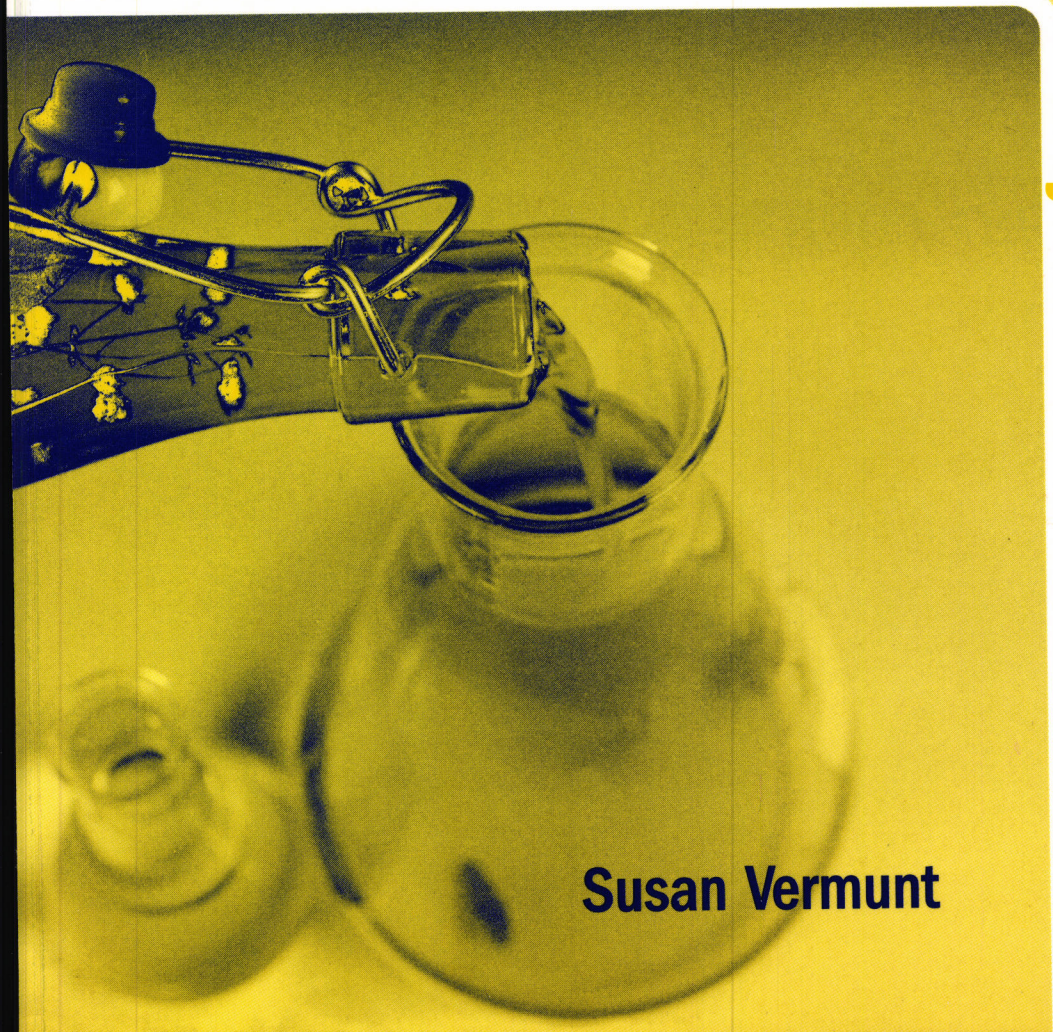
Metabolic Aspects of Cis and Trans Polyunsaturated Fatty Acids from the Diet



Susan Vermunt

Metabolic aspects of *cis* and *trans* polyunsaturated fatty acids from the diet

The study presented in this thesis was performed at the Nutrition and Toxicology Research Institute Maastricht (NUTRIM), which participates in the Graduate School VLAG-2 (Food Technology, Agrobiotechnology, Nutrition and Health Sciences) accredited by the Royal Netherlands Academy of Arts and Sciences.

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Stellingen

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Metabolic aspects of *cis* and *trans* polyunsaturated fatty acids from the diet

1. *Trans* α-linoleenzuur uit de voeding verhoogt de verhouding tussen totaal en HDL cholesterol in het bloed ten opzichte van *cis* α-linoleenzuur (dit proefschrift)

2. α-Linoleenzuur uit de voeding verlaagt de relatieve omzetting van dit vetzuur in vetzuurderivaten met een langere keten en meer dubbele bindingen (dit proefschrift)

3. De oxidatie van α-linoleenzuur uit de voeding is negatief gecorreleerd met de omzetting van α-linoleenzuur in vetzuren met een langere keten en meer dubbele bindingen (dit proefschrift)

4. Delta 6 en Δ9 desaturase mRNA niveaus in witte bloedcellen kunnen worden gebruikt om de mRNA niveaus van deze enzymen in de lever te voorspellen (dit proefschrift)

5. Het gebruik van afkortingen is een goed voorbeeld van suboptimalisatie: de tijdswinst door het gebruik weegt niet op tegen het tijdsverlies bij de interpretatie

6. Naast het reviewen van artikelen, zouden tijdschriften ook hun reviewers moeten reviewen

7. Een zwangere promovenda heeft het voorrecht om te mogen worden bijgestaan door tenminste drie paranimfen

8. Voor een assistent in opleiding (AIO) is niets zo frustrerend als de vraag wanneer je afstudeert

9. Zonder voorlichting zijn functional foods niet functioneel

Susan Vermunt
Maastricht, 27 september 2001

Metabolic aspects of *cis* and *trans* polyunsaturated fatty acids from the diet

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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Susanne Hanneke Francisca Vermunt

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Aan mijn ouders

Voor Paul

Contents

1

General introduction

Fat contributes for on average 36% of the energy intake in the total Dutch population, of which 7% of energy is from polyunsaturated fatty acids (PUFAs) (1). Dietary PUFA, which are mainly incorporated into triacylglycerols and phospholipids, are essential components of membranes, play a role in the production of energy, and some are precursors of eicosanoids (2). Furthermore, PUFAs may have beneficial effects on serum lipids and lipoproteins and their overall effect is that they may reduce the risk for coronary heart disease (CHD) (3-5). In contrast, a high total or saturated fat intake may increase the risk for CHD (4,6). In the Netherlands, it is therefore advised to consume maximal 30-35% of energy from fat, of which less than 10% from saturated fat (7). Moreover, an expert panel recently advised to eat fish once a week or to consume 200 mg marine n-3 PUFA (eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA)) daily (5). A daily intake of 2 g or 1% of energy α-linolenic acid has been recommended as well (5).

The aim of this chapter is to introduce the essential fatty acids linoleic acid and α-linolenic acid. First, the structure and nomenclature of these fatty acids are explained, and the synthesis of longer chain polyunsaturated fatty acids (LCPs) derived from essential fatty acids as well as the degradation of essential fatty acids by β-oxidation are discussed. Special attention will be paid to nutritional factors that may influence essential fatty acid metabolism. Because stable isotopes are pre-eminently suitable for studying human *in vivo* fatty acid metabolism, advantages and disadvantages of this technique are discussed. Furthermore, determination of desaturase gene expression in human tissues is considered. Finally, the contribution of *trans* fatty acids to the diet and the effects of *trans* fatty acids on plasma lipids are discussed briefly. An extensive overview of the health aspects of *trans* fatty acids is given in chapter 6. This chapter will be closed by giving an outline of this thesis.

FATTY ACIDS

Fatty acids consist of a chain of carbon atoms (C-atoms) connected by single or double bonds, with at one end a methyl group (CH_3-group) and at the other end a carboxyl-group (COOH-group). Saturated fatty acids have no double bonds, monounsaturated fatty acids have one double bond and PUFAs have two or more double bonds. The unsaturated fatty acids belong to either the α-linolenic (n-3), linoleic (n-6), palmitoleic (n-7), or oleic (n-9) acid family, in which the first double bond is located between the 3^{rd} and 4^{th}, 6^{th} and 7^{th}, 7^{th} and 8^{th}, or 9^{th} and 10^{th} carbon atom, respectively, as counted from the methyl-end of the carbon chain. The position of the double bonds may also be counted from the carboxyl-end of the molecule, which is symbolized as 'Δ'. Chain elongation and desaturation always occur between the carboxyl-group and its nearest double bond. Consequently, the position of the first double bond from the methyl-end will not change during conversion reactions, and fatty acids will not change in 'families' as a result of metabolism.

Double bonds may occur in two configurations, the *cis*- and the *trans*-configuration. In the *cis*-configuration the hydrogen atoms (H-atoms) adjacent to the double bond are located at the same side of an imaginary 'plane' through the double bond. This form is the most common in nature. In the *trans*-configuration both H-atoms adjacent to the double bound are located at opposite sides of the double bond. Figure 1.1 shows linoleic acid (C18:2n-6), which belongs to the n-6 family, α-linolenic acid (C18:3n-3), which belongs to the n-3 family, and one of the *trans* isomers of α-linolenic acid (C18:3n-3 Δ9*c*,12*c*,15*tr*).

Fatty acids of the n-7 and n-9 family can be synthesized *de novo* in most cells. Linoleic and α-linolenic acid can not be synthesized *de novo* by human cells, because they lack Δ12- and Δ15 desaturases. Therefore, these fatty acids are called essential fatty acids (EFAs) and must be provided in the diet. *Trans* fatty acids are only formed *de novo* as an intermediate in the β-oxidation. Nearly all *trans* fatty acids in human tissues originate from the diet.

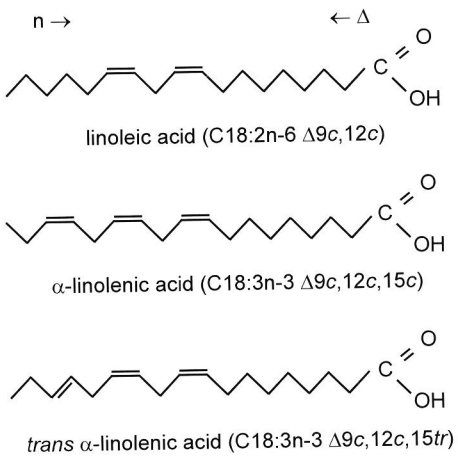

Figure 1.1 Chemical structure of linoleic, and *cis*- and *trans* α-linolenic acid

METABOLISM OF ESSENTIAL FATTY ACIDS

Desaturation and elongation

In the human body, fatty acids can by converted by alternate desaturation and elongation reactions. The conversions of the α-linolenic, linoleic, palmitic, and stearic acids into their longer chain metabolites are shown in figure 1.2.

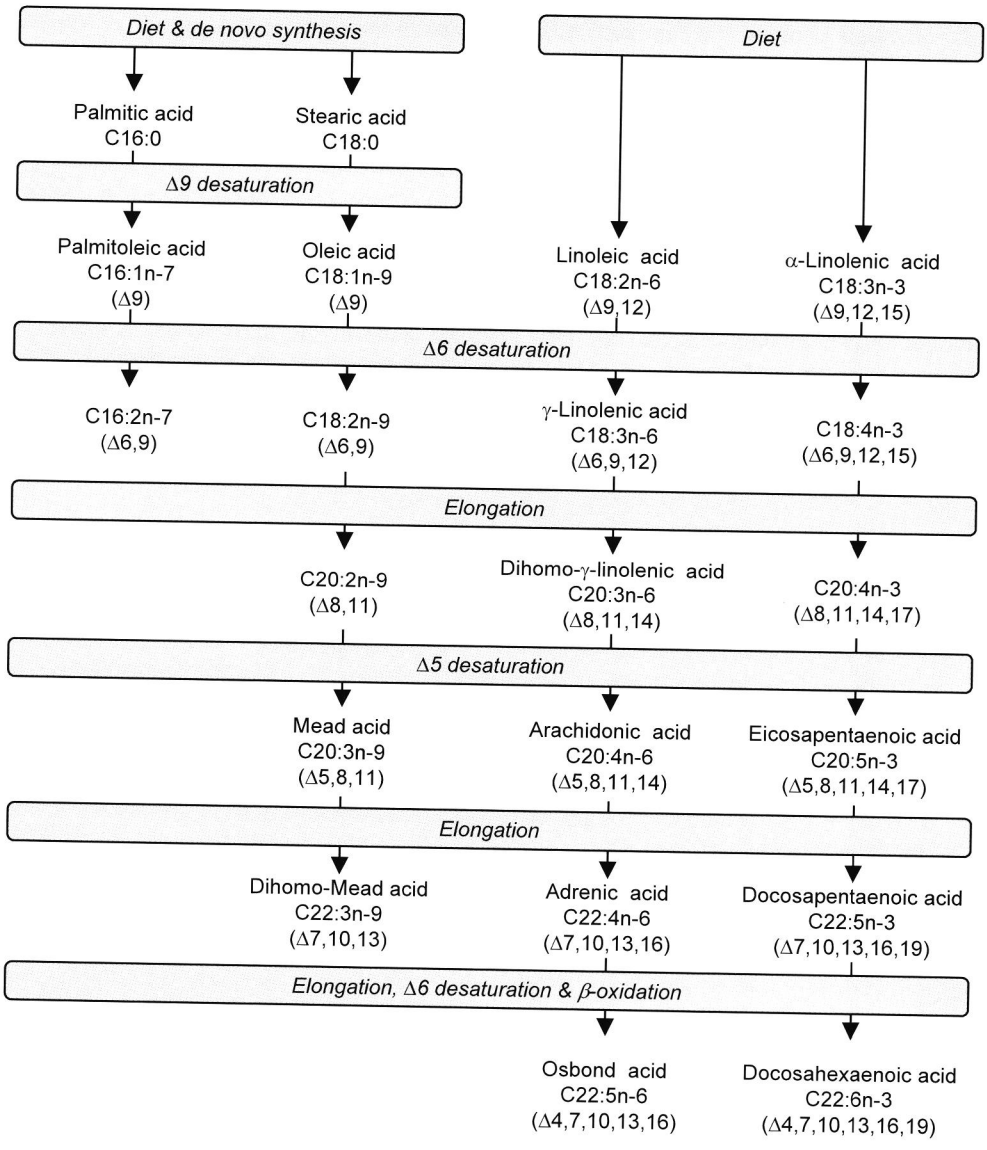

Figure 1.2 Conversion pathways of fatty acids

Humans have three desaturase enzymes, Δ9-, Δ6-, and Δ5-desaturases, of which Δ9 desaturase is not involved in the conversion of essential fatty acids. Delta 6 desaturase introduces a double bond at the Δ6 position when this fatty acid has already a double bond at the Δ9 position, while Δ5 desaturase introduces a double

bond at the Δ5 position when the Δ11 and Δ8 positions are already desaturated. The desaturases are each part of three separate desaturase systems. These desaturase systems are embedded in microsomal membranes, and involve three integral components, the desaturase, NADH cytochrome-b_5 reductase and cytochrome-b_5. The desaturase is the rate limiting reaction in the desaturase system (8).

Recently, the human Δ6 and Δ5 desaturase genes have been sequenced (9,10), and mRNAs from these genes were found in many human tissues including skeletal muscle, lung, placenta, kidney and pancreas. mRNA levels of both desaturases, however, are highest in the liver, heart and brain (10). Also, *in vitro* animal studies, using radioactive labeled fatty acids, showed high desaturation activity in the liver (11). The Δ6 desaturase activity has been reported to be the rate limiting reaction for the synthesis of arachidonic acid and EPA from linoleic acid and α-linolenic acid, respectively (12-14). C22:4n-6 and C22:5n-3 are not converted into C22:5n-6 and C22:6n-3 by a Δ4 desaturase and elongase, but by three reactions, two elongation steps followed by Δ6-desaturation in microsomes, and a peroxisomal shortening (15). It has been suggested that this Δ6 desaturase may be different from the one that converts α-linolenic and linoleic acid (16).

Elongation results in the incorporation of a two-carbon unit from malonylCoA into the activated fatty acid chain proximal to the carboxyl group (17). The elongation system is located on the membrane of endoplasmatic reticulum of many animal tissues, like liver, brain, kidney, small intestine, placenta, lung etcetera (17,18). In addition, *in vitro* elongation has been observed in many mammalian cell lines (19,20). Besides in endoplasmatic reticulum, elongation has also been observed in mitochondria and peroxisomes of rats (21,22). The elongase enzyme system requires several enzymes, anaerobic conditions, adenosine triphosphate (ATP), and a reduced pyridine nucleotide (NADPH) (23). Apparently, PUFAs and saturated fatty acids are elongated by different enzymes (24). The rate of elongation is faster with an increased degree of unsaturation and with shorter chain length (25).

Conversion of essential fatty acids has been investigated mainly in *in vitro* and animal studies, while human *in vivo* studies are limited. In one of the first human *in vivo* studies ^{14}C labeled linoleic acid was used to investigate its conversion into arachidonic acid in normo- and hyperlipidemic adult males (26). A later study used deuterated fatty acids to investigate incorporation of linoleic and α-linolenic acid and their LCPs in plasma lipids of healthy adults (27). In infants, ^{13}C enrichment of dihomo-γ-linolenic acid and arachidonic acid in plasma was observed after administration of corn oil (28), which has a relatively high ^{13}C linoleic acid content. This study showed that infants are also capable to convert linoleic and α-linolenic acid into LCPs. None of the previous studies combined the conversion and oxidation of labeled fatty acids, which is however only possible when ^{13}C labeled fatty acids are used, neither did they examine the incorporation of fatty acids into tissue membranes.

The longer chain more unsaturated fatty acid products of α-linolenic and linoleic acid can be incorporated into intracellular and plasma membranes, and are precursors of a wide range of eicosanoids. Dietary n-3 and n-6 fatty acids are incorporated into membranes as phospholipids and its content in cellular membranes reflects dietary intake (29,30). Both n-3 and n-6 fatty acids are essential as structural components and are related to membrane functions like membrane fluidity, osmotic fragility, permeability for metabolites, degranulation of vesicles, activity of membrane bound enzymes and receptors and the transduction of electrical signals.

Eicosanoids (prostanoids, leukotrienes and hydroxy fatty acids) are formed enzymatically by oxygenation of the C20 fatty acids dihomo-γ-linolenic acid (C20:3n-6), arachidonic acid (C20:4n-6), and EPA (C20:5n-3). Figure 1.3 shows the formation of eicosanoids from arachidonic acid. Eicosanoids are active in the cardiovascular system. For example, the antithrombotic prostacyclin (PGI_2) is a highly potent vasodilator and inhibitor of platelet aggregation, while the prothrombotic thromboxane A_2 (TxA_2) is a strong vasoconstrictor causing aggregation of platelets. In normal hemostasis, the syntheses of these eicosanoids are in balance.

Factors influencing desaturase activity

Conversion rates of α-linolenic and linoleic acid may be affected by nutritional and hormonal factors, and by age. Inhibition of desaturases may result in decreased levels of LCPs, which may even become inadequate. Knowledge of factors that affect activities of desaturases is therefore needed to determine appropriate intakes of α-linolenic and linoleic acid and their LCPs. Indeed, it may be necessary to increase the intake of LCPs in several populations at risk (5).

Effects of nutritional factors influencing desaturase activities have been reviewed several times (2,8,23,31). The studies described in these reviews, however, were mainly animal and *in vitro* studies. Briefly, Δ6 desaturase activity is activated by essential fatty acid deficiency, while it is depressed by fasting, and n-3 and n-6 LCPs. Competition between fatty acids exists, since n-3 and n-6 fatty acids are converted by the same desaturase enzymes, however, Δ6 desaturase has a higher affinity for α-linolenic acid as for linoleic acid. It was also reported that Δ5 desaturase activity is increased on essential fatty acid deficiency. Results, however, were contradictory.

Only one human *in vivo* study investigated diet effects on conversion rates of essential fatty acids. It was reported that dietary linoleic acid reduced the conversion of both deuterated linoleic and α-linolenic acid in healthy adults (27).

β-Oxidation

Fatty acids can be degraded by β-oxidation and are thus an important source of energy for the human body. For β-oxidation, the activated fatty acids are dehydrogenated at the carboxyl side, after which two carbons (acetyl-CoA) are split off. Thereafter, the acyl-CoA residue can be dehydrogenated again until the fatty acid is degraded completely. The degradation of unsaturated fatty acids needs two

additional enzymes, Δ3-*cis*-Δ2-*trans* enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase. AcetylCoA can enter the Krebs cycle to be oxidized to CO_2 and H_2O. The electrons removed in the two hydrogenation steps move to oxygen via the respiratory chain, accompanied by oxidative phosphorylation of ADP (32).

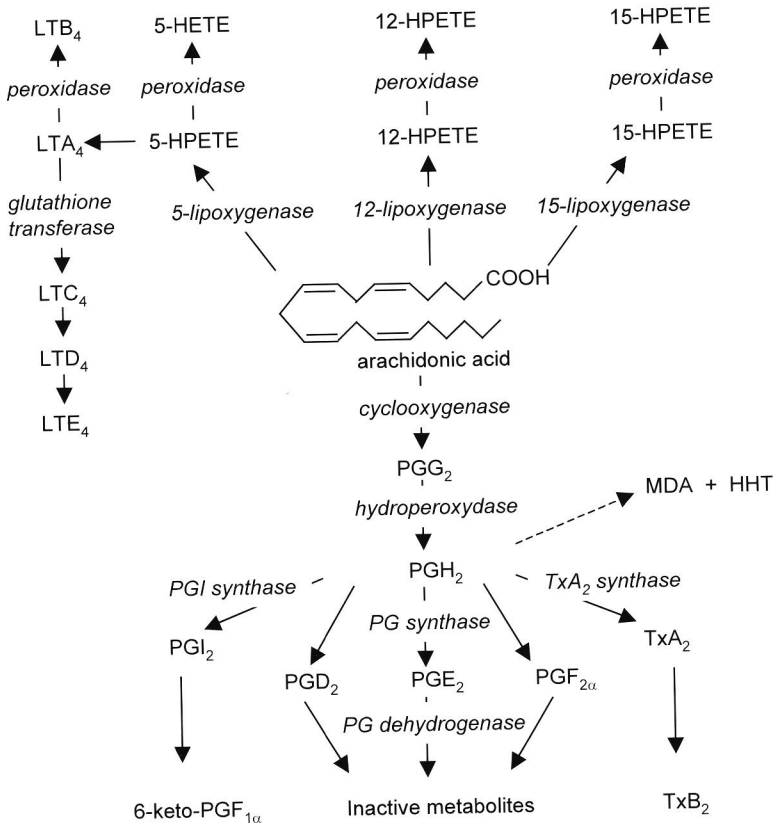

Figure 1.3 Formation of eicosanoids from arachidonic acid

Most dietary fatty acids are oxidized for energy via β-oxidation in mitochondria, while the peroxisomal system is used for the β-oxidation of very long chain fatty acids, prostaglandines and dicarboxylic acids (32,33). Indeed, n-3 and n-6 C22 and C24 fatty acids are retroconverted into C20 fatty acids by peroxisomes of human fibroblasts via β-oxidation (34). Whole body oxidation of fatty acids may vary (35). ^{13}C Oleic acid is oxidized at a faster rate than ^{13}C linoleic acid, which is oxidized at a faster rate than ^{13}C stearic acid.

TECHNIQUES TO STUDY *IN VIVO* FATTY ACID METABOLISM IN HUMANS

Stable isotopes

Only until the introduction of stable isotopes, human fatty acid metabolism was mainly measured in *in vitro* studies. Emken and coworkers were the first who used stable isotopes of hydrogen (deuterium) to study the conversion of series of *trans* isomers of oleic acid (36-40). Isotopes differ from their normal counterparts (tracee) by a different number of nuclear neutrons. The stable isotope of carbon (^{12}C) is ^{13}C, in which the superscript refers to the atomic mass. Molecules in which all C-atoms are ^{13}C labeled, are called uniformly (U) labeled. Except for the lack of exposure to radiation, using stable isotopes in *in vivo* studies has other advantages (41). The isotopic effects will be smaller than for ^{14}C because the mass differences between the naturally predominating isotope are smaller. Furthermore, it is possible to use different tracers simultaneously and to repeat dosing. A disadvantage is that the natural abundance of ^{13}C is as high as 1.11%. However, one can easily correct for this by collecting baseline samples. Using ^{13}C appears to be safe, since unwanted side effects or biological effects of the ^{13}C have never been detected (41).

When ^{13}C labeled fatty acids are given to subjects, ^{13}C will appear in for example plasma, erythrocytes, and breath. ^{13}C Enrichment ($\delta^{13}C$) in these components can be measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Fatty acids from plasma and erythrocytes are first separated by the GC, and thereafter combusted by the interface. The formed CO_2 can then be measured as isotope ratio ($^{13}C/^{12}C$) (42). The true isotope ratio can be calculated by normalizing the isotopic enrichment against the international standard Pee Dee Belemate limestone (PDB), which has a $^{13}C/^{12}C$ ratio of 0.0112372. The fatty acid concentration in plasma and erythrocytes can be measured by gas chromatography coupled with a flame ionization detector (GC/FID), which makes it possible to calculate the tracer concentration. Oxidation of ^{13}C labeled fatty acids may be measured by taking breath samples (43). This is non-invasive and painless for volunteers, and very easy to perform for the investigator. By analyzing also total CO_2 production, oxidation of the ^{13}C labeled fatty acids can be calculated as well.

Desaturase gene expression in human tissues

Since desaturases and elongase are essential for the conversion of essential fatty acids into LCPs and eventually into eicosanoids, studying their gene expressions may be helpful to clarify fatty acid metabolism. Human $\Delta 9$, $\Delta 6$, and $\Delta 5$ desaturase have been cloned and characterized (9,10,44). As reported, desaturase mRNAs are found in many human tissues, but are most abundant in the liver (10). This and several other organs and tissues are, however, not suitable to investigate in human studies. Alternatively, desaturase mRNAs can be measured in human leukocytes, in which $\Delta 6$ desaturase activity has been found (45). It is, however important to know whether the gene expressions in liver and leukocytes are related. If so, regulation of

Δ6 desaturase in leukocytes may be measured in future research in order to predict regulation of Δ6 desaturase in the liver. The amino acid sequence of human elongase has not been reported yet.

TRANS FATTY ACIDS

Intake of trans *fatty acids*

The daily total *trans* fatty acid intake in the Netherlands is about 4.8 g (1.5% of energy) in men and 3.8 g (1.6% of energy) in women (46). In Europe, the daily total *trans* fatty acid intake varies between 0.5% of energy in Italy to 2.0% of energy in Iceland (46). Most *trans* fatty acids are *trans* monounsaturated fatty acids. In fact, more than 80% of total *trans* fatty acids is elaidic acid (C18:1 Δ9*tr*). Furthermore, more than 10% of the *trans* fatty acids is *trans* linoleic acid. *Trans* α-linolenic acid and *trans* monounsaturated fatty acids other than elaidic acid also contribute to the *trans* intake. *Trans* monounsaturated fatty acids are mainly formed by bacteria in the first stomach (rumen) of ruminant animals and by industrial hydrogenation, while *trans* polyunsaturated fatty acids are mainly formed during the deodorization process. In US vegetable oils, the degree of *trans*-isomerization of linoleic acid and α-linolenic acid may be up to 3.3% and 37.1%, respectively (47). In addition, some *trans* polyunsaturated fatty acids are formed during deep-frying (48). Increment of the dietary α-linolenic acid intake as recommended (5), may thus increase the intake of *trans* α-linolenic acid.

Effects of trans *fatty acids on the plasma lipid profile*

A high plasma total cholesterol level is associated with an increased risk for CHD. In addition, the distribution of cholesterol among lipoproteins is important: low density lipoprotein (LDL) cholesterol increases CHD risk, and high density lipoprotein (HDL) cholesterol is associated with a decreased risk (49). An important parameter to predict risk for CHD is the total to HDL cholesterol ratio (50). This ratio reflects the risk when an increased total or LDL cholesterol level is combined with a lowered HDL cholesterol level. In the Netherlands, this ratio is used to predict CHD risk (7). Increased fasting triacylglycerol concentrations are also associated with an increased CHD risk (51).

Trans monounsaturated fatty acids have been found to influence plasma lipoprotein levels negatively. In fact, each additional percent of dietary energy as *trans* monounsaturated fatty acids at expense of oleic acid results in an increase in LDL cholesterol levels of 0.040 mmol/l, a decrease in HDL cholesterol levels of 0.013 mmol/l, and an increase in triacylglycerol levels by 0.013 mmol/l (52). The effects of *trans* polyunsaturated fatty acids on serum lipids and lipoproteins are not yet known.

OUTLINE OF THE THESIS

The *in vivo* essential fatty acid metabolism has not been extensively studied in human subjects, neither did previous studies investigate both oxidation and elongation. Furthermore, effects of *trans* polyunsaturated fatty acids on risk parameters of CHD have not been investigated yet.

Therefore, this thesis was aimed at:

- ❖ investigating the *in vivo* metabolism of n-3 and n-6 fatty acids in healthy subjects
- ❖ examining the effects of *trans* α-linolenic acid on plasma lipids and lipoproteins

These issues have been investigated in four studies. The first study focused on linoleic acid metabolism, while effects of dietary composition on α-linolenic acid metabolism has been investigated in the second study. The third study investigated mRNA levels for Δ5, Δ6 and Δ9 desaturases in human liver and mononuclear blood cells. Finally, the fourth study was a large intervention study, which examined the nutritional and health impact of *TRANS* α-Linolenic acid in European men: the *TRANS*LinE Study.

Chapter 2 focuses on the oxidation and conversion of ^{13}C linoleic acid into its long chain metabolites in healthy subjects. **Chapter 3** describes the effects of dietary α-linolenic acid on the oxidation and conversion of ^{13}C α-linolenic acid into its longer chain more unsaturated fatty acids. Enrichments of ^{13}C labeled fatty acids in plasma and ^{13}C recovery in breath CO_2 are presented. Incorporation of ^{13}C labeled fatty acids into erythrocytes after intake of a single bolus of ^{13}C α-linolenic acid is discussed in **chapter 4**.

In **chapter 5**, the relationship between mRNA levels for Δ5, Δ6 and Δ9 desaturase in mononuclear blood cells and the liver are presented.

Chapter 6 gives an overview of the metabolism and health effects of *trans* fatty acids. **Chapter 7** describes the effects of dietary *trans* α-linolenic acid on plasma lipids and platelet fatty acid composition of the *TRANS*LinE-study. In **chapter 8**, effects of *trans* α-linolenic acid on plasma lipids and lipoproteins of the *TRANS*LinE-study in healthy men are discussed.

Finally, the main results of the four studies are discussed in **chapter 9**.

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2

The metabolism of linoleic acid in healthy subjects after intake of a single dose of ^{13}C linoleic acid

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ABSTRACT

The objective of the study was to investigate the *in vivo* oxidation of ^{13}C linoleic acid ($^{13}C18:2n-6$) and its conversion into longer chain polyunsaturates (LCPs) in healthy subjects. Blood samples were collected from six subjects, three men and three women, before (fasted) and 7, 11 (non-fasted), 24, 48, 72, 168 and 336 hours (fasted) after ingestion of a single bolus of 45 mg uniformly labeled ^{13}C linoleic acid dissolved in 8 g olive oil. In three subjects, breath was also sampled and CO_2 production measured every hour during the first 12 hours. Subjects consumed their habitual diets. Plasma ^{13}C enrichments were measured by gas chromatography-combustion-isotope ratio mass spectrometry and fatty acid compositions by gas chromatography coupled with a flame ionization detector.

The tracer/tracee ratio (TTR) of C18:2n-6 in plasma total lipids was already increased 5 hours after tracer intake. The mean peak amount (± sem) of $^{13}C18:2n-6$ (3.4 ± 0.8 mg; 7.6% of tracer dose) was found after about 17 hours, $^{13}C18:3n-6$ (0.018 ± 0.008 mg; 0.04% of tracer dose) after 7 to 48 hours, and $^{13}C20:3n-6$ (0.028 ± 0.011 mg; 0.06% of tracer dose) after 48 to 336 hours. Time to peak TTRs of arachidonic acid (C20:4n-6) varied between subjects and peak amounts were on average 0.05% of tracer dose. The proportion of ^{13}C linoleic acid recovered in breath after 12 hours ranged between 16.8% and 25.1%.

These findings suggest that a single bolus of 45 mg ^{13}C linoleic acid can be used to study the oxidation of ^{13}C linoleic acid. However, because of the low TTRs for C20:4n-6, a higher dose is recommended for studying the conversion of ^{13}C linoleic acid into arachidonic acid. In addition, since only about 35% of the tracer was found in plasma total lipids and as $^{13}CO_2$ in breath, it might be necessary to study other accessible lipid fractions as well to determine the overall conversion of linoleic acid.

INTRODUCTION

Arachidonic acid (C20:4n-6) is a major precursor of eicosanoids, which have important physiological and pathological functions in humans like in the regulation of inflammatory processes, immunological reactions and platelet aggregation. Arachidonic acid can be derived from the diet, but can also be synthesized, mainly in the liver, by alternate desaturation and elongation of dietary linoleic acid (C18:2n-6). These conversion reactions, however, have been investigated mainly in *in vitro* and animal studies. To what extent linoleic acid is converted into its longer chain more unsaturated metabolites in man is only partially known.

A few studies have examined the *in vivo* metabolism of linoleic acid in humans. In 1967, Nichaman et al. reported the *in vivo* oxidation and conversion of ^{14}C labeled linoleic acid into arachidonic acid in normo- and hyperlipidemic males (1). More recently, the conversion of deuterated linoleic acid has been demonstrated in three human studies (2-4). In these studies, however, large doses of deuterated linoleic acid were administered, which may have decreased the dietary n-3 to n-6 fatty acid ratio considerably. Because linoleic acid and α-linolenic acid compete for the same $\Delta 6$ desaturase, the key enzyme in the conversions of linoleic and α-linolenic acids into their respective longer chain polyunsaturated fatty acids (LCPs), high doses of labeled linoleic acid may have an unwanted impact on conversion reactions and, consequently, substrate inhibition may occur (2).

Other previous studies have used ^{13}C labeled linoleic acid to investigate in lactating women linoleic acid oxidation and the appearance of n-6 fatty acids in milk (5). Also, the conversion of ^{13}C linoleic acid has been studied in infants (6-10). How the results in these special groups compare to those of the general population is not known. In addition, previous studies did not combine the conversion and appearance in plasma, and oxidation of ^{13}C linoleic acid. Therefore, the aim of this study is to examine both oxidation of ^{13}C linoleic acid and its conversion into LCPs in normal healthy adults.

SUBJECTS AND METHODS

Subjects

Six healthy volunteers, three men and three women, participated in this study. The subjects were aged between 21 and 30 years (mean ± sem; 25.7 ± 1.2 years), weighed 58.1 to 78.1 kg (mean 68.3 ± 3.3 kg) and had body mass indexes (BMI) between 18.8 and 28.3 kg/m^2 (mean 22.2 ± 1.3 kg/m^2). None of the subjects used medication known to affect lipid metabolism. All subjects were apparently healthy, as indicated by a general questionnaire. Three of the participants smoked, one man and two women. Furthermore, two women used oral contraceptives. The protocol had

been approved by the local ethical committee and all subjects gave their written informed consent. The study was performed during the summer of 1996.

Experimental design

After an overnight fast, subjects came to the department at 8.00 a.m. First, a blood sample of 10 ml was collected. Thereafter, the CO_2 production of three subjects was measured by indirect calorimetry (Sensormedics 2900; Sensormedics, Anaheim, CA, USA or Oxycon β; Mijnhardt, the Netherlands), after which a breath sample was taken. Breath samples were stored in vacutainers (Becton & Dickinson, Meylan, France). All six subjects then received a single bolus of uniformly labeled ^{13}C linoleic acid (isotopic purity >98% ^{13}C18:2n-6, chemical purity >95%; Martek Biosciences Corporation, Columbia, MD) as methylester at a dose of 45 mg dissolved in 8 g olive oil. Blood samples were collected every 2 hours, and breath samples (three subjects) hourly during the first 12 hours. Further fasting blood samples were collected after 24, 48, 72, 96 and 168 hours, and after 336 hours a final blood sample was collected.

Two hours after receiving the ^{13}C linoleic acid, subjects were allowed to eat breakfast ad libitum. During the first day, they stayed at the department and food, which subjects were allowed to consume ad libitum, was available for the whole day. Use of coffee or drinks containing caffeine was prohibited during the first day only, because of the possible stimulating effect of caffeine on lipolysis and lipid oxidation (11).

Blood sampling and analysis

Until 12 hours after ^{13}C linoleic acid administration, blood was sampled in an EDTA containing tube through a Teflon catheter (Baxter Quick Cath, Dupont, Ireland) or in a vacuum tube using a 1.2-mm needle (Strauss Kanule; Luer, Wächtersbach, Germany) with the volunteer in a recumbent position. Subjects were free to choose the method of sampling. The catheter was flushed every hour with 1 ml of a heparin solution (0.5 IU heparin-sodium; Leo Pharmaceutical Products BV, Weesp, the Netherlands). The other fasting blood samples were taken with a vacuum tube. Plasma for ^{13}C enrichment analyses and analyses of fatty acid composition of plasma total lipids was obtained by centrifugation at 2000 × g for 15 minutes at 4°C, within one hour of venipuncture. Plasma samples were quickly snap-frozen and stored at -80°C.

Lipids from plasma were extracted (12), using tri-heptadecanoic acid (C17:0) as an internal standard. Thereafter, lipid extracts were hydrolyzed and the resulting fatty acid methylated (13). The absolute fatty acid composition in plasma total lipids was analyzed using a gas chromatograph with a flame ionization detector (GC/FID) (Perkin Elmer, Norwalk, Connecticut, USA) as described elsewhere (14).

^{13}C Enrichments of individual fatty acid methyl esters (FAMEs) in total lipids from plasma and in breath were analyzed with a gas chromatograph (Varian 3400) coupled with an isotope ratio mass spectrometer (GC-C-IRMS; Finnigan MAT 252,

Bremen, Germany) via a combustion interface. Samples were injected on a 50 m column (BPX70, 0.33 mm, 0.25 μm film thickness; SGE, Austin, Texas, USA) at a temperature of 250°C. Helium gas was used as a carrier (injector inlet pressure 124 kPa). Enrichments of C18:2n-6, C18:3n-6, C20:3n-6, C20:4n-6, C22:4n-6 and C22:5n-6 were determined in one run. The GC-oven was programmed at 160°C for 10 min (rise of 1°C min^{-1}), then 190°C for 14 min (rise of 30°C min^{-1}), and finally 260°C for 25 min.

Calculations

The difference between the tracer/tracee ratio (TTR; $^{13}C/^{12}C$ ratio) of the individual FAMEs and a known reference standard was expressed as the delta ($\delta^{13}C$). The true TTR of the FAME was then calculated by normalizing the isotopic enrichment ($\delta^{13}C$) against the international standard Pee Dee Belemate limestone (PDB), which has a TTR of 0.0112372. The concentration of a ^{13}C labeled fatty acid was calculated with the true TTR and the plasma concentration of that particular fatty acid. It was assumed that during fatty acid elongation the two carbon-atom fragments added after elongation were not enriched above background. Absolute amounts (in mg) of plasma fatty acids in the body were calculated based on a plasma volume of 4.5% of body weight (15).

Recovery of ^{13}C in breath derived from ^{13}C linoleic acid during the first 12 hours after tracer intake was calculated from the TTR in breath and the absolute CO_2 production.

Values of subjects were averaged, and presented as means ± standard errors.

RESULTS

On the basis of the first three subjects, whose results are presented in figure 2.1, it was decided to measure the ^{13}C enrichments of LCPs in plasma total lipids only at t=0, 7 (plasma of t=6 and t=8 hours pooled), 11 (plasma of t=10 hours and t=12 hours pooled), 24, 48, 72, 168 and 336 hours for the other three subjects so as to reduce the number of analyses to a more manageable number. In order to compare the results of the first three subjects with the others, the data from t=6 hours and t=8 hours and from t=10 hours and t=12 hours were averaged.

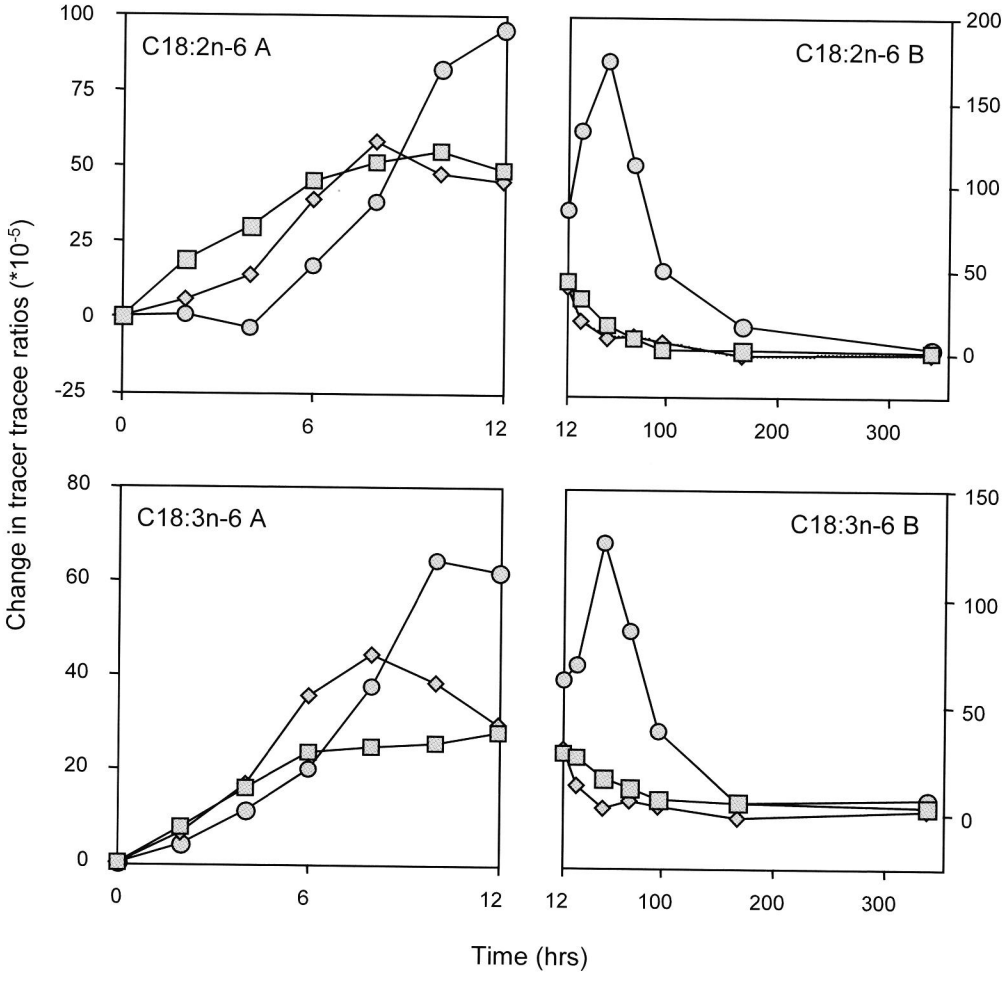

Figure 2.1 Plasma ^{13}C enrichments, corrected for baseline values, of C18:2n-6 and C18:3n-6 after a single dose of ^{13}C linoleic acid for three subjects over the first 12 hours (A) and in the period between 12 and 336 hours (B)

Tracer/tracee ratios

Peak tracer/tracee ratios (TTR) of C18:2n-6 in plasma total lipids were obtained within the first 12 hours for five subjects and after 48 hours for one subject, with a mean of $107.8 * 10^{-5} \pm 22.6 * 10^{-5}$ after 17 hours (median 11 hours; table 2.1). Mean peak TTR was the highest for C18:2n-6. Peak TTRs of C18:3n-6 were reached after 7 to 48 hours, with a mean of $46.3 * 10^{-5} \pm 16.9 * 10^{-5}$ after 25 hours (median 18 hours). Peak TTRs of C20:3n-6 were reached after 48 to 72 hours for five subjects

and after 336 hours for one subject, and was on average 16.3 * 10^{-5} ± 5.0 * 10^{-5}. Time to peak TTRs of C20:4n-6 varied between the subjects and were rather low. TTRs of C22:4n-6 and C22:5n-6 were very low as well, and TTRs exceeding background enrichments were observed in three subjects only.

Table 2.1 Mean (± sem) peak tracer/tracee ratios (TTR) and time to peak TTR (hours) of C18:2n-6 and its longer chain more unsaturated fatty acids after a bolus dose of 45 mg ^{13}C linoleic acid (n=6)

	TTR (*10^{-5})			Time to peak TTR (hours)			
Fatty acid	Mean ± sem	Range		Mean ± sem	Range		Median
C18:2n-6	107.8 ± 22.6	48.7	to 196.6	16.5 ± 6.3	7	to 48	11
C18:3n-6	46.3 ± 16.9	14.3	to 125.7	24.8 ± 7.7	7	to 48	18
C20:3n-6	16.3 ± 5.0	6.6	to 39.0	104.0 ± 46.6	48	to 336	60
C20:4n-6	3.0 ± 0.6	1.1	to 5.3	154.3 ± 62.3	7	to 336	120
C22:4n-6 [a]	2.5 ± 0.5	1.5	to 3.3	75.7 ± 47.4	48	to 96	48
C22:5n-6 [a]	4.0 ± 1.4	2.5	to 6.8	80.0 ± 16.0	11	to 168	96

[a] Detected in three subjects only

Plasma fatty acid concentrations

Mean fasted n-6 fatty acid concentrations in plasma total lipids were the highest for C18:2n-6 (953 ± 52 mg/l; 32.54 ± 1.12% of total fatty acids) and C20:4n-6 (195 ± 10 mg/l; 6.73 ± 0.40% of total fatty acids). Mean fasted plasma concentrations were 9.3 ± 1.4 mg/l (0.31 ± 0.02% of total fatty acids) for C18:3n-6, 44.0 ± 3.0 mg/l (1.50 ± 0.09% of total fatty acids) for C20:3n-6, 6.3 ± 0.2 mg/l (0.22 ± 0.01% of total fatty acids) for C22:4n-6, and 6.9 ± 0.5 mg/l (0.24 ± 0.03% of total fatty acids) for C22:5n-6.

Absolute amounts of plasma ^{13}C labeled fatty acids

Absolute amounts (in mg) of ^{13}C18:2n-6 started to increase almost directly after tracer intake (figure 2.2). After 168 hours most of the ^{13}C18:2n-6 had disappeared from the plasma, but only after 336 hours values had nearly returned to baseline. Appearance of ^{13}C18:3n-6 in the plasma occurred almost at the same time as that of ^{13}C18:2n-6. At the end of the study, plasma levels of ^{13}C18:3n-6 had almost returned to baseline. The mean amount of plasma ^{13}C20:3n-6 increased slowly over time until after 72 hours, whereafter it declined. After 336 hours, most subjects had still considerable amounts of ^{13}C20:3n-6 in plasma as compared to their peak. ^{13}C20:4n-6 appeared rather quickly in plasma and continued to increase until after 336 hours. Amounts of ^{13}C22:4n-6 and ^{13}C22:5n-6 were very small, and are, therefore, not shown in figure 2.2.

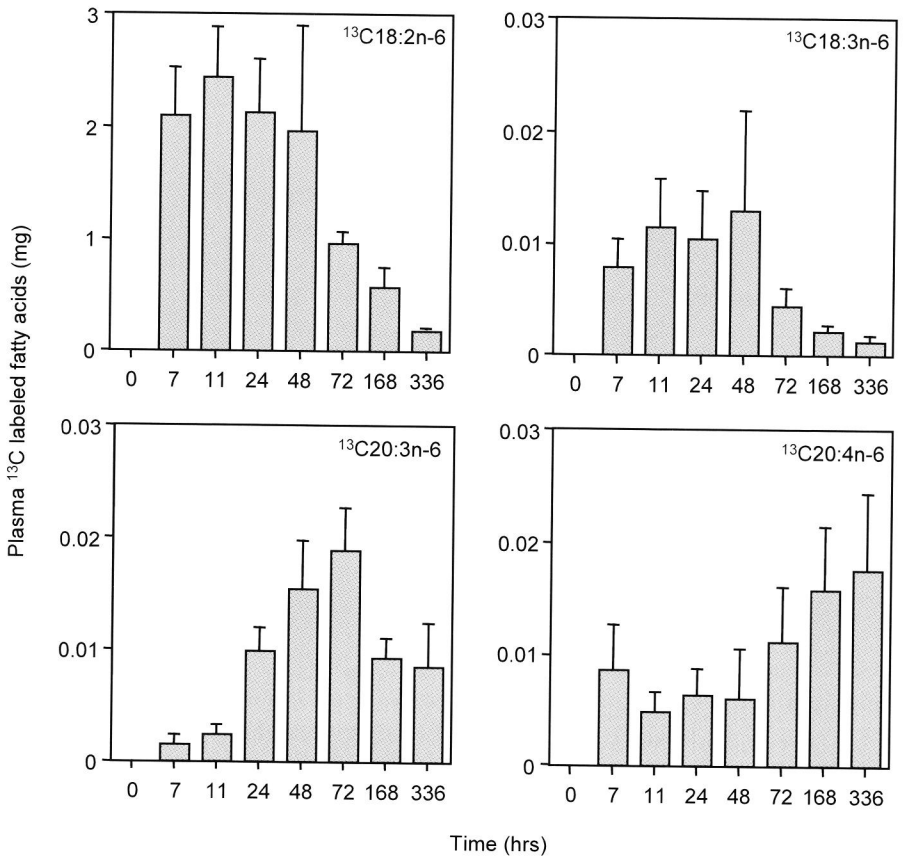

Figure 2.2 Mean (± sem) absolute amounts (in mg) of plasma ^{13}C18:2n-6, ^{13}C18:3n-6, ^{13}C20:3n-6 and ^{13}C20:4n-6 after a single dose of ^{13}C linoleic acid during the course of the study. Values were calculated based on a plasma volume of 4.5% of body weight (15).

The mean peak absolute amount of ^{13}C18:2n-6 in plasma was 3.4 ± 0.8 mg, which was 7.6% of the tracer dose (figure 2.3). Peak amounts of ^{13}C18:3n-6 were 0.003 to 0.057 mg, with a mean amount of 0.018 ± 0.008 mg (0.04% of tracer dose). The mean peak amount of ^{13}C20:3n-6 was 0.028 ± 0.011 mg, which was only about 0.06% of the tracer dose. The peak amount of ^{13}C20:3n-6 varied between 0.010 and 0.079 mg. Peak amounts of ^{13}C20:4n-6 ranged between 0.009 and 0.048 mg, which was on average only 0.04% of the given dose of ^{13}C linoleic acid. The peak amount of ^{13}C18:3n-6 plus ^{13}C20:3n-6 plus ^{13}C20:4n-6 was 0.15% of the tracer dose and 2.0% of the peak amount of ^{13}C18:2n-6.

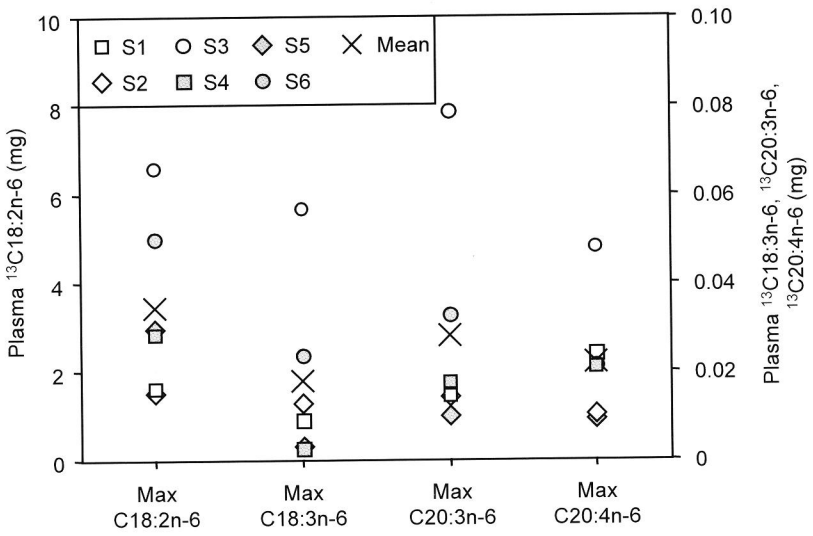

Figure 2.3. Individual (S1 to S6) and mean (± sem) peak amounts (in mg) of plasma ^{13}C18:2n-6, ^{13}C18:3n-6, ^{13}C20:3n-6 and ^{13}C20:4n-6 after a single dose of ^{13}C linoleic acid. Values were calculated based on a plasma volume of 4.5% of subjects body weight (15).

Oxidation

Breath samples were measured in three subjects. Peaks in ^{13}C enrichment in breath were reached after 3 to 5 hours and ranged from 7‰ to 12‰ (data not shown). Figure 2.4 shows the recovery of ^{13}C in breath derived from ^{13}C linoleic acid at various time points during the first 12 hours after tracer intake. After 6 hours, total ^{13}C recovery ranged between 11.9% and 17.4%, while after 12 hours total recovery of the tracer had increased to 16.8% to 25.1%.

DISCUSSION

This study demonstrates that conversion of ^{13}C18:2n-6 into ^{13}C18:3n-6 starts almost immediately after tracer intake. The peak TTR of ^{13}C18:3n-6 was much higher than that of ^{13}C20:3n-6. However, because of the smaller pool of plasma C18:3n-6 relative to C20:3n-6, absolute peak amounts of ^{13}C18:3n-6 were lower than those of ^{13}C20:3n-6. The small pool of C18:3n-6 may be due to the rapid elongation step of C18:3n-6 into C20:3n-6, as suggested from rat studies (16). From animal studies using radioactive isotopes, it is known that C20:4n-6 can be converted into C22:4n-6 and C22:5n-6 (16,17). However, we hardly found any ^{13}C enrichment in plasma of these latter two fatty acids. This is in agreement with results of Emken et al. (2,3).

These findings may be due to the preferential β-oxidation of C22:4n-6 rather than esterification, and the preferential esterification of C20:4n-6 rather than β-oxidation (18-20). In addition, the failure to find measurable amounts of C22:5n-6 may have been caused by the very slow rate of conversion of C22:4n-6 into C22:5n-6 as found for rats (16).

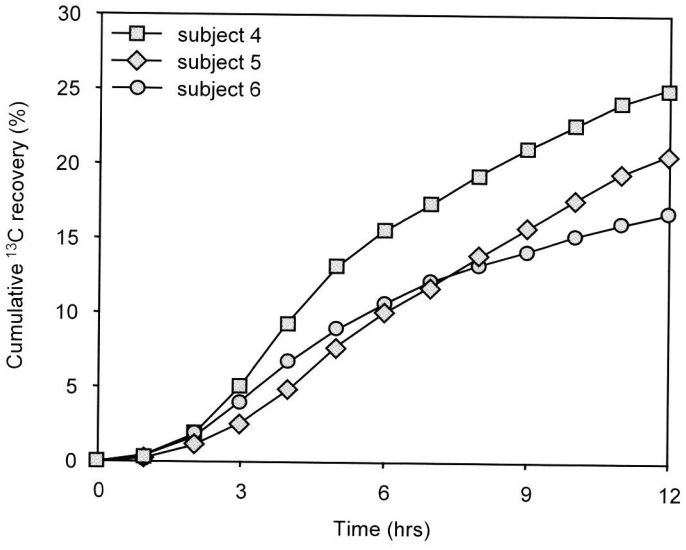

Figure 2.4 Cumulative ^{13}C recovery in breath after a single dose of ^{13}C linoleic acid for three subjects during the first 12 hours

Mean time to peak TTR was 17 hours for C18:2n-6, and 104 hours for C20:3n-6. The mean peaks of these fatty acids, however, were both affected by one extreme value from different subjects. Without these outliers, mean time to peak TTRs were 10 and 58 hours. Medians were 11 and 60 hours, respectively. Peak ^{13}C enrichment of C18:2n-6 in milk of lactating women was reached 12 hours after ingestion of 1 mg ^{13}C linoleic acid/kg body weight (5), which corresponds with our plasma results. Time to peak ^{13}C enrichment of C20:3n-6 was 12 to 24 hours in milk of lactating women (5). Thus, ^{13}C18:2n-6 seems to appear at the same time in plasma and human milk, while the peak in ^{13}C20:3n-6 was reached earlier in human milk (5) as compared to plasma. Appearance of the ^{13}C labeled fatty acids in plasma of lactating women, which is not investigated in the study of Demmelmair et al (5), may however be different from that of non-lactating subjects.

Peak ^{13}C enrichments in breath were obtained 3 to 5 hours after intake of the labeled linoleic acid, which agree well with the time to peak ^{13}C enrichments in breath after ^{13}C linoleic acid administration to lactating women (5). Total recovery in breath CO_2 for lactating women was 18% to 24%, depending on the week of lactation (5), which is comparable with our results.

In the present study, about 21% of the ingested ^{13}C linoleic acid was recovered in breath, while the peak amount of ^{13}C linoleic acid in plasma was about 8%. Furthermore, a small percentage was detected as its longer chain metabolites in plasma. Since Emken et al. have demonstrated that on average 92% of the linoleic acid may be absorbed (2), a large part of the tracer is still missing. Parts of the tracer may be incorporated into membranes or stored in adipose tissue reserves. Recently, it has been demonstrated that many human tissues express $\Delta 5$ and $\Delta 6$ desaturase, of which liver, brain and heart are the most abundant (21). This may thus suggest that these tissues contain part of the tracer as well. Furthermore, one third of the $^{13}CO_2$ derived from tracer oxidation may not be excreted in breath (22). Part of the tracer may be incorporated into products of the tricarboxylic acid cycle or fixated in the bicarbonate pool (23,24).

All participants had received the same dose of ^{13}C linoleic acid. However, amounts of ^{13}C linoleic acid and its ^{13}C labeled LCPs varied considerably between the subjects. In a recent similar study with ^{13}C α-linolenic acid (14), we found a significant negative correlation between plasma ^{13}C LCPs and the ^{13}C elimination with breath CO_2. In the present study, in the three subjects of whom ^{13}C elimination was known, the subject with the highest amounts of ^{13}C LCPs had also the lowest ^{13}C elimination by breath and vise versa. Variation in plasma ^{13}C LCPs between subjects may thus, at least partly, be explained by variation in ^{13}C elimination by breath. Furthermore, time courses of the appearance of ^{13}C labeled fatty acids were different between subjects. In the subject with the highest amounts of ^{13}C labeled n-6 fatty acids, time to peak amounts was the longest. Possibly, absorption of essential fatty acids and/or activity of desaturase or elongase enzymes is different from that of the other subjects.

In conclusion, a single bolus of 45 mg of ^{13}C linoleic acid can be used to study the oxidation of ^{13}C linoleic acid. Plasma ^{13}C enrichments of C20:4n-6, however, were rather low and even near detection limit in some subjects. A higher dose of ^{13}C linoleic acid is therefore recommended for the study of the conversion into arachidonic acid. Results of plasma total lipids and $^{13}CO_2$ recovery in breath suggest that a considerable part of the tracer is still missing. In order to detect larger parts of the tracer, other accessible lipid fractions, like erythrocytes should also be included in future research. In addition, since $\Delta 6$ desaturation is the rate-limiting step in the conversion of linoleic and α-linolenic acid, measurement of mRNA levels of $\Delta 6$ desaturase, which has been sequenced recently (25), may help to study determinants of the n-3 and n-6 fatty acid metabolism in future research.

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3

Effects of dietary α-linolenic acid on the conversion and oxidation of ^{13}C α-linolenic acid

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ABSTRACT

The effects of a diet rich in α-linolenic acid versus an oleic acid rich diet on the oxidation of uniformly labeled ^{13}C α-linolenic acid and its conversion into longer chain polyunsaturates (LCPs) were investigated *in vivo* in healthy human subjects. Volunteers received a diet rich in oleic acid (n=5) or a diet rich in α-linolenic acid (n=7; 8.3 gram/day) for 6 weeks prior as well as during the study. After 6 weeks, subjects were given 45 mg of ^{13}C α-linolenic acid dissolved in olive oil. Blood samples were collected at t = 0, 5, 11, 24, 96, and 336 hours. Breath was sampled and CO_2 production was measured each hour for the first 12 hours.

The mean (± sem) maximal absolute amount of ^{13}C-eicosapentaenoic acid (EPA) in plasma total lipids was 0.04 ± 0.01 mg in the α-linolenic acid group, which was significantly lower (P=0.01) than the amount of 0.12 ± 0.03 mg ^{13}C-EPA in the oleic acid group. Amounts of ^{13}C-docosapentaenoic acid (DPA) and ^{13}C-docosahexaenoic acid (DHA) tended to be lower as well. The mean proportion of labeled α-linolenic acid recovered as $^{13}CO_2$ in breath after 12 hours was 20.4% in the α-linolenic acid group and 15.7% in the oleic acid group, which was not significantly different (P=0.12). The cumulative recovery of ^{13}C from ^{13}C α-linolenic acid in breath during the first 12 hours was negatively correlated with the maximal amounts of plasma ^{13}C-EPA (r=-0.58, P=0.047) and ^{13}C-DPA (r=-0.63, P=0.027), but not of ^{13}C-DHA (r=-0.49, P=0.108).

In conclusion, conversion of ^{13}C α-linolenic acid into its LCPs may be decreased on diets rich in α-linolenic acid, while oxidation of ^{13}C α-linolenic acid is negatively correlated with its conversion into LCPs. In a few pilot samples, low ^{13}C enrichments of n-3 LCPs were observed on a diet rich in EPA/DHA as compared to oleic acid.

INTRODUCTION

Eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) are important structural membrane components and play a major role in many physiological processes (1). These longer chain polyunsaturated fatty acids (LCPs) are not only provided by the diet, but can also be formed by alternate desaturation and elongation of α-linolenic acid (C18:3n-3), the parent essential fatty acid from the n-3 family. However, it is largely unknown to what extent this happens in man.

From *in vitro* and rat studies with radioactive isotopes it has been suggested that the conversion of α-linolenic acid is positively related to the amount of substrate (α-linolenic acid), but negatively to that of their LCPs (EPA and DHA) (2,3). These results, however, have not been confirmed by others (4,5).

In man, EPA and docosapentaenoic acid (DPA; C22:5n-3) concentrations increase after α-linolenic acid consumption (6,7), while DHA concentrations are hardly affected (8). Fatty acid compositions of tissues, however, give no information about the origin of the fatty acids or their availability for conversion reactions. The introduction of stable isotopes has now created better opportunities to examine the *in vivo* metabolism of essential fatty acids in humans. So far, a few studies with deuterated α-linolenic acid in adults (9-11) and in infants (12), and some studies with ^{13}C labeled α-linolenic acid in infants (13-15) have been carried out. These studies, however, only focused on the chain elongation and desaturation of α-linolenic acid, although it is also possible to measure oxidation at the same time when ^{13}C labeled fatty acids are used.

In the present study, we have therefore investigated the *in vivo* conversion of uniformly labeled ^{13}C α-linolenic acid into its LCPs and its oxidation in healthy adults. In particular, the effects of a diet rich in α-linolenic acid versus a diet rich in oleic acid on ^{13}C α-linolenic acid metabolism have been examined. Finally, the relationship between the oxidation of ^{13}C α-linolenic acid and the conversion into LCPs was studied. The effects of dietary EPA plus DHA on ^{13}C α-linolenic acid metabolism were examined in a pilot study.

SUBJECTS AND METHODS

Subjects

Fifteen subjects, both men and women (age between 21 and 66 years), who participated in an intervention trial on the effects of α-linolenic acid and EPA/DHA on various cardiovascular risk markers (16), were asked to participate in this study. All subjects were healthy as indicated by a medical questionnaire and had fasting serum total cholesterol concentrations below 7.5 mmol/l and serum triacylglycerols below 3.0 mmol/l. None of the volunteers used medication or a diet known to affect fatty acid metabolism. Five of the participants smoked. Three younger women used oral

contraceptives, while the elderly women were post menopausal. The protocol had been approved by the local ethical committee and informed written consent was obtained from each volunteer.

Diets and experimental design

After a run-in period of 3 weeks, in which all subjects consumed an oleic acid rich diet, the subjects were randomly allocated to three groups. The first group received for the next 7 weeks the same diet as during the run-in period (n=5), the second group received a diet rich in α-linolenic acid (n=7), while the third group received a diet rich in EPA plus DHA (n=3). α-Linolenic acid and EPA/DHA replaced oleic acid. Fatty acids were provided as margarine from which also pies, cakes and chocolate-paste were prepared. Subjects had to consume 30 g of the experimental fat daily. In order not to change energy intakes, the volunteers were required to replace their usual margarine, pastries and sandwich spread by the experimental products provided in this study. Subjects in the oleic acid and α-linolenic acid groups were not allowed to consume fatty fish during the whole study. Furthermore, subjects were urged not to change their background diets. The fatty acid compositions of the experimental margarines, which were prepared by Unilever Research Vlaardingen, the Netherlands, have been presented elsewhere (16).

During the run-in and the experimental period, subjects recorded their food intake during the whole day for 3 consecutive days, 1 weekend and 2 week days. After the food diary had been checked by a dietitian, foods were coded, and energy and nutrient intakes were calculated using the Dutch food composition table (17).

At week 9, subjects came to the department at 8.00 a.m. after a fasting period of 12 hours. First, a 10 ml blood sample was taken, whereafter 45 mg of uniformly labeled ^{13}C α-linolenic acid (isotopic purity >95% ^{13}C18:3n-3) was given as a methylester (Martek Biosciences Corporation, Columbia, MD, USA) dissolved in 8 g olive oil. Subjects were then given a breakfast, which consisted of two slices of white bread with jam. Two hours after ^{13}C α-linolenic acid intake, a second blood sample was taken, after which the subjects were allowed to eat and drink according to their respective dietary regimes. During the day, subjects stayed at the department and food was provided for the whole day. Other, non fasting, blood samples were collected 2, 4, 6, 8, 10 and 12 hours after the intake of labeled α-linolenic acid, while fasting samples were taken after 24, 48, 72, 96, and 168 hours. Subjects then returned to their usual diets and one week later a final fasting blood sample was taken at t=336 hours. At the same time points of blood sampling (except at t=336 hours and during the first day at t=3, 5, 7, 9 and 11 hours) a breath sample was taken. For this, subjects breathed for 5 minutes into the SensorMedics (SensorMedics 2900 analyzer, Anaheim, CA, USA) in order to stabilize their breathing. For the next 5 minutes, CO_2 production was registered after which a breath sample was taken. Breath samples were stored in vacutainers (Becton & Dickinson, Meylan, France).

Blood sampling and analysis

Until 12 hours after ^{13}C α-linolenic acid administration, blood (10 ml) was sampled in an EDTA tube through a Teflon catheter (Baxter Quick Cath Dupont, Ireland) or a vacuum tube using a 1.2-mm needle (Strauss Kanule; Luer, Wächtersbach, Germany) with the volunteer in a recumbent position. The catheter was flushed every hour with 1 ml heparin solution (0.5 IU heparin-sodium; Leo Pharmaceutical Products BV, Weesp, the Netherlands). The next fasting blood samples were taken with a vacuum tube. Plasma for ^{13}C enrichment analyses and analyses of fatty acid composition of total plasma lipids was obtained by centrifugation at 2000 × g for 15 minutes at 4°C, within one hour of venipuncture. Plasma samples were snap-frozen and stored at -80°C.

Lipids from plasma were extracted (18), using tri-heptadecanoate (C17:0) as an internal standard. Thereafter, lipid extracts were hydrolyzed and the resulting fatty acids methylated (19). The absolute fatty acid composition in plasma total lipids was analyzed using a gas chromatograph with a flame ionization detector (GC/FID) (Perkin Elmer Autosystem, Norwalk, Connecticut, USA). Fatty acid methyl esters (FAMEs) were separated using a 50 m column (CP-SIL88, 0.25 mm, 0.20 μm film thickness; Chrompack, Middelburg, the Netherlands) with an injection temperature of 300°C. Helium gas was used as a carrier (injector inlet pressure 130 kPa). The oven was programmed from 160°C to 230°C in 3 temperature steps (160°C for 10 min, rise of 3.2°C min^{-1}, 190°C for 15 min, rise of 5°C min^{-1}, 230°C for 37 min).

^{13}C enrichments of individual FAMEs in total lipids from plasma and of CO_2 in breath were analyzed with a gas chromatograph (Varian 3400) coupled to an isotope ratio mass spectrometer (IRMS; Finnigan MAT 252, Bremen, Germany) via a combustion interface. Samples were injected on a 50 m column (BPX70, 0.33 mm, 0.25 μm film thickness; SGE, Austin, Texas, USA) at a temperature of 250°C. Helium gas was used as a carrier (injector inlet pressure 124 kPa). For complete separation of the peaks, enrichments of the FAMEs were determined in two separate runs, as shown in the chromatograms in figure 3.1. For C18:3n-3, C20:5n-3 and C22:6n-3, the oven was programmed from 185°C for 35 min (rise of 30°C min^{-1}) to 260°C for 33 min (figure 3.1A), and for C22:5n-3 from 205°C for 20 min (rise of 30°C min^{-1}) to 260°C for 25 min (figure 3.1B). The $^{13}C/^{12}C$ ratios were determined with a precision of δ_{PDB}<1‰, while the reproducibility of the standard was δ_{PDB}<1‰.

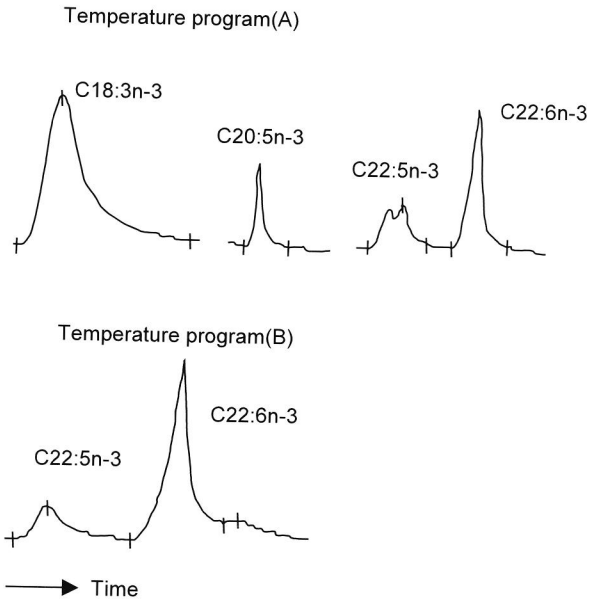

Figure 3.1 Chromatograms showing the complete separation of the C18:3n-3, C20:5n-3, 22:5n-3 and C22:6n-3 peaks using GC-C-IRMS. Temperature program (A): from 185°C for 35 min (rise of 30°C min^{-1}) to 260°C for 33 min for separation of C18:3n-3, C20:5n-3 and C22:6n-3; Temperature program (B): from 205°C for 20 min (rise of 30°C min^{-1}) to 260°C for 25 min for separation of C22:5n-3.

Calculations

The difference between the $^{13}C/^{12}C$ ratio of the samples and a known reference standard was expressed as the delta ($\delta^{13}C$). The true ratio of the sample was then calculated by normalizing the isotopic enrichment ($\delta^{13}C$), against the international standard Pee Dee Belemate limestone (PDB), which has a $^{13}C/^{12}C$ ratio of 0.0112372. The concentration of a ^{13}C labeled fatty acid was calculated by correction for the plasma concentration of this particular fatty acid. It was assumed that during fatty acid elongation the two carbon-atom fragments added after elongation were not enriched above background. Absolute amounts of total body plasma fatty acids were calculated based on a plasma volume of 4.5% of body weight (20).

Oxidation of ^{13}C α-linolenic acid was calculated from the $^{13}C/^{12}C$ ratio in breath and the absolute CO_2 production. From the second day after intake of ^{13}C α-linolenic acid onwards, the CO_2 production was estimated only once a day. Since the determination of total $^{13}CO_2$ production during the day requires regular measurements of both ^{13}C enrichment and CO_2 production during the day, $^{13}CO_2$ production was calculated for the first 12 hours only.

Statistical analyses

Results for the EPA/DHA group were not statistically analyzed as plasma ^{13}C enrichments in n-3 fatty acids of only two subjects could be measured. Values of subjects within the other groups were averaged, and presented as means ± standard errors. For each subject changes were calculated by subtracting baseline values (t=0). Differences between the oleic acid group and the α-linolenic acid group were tested with the Mann Whitney U test. Spearman correlation coefficients were calculated to examine the relationship between the cumulative recovery of $^{13}CO_2$ in breath, derived from α-linolenic acid, and the maximal amount of plasma ^{13}C labeled n-3 fatty acids. P-values are two-tailed, and differences were considered statistically significant when $P<0.05$.

RESULTS

The mean daily energy and nutrient intakes are shown in table 3.1. The mean supplemented daily α-linolenic acid intake was significantly higher in the α-linolenic acid group (8.3 ± 1.3 g; 3.1 ± 0.4% of energy) as compared to the oleic acid group. Furthermore, the intake of protein and total polyunsaturated fatty acids differed between the groups.

First, plasma ^{13}C enrichments of all 13 plasma samples of four randomly chosen subjects, one or two from each intervention group, were analyzed. Results of one subject from the α-linolenic acid group and one of the oleic acid group are shown in figure 3.2. For α-linolenic acid, results are presented for the first 24 hours only, because values had nearly returned to baseline within one day. Results of EPA, DPA and DHA are presented for 336 hours. Based on these results it was decided to analyze only samples from t=0, 24, 96 and 336 hours for the other 11 subjects so as to reduce the number of analyses to a more manageable quantity. In addition, samples from t=4 hours and t=6 hours, and from t=10 hours and t=12 hours were pooled and analyzed. These samples are denoted as t=5 hours and t=11 hours. In order to compare the results of the first four subjects with the others, the data from t=4 hours and t=6 hours, and from t=10 hours and t=12 hours were averaged.

Table 3.1 Mean (± sem) energy and nutrient intake at baseline on diets rich in oleic acid or α-linolenic acid

	Oleic acid group (n=5)	ALA group (n=7)
Energy (MJ/day)	9.5 ± 1.1	9.9 ± 0.6
Protein (En%)	15.0 ± 0.5	12.4 ± 0.8*
Fat (En%)	36.0 ± 2.7	38.3 ± 0.8
SAFA	12.1 ± 1.2	12.1 ± 0.5
MUFA	15.5 ± 1.6	14.5 ± 0.7
PUFA	6.0 ± 0.7	8.8 ± 0.7*
Linoleic acid	4.1 ± 0.7	4.5 ± 0.9
Carbohydrates (En%)	43.1 ± 3.1	46.9 ± 1.6
Alcohol (En%)	6.1 ± 1.5	2.5 ± 0.7
Cholesterol (mg/MJ)	16.1 ± 2.1	16.8 ± 3.1
Dietary fiber (g/MJ)	2.6 ± 0.5	1.8 ± 0.2

* Denotes a significant difference between groups: $P<0.05$.

Enrichments

The changes in ^{13}C enrichments of α-linolenic acid, EPA, DPA and DHA are shown in figure 3.3. Maximum changes in ^{13}C enrichment of α-linolenic acid were reached after 5 hours, followed by a rapid decrease. After 24 hours, changes in ^{13}C enrichment were still about 15% of the maximal changes. Values had returned to baseline after 336 hours (data not shown). The mean maximum ^{13}C enrichment of α-linolenic acid relative to baseline in the α-linolenic acid group was only 37% of that in the oleic acid diet. The ^{13}C enrichment peaks of EPA relative to baseline were much lower and were reached 19 hours later than those of α-linolenic acid. In the α-linolenic acid group, maximum changes in ^{13}C enrichments of EPA were 43% of those in the oleic acid group. The ^{13}C enrichments of DPA hardly changed between 24 and 96 hours in the α-linolenic acid group. As compared to the oleic acid group, maximum changes in ^{13}C enrichment were 40% in the α-linolenic acid group. After 336 hours, ^{13}C enrichments of EPA and DPA had nearly returned to baseline values in both groups. Ninety-one hours after the ^{13}C enrichment peak of α-linolenic acid, ^{13}C enrichment of DHA plateaued in both groups until after 336 hours. The order of appearance of ^{13}C labeled α-linolenic acid, EPA, DPA and DHA in plasma was thus in accordance with the conversion of ^{13}C α-linolenic acid into its LCPs.

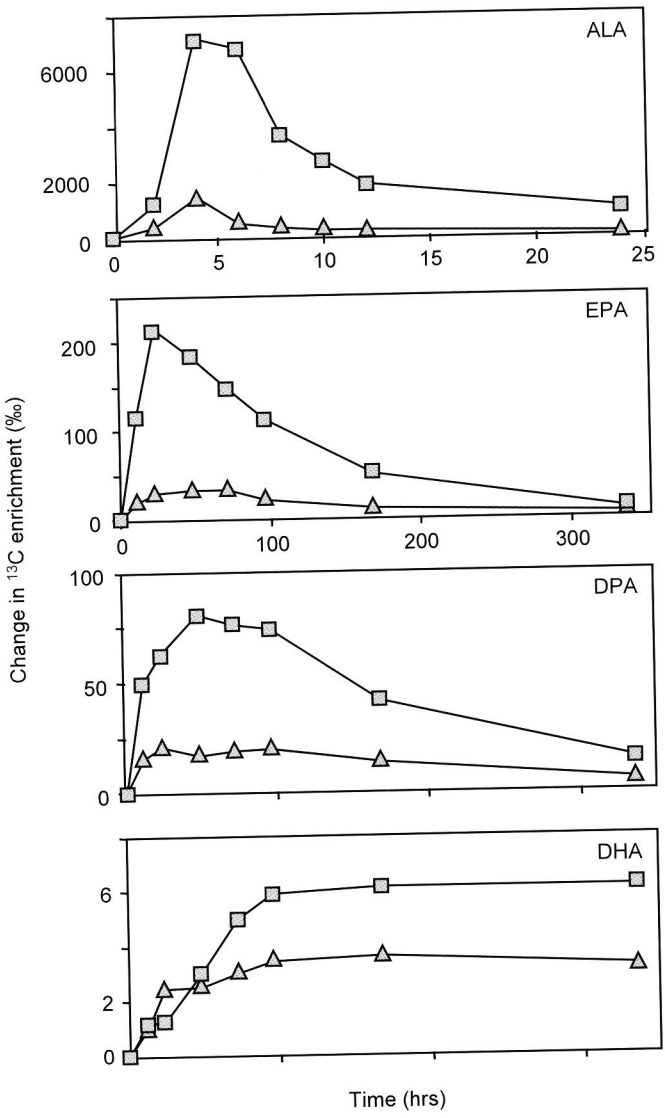

Figure 3.2 Changes in plasma ^{13}C enrichments of α-linolenic acid, EPA, DPA and DHA after intake of a single dose of ^{13}C α-linolenic acid on diets rich in oleic acid (□), or α-linolenic acid (△) of two randomly chosen volunteers

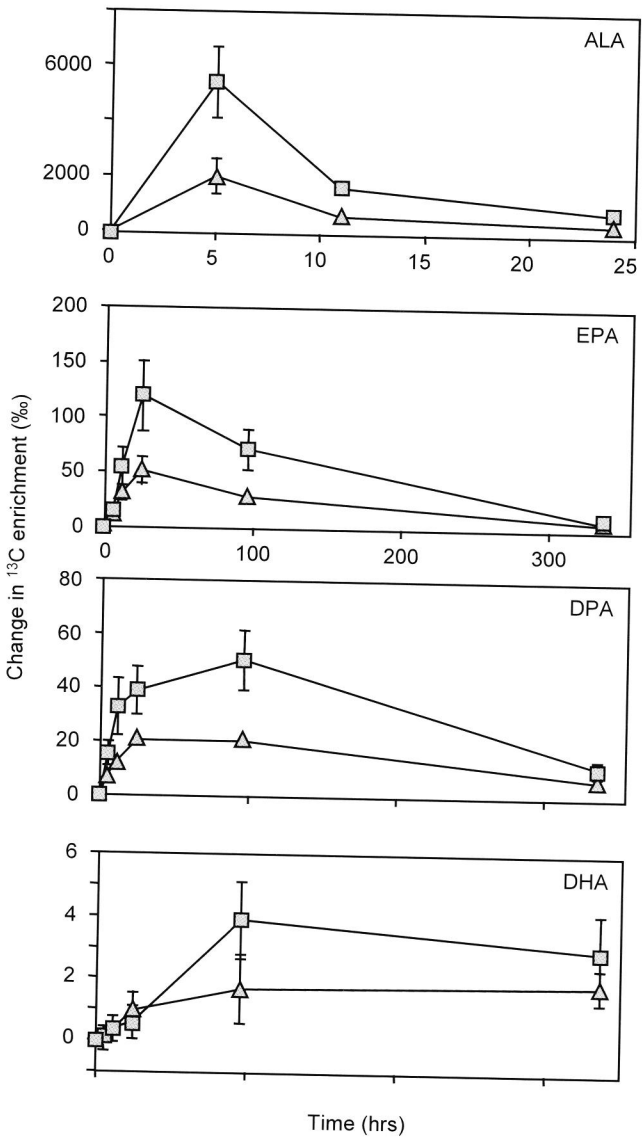

Figure 3.3 Mean (± sem) changes in plasma ^{13}C enrichments of α-linolenic acid, EPA, DPA and DHA after intake of a single dose of ^{13}C α-linolenic acid on diets rich in oleic acid (□) or α-linolenic acid (△)

Plasma total fatty acid concentrations

Proportions of total α-linolenic acid in plasma total lipids at baseline were 0.91 ± 0.35% of total fatty acids in the oleic acid group and 1.12 ± 0.17% of total fatty acids

in the α-linolenic acid group. Plasma total EPA, DPA and DHA concentrations were 0.77 ± 0.12, 0.55 ± 0.06, and 1.61 ± 0.12% of total fatty acids for the oleic acid group and 0.63 ± 0.11, 0.48 ± 0.07, and 1.37 ± 0.13% of total fatty acids for the α-linolenic acid group, respectively.

Absolute amounts of plasma labeled fatty acids

Figure 3.4 shows the individual maximal absolute amounts of whole body plasma ^{13}C n-3 fatty acids. The mean maximal amounts of plasma ^{13}C α-linolenic acid were 4.4 ± 0.7 mg (9.8% of the given dose) on the oleic acid diet and 2.8 ± 0.6 mg (6.2%) on the α-linolenic acid diet (P=0.22). Mean maximal amounts of ^{13}C-EPA were significantly lower in the α-linolenic acid group (0.04 ± 0.01 mg) as compared to the oleic acid group (0.12 ± 0.03 mg) (P=0.01). Values of ^{13}C-DPA and ^{13}C-DHA were very low, and were somewhat lower on the α-linolenic acid rich diet compared to the oleic acid rich diet (P=0.17 and P=0.17, respectively).

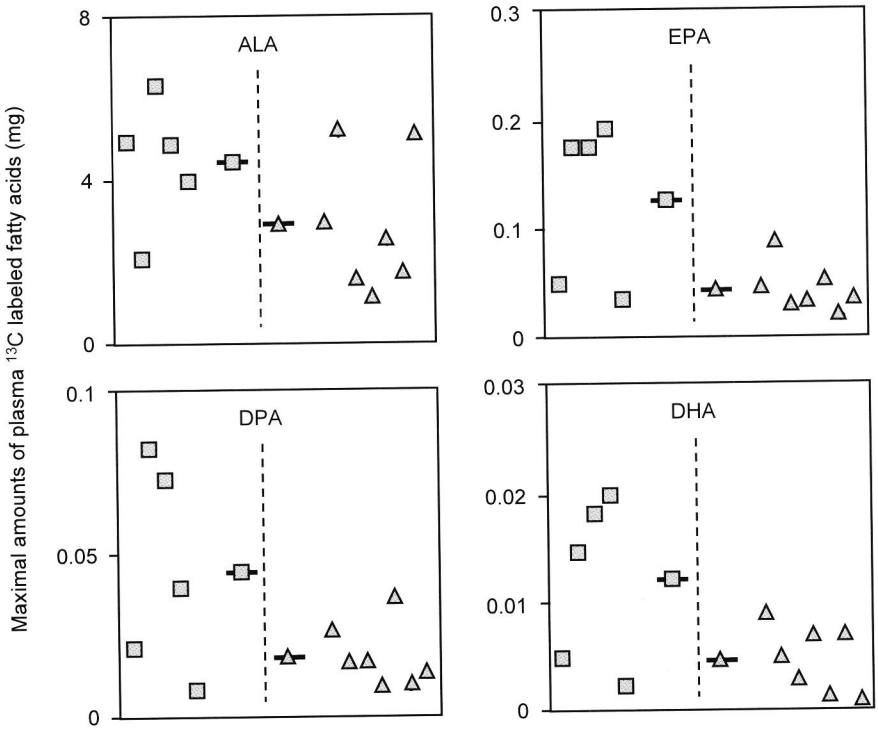

Figure 3.4 Individual absolute amounts of plasma ^{13}C labeled α-linolenic acid, EPA, DPA and DHA after intake of a single dose of ^{13}C α-linolenic acid on diets rich in oleic acid (□; mean -□-) or α-linolenic acid(△ ; mean -△-)

Oxidation

During the first 12 hours maximum ^{13}C enrichments in breath were 11.2 ± 2.5‰ in the oleic acid group and 12.6 ± 1.7‰ in the α-linolenic acid group, and were reached after about 5 hours. Thereafter, ^{13}C enrichment decreased, until 3.8 ± 0.5‰ and 4.7 ± 0.9‰ respectively after 12 hours (data not shown). The cumulative recovery of ^{13}C in breath derived from ^{13}C α-linolenic acid during the first 12 hours after intake is presented in figure 3.5. After 12 hours, the mean recovery of ^{13}C was not significantly different on the α-linolenic acid rich diet (20.4 ± 0.8%) as compared to the oleic acid rich diet (15.7 ± 2.3%) (P=0.12).

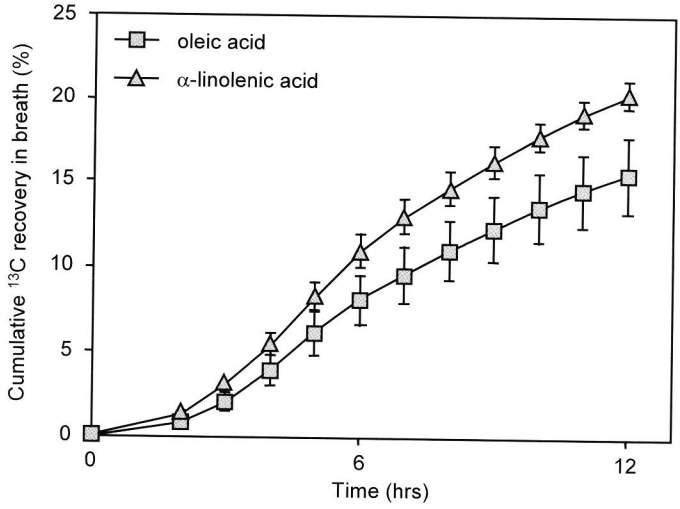

Figure 3.5 Cumulative recovery of $^{13}CO_2$ in breath derived from α-linolenic acid (mean ± sem) after intake of a single dose of ^{13}C α-linolenic acid on diets rich in oleic acid (n=5) or α-linolenic acid (n=7)

Correlations

Spearman correlation coefficients were calculated between the cumulative recovery of ^{13}C in breath derived from ^{13}C α-linolenic acid during the first 12 hours after tracer intake and the maximal amount of total body plasma ^{13}C labeled n-3 fatty acids for all subjects. The cumulative recovery of $^{13}CO_2$ in breath was negatively correlated with plasma ^{13}C-EPA (r=-0.58, P=0.047) and ^{13}C-DPA (r=-0.63, P=0.027), but not with ^{13}C α-linolenic acid (r=0.05, P=0.888) and ^{13}C-DHA (r=-0.49, P=0.108). The maximal amounts of plasma ^{13}C α-linolenic acid, ^{13}C-EPA, ^{13}C-DPA and ^{13}C-DHA did not correlate with each other.

EPA/DHA-group

The daily supplemented intake in the EPA/DHA group was 0.9 g (0.5% of energy) for EPA and 0.5 g (0.3% of energy) for DHA.

Conversion of ^{13}C α-linolenic acid could not be measured in one woman of the EPA/DHA group, due to problems during preparation of FAMEs. Maximum changes in ^{13}C enrichment of α-linolenic acid (2427‰) and EPA (5.1‰) were reached at the same time as in the α-linolenic and oleic acid group, while changes were lower for EPA. Values had returned to baseline after 336 hours. Changes in ^{13}C enrichments of DPA were very low (2.8‰), and that of DHA were zero.

The proportions of total α-linolenic acid, EPA, DPA and DHA in plasma total lipids at baseline were 0.56, 3.09, 0.71, and 2.40%, respectively. Maximal amounts of ^{13}C α-linolenic acid, ^{13}C-EPA, and ^{13}C-DPA were 2.1 mg, $3.4 * 10^{-2}$ mg, and $5.1 * 10^{-3}$ mg, respectively. The cumulative recovery of ^{13}C α-linolenic acid in breath during the first 12 hours after intake was 24.8%.

DISCUSSION

In this study with healthy adults, maximal absolute amounts of labeled EPA in plasma were significantly lower in the α-linolenic acid group as compared to the oleic acid group, after intake of a single oral dose of uniformly labeled ^{13}C α-linolenic acid. Although maximal absolute amounts of labeled DPA and DHA were not lowered on the α-linolenic acid rich diet, our conversion data may suggest that the metabolism of dietary α-linolenic acid depends on its intake.

Effects of α-linolenic acid on the conversion of α-linolenic acid *in vivo* have never been reported in humans before. Dietary linoleic acid, however, inhibits the conversion of deuterated linoleic acid (9), while a diet rich in arachidonic acid has no significant effect on the conversion of deuterated linoleic acid into arachidonic acid (21). This suggests that the reduced conversion of deuterated linoleic acid is caused by linoleic acid from the diet.

α-Linolenic acid is converted into C18:4n-3 by Δ6 desaturation, subsequently elongated into C20:4n-3, from which EPA can be formed by Δ5 desaturation. EPA is converted into DHA via two elongation steps, followed by another Δ6 desaturation step and one cycle of β-oxidation (22). The Δ6 desaturation of α-linolenic acid has been suggested to be the rate limiting step in the conversion of α-linolenic acid. We are unable to decide whether or not α-linolenic acid affects the Δ6 or Δ5 desaturase activity, because plasma concentrations of C18:4n-3 and C20:4n-3 were too low to detect ^{13}C enrichments.

Our experimental design did not allow to quantify the conversion of α-linolenic acid. One commonly used method is to calculate area under the curves (21). We think, however, that this method is not applicable in our study, since after administration of a single dose of ^{13}C labeled α-linolenic acid the distribution of ^{13}C

n-3 fatty acids over the various tissue lipids have not reached a steady state. In addition, the slow appearance and disappearance rates of the tracer in plasma may indicate that these processes are not sequential, but overlap. Therefore, the area under the curve does not reflect the amount of ^{13}C labeled fatty acids converted (23).

From rat studies it is known that the activity of enzymes involved in the mitochondrial and peroxisomal fatty acid oxidation are increased by dietary EPA (24). The effects of dietary α-linolenic acid on α-linolenic acid oxidation in men are, however, not known. In the present study, the mean proportion of ^{13}C labeled α-linolenic acid recovered as $^{13}CO_2$ in breath after 12 hours was slightly, although not significantly, higher in the α-linolenic acid group compared to the oleic acid group, suggesting that the oxidation of ^{13}C α-linolenic acid may not be inhibited by dietary α-linolenic acid.

We hardly found any ^{13}C enrichment of n-3 LCPs on a diet rich in EPA/DHA. However, the plasma total EPA and DHA concentrations were increased on an EPA/DHA rich diet as compared to values on an oleic acid or α-linolenic acid rich diet. This suggests that plasma EPA and DHA were mainly derived from dietary sources. Absolute amounts of ^{13}C-LCPs were very low, suggesting that conversion of ^{13}C α-linolenic acid may be lowered on dietary EPA/DHA. As suggested before (25), the conversion of ^{13}C α-linolenic acid may be inhibited by dietary EPA plus DHA. However, because of the small number of subjects in this group, results should be interpreted with caution. Recently, our suggestion was confirmed by Emken et al., who found decreased accumulation of deuterated n-3 LCPs on a diet enriched in DHA (11).

We observed negative correlations between the recovery in breath of $^{13}CO_2$ derived from ^{13}C α-linolenic acid after 12 hours and the maximal amounts of plasma ^{13}C-EPA and ^{13}C-DPA in plasma total lipids. The correlation between the $^{13}CO_2$ recovery and ^{13}C-DHA amounts failed to reach statistical significance. If results of the EPA/DHA group are, however, also incorporated into the analysis, the negative correlations between the oxidation and conversion of ^{13}C α-linolenic acid become stronger. Even ^{13}C-DHA is now significantly correlated with the $^{13}CO_2$ recovery ($r=-0.63$, $P=0.008$). The lack of significantly lower amounts of ^{13}C-DPA and ^{13}C-DHA on the α-linolenic acid group as compared to the oleic acid group, could also be due to the limited number of subjects.

Except for α-linolenic acid, differences in energy and nutrient intakes between the treatment groups were small. We, therefore, suggest that conversion of ^{13}C α-linolenic acid into LCPs may be lowered by dietary α-linolenic acid, while oxidation of ^{13}C α-linolenic acid is negatively correlated with its conversion into LCPs. The preliminary results on an EPA/DHA rich diet showed very low amounts of ^{13}C-LCPs, suggesting that conversion of ^{13}C α-linolenic acid may also be lowered on dietary EPA/DHA. Therefore, it seems very interesting to examine this issue in more detail in further studies.

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4

Incorporation of ^{13}C α-linolenic acid and its longer chain metabolites into human erythrocytes

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ABSTRACT

Effects of dietary α-linolenic acid versus oleic acid on the incorporation of ^{13}C α-linolenic acid and its longer-chain polyunsaturates (LCPs) into human erythrocytes were investigated *in vivo* in healthy subjects. Volunteers received diets rich in α-linolenic (n=7) or oleic acid (n=5) for 6 weeks prior as well as during the study. After 6 weeks, subjects were given 45 mg ^{13}C α-linolenic acid dissolved in olive oil. Blood samples were collected at t = 0, 5, 11, 24, 96, and 336 hours.

Baseline proportions of C18:3n-3 were higher for the α-linolenic acid group (0.33% of total fatty acids) than for the oleic acid group (0.10% of total fatty acids; P<0.0001). Median proportions of $^{13}C18:3n-3$, $^{13}C20:5n-3$, $^{13}C22:5n-3$ and $^{13}C22:6n-3$ were highest after 11, 48, 336 and 336 hours, respectively. Median proportions in the oleic acid and α-linolenic acid group were $1.06 * 10^{-3}$ and $0.83 * 10^{-3}$ % of total fatty acids for $^{13}C18:3n-3$, $0.31 * 10^{-3}$ and $0.09 * 10^{-3}$ % of total fatty acids for $^{13}C20:5n-3$, $0.17 * 10^{-3}$ and $0.16 * 10^{-3}$ % of total fatty acids for $^{13}C22:5n-3$, and $0.04 * 10^{-3}$ and $0.05 * 10^{-3}$ % of total fatty acids for $^{13}C22:6n-3$, respectively. These proportions did not significantly differ between the groups.

We conclude that ^{13}C α-linolenic acid and its LCPs are incorporated into human erythrocytes, which is already evident after 5 hours for ^{13}C α-linolenic acid. Values of ^{13}C labeled α-linolenic acid, $^{13}C20:5n-3$ and $^{13}C22:5n-3$ were higher in the oleic acid group, but differences with the α-linolenic acid group did not reach statistical significance. From a few pilot samples the effect of a diet rich in eicosapentaenoic plus docosahexaenoic acid was not obvious. Results of a separate experiment, demonstrated that at least a part of the ^{13}C n-3 fatty acids was truly incorporated into the erythrocyte and not simply adsorbed as nonesterified fatty acids to the membrane.

INTRODUCTION

α-Linolenic acid is the ultimate dietary precursor of the longer chain polyunsaturated fatty acids (LCPs) of the n-3 family, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These essential fatty acids are important for membrane structure as major components of phospholipids, while EPA is also a precursor of eicosanoids. Except for being incorporated into tissue phospholipids or converted into longer chain metabolites and/or into eicosanoids, dietary n-3 fatty acids may also be stored in adipose tissue or oxidized via β-oxidation (1,2). However, not all of these processes can be extensively investigated in humans, because of the limited accessibility of certain tissues.

One way to investigate the incorporation of dietary essential fatty acids into membranes is to determine the fatty acid composition of erythrocytes, which can be collected in a relatively easy way. From human intervention studies it is now known that fish oil consumption increases the proportions of EPA and DHA in erythrocytes (3,4). In neonatal baboons dietary DHA appeared more efficiently in erythrocytes as compared to DHA derived from dietary α-linolenic acid (5). Whether n-3 longer chain metabolites derived from dietary α-linolenic acid are incorporated into human erythrocytes is not known. In addition, effects of dietary background on the incorporation of n-3 fatty acids into erythrocytes have not been investigated.

In a previous study, we have examined the effects of dietary α-linolenic acid on the conversion and oxidation of ^{13}C α-linolenic acid in healthy volunteers (6). Based on plasma analyses, we concluded that α-linolenic acid is converted into its LCPs in humans. Furthermore, dietary α-linolenic acid decreased the absolute amount of EPA derived from ^{13}C α-linolenic acid. Because of an increased elimination of $^{13}CO_2$ in breath after tracer ingestion, we concluded that part of the ^{13}C labeled α-linolenic acid had been oxidized as well. However, only about 25% of the tracer was found in plasma total lipids and breath CO_2 (6), while about 92% of the α-linolenic acid is absorbed (7). Thus, a substantial part of the tracer is missing, and we hypothesized that some ^{13}C labeled fatty acids had been incorporated into membranes. Fortunately, we had also collected erythrocytes during this study (6) and we now present results of the incorporation of ^{13}C α-linolenic acid and its longer chain metabolites into erythrocytes. In addition, effects of dietary α-linolenic acid on this incorporation were studied as well.

SUBJECTS AND METHODS

Subjects

Fifteen men and women (age between 21 and 66 years), participated (6). All subjects were healthy as indicated by a medical questionnaire and had normal serum total cholesterol and triacylglycerol levels. None of the volunteers used medication or a

diet known to affect serum lipids. Five of the participants smoked. Three women used oral contraceptives, while five women were post menopausal. The protocol had been approved by the local ethical committee and informed written consent was obtained from each volunteer.

Diets and experimental design

The study consisted of two consecutive periods. During a 3-weeks run-in period, subjects consumed an oleic acid-rich diet. Thereafter, subjects were randomly allocated to three groups. The first group received for the next 7 weeks the same diet as during the run-in period (n=5), the second group received a diet rich in α-linolenic acid (8.3 gram/day; n=7), while the third group received a diet rich in EPA (0.9 gram/day) plus DHA (0.5 gram/day; n=3). α-Linolenic acid and EPA/DHA replaced oleic acid. Fatty acids were provided as margarine from which also pies, cakes and chocolate-paste were prepared.

At week 9, subjects came to the department at 8.00 a.m. after a fasting period of 12 hours. First, a 10 ml blood sample was taken, whereafter 45 mg of uniformly labeled ^{13}C α-linolenic acid (isotopic purity >98% $^{13}C18:3n-3$, chemical purity >95%) was given as a methylester (Martek Biosciences Corporation, Columbia, MD, USA) dissolved in 8 g olive oil. Further blood samples were collected every 2 hours during the first 12 hours (non-fasted) and after 24, 48, 72, 96, 168, and 336 hours (overnight-fasted). After 168 hours, subjects returned to their usual diets. After tracer intake, a breakfast consisting of two slices of white bread with jam was given. Two hours after tracer intake, subjects were allowed to eat and drink according to their respective dietary regimen. During the first day, the volunteers stayed at the department and food was provided during the whole day. Use of coffee or drinks containing caffeine was prohibited during the first day only, because of the possible stimulating effect of caffeine on lipolysis and lipid oxidation (8). A detailed description of the study design has been published before (6).

Blood sampling and analysis

Until 12 hours after tracer administration, blood was sampled in an EDTA tube through a Teflon catheter (Baxter Quick Cath, Dupont, Ireland) or in a vacuum tube using a 1.2-mm needle (Strauss Kanule; Luer, Wächtersbach, Germany) with the volunteer in a recumbent position. Subjects were free to choose the method of sampling. The catheter was flushed every hour with a 1 ml heparin solution (0.5 IU heparin-sodium; Leo Pharmaceutical Products BV, Weesp, the Netherlands). The other fasting blood samples were taken with a vacuum tube. Erythrocytes for ^{13}C enrichment analyses and analyses of fatty acid composition of erythrocyte total lipids were obtained by centrifugation at 2000 $\times$ g for 15 minutes at 4°C, within one hour of venipuncture. After plasma was separated, the erythrocytes were washed twice with a physiological salt solution. Washed erythrocyte samples were snap-frozen and stored at -80°C.

Within 1 week, lipids from erythrocytes were extracted (9), using tri-heptadecanoic acid (C17:0) as an internal standard. Thereafter, lipid extracts were hydrolyzed and the resulting fatty acids methylated (10). The fatty acid composition in erythrocyte total lipids was analyzed using a gas chromatograph with a flame ionization detector (GC/FID) (Perkin Elmer, Norwalk, Connecticut, USA) as described before (6).

^{13}C Enrichments of individual fatty acid methyl esters (FAMEs) in total lipids from erythrocytes were analyzed with a gas chromatograph (Varian 3400) coupled with an isotope ratio mass spectrometer (GC-C-IRMS; Finnigan MAT 252, Bremen, Germany) via a combustion interface, as described before (6).

Calculations

The difference between the $^{13}C/^{12}C$ ratio of the individual FAME and a known reference standard was expressed as the delta ($\delta^{13}C$). The true ratio of the FAMEs was then calculated by normalizing the isotopic enrichment ($\delta^{13}C$), against the international standard Pee Dee Belemate limestone, which has a $^{13}C/^{12}C$ ratio of 0.0112372. Proportions of ^{13}C labeled fatty acids were calculated by correction for the proportions of that particular fatty acid in the erythrocyte. It was assumed that during fatty acid elongation the two carbon-atom fragments added after elongation were not enriched above background.

Statistical analyses

For each subject, changes in $^{13}C/^{12}C$ ratios were calculated by subtracting baseline values (t=0 hours). Differences between the oleic acid group and the α-linolenic acid group were tested with the Mann Whitney U test. Spearman correlation coefficients were calculated to examine the relationship between the maximal proportion of erythrocyte ^{13}C labeled n-3 fatty acids (as % of total fatty acids), and the cumulative recovery of $^{13}CO_2$ derived from ^{13}C α-linolenic acid in breath and the maximal proportion of plasma ^{13}C labeled n-3 fatty acids (as % of total fatty acids). Maximal proportions represent the proportion of ^{13}C fatty acids (as % of total fatty acids) at the time of maximal enrichment. Results of breath (as % recovery) and plasma (in mg) have been published before (6). P-values are two-tailed, and differences were considered statistically significant when $P<0.05$. Because of small group size (n=3), results of the EPA/DHA group were not statistically analyzed. Values are presented as medians and as means ± standard errors.

Additional experiment

In a small additional experiment, we examined whether the ^{13}C labeled fatty acids were indeed incorporated into the erythrocytes, or adsorbed to the erythrocyte membrane as non-esterified fatty acids. For this purpose, one healthy woman (aged 29, BMI 19.6 kg/m^2, non-smoker), consuming her habitual diet, participated. After a fasting period of 12 hours, the subject came to the department at 8.00 a.m. for a

blood sample (30 ml). Thereafter, 45 mg of uniformly labeled ^{13}C α-linolenic acid was given dissolved in 8 g olive oil, similarly as in the main study. During the whole day, the subject was allowed to eat and drink according to her self-chosen dietary regimes. Further blood samples were collected in vacuum EDTA tubes at t=5 hours (non fasted) and t=24 hours (overnight-fasted). Erythrocytes were obtained by centrifugation at 2000 × g for 15 minutes at 4°C, within 1 hour of venipuncture. After plasma was separated, the erythrocytes were washed three times on ice with a physiological salt solution containing fatty acid free bovine serum albumin (BSA) (2 g/l) (Sigma-Aldrich, Steinheim, Germany) and phloretin (0.4 mM) (Sigma-Aldrich, Steinheim, Germany). Since phloretin blocks the influx and efflux of fatty acids across the membrane (11-13), addition of fatty acid free BSA plus phloretin to the physiological salt solution will only remove the non-esterified fatty acids adsorbed to the erythrocyte membrane. Consequently, detected ^{13}C labeled fatty acids will reflect the fatty acids truly incorporated into the erythrocyte. Washing erythrocytes with a physiological salt solution only, as done in the main study, will not remove adsorbed fatty acids. Washed erythrocytes samples were snap-frozen and stored at -80°C. Total lipids in erythrocytes were analyzed as described above.

RESULTS

Based on ^{13}C enrichments in plasma, as published before (6), we decided to analyze only erythrocyte samples of t=0, 24, 48, and 336 hours. Furthermore, samples from t=4 hours and t=6 hours, and from t=10 hours and t=12 hours were pooled before analyses, and named t=5 hours and t=11 hours, respectively.

Erythrocyte fatty acid compositions

At baseline (t=0), proportions of C18:3n-3 were higher in the α-linolenic acid group (median of 0.31% of total fatty acids) than in the oleic acid group (0.10% of total fatty acids; P<0.0001) (table 4.1). Proportions of C20:5n-3, C22:5n-3 and C22:6n-3, as well as of saturated fatty acids, monounsaturated fatty acids and total *trans* fatty acids did not differ significantly between the groups.

Table 4.1 Baseline fatty acid compositions of erythrocytes (% of total fatty acids) on diets rich in oleic acid or α-linolenic acid

Fatty acids	Oleic acid n=5	ALA n=7
SAFA	43.03 (43.41 ± 0.29)	43.23 (43.20 ± 0.35)
MUFA	19.05 (19.05 ± 0.47)	19.13 (18.76 ± 0.31)
total n-6	29.22 (29.24 ± 0.39)	30.00 (29.66 ± 0.79)
C18:3n-3 (ALA)	0.10 (0.10 ± 0.01)	0.31 (0.33 ± 0.03)*
C20:5n-3 (EPA)	0.58 (0.64 ± 0.06)	0.69 (0.68 ± 0.10)
C22:5n-3 (DPA)	2.64 (2.61 ± 0.13)	2.65 (2.63 ± 0.13)
C22:6n-3 (DHA)	3.66 (4.06 ± 0.37)	4.08 (3.82 ± 0.33)
total n-3	6.96 (7.45 ± 0.52)	7.56 (7.49 ± 0.42)
total *trans*	0.78 (0.73 ± 0.09)	0.82 (0.78 ± 0.04)

Values are medians (means ± sem)

*Denotes a significant difference from the oleic acid group

Proportions of ^{13}C labeled fatty acids

Figure 4.1 shows the median proportions of ^{13}C labeled fatty acids during the course of the study. Proportions of ^{13}C18:3n-3 increased during the first 11 hours after tracer intake, while after 336 hours proportions of ^{13}C18:3n-3 had almost returned to baseline. After 48 hours, median proportions of ^{13}C20:5n-3 were highest, and after 336 hours proportions were still considerable as compared to after 48 hours. Proportions of ^{13}C22:5n-3 and ^{13}C22:6n-3 increased until 336 hours after tracer intake.

Median maximal proportions of ^{13}C18:3n-3, ^{13}C20:5n-3, ^{13}C22:5n-3, ^{13}C22:6n-3, total ^{13}C labeled n-3 fatty acids (^{13}C18:3n-3 + ^{13}C20:5n-3 + ^{13}C22:5n-3 + ^{13}C22:6n-3) and ^{13}C n-3 LCPs (^{13}C20:5n-3 + ^{13}C22:5n-3 + ^{13}C22:6n-3) for the oleic and α-linolenic acid groups are shown in table 4.2. No significant differences in proportions of ^{13}C labeled fatty acids between the two groups were observed.

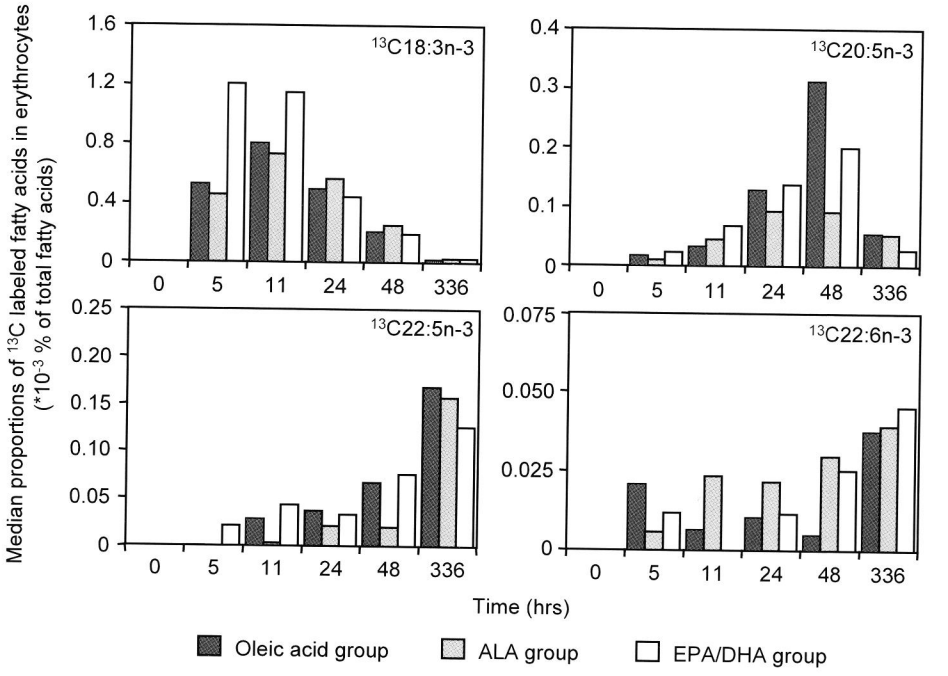

Figure 4.1 Median proportions (in 10^{-3} % of total fatty acids) of erythrocyte ^{13}C18:3n-3, ^{13}C20:5n-3, ^{13}C22:5n-3 and ^{13}C22:6n-3 after a single dose of ^{13}C18:3n-3 on diets rich in oleic acid, α-linolenic acid (ALA) or eicosapentaenoic/docosahexaenoic acid (EPA/DHA) during the course of the study

Table 4.2 Median maximal proportions of ^{13}C labeled fatty acids in erythrocytes (% of total fatty acids) on diets rich in oleic acid or α-linolenic acid

Fatty acids	Maximal proportion (*10^{-3})		Time to maximal proportion (hours)	
	Oleic acid n=5	ALA n=7	Oleic acid n=5	ALA n=7
^{13}C18:3n-3	1.06 (1.64 ± 0.72)	0.83 (4.11 ± 3.21)	11 (11.2 ± 3.5)	11 (13.9 ± 2.7)
^{13}C20:5n-3	0.31 (0.27 ± 0.08)	0.09 (0.17 ± 0.06)	48 (48.0 ± 0.0)	48 (41.1 ± 4.4)
^{13}C22:5n-3	0.17 (0.19 ± 0.06)	0.16 (0.18 ± 0.05	336 (278.4 ± 57.6)	336 (288.7 ± 47.3)
^{13}C22:6n-3	0.04 (0.04 ± 0.01)	0.05 (0.07 ± 0.02)	336 (253.3 ± 82.8)[c]	48 (168.0 ± 59.5)
^{13}C n-3 FA [a]	1.72 (2.14 ± 0.77)	1.48 (4.52 ± 3.21)		
^{13}C n-3 LCPs [b]	0.55 (0.50 ± 0.13)	0.42 (0.41 ± 0.12)		

Values are medians (means ± sem)

[a] ^{13}C n-3 FA (^{13}C18:3n-3 + ^{13}C20:5n-3 + ^{13}C22:5n-3 + ^{13}C22:6n-3)

[b] ^{13}C n-3 LCPs (^{13}C20:5n-3 + ^{13}C22:5n-3 + ^{13}C22:6n-3)

[c] Only for four subjects. In one subject no enrichment was detected.

Correlations

In the present study, ^{13}C enrichments in breath and plasma were also measured. We have now calculated correlations between maximal proportions of ^{13}C labeled fatty acids in erythrocytes (as % of total fatty acids), and total $^{13}CO_2$ in breath for the first 12 hours or maximal proportions of ^{13}C labeled fatty acids in plasma (as % of total fatty acids) after intake of ^{13}C α-linolenic acid.

Results of the total ^{13}C recovery in breath ($^{13}CO_2$) derived from α-linolenic acid and the appearance of ^{13}C labeled n-3 fatty acids in plasma (in mg) have been reported before (6). Median maximal proportions of $^{13}C18:3n-3$ in plasma (as % of total fatty acids) were 33.5 * 10^{-3} % in the oleic acid group and 20.9 * 10^{-3} % in the α-linolenic acid group. Median maximal proportions in plasma total lipids were 1.01 * 10^{-3} % and 0.45 * 10^{-3} % for $^{13}C20:5n-3$, 0.34 * 10^{-3} % and 0.19 * 10^{-3} % for $^{13}C22:5n-3$, and 0.08 * 10^{-3} % and 0.05 * 10^{-3} % for $^{13}C22:6n-3$ in the oleic acid and α-linolenic acid group, respectively. No significant differences between the groups were observed.

Maximal proportions of $^{13}C20:5n-3$ (r=0.73, P=0.007), $^{13}C22:5n-3$ (r=0.62, P=0.033), and total ^{13}C n-3 LCPs (r=0.80, P=0.002) in erythrocytes correlated with maximum proportions of $^{13}C20:5n-3$ in plasma, but not with other plasma ^{13}C n-3 LCPs. In addition, ^{13}C recovery in breath was not significantly correlated with maximal proportions of ^{13}C labeled fatty acids in erythrocytes.

EPA/DHA group

At baseline (t=0), proportions of total n-3 fatty acids were higher on the EPA/DHA group (median of 10.68% of total fatty acids) as compared to the oleic acid (6.96% of total fatty acids) and α-linolenic acid group (7.56% of total fatty acids), which was caused by higher proportions of C20:5n-3 (1.99, 0.58, and 0.69% of total fatty acids for the EPA/DHA, oleic acid, and α-linolenic acid groups, respectively), C22:5n-3 (3.40, 2.64, and 2.65% of total fatty acids, respectively) and C22:6n-3 (5.00, 3.66, and 4.08% of total fatty acids, respectively). Proportions of total n-6 fatty acids were lower on the EPA/DHA rich diet (26.0% of total fatty acids) than on the oleic acid rich (29.2% of total fatty acids) and α-linolenic acid rich diets (30.0% of total fatty acids).

The median maximal proportion of $^{13}C18:3n-3$ was 0.66 * 10^{-3} % of total fatty acids was reached after 11 hours, and had almost returned to baseline after 336 hours. Maximal proportion of $^{13}C20:5n-3$ was reached after 48 hours and was 0.09 * 10^{-3} % of total fatty acids. The proportion of $^{13}C22:5n-3$ was highest after 336 hours, and was 0.12 * 10^{-3} % of total fatty acids. The proportion of $^{13}C22:6n-3$ increased until 0.05 * 10^{-3} % of total fatty acids after 336 hours.

Additional experiment

^{13}C Enrichments in C18:3n-3, after washing the erythrocytes with a physiological salt solution containing fatty acid free BSA and phloretin, were 146 and 233‰ after 5 and 24 hours, respectively. In addition, ^{13}C enrichments were detected for C20:5n-3 as

well. These results indicated that at least a part of the ^{13}C labeled fatty acids were incorporated into the erythrocyte membrane.

DISCUSSION

After ingestion of a single bolus of ^{13}C α-linolenic acid, we found in human erythrocytes ^{13}C enrichments in C18:3n-3 and its LCPs, C20:5n-3, C22:5n-3 and C22:6n-3. This shows that not only dietary EPA and DHA are incorporated into erythrocytes (3,4,14,15), but also n-3 longer chain metabolites derived from dietary α-linolenic acid. Incorporation of ^{13}C α-linolenic acid as well as ^{13}C-EPA and ^{13}C-DHA into human erythrocytes was very fast and already observed after 5 hours.

As expected, after 6 weeks on an α-linolenic acid rich diet providing 8.3 gram α-linolenic acid extra per day, proportions of erythrocyte C18:3n-3 were higher in the α-linolenic acid group as compared to the oleic acid group. However, no significant changes were observed in the proportions of n-3 LCPs. As in other studies (3,4,14), proportions of C20:5n-3 and C22:6n-3 were higher on the EPA/DHA rich diet as compared to the α-linolenic and oleic acid rich diets.

We further examined effects of dietary α-linolenic acid on incorporation of ^{13}C fatty acids into erythrocytes. The high mean maximal proportion of ^{13}C18:3n-3 in the α-linolenic acid group (table 4.2) was caused by values of one subject, and medians are therefore more informative. Maximal proportions of ^{13}C20:5n-3 were, although not significantly, lower on the α-linolenic acid diet as compared to the oleic acid diet. For plasma total lipids, differences in maximal amounts of ^{13}C20:5n-3 (in mg) did reach statistical significance (6), but differences in maximal proportions of plasma ^{13}C20:5n-3 were not significant. Furthermore, we found a significant correlation between maximal proportions of ^{13}C20:5n-3 in erythrocytes with those in plasma. Maximal proportions of ^{13}C20:5n-3 in erythrocytes were borderline significantly correlated with absolute maximal amounts of ^{13}C20:5n-3 in plasma (in mg) (P=0.07). Although suggestive, our results are not conclusive whether dietary α-linolenic acid affects incorporation of LCPs derived from ^{13}C α-linolenic acid into erythrocytes.

In a small additional experiment, we have examined whether the ^{13}C labeled fatty acids were indeed incorporated into the erythrocytes or were only adsorbed to the erythrocyte membrane as non-esterified fatty acids. After adsorption, non-esterified fatty acids may cross the membrane (16-19) to become incorporated into phospholipids at the inner leaflet of the membrane (20) or bound to haemoglobin in the cytoplasm (21). A previous *in vitro* study with ^{14}C labeled palmitic, oleic and linoleic acid has shown that fatty acid free BSA may remove up to 88% of the non-esterified fatty acids adsorbed to the human erythrocyte membrane (22). Non-esterified fatty acids from the cytosol are hardly removed by fatty acid free albumin (21). *In vitro* the influx and efflux of ^{14}C-oleic acid in rat adipocytes (12) and hepatocytes (13) is blocked by phloretin. In the additional experiment, we have also

used phloretin to block the translocation of fatty acids across the membrane. The adsorbed non-esterified fatty acids were then removed from the membrane by using fatty acid free BSA. Consequently, these results reflected the ^{13}C-labeled fatty acids truly incorporated into the erythrocyte, either esterified or as free fatty acid. We did find ^{13}C enrichments in C18:3n-3 and C20:5n-3, suggesting that at least a part of these fatty acids was truly incorporated into the erythrocyte and not simply adsorbed as nonesterified fatty acids to the membrane.

In conclusion, we found ^{13}C α-linolenic acid as well as its longer chain more unsaturated fatty acids in human erythrocytes. This suggests that n-3 longer chain metabolites derived from dietary α-linolenic acid are also incorporated into erythrocytes. These incorporations were already evident after 5 hours. Whether incorporation of ^{13}C LCPs into erythrocytes is affected by dietary α-linolenic acid is not clear.

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5

Relationship between mRNA levels for Δ5, Δ6 and Δ9 desaturases in mononuclear blood cells and liver tissue in man

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ABSTRACT

Desaturase activity is mainly located in the liver, but has also been demonstrated in mononuclear blood cells. Since the liver is not easily accessible to investigate in humans, in the present study, desaturase mRNA levels were measured in both liver and mononuclear blood cells in order to examine the possibility to predict regulation of desaturases in the liver by measuring those in mononuclear blood cells. Patients who underwent cholecystectomy for symptomatic gallstones (n=1), hemi-hepatectomie (n=2), or with morbid obesity who underwent gastroplasty (n=11) participated. During surgery, fasting venous blood was sampled, and a liver sample was collected. Total RNA was isolated from mononuclear blood cells and liver, and mRNA levels of Δ5, Δ6, and Δ9 desaturase were determined by reverse-transcription polymerase chain reaction. mRNA levels were expressed as ratios relative to β-actin.

Delta 6 desaturase mRNA in mononuclear blood cells was negatively correlated with Δ6 desaturase mRNA in liver (r=-0.64; P=0.015), and Δ9 desaturase mRNAs in mononuclear blood cells was negatively correlated with Δ9 desaturase mRNA in liver (r=-0.83; P=0.001). Delta 5 desaturase mRNA in mononuclear blood cells was not correlated with that in liver. Within liver tissue, Δ5 and Δ6 desaturase mRNA, and Δ6 and Δ9 desaturase mRNA were positively correlated (r=0.71, P=0.007, and r=0.59, P=0.034, respectively). Within mononuclear blood cells, desaturases were not correlated with each other.

We conclude that Δ6 and Δ9 desaturase mRNAs in mononuclear blood cells may predict mRNA levels in the liver.

INTRODUCTION

In men, fatty acids can be converted by alternate desaturation and elongation reactions into fatty acids with a higher degree of unsaturation and a longer chain length. The first desaturation step of most saturated fatty acids is often mediated by the Δ9 desaturase enzyme. In this way, oleic acid (C18:1n-9 Δ9) is formed from stearic acid (C18:0). The essential fatty acids α-linolenic acid (C18:3n-3 Δ9,12,15) and linoleic acid (C18:2n-6 Δ9,12) cannot be synthesized *de novo* by mammals, because they lack the required desaturase enzymes. However, they can be converted to longer chain more unsaturated fatty acids (LCPs) of the n-3 and n-6 families, respectively, which are important structural membrane components and play a major role in many physiological processes (1). In the further desaturation of oleic-, linoleic- and α-linolenic acids, Δ6 and Δ5 desaturases are involved.

Desaturase activity is mainly located in the liver, which, for obvious reasons, is not easily accessible for studies in humans. Cunnane et al., however, have shown that human leukocytes also convert ^{13}C linoleic acid into di-homo-γ-linolenic acid (C18:3n-6) and arachidonic acid (C20:4n-6), indicating the presence of Δ6 and Δ5 desaturases in leukocytes (2). Therefore, it is likely that desaturase mRNA is present in leukocytes as well. If so, effects of drugs, diets, diseases, etcetera on expression of desaturases can possibly be investigated in leukocytes in stead of in liver. This requires, however, that desaturase regulation in human leukocytes relates to desaturase regulation in the liver. Such activity relationships between human mononuclear blood cells and liver have indeed been observed, for instance for the low density lipoprotein (LDL) receptor and for 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoA) (3).

Recently, human Δ5 and Δ6 desaturase cDNAs have been cloned and characterised (4,5). cDNA of human Δ9 desaturase has been sequenced as well (6). This gives the possibility to examine in humans the relationships between mRNA levels of Δ5, Δ6 and Δ9 desaturase in mononuclear blood cells with those in the liver.

SUBJECTS AND METHODS

Subjects

The study was carried out at the University Hospital in Maastricht. Patients aged <18 years were excluded. Fourteen subjects (six men and eight women), who were aged between 25 and 74 years (mean ± sem: 39.0 ± 3.6 years), weighed between 68 and 200 kg and had a body mass index between 24.8 and 57.2 kg/m^2 participated in this study (table 5.1). Patients with morbid obesity underwent gastroplasty (n=11), while two subjects underwent hemi-hepatectomy and one cholecystectomy for symptomatic gallstones. The patient who underwent cholecystectomy had diabetes mellitus type 2. The study was approved by the Medical Ethical Committee of the

Maastricht University Hospital. Before surgery, the protocol was fully explained to the patients, who have all given their written informed consent.

Table 5.1 Characteristics of the patients

Patient	Gender	Age (yr)	Weight (kg)	Height (m)	BMI (kg/m^2)	Operation
1	M	38	132	1.65	48.4	gastroplasty
2	F	56	86	1.70	29.8	hemi-hepatectomy
3	M	28	200	1.87	57.2	gastroplasty
4 [a]	M	74	85	1.85	24.8	cholecystectomy
5	F	34	91	1.53	38.7	gastroplasty
6	F	30	140	1.70	48.4	gastroplasty
7	F	50	68	1.64	25.3	hemi-hepatectomy
8	F	25	127	1.72	42.9	gastroplasty
9	M	40	140	1.82	42.3	gastroplasty
10	M	37	155	1.87	44.3	gastroplasty
11	F	34	111	1.61	42.8	gastroplasty
12	F	33	136	1.76	43.9	gastroplasty
13	M	41	179	1.87	51.2	gastroplasty
14	F	26	142	1.77	45.3	gastroplasty

[a] Patient with Diabetes Mellitus type 2

Sample collection

During surgery, fasting venous blood (about 30 ml) was sampled, and a liver tissue biopsy (about 30 mg) was taken from the caudal edge of segment III of the left lobe. Hemostasis was achieved in all patients with diathermia only. No complications related to the biopsies were observed. Immediately after blood was sampled in a syringe, about 6 ml blood was transferred into a serum tube for measurement of liver enzymes, and the remainder into EDTA tubes for isolation of total RNA and for analysis of haematological variables (number of leukocytes, erythrocytes, and platelets, packed cell volume and haemoglobin concentration). Serum gamma-glutamyltranspeptidase (γ-GTP), alanineaminotransferase (ALAT) and total bilirubine levels showed no significant deviations from normal values. Liver samples were used for isolation of total RNA. After collection, liver tissues were directly placed in RNA*later* (RNA*later*™; Ambion, Austin TX) for about 24 hours.

RNA preparation

Mononuclear blood cells were isolated by lymphoprep™ (Nycomed Pharma As, Oslo, Norway) according to the manufacturer's instructions. Liver samples were homogenized in RNA lysis buffer (Promega Corporation, Madison, WI, USA) with a homogenizer. Total RNA from mononuclear blood cells and liver was isolated using a

kit of Promega (Promega Corporation, Madison, WI, USA). RNA was dissolved in nuclease-free water (Promega Corporation, Madison, WI, USA) and stored at –20°C until polymerase chain reaction (PCR) amplification. Before PCR amplification, RNA concentrations were measured spectrophotometrically at 260 nm.

PCR amplification

In both mononuclear blood cells and liver samples, the amounts of mRNA for Δ5, Δ6, and Δ9 desaturase were measured by reverse-transcription (RT) PCR. 600 ng RNA from mononuclear blood cells or 1 μg RNA from liver was first heated at 85°C for 5 minutes. Then, RNA was reverse transcribed into cDNA in a 20 μl mixture of RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 10 mM dithiothreitol (DTT; Gibco, Life Technologies, Breda, the Netherlands), 200 U Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Gibco, Life Technologies, Breda, the Netherlands), 10 U/μl RNase inhibitor (Gibco, Life Technologies, Breda, the Netherlands), deoxyribonucleoside triphosphates (dNTP; 0.125 mmol of each nucleotide; dATP, dCTP, dGTP and dTTP; Pharmacia Biotech, Roosendaal, the Netherlands), and 5 μM random hexamers (Perkin Elmer, Applied Biosystems, Foster City, CA, USA). Samples were heated for 10 minutes at 22°C, 90 min at 37°C and 3 min at 95°C, and then chilled at 4°C. 5μl cDNA was amplified using a 50 μl volume of PCR buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 15 mM $MgCl_2$, 0.1% Triton X-100), 0.05 mM dNTP, and 30 pmol of each primer (table 5.2) with an Amplitron® II Thermolyne (Barnstead, Thermolyne Corporation, Dubuque, USA). After an annealing step of 2 min at 94°C, 2.5 U taq polymerase (Pharmacia Biotech, Roosendaal, the Netherlands) was added.

Before start of the study, mRNA levels of the three desaturases and of β-actin in mononuclear blood cells were measured at 22, 24, 26, 28, 30, and 32 cycles to determine the linear phase of the amplification curve. In the liver, mRNA levels were measured at 16 and 17 cycles (only for β-actin) and at 18, 20, 22, 24, 26, 28, and 30 cylces for the desaturases and β-actin. For the study, mRNA levels in respectively mononuclear blood cells and liver were determined at three different cycles which were part of the linear phase: 28, 29, 30 and 18, 19, 20 cycles for Δ9 desaturase, and 28, 29, 30 and 20, 21, 22 cycles for Δ5 and Δ6 desaturase. In addition, β-actin was quantified in triplicate using 23 cycles in mononuclear blood cells and 18 cycles in liver tissue.

PCR for measurement of desaturases consisted of denaturation at 94°C for 30 s, primer annealing at 60°C for Δ9 desaturase and at 50°C for Δ5 and Δ6 desaturase for 30 s and extension at 72°C for 1 min. PCR for measurement of β-actin consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 45 s and extension at 72°C for 2 min.

Table 5.2 Primer sequences for amplification of human RNA

Gene	5' Primers	3' Primers	Size of PCR product (bp)
Δ5 D [a]	5'-GAATAAAGAGCTGACAGATGAG-3'	5'-CCTGAACTGCACTGAGCA-3'	202
Δ6 D [a]	5'-GGCAAGAACTCAAAGATCAC-3'	5'-GAGAGGTAGCAAGAACAAAG-3'	204
Δ9 D [b]	5'-GGAAGACGACATTCGCCCTG-3'	5'-TGGTCACGAGCCCATTCATAGAC-3'	347
β-actin [c]	5'-ACTGACTACCTCATGAAGAT-3'	5'-CGTCATACTCCTGCTTGCTGAT-3'	535

[a] Primers as indicated in Cho et al. (5)

[b] Primers were made by the *Amplify* program (7) and checked by *CPrimers* (University of California, USA, 1995) on melting points and interfering structures

[c] Primers as indicated in Schrauwen et al. (8)

PCR product detection

PCR products were fractionated by electrophoresis on a 3% agarose gel in TBE (Tris-borate-EDTA) buffer (both containing 5% ethidium bromide) for 1.5 h at 125 V, and quantitation of the PCR products were performed via densitometry (Imagemaster® VDS; Pharmacia Biotech). Figure 5.1 shows PCR products of liver Δ5, Δ6, and Δ9 desaturase.

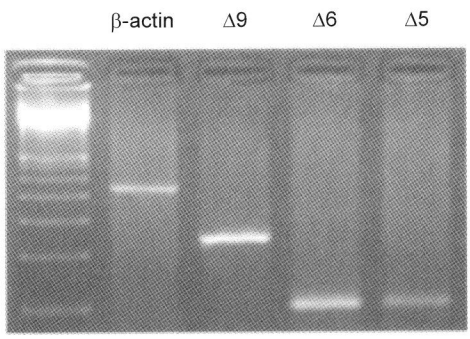

Figure 5.1 PCR products of liver Δ5 (202 bp), Δ6 (204 bp), and Δ9 desaturase (347 bp), and β-actin (535 bp). These mRNAs were determined using 19 cycles for Δ9 desaturase, 21 cycles for Δ5 and Δ6 desaturase, and 18 cycles for β-actin.

Calculations and statistical analyses

Signal intensities of the PCR products were plotted against the number of cycles. Then, regression lines were calculated and checked for linearity. If regression lines were not linear, analyses were repeated. Signal intensities of 29 and 19 cycles for the Δ9 desaturase, and 29 and 21 cycles for Δ5 and Δ6 desaturase for mononuclear

blood cells and liver, respectively, were calculated using the regression equation. Levels of mRNA were expressed as the ratio of signal intensity for the Δ5, Δ6, or Δ9 desaturase PCR products relative to that of the PCR product of the house-keeping gene β-actin (9).

Pearson correlation coefficients were calculated using the Statistical Package for the Social Sciences (SPSS; SPSS Inc., Chicago, IL, USA) to determine the relationships between mRNA levels of Δ5, Δ6 and Δ9 desaturase in mononuclear blood cells with those in the human liver, between age and BMI with mRNA levels of Δ5, Δ6 and Δ9 desaturase in mononuclear blood cells and liver, and to determine the interrelationships between mRNA levels of the three different desaturases in both mononuclear blood cells and liver. Gender differences were tested by unpaired t-tests. Differences between hepatic Δ5 and Δ6 desaturase mRNAs and between Δ5, Δ6 and Δ9 desaturase mRNAs in mononuclear blood cells were tested by paired t-tests. Hepatic Δ9 desaturase mRNA could not be compared with Δ5 and Δ6 desaturase mRNAs because of different cycle numbers, as applied during the PCR amplification.

RESULTS

Mean (± sem) Δ5 desaturase mRNA relative to β-actin was 0.94 ± 0.02 in liver and 0.90 ± 0.02 in mononuclear blood cells (table 5.3). Mean Δ6 desaturase mRNAs were 1.00 ± 0.03 and 0.90 ± 0.02, and mean Δ9 desaturase mRNAs were 1.01 ± 0.03 and 0.83 ± 0.01 in liver and mononuclear blood cells, respectively. Delta 9 desaturase mRNA in mononuclear blood cells was significantly lower than Δ5 and Δ6 desaturase mRNA levels in mononuclear blood cells (P=0.003). The difference in hepatic Δ5 and Δ6 desaturase mRNA levels nearly reached statistical significance (P=0.084). Desaturase mRNA levels did not differ between men and women.

Table 5.3 Mean (± sem) mRNA levels of desaturase-enzymes (D) relative to β-actin in human liver and mononuclear blood cells (n=14)

Enzyme	Liver	Mononuclear blood cells
Δ5 D	0.94 ± 0.02	0.90 ± 0.02
Δ6 D	1.00 ± 0.03	0.90 ± 0.02
Δ9 D	1.01 ± 0.03	0.83 ± 0.01 [a]

[a] Significantly different from Δ5 and Δ6 desaturase mRNA in mononuclear blood cells; P=0.003

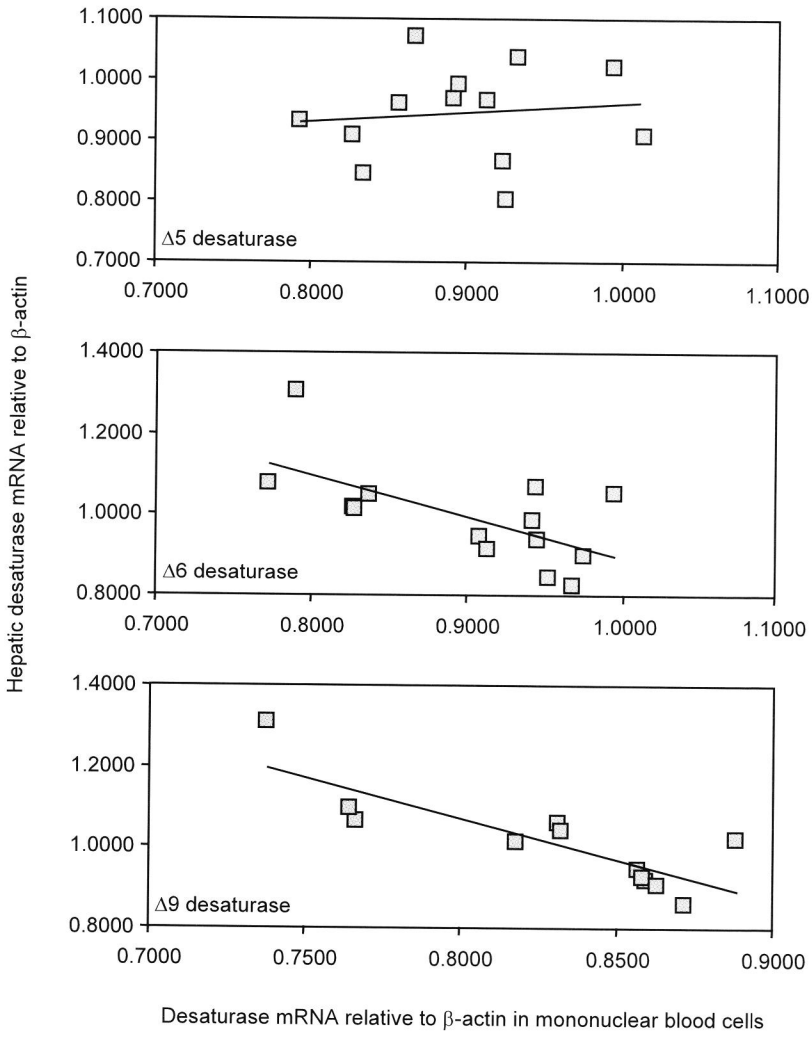

Figure 5.2 Relationships between Δ5, Δ6 and Δ9 desaturase mRNA relative to β-actin in mononuclear blood cells and liver. mRNAs were determined with 29 and 19 cycles for the Δ9 desaturase, and 29 and 21 cycles for Δ5 and Δ6 desaturase for mononuclear blood cells and liver, respectively.

Delta 6 and Δ9 desaturase mRNAs in mononuclear blood cells were negatively correlated with Δ6 desaturase (r=-0.64, P=0.015) and Δ9 desaturase mRNAs (r=-0.83, P=0.001) in liver, respectively (figure 5.2). No significant relationship was found for Δ5 desaturase mRNA in mononuclear blood cells and liver (r=0.11, P=0.725).

Correlations between desaturases within the liver and within mononuclear blood cells were also calculated. Within the liver, Δ5 and Δ6 desaturase mRNA, as well as Δ6 and Δ9 desaturase mRNA were positively correlated (r=0.71, P=0.007, and r=0.59, P=0.034, respectively). The relationship between liver Δ5 and Δ9 desaturase mRNAs was of borderline significance (r=0.51, P=0.076). Desaturases were not significantly correlated with each other within the mononuclear blood cells.

BMI was not correlated with any of the desaturases, while age was positively correlated with Δ6 desaturase mRNA in liver (r=0.60, P=0.023). However, when subject 4 (patient with Diabetes Mellitus type 2) was excluded from the analysis, age was not correlated with hepatic Δ6 desaturase mRNA.

DISCUSSION

The pivotal role of the liver in the conversion of fatty acids (10) is difficult to study in humans, because the liver is not an easily accessible organ. Circulating mononuclear blood cells, which are of course much easier to sample, may serve as a useful alternative, as these cells also elongate and desaturate fatty acids (2). It is not known, however, whether elongation and desaturation of fatty acids in these cells reflect that in the liver. In the present study, we have therefore examined in humans the relationships between mRNAs levels of the three desaturases in mononuclear blood cells with those in the liver.

Surprisingly, both Δ6 and Δ9 desaturase mRNA levels in mononuclear blood cells were negatively correlated with those in the liver. We do not have a clear explanation for this finding, but it can be speculated that an increased supply of precursor fatty acid will increase hepatic desaturase mRNA levels. This may result in increased hepatic LCP-formation and increased LCP-output via very low density lipoproteins (VLDL). In extra hepatic tissues and cells, these LCPs may downregulate the levels of extrahepatic Δ6 and Δ9 desaturase mRNAs. Studies with stable isotopes are not helpful to accept or refute this concept. It has been suggested that the relative conversions of ^{13}C α-linolenic acid (11) and of linoleic acid (12) are inhibited by the substrate itself. Likewise, the relative conversions of total linoleic acid and α-linolenic acid are decreased when the diets are enriched with their elongation and desaturation products arachidonic acid and DHA, respectively (13,14). This, however, does not necessarily mean that the absolute conversion - expressed as the absolute amount of the precursor fatty in the diet that is converted - is also decreased. Unfortunately, fatty acid conversions can not reliably be quantified after adminstration of a single dose of stable isotope (15), as done in these studies (11-14). The concept that an increased output of LCPs by the liver may downregulate levels of extrahepatic Δ6 and Δ9 desaturase mRNAs seems to be contradicted, however, by the results of a short-term study in rats by Cho et al., who reported a

decrease in hepatic Δ5 and Δ6 desaturase mRNA after supplementation of a high glucose, fat free diet with safflower oil (65% linoleic acid) (5).

The negative correlation between Δ6 and Δ9 desaturase mRNAs in mononuclear blood cells with those in the liver does not necessarily mean that desaturase mRNAs in mononuclear blood cells can not be used to predict those in the liver. Whether mRNA levels in mononuclear blood cells can be used to examine effects of external factors, e.g. diets, on hepatic desaturase mRNA levels need to be examined in future research. In these studies, diet effects on mononuclear blood cell desaturase mRNA levels should be combined with results from stable isotope conversion studies.

We did not find a correlation between Δ5 desaturase mRNAs in the liver and in the mononuclear blood cells. Delta 5 desaturase mRNA in the liver was, however, significantly correlated with hepatic Δ6 desaturase and almost significantly correlated with hepatic Δ9 desaturase, while desaturases were not significantly correlated with each other within the mononuclear blood cells. Thus, the lack of a significant relationship between Δ5 desaturase mRNA in liver and mononuclear blood cells seems to be caused by mRNA levels in the mononuclear blood cells. This suggests that, unlike Δ6 and Δ9 desaturase in mononuclear blood cells and Δ5, Δ6 and Δ9 desaturase in liver tissue, Δ5 desaturase mRNA synthesis in mononuclear blood cells is regulated in a different way. Furthermore, if there is truly no relationship between Δ5 desaturase in mononuclear blood cells and in the liver, this would mean that results of mononuclear blood cells regarding this desaturase can not be extrapolated to liver. It should also be noted that most of our subjects were heavily obese. Whether this may have influenced the relationships observed, is not known, but certainly needs further investigation.

Delta 5 and Δ6 desaturases are both involved in the conversion of n-3 and n-6 fatty acids, and their gene expressions may differ between tissues. Gene expressions of Δ5 and Δ6 desaturases are highest in human liver (5). Furthermore, in human heart, brain and lung also considerable amounts of mRNA are detected, whereas amounts in placenta, skeletal muscle, kidney and pancreas are small. In mononuclear blood cells, mRNA levels of Δ6 desaturase did not significantly differ from Δ5 desaturase mRNA, while in the liver the difference nearly reached statistical significance. Cho et al. reported higher Δ6 desaturase mRNA levels as compared to Δ5 desaturase mRNA levels in various human tissues (5).

We have observed a positive relationship between Δ5 and Δ6 desaturase mRNAs in the liver, which suggests that in man the expression of these two enzymes is regulated in parallel. This notion is supported by studies in rats, in which supplementation of a high glucose fat free diet with safflower or fish oil decreased hepatic mRNA levels of both Δ5 and Δ6 desaturase (5).

In mice, a polyunsaturated fatty acid diet reduced hepatic Δ6 desaturase mRNA (4). This was associated with a suppressed enzyme activity, which was measured *in vitro* in isolated microsomes using ^{14}C labeled linoleic acid. This

suggests that - at least in mice - diet-induced changes in Δ6 desaturase mRNA levels affects Δ6 desaturase activity into the same direction. It should be kept in mind, however, that mRNA levels do not necessarily reflect the amounts of enzymes formed or enzyme activity.

To summarize, we found Δ5, Δ6 and Δ9 desaturase mRNAs in both human liver and mononuclear blood cells. Delta 6 and Δ9 desaturase mRNAs in mononuclear blood cells were negatively correlated with those in the liver, while Δ5 desaturase mRNA in liver was not correlated with that in the mononuclear blood cells. Hepatic Δ5, Δ6 and Δ9 desaturase mRNAs were positively correlated with each other. No such intercorrelations were observed within mononuclear blood cells. We conclude that Δ6 and Δ9 desaturase mRNAs in mononuclear blood cells may predict mRNA levels in the liver, however, further research is necessary. Combining mononuclear blood cell desaturase mRNA results with stable isotope studies can be expected to extend our knowledge of the conversion pathways of both essential and non-essential fatty acids.

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6

Health effects of *trans* fatty acids

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INTRODUCTION

Trans fatty acids differ from their *cis* counterparts in the *trans* configuration of at least one double bond. This means that both carbon (C)-atoms adjacent to the double bound are located at opposite sides of an imaginary 'plane' through the double bond. The position of the double bond in the carbon-chain can be counted from the carboxyl-end (-COOH) of the molecule, which is symbolized as 'Δ', or from the methyl-end (-CH_3), which is symbolized as 'n minus'. Thus, 'n-3' means that the double bond is located at the third carbon atom from the methyl-end. Chain elongation and desaturation always occur at the carboxyl-end of the fatty acid molecule. Consequently, the position of the first double bond at the methyl-end will not change during conversion reactions. Figure 1.1 shows α-linolenic acid (C18:3n-3), which belongs to the (n-3) family, and one of its *trans* isomers C18:3n-3 Δ9*c*,12*c*,15*tr*. These two molecules are geometrical isomers.

Trans fatty acids can be formed from dietary (poly-) unsaturated fatty acids by bacteria in the first stomach (rumen) of ruminant animals and by industrial hydrogenation and deodorization. In order to improve the chemical, physical and sensory characteristics, vegetable oils are often hydrogenated to make them suitable for food. During this industrial process, the *cis* double bonds of polyunsaturated fatty acids in particular are saturated (hydrogenated) by adding hydrogen to the oil. When all double bonds are hydrogenated, a saturated fatty acid is formed. However, the *cis* configuration may also isomerize without net uptake of hydrogen, which results in *trans* double bonds. Furthermore, the double bonds can migrate along the molecule, resulting in positional isomers. In this way, linoleic acid can be converted into many different molecules (figure 6.1). *Trans* monounsaturated fatty acids in particular are formed by hydrogenation.

Deodorization is the last step in the refining process, during which oils are prepared for use as an ingredient in margarine, shortening, cooking oil, etcetera *Trans* polyunsaturated fatty acids can be easily formed by deodorization of oil (1,2). Finally, *trans* polyunsaturated fatty acids can be formed during heat or frying treatments (3).

This chapter focuses on the effects of *trans* fatty acids on various risk parameters for coronary heart disease (CHD), including lipids and lipoproteins, low density lipoprotein (LDL) oxidation and hemostasis, and on cancer. Furthermore, the metabolism of *trans* fatty acids and their effects on the desaturation and elongation of other fatty acids is discussed.

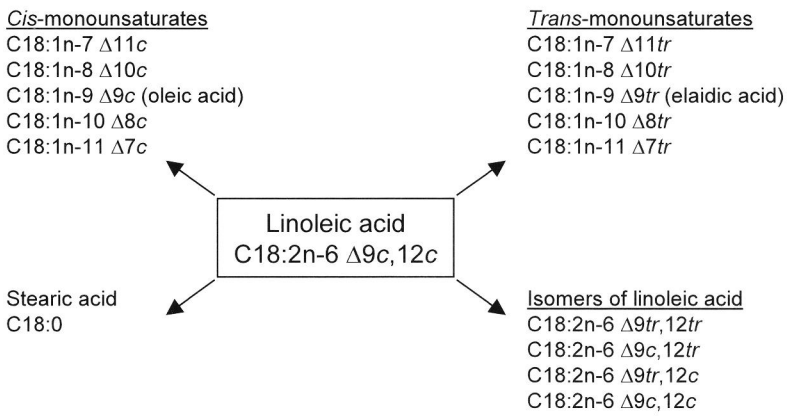

Figure 6.1 Potential conversions of linoleic acid into its positional and geometrical isomers

TRANS FATTY ACIDS IN FOODS

The *trans* fatty acid intake of the United States (US)-population has been estimated using availability or disappearance data, food frequency questionnaires, analysis of self selected diets, duplicate portion analysis and/or adipose tissue composition data. The most recent estimate of the daily total *trans* fatty acid intake in the US is 2.6% of energy or 7.4% of total fat intake for the total population aged 3 years and older (4). Estimates ranged from 2.6% to 2.8% and from 7.1% to 7.9%, respectively across different age and gender groups. Values were based on results of the 1989-1991 Continuing Survey of Food Intakes by Individuals (CSFII) of the US Department of Agriculture (USDA), and the *trans* fatty acid contents of specific foods calculated from a database compiled by the USDA. In Europe, the daily total *trans* fatty acid intake varied between 0.5% of energy in Italy to 2.0% of energy in Iceland (5). These data were based on analysis of food samples and food consumption-survey data, which were collected in 1995-1996 in 14 European countries.

Trans monounsaturated fatty acids contribute about 85% of total *trans* fatty acids (4). *Trans* monounsaturates from milk and meat fat are produced by bacteria in the rumen of animals. The major *trans* isomer in fat from ruminants is vaccenic acid (C18:1n-7 Δ11*tr*), although *trans* double bonds at the Δ5 to Δ16 positions are also present. Non-ruminants that are fed diets containing *trans* C18:1 also have *trans* C18:1 isomers in their tissues.

More important sources of *trans* monounsaturated fatty acids, however, are partially hydrogenated vegetable oils and products made from these oils. Fried foods and stick margarine are the major contributors to the *trans* fatty acid intake in the US, as shown in table 6.1. Most *trans* double bonds are at the Δ10, Δ9 or Δ8 position.

Trans polyunsaturated fatty acids are mainly formed during the deodorization process. In US vegetable oils, the degree of isomerisation of linoleic acid and α-linolenic acid range from 0.3% to 3.3% and from 6.6% to 37.1%, respectively (6). Most *trans* polyunsaturated fatty acids are mono-*trans* isomers of linoleic and α-linolenic acid, while only a few di-*trans* isomers are detected. Finally, *trans* polyunsaturated fatty acids are formed during heat treatment of vegetable oils, like during deep frying processes (3). Again most *trans* isomers are mono-*trans* isomers.

Table 6.1 Per capita consumption of *trans* fatty acids from primary food sources

Food source	*Trans* fatty acids g/day	Total fatty acids g/day	*Trans* fatty acids % of total fatty acids
Fried foods	0.8	3.9	20.5
Margarine, stick	0.5	1.7	29.4
Bread, commercial	0.3	4.0	7.5
Cakes and related baked goods	0.3	2.9	10.3
Savory snacks	0.3	2.3	13.0
Margarine, soft and spreads	0.2	1.2	16.7
Cookies	0.2	1.2	16.7
Milk	0.2	5.5	3.6
Butter	0.1	1.3	7.7
Crackers	0.1	0.5	20.0
Household shortenings	0.1	0.4	25.0
Ground beef	0.1	3.4	2.9

Values are based on results of the 1989-1991 CSFII of the USDA, and the *trans* fatty acid composition data is adapted from Nutrient Data Bank Bulletin Board (USDA)

METABOLISM

Digestion, absorption and incorporation into blood lipids

Trans fatty acids in margarines and dairy fats are incorporated in triacylglycerols, while in meat *trans* fatty acids are found in phospholipids. Figure 6.2 shows the metabolic pathways of *trans* fatty acids after dietary intake. After ingestion, triacylglycerols are split by pancreatic lipase into free fatty acids and sn2-monoglycerides. Small portions remain as diglycerides. Phospholipids are split by pancreatic phospholipases, which remove the fatty acid from the sn1 or sn2 position of the phospholipid. Consequently, lysophospholipids and free fatty acids are formed. The digestion of *trans* monounsaturated fatty acids does not differ from that of their *cis*-isomers (7).

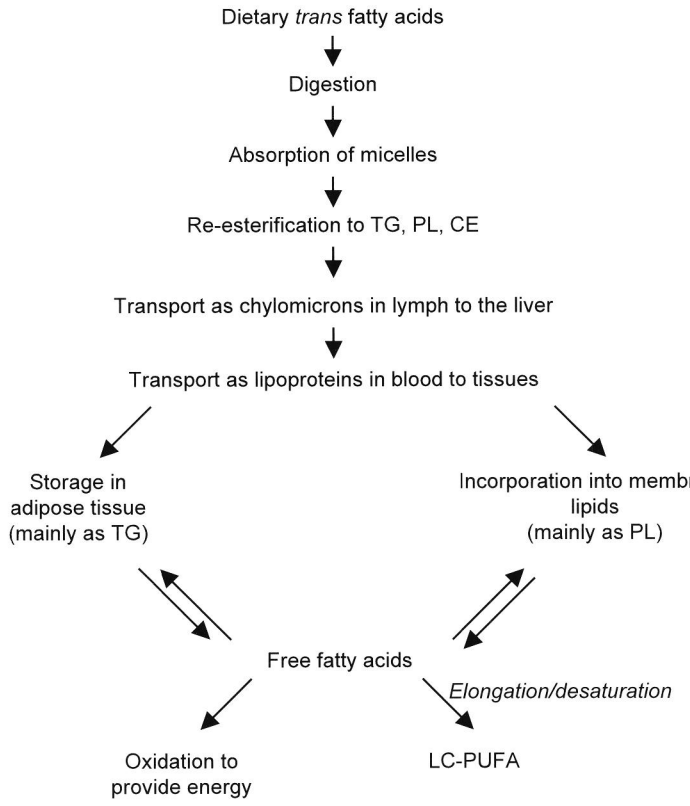

Figure 6.2 Simplified scheme of *trans* fatty acid metabolism in humans. TG, triacylglycerols; PL, phospholipids; CE, cholesteryl esters; LC-PUFA, long chain polyunsaturated fatty acids

After ingestion, fatty acids are absorbed by the enterocyte after formation of micelles. Absorption of oleic acid and its positional and geometrical isomers is comparable (8-11). In the enterocyte, free fatty acids, sn2-monoglycerides and diglycerides are re-esterified to triacylglycerols, cholesteryl esters and phospholipids. The incorporation of positional and geometrical isomers of C18:1n-9 $\Delta 9c$ in triacylglycerols, cholesteryl esters and phospholipids may differ from that of C18:1n-9 $\Delta 9c$ (11). After re-esterification, lipids may be taken up by chylomicrons, which transport the fatty acids through the lymph to the liver. Then, *trans* fatty acids are transported by lipoproteins through the blood stream. The incorporation of *trans* isomers of C18:1n-9 $\Delta 9$ into very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) is lower than that of C18:1n-9 $\Delta 9c$ (9-11).

Trans fatty acids may also be incorporated into tissue phospholipids, stored in adipose tissue, desaturated and elongated into longer chain polyunsaturated fatty acids or oxidized via β-oxidation and peroxisomal systems.

Tissue levels

In the human body, *trans* fatty acids are found in triacylglycerols, mainly at positions 1 and 3. The *trans* fatty acid content of adipose tissue for US subjects is about 4% of total fatty acids (12). Approximately 70% of the *trans* fatty acids are C18:1 isomers, which have their double bonds on the Δ8, 9, 10, 11, 12 or 13 position. *Trans* C16:1 isomers, which originate mainly from dairy fat, are also found in adipose tissue. About 20% of the *trans* isomers in adipose tissue are *trans* C18:2n-6 isomers. Both mono-*trans* (Δ9*c*,12*tr* and Δ9*tr*,12*c*) and di-*trans* (Δ9*tr*,12*tr*) C18:2n-6 isomers, and several *trans* C18:3 isomers have been detected in adipose tissue.

Trans fatty acids in other tissues than adipose tissue are incorporated mainly into phospholipids predominantly at position 1. *Trans* C18:1 and C18:2 fatty acids have been found in human kidney, brain, heart, liver, aorta, jejunum and human milk (13,14), while *trans* C18:3 has been detected in platelets and human milk (15,16).

Desaturation and elongation

Human liver microsomal complexes contain 3 different desaturation enzymes: Δ9, Δ6 and Δ5 desaturases, which insert double bonds at the Δ9, Δ6 or Δ5 position of the fatty acid molecule, respectively. Furthermore, fatty acids can be elongated by addition of a two-carbon unit to the fatty acids. In this way, stearic acid, linoleic acid and α-linolenic acid are converted into their longer chain metabolites (figure 1.2), which play an important role in many physiological processes (17).

Trans fatty acids are desaturated by the same enzymes as *cis* fatty acids. Except for Δ8, Δ9 and Δ10 *trans* isomers, C18:1 positional isomers are good substrates for Δ9 desaturase (18). Consequently, *cis*,*trans* and *trans*,*cis* fatty acids are formed. Some *trans* isomers of C18:1 are also substrates for Δ6 and Δ5 desaturase (19). In addition, C18:1 *trans* isomers can be elongated into C20 and C22 fatty acids.

Trans linoleic acid can be converted into *trans* isomers of arachidonic acid (C20:4n-6), however, this occurs at a lower rate than all-*cis* C18:2n-6 (20). *Trans* isomers of α-linolenic acid can be converted into *trans* isomers of docosahexaenoic acid (DHA; C22:6n-3) (21,22). Observations, however, are mainly based on animal and *in vitro* studies and may be different for the *in vivo* situation in man.

Influence of trans *fatty acids on desaturation and elongation*

Because *trans* and *cis* fatty acids can be converted by the same desaturase and elongase enzymes, competition between fatty acids exists. Both the *cis* and *trans* monoenoic positional isomers have been reported to inhibit the conversion of linoleic acid into arachidonic acid in cultured glioma cells (23). Furthermore, in human skin

fibroblasts, C18:1n-9 Δ9*tr* increased Δ9 desaturation of stearic acid, while C18:1n-7 Δ11*tr* and C18:2n-6 Δ9*tr*,12*tr* had no effect (24). In addition, C18:1n-9 Δ9*tr* and C18:2n-6 Δ9*tr*,12*tr* inhibited Δ6 desaturation. However, because levels of *cis* n-3 and n-6 fatty acids in human tissues are much higher than levels of *trans* fatty acids, these fatty acids are physiologically more important competitive inhibitors for the conversion of linoleic acid into arachidonic acid and α-linolenic acid into DHA as compared to *trans* isomers.

Oxidation

Only a few human studies have been carried out to examine the effects of *trans* fatty acids on fatty acid oxidation. So far, it is only known that the whole body oxidation, measured with ^{13}C labeled fatty acids, and oxidation rates by human heart homogenates were equal between the *trans* fatty acid elaidic acid and the non-*trans* fatty acid with equivalent chain length oleic acid (25,26).

EFFECTS OF *TRANS* MONOUNSATURATED FATTY ACIDS ON SERUM LIPIDS AND LIPOPROTEINS

Total cholesterol, LDL and HDL cholesterol

The effect of dietary *trans* monounsaturated fatty acids on serum cholesterol concentrations has been investigated since the early 1960s. Most studies have found increased serum total cholesterol levels in humans consuming partially hydrogenated vegetable oil. Results, however, were not uniform (27). In addition, these earlier studies did not examine the relationship between dietary *trans* monounsaturated fatty acids and the distribution of cholesterol among lipoproteins. The first controlled intervention trial in which the effects of *trans* monounsaturated fatty acid intake on serum LDL and HDL cholesterol were examined, was reported in 1990 by Mensink and Katan (28). Twenty-five men and 34 women were fed a diet high in oleic acid, high in *trans* isomers of oleic acid or high in a mixture of the saturated fatty acids lauric and palmitic acid. Each diet was fed for 3 weeks. The level of *trans* C18:1 in the *trans* diet was 11% of total energy. Results showed that *trans* C18:1 significantly raised total and LDL cholesterol levels by 0.26 mmol/l and 0.37 mmol/l, respectively, and lowered HDL cholesterol levels by 0.17 mmol/l as compared to oleic acid. Later studies investigated effects of lower intakes of *trans* fatty acids (table 6.2) (29-33). The general conclusion is that *trans* fatty acids and saturated fatty acids have similar effects on LDL cholesterol, but increase LDL cholesterol as compared to monounsaturated fatty acids and polyunsaturated fatty acids. Also, *trans* fatty acids lower HDL cholesterol as compared to the other major categories of fatty acids. Because of differences in *trans* fatty acid intake, intakes of other dietary fatty acids and experimental designs, results of studies are difficult to compare. Therefore, results of five studies have been combined and effects of *trans* fatty acids on

lipoproteins were compared with those of oleic acid (27). It is suggested that each additional percent of dietary energy as *trans* monounsaturated fatty acids at expense of oleic acid results in an increase in LDL cholesterol levels of 0.040 mmol/l and a decrease in HDL cholesterol levels of 0.013 mmol/l (figure 6.3) (27). Such changes in LDL and HDL cholesterol are associated with an increased risk for CHD.

Lipoprotein(a)

Lipoprotein(a) (Lp(a)) is a LDL molecule with an additional glycoprotein (apoprotein (a)), attached through a disulfide link. High levels of Lp(a) are associated with an increased risk for CHD (34). Therefore, effects of *trans* monounsaturated fatty acids on Lp(a) have been investigated in several intervention studies. Nestel et al. found that Lp(a) levels were significantly higher on a *trans* diet, as compared to palmitic acid, but not as compared to oleic acid (35). Mensink et al. reported significantly higher Lp(a) levels on *trans* fatty acids compared to saturated fatty acids, oleic acid, stearic acid and linoleic acid (36). Lichtenstein et al. did not find an increase in Lp(a) concentrations (37), while Almendingen et al. found increased Lp(a) levels when butter was exchanged for partially hydrogenated soybean oil or fish oil (29). Aro et al. observed increased Lp(a) levels on a *trans* fatty acid diet as compared to a dairy fat based diet (30). In conclusion, most studies showed that *trans* monounsaturated fatty acids may raise Lp(a) levels, in particular in subjects who already have high Lp(a) levels (36).

Triacylglycerols

An elevated fasting triacylglycerol level is also a risk marker for CHD (38). Katan et al. summarized studies concerning the *trans* monounsaturated fatty acid effect on fasting triacylglycerol levels. It was calculated that each additional percent of energy as *trans* monounsaturated fatty acids at the expense of oleic acid increases triacylglycerol levels by 0.013 mmol/l (27).

Conclusion

Trans monounsaturated fatty acids raise serum LDL cholesterol, Lp(a) and triacylglycerol levels, while HDL cholesterol levels are lowered. The effects of *trans* polyunsaturated fatty acids on serum lipids and lipoproteins are not yet known.

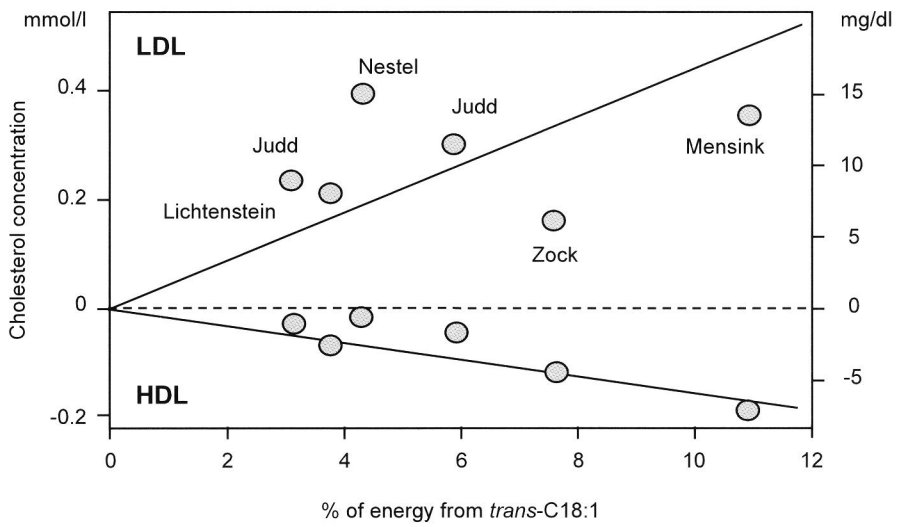

Figure 6.3 Effects of exchanging oleic acid for *trans* monounsaturated fatty acids on LDL and HDL cholesterol levels. Data are derived from six previous studies. Regression coefficients are 0.040 mmol/l for LDL and –0.013 mmol/l for HDL cholesterol, which represent the predicted changes in serum LDL and HDL cholesterol levels if 1% of daily energy intake from oleic acid is replaced by *trans* monounsaturated fatty acids (27).

Mechanism

Although a number of studies have shown that dietary *trans* fatty acids affect serum LDL and HDL cholesterol levels, the underlying mechanisms for these effects are still not understood. It has been hypothesized that cholesteryl ester transfer protein (CETP) is involved. CETP transfers cholesteryl esters from HDL to the apolipoprotein B-containing lipoproteins LDL and VLDL in exchange for triacylglycerol. Indeed, an increased CETP activity has been found in volunteers who consumed a *trans* fatty acid enriched diet (39,40), and after addition of *trans* fatty acids to human plasma *in vitro* (41). However, some other studies did not confirm these results (30,42).

Lecithin:cholesterol acyltransferase (LCAT) has also been suggested to be involved. LCAT, which is bound to HDL, esterifies free cholesterol from tissues by the transfer of an acyl group from phosphatidyl choline (PC). Since a significant part of the dietary *trans* fatty acids is incorporated into PC (11), a few studies have investigated the effect of *trans* fatty acids on LCAT. Subbaiah et al. reported a decrease of LCAT activity on *trans* fatty acids (43). Van Tol et al., however, did not demonstrate an effect of *trans* fatty acids on LCAT activity (40).

Table 6.2 Effects of *trans* fatty acids on serum LDL and HDL cholesterol levels

Reference	Year	No. of subjects		Design	Days of test period	Test Diet	Fatty acid content of test diet (% of daily energy intake)				Diet effects (mmol/l)	
		Men	Women				S	M	P	T	HDL	LDL
Almendingen et al. (29)	1995	31		×	21	I	15.7	8.1	5.9	0.9	1.05	3.81
						II	10.5	9.2	6.5	8.5	1.05	3.58[b]
						III	10.8	5.7	7.0	8.0	0.98[b,c]	3.94[c]
Aro et al. (30)	1997	29	29	//	35	C	13.8	12.2	3.4	0.8		
						I	15.0	12.2	3.5	0.4	1.42	2.89
						II	7.1	21.2	2.9	8.7	1.36[a,b]	3.06[a,b]
Müller et al. (31)	1998		27	×	17	I	11.2	11.8	5.7	0.1	1.47	2.90
						II	3.8	10.6	5.5	7.0	1.32[b]	2.88
						III	6.1	11.7	10.2	0.2	1.43[c]	2.61[b,c]
Judd et al. (32)	1998	23	23	×	35	I	11.2	10.8	7.2	2.7	1.27	3.44
						II	7.9	11.2	9.0	3.9	1.24	3.27[b]
						III	8.3	10.4	10.8	2.4	1.24	3.21[b,c]
Lichtenstein et al. (33)	1999	18	18	×	35	I	16.7	8.1	2.4	1.3	1.16	4.58
						II	7.3	8.1	12.5	0.6	1.11	3.98[b]
						III	8.6	8.1	13.5	0.9	1.11	4.01[b]
						IV	8.4	8.0	11.1	3.3	1.11	4.11[b]
						V	8.6	9.9	8.1	4.2	1.11	4.24[b,c]
						VI	8.5	8.5	6.3	6.7	1.09[b]	4.34[b,c,d]

Only studies with a thorough control of food intake, with dietary fatty acids being the sole variable, and designs that eliminated the effect of nonspecific drifts of the outcome variables with time, were selected. C, control diet; S, saturated fatty acids; M, monounsaturated fatty acids; P, polyunsaturated fatty acids; T, *trans* fatty acids.

× Cross-over or Latin square design

// Parallel design

[a] Corrected for differences in lipid values between groups when on the control diets

[b] for comparison with I

[c] for comparison with II

[d] for comparison with III

In the liver, fatty acids from chylomicrons are esterified to free cholesterol by acyl-CoA:cholesterol acyl transferase (ACAT). The affinity of ACAT differs for different fatty acids. A low affinity of the enzyme for a particular fatty acid may result in an increase of free cholesterol in the liver. This causes a decrease of the LDL receptor activity. Since the LDL receptor is involved in the uptake of LDL from plasma, a decreased LDL receptor activity may result in higher LDL concentrations in plasma. Thus, through this pathway dietary fatty acids may influence the plasma LDL cholesterol concentration. In a hamster study, Woollett et al. demonstrated that *trans* C18:1 decreased the cholesteryl ester concentration in the liver relative to oleic acid (44). This could suggest that the affinity of ACAT is lower for *trans* C18:1 as compared to oleic acid, which may result in higher free cholesterol levels in the liver. In addition, the plasma LDL cholesterol concentration was shown to be increased and the LDL receptor activity was decreased on *trans* C18:1. Possibly, *trans* C18:1 affects plasma LDL cholesterol via the pathway described above. This mechanism, however, has not been investigated in man. Finally, *de novo* cholesterol synthesis did not differ between a high *trans*-hydrogenated corn oil rich diet as compared to an unhydrogenated corn oil rich diet low in *trans* fatty acids in healthy volunteers (45).

EFFECTS OF *TRANS* FATTY ACIDS ON OTHER RISK FACTORS FOR CORONARY HEART DISEASE

LDL oxidation

Free radicals may initiate oxidation of polyunsaturated fatty acids in the LDL particle, which eventually may lead to formation of an atherosclerotic plaque. The fatty acid composition of the LDL particle is a reflection of the fatty acid composition of the diet. Consequently, increased dietary *trans* fatty acid intake leads to higher proportions of *trans* fatty acids in the LDL particle. Whether this influences the susceptibility for LDL to oxidation has been investigated in several intervention trials. However, no effect on *in vitro* LDL oxidation was found after consumption of *trans* monounsaturated fatty acids as compared to oleic acid or palmitic acid (35), of hydrogenated as compared to unhydrogenated corn oil (45), or of partially hydrogenated fish oil or partially hydrogenated soybean oil as compared to butter-fat (46). Therefore, it appears that, at least *in vitro*, *trans* fatty acids have not a major impact on LDL-oxidizability.

Hemostasis

Human studies investigating the effects of *trans* fatty acids on platelet aggregation, coagulation and fibrinolysis are limited. Almendingen et al. have found that a diet enriched in partially hydrogenated soybean oil increased concentrations of plasminogen activator inhibitor Type 1 (PAI-1) antigen and PAI-1 activity as compared with a diet high in partially hydrogenated fish oil or butter fat (47). This indicates an inhibition of the fibrinolytic pathway. Fibrinogen levels were increased on

butter compared to partially hydrogenated fish oil, while levels of factor VII were not affected by the diets. It was therefore concluded that partially hydrogenated fish oil had the least unfavorable hemostatic effects, while partially hydrogenated soybean oil had the worst. Mutanen and Aro did not see differences in concentrations of fibrinogen, factor VII coagulation activity, tissue type plasminogen activity and PAI-1 activity between dietary *trans* fatty acids from partially hydrogenated vegetable oil and stearic acid in healthy subjects (48). However, collagen-induced platelet aggregation was significantly decreased on *trans* fatty acids, as compared to stearic acid (49). No effects of *trans* fatty acids were found on *in vitro* tromboxane B_2 production, adenosine diphosphate (ADP)-induced aggregation, and production of endothelial prostacyclin (PGI_2) (49).

Few other studies have investigated the effects of *trans* polyunsaturated fatty acids on human platelet aggregation. Collagen-induced platelet aggregation was less inhibited on *trans* isomers of the long chain polyunsaturated fatty acids from fish, eicosapentaenoic acid (EPA) and DHA as compared to their *cis* isomers when these fatty acids were incorporated into platelets (15). O'Keefe et al. has also shown this for EPA in arachidonic acid-induced platelet aggregation, but not for DHA (50).

In conclusion, the effects of *trans* fatty acids on aggregation, coagulation and fibrinolysis have hardly been investigated. At present, however, it does not seem that *trans* fatty acids have significant effects on hemostasis.

EPIDEMIOLOGICAL STUDIES

Various approaches have been used in epidemiological studies to examine the relationship between the intake of *trans* fatty acids and the risk for CHD. Because serum LDL cholesterol levels are negatively associated with CHD and high levels of HDL cholesterol are thought to be anti-atherogenic, some epidemiological studies have focussed on the relation between the proportion of *trans* fatty acids in tissues, which reflects dietary intakes, or the dietary *trans* fatty acid intake and the lipid profile (51-53). In the cross-sectional study of Hudgins et al. no significant correlations between total *trans* fatty acids in adipose tissue and plasma HDL cholesterol, total to HDL and LDL to HDL cholesterol ratios, and triacylglycerol were observed in Caucasian men (51). On the contrary, in another cross-sectional study the energy adjusted *trans* fatty acid intake (3.4 ± 1.2 g/day, mean ± sd) was positively related to serum LDL cholesterol, and total to HDL and LDL to HDL cholesterol ratios and inversely related to HDL cholesterol (52). In the case-control study of Siguel and Lerman, the relationships between plasma *trans* fatty acids and lipid profile, and between plasma *trans* fatty acids and risk for CHD, were investigated (53). It was shown that *trans*-16:1 was positively related to plasma total and LDL cholesterol, triacylglycerol and risk for CHD, and negatively related to HDL cholesterol and the HDL to total cholesterol ratio. Altogether, results of these studies are in agreement

with dietary intervention studies reporting that *trans* fatty acids have a negative impact on the lipid profile. Most of these studies, however, did not address the question if high intakes of *trans* fatty acids are related to the risk for CHD.

In case control studies, the proportion of *trans* fatty acids in tissues or the dietary *trans* fatty acid intake of case subjects, e.g. subjects with CHD, has been compared with that of control subjects, e.g. subjects without CHD (13,54-60). Some studies suggested higher *trans*-C16:1 (54,55) or total *trans* fatty acid intakes (60) in cases as compared to controls, while other studies have not found any association between *trans* fatty acids and CHD-risk (13,56,57,59,61). One study even showed that the proportion of *trans*-18:1 in adipose tissue was negatively associated with the risk for CHD (58). Case-controls studies, however, have the disadvantage that they are sensitive to information bias, selection bias and confounding.

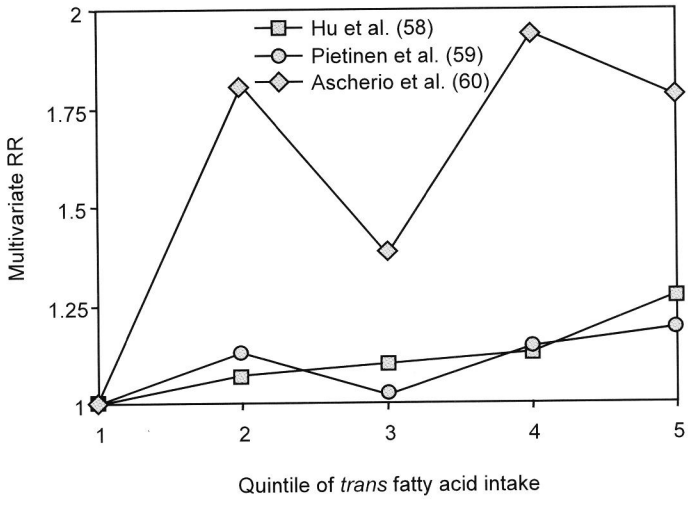

Figure 6.4 Multivariate relative risk of coronary heart disease according to the quintiles of *trans* fatty acid intake of 3 cohort studies: The Nurses Health study of Hu et al. (62), the α-Tocopherol, β-Carotene Cancer Prevention Study of Pietinen et al. (63), and The Health Professionals Follow-up Study of Ascherio et al. (64).

Prospective cohort studies suffer from less of the disadvantages attributed to case control studies. Four cohort studies have examined the potential relationship between the *trans* fatty acid intake and the risk for CHD. In the Nurses' Health Study, the relative risk (RR) of CHD was 1.27 (95% confidence interval (CI), 1.03 to 1.56) for the highest quintile of *trans* fatty acid intake (mean intake of 2.9% of energy) relative to the lowest (mean intake of 1.3% of energy) after adjustment for age and other

CHD risk factors (62). This means that women who consume 2.9% of energy as *trans* fatty acids per day, have a 27% higher risk for CHD as compared to women with a *trans* fatty acid intake of 1.3% of energy. Pietinen et al. found in the Finnish α-Tocopherol, β-Carotene Cancer Prevention Study a positive association between the *trans* fatty acid intake and risk of coronary death after adjustment for age, supplementation group and several coronary risk factors (63). The RR for CHD was 1.39 (95% CI,1.09 to 1.78) for the highest quintile of *trans* fatty acid intake (median intake of 5.6 g/day) as compared to the lowest (median intake of 1.3 g/day). In addition, in the Health Professionals Follow-up Study, subjects in the highest quintile of *trans* intake (median intake of 4.3 g/day) had a 78% (RR of 1.78; 95% CI, 1.11 to 1.84) higher risk on fatal CHD as compared to subjects in the lowest quintile of *trans* intake (median intake of 1.5 g/day) after controlling for age and several coronary risk factors (64). Multivariate RRs of these three cohort studies are summarized in figure 6.4, which shows that multivariate RRs increase with *trans* fatty acid intakes. Finally, Kromhout et al. have reported that *trans* fatty acid intake was positively related to 25-year mortality from CHD in the Seven Countries Study (65).

In summary, results from case control studies are inconsistent. In the cohort studies, results are more uniform: a high intake of *trans* fatty acids was associated with an increased risk of CHD. Therefore, based on the results of the cohort studies, it seems advisable to avoid high intakes of *trans* fatty acids.

CANCER

The association between fat intake and carcinogenesis has been investigated in many animal and human studies. Only a few human studies, however, have focussed on *trans* fatty acids and cancer, in particular the risk for breast cancer. Holmes et al. found in the Nurses' Healthy Study, a large prospective cohort study, a relative risk on breast cancer of 0.92 (95% CI, 0.86-0.98) when the *trans* fatty acid intake would increase with 1% of energy (66).

Three case-control studies have related the *trans* fatty acid content of adipose tissue to cancer risk. In the EURAMIC study, the *trans* fatty acid content of subcutaneous adipose tissue was positively associated with risk for breast cancer (67). On the contrary, in women with breast cancer the *trans* fatty acid content in adipose tissue was negatively associated with having breast cancer as well as cancer in lymph nodes (68). Finally, London et al. did not find an association between the *trans* fatty acid content of subcutaneous adipose tissue and breast cancer risk (69).

Human studies investigating the relationship between *trans* fatty acids and other classes of cancers are scarce. In the EURAMIC study, a significant correlation was found between the amount of *trans* fatty acids in adipose tissue and the incidence of colon cancer. No association was found with prostate cancer (70). In

conclusion, there is not a consistent relationship from human studies between the intake and/or tissue levels of *trans* monounsaturated fatty acids and cancer risk.

CONCLUSIONS AND RECOMMENDATIONS

The digestion and absorption of *trans* monounsaturated fatty acids are comparable with those of their *cis* isomers. However, incorporation of various *trans* monoenoic isomers into lipid classes and lipoproteins appears to be lower than that of the *cis* isomers. Furthermore, both *trans* monounsaturated and polyunsaturated fatty acids can be desaturated and elongated into longer chain more unsaturated metabolites. However, conversion of *trans* linoleic acid may be less than that of linoleic acid. *Trans* monounsaturated fatty acids inhibit the conversion of linoleic acid, while conversion of stearic acid increases. The oxidation seems to be similar between *cis* and *trans* isomers. These processes, however, have mainly been investigated in animal and *in vitro* studies. More human *in vivo* studies are therefore necessary.

Most studies on the effects of *trans* fatty acids and health have focussed on *trans* monounsaturated fatty acids, and effects of *trans* polyunsaturated fatty acids are hardly known. *Trans* monounsaturated fatty acids increase serum LDL cholesterol, triacylglycerols and Lp(a) concentrations and decrease serum HDL cholesterol as compared with oleic acid. The underlying mechanisms for these effects are not exactly known. Furthermore, *trans* monounsaturated fatty acids seem to have no major impact on LDL-oxidizability, platelet aggregation, coagulation and fibrinolysis. Several prospective epidemiological studies have shown no correlation between *trans* fatty acids and cancer risk, while a positive association between *trans* fatty acids and the risk for CHD was reported. Therefore, a limitation of the *trans* fatty acid intake as recommended by the American Heart Association is justified (71).

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The effect of dietary *trans* α-linolenic acid on plasma lipids and platelet fatty acid composition: the *Trans*LinE Study

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ABSTRACT

The aim of the study was to investigate the incorporation of *trans* α-linolenic acid into plasma and platelet lipids as part of a multicenter study examining the effect of dietary *trans* α-linolenic acid on a variety of risk factors for coronary heart disease.

Eighty-eight male volunteers from three European countries (France, Scotland and the Netherlands) were recruited and the habitual diet assessed by a 4-day weighed record. Fatty acid composition of plasma and platelet lipids were determined by gas chromatography at baseline. After a 6 week run-in period on a *trans* free diet, volunteers were randomized to consume 0.6% of energy *trans* α-linolenic acid or to continue with a diet low in *trans* α-linolenic acid for 6 weeks. Rapeseed oil was deodorized especially to produce the *trans* free and high *trans* foods for this study. The incorporation *trans* α-linolenic acid and its longer chain more unsaturated metabolites into plasma and platelet lipids was assessed by gas chromatography, and dietary compliance was verified by 4-day weighed record.

Less *trans* α-linolenic acid isomers are present into human plasma lipids in French volunteers than in Dutch or Scottish volunteers consuming their habitual diets. The high *trans* diet provided 1410 ± 42 mg/d *trans* isomers of α-linolenic acid, whilst the low *trans* group consumed 60 ± 75 mg/d. The change in plasma lipid and platelet fatty acid composition documented that *trans* α-linolenic isomers are incorporated and converted to a *trans* isomer of eicosapentaenoic acid. Only the Δ15*trans* α-linolenic acid is incorporated into plasma cholesteryl esters. The group consuming low *trans* diet had a slightly higher intake of fat, especially saturated and monounsaturated fat.

In conclusion, dietary *trans* isomers of α-linolenic acid are incorporated in plasma lipids and converted to long chain polyunsaturated fatty acids.

INTRODUCTION

Dietary *trans* isomers of monounsaturated fatty acids, formed during partial hydrogenation of edible oils or by intestinal fermentation in ruminants, have been linked with an increased risk of coronary heart disease (CHD) (1-4). This is perhaps due to the fact that *trans* monounsaturated fatty acids when compared with *cis* monounsaturates raise serum low density lipoprotein (LDL) and reduce high density lipoprotein (HDL) cholesterol (5). The relative amount of *trans* fatty acids in adipose tissue and plasma phospholipids, arguably a measure of the average intake, has not been associated with an increased risk of coronary heart disease however (6-8) and some of the reasons for the contradictory results have been discussed (4,9,10).

Trans isomers of polyunsaturated fatty acids are formed during deodorization of edible oils and numerous animal studies have shown that isomers of linoleic and linolenic acids are incorporated in rat tissues (11). Δ9*tr*,12*c*,15*c* -, Δ9*c*,12*tr*,15*c* - and Δ9*c*,12*c*,15*tr* - α-linolenic acid are converted by successive desaturation and elongation into *trans* isomers of eicosapentaenoic acid (EPA; C20:5n-3) (12,13) and compete with α-linolenic acid (C18:3n-3) at the Δ6 desaturation step (14). Dietary *trans* α-linolenic acid can also inhibit the Δ5 desaturase and hence the biosynthesis of arachidonic acid (C20:4n-6) (15). *Trans* isomers of C20 or C22 n-3 fatty acids occur in up to 1% of fatty acids in all examined tissues, including the liver and the heart of the rat (12).

Much less is known about the metabolism of dietary *trans* polyunsaturated fatty acids in man. Human platelets do contain small amounts of these *trans* isomers of EPA and docosahexaenoic acid (DHA; C22:6n-3) (16). *Trans* isomers of α-linolenic acid occur in human serum (17) and maternal milk (18,19). This is not surprising since *trans* polyunsaturated fatty acids are found in heated vegetable oils and in numerous human food items (20), such as low calorie spreads (21) and infant formulas (22-24). The average intake of *trans* α-linolenic acid in European men and women is not precisely known (because of difficulties separating *trans* C18:3n-3 and C20:1 by capillary gas chromatography in some laboratories). Nevertheless, the intake of the sum of these two isomers is low in comparison to *trans* isomers of C18:1 (25). As much as 50% of α-linolenic acid can be in the *trans* form (19), suggesting the intake of *trans* α-linolenic acid is likely to increase if more α-linolenic acid would be consumed to prevent coronary heart disease (up to 1% of energy) as advised by an expert committee (26).

In view of these changing trends it is imperative to collect baseline data concerning the presence of *trans* fatty acids in plasma and platelet lipids and to study the effects of a diet low or high in *trans* α-linolenic acid.

SUBJECTS AND METHODS

Design

The purpose of the *Trans*LinE (*trans* α-linolenic acid in Europe) project in three European centers: Clermont-Ferrand (France), Edinburgh (Scotland) and Maastricht (the Netherlands) was to assess the effect of *trans* α-linolenic acid on risk factors for CHD in male volunteers. A parallel design comparing low *trans* and high *trans* α-linolenic acid in the diet was adopted (see protocol below). The aim was to consume 0.6% of energy as *trans* at expense of all *cis* α-linolenic acid from refined canola oil. Furthermore, subjects were asked to reduce the consumption of foods containing *trans* fatty acids as much as possible for the duration of the entire project. Consequently, the intake of ruminant meat, cheese (except goat cheese: low in *trans*), as well as foods containing hydrogenated fats were avoided. The main part of the *trans* fatty acid intake was reduced by replacing subjects usual oil and margarine by experimental products. In all other aspects the participants were encouraged to continue their normal lifestyle and physical activity.

The men gave informed consent and approval had been obtained from the 3 local ethical committees.

Recruitment of volunteers

Men were recruited from a panel of registered volunteers (Clermont-Ferrand), or local advertisement campaigns in Edinburgh (students and staff of the University) and in Maastricht (general population). Only clinically healthy subjects with a normal energy intake, body mass index (BMI <27 kg/m^2) and plasma cholesterol levels (<6.5 mmol/l measured on two separate occasions at least one week apart) were enrolled. A total of 91 men entered the study: 32 in Clermont-Ferrand, (16 during the spring and 16 during the autumn of 1996), 28 in Edinburgh (all during the spring of 1996) and 31 in Maastricht (all during the autumn of 1996). Thirty-one subjects completed the study in Clermont-Ferrand, whereas 26 subjects completed the study in Edinburgh. All the 31 volunteers completed the study in Maastricht. The French dropout used anti-inflammatory medication interfering with the protocol and the two who dropped out in Edinburgh found the constraints of the study too hard. Baseline characteristics of the subjects at entry did not differ between the centers, except for a small difference in systolic blood pressure and smoking habits (table 7.1). The difference in these smoking habits was due to the fact that the target of 40% smokers was reached only in Clermont-Ferrand.

Preparation of experimental oils

The technology for the preparation of the experimental oils and fats, low or high in *trans* α-linolenic acid, had to be developed for this project (27). Canola oil (360 kg) containing 11.9% of α-linolenic acid was deodorized at a low temperature (180°C) for

5 hours under a blanket of nitrogen at 2-3 mbar pressure (*trans* free). Another portion of the same batch of canola oil (350 kg) was deodorized in a pilot plant for 52.5 hours at 205°C under 3 mbar pressure. These conditions were optimized to yield a high amount of *trans* α-linolenic acid with as little *trans* linoleic acid (C18:2n-6) as possible (27). Under these conditions 36.5% of α-linolenic acid was converted to the *trans* form. The relative amount of *trans* linoleic acid rose from undetectable levels to 0.5% of fatty acids. Therefore, *trans* linoleic acid was added in the form of a highly isomerized sunflower oil (deodorized at 270°C for 18 hours) to the *trans*-free oil to arrive at the low *trans* oil. One batch of oil was rejected in the production of margarine by a tasting panel, but generally the oil had good sensory properties. The final fatty acid composition of the mixture of 5 batches used for the study is presented in table 7.2. Vitamin E in the form of the free alcohol was added to the high *trans* oil in order to have the same amount of tocopherols in both the high *trans* and the low *trans* oils.

Table 7.1 Selected characteristics and fasting plasma lipid concentrations of the volunteers from Clermont-Ferrand, Edinburgh and Maastricht at baseline. Values are means ± sd.

	Clermont-Ferrand (n=31)	Edinburgh (n=26)	Maastricht (n=31)
Age (y)	32.4 ± 8.3	33.6 ± 11.5	34.2 ± 9.4
Height (m)	1.78 ± 0.06	1.78 ± 0.08	1.81 ± 0.08
Weight (kg)	73.7 ± 9.3	74.1 ± 9.7	75.3 ± 8.5
BMI (kg/m^2)	23.3 ± 2.5	23.2 ± 2.7	23.0 ± 2.2
Blood Pressure (mmHg)			
systolic	127 ± 9	121 ± 8	122 ± 8
diastolic	72 ± 10	72 ± 8	74 ± 7
Cholesterol (mmol/l)			
Total	4.77 ± 0.86	4.45 ± 0.54	4.77 ± 0.89
HDL	1.18 ± 0.28	1.22 ± 0.23	1.25 ± 0.33
LDL [a]	3.16 ± 0.78	2.86 ± 0.52	3.09 ± 0.80
Triacylglycerols (mmol/l)	0.93 ± 0.41	0.75 ± 0.27	0.94 ± 0.63
Smokers (n)	12	1	3

[a] Derived by Friedewald equation

All between center comparisons are non-significant except systolic blood pressure CF vs E, $P<0.05$

These oils were used to prepare margarine, cheese, biscuits and muffins. In Finland (Raisio Margarinii, Raisio), margarine was prepared by mixing the oils with a hard fat component, devoid of *trans* fatty acids. The fat mixture consisted of 77% of oil and 23% of the hard fat component. The margarines contained 70% fat and 0.5% salt with milk protein. The fatty acid composition of the margarine is given in table 7.3. Cheese was produced from homogenized pasteurized fat free milk, lecithin and

experimental oil (30%). The final fat content of the cheeses (200 g each), matured for 30 days, was 20% (Duboz & Grappin, Poligny, France). Finally, some chocolate flavored muffins and orange flavored biscuits (CRNH, Clermont-Ferrand) were produced to vary the sources of oils in the diet. These were prepared using 13.70 g margarine /100 g biscuits or cookies. In Maastricht cheese, muffins and biscuits were not provided, but a fruit pie (Limburgse vlaai), cakes and biscuits prepared by a local baker using the experimental margarines were given. All oils and food items were color coded (yellow *trans* free, orange and green for the high and low *trans*, respectively, to maintain the double-blind nature of the study).

Table 7.2 Fatty acid composition of experimental oil and margarine fat blends

	Oil			Margarine		
Individual FA	*Trans* free	Low *trans*	High *trans*	*Trans* free	Low *trans*	High *trans*
C16:0	3.4	3.5	3.5	8.5	8.5	8.5
C18:0	1.7	1.8	1.7	8.1	8.1	8.1
C18:1	59.0	58.6	59.2	45.7	45.5	46.1
C18:2 *cis*	21.5	21.6	21.1	16.5	16.8	16.4
C18:2 *trans*	-	0.5	0.5	-	0.4 [d]	0.4
C18:3 *cis* [a]	12.6	12.4	7.8	9.4	9.4	6.0
C18:3 *trans*	0.1	0.1	4.5	0.1	0.1	3.4
Σ Saturated	5.9	6.2	6.0	27.7 [c]	27.2	27.2
Σ Monounsaturated [b]	59.6	59.2	59.8	46.1	46.0	46.6
Σ Polyunsaturated [b]	34.1	34.1	29.0	25.9	26.2	22.4
Σ *trans*	0.1	0.6	5.1	0.2	0.6	3.9

[a] Including C20:1 in margarines

[b] *cis* only

[c] The amount of C6:0 to C14:0 in margarines ranged from 9.7-10.2% of fatty acids and did not differ between the 3 types

[d] *Trans* linoleic acid added to same level as in high *trans* oil or margarine

Laboratory methods

The principle of centralized laboratory analyses was adopted to avoid systematic bias in results from the three countries, except that the platelet aggregation studies were carried out immediately after blood collection using a standardized method. Determination of plasma total, LDL and HDL cholesterol, and triacylglycerols will be described elsewhere. Fatty acid composition of plasma lipids was determined in Dijon. Plasma lipids were extracted according to Moilanen & Nikkari (28) and were separated by thin layer chromatography on precoated silica gel plates (SDS, Peypin, France). Phospholipids (remaining at the origin), triacylglycerols (Rf value 0.35) and cholesteryl esters (Rf 0.45) were scraped off the plates and the fatty acid methyl esters obtained by *trans*-methylation according to Morrison & Smith (29). Methyl

esters were prepared by direct *trans*-methylation of extracted lipids from the oil, margarine, cheese and biscuit used for the study. The methyl esters were then analyzed by capillary gas chromatography using Hewlett Packard 6890 GC with an automatic injector, a BPX 70 column (50 m x 0.25 mm internal diameter, SGE, Melbourne, Australia) and a flame ionization detector. The error in the determination of fatty acid methyl esters depends on the relative amount of a particular component in the sample. Thus for peaks representing >1% of fatty acids, the coefficient of variation is less then 10%. For smaller peaks of 0.2 or <0.1% of fatty acids these errors are in the order of 40 and 100% respectively. Fractionation of the lipid fractions by high pressure liquid chromatography could have reduced the errors, but this was not possible for this project due to the large number of samples.

Dietary analyses

The fatty acid composition of the diet (weighed record) was calculated separately for the three centers, using country specific food tables and the composition of the experimental fats, oils and foods. The dietary record was made over 4 days including at least 1 weekend day. In Edinburgh Comp-Eat4 (Nutrition Systems, London, UK) was used and the fatty acid composition including *trans* was obtained from manufacturers, existing food tables (Foodbase, Institute of Brain Chemistry and Human Nutrition, London, UK) and laboratory analyses. However, there are still a number of foods with missing and/or doubtful *trans* fatty acid data. The intake of fat from these represents some 10 to 15% of total fat consumption. In Maastricht and Clermont-Ferrand the food composition was calculated in a similar manner using the Dutch Food Composition Table (31) and the Répertoire Général des Aliments in France (32), respectively. The energy intake of *trans* fatty acids could not be calculated as these two food tables do not list *trans* fatty acid contents. The intake of *trans* α-linolenic acid is, however, based on the analyses of the fatty acid composition of the experimental foods.

Protocol

After recruitment and analysis of their habitual diet by a 4-day weighed record, the men attended the clinic each week for the duration of the study (12 weeks) to collect food items and also to be weighed without shoes and heavy clothes. If necessary, energy intake was adjusted to maintain stable body weight. On the first visit, the men attended in the fasting state (from at least 10:00 p.m.), blood pressure (after 5 min rest) was recorded before the collection of blood samples (EDTA) for the analysis of plasma lipids and lipoproteins. A large needle (1.2 mm diameter; Strausz cannula, Süddeutsche Feinmechanik, D6480 Wächtersbach, Germany) was used without stasis and the first 5 ml blood discarded. In addition, a large volume of citrated blood (100 ml) was collected for the isolation of platelets. Aliquots were stored for the analysis of fatty acid composition of plasma and platelets. All men then were instructed how to avoid foods containing *trans* fatty acids and consumed foods

prepared with *trans* free rapeseed oil or margarine for a period of 6 weeks (run-in). Blood samples for lipids were collected again at weeks 3, 5 and 6. A second large blood sample was collected to isolate platelets at week 6 (figure 7.1). Blood pressure measurement was repeated and a second 4-day weighed dietary record was made during week 6.

The men stratified by age, BMI and smoking in the three cities, were then randomly allocated to either a low *trans* or high *trans* diet by the Edinburgh center. The test period lasted 6 weeks and all measurements and blood sampling after overnight fast were repeated at identical intervals as described for the run-in period. During week 12 a third and final 4-day weighed dietary record was made (figure 7.1).

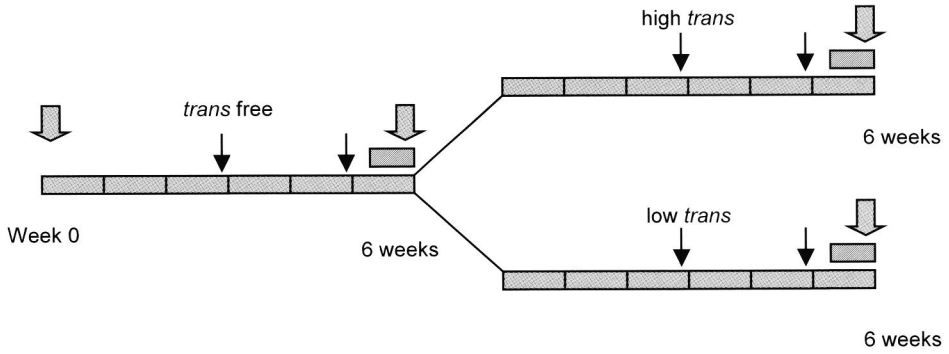

Figure 7.1 The schematic diagram of the protocol of the human feeding study.
The double-blind feeding study comprised two successive periods of 6 weeks. After the first run-in period (0-6 weeks), when all subjects consumed a *trans* free diet, the volunteers were randomly switched to a low or a high *trans* α-linolenic acid diet (0.6% of energy) for the second 6 week period. Small arrows denote fasting blood samples for the measurement of lipids (and haemostatic factors), whilst big arrows denote a larger blood sample that allowed also platelet fatty acid composition (and function) to be determined. Hatched bar indicates a 4-day weighed dietary record in that week.

Statistical methods

Differences between centers were tested by chi-squared or Mann-Whitney tests and differences between the randomized treatment groups after the test period were tested by analysis of covariance adjusting for the level of the corresponding measurement at the end of the first 6 weeks on a *trans* free diet (run-in period).

Table 7.3 Selected fatty acids (wt%) in plasma triacylglycerols, phospholipids, cholesteryl esters and platelets of subjects from Clermont-Ferrand, Edinburgh, and Maastricht at baseline. Values are means ± sd

	CF (n=28)	E (n=25)	M (n=29)	Significance		
				CF vs E	CF vs M	E vs M
Triacylglycerols						
C18:1 *trans*	1.17 ± 0.64	1.71 ± 0.82	1.52 ± 0.81	**		
C18:2 *cis*	19.3 ± 6.71	18.4 ± 4.86	20.9 ± 4.78			
C18:2 *trans*	0.64 ± 0.21	0.51 ± 0.18	0.89 ± 0.32		***	***
C18:3 *cis*	0.70 ± 0.40	1.02 ± 0.39	1.06 ± 0.32	**	***	
C18:3 *trans*	0.10 ± 0.04	0.16 ± 0.12	0.18 ± 0.09		***	
∑ Saturated	30.4 ± 4.48	30.7 ± 4.83	27.2 ± 4.26		**	*
∑ Monounsaturated	42.3 ± 4.00	42.8 ± 4.08	42.9 ± 4.26			
∑ n-3	2.32 ± 0.72	2.56 ± 1.22	2.61 ± 0.90			
∑ n-6	22.8 ± 6.97	21.1 ± 6.06	24.5 ± 5.21			**
∑ *trans*	1.91 ± 0.77	2.41 ± 0.82	2.58 ± 1.01	*	**	
Phospholipids						
C18:1 *trans*	0.51 ± 0.28	0.80 ± 0.44	0.80 ± 0.36	**	**	
C18:2 *cis*	21.7 ± 2.84	23.7 ± 3.02	24.0 ± 2.68		**	
C18:2 *trans*	0.22 ± 0.07	0.26 ± 0.10	0.27 ± 0.08			
C18:3 *cis*	0.14 ± 0.06	0.21 ± 0.11	0.20 ± 0.06	**	***	
C18:3 *trans*	0.02 ± 0.02	0.06 ± 0.03	0.04 ± 0.04	***		
∑ Saturated	42.3 ± 1.16	41.6 ± 1.31	41.7 ± 0.99			
∑ Monounsaturated	12.7 ± 1.64	12.9 ± 1.58	2.4 ± 1.48			
∑ n-3	5.81 ± 1.47	5.71 ± 1.64	5.48 ± 1.06			
∑ n-6	34.5 ± 2.13	37.6 ± 2.67	38.6 ± 1.65		***	
∑ *trans*	0.76 ± 0.33	1.13 ± 0.52	1.11 ± 0.41	**	**	
Cholesteryl esters						
C18:1 *trans*	0.15 ± 0.09	0.21 ± 0.11	0.39 ± 0.14		**	
C18:2 *cis*	53.6 ± 4.06	54.9 ± 7.29	56.0 ± 3.73			
C18:2 *trans*	0.25 ± 0.06	0.29 ± 0.13	0.43 ± 0.17		***	**
C18:3 *cis*	0.41 ± 0.10	0.52 ± 0.17	0.55 ± 0.13		***	
C18:3 *trans*	0.08 ± 0.04	0.11 ± 0.05	0.15 ± 0.07	**	***	
∑ Saturated	12.8 ± 1.22	12.9 ± 2.46	11.8 ± 1.38		**	
∑ Monounsaturated	22.0 ± 2.69	21.3 ± 4.43	20.4 ± 2.51			
∑ n-3	1.91 ± 0.74	2.00 ± 0.89	1.91 ± 0.59			
∑ n-6	62.1 ± 3.74	62.7 ± 7.06	64.4 ± 3.14			
∑ *trans*	0.48 ± 0.13	0.62 ± 0.23	0.82 ± 0.32	*	***	
Platelets						
C18:1 *trans*	0.76 ± 0.25	1.25 ± 0.58	1.15 ± 0.69	***	***	
C18:2 *cis*	5.87 ± 0.92	6.79 ± 1.23	8.12 ± 2.08	**	***	
C18:2 *trans*	0.29 ± 0.14	0.30 ± 0.23	0.62 ± 0.38		***	**
C18:3 *cis*	0.02 ± 0.02	0.03 ± 0.03	0.10 ± 0.06		***	***
C18:3 *trans*	0.00 ± 0.00	0.02 ± 0.06	0.00 ± 0.00			
C20:4 *cis*	22.1 ± 2.65	23.1 ± 2.24	19.6 ± 4.01			***
C20:5 *cis*	0.34 ± 0.16	0.37 ± 0.29	0.40 ± 0.13			
C20:5 *trans*	0.03 ± 0.03	0.00 ± 0.00	0.03 ± 0.04	***		***
∑ Saturated	42.8 ± 4.17	39.8 ± 2.75	42.5 ± 3.80	**	***	**
∑ Monounsaturated	16.2 ± 1.05	18.4 ± 1.58	18.0 ± 1.91	***	***	
∑ n-3	32.1 ± 3.49	34.4 ± 2.97	32.0 ± 3.35			*
∑ n-6	3.93 ± 1.12	4.43 ± 1.10	3.56 ± 1.17			
∑ *trans*	1.09 ± 0.32	1.57 ± 0.64	1.80 ± 0.82	**		

* P<0.017, ** P<0.01, *** P<0.001. CF, Clermont-Ferrand; E, Edinburgh; M, Maastricht

RESULTS

Plasma and platelet fatty acid composition during habitual diet

Trans isomers of individual fatty acids and the total amount of *trans* fatty acids in plasma lipid fractions differed between the three countries. The relative amounts of *trans* α-linolenic acid in plasma triacylglycerols was higher in Maastricht and in phospholipids was higher in Edinburgh than in Clermont-Ferrand. Values between Edinburgh and Maastricht were not significant. The relative amounts of *cis* α-linolenic acid in plasma lipids and platelets showed similar trends: similar values in Dutch and Scottish and lower levels in the French volunteers (table 7.3). Only traces of *trans* α-linolenic acid were found in platelet membranes. Despite this, slightly higher amounts of *trans* EPA were observed in platelet membranes of Dutch and French volunteers (table 7.3). The levels of total *trans* fatty acids were higher in plasma triacylglycerols than in cholesteryl esters or phospholipids. As expected the major *trans* fatty acids in plasma triacylglycerols, phospholipids and platelets were *trans* monounsaturated fatty acids (*trans* C18:1). Only in the cholesteryl ester fraction were the relative amounts of *trans* isomers from linoleic and α-linolenic acid higher or similar to those of *trans* C18:1.

Nutrient intake in the intervention trial

First careful, low temperature deodorization of a canola oil containing some 12.4% α-linolenic acid did not result in *trans* α-linolenic acid (~ 0.1% of fatty acids). Almost 40% of α-linolenic acid was isomerized during the production of the high *trans* canola oil.

Eighty-eight volunteers completed the dietary intervention trial. At the end of the run-in period, when all the volunteers had been consuming a *trans* free diet for 6 weeks, the nutrient intake did not differ between the two groups assigned to the low or high *trans* diet for the next 6 weeks. The average intake of fat was in the order of 35% of energy and contained little *trans* fatty acids (table 7.4). After 6 weeks on the high *trans* diet, the intake of *trans* α-linolenic acid increased some 20 fold, but did not change in the group consuming the low *trans* diet (table 7.4). These changes in *trans* α-linolenic acid intake did not differ significantly between the three centers (not shown). The intake of fat, particularly of saturated and monounsaturated fat increased slightly in the group on the low *trans* diet, whereas there was the opposite trend in the high *trans* group. The difference was significant for the total, saturated and monounsaturated fat intake. The intake of energy and other nutrients did not differ between the two dietary groups.

Table 7.4 Mean daily energy and nutrient intakes at the end of the run-in period and test period with a low or high *trans* diet. Values are means ± sd.

	Low *trans* (n=44)	High *trans* (n=44)	Significance[a]
Energy (MJ/day)			
run-in	11.1 ± 2.5	11.0 ± 1.8	
test period	11.1 ± 2.8	10.7 ± 1.9	
change	0.0 ± 2.2	-0.3 ± 1.8	
Protein (En%)			
run-in	15.7 ± 3.2	15.1 ± 2.7	
test period	15.2 ± 3.2	15.4 ± 3.3	
change	-0.5 ± 2.6	0.3 ± 2.3	
Fat (En%)			
run-in	32.4 ± 6.3	35.3 ± 5.9	
test period	34.5 ± 6.9	34.1 ± 6.1	
change	2.1 ± 5.7	-1.1 ± 5.4	*
SAFA			
run-in	8.6 ± 3.4	9.9 ± 3.3	
test period	9.6 ± 3.9	9.4 ± 3.3	
change	1.1 ± 2.2	-0.5 ± 1.9	**
MUFA			
run-in	14.1 ± 2.8	15.1 ± 2.5	
test period	15.2 ± 3.0	14.5 ± 2.7	
change	1.1 ± 2.7	-0.5 ± 2.3	*
PU FA			
run-in	7.1 ± 1.4	7.7 ± 1.8	
test period	7.2 ± 1.8	7.4 ± 1.6	
change	0.1 ± 1.9	-0.3 ± 1.5	
Trans C18:1 (mg)[b]			
run-in	364 ± 252	496 ± 484	
test period	269 ± 161	366 ± 213	
change	-95 ± 322	-130 ± 365	
Trans C18:3n-3 (mg)[b]			
run-in	49 ± 53	66 ± 102	
test period	60 ± 75	1410 ± 427	
change	10 ± 67	1344 ± 428	***
Carbohydrates (En%)			
run-in	49.1 ± 6.9	45.8 ± 6.9	
test period	47.4 ± 6.8	46.7 ± 6.6	
change	-1.7 ± 6.3	0.9 ± 5.6	
Alcohol (En%)			
run-in	2.7 ± 4.1	3.7 ± 5.5	
test period	2.8 ± 3.1	3.7 ± 5.1	
change	0.1 ± 4.0	-0.1 ± 2.6	
Cholesterol (mg/MJ)			
run-in	24.8 ± 14.2	25.2 ± 14.9	
test period	27.5 ± 16.1	25.4 ± 11.4	
change	2.7 ± 13.7	0.2 ± 12.1	
Dietary fiber (g/MJ)			
run-in	2.0 ± 0.5	1.9 ± 0.6	
test period	1.9 ± 0.5	2.0 ± 0.6	
change	-0.1 ± 0.5	0.1 ± 0.4	

[a] Low vs high *trans* *P<0.05, **P<0.01, ***P<0.001

[b] *Trans* C18:1 fatty acid and *trans* α-linolenic acid intakes of Clermont-Ferrand and Maastricht are only from the experimental products because the French and Dutch food composition tables do not contain values for *trans* fatty acids

Mean body weight of the volunteers decreased slightly in the group consuming the low *trans* diet, whereas it increased in the high *trans* group: -0.4 ± 1.2 vs 0.2 ± 1.1 kg. The difference between the two dietary groups was significant ($P<0.05$) after adjustment for the initial body weight (at the end of the run-in period). Changes in body weight of volunteers consuming the low or high *trans* diet differed between the three centers ($P<0.01$). In Clermont-Ferrand both dietary groups tended to lose weight, whereas in Edinburgh and Maastricht there was a tendency to lose weight on the low and to gain weight on the high *trans* diet (not shown). The mean systolic and diastolic pressures varied between 120 and 124 mm Hg, and 70 and 73 mm Hg, respectively, without any significant difference before and during the 6-week experimental period.

Table 7.5 Change in n-3 fatty acid composition (wt%) of plasma triacylglycerols, phospholipids and cholesteryl esters in healthy volunteers consuming a low *trans* or a high *trans* α-linolenic acid diet for 6 weeks. Values are means ± sd.

	Low *trans* (n=44)	High *trans* (n=44)	Significance[a]
Triacylglycerols			
C18:3 *cis* [b]	-0.29 ± 1.00	-0.33 ± 0.84	
C18:3 *trans*	-0.02 ± 0.09	0.37 ± 0.28	***
C20:5 *cis*	-0.02 ± 0.25	-0.03 ± 0.36	
C20:5 *trans*	-0.01 ± 0.03	0.03 ± 0.05	*
Phospholipids			
C18:3 *cis*	0.06 ± 0.14	-0.03 ± 0.12	*
C18:3 *trans*	0.00 ± 0.02	0.14 ± 0.10	***
C20:5 *cis*	0.08 ± 0.64	-0.14 ± 0.64	
C20:5 *trans*	0.00 ± 0.01	0.07 ± 0.04	***
Cholesteryl esters			
C18:3 *cis*	-0.02 ± 0.25	-0.24 ± 0.22	***
C18:3 *trans*	0.00 ± 0.07	0.26 ± 0.20	***
C20:5 *cis*	0.03 ± 0.66	-0.12 ± 0.44	
C20:5 *trans*	0.00 ± 0.01	0.02 ± 0.04	***

[a] Low vs high *trans* *P<0.05, **P<0.01, ***P<0.001

[b] *Cis* isomers only

Changes in fatty acid composition

The changes in the *cis* and *trans* n-3 fatty acid composition were as expected. The relative amount of *trans* α-linolenic acid increased in plasma triacylglycerol, phospholipid and cholesteryl ester fractions of volunteers consuming the high *trans* diet (table 7.5). *Trans* α-linolenic acid comprised one third of the total amount of α-linolenic acid in plasma phospholipids and cholesteryl esters. Interestingly the only

trans isomer of α-linolenic acid that was incorporated in the cholesteryl ester fraction was the Δ9*c*,12*c*,15*tr*-form (figure 7.2). At the same time the percentage of *cis* α-linolenic acid decreased in these fractions, except in plasma triacylglycerol fraction. The n-3 fatty acid composition was stable in the group consuming the low *trans* diet and the difference between the low and high *trans* groups was significant. There was an increase in the small amount of *trans* EPA in plasma triacylglycerols, phospholipids and cholesteryl esters, but without a change in the amount of *cis* EPA. The relative amounts of *trans* isomers of oleic and linoleic acid in these fractions remained stable during the experimental dietary periods (not shown).

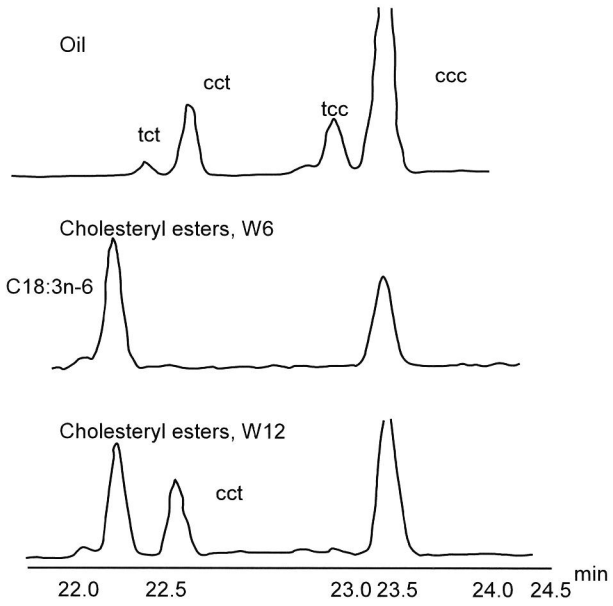

Figure 7.2 The capillary gas chromatogram demonstrating the separation of the different *trans* isomers of α-linolenic acid as the methyl esters in a sample of the high *trans* oil (top panel), and of the plasma cholesteryl ester fraction of the same male volunteer at the end of a period of 6 weeks on the *trans* free diet (run-in period; middle panel) and at the end of a 6 week period on the high *trans* fat diet (bottom panel). Note the absence of the Δ9*tr*,12*c*,15*c*- isomer of α-linolenic acid in the cholesteryl ester fraction.
The identifications *tct*, *cct*, *tcc* refer to Δ9*tr*,12*c*,15*tr*-, Δ9*c*,12*c*,15*tr*- and Δ9*tr*,12*c*,15*c*-isomers of α-linolenic acid (*ccc)* respectively. γ-Linolenic methyl ester peak in the cholesteryl ester fraction is labeled with C18:3n-6.

As expected the relative amount of *trans* isomers of C18:1 in plasma triacylglycerol and cholesteryl esters in the volunteers consuming the *trans* fat free

diet for 6 weeks decreased from 1.37 ± 0.08 to 0.97 ± 0.09 and from 0.25 ± 0.05 to 0.14 ± 0.02% of fatty acids, respectively (both $P<0.05$). However the level did not decline further in the group continuing with the low *trans* diet for 6 weeks with values of 0.91 ± 0.09 and 0.11 ± 0.01% of fatty acids respectively, nor did the levels decline in the group switched to the high *trans* diet (not shown). What is perhaps surprising is that levels of these *trans* isomers in plasma phospholipids never changed at all: 0.69 ± 0.04 (control), 0.63 ± 0.04 (6 weeks *trans* free) and 0.59 ± 0.04% of fatty acids (*trans* free followed by 6 weeks low *trans*).

The high *trans* diet did not influence the relative amounts of n-6 polyunsaturated fatty acids linoleic, γ-linolenic, dihomo-γ-linolenic and arachidonic acid (not shown).

DISCUSSION

Trans *fatty acids at baseline*

Our results confirm earlier observations (6,25) that the relative amount of *trans* fatty acids in plasma lipids, adipose tissue or in the diet differ between European populations. Our French volunteers had lower *trans* C18:1 levels than their Dutch or Scottish counterparts and almost certainly this reflects the higher intake of *trans* fats and of hydrogenated oils in Northern Europe than in the Southern countries (21,25). The same geographical trend across Europe is not observed for the *trans* isomers of linoleic and α-linolenic acid. *Trans* isomers of these fatty acids are formed during the deodorization of linoleic and α-linolenic acid-rich vegetable oils at high temperatures (27,33). It is known that there are large variations in the relative amount of α-linolenic acid isomerized in vegetable oils (21,24). In fact one may think that the quantity of these *trans* polyunsaturated fatty acids in biological samples may reflect primarily the quality, rather than the quantity of the ingested refined oil and ultimately they reflect the conditions under which the oil was refined. Thus if well-deodorized, the intake of *trans* α-linolenic acid could be negligible. What this project did show is that it is indeed possible to obtain well-refined oils rich in α-linolenic acid with good organoleptic properties and yet low in *trans* polyunsaturated fatty acids.

The information about *trans* α-linolenic acid in foods is virtually non-existent. However, the average levels of *trans* α-linolenic acid in plasma lipids at baseline when compared with the increase during the experimental period when given 1.4 g/d should indicate the relative amount consumed habitually. The estimates for the Dutch and Scottish subjects average some 0.6, 0.7 and 0.5 g/d using the fatty acid composition of plasma triacylglycerol, cholesteryl ester and phospholipid data, respectively. For the French subjects the *trans* α-linolenic acid intake is estimated to be in the order of 0.2 to 0.4 g/d. However, these approximations depend on small concentrations of *trans* α-linolenic acid, which are determined with relatively large analytical errors. We also assume a linear relation between intake and incorporation

into plasma lipid fractions. This is not entirely the case, as certain *trans* α-linolenic acid isomers are not incorporated into the cholesterol ester fraction (see figure 7.2). Nevertheless the similarity in estimates based on plasma triacylglycerol, cholesterol ester and phospholipid composition suggests that they are a reasonable first estimate.

One word of caution is necessary. Our volunteers were recruited from a panel of volunteers in Clermont-Ferrand and from an advertising campaign in Maastricht and in Edinburgh. Obviously they were self-selected and do not represent a random population sample. Therefore, the levels of *trans* fatty acids in these volunteers do not necessarily reflect those of the French, Dutch and Scottish populations with great accuracy. Nevertheless the results are in line with those of the Transfair project which specifically examined dietary intake of *trans* fatty acids in European populations by analyzing the *trans* fatty acid content in up to 100 food samples representative of the total fat intake of the 14 countries (34).

Effects of a high and low trans *fat diet*

All volunteers consumed a diet low in *trans* fatty acids for 6 weeks to decrease the relative amounts of *trans* fatty acids in plasma lipids (and readily exchangeable tissue pools). Obviously starting from a low baseline would make it easier to demonstrate any putative effect of *trans* α-linolenic acid. The question is whether our run-in period was long enough if we aimed to reduce all *trans* fatty acids, in particular *trans* C18:1. Interestingly the reduced level of *trans* C18:1 in plasma triacylglycerol and cholesteryl esters did not fall significantly further in the group that continued with the low *trans* diet for another 6 weeks and the levels of these C18 *trans* isomers in phospholipids were not influenced by the low *trans* diet at all. Perhaps some of these *trans* isomers originated from adipose tissue. Another source might be biscuits (prepared with partially hydrogenated oils) consumed by the subjects. It is possible that despite our careful instructions our volunteers did continue to consume, perhaps unwittingly *trans* fatty acids. Indeed our dietary analyses in Edinburgh men where the intake of *trans* C18:1 could be assessed support the view that this may have been so and only in a study conducted under strict metabolic ward conditions would one be able to obtain perfect compliance. It must be remembered that some partners of these volunteers actually prepared the food. As this was expected, free food was also offered to the entire family to prevent inadvertently mixing of our experimental oils and fats with similar products kept in the household. The volunteers were actively encouraged to discard or return all other fats and oils at the start of each dietary period.

Whatever the reason for the relatively steady C18:1 *trans* levels after the 6 week run-in period, the biological effects in our study are not affected by changes in C18:1 *trans. Trans* isomers of C20:5 did also not change further in the group consuming low *trans.* Thus their production was close to a steady state.

Our dietary record also documented that the amount of *trans* α-linolenic acid consumed was on average close to what was intended. What was surprising was that

the group consuming a low *trans* diet tended to increase the intake of dietary fat, whereas the opposite was true for the high *trans* group. The difference was significant and can affect the outcome of our study. It is well known that a saturated fat diet increases total cholesterol and a fatty diet can raise high density cholesterol (35,36). However statistical analysis of covariance can easily adjust for this unexplained finding.

An important aspect is the dose of *trans* α-linolenic acid we used. Some 40% of the total dietary α-linolenic acid was in the *trans* form, close to highest values observed in human milk samples from a large Canadian study (19). On that basis it may appear that our study tested rather an extreme dietary amount of *trans* α-linolenic acid. However, the amount is still within the range that can be observed in the free-living population. The level in human milk samples could be misleading if there is a marked discrimination against *trans* α-linolenic acid during the absorption and incorporation into milk and perhaps also into lipids.

Our data already shows that after 6 weeks on a diet rich in *trans* α-linolenic acid isomers, there was an increase in these *trans* isomers as well as that of *trans* EPA in all plasma lipid classes. These results confirm that the conversion of Δ15*trans* α-linolenic to a *trans* isomer of EPA is not restricted to the rat (12). Most interestingly, while the different *trans* isomers were incorporated into plasma triacylglycerol and phospholipids, only the Δ15*trans* α-linolenic acid was detected in plasma cholesteryl esters. A similar preferential incorporation of this *trans* isomer into cardiolipin, a phospholipid rich in linoleic acid, suggests that this *trans* isomer containing three carbon-carbon double bonds, is recognized as linoleic acid (37). Preferential incorporation of Δ15*trans* α-linolenic acid into plasma cholesteryl esters could be due to a higher affinity of lecithin cholesterol acyl transferase (LCAT) for this than for the other *trans* isomers. It is well known that this enzyme selectively transfers linoleic acid from lecithin to cholesterol (38). The fatty acid composition of plasma cholesteryl esters and/or phospholipids are used as a marker of compliance of fatty acid intake (39-43). Our data suggest that caution should be taken because of the selectivity of LCAT. Interestingly, these *trans* isomers do not appear to be incorporated into plasma lipids at the expense of α-linolenic acid, illustrated by the fact that the total content of α-linolenic acid (*cis*+*trans*) remains constant irrespective of whether volunteers were consuming a low or high *trans* diet. Also the amount of *trans* monounsaturated fatty acids in plasma phospholipids (unlike in triacylglycerol and cholesteryl esters) did not decline in volunteers consuming a diet low in these *trans* isomers for 12 weeks altogether.

Our results do not support the view that *trans* α-linolenic acid inhibits the activity of Δ6 desaturation of linoleic acid, since there were no changes in the composition of n-6 polyunsaturated fatty acids in plasma lipids. However, this indirect index of Δ6 desaturation has been criticized previously. The conversion of stable isotope labeled linoleic acid in volunteers consuming alternatively a diet low and high in *trans* α-linolenic acid will be examined.

In conclusion, *trans* isomers are absorbed from the diet, incorporated in plasma lipids and some *trans* isomers are converted to long chain polyunsaturated fatty acids.

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8

Dietary *trans* α-linolenic acid from deodorized rapeseed oil and plasma lipids and lipoproteins in healthy men: the *Trans*LinE Study

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ABSTRACT

Trans isomers of α-linolenic acid, which are formed by deodorization of refined vegetable oils, can be found in significant amounts in edible oils. Effects of *trans* α-linolenic acid on plasma lipoproteins are unknown. We, therefore, investigated the effects of *trans* α-linolenic acid on plasma lipids and lipoproteins in healthy European men.

Eighty-eight healthy men from three European countries (France, Scotland and the Netherlands) first consumed for 6 weeks a diet with experimental oils 'free' of *trans* fatty acids (run-in period). For the next 6 weeks, they were randomly allocated to a diet with experimental oils 'high' or 'low' in *trans* α-linolenic acid. Daily total *trans* α-linolenic acid intake in the high *trans* group was 1410 (Range 583-2642) mg. Experimental oils were provided as such, or incorporated into margarines, cheeses, muffins and biscuits.

The high *trans* α-linolenic acid diet significantly increased the plasma low density lipoprotein (LDL) to high density lipoprotein (HDL) cholesterol ratio by 8.1% (95% CI 1.4, 15.3, P=0.02), and the total to HDL cholesterol ratio by 5.1% (95% CI 0.4, 9.9, P=0.03), compared to the low *trans* diet. This was largely explained by an increase in LDL cholesterol on the high *trans* diet, while no change was observed in the low *trans* group (mean treatment effect of 4.7% (95% CI -0.8, 10.5), P=0.10). No effects were found on total and HDL cholesterol, triacylglycerols, lipoprotein B and A-1, and lipoprotein(a) concentrations.

In conclusion, *trans* α-linolenic acid may increase plasma LDL to HDL cholesterol and total to HDL cholesterol ratios. Whether diet-induced changes in these ratios truly affects the risk for coronary heart disease remains to be established.

INTRODUCTION

Trans monounsaturated fatty acids from industrially hydrogenated oils increase cholesterol concentrations in the atherogenic serum low density lipoproteins (LDL) and decrease those of the anti-atherogenic high density lipoproteins (HDL). Therefore, a reduction in the intake of hydrogenated oils has been advocated (1). However, not only *trans* monounsaturated fatty acids, which represent most of the intake of *trans* fatty acids, are found in processed fats. *Trans* isomers of polyunsaturated fatty acids are also formed during oil processing (2). In particular, α-linolenic acid is easily converted into *trans* α-linolenic acid.

Soybean oil and canola oil (low erucic acid rapeseed oil) are important sources of α-linolenic acid (3) and *trans* isomers may represent up to 40% of α-linolenic acid (2,4-6). There is an increasing recognition that the intake of n-3 fatty acids (such as α-linolenic acid and fish oil fatty acids) should increase. The growing consumption of n-3 fatty acid containing vegetable oils over the last few years (7) may therefore result in an increased intake of *trans* α-linolenic acid from refined oils. Although such *trans* n-3 polyunsaturated fatty acids are absorbed and incorporated into tissue lipids by man (8-10), their health effects have hardly been investigated. Therefore, we have studied the effects of a high *trans* α-linolenic acid diet vs a low *trans* α-linolenic acid diet on plasma lipid and lipoprotein concentrations in healthy men.

SUBJECTS AND METHODS

Experimental design

The study was designed as a controlled, parallel intervention trial, which was carried out under standardized conditions in three different centers: 1) Université d'Auvergne, Laboratoire de Nutrition Humaine, Clermont-Ferrand, France, 2) Cardiovascular Research Unit, University of Edinburgh, Scotland, and 3) Department of Human Biology, Maastricht University, the Netherlands. The study was a part of the *Trans*LinE project (*Trans* α-Linolenic acid in Europe), in which the health impact of *trans* polyunsaturated fatty acids in European populations was examined.

The experiment consisted of two consecutive periods of 6 weeks. During the first period (run-in), all subjects received experimental products made of oil free of *trans* isomers of oleic, linoleic and α-linolenic acid (<0.1% *trans* fatty acids) (*trans* 'free' diet). Thereafter, subjects were randomly allocated to a 'high' or 'low' *trans* diet. For the next 6 weeks, subjects in the high *trans* group received on average 1.4 gram *trans* α-linolenic acid per day (assessed by food diaries), which was provided by an oil containing 4.5 g/100 g product *trans* α-linolenic acid and a margarine containing 3.4 g/100 g product *trans* α-linolenic acid. Also, foods prepared from the oil or margarine were provided (cheese, muffins, pies and biscuits). Identical experimental

products free of *trans* α-linolenic acid were given to the low *trans* group. The high *trans* α-linolenic acid products had a reduced all-*cis* α-linolenic acid content, but in all other aspects the high and low *trans* products did not differ (11). During the preparation of the *trans* α-linolenic acid rich oil, formation of *trans* linoleic acid could not be avoided (12). Therefore, for the low *trans* group a *trans*-free rapeseed oil was mixed with an isomerized sunflower oil (50:1 v/v), in order to compensate for the unavoidable formation of 0.5% *trans* isomers of linoleic acid during deodorization of the rapeseed oil for the high *trans* group. All products were color coded so as to blind the subjects.

For the duration of the study, subjects were advised to avoid consuming *trans* fatty acids: intake of ruminant meat, cheese (except for goat cheese, which is low in *trans*), and foods containing hydrogenated fats were avoided. The main part of the *trans* fatty acid intake was, however, reduced by replacing subjects usual oil and margarine by experimental products. Before and during week 5 and week 11, subjects recorded their food intake for 4 days, of which at least 1 was in the weekend, to allow us to estimate their energy and nutrient intakes. Energy and nutrient composition of the diets were calculated separately for the three centers using country specific food tables and the composition of the experimental fats, oils and products. Unfortunately, no reliable information on the *trans* fatty acid content of French and Dutch products was available. Therefore, it was not possible to estimate the amount of *trans* fatty acid in the background diet of subjects from Clermont-Ferrand and Maastricht. *Trans* C18:1 intake of the Scottish subjects was about 400 mg (11). Overall, 30% of the fat of the habitual diet was replaced by foods prepared with the experimental fats and oils. Subjects visited the dietician of the department once every week to receive a new supply of products and to measure their body weights.

Subjects recorded in diaries any signs of illness, medication used and any deviations from the protocol. In addition, except for avoid consuming *trans* fatty acids, subjects were urged not to change their background diets, level of physical exercise, smoking habits, or use of alcohol during the study. The protocol and the aim of the study were fully explained to the subjects, who gave their written informed consent. The study had been approved by the local Medical Ethical Committees.

Subjects

A total of 91 men, recruited from a panel of volunteers (Clermont-Ferrand, n=32), university staff and students (Edinburgh, n=28) and from the general population (Maastricht, n=31), entered the study. Volunteers were aged between 18 and 55 years, non-obese, clinically healthy, normotensive and normolipidaemic. During the study, one subject from Clermont-Ferrand dropped out because of use of a deviation from the protocol interfering with another aspect of this study, and two subjects from Edinburgh because of the constraints of the study. A detailed description of the study design has been reported elsewhere (11).

Blood sampling and analyses

Free-flowing blood was sampled after an overnight fast at weeks 0, 3, 5, 6, 9, 11 and 12 for lipid and lipoprotein analyses, and at weeks 0, 6 and 12 for measurement of fatty acid composition of plasma cholesteryl esters. Additional measurements will be reported elsewhere. In each center, all punctures were performed by the same technician, at the same location, and as much as possible at the same time of the same day of the week. All blood samples were taken before 11 a.m.

Plasma was obtained by centrifugation at 2000 × g for 30 minutes at 4°C and stored at -80°C. Plasma total and HDL cholesterol (CHOD-PAP method; Monotest cholesterol, Roche, Germany) and triacylglycerols (GPO-Trinder; Sigma Diagnostics, St Louis, USA) were analyzed enzymatically. LDL cholesterol was calculated with the Friedewald equation (13). Plasma apolipoprotein A-1 (apoA-1) and apolipoprotein B (apoB) were measured using an immunoturbidimetric reaction (UNI-KIT ApoA-1 and UNI-KIT ApoB, Roche, Switzerland) with antiserum raised in sheep and rabbits, respectively. Lipoprotein(a) (Lp(a)) was measured using a kit from Biopool (Biopool, TintElize; Umeå, Sweden). The technicians were blinded to treatment status. All lipid and lipoprotein analyses were centralized in Maastricht in order to eliminate any possible systematic analytical bias between centers. All samples from one subject were analyzed within one run, while each run contained samples from each center.

To monitor dietary compliance, the fatty acid composition of plasma cholesteryl esters was determined gas chromatographically by the INRA (Dijon, France) as reported elsewhere (11)

Statistical analyses

Analysis of covariance was used using the Statistical Package for the Social Sciences (SPSS; SPSS Inc., Chicago, IL, USA) to examine the effect of a high *trans* diet versus a low *trans* diet (treatment effect) on the week 11 + 12 means, adjusted for the week 5 + 6 means, and to check whether results were consistent across the three centers. Because the distributions of triacylglycerols, Lp(a), LDL to HDL cholesterol ratio, total to HDL cholesterol ratio and apoB to apoA-1 ratio were skewed, a logarithmic transformation was applied to these measurements before analysis. Treatment effects were then expressed as percent changes and their 95% confidence intervals (95% CI) by taking antilogs. For the sake of uniformity, a similar procedure was used for the other lipid related variables. All P-values are two-tailed, and differences were considered statistically significant when $P<0.05$.

RESULTS

The intake of *trans* α-linolenic acid, as estimated by food records, increased from run-in levels of 66 mg (Range 19-585) (0% of energy (Range 0-0.2)) to 1410 mg (Range 583-2642) (0.5% of energy (Range 0.3-0.8)) on the high *trans* diet (increase

of 1344 mg (Range 543-2612) (0.5% of energy (Range 0.2-0.8)). On the low *trans* diet, *trans* α-linolenic acid intake increased from run-in levels of 49 mg (Range 16-310) (0.01% of energy (Range 0-0.1)) to 60 mg (Range 2-404) (0% of energy (0-0.2)) (increase of 10 mg (Range -236-300) (0% of energy (-0.1-0.2)) (P<0.01). As expected, the proportion of *trans* α-linolenic acid (after adjustment for week 6 values, the mean treatment effect was 0.26 g/100 g fatty acid (95% CI 0.21, 0.32), P<0.001) in plasma cholesteryl esters increased significantly on the high *trans* diet (from 0.11 g/100 g fatty acid (Range 0-0.39) to 0.36 g/100 g fatty acid (Range 0.02-0.74)) compared to the low *trans* diet (from 0.11 g/100 g fatty acid (Range 0.03-0.28) to 0.10 g/100 g fatty acid (Range 0-0.21)) (figure 8.1). What was surprising was that the men consuming a low *trans* diet following the run-in period tended to increase the intake of dietary fat (2.1 ± 5.7% of energy) whereas the opposite was true for the men of the high *trans* group (-1.1 ± 5.4% of energy). The difference in changes nearly reached statistical significance (P=0.051). Changes in the intakes of saturated fatty acids and monounsaturated fatty acids also differed (table 8.1). The changes in protein and carbohydrate intake did not differ between the low *trans* (-0.5 ± 2.6% of energy and -1.7 ± 6.3% of energy, respectively) and the high *trans* group (0.3 ± 2.3% of energy and 0.9 ± 5.6% of energy, respectively). Further details of energy and nutrient intakes have been reported elsewhere (11).

Mean body weight of the subjects was increased by 0.2 ± 1.1 kg in the high *trans* group, which differed significantly from the decrease of 0.4 ± 1.2 kg in the low *trans* group (mean treatment effect of 0.6 ± 2.4 kg (95% CI 0.1, 1.1), P=0.03).

Changes in plasma lipids and lipoproteins were corrected for weight changes and changes in the intakes of saturated and monounsaturated fatty acids. The consumption of *trans* α-linolenic acid did not change plasma total cholesterol or triacylglycerol concentrations (table 8.2). LDL cholesterol concentrations tended to increase in the men consuming the high *trans* α-linolenic acid diet, whilst it remained unchanged in the group who consumed the low *trans* diet following the run-in period. However, the treatment effect of 4.7% failed to reach significance (P=0.10). The reverse was observed for plasma HDL cholesterol concentrations, where the concentrations tended to increase slightly in the low *trans* group, whilst there was no change in the men consuming the high *trans* α-linolenic acid diet. The treatment effect did not reach statistical significance. However, the ratios of total to HDL and LDL to HDL cholesterol increased significantly by 5.1% (P=0.03) and 8.1% (P=0.02) on the high *trans* diet compared to the low *trans* diet.

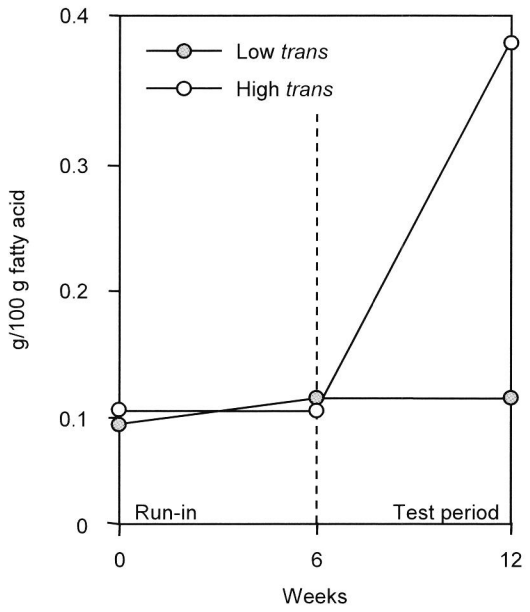

Figure 8.1 Median proportions of *trans* α-linolenic acid in plasma cholesteryl esters during the run-in period (0-6 weeks) (n=43 in both groups on t=0; n=44 in both groups on t=6) and test period of low (n=44) or high *trans* α-linolenic acid (n=42) (6-12 weeks)

Table 8.1 Mean daily energy and nutrient intake

	Low *trans* (n=44)			High *trans* (n=44)		
Fat (En%)						
run-in	32.4	±	6.3	35.3	±	5.9
change	2.1	±	5.7	-1.1	±	5.4
SAFA						
run-in	8.6	±	3.4	9.9	±	3.3
change	1.1	±	2.2	-0.5	±	1.9 **
MUFA						
run-in	14.1	±	2.8	15.1	±	2.5
change	1.1	±	2.7	-0.5	±	2.3*
PUFA						
run-in	7.1	±	1.4	7.7	±	1.8
change	0.1	±	1.9	-0.3	±	1.5

SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids.
Differences between diet groups *P<0.05, **P<0.01

Table 8.2 Plasma lipids and lipoproteins concentrations after 6 weeks of a diet low or high in *trans* α-linolenic acid (mean values and standard deviations, and 95% confidence intervals for treatment effect (high vs low *trans*))

	Low *trans* (n=44)	High *trans* (n=44)	Mean (95% CI)	P-value
Total cholesterol				
run-in (mmol/l)	4.45 ± 0.76	4.21 ± 0.80		
treatment effect (%)	1.1	2.7	1.7 (-2.2 to 5.7)	0.41
LDL cholesterol				
run-in (mmol/l)	2.79 ± 0.65	2.59 ± 0.71		
treatment effect (%)	-0.4	4.0	4.7 (-0.8 to 10.5)	0.10
HDL cholesterol				
run-in (mmol/l)	1.27 ± 0.28	1.22 ± 0.26		
treatment effect (%)	3.8	0.3	-2.7 (-6.8 to 1.6)	0.22
Triacylglycerols				
run-in (mmol/l)	0.85 ± 0.38	0.87 ± 0.44		
treatment effect (%)	-1.4	-1.6	2.6 (-9.1 to 15.8)	0.68
LDL:HDL cholesterol ratio				
run-in	2.30 ± 0.75	2.25 ± 0.82		
treatment effect (%)	-4.0	3.6	8.1 (1.4 to 15.3)	0.02
Total:HDL cholesterol ratio				
run-in	3.63 ± 0.91	3.60 ± 0.98		
treatment effect (%)	-2.6	2.3	5.1 (0.4 to 9.9)	0.03

Treatment effects are expressed as percent changes.
Treatment effects did not differ between the three centers.
Treatment effects (high versus low *trans*) were corrected for changes in dietary saturated and monounsaturated fatty acid intake and in body weight.

Trans α-linolenic acid did not affect plasma apoB and apoA-1 concentrations and their ratio (table 8.3). Lp(a) concentrations tended to fall in men consuming the low *trans* α-linolenic acid diet, whilst concentrations remained constant in the group that was switched to a high *trans* α-linolenic acid diet (data not shown). The treatment effect did not reach statistical significance.

Table 8.3 Plasma apolipoprotein A-1 and B after 6 weeks of a diet low or high in *trans* α-linolenic acid (Mean values and standard deviations, and 95% confidence intervals for treatment effect (high vs low *trans*))

	Low trans (n=44)	High trans (n=44)	Mean (95% CI)	P-value
ApoB				
run-in (mg/dl)	82.5 ± 20.2	76.8 ± 20.6		
treatment effect (%)	1.8	0.7	-1.8 (-7.7 to 4.4)	0.56
ApoA-1				
run-in (mg/dl)	138.2 ± 24.2	134.7 ± 23.6		
treatment effect (%)	1.3	-2.1	-3.3 (-8.2 to 1.8)	0.21
apoB:apoA-1 ratio				
run-in	0.6 ± 0.1	0.6 ± 0.2		
treatment effect (%)	0.6	2.8	2.3 (-4.4 to 9.6)	0.51

Treatment effects are expressed as percent changes

Treatment effects did not differ between the three centers

Treatment effects (high versus low *trans*) were corrected for changes in dietary saturated and monounsaturated fatty acid intake and in body weight

DISCUSSION

In this multi-center study with healthy normolipidaemic male volunteers, we did not find any unfavorable effects of high, but not unrealistic intakes of *trans* isomers from α-linolenic acid on plasma total or HDL cholesterol concentrations. LDL cholesterol concentrations, however, tended to increase, while the ratios of LDL to HDL and total to HDL cholesterol were significantly increased on the high *trans* diet compared to the low *trans* diet. These effects were observed in each center and were already evident after 3 weeks consumption of the experimental diets (data not shown). Results were corrected for differences in fatty acid intake and body weight between the two groups.

We found a difference in the total to HDL cholesterol ratio of 0.15 between the low and the high *trans* group. This ratio may predict the risk for CHD even better than total or LDL cholesterol concentrations (14,15). It has been suggested that an increase of 1 unit in the serum cholesterol over HDL ratio may increase the risk of myocardial infarction by 53% (16). This would suggest that the risk for CHD would decrease by 8%, when 1334 mg *trans* α-linolenic acid is replaced by dietary *cis* α-linolenic acid. It remains to be established, however, if diet-induced changes in HDL to total cholesterol ratio affect coronary heart disease risk.

So far, most previous studies have focused on the effects of *trans* monounsaturated fatty acids on the serum lipoprotein profile (17). *Trans* monounsaturates have been found to increase LDL cholesterol and to decrease HDL concentrations relative to its *cis* isomers (17). As a consequence *trans*

monounsaturated fatty acid increase the total to HDL cholesterol ratio as in our study. Triacylglycerol concentrations were also increased on diets high in *trans* monounsaturated fatty acids (17). However, we did not observe effects of *trans* α-linolenic acid on triacylglycerols. Possibly, intakes of *trans* isomers were even be too low, resulting in a limited power of the present study to detect such effects. Alternatively, *trans* α-linolenic acid may act differently than *trans* monounsaturated fatty acids. Lp(a) was also not affected by an intake of 1.4 g *trans* α-linolenic acid. Studies using *trans* monounsaturated fatty acids at high intakes, however, showed increased Lp(a) levels (17). One study has reported that partially hydrogenated fish oil, which contains *trans* isomers of the longer chain polyunsaturated fatty acids, elevated plasma total cholesterol, LDL cholesterol, and the LDL cholesterol to HDL cholesterol ratio relative to a partially hydrogenated soy-bean oil rich in *trans* monounsaturated fatty acids (18). However, results from that study are difficult to interpret because of differences in the composition of other fatty acids between the two hydrogenated oil diets.

Studies with *trans* monounsaturated fatty acids found that effects were similar for women and men (19-21) and for subjects with normal and mildly elevated triacylglycerol levels (22,23). Whether this is also true for *trans* polyunsaturated fatty acids, should be examined in future studies. Furthermore, it remains to be determined what would be the longer-term effects of a high intake of *trans* α-linolenic acid.

In our study subjects consumed on average 1410 mg *trans* α-linolenic acid. Hulshof et al. reported that for men the mean daily *trans* α-linolenic acid plus *trans* 20:1 intake - these two fatty acids could not be separated by gas chromatography - in 14 Western European countries varied between 20 mg in Italy and 490 mg in Iceland (24). Based on the fatty acid composition of cholesteryl esters, we considered as a first reasonable estimate that the mean habitual diet in Edinburgh and Maastricht provided approximately 600 mg *trans* α-linolenic acid per day and in Clermont-Ferrand about 300 mg (11). Proportions of α-linolenic acid in serum cholesteryl esters from the Dutch cohort was about 1.7 times higher as in 1990 (25) (0.55% vs. 0.32%), suggesting that α-linolenic acid intake has increased during the last years. In general, data on the intake of α-linolenic acid does not suggest that the habitual intake of *trans* α-linolenic acid is as high as we have used. Thus, the amount of *trans* α-linolenic acid (1.4 g/d) seemed to be high but not unrealistic. Standard deviations and ranges in *trans* α-linolenic acid intake were rather large. This was due to inaccuracies inherent to the method, used to estimate food intake (food record) and to differences in compliance. The oil for this study was deodorized especially for this study, but the composition of the different *trans* isomers (14% *trans, cis, cis-*, 19% *cis, cis, trans-* and 3.2% *trans, cis, trans-* α-linolenic acid) is typical for commercially available oils (5,26).

In conclusion, our study suggests that a high, but not unrealistic intake of *trans* α-linolenic acid, which is formed during oil processing, may influence the total to HDL

cholesterol ratio in an unfavorable way. Increasing the dietary intake of *cis, cis, cis*-α-linolenic acid has been recommended by an expert panel (27) and obviously it does not make sense to provide α-linolenic acid in the *trans* form. Careful deodorization prevents the formation of *trans* α-linolenic acid (12) and may help to improve the diet, deliver more *cis* α-linolenic acid and thereby possibly reduce ischaemic heart disease (28).

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9

General discussion

The studies described in this thesis examined metabolic aspects of dietary *cis* and *trans* polyunsaturated fatty acids. The two essential *cis* polyunsaturated fatty acids, linoleic and α-linolenic acid, have to be provided by the diet and are converted in the human body into longer chain more unsaturated fatty acids (LCPs) by alternate desaturation and elongation. *In vivo* conversion of these fatty acids as well as their oxidation have, however, hardly been studied in humans. Furthermore, since cDNAs of human desaturases have only recently been cloned, gene expressions of these enzymes have not been extensively investigated in humans.

Dietary unsaturated fatty acids occur in two configurations: the *cis* and *trans* form. *Trans* monounsaturated fatty acids are formed by bacteria in the first stomach of ruminants and by industrial hydrogenation, and have a negative impact on serum lipoproteins. *Trans* polyunsaturated fatty acids are mainly formed during the deodorization process, but their effects on risk parameters of coronary heart disease (CHD) have not been investigated. Since an increment of α-linolenic acid intake has been recommended (1), which could result in an increased intake of *trans* α-linolenic acid, it is necessary to investigate the health effects of *trans* isomers of α-linolenic acid as well.

This thesis describes four studies investigating (i) the *in vivo* metabolism of essential fatty acids (linoleic and α-linolenic acid) in healthy subjects using ^{13}C tracers (chapters 2 and 3), (ii) effects of dietary α-linolenic acid on the metabolism of ^{13}C α-linolenic acid (chapter 3), (iii) incorporation of ^{13}C α-linolenic acid and its longer chain metabolites into human erythrocytes (chapter 4), (iv) the relationship between Δ5, Δ6, and Δ9 desaturase mRNAs in human liver and mononuclear blood cells (chapter 5), (v) the incorporation of *trans* α-linolenic acid into plasma and platelet lipids (chapter 7) and (vi) effects of *trans* α-linolenic acid on plasma lipids and lipoproteins (chapter 8).

IN VIVO CONVERSION AND OXIDATION OF N-3 AND N-6 FATTY ACIDS

Design of the tracer-studies

The metabolism of both linoleic acid and α-linolenic acid has been studied *in vivo* in healthy subjects using a single bolus of 45 mg uniformly ^{13}C labeled fatty acids dissolved in olive oil. The dose of 45 mg was established by a pilot study, in which three subjects were given 15, 45 or 60 mg ^{13}C linoleic acid (^{13}C18:2n-6). We detected $^{13}CO_2$ in breath (figure 9.1), and ^{13}C18:2n-6, ^{13}C18:3n-6, ^{13}C20:3n-6, and ^{13}C20:4n-6 (arachidonic acid) in plasma total lipids (figure 9.2) at all doses. Plasma ^{13}C enrichments of these fatty acids were highest on a dose of 60 mg, while differences between doses of 15 and 45 mg were only marginal. On the other hand, we could detect ^{13}C22:4n-6 and ^{13}C22:5n-6 with 45 and 60 mg tracer, but not with 15 mg.

Therefore, we concluded that 45 mg could be used to study the oxidation of ^{13}C linoleic acid and its conversion into LCPs (chapter 2).

After plasma analyses of all volunteers in the linoleic acid study, however, ^{13}C enrichments of C20:4n-6 were low in three of the six subjects (chapter 2). Therefore, a dose of 45 mg was on the low side to study conversion of ^{13}C linoleic acid into arachidonic acid. Demmelmair et al. used a bolus of 1 mg ^{13}C linoleic acid per kilogram body weight, corresponding to a mean dose of about 62 mg, to study linoleic acid metabolism in healthy adults (2). Reported plasma labeled linoleic acid concentrations were comparable with our results (chapter 2), while levels of arachidonic acid also seems to be very low in some subjects (2). Based on results of both studies it may therefore be recommended to use a three to fourfold higher dose of ^{13}C linoleic acid when using a single bolus. Furthermore, since ^{13}C enrichments of some fatty acid metabolites still increased 2 weeks after tracer ingestion (chapter 2), it seems necessary to sample over a longer time period.

Plasma pools of n-3 fatty acids are lower than those of n-6 fatty acids. Consequently, 45 mg of ^{13}C α-linolenic acid would result in higher ^{13}C enrichments in n-3 fatty acids as compared to ^{13}C enrichments in n-6 fatty acids after 45 mg of ^{13}C linoleic acid. We, therefore, reasoned that 45 mg ^{13}C α-linolenic acid should be adequate to study α-linolenic acid metabolism (chapter 3 and 4). Indeed, ^{13}C α-linolenic acid metabolism could be studied well using 45 mg tracer.

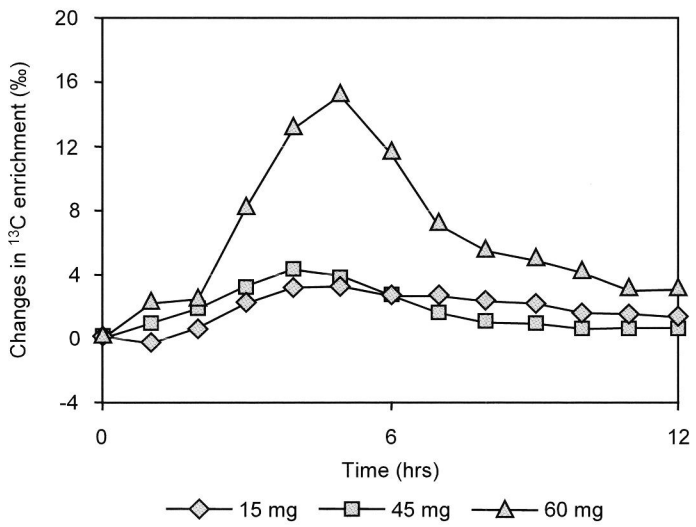

Figure 9.1 Changes in ^{13}C enrichment in breath (‰) after intake of a single dose of ^{13}C linoleic acid

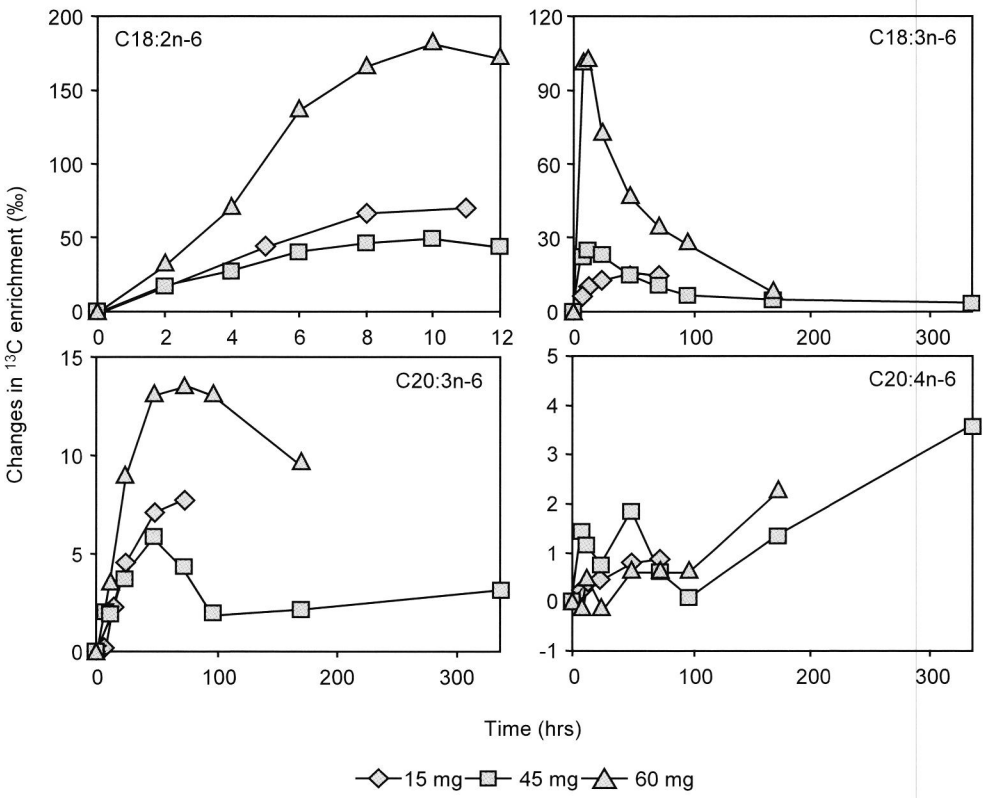

Figure 9.2 Changes in ^{13}C enrichments of plasma n-6 fatty acids after a single dose of 15, 45, and 60 mg ^{13}C linoleic acid

We have used ^{13}C labeled fatty acids, which has several advantages above deuterated fatty acids. Firstly, conversion and oxidation of fatty acids can be investigated at the same time, which will give a more complete picture of the metabolism of labeled fatty acid as compared to deuterated fatty acids, which can only be used to measure conversion. Secondly, detection of deuterated fatty acids by gas chromatography mass spectrometry (GC-MS) requires higher tracer doses as compared to ^{13}C tracer detection by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Therefore, ^{13}C labeled fatty acids, in contrast to deuterated fatty acids will hardly influence the n-3 and n-6 fatty acid intake. This may be of importance because of the competition of n-3 and n-6 fatty acids for desaturases. In a previous study, participants were provided with 3.0 g of deuterated linoleic acid (3), which is about 23% of the daily intake in US (4). In some subjects, this was combined with 3.0 or 3.5 g deuterated α-linolenic acid (3), which is more

than 100% of the daily α-linolenic acid intake (4). This certainly affected the absolute amounts of n-3 and n-6 fatty acids in the diet. Whether it also affects conversion reactions - and consequently interpretation of the results - remains to be established.

Human tracer studies are regularly carried out with only a small number of subjects. Previous fatty acid studies have used 2 to 13 subjects in each study group (3,5-9), and some of them found differences between groups with only 2 or 3 subjects in each group (3,6-8). As in other studies (5,9), standard errors in tracer enrichments between subjects were rather large in our studies. The number of 5 to 7 subjects in our studies seems not unusual, and significant diet effects on the conversion of ^{13}C α-linolenic acid were found (chapter 3). Although this may suggest that diet effects can be rather strong, it is advicable to look for strategies to reduce the variability between and within subjects, e.g. by using a cross-over design or repeated measures.

Oxidation of ^{13}C labeled linoleic and α-linolenic acid

Figure 9.3 shows the individual cumulative ^{13}C recovery of both ^{13}C linoleic acid (chapter 2) and ^{13}C α-linolenic acid (chapter 3) in breath during the first 12 hours after intake of a single bolus of 45 mg tracer. Cumulative ^{13}C recovery of ^{13}C α-linolenic acid in breath after 12 hours was not significantly affected by dietary n-3 fatty acids (20.4% on an α-linolenic acid rich diet, 15.7% on an oleic acid rich diet, and 24.8% on an eicosapentaenoic acid (EPA) / docosahexaenoic acid (DHA) rich diet), and was on average 19.7 ± 1.4% (mean ± sem) for the whole group. Mean cumulative ^{13}C recovery of ^{13}C linoleic acid in breath was 20.9%. This may suggest that α-linolenic acid and linoleic acid are oxidized at the same rate. A recent study using ^{13}C linoleic acid in lactating women, reported a recovery of 17.7 to 24.0%, depending on the week of lactation (5), which agrees with our results. In newborn infants, ^{13}C recovery during the first 6 hours after ^{13}C linoleic acid ingestion was 7.4% (10), which was lower than in our study (12% after 6 hours). This suggest that age may affect oxidation of ^{13}C linoleic acid. It would therefore be interesting to further investigate oxidation of ^{13}C labeled fatty acids in different age-groups. Peak ^{13}C enrichments in breath were obtained 3-5 hours after tracer intake for both ^{13}C labeled α-linolenic acid and linoleic acid, which corresponds to the time to peak ^{13}C enrichments in breath of lactating women (5).

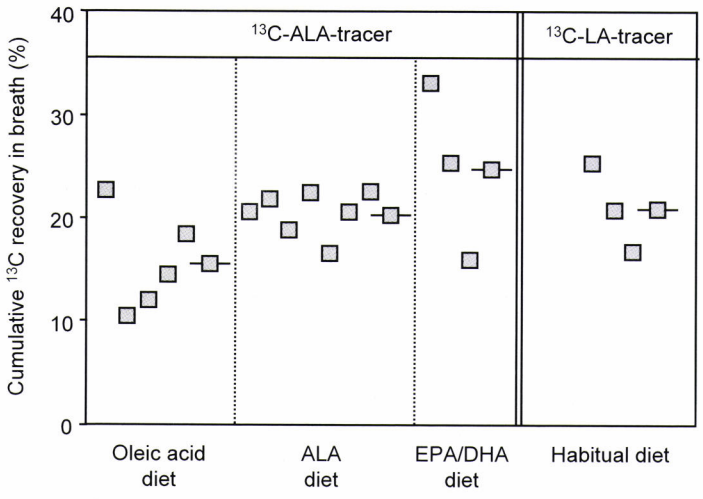

Figure 9.3 Individual cumulative ^{13}C recovery of ^{13}C linoleic acid and ^{13}C α-linolenic acid in breath during the first 12 hours after intake of a single bolus of 45 mg tracer

Incorporation of ^{13}C labeled linoleic and α-linolenic acid into plasma total lipids

Maximal amounts of plasma ^{13}C α-linolenic acid in the α-linolenic acid study, which were 5 to 10% of the given dose, were not affected by dietary n-3 fatty acids (chapter 3). Emken et al. also found no effect of dietary DHA on deuterated α-linolenic acid concentrations in plasma total lipids, after intake of a single bolus of deuterated α-linolenic acid, as compared to a low DHA diet (8). Dietary linoleic acid, however, decreased deuterated α-linolenic acid concentrations in plasma total lipids as compared to dietary saturated fatty acids (3).

The mean maximal amount of ^{13}C linoleic acid (7% of given dose) was not essentially different from the amount of ^{13}C α-linolenic acid in the α-linolenic acid group (6% of given dose), and the amount of ^{13}C α-linolenic acid in the oleic acid group (10% of given dose), as shown in figure 9.4. Thus, incorporation of ^{13}C linoleic acid in plasma total lipids seems to be nearly equal to that of ^{13}C α-linolenic acid.

Conversion of ^{13}C labeled linoleic and α-linolenic acid into their LCPs

Several previous *in vivo* studies have shown longer chain more unsaturated metabolites after administration of ^{14}C labeled, ^{13}C labeled or deuterated linoleic and α-linolenic acid in plasma of adults (3,7,9,11), in plasma of infants (10,12-15), and in milk of lactating women (5). Human *in vivo* studies reporting diet effects on conversion of essential fatty acids are, however, limited. We found significantly lower mean maximal amounts of ^{13}C-EPA in the α-linolenic acid group (0.04 mg) as compared to the oleic acid group (0.12 mg). In addition, although not statistically tested, amounts of ^{13}C-EPA were lower in the EPA/DHA group (0.03 mg) as

compared to the other groups (chapter 3), suggesting that n-3 LCPs decrease the conversion of ^{13}C α-linolenic acid. Hence, the relative conversion of plasma total α-linolenic acid (^{13}C plus ^{12}C) may be inhibited by the substrate itself or by its products. Because of a higher quantity of substrate in the α-linolenic acid group, however, the absolute conversion of plasma total α-linolenic acid is still higher in this group as compared to the oleic acid group. Emken et al. reported lowered deuterated n-3 LCP levels after deuterated α-linolenic acid administration on a high DHA diet as compared to a low DHA diet (8), which agrees with our study. In an intervention study with infants no effects of dietary α-linolenic acid on the conversion of ^{13}C α-linolenic acid into DHA was found, while conversion of ^{13}C linoleic acid into arachidonic acid was inhibited (13). In addition, dietary arachidonic acid decreased the conversion of deuterated linoleic acid into arachidonic acid (7). Conversion of both deuterated linoleic and α-linolenic acid is reduced by dietary linoleic acid in adult males (3). Figure 9.5 shows the individual maximal amounts of ^{13}C EPA ($^{13}C20:5n-3$) and ^{13}C arachidonic acid ($^{13}C20:4n-6$) after a dose of ^{13}C labeled α-linolenic acid and linoleic acid, respectively. Mean peak amount of ^{13}C EPA was 0.3% of the given dose on the oleic acid diet and 0.09% on the α-linolenic acid diet, while mean peak amount of ^{13}C arachidonic acid was 0.05% of the given dose. Results are difficult to compare due to differences in background diets, which may have affected fatty acid conversions.

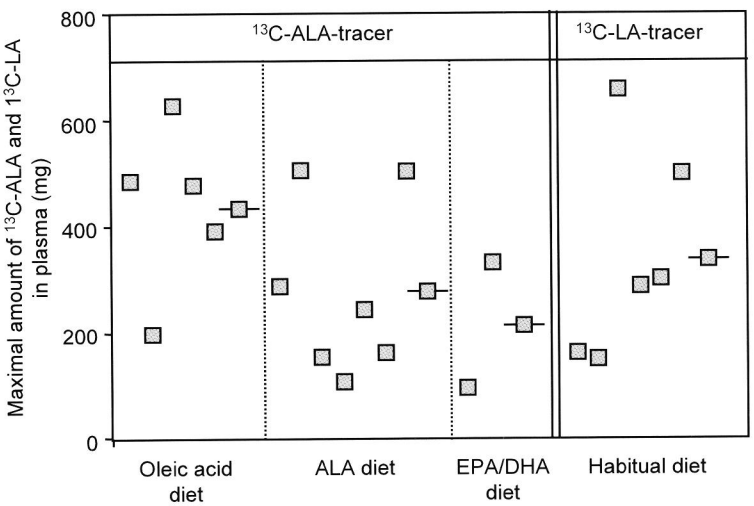

Figure 9.4 Individual maximal amounts of ^{13}C α-linolenic acid in plasma on diet rich in oleic acid (n=5), α-linolenic acid (n=7), or EPA/DHA (n=3) and of ^{13}C linoleic acid on an habitual diet (n=6) after intake of a single bolus of ^{13}C α-linolenic acid and ^{13}C linoleic acid, respectively

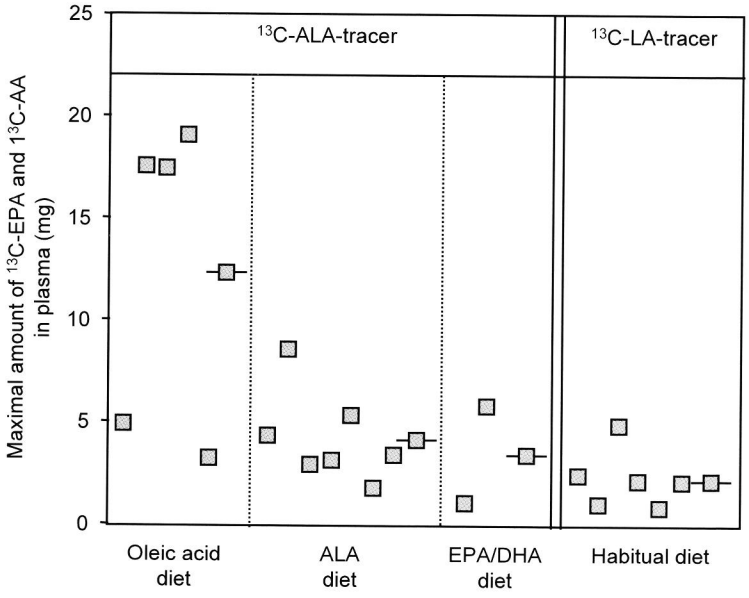

Figure 9.5 Individual maximal amounts of ^{13}C-EPA in plasma on diet rich in oleic acid (n=5), α-linolenic acid (n=7), or EPA/DHA (n=3) and of ^{13}C arachidonic acid on an habitual diet (n=6) after intake of a single bolus of ^{13}C α-linolenic acid and ^{13}C linoleic acid, respectively

To elucidate whether ^{13}C-atoms derived from ^{13}C α-linolenic acid are used for synthesis of non-essential fatty acids, we have also measured ^{13}C enrichment in myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1n-7) and oleic acid (C18:1n-9) in plasma total lipids after administration of ^{13}C α-linolenic acid in three subjects (two from the α-linolenic acid group, and one from the oleic acid group; chapter 3). We detected ^{13}C enrichments in all of these fatty acids, and ^{13}C palmitoleic acid was the most abundant (mean maximum change in ^{13}C enrichment of 11‰) (table 9.1). Peak ^{13}C enrichments of myristic, palmitoleic, and oleic acid were reached within the first day, while only after almost 2 weeks ^{13}C enrichments of palmitic, and stearic acid peaked. These saturated, n-9 and n-7 fatty acids may have been formed from degradation products of ^{13}C α-linolenic acid or its LCPs. In this study, only about 0.2% of the tracer was found as myristic, palmitic, stearic, palmitoleic or oleic acid. Therefore, the low recovery of ^{13}C α-linolenic acid (chapter 3 and 4) may not be explained by its conversion into non-essential fatty acids.

Table 9.1 Maximal ^{13}C enrichment of myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1n-7) and oleic acid (C18:1n-9) in plasma total lipids after administration of ^{13}C α-linolenic acid in three subjects (two after an α-linolenic acid rich diet, and one after an oleic acid rich diet)

	Maximal ^{13}C enrichment (‰)		Time to peak ^{13}C enrichment (hours)	
Fatty acid	Median	Mean	Median	Mean
C14:0	3.35	3.08	5	7
C16:0	1.17	1.92	336	232
C18:0	0.81	2.27	336	232
C16:1n-7	10.85	11.17	5	5
C18:1n-9	1.35	1.55	24	20

In order to gain our insight into the distribution of labeled fatty acids among the human body, incorporation of ^{13}C α-linolenic acid and its LCP into erythrocytes has been investigated. We did not found differences in ^{13}C n-3 fatty acid incorporation between the α-linolenic acid and oleic acid group (chapter 4). We have also checked for incorporation of ^{13}C linoleic acid and its metabolites into human erythrocytes in three subjects of the linoleic acid study (chapter 2), and we found ^{13}C enrichments of linoleic acid, C20:3n-6, arachidonic acid (C20:4n-6), and C22:4n-6 (C18:3n-6 and C22:5n-6 were not measured). Table 9.2 shows median and mean maximal proportions of n-3 fatty acids after ^{13}C α-linolenic acid intake (chapter 4) and that of n-6 fatty acids after ^{13}C linoleic acid intake (from the study described in chapter 2). As already described in chapter 4, the high mean proportion of ^{13}C18:3n-3 in the α-linolenic acid group was caused by one subject. Median proportions of ^{13}C linoleic acid seem to be higher than of ^{13}C α-linolenic acid. The mean maximal proportion of ^{13}C20:4n-6 was the highest of all n-6 and n-3 LCPs. Incorporation of ^{13}C linoleic acid into erythrocytes, however, occurred at a slower rate as compared to that of ^{13}C α-linolenic acid, as evidenced from the time to peak proportions of 48 hours for ^{13}C linoleic acid and of 11 hours for ^{13}C α-linolenic acid.

Table 9.2 Maximal proportions of ^{13}C labeled fatty acids in erythrocytes (% of total fatty acids) after intake of ^{13}C α-linolenic acid (ALA; on diets rich in oleic acid or α-linolenic acid) (chapter 4) and after intake of ^{13}C linoleic acid (LA; habitual diet) (chapter 2)

Maximal proportions (*10^{-3} % of total fatty acids)				
	^{13}C-ALA tracer		^{13}C-LA tracer	
	Oleic acid (n=5)	ALA (n=7)		(n=3)
$^{13}C18:3n-3$	1.06 (1.64 ±0.72)	0.83 (4.11 ± 3.21)	$^{13}C18:2n-6$	2.17 (4.01 ± 2.53)
$^{13}C20:5n-3$	0.31 (0.27 ±0.08)	0.09 (0.17 ± 0.06)	$^{13}C20:3n-6$	0.08 (0.13 ± 0.06)
$^{13}C22:5n-3$	0.17 (0.19 ±0.06)	0.16 (0.18 ± 0.05)	$^{13}C20:4n-6$	0.55 (0.55 ± 0.28)
$^{13}C22:6n-3$	0.04 (0.04 ±0.01)	0.05 (0.07 ± 0.02)	$^{13}C22:4n-6$	0.11 (0.11 ± 0.06)

Values are medians (means ± sem)

Conclusion tracer-studies

To summarize, after administration of a single bolus of 45 mg ^{13}C α-linolenic acid to healthy subjects, about 20% was recovered as $^{13}CO_2$ in breath after 12 hours, and at least 5 to 10% appeared as precursor and 0.1-0.3% as LCPs in plasma total lipids. In addition, a small part of the tracer was detected as precursor or metabolite in erythrocytes, and at least 0.2% may have been converted into non-essential fatty acids. This means that a large part of the tracer is still missing. In our studies, we have only investigated oxidation, and incorporation of the tracer in plasma and erythrocyte total lipids. The labeled fatty acids may also have been incorporated into other tissues. More importantly, the recovery of the tracer may have been underestimated because our data were based on one single time point (maximal changes).

When a single bolus is used to investigate fatty acid conversion, like in our studies, quantification of conversion of fatty acids is difficult because the appearance and disappearance of fatty acid tracers in plasma may overlap. Demmelmair et al. have compared a single bolus of ^{13}C linoleic acid with fractionated tracer doses (9 equal portions over 3 days) (2), and used three methods to describe conversion: compartmental modeling, integrating tracer concentration-time curves, and tracer concentrations of LCPs relative to linoleic acid at specific time points. Fractional conversion of ^{13}C linoleic acid into $^{13}C20:3n-6$ was significantly higher in the group which received the fractionated doses (2.1% per day) than in the bolus group (1.5% per day). When calculating area under the curves, conversions were 4.1% and 5.1%, respectively. The authors suggested that the area under the curve overestimate the amount of ^{13}C labeled fatty acids converted (2). Using specific time points to describe conversion has the disadvantage that tracer enrichments are dependent on the time of sampling and turn-over times of the fatty acids. When we would have used one specific time point in the ^{13}C α-linolenic acid study (chapter 3), for example t=5 hours - ^{13}C α-linolenic acid enrichments are maximal at this point - we would not have been able to detect differences in ^{13}C enrichments of LCPs between the α-linolenic acid

rich and the oleic acid rich diet (figure 3.3). So far, there is no optimum method to describe *in vivo* conversion of fatty acids.

Desaturase mRNAs in human mononuclear blood cells and liver tissue

Three desaturase enzymes are involved in the conversion of essential and non-essential fatty acids: Δ5, Δ6 and Δ9 desaturase. Desaturase activity mainly takes place in the liver, but this is not an easy accessible organ to investigate in humans. Since a previous study has demonstrated that ^{13}C linoleic acid is converted into its LCPs in human leukocytes (16), we reasoned that desaturase mRNA could be present in human leukocytes as well. In order to predict desaturase mRNA levels of liver by measuring those in leukocytes, desaturase regulation in human leukocytes should be related to desaturase regulation in the liver. Such a relationship has been found between low density lipoprotein (LDL) receptor mRNA and 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoA) mRNA levels in mononuclear blood cells with those in human liver, suggesting that regulation of gene expressions of the LDL receptor and HMGCoA in leukocytes parallels that in the liver (17).

Desaturase mRNA levels were measured in patients with morbid obesity who underwent gastroplasty, patients who underwent hemi-hepatectomy, and patients who underwent cholecystectomy for symptomatic gallstones (chapter 5). Although absolute amounts of desaturase mRNA in these patients may deviate from that of healthy subjects, there is no need to assume that the correlation of desaturase gene expression between liver and mononuclear blood cells - if any - is different. These patients underwent an operation close to the liver, which makes the liver accessible for liver biopsy. An extra operation is not necessary. These patients are therefore more suitable for participating in this study than any other population. One disadvantage is, however, that the *in vivo* enzyme activity, measured by stable isotopes, could not be investigated at the same time, because this would have been too demanding for the patients. *In vitro* measurement of enzyme activity in liver was not possible, because 3-4 g liver is needed for isolation of an adequate amount of microsomes (18; personal communication), which is unethical to collect.

We found Δ5, Δ6 and Δ9 desaturase mRNAs in both human liver and human mononuclear blood cells (chapter 5). Delta 6 and Δ9 desaturase mRNAs in mononuclear blood cells were negatively correlated with Δ6 and Δ9 desaturase mRNAs in the liver, while Δ5 desaturase mRNA in liver was not correlated with that in the mononuclear blood cells. We do not have a clear explanation for these negative correlations. Results of our study (chapter 3) and of other previous studies (3,7,8) suggest that the relative conversions of linoleic and α-linolenic acid - expressed as the proportion of the total amount of linoleic or α-linolenic acid (^{13}C plus ^{12}C) in the diet that is converted - may be inhibited by the substrate itself as well as by its products. Absolute conversions - expressed as the absolute amount of α-linolenic acid in the diet that is converted - may, however, still be higher because of the higher

quantity of substrate in the diet. We speculated that an increased supply of substrate will first reach the liver via chylomicron and chylomicron remnants and may here increase mRNA levels of Δ6 desaturase. This will result in increased LCP-formation and LCP-output via very low density lipoprotein (VLDL). These LCPs may subsequently lower mRNA of Δ6 desaturase in extra hepatic tissues and cells. This pathway may result in a negative correlation between hepatic and extra hepatic desaturase levels, as observed in our study (chapter 5).

EFFECTS OF *TRANS* α-LINOLENIC ACID ON PLASMA LIPIDS AND LIPOPROTEINS

Study performance

The *TRANS*LinE study investigated the incorporation of fatty acids into plasma lipids and platelets, and effects on plasma lipids after a *trans* α-linolenic acid enriched diet in male subjects. The study was performed in three centers located in three European Countries, France (Clermont-Ferrand), Scotland (Edinburgh) and the Netherlands (Maastricht), under comparable conditions. In each center, all punctures were performed by the same technician, at the same location, and as much as possible at the same time of the same day of the week. Laboratory analyses were centralized in order to eliminate any possible systematic analytical bias between centers.

The *TRANS*LinE study was well controlled: Subjects visited the centers every week to control for body weight and to collect experimental products. If necessary, energy intake was adjusted to maintain a stable body weight. Furthermore, subjects were urged not to change smoking habits, background diets, alcohol use or physical exercise during the study, and any deviations from the protocol, medication used and any signs of illness were recorded in diaries. All subjects were assigned a random number, which was used for labeling the tubes and cups. In addition, food items were color coded. In this way, both subjects and technicians were blinded. Compliance was checked by food diaries and by changes in the fatty acid compositions of plasma cholesteryl esters, phospholipids, triacylglycerols and platelets.

The high *trans* α-linolenic acid diet provided on average 0.5% of energy (or 1410 mg) from *trans* α-linolenic acid. A previous study reported daily intakes of *trans* α-linolenic acid plus *trans* 20:1 between 20 mg and 490 mg in Western European countries (19). Based on the fatty acid composition of cholesteryl esters of the subjects before the start of the study, we considered 300 mg (in Clermont-Ferrand) to 600 mg (in Edinburgh and Maastricht) *trans* α-linolenic acid per day as a reasonable estimate (chapter 7 and 8).

Results

Proportions of *trans* fatty acids differed between European countries (20,21); *trans* monounsaturated were the highest in the Northern European countries. In our study, the total *trans* fatty acid contents in plasma triacylglycerols, phospholipids, cholesteryl esters and platelets at baseline were higher in Edinburgh and Maastricht as compared to Clermont-Ferrand (chapter 7). Proportions of *trans* monounsaturated fatty acids were lowest in Clermont-Ferrand, while no differences between Edinburgh and Maastricht were observed. In Edinburgh, subjects were recruited by advertisement in the university bulletin and posters, while French and Dutch subjects were recruited mainly from the general population (among a panel in Clermont Ferrand and through announcements in university and local newspapers in Maastricht). Different backgrounds of the subjects may have biased comparisons of fatty acid compositions at baseline. Subjects in Maastricht had higher proportions of *trans* linoleic acid in plasma triacylglycerols and cholesteryl esters compared to Clermont-Ferrand and Edinburgh, and higher proportions of *trans* α-linolenic acid in plasma triacylglycerols, and cholesteryl esters compared to Clermont-Ferrand (chapter 7). The EURAMIC study also showed that in 1991/92, the Dutch cohort had the highest proportions of the *trans* total fatty acids in adipose tissue (22), which reflects dietary intakes (23). Since 1995, the *trans* fatty acid content of Dutch margarines and spreads is reduced (24), which should result in a lower intake of *trans* fatty acids. Our study now suggest that intakes of both *trans* monounsaturates and polyunsaturates are relatively high in the Netherlands.

After a diet with experimental oils high in *trans* α-linolenic acid, the relative amount of *trans* α-linolenic acid increased in plasma triacylglycerols, phospholipids and cholesteryl esters (chapter 7). Furthermore, on the high *trans* α-linolenic acid diet *trans* eicosapentaenoic acid was raised by 0.04% of total fatty acids in plasma triacylglycerols, by 0.07% of total fatty acids in plasma phospholipids and by 0.02% of total fatty acids in plasma cholesteryl esters as compared to the low *trans* diet. This suggests that *trans* isomers of α-linolenic acid are elongated and desaturated by man. *Trans* isomers of oleic acid and linoleic acid, as well as other *cis* polyunsaturated fatty acids in plasma cholesteryl esters, were not affected by the diets (chapter 7).

In the *TRANS*LinE study, we found increased total to HDL cholesterol and LDL to HDL cholesterol ratios on *trans* α-linolenic acid as compared to *cis* α-linolenic acid. *Trans* α-linolenic acid did not affect other plasma lipids (chapter 8). On the contrary, *trans* monounsaturates did affect plasma LDL and HDL cholesterol, triacylglycerol and in some studies lipoprotein (a) (24).

Effects of *trans* monounsaturated fatty acids on plasma lipids have been reported in several previous studies (24). Katan et al. have combined results of five studies, and suggested that each additional percent of dietary energy as *trans* monounsaturated fatty acids at expense of oleic acid results in an increase in LDL

cholesterol levels of 0.040 mmol/l and a decrease in HDL cholesterol levels of 0.013 mmol/l (24). From a previous study of Almendingen et al. it can be calculated that each percent of dietary energy as *trans* isomers of the LCPs from partially hydrogenated fish oil elevated plasma LDL cholesterol by 0.071 mmol/l, and decreased HDL cholesterol concentrations by 0.001 mmol/l relative to carbohydrates (25). We found an increase in LDL cholesterol of 0.22 mmol/l and a decrease in HDL cholesterol of 0.04 mmol/l on each percent of dietary energy on *trans* α-linolenic acid as compared to *cis* α-linolenic acid. Figure 9.6 shows the effects of these three *trans* fatty acids relative to carbohydrates (27). Results suggest that the relative effects of *trans* α-linolenic acid may even be stronger than that of *trans* monounsaturated fatty acids. Intake of *trans* monounsaturated fatty acids will, however, be higher than that of *trans* polyunsaturated fatty acids.

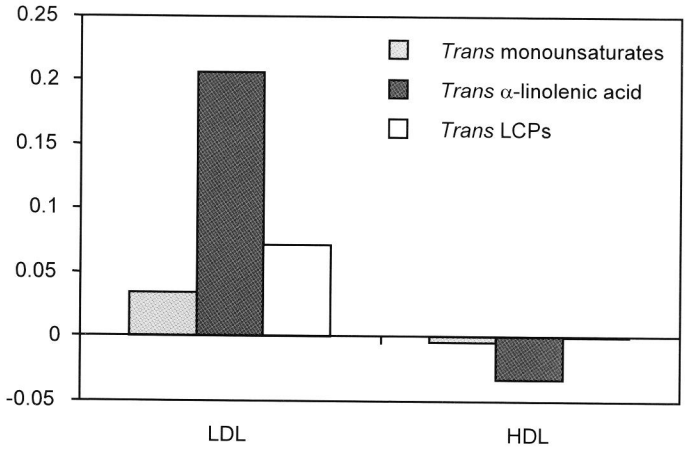

Figure 9.6 Changes in LDL and HDL cholesterol levels on each percent of dietary energy as *trans* monounsaturated fatty acids (24), *trans* α-linolenic acid (chapter 8), and partially hydrogenated fish oil rich in *trans* polyunsaturated fatty acids (LCPs) (25) relative to carbohydrates

The underlying mechanisms for effects of both *trans* mono- and polyunsaturated fatty acids are still not understood. Cholesteryl ester transfer protein (CETP) and lecithin:cholesterol acyltransferase (LCAT) have been suggested to be involved. CETP transfers cholesteryl esters from HDL to the apolipoprotein B-containing lipoproteins LDL and VLDL in exchange for triacylglycerol. Some previous studies found an increased CETP activity in volunteers who consumed a *trans* fatty acid enriched diet (28,29), and after addition of *trans* fatty acids to human plasma *in vitro*

(30), while others did not confirm these results (31,32). LCAT is bound to HDL and esterifies free cholesterol from tissues. A decrease of LCAT activity on *trans* fatty acids has been reported (33), although this was not supported by another study (34). Furthermore, a low affinity of acyl-CoA:cholesterol acyl transferase (ACAT), which esterifies fatty acids from chylomicrons, for *trans* C18:1 have been reported (35), which may eventually result in increased levels of LDL cholesterol by a decrease of the liver LDL receptor activity. Further research is needed to investigate these mechanisms.

CONCLUSIONS AND RECOMMENDATIONS

The studies described in this thesis showed that after ingestion of ^{13}C labeled linoleic and α-linolenic acid to healthy volunteers, the precursors as well as their longer chain n-6 and n-3 fatty acids are incorporated into plasma total lipids. This suggests that ^{13}C linoleic and α-linolenic acid are converted into longer chain more unsaturated fatty acids in humans. The conversion of ^{13}C α-linolenic acid was decreased by an α-linolenic acid rich diet, as compared to an oleic acid rich diet. Part of the ^{13}C linoleic and α-linolenic acid is oxidized and excreted by breath as $^{13}CO_2$. Another part of the tracer is incorporated into erythrocytes as precursor and as metabolite. Incorporation of ^{13}C α-linolenic acid into erythrocytes was not affected by dietary α-linolenic acid. We detected only about 30% of the tracer, which means that a large part of the tracer is still missing.

Linoleic and α-linolenic acid are converted by alternate elongation and desaturation. We have now detected Δ5 and Δ6 desaturase mRNA in human liver and mononuclear blood cells. In addition, mRNA of Δ9 desaturase, which is involved in the conversion of non-essential fatty acids, was measured. We found a negative correlation between Δ6 and Δ9 desaturase mRNA in mononuclear blood cells with those in the liver, which may be explained as follows. Absolute conversion of fatty acids may be higher when more substrate is supplied. An increased supply of substrate may increase hepatic Δ6 desaturase mRNA, which results in increased LCP-formation and LCP-output via VLDL. This may subsequently lower Δ6 desaturase mRNA in extra hepatic tissue. It would now also be interesting to examine effects of external factors, e.g. diets, on desaturase mRNA levels in mononuclear blood cells, in order to examine whether desaturase mRNAs in mononuclear blood cells can indeed be used to examine fatty acid conversions. Combining the stable isotope technique with determinations of desaturase mRNAs and possible other enzymes involved in the metabolism of essential and non-essential fatty acids, as well as qualification of the amounts of enzymes in human tissues by using antibodies seem to be useful to increase our understanding on the regulation of essential fatty acid metabolism.

Dietary *trans* isomers of α-linolenic acid are incorporated into plasma lipids and converted into longer chain polyunsaturated fatty acids. Furthermore, *trans* α-linolenic acid increases plasma LDL to HDL cholesterol and total to HDL cholesterol ratios, which are important risk parameters for CHD. Increasing the *cis* α-linolenic acid intake, as recommended, may also increase the intake of α-linolenic acid in the *trans* form. Careful deodorisation may prevent the formation of *trans* α-linolenic acid and may help to improve the diet, deliver more *cis* α-linolenic acid and thereby possibly reduce risk for CHD.

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Fatty acid nomenclature and abbreviations

Fatty acid nomenclature

Cis fatty acids

C14:0	myristic acid
C16:0	palmitic acid
C16:1n-7 (Δ9)	palmitoleic acid
C17:0	triheptadecanoic acid
C18:0	stearic acid
C18:1n-9 (Δ9*c*)	oleic acid
C20:3n-9 (Δ5,8,11)	Mead acid
C22:3n-9 (Δ7,10,13)	dihomo-Mead acid
C18:2n-6 (Δ9,12)	linoleic acid
C18:3n-6 (Δ6,9,12)	γ-linolenic acid
C20:3n-6 (Δ8,11,14)	dihomo-γ-linolenic acid
C20:4n-6 (Δ5,8,11,14)	arachidonic acid
C22:4n-6 (Δ7,10,13,16)	adrenic acid
C22:5n-6 (Δ4,7,10,13,16)	Osbond acid
C18:3n-3 (Δ9,12,15)	α-linolenic acid
C20:5n-3 (Δ5,8,11,14,17)	eicosapentaenoic acid
C22:5n-3 (Δ7,10,13,16,19)	docosapentaenoic acid
C22:6n-3 (Δ4,7,10,13,16,19)	docosahexaenoic acid

Trans fatty acids

C18:1n-9 (Δ9*tr*)	elaidic acid
C18:1n-7 (Δ11*tr*)	vaccenic acid

Abbreviations

ACAT	acyl-CoA:cholesterol acyltransferase
ADP	adenosine diphosphate
ALA	α-linolenic acid
Apo	apolipoprotein
ATP	adenosine triphosphate
BMI	body mass index

BSA	bovine serum albumin
CETP	cholesteryl ester transfer protein
CI	confidence interval
CHD	coronary heart disease
CSFII	Continuing Survey of Food Intakes by Individuals
EDTA	ethylenediaminetetracetic acid
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
dNTP	deoxyribonucleoside triphosphate
DPA	docosapentaenoic acid
EFA	essential fatty acid
FA	fatty acid
FAME	fatty acid methyl ester
GC-C-IRMS	gas chromatography-combustion-isotope ratio mass spectrometry
GC/FID	gas chromatography coupled with a flame ionization detector
HDL	high density lipoprotein
HETE	hydroxyeicosatetraenoic acid
HHT	12-hydroxy heptadecatrienoic acid
HPETE	hydroxyperoxyeicosatetraenoic acid
LCAT	lecithin:cholesterol acyltransferase
LCP	long chain polyunsaturated fatty acid
LDL	low density lipoprotein
Lp(a)	lipoprotein(a)
LT	leukotriene
MDA	malondialdehyde
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid
PAI-1	plasminogen activator inhibitor type 1
PC	phosphatidylcholine
PDB	Pee Dee Belemate
PG	prostaglandin
PGI_2	prostacyclin
PUFA	polyunsaturated fatty acid
RR	relative risk
RT-PCR	reverse transcription-polymerase chain reaction
SAFA	saturated fatty acid
SD	standard deviation
SEM	standard error of the mean
TTR	tracer/tracee ratio
Tx	tromboxane
USDA	US Department of Agriculture
VLDL	very low density lipoprotein

Summary

Dietary polyunsaturated fatty acids are mainly incorporated into triacylglycerols and phospholipids. Triacylglycerols are an important source of energy, while phospholipids are essential components of membranes. Some polyunsaturated fatty acids are precursors of eicosanoids, which have important physiological functions in humans like in inflammatory processes, immunological reactions and platelet aggregation. The polyunsaturated fatty acids linoleic (C18:2n-6) and α-linolenic acid (C18:3n-3) are essential fatty acids, which can be converted into their longer chain more unsaturated fatty acids (LCPs) by chain elongation and desaturation. *In vivo* conversion of these fatty acids as well as their oxidation have, however, hardly been studied in humans.

The double bonds in dietary linoleic and α-linolenic acid have a *cis* configuration. However, *trans* polyunsaturated fatty acids are also part of the diet. It is known that *trans* monounsaturated fatty acids have a negative impact on serum lipoproteins, but effects of *trans* polyunsaturated fatty acids on risk parameters of coronary heart disease (CHD) have not been extensively investigated. Since increment of dietary α-linolenic acid intake has been recommended, which could result in an increased intake of *trans* α-linolenic acid, it would be interesting to investigate the health effects of *trans* isomers of α-linolenic acid as well. Therefore, the studies presented in this thesis were aimed at examining the metabolic aspects of dietary *cis* and *trans* polyunsaturated fatty acids.

The *in vivo* conversion and oxidation of fatty acids can be measured safely in humans by using stable isotopes. The stable isotope of carbon (^{12}C) is ^{13}C, which has a natural abundance of 1.11%. **Chapter 2** examines the *in vivo* conversion of 45 mg ^{13}C linoleic acid in 6 healthy volunteers. Blood was sampled before and at several time points up to 2 weeks after ^{13}C linoleic acid ingestion. About 17 hours after intake of ^{13}C linoleic acid, amounts of ^{13}C linoleic acid in plasma total lipids were maximal and were 8% of the given dose. Also ^{13}C18:3n-6, ^{13}C20:3n-6, ^{13}C20:4n-6 (arachidonic acid), ^{13}C22:4n-6 and ^{13}C22:5n-6 were detected in plasma total lipids, suggesting that ^{13}C linoleic acid is converted into its LCPs. Amounts of ^{13}C20:4n-6 increased until 2 weeks, but were rather low. The C22 fatty acids ^{13}C22:4n-6 and ^{13}C22:5n-6 could not be detected in all subjects. The peak amount of ^{13}C LCPs (^{13}C18:3n-6 + ^{13}C20:3n-6 + ^{13}C20:4n-6) was 0.15% of the ^{13}C linoleic acid dose. We therefore recommend to use a higher dose of ^{13}C linoleic acid to investigate its conversion into arachidonic acid. After 12 hours, about 21% of the ^{13}C linoleic acid was recovered as $^{13}CO_2$ in breath.

Effects of a diet rich in α-linolenic acid (8.3 gram/day for 6 weeks) versus an oleic acid rich diet on the oxidation and the *in vivo* conversion of 45 mg ^{13}C α-linolenic acid into LCPs in 12 healthy subjects is described in **chapter 3**. Six percent of the ^{13}C α-linolenic acid on the α-linolenic acid rich diet and 10% of the ^{13}C α-linolenic acid on the oleic acid rich diet was found as ^{13}C α-linolenic acid in plasma total lipids. Furthermore, eicosapentaenoic acid (EPA; ^{13}C20:5n-3), ^{13}C docosapentaenoic acid (DPA; ^{13}C22:5n-3) and ^{13}C docosahexaenoic acid (DHA; ^{13}C22:6n-3) were detected in plasma total lipids. Maximal amounts of ^{13}C-EPA in plasma total lipids were significantly lower in the α-linolenic acid group (0.04 mg) than in the oleic acid group (0.12 mg). Amounts of ^{13}C-DPA and ^{13}C-DHA tended to be lower as well. These findings suggest that the relative conversion of ^{13}C α-linolenic acid into its LCPs may be decreased on diets rich in α-linolenic acid. The proportion of tracer recovered as $^{13}CO_2$ in breath after 12 hours was 20% in the α-linolenic acid group and 16% in the oleic acid group, which was not significantly different. In addition, part of the ^{13}C α-linolenic acid and ^{13}C LCPs was incorporated into human erythrocytes (**chapter 4**). This incorporation does not seems to be affected by dietary α-linolenic acid. Because about 20% of the tracer was recovered as $^{13}CO_2$ in breath, 5-10% appeared as precursors and 0.1-0.3% as LCPs in plasma total lipids, and a small part was detected as precursor or metabolite in erythrocytes, it is concluded that an important part of the tracer is still missing which may be incorporated into other tissues.

Linoleic and α-linolenic acids are converted into their LCPs by elongase and Δ5 and Δ6 desaturases, which occur mainly in the liver. In addition, Δ9 desaturase is involved in the conversion of non-essential fatty acids. The liver is not an easy accessible organ to investigate in humans, while leukocytes can be collected in a relatively easy way. Therefore, it was investigated whether gene expressions of these desaturases in human leukocytes reflects that in the liver. Both mononuclear blood cells and human liver samples were collected from 14 patients who underwent an operation close to the liver (**chapter 5**). Delta 6 and Δ9 desaturase mRNAs in mononuclear blood cells were negatively correlated with Δ6 (r=-0.64) and Δ9 desaturase mRNAs (r=0.83) in liver, respectively. This does, however, not necessarily mean that desaturase mRNAs in mononuclear blood cells can not be used to predict those in the liver. Hepatic Δ5 and Δ6 desaturase mRNA (r=0.71), and Δ6 and Δ9 desaturase mRNA (r=0.59) were positively correlated.

Most dietary *trans* fatty acids are *trans* monounsaturated fatty acids, which are formed by bacteria in the first stomach of ruminant animals and by industrial hydrogenation. *Trans* polyunsaturated fatty acids are mainly formed during the deodorization process. Effects of *trans* α-linolenic acid from deodorized rapeseed oil on its incorporation into plasma lipids (**chapter 7**) and on plasma lipids and lipoproteins (**chapter 8**) have now been investigated in a large multicenter study. Eighty-eight male volunteers from three European university research departments, Clermont-Ferrand, Edinburgh and Maastricht, received 0.5% of energy (1.4

gram/day) *trans* α-linolenic acid for 6 weeks versus a low *trans* fatty acid diet (60 mg/day). Less total *trans* fatty acids were incorporated into plasma triacylglycerols, phospholipids, cholesteryl esters and platelets in French volunteers (1.9, 0.8, 0.5, and 1.1% of total fatty acids, respectively) than in Dutch (2.6, 1.1, 0.8, and 1.8% of total fatty acids, respectively) or Scottish volunteers (2.4, 1.1, 0.6 and 1.6% of total fatty acids, respectively) consuming their habitual diets. In addition, *trans* isomers of α-linolenic acid and monounsaturated fatty acids were also lowest in plasma triacylglycerols, phospholipids and cholesteryl esters, and *trans* monounsaturated fatty acids in platelets in the Clermont-Ferrand. Results suggest that *trans* fatty acid intake still differs between European countries. Changes in plasma lipid and platelet fatty acid composition documented that *trans* α-linolenic isomers were incorporated and converted to a *trans* isomer of EPA. The high *trans* α-linolenic acid diet significantly increased the plasma low density lipoprotein (LDL) to high density lipoprotein (HDL) cholesterol ratio by 8.1%, and the total to HDL cholesterol ratio by 5.1%, compared to the low *trans* diet. This was largely explained by an increase in LDL cholesterol on the high *trans* α-linolenic acid diet, while no change was observed on the low *trans* diet. No effects were found on total and HDL cholesterol, triacylglycerols, lipoprotein B and A-1, and lipoprotein(a) concentrations. It is concluded that dietary *trans* isomers of α-linolenic acid are incorporated in plasma lipids and converted to LCPs. In addition, *trans* α-linolenic acid may increase plasma LDL to HDL cholesterol and total to HDL cholesterol ratios, which are important risk parameters for coronary heart disease. It is recommended, in addition to a reduction of *trans* monounsaturated fatty acids, to reduce consumption of *trans* polyunsaturated fatty acids.

In **chapter 9** findings from the four studies described are discussed. The *in vivo* metabolism of essential fatty acids has been investigated by using two methods, the stable isotope technique and molecular biology. Whether mononuclear blood cells are suitable for investigation of fatty acids conversion should be investigated in future research focussing on effects of external factors, e.g. diets, on desaturase mRNA levels in mononuclear blood cells. In addition, combining different techniques seems to be useful in future research.

Samenvatting

Meervoudig onverzadigde vetzuren uit de voeding worden in het lichaam met name ingebouwd in triglyceriden en fosfolipiden. Triglyceriden spelen een belangrijke rol als energieleverancier, terwijl fosfolipiden een belangrijke structurele component zijn van celmembranen. Daarnaast kunnen sommige meervoudig onverzadigde vetzuren worden omgezet in eicosanoïden, die betrokken zijn bij het aansturen van vele lichaamsprocessen. De meervoudig onverzadigde vetzuren linolzuur (C18:2n-6) en α-linoleenzuur (C18:3n-3) zijn essentiëel, en kunnen in het lichaam door elongatie en desaturatie worden omgezet in vetzuren met een langere ketenlengte en met meer dubbele bindingen, de zogenaamde 'long chain polyunsaturated fatty acids' (LCPs). Over de *in vivo* omzetting en oxidatie van essentiële vetzuren bij de mens is nog niet veel bekend.

De dubbele bindingen in linolzuur en α-linoleenzuur hebben de zogenaamde *cis* vorm. In de voeding komen echter ook meervoudig onverzadigde vetzuren voor met de *trans* vorm. Het is reeds bekend dat *trans* enkelvoudig onverzadigde vetzuren een negatieve invloed hebben op lipoproteïnenconcentraties in serum. Naar de effecten van *trans* meervoudig onverzadigde vetzuren op risicofactoren voor hart- en vaatziekten is veel minder onderzoek gedaan. De huidige voedingsrichtlijnen zijn er onder andere op gericht om de inname van α-linoleenzuur te verhogen, hetgeen kan resulteren in een toename in de *trans* α-linoleenzuurinname. Het is daarom van belang om ook de gezondheidseffecten van *trans* isomeren van α-linoleenzuur te bestuderen. De studies uit dit proefschrift hebben dan ook tot doel om meer inzicht te krijgen in de metabole aspecten van *cis* en *trans* meervoudig onverzadigde vetzuren.

Een veilige manier om bij de mens de *in vivo* omzetting en oxidatie van vetzuren te bestuderen is met behulp van stabiele isotopen. Het stabiele isotoop van koolstof (^{12}C) is ^{13}C en beslaat in de natuur 1,11% van het totaal aantal koolstofatomen. In **hoofdstuk 2** wordt de *in vivo* omzetting van 45 mg ^{13}C gelabeld linolzuur bij 6 gezonde vrijwilligers beschreven. Tot 2 weken na inname van het ^{13}C linolzuur werd op verschillende tijdstippen bloed afgenomen, waarin verrijkingen van ^{13}C gelabelde vetzuren werden bepaald. Ongeveer 17 uur na inname van ^{13}C linolzuur werd een maximum bereikt in de hoeveelheid ^{13}C linolzuur in plasma. Dit maximum was 8% van de toegediende dosis. Daarnaast werden ook de volgende LCPs in plasma gevonden: $^{13}C18:3n-6$, $^{13}C20:3n-6$, $^{13}C20:4n-6$ (arachidonzuur), $^{13}C22:4n-6$ en $^{13}C22:5n-6$. De hoeveelheden $^{13}C20:4n-6$ waren erg klein, en stegen tot 2 weken na inname van ^{13}C linolzuur. De C22 vetzuren $^{13}C22:4n-6$ en $^{13}C22:5n-6$ konden niet bij alle personen worden aangetoond. De totale maximale hoeveelheid ^{13}C gelabelde LCPs ($^{13}C18:3n-6$ + $^{13}C20:3n-6$ + $^{13}C20:4n-6$) was slechts 0,15% van de

toegediende dosis. De resultaten wijzen erop dat ^{13}C linolzuur wordt omgezet in langere keten vetzuren. Om echter de omzetting in arachidonzuur te besturen, wordt een hogere dosis aanbevolen. Na 12 uur werd bovendien ongeveer 21% van het ^{13}C linolzuur teruggevonden als $^{13}CO_2$ in de uitademingslucht.

De effecten van een α-linoleenzuurrijke voeding (8,3 gram/dag gedurende 6 weken) versus een oliezuurrijke voeding op de oxidatie en *in vivo* omzetting van 45 mg ^{13}C α-linoleenzuur in LCPs bij 12 gezonde proefpersonen worden beschreven in **hoofdstuk 3**. Na de α-linoleenzuurrijke voeding werd 6% en na de oliezuurrijke voeding werd 10% van het toegediende ^{13}C α-linoleenzuur teruggevonden als ^{13}C α-linoleenzuur in het plasma. Daarnaast waren er ook hoeveelheden ^{13}C gelabeld eicosapentaeenzuur (EPA; ^{13}C20:5n-3), ^{13}C gelabeld docosapentaeenzuur (DPA; ^{13}C22:5n-3) en ^{13}C gelabeld docosahexaeenzuur (DHA; ^{13}C22:6n-3) in plasma aanwezig. De absolute maximale hoeveelheid ^{13}C-EPA in plasma was significant lager in de α-linoleenzuurgroep (0,04 mg) dan in de oliezuurgroep (0,12 mg). Hoeveelheden ^{13}C-DPA en ^{13}C-DHA leken ook verlaagd. Deze resultaten suggereren dat de relatieve omzetting van ^{13}C α-linoleenzuur in zijn LCPs verlaagd wordt door een α-linoleenzuurrijke voeding. Na 12 uur werd 20% van de tracer in de α-linoleenzuurgroep en 16% in de oliezuurgroep als $^{13}CO_2$ teruggevonden in de uitademingslucht. Dit verschilde niet significant tussen de groepen. Daarnaast werd een deel van de ^{13}C α-linoleenzuur en ^{13}C LCPs ingebouwd in de erythrocyten (**hoofdstuk 4**). Deze inbouw leek niet te worden beïnvloed door de hoeveelheid α-linoleenzuur uit de voeding. Samenvattend kan worden gesteld dat in deze studie slechts ongeveer 20% van het ^{13}C α-linoleenzuur als $^{13}CO_2$ in de uitademingslucht, en 5-10% als precursor en 0,1-0,3% als LCPs in het plasma is teruggevonden, terwijl slechts een klein deel als precursor of metaboliet in de erythrocyten werd ingebouwd. Een deel van het ^{13}C α-linoleenzuur hebben we dus niet aan kunnen tonen, en zal mogelijk zijn opgenomen in andere weefsels van het lichaam.

Linolzuur en α-linoleenzuur worden omgezet in hun LCPs door elongase en Δ5- en Δ6 desaturase. Deze reacties vinden voornamelijk in de lever plaats. Daarnaast is Δ9 desaturase betrokken bij de omzetting van niet-essentiële vetzuren. Aangezien de lever vaak niet toegankelijk is om omzettingen van vetzuren te bestuderen, terwijl het verzamelen van leukocyten niet erg belastend is, is er onderzocht of de gen-expressies van deze desaturases in de leukocyten van de mens de gen-expressies in de lever weerspiegelen. Hiertoe werden zowel mononucleaire bloedcellen als humaan leverweefsel van 14 patiënten verzameld. Deze patiënten ondergingen een operatie vlak bij de lever, zodat het mogelijk was om een leverbiopt te nemen (**hoofdstuk 5**). In tegenstelling tot de verwachting waren Δ6 en Δ9 desaturase mRNAs in mononucleaire bloedcellen negatief gecorreleerd met respectievelijk Δ6 (r=-0,64) en Δ9 desaturase (r=-0,83) mRNAs in de lever. Mogelijk kunnen desaturase mRNAs in mononucleaire bloedcellen echter wel gebruikt worden om waarden in de lever te voorspellen. In deze studie bleken de

hepatische Δ5 en Δ6 desaturase mRNAs (r=0,71), en Δ6 en Δ9 desaturase mRNAs (r=0,59) positief te zijn gecorreleerd.

De meeste *trans* vetzuren uit de voeding zijn *trans* enkelvoudig onverzadigde vetzuren, welke worden gevormd door bacteriën in de lebmaag van herkauwers en bij industriële hydrogenatie. *Trans* meervoudig onverzadigde vetzuren worden voornamelijk gevormd tijdens het deodorizeren van oliën. Effecten van *trans* α-linoleenzuur uit gedeodoriseerde raapzaadolie op de inbouw van plasmalipiden (**hoofdstuk 7**) en op plasmalipiden en -lipoproteïnenconcentraties (**hoofdstuk 8**) zijn onderzocht in een grote multicenter studie. Achtentachtig mannelijke vrijwilligers uit drie Europese steden, Clermont-Ferrand, Edinburgh en Maastricht, kregen 0,5 energie% (1,4 gram/dag) *trans* α-linoleenzuur gedurende 6 weken versus een 'laag *trans* vetzuren voeding' (60 mg/dag). Voor aanvang van de studie waren de totale *trans* vetzurenconcentraties in plasma triglyceriden, fosfolipiden en cholesteryl esters, en bloedplaatjes lager bij de Franse vrijwilligers (respectievelijk 1,9, 0,8, 0,5, en 1,1% van totaal vetzuren) in vergelijking met die van de Nederlandse (respectievelijk 2,6, 1,1, 0,8, en 1,8% van totaal vetzuren) en Schotse vrijwilligers (respectievelijk 2,4, 1,1, 0,6 en 1,6% van totaal vetzuren). Bovendien waren ook het *trans* α-linoleenzuur en de *trans* enkelvoudig onverzadigde vetzuren in de plasmalipidenfracties en *trans* enkelvoudig onverzadigde vetzuren in bloedplaatjes het laagst in Clermont-Ferrand. Aangezien de waarden bij aanvang van de studie de samenstelling van de gebruikelijke voeding weergaven, blijken er dus verschillen te bestaan in *trans* vetzureninname tussen Europese landen. Uit de veranderingen in de vetzuursamenstelling van plasmalipiden en bloedplaatjes na de 6 weken durende *trans* α-linoleenzuurrijke voeding bleek dat *trans* α-linoleenzuur werd ingebouwd en werd omgezet in *trans* isomeren van EPA. De *trans* α-linoleenzuurrijke voeding leidde bovendien tot een significante verhoging van de plasma 'low density lipoprotein' (LDL) / 'high density lipoprotein' (HDL) cholesterol ratio met 8,1%, en de totaal/HDL cholesterol ratio met 5,1%, in vergelijking met de 'laag *trans* vetzuren voeding'. Dit werd grotendeels veroorzaakt door een toename in LDL cholesterol na de *trans* α-linoleenzuurrijke voeding, terwijl er geen verandering optrad na de 'laag *trans* vetzuren voeding'. Er werden geen effecten van *trans* α-linoleenzuur uit de voeding gevonden op het totaal en HDL cholesterol, triglyceriden, lipoproteïne B and A-1, and lipoproteïne (a). Uit de resultaten blijkt dat de *trans* isomeren van α-linoleenzuur uit de voeding worden ingebouwd in plasmalipiden en omgezet in LCPs. Bovendien verhoogt *trans* α-linoleenzuur de plasma LDL/HDL cholesterol en de totaal/HDL cholesterol ratios, twee belangrijke risicoparameters voor hart- en vaatziekten. Het wordt daarom aanbevolen naast de inname van *trans* enkelvoudig onverzadigde vetzuren, ook zo min mogelijk *trans* meervoudig onverzadigde vetzuren in te nemen.

In **hoofdstuk 9** worden de bevindingen uit de verschillende studies bediscussieerd. Met behulp van twee onderzoekstechnieken (stabiele isotopen onderzoek en bepaling van desaturase mRNA), is het *in vivo* metabolisme van de essentiële vetzuren bestudeerd. Toekomstig onderzoek, waarin effecten van externe factoren, zoals bijvoorbeeld voeding, op desaturase mRNA in mononucleaire bloedcellen worden onderzocht, zal verder uit moeten wijzen of leukocyten geschikt zijn om bij bestudering van vetzuuromzettingen te gebruiken. Daarnaast lijkt het waardevol om, binnen een studie, bovengenoemde onderzoekstechnieken te combineren.

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Susan

Curriculum vitae

Susanne Hanneke Francisca Vermunt was born on July 14 1970, in Prinsenbeek, the Netherlands. After graduation from secondary education at 'Markenhage' in Breda, she completed a higher education for dietician at the 'Hogeschool Nijmegen' in Nijmegen in 1993. As part of the training she spent 5 months of practical research at TNO Nutrition and Food Research of the Netherlands Organization for Applied Scientific Research (TNO) in Zeist. In 1993, she started her academic education at Maastricht University in Maastricht, where she studied Biological Health Sciences. As part of the training she spent 5 months of practical research at the Department of Urology at the University Hospital in Maastricht, during which she studied the effects of an animal protein and sodium restricted diet on the composition of urine in patients with hypercalciuria. She graduated in 1995. For a period of 6 months she worked as a research assistant at the Department of Human Biology, Maastricht University, during which the effects of a fermented milk product on serum lipoproteins in healthy subjects were studied. She started in March 1995 as a PhD-fellow at the Department of Human Biology, Maastricht University. She conducted an international intervention study with healthy men on the effects of *trans* polyunsaturated fatty acids on lipoproteins. Furthermore, she conducted stable isotope studies with healthy subjects on the metabolism of polyunsaturated fatty acids and studies on mRNA levels of desaturase enzymes by molecular biology. Parts of research findings have been presented at international conferences in Maastricht (the Netherlands), Edinburgh (Scotland) and Lyon (France). From May 2000 on, she appointed at TNO Nutrition and Food Research in Zeist.

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- Vermunt SHF, Greve JW, Mensink RP, and Hornstra G. Relationship between mRNA levels for $\Delta 5$, $\Delta 6$ and $\Delta 9$ desaturases in mononuclear blood cells and the liver.
- Vermunt SHF, Mensink RP, Simonis AMG, and Hornstra G. Incorporation of ^{13}C α-linolenic acid and its longer chain metabolites into human erythrocyte.

Metabolic Aspects of Cis and Trans Polyunsaturated Fatty Acids from the Diet