in het bloed anders is bij kankerpatiënten dan bij gezonde mensen;
3. of de afbraak van vet bij kankerpatiënten en bij gezonde vrijwilligers geremd wordt door het geven van EPA;
4. of het geven van EPA gedurende lange tijd invloed heeft op het gewichtsverloop bij kankerpatiënten.

Men vermoedt dat de afbraak van vetweefsel bij kankerpatiënten hoger is dan bij gezonde mensen. Het is echter bekend dat de afbraaksnelheid van vet in het lichaam mede bepaald wordt door de energie-inname en de lichaamssamenstelling. Doordat deze factoren zouden kunnen verschillen tussen kankerpatiënten en gezonde mensen, is het mogelijk dat de uitkomsten van eerdere studies hierdoor vertekend zijn. Het doel van onze eerste studie *(Hoofdstuk 2)* was na te gaan of de vetafbraak bij kankerpatiënten met gewichtsverlies hoger is dan bij gezonde mensen na correctie voor voedselinname en lichaamssamenstelling. Achttien patiënten met verschillende soorten kanker en met meer dan 5% gewichtsverlies en zestien gezonde vrijwilligers namen deel aan het onderzoek. Eerst schreven zij gedurende vier dagen op wat zij eten en dronken. Na een nacht vasten werd de lichaamssamenstelling bepaald (bioelectrische impedantie analyse) en werd de snelheid van de vetafbraak gemeten met behulp van een continu infuus met een gelabeld vetzuur. Na correctie voor energie-inname was de snelheid van de vetafbraak significant hoger bij kankerpatiënten dan bij gezonde mensen. Ook de verbranding van vet was

significant hoger bij patiënten dan bij de controlepersonen. Het verschil in vetafbraak tussen kankerpatiënten en gezonde vrijwilligers werd niet verklaard door verschillen in lichaamssamenstelling, aangezien deze vergelijkbaar was tussen de twee groepen.

Recent hebben onderzoekers in de urine van kankerpatiënten met gewichtsverlies een stof geïsoleerd die de vetafbraak in vetcellen stimuleert en die bij muizen gewichtsverlies veroorzaakt. Of deze stof aanwezig is in het bloed van mensen kan bepaald worden door het meten van de zogenaamde 'lipolytische activiteit' van het bloed. Hiervoor worden vetcellen van proefdieren gedurende twee uur geïncubeerd met het bloed van de patiënt, waarna gemeten wordt hoeveel glycerol (een bestanddeel van vet) er uit de cellen is vrijgekomen. Omdat niet bekend is in hoeverre deze *in vitro* bepaling van de vetafbraak dezelfde uitkomst geeft als de bepaling van de vetafbraak bij patiënten met kanker, is in de studie van *Hoofdstuk 3* onderzocht in hoeverre deze twee maten met elkaar gecorreleerd zijn. De vetcellen werden hiervoor geïncubeerd met het bloed van de kankerpatiënten en gezonde vrijwilligers uit *Hoofdstuk 2*. De afbraak van vet in de cellen was significant hoger bij het bloed van kankerpatiënten dan van gezonde mensen. De mate van vetafbraak in deze cellen was echter niet gecorreleerd met de mate van vetafbraak in het totale lichaam, ook niet na opsplitsing van de groep in patiënten en gezonde controlepersonen. Dit wijst erop dat ook andere factoren zoals hormonale veranderingen invloed hebben op de snelheid van de vetafbraak bij kankerpatiënten.

Omdat kankerpatiënten vaak minder eten, de opname in de darm verminderd kan zijn en bij veel patiënten de stofwisseling verhoogd is, lopen zij een verhoogd risico op het ontwikkelen van tekorten aan bepaalde vetzuren in het lichaam. In het onderzoek beschreven in *Hoofdstuk 4* is nagegaan of de concentraties van n-3 vetzuren (de vetzuren uit vette vis) in het bloed lager zijn bij kankerpatiënten dan bij gezonde mensen. De concentraties van n-3 vetzuren in het bloed waren aanzienlijk verlaagd bij patiënten met pancreaskanker, met name bij die patiënten die geen suikerziekte hadden. Bij de patiënten met longkanker was de concentratie van n-3 vetzuren eveneens verlaagd, hoewel het verschil ten opzichte van gezonde personen niet statistisch significant was. Deze verlaging werd met name gevonden bij de longkankerpatiënten met gewichtsverlies. Bij patiënten met slokdarmkanker waren de concentraties van n-3 vetzuren niet afwijkend van die van gezonde mensen. Verder bleek dat de n-3 vetzuren in het bloed significant lager waren bij kankerpatiënten bij wie sprake was van een ontstekingsreactie dan bij kankerpatiënten zonder ontstekingsreactie.

Wanneer n-3 vetzuren gegeven worden in de vorm van capsules, heeft men de keuze uit verschillende visolie-supplementen. In 'natuurlijke visolie' komen n-3 vetzuren

voor als vetten (oftwel triglyceriden), waarbij echter de concentratie van n-3 vetzuren relatief laag is. Daarom wordt vaak gekozen voor het gebruik van ethyl-esters, een kunstmatige vorm van visolie. Wanneer ethyl-esters gebruikt worden, is het mogelijk om n-3 vetzuren in zuivere vorm en in hoge concentratie aan te bieden. Het is echter niet duidelijk of de opname van ethyl-esters door de darm wel goed is. In *Hoofdstuk 5* wordt beschreven in welke mate de concentraties van EPA en DHA in het bloed omhoog gaan wanneer gezonde mensen zeven dagen lang visolie in de vorm van ethyl-esters slikken. Uit deze studie bleek dat de stijging van EPA in het bloed relatief groot was en snel verliep, terwijl de stijging van DHA aanmerkelijk kleiner en langzamer was. Ook waren er duidelijke verschillen tussen de diverse vetfracties in het bloed. De belangrijkste conclusie uit dit onderzoek was dat de opname van EPA in het lichaam goed is wanneer n-3 vetzuren in de vorm van ethyl-esters gegeven worden.

Hoofdstuk 6 beschrijft het effect van EPA ethyl-esters op de afbraak van vet en de vetverbranding bij gezonde mensen. Zestien gezonde vrijwilligers werden door middel van loting verdeeld over twee groepen. De eerste groep kreeg EPA ethyl-esters (6 g/dag) en de tweede groep kreeg een placebosupplement (oliezuur ethyl-esters; 6 g/dag) gedurende zeven dagen. De proefpersonen wisten zelf niet in welke groep zij ingedeeld waren. Bij aanvang van de studie en na twee en zeven dagen werd de vetafbraak gemeten met behulp van een infuus met een gelabeld vetzuur. De afbraak van vet en de vetverbranding verschilden niet significant tussen de twee behandelingsgroepen. Het was echter wel opvallend dat zowel de vetafbraak als de vetverbranding in beide behandelingsgroepen, dus ook in de placebogroep, leek te dalen.

In *Hoofdstuk 7* wordt het effect van EPA ethyl-esters op de vetafbraak en de vetverbranding bij kankerpatiënten met gewichtsverlies beschreven. Zeventien patiënten met verschillende soorten kanker werden door middel van loting verdeeld over een EPA ethyl-ester groep en een placebogroep. Bij aanvang van het onderzoek en na twee en zeven dagen slikken van de capsules werd de snelheid van de vetafbraak gemeten. Ook hier waren de vetafbraak en de vetverbranding niet significant verschillend tussen de twee behandelingsgroepen. Ook het energieverbruik tijdens rust en de mate van ontsteking in het lichaam verschilden niet significant tussen de twee groepen. Net als bij de gezonde vrijwilligers leek de vetafbraak in beide behandelingsgroepen te dalen.

In eerder onderzoek leidde het geven van visolie aan gezonde mensen tot een daling van de concentraties van vrije vetzuren in het bloed. In bovenstaande studies *(Hoofdstukken 6 en 7)* werd deze daling echter niet gevonden. Dit heeft mogelijk te maken met het gebruik van verschillende soorten visolie in de diverse studies. Daarom is

onderzocht in hoeverre de effecten van de diverse soorten visolie op de vetconcentraties in het bloed verschillen *(Hoofdstuk 8)*. Negenenveertig gezonde vrijwilligers werden door middel van loting verdeeld over vijf behandelingsgroepen: drie groepen met visolie en twee groepen met olijfolie. Groep I kreeg een supplement met zowel EPA als DHA in de vorm van triglyceriden, groep II kreeg EPA en DHA in de vorm van ethyl-esters en groep III kreeg zuivere EPA in de vorm van ethyl-esters. De groepen IV en V kregen placebosupplementen in de vorm van olijfolie. De personen slikten deze supplementen gedurende zeven dagen. Bij aanvang van de studie en na twee en zeven dagen werd er 's morgens nuchter bloed afgenomen. De concentratie triglyceriden in het bloed daalde significant bij alle visoliegroepen. Echter, de mate van daling was significant groter bij de mensen die EPA en DHA in de vorm van triglyceriden kregen dan bij de personen die zuivere EPA in de vorm van ethyl-esters kregen. De concentratie van totaal cholesterol in het bloed daalde significant in de groep die EPA plus DHA in de vorm van triglyceriden kreeg, maar niet in de andere groepen met visolie of olijfolie. Het HDL-cholesterol gehalte steeg daarentegen alleen in de groep die EPA plus DHA in de vorm van ethyl-esters. Hierdoor daalde de ratio van totaal en HDL-cholesterol in de twee groepen die een mengsel van EPA en DHA kregen, terwijl deze ratio niet veranderde in de groep met alleen EPA. Deze resultaten laten zien dat er duidelijke verschillen in effect bestaan tussen de verschillende soorten visolie.

Tot slot is in een pilotstudie geprobeerd een indruk te krijgen van de klinische effecten van EPA op lange termijn (*Hoofdstuk 9*). In dit onderzoek werd onderzocht in hoeverre het geven van EPA het gewichtsverloop, de lichaamssamenstelling en de enkele bloedparameters kan beïnvloeden bij kankerpatiënten met gewichtsverlies. Daartoe werd aan de patiënten uit ons onderzoek (beschreven in *Hoofdstuk 7*) gevraagd om, na de eerste week van metingen van de vetafbraak, door te gaan met het slikken van de capsules gedurende maximaal twaalf weken. Bij aanvang van de studie en na 1, 4, 8 en 12 weken werden de patiënten gewogen, werd de lichaamssamenstelling bepaald (huidplooimetingen) en werd bloed afgenomen. Voorafgaand aan elke meting schreven de patiënten gedurende zeven dagen op wat zij aten en dronken. Als gevolg van overlijden, voortschrijding van de ziekte of operaties waren er na vier weken nog slechts 11 van de 19 patiënten beschikbaar voor de effectmetingen (4 patiënten in de EPA groep; 7 patiënten in de placebogroep). Van de overgebleven patiënten waren het lichaamsgewicht en de hoeveelheid vetweefsel significant hoger bij de patiënten met EPA dan bij de patiënten met het placebosupplement. Daarnaast was de ontstekingsreactie significant lager in de EPA groep dan in de controlegroep. Hoewel het mogelijk is dat deze resultaten beïnvloed zijn door de relatief hoge uitval van patiënten in de eerste vier weken, komen deze resultaten overeen met

gegevens in de literatuur dat EPA een positief effect heeft op het lichaamsgewicht, de hoeveelheid vetweefsel en de ontstekingsreactie.

De conclusies van het huidige onderzoek zijn:

- De afbraak van vet is hoger bij kankerpatiënten met gewichtsverlies dan bij gezonde mensen.
- De concentraties van n-3 vetzuren (vetzuren uit visolie) in het bloed zijn verlaagd bij patiënten met pancreaskanker en waarschijnlijk ook bij patiënten met longkanker, maar zijn niet veranderd bij patiënten met slokdarmkanker ten opzichte van gezonde vrijwilligers.
- Toediening van EPA gedurende zeven dagen heeft geen effect op de vetafbraak of de vetverbranding bij kankerpatiënten met gewichtsverlies of bij gezonde vrijwilligers, in vergelijking met olijfolie als een placebosupplement.
- Verschillende soorten visolie hebben verschillende effecten op de vetconcentraties in bloed.
- Het geven van EPA gedurende langere tijd lijkt een positief effect te hebben op het lichaamsgewicht, de hoeveelheid vetweefsel en de ontstekingsreactie.

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Tot slot ...

Het moment is nu dan toch daar: mijn boekje is af! Ik moet zeggen dat dat een zeer prettig gevoel geeft. Maar... het feit dat mijn naam mag prijken op de kaft betekent natuurlijk niet dat ik dit boekje in mijn eentje gemaakt heb. Zonder de hulp van vele mensen om mij heen had ik dit onderzoek namelijk nooit tot een goed einde kunnen brengen. Ik maak dan ook graag van deze gelegenheid gebruik om iedereen te bedanken die mij in de afgelopen vier jaar met mijn onderzoek geholpen heeft.

Mijn begeleider en copromotor Pieter Dagnelie heeft zich in de afgelopen vier jaren volledig ingezet voor het onderzoek. Pieter, jouw enthousiasme, je grote betrokkenheid (zelfs vanuit het verre Maastricht) en jouw snelheid wat betreft lezen zullen weinig mensen kunnen evenaren.

Professor Wilson was meestal het grote brein op het gebied van de goede ideeën en geschikte oplossingen. Zijn positieve kijk en grote vertrouwen hebben mij tijdens de, soms moeizame, uitvoering van het onderzoek vaak een hart onder de riem gestoken.

Binnen het laboratorium van de Inwendige Geneeskunde II was Wim van den Berg op vele gebieden onmisbaar. Wim, ik bewaar zeer goede herinneringen aan onze talloze besprekingen over de stofwisseling en de laboratoriumtechnieken, aan jouw gezelligheid en vooral ook aan jouw oppeppende woorden wanneer ik even in een dipje zat.

Roel Swart was als clinicus en hoofd van de sectie 'Voeding en Metabolisme' altijd bereid tot het voeren van interessante discussies over het onderzoek en de klinische voeding in het algemeen. Roel, jouw klinische inbreng en kritische opmerkingen hielden mij scherp en hebben mij veel geleerd over de klinische praktijk.

De vele laboratoriumanalyses hadden niet uitgevoerd kunnen worden zonder de grote inzet van Trinet Rietveld en Darcos Wattimena. Trinet, naast de vele dingen die je mij geleerd hebt over het werken in een laboratorium, was jouw gezelschap op het lab erg aangenaam. Darcos, het is misschien maar goed dat ik geen idee heb hoe vaak jij 's avonds, 's nachts en in het weekend op het lab geweest bent om de analyses van onze monsters op de massaspectrometer tot een goed einde te brengen.

De gezelligheid in ons knusse kamertje had ik te danken aan mijn kamergenoten Erik Agteresch en Anne Marie Petersen-Westerman. Erik, als arts heb jij voor mij talloze infuusnaalden geprikt en vele patiënten beoordeeld op hun geschiktheid voor deelname aan het onderzoek. Anne Marie, je had altijd belangstelling voor mijn onderzoek en onze gezellige 'klets-uurtjes' waren een aangename afleiding.

De congressen kregen een extra kleurtje door de altijd vrolijke Susanne Leij-Halfwerk. Susanne, ik heb er buitengewoon veel zin in om samen met jou aan de slag te gaan bij Numico Research in Wageningen.

Ook alle andere collega's van het laboratorium Inwendige Geneeskunde II hebben bijgedragen aan de prettige werksfeer op het lab. De gezellige koffiepauzes, de vele helpende handen en de nooit aflatende belangstelling voor zowel werk- als privé-aangelegenheden zal ik zeker niet vergeten.

Manon van Seters, Michiel van der Heijden en David Dezentjé hebben als studenten een belangrijke steen bijgedragen aan de praktische uitvoering van het onderzoek. Manon, ik vertrouwde de proefpersonen en patiënten tijdens de metingen graag aan jouw zorg toe. Michiel, je gedrevenheid, sociale vaardigheden en kritische blik hebben een belangrijke rol gespeeld bij het slagen van jullie onderzoek. David, zelfs na het voltooien van jouw verplichte stageperiode bleef je ons helpen, waardoor we veel extra patiënten in het onderzoek hebben kunnen insluiten.

Voor de uitvoering voor mijn onderzoek was de samenwerking met de afdeling Interne Oncologie zeer belangrijk. Dokter Splinter, Ate van der Gaast, Tjebbe Kok, André Vos en Leon Kerkhofs hebben zich twee jaar lang ingezet bij de werving van patiënten. Daarnaast was de medewerking van Gea, Mildred, Annie, Marian, Rosa, Elma en alle andere assistentes van de afdeling Oncologie / Hematologie zeer prettig bij het zoeken naar patiënten, het gebruik van kamers voor de metingen en het prikken van infusen.

Ook de artsen van de afdeling Radiotherapie hebben enige tijd meegewerkt aan de patiëntenwerving. Bij Yvonne en Astrid als assistentes van de afdeling Radiotherapie kon ik altijd terecht voor hulp en een gezellig kletspraatje.

Naast de bovengenoemde afdelingen hebben ook vele artsen, verpleegkundigen en assistentes van de afdelingen Longziekten, Heelkunde en het Thoraxcentrum zich ingezet bij onze patiëntenwerving en het verzamelen van patiëntenmateriaal.

De verpleging van de afdeling 4-Midden was altijd bereid tot assistentie bij het prikken van infuusnaalden en tot het ter beschikking stellen van de onderzoekskamer. Yvonne, door jouw vrolijkheid vergaten de patiënten dat het afnemen van bloed eigenlijk iets vervelends is.

De dagelijkse bereiding van de infuusvloeistof en het uitvullen van capsules kwam voor rekening van de medewerkers van de apotheek van het Dijkzigt Ziekenhuis. De inzet van Lidwien Hanff, Marien Pluim en Frederike Engels hebben er mede voor gezorgd dat de praktische uitvoering van het onderzoek goed kon verlopen.

De randomizatie en registratie van de patiënten en vrijwilligers in mijn onderzoek is verzorgd door de medewerkers van het Trialbureau van de Daniel den Hoed Kliniek onder leiding van Dr. P. Schmitz.

Voor adviezen omtrent de ingewikkelde 'repeated measures' methode in het statistische programma SAS kon ik terecht bij Professor Th. Stijnen van de afdeling Epidemiologie en Biostatistiek.

De hulp van Alies van Lier bij het uitrekenen van alle voedingsdagboekjes heeft mij zeer veel tijd uitgespaard.

I would like to thank professor M.J. Tisdale, Allison Whitehouse and William Field from the CRC Nutritional Biochemistry Research Group, Aston University, Birmingham, United Kingdom, for there willingness to cooperate in a study to determine the correlation between *in vitro* lipolytic activity of our plasma samples and whole-body lipolysis in our study.

Dit onderzoek is financieel mogelijk gemaakt door Numico Research B.V. te Wageningen. De medewerking en het niet aflatende vertrouwen van Dr. J.G. Bindels van Numico Research in een goede afronding van het onderzoek waren een essentiële factor bij de succesvolle voltooiing van dit project.

Natuurlijk had dit onderzoek nooit uitgevoerd kunnen worden zonder de medewerking van vele, veelal ernstig zieke kankerpatiënten, hun familieleden en de vele gezonde vrijwilligers die aan dit onderzoek meegewerkt hebben. De meeste patiënten kunnen de afronding van dit onderzoek helaas niet meer meemaken, maar ik hoop dat hun grote inzet mag bijdragen aan de ontwikkeling van nieuwe behandelmethoden van kankerpatiënten in de toekomst.

Tot slot is ook alle steun en belangstelling voor mijn onderzoek van familie en vrienden in de afgelopen vier jaren zeer belangrijk voor mij geweest. Maar van al die mensen was jij, Remco, toch echt de belangrijkste. Naar gelang de situatie bood jij mij een luisterend oor, een kritisch oog, een brede schouder of een helpende hand. Daarnaast heb je mij laten zien hoe leuk het verbouwen van een huis kan zijn. Ons huis in Delft is nu af, dus wat dacht je van een mooi oud huis in de regio Wageningen?

Curriculum Vitae

Sonja Zuijdgeest-van Leeuwen werd geboren op 1 augustus 1972 te Delft. Hier behaalde zij in 1990 haar Gymnasium β diploma op het Sint Stanislas College, waarna zij Voeding van de Mens ging studeren aan de Landbouwuniversiteit te Wageningen. Haar eerste afstudeervak deed zij in het Academisch Ziekenhuis te Utrecht, waar zij onder begeleiding van prof. dr. L.M.A. Akkermans op de afdeling Gastroenterologie onderzoek deed naar de relatie tussen voeding en gastro-oesofageale reflux. Haar tweede afstudeervak vond plaats op het RIKILT-DLO te Wageningen in samenwerking met de Vakgroep Humane Voeding van de Landbouwuniversiteit. Tijdens dit onderzoek bestudeerde zij, onder begeleiding van dr. ir. P.C.H. Hollman en prof. dr. M.B. Katan, de intestinale absorptie van flavonoïden bij gezonde vrijwilligers met een ileostoma. Haar derde onderzoeksproject vond plaats in het Hadassah Hospital te Jerusalem, Israel, waar zij onder begeleiding van professor E.M. Berry onderzoek deed naar de invloed van stress op het eetgedrag bij muizen. Na het behalen van haar diploma begon zij in augustus 1995 in het Academisch Ziekenhuis te Rotterdam met het promotieonderzoek dat beschreven staat in dit proefschrift. Vanaf 1 januari 2000 is zij werkzaam als onderzoeker bij Numico Research B.V. te Wageningen.

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