

Dietary C18 fatty acids : effects on cardiovascular risk markers and fatty acid metabolism

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Dietary C18 fatty acids:

*effects on cardiovascular risk
markers and fatty acid metabolism*



nutrim



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

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1

General Introduction

GENERAL INTRODUCTION

Besides carbohydrates and proteins, fatty acids are major macronutrients of the diet (**Table 1.1**). Data from the most recent food consumption survey indicated that on average 34% of the daily dietary energy intake is provided by fatty acids as present in fats and oils, whereas carbohydrates and proteins provided about 48% and 14% of energy in the Dutch diet (1). In contrast to the food consumption survey of 1997-1998, intakes of the individual fatty acids were not reported. In this earlier survey, 37%, 45% and 15% of energy was provided by respectively fatty acids, carbohydrates, and proteins (2). With respect to the fatty acids, approximately 14% of energy was derived from saturated fatty acids. The saturated fatty acids lauric acid, myristic acid, and palmitic acid provided approximately 9% of energy, whereas stearic acid provided 3-4% of energy. Oleic acid and linoleic acid, the major monounsaturated and polyunsaturated fatty acids, contributed for 10-11% and 7-8% to the total daily energy intake, respectively (3). Next to its well-recognised role in energy metabolism, dietary fat and fatty acids have profound effects on other metabolic processes due to their effects on for example membrane lipid composition and gene expression. Dependent on their structure and function, fatty acids exert differential effects on risk markers of cardiovascular diseases.

Cardiovascular diseases are a major cause of morbidity and mortality in western and developed countries. In the Netherlands, coronary heart disease, cerebrovascular diseases and peripheral arterial diseases are responsible for approximately 34% of all deaths (4). Because a diversity of risk markers is related with its prevalence, cardiovascular diseases have a multifactorial aetiology. Increased age, male gender, personal and family history of cardiovascular diseases and genetic background are all associated with an increased cardiovascular disease risk. Other major risk markers are smoking, physical inactivity, hypertension, obesity, diabetes mellitus, an unfavourable lipoprotein profile, increased thrombotic tendency, increased oxidative stress, and low-grade inflammation (5,6). These latter factors are amenable to prevention by lifestyle changes such as the fatty acid composition of the diet.

In this thesis, the effects of dietary fatty acids are described on cardiovascular disease risk markers. In the present chapter, the structure, function, and metabolism of fatty acids are introduced, and the pathogenesis of cardiovascular diseases and risk markers of cardiovascular diseases are described. Furthermore, studies which evaluated the relationships between fatty acids, lipid and lipoprotein concentrations and cardiovascular diseases are briefly introduced.

Table 1.1 Average daily intakes of energy and nutrients in The Netherlands.

| Food consumption survey Nutrient | 1997-1998 ¹ | | 2003 ² | |
|-------------------------------------|------------------------|-------|-------------------|-------------------|
| | Men | Women | Young men | Young women |
| Energy (kcal) | 2668 | 2031 | 2760 | 1921 |
| Energy (kJ) | 11192 | 8519 | 11581 | 8063 |
| Carbohydrates (% of energy) | 44.6 | 45.0 | 47.2 | 49.1 |
| Proteins (% of energy) | 14.5 | 15.3 | 14.0 | 14.6 |
| Fats (% of energy) | 36.5 | 37.0 | 34.4 | 34.3 |
| Saturated fatty acids | 13.7 | 14.1 | 12.7 | 13.0 |
| Lauric acid (C12:0) | 0.8 | 0.8 | | |
| Myristic acid (C14:0) | 1.3 | 1.4 | | |
| Palmitic acid (C16:0) | 6.8 | 7.0 | | |
| Stearic acid (C18:0) | 3.4 | 3.5 | | |
| Monounsaturated fatty acids | 10.4 | 10.6 | 18.1 ³ | 17.6 ³ |
| Oleic acid (C18:1n-9) | 9.7 | 9.9 | | |
| Polyunsaturated fatty acids | 7.6 | 7.4 | | |
| Linoleic acid (C18:2n-6) | 6.6 | 6.3 | | |
| α -Linolenic acid (C18:3n-3) | 0.7 | 0.6 | | |
| EPA (C20:5n-3) | 0.01 | 0.01 | | |
| DHA (C22:6n-3) | 0.02 | 0.02 | | |
| Trans fatty acids | 1.2 | 1.4 | 1.0 | 1.1 |
| Alcohol (% of energy) | 4.5 | 2.6 | 4.3 | 1.8 |
| Cholesterol (mg/MJ) | 22.2 | 23.8 | | |
| Dietary fibre (g/MJ) | 2.2 | 2.4 | 2.0 | 2.1 |

¹Data are derived from the national food consumption survey 1997-1998 in men and women aged between 19 and 50 y in The Netherlands (2,3).

²Data are derived from the national food consumption survey 2003 in young adults aged between 19 and 30 y in The Netherlands (1).

³Total % of energy as provided by monounsaturated and polyunsaturated fatty acids.

STRUCTURE AND FUNCTION OF FATTY ACIDS

With respect to their structure, all fatty acids consist of a skeleton of carbon (C) atoms with at one end a methyl (CH₃) group and at the other end a carboxyl (COOH) group. This carbon chain varies in length and degree of unsaturation. Moreover, the position and configuration of the double bonds in the carbon chain may differ between fatty acids (**Figure 1.1**). According to their structure, fatty acids are denoted as Ca:bn-c, in which 'a' indicates the number of carbon atoms in the fatty acid chain, 'b' the number of double bonds, and 'c' the position of the first double bond as counted from the methyl group. Based on the number of double

bonds, fatty acids without any double bond are classified as saturated fatty acids, while fatty acids with one double bond belong to the monounsaturated fatty acids and those with two or more double bonds to the polyunsaturated fatty acids (**Figure 1.1**). Stearic acid is an example of a saturated fatty acid, oleic acid of a monounsaturated fatty acid and linoleic acid of a polyunsaturated fatty acid.

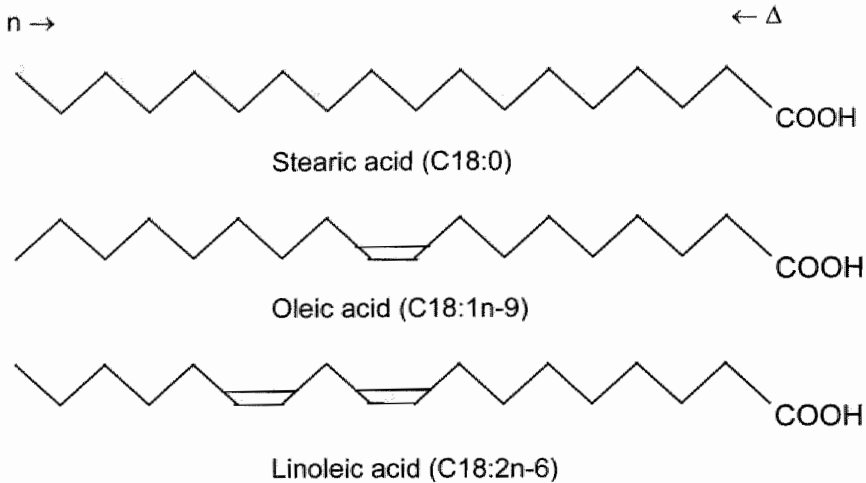


Figure 1.1 Chemical structures of stearic acid (a saturated fatty acid), oleic acid (a monounsaturated fatty acid) and linoleic acid (a polyunsaturated fatty acid).

According to the position of the double bonds, unsaturated fatty acids are subdivided into various families. As counted from the methyl end of the carbon chain (n -designation), unsaturated fatty acids either belong to the n -3, n -6, n -7 or n -9 families. In contrast, the position of the double bonds may also be counted from the carboxyl end group and is then noted as ' Δ ' (**Figure 1.1**). Because fatty acid chain desaturation and elongation always occur between the carboxyl group and its nearest double bond, fatty acids do not change families. The major family parents of the n -3, n -6, n -7 and n -9 families are α -linolenic acid (C18:3n-3), linoleic acid (C18:2n-6), palmitoleic acid (C18:1n-7) and oleic acid (C18:1n-9), respectively.

The configuration or stereochemistry of the double bonds may be *cis* or *trans* (**Figure 1.2**). Fatty acids, as present in natural foods mostly contain *cis* double bonds, whereas *trans* bonds result from industrial hydrogenation to harden fats or from microbial metabolism in ruminants. Conjugated linoleic acids (CLA) contain a *cis* as well as a *trans* double bond.

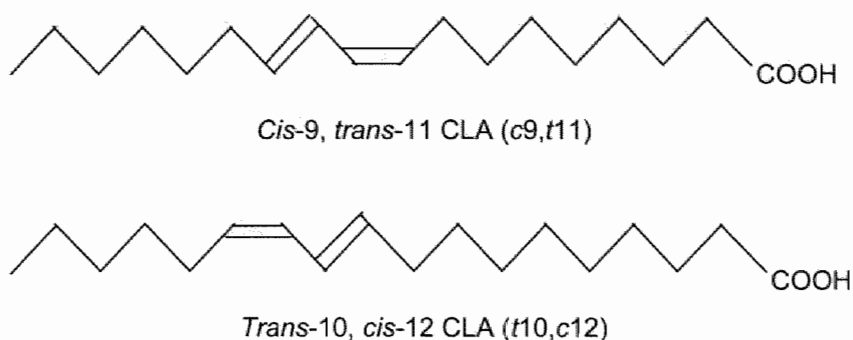


Figure 1.2 Chemical structures of *cis-9, trans-11 (c9,t11)* and *trans-10, cis-12 (t10,c12)* conjugated linoleic acid (CLA).

As already mentioned, fatty acids play a major role in many physiological processes. Fatty acids are components of biological membranes, function as energy suppliers and are precursors of several signalling molecules, including eicosanoids such as prostaglandins, thromboxanes, and leukotrienes. Moreover, fatty acids influence transcription of genes involved in metabolism (7). Unlike saturated fatty acids (e.g. stearic acid and palmitic acid) and monounsaturated fatty acids (e.g. oleic acid) which can be synthesised *de novo* in most human cells, polyunsaturated fatty acids such as linoleic acid and α -linolenic acid can not be synthesised *de novo*. Therefore, these latter fatty acids of the n-6 and n-3 families have to be provided by the diet and are called essential fatty acids. The availability of long-chain polyunsaturated fatty acids in human cells greatly depends on the activity of enzymes - desaturases and elongases - involved in fatty acid biosynthesis and metabolism (8).

METABOLISM OF FATTY ACIDS

The hepatic desaturase and elongase enzymes are importantly involved in fatty acid metabolism and lipid synthesis (8,9). Desaturation and elongation are involved in the maintenance of membrane fatty acid composition and fluidity, the generation of precursors for signalling molecules like eicosanoids, and the regulation of nuclear receptors. Fatty acid desaturases and elongases are involved in the conversion of fatty acids into fatty acids with a higher degree of unsaturation and a longer chain length. While fatty acid desaturases introduce a double bond in fatty acids between the carboxyl group and its nearest double bond, elongases lengthen fatty acids with a two-carbon unit from malonyl-coenzyme A to the carboxyl end of a fatty acid chain

(8,10). In **Figure 1.3** the biosynthesis of long-chain polyunsaturated fatty acids is shown.

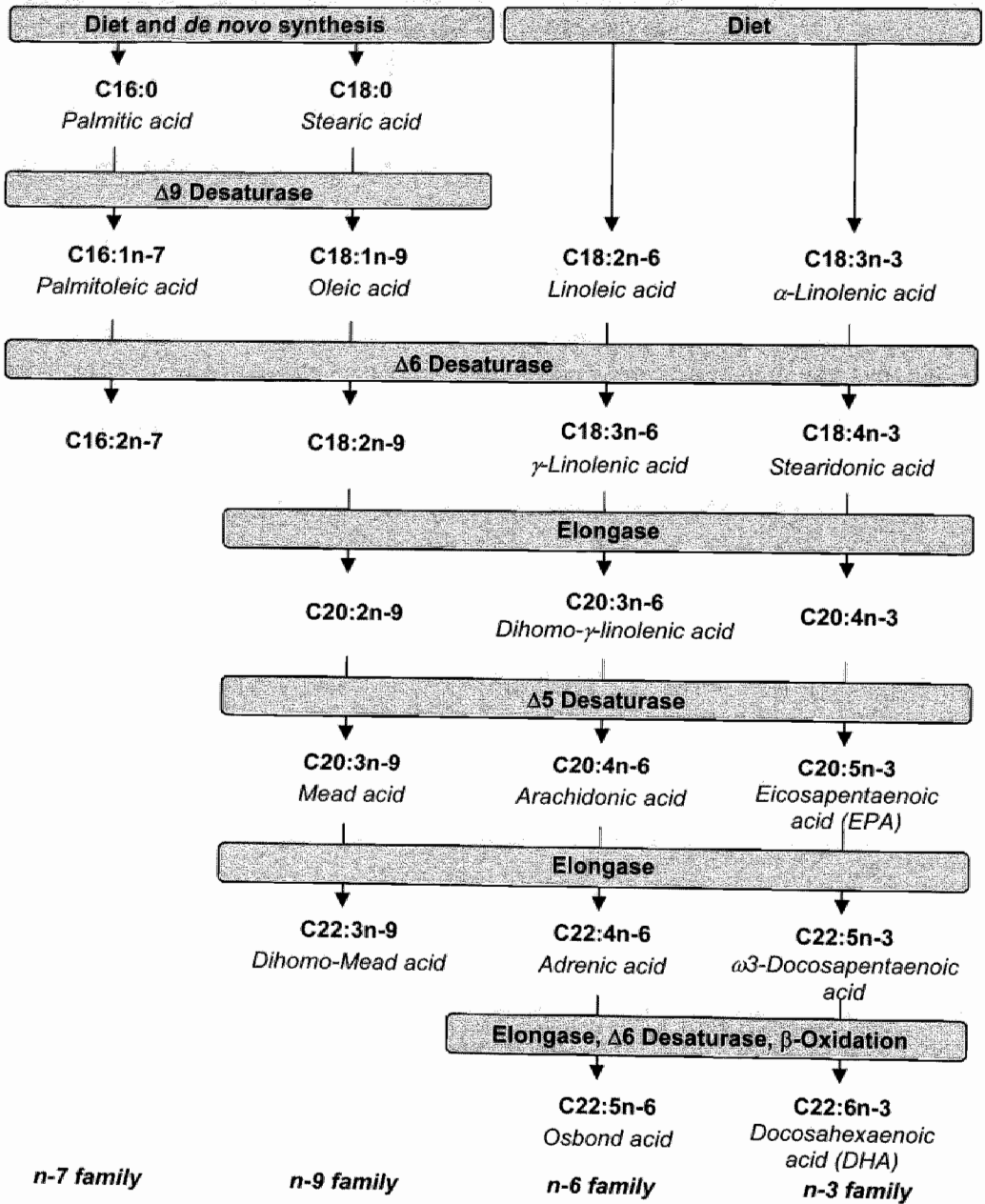


Figure 1.3 Desaturation and elongation of fatty acids.

Desaturation

In humans, three kinds of fatty acid desaturases are present, namely $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases. All three desaturases are part of a desaturase system, which includes the desaturase, NADH-cytochrome-b5 reductase and cytochrome-b5. In this system, the desaturase enzyme is the rate-limiting enzyme. Because human desaturases are present in microsomal membranes (endoplasmatic reticulum, mitochondria and peroxisomal membranes) and use fatty acids esterified to coenzyme A as substrate, they belong to the group of membrane-bound desaturases and are also called acyl-coenzyme A (CoA) desaturases (9).

The $\Delta 9$ desaturase enzyme, also called stearoyl-CoA desaturase (SCD), mediates the desaturation step of saturated fatty acids into monounsaturated fatty acids (**Figure 1.3**). Because desaturase enzymes are specific for the location, number, and stereochemistry of double bonds, $\Delta 9$ desaturase introduces the first *cis*-double bond at the $\Delta 9$ position from the carboxyl end of fatty acids (9). Since $\Delta 9$ desaturase is the rate-limiting enzyme in the cellular synthesis of monounsaturated fatty acids and a proper ratio of saturated to monounsaturated fatty acids contributes to membrane fluidity, and monounsaturated fatty acids are required in the liver for the synthesis of triacylglycerols and cholesteryl esters, $\Delta 9$ desaturase has been implicated in the regulation of cell growth and differentiation through effects on membrane fluidity and signal transduction (9,11).

$\Delta 5$ and $\Delta 6$ desaturases are involved in the conversion of fatty acids into the long-chain polyunsaturated members of the n-9, n-6 and n-3 families. Both $\Delta 5$ and $\Delta 6$ desaturases are called front-end desaturases because they introduce a double bond between the pre-existing double bond and the carboxyl front end of the fatty acid (9). $\Delta 6$ Desaturase introduces a double bond at the $\Delta 6$ position of fatty acids that contain already a double bond in the $\Delta 9$ position. The products of $\Delta 6$ desaturation are then converted by an elongase and subsequently desaturated by $\Delta 5$ desaturase, which is specific to desaturate C20 fatty acids at the $\Delta 5$ position when the fatty acid is already desaturated at the $\Delta 8$ and $\Delta 11$ positions (**Figure 1.3**).

The human $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturase genes have been identified and cloned recently. Although four isoforms of $\Delta 9$ desaturase have been identified in mice (SCD-1, SCD-2, SCD-3 and SCD-4) only one functional SCD gene that is highly homologous to mouse SCD-1 is known in humans (12). The human $\Delta 9$ desaturase gene has been localised to chromosome 10 and consists of 6 exons and 5 introns. A second known SCD locus in humans on chromosome 17 is a fully processed, transcriptionally inactive pseudogene (13). The human $\Delta 5$ and $\Delta 6$ desaturase genes are localised in a cluster on chromosome 11. Both genes consist of 12 exons and 11

introns spanning the 17 kb and 39 kb regions, respectively. Both genes encode 444 amino acids and possess 61% amino acid identity and 75% nucleotide homology to each other (14,15).

Elongation

As the desaturation steps have for long been considered to be the rate-limiting steps for the biosynthesis of long-chain polyunsaturated fatty acids, only recently interest has increased to study the role and mechanisms of elongation. The elongase system is composed of 4 enzymes namely a condensing enzyme (β -ketoacyl coenzyme A synthase), β -ketoacyl coenzyme A reductase, β -hydroxyacyl coenzyme A dehydrase and trans-2-enoyl coenzyme A reductase. In human cells, multiple microsomal elongase enzymes have been identified with different chain length specificities. Corresponding gene sequences have been classified for the gene family of elongases (ELOVL) involved in the biosynthesis of very-long-chain fatty acids (8). ELOVL2 and ELOVL4 have been found to convert C20 as well as C22 polyunsaturated fatty acids, whereas γ -linolenic acid and α -linolenic acid are substrates for ELOVL1, ELOVL3 and ELOVL5. In addition, ELOVL1 and ELOVL5 (or HELO1) convert arachidonic acid and EPA, and ELOVL5 also stearidonic acid (8). The human ELOVL5 gene has been localised to chromosome 6 and consists of 7 exons and 6 introns. The ELOVL5 gene encodes 299 amino acids and shares 56% amino acid identity with ELOVL2 (16,17).

Regulation of desaturases and elongases by fatty acids

As the liver plays a central role in the maintenance of systemic lipid homeostasis, desaturases and elongases are predominantly expressed in the liver. However, also other human tissues including heart, brain, lung, and kidney tissues express human desaturases (13,15) and elongases (17). Several nutritional and hormonal factors are known to regulate the activity of desaturases and elongases (9,12,18). Polyunsaturated fatty acids, especially those of the n-6 and n-3 families, have been reported to suppress the activity of desaturases and elongases (19-21). Moreover, CLA isomers, in particular Δ^{10},Δ^{12} CLA, may inhibit the expression or activity of Δ^9 desaturase (22-26). Also the expression of Δ^5 and Δ^6 desaturases is suppressed by linoleic acid and fish fatty acids (14,15). Previous studies reported less or even no effects of saturated and monounsaturated fatty acids on the expression of desaturases and elongases (20,21,27).

Most of the studies, which investigated the regulation of desaturases and elongases by fatty acids, were performed in rats and mice. For human studies, however, liver cells are not easily accessible. Probably peripheral blood

mononuclear cells (PBMC) obtained by venipuncture may reflect gene expression of desaturases and elongases. Whether human desaturases and elongases are regulated in a similar way as in animals is unknown.

PATHOGENESIS OF CARDIOVASCULAR DISEASES

Atherosclerosis is importantly involved in the pathogenesis of cardiovascular diseases. Morbidity and mortality from cardiovascular diseases including coronary heart disease (angina pectoris, myocardial infarction), cerebrovascular diseases (ischemic stroke) and peripheral arterial disease (gangrene, intermittent claudicatio) can largely be explained by the development of an atherosclerotic plaque in one or more coronary, cerebrovascular or peripheral arteries (5,28).

Atherosclerosis is characterised by the accumulation of lipids in the arterial wall. Uptake, modification, and the subendothelial retention of cholesterol-rich, atherogenic lipoproteins within the arterial wall are thought to be initiating events in atherosclerosis (28). Through ionic interactions between positively charged residues of the lipoproteins and negatively charged residues in the extracellular matrix molecules, in particular of proteoglycans, atherogenic lipoproteins that gain entry to the subendothelial space are bound and retained in the subendothelium (29,30). As a result of exposure to free radicals or reactive oxygen species, lipids and in particular low-density lipoproteins (LDL) become modified by oxidation, ultimately resulting in lipid peroxidation (31). Such modifications initially give rise to mildly oxidised LDL, which is recognised by the normal LDL receptor but not by the scavenger receptors of macrophages. Further oxidation of the LDL particles leads to highly oxidised LDL, which can be recognised by both receptor species mediating internalisation into macrophages resulting in the formation of foam cells (32,33).

In the last decades, evidence emerged that atherosclerosis is characterised by a chronic condition of low-grade inflammation. Several inflammatory cells including monocyte-derived macrophages, T-lymphocytes and smooth muscle cells have been found to accumulate at the site of an atherosclerotic plaque (28). In the activation and chemotaxis of these cells, adhesion molecules expressed on endothelial cells as well as immune cells and chemoattractant molecules play an important role (28,34,35).

Finally, this chronic inflammatory process may result in an acute clinical event. Most common complications of an atherosclerotic plaque result from plaque rupture and arterial thrombus formation (28). Arterial thrombus formation at the site of an atherosclerotic plaque results from a misbalance between thrombogenesis and thrombolysis. Platelet adhesion, activation and aggregation as well as blood

coagulation, resulting in fibrin formation, and fibrinolysis are involved in the regulation of the thrombotic tendency (28,34-36).

CARDIOVASCULAR DISEASE RISK MARKERS

Lipids and lipoproteins

The concentrations of total cholesterol are positively associated with mortality rates from cardiovascular diseases (37). Because cholesterol is transported in human blood by lipoproteins, in particular by the atherogenic low-density lipoproteins (LDL), LDL particles are a major determinant of serum total cholesterol concentrations. In contrast to LDL cholesterol, increased concentrations of cholesterol in the high-density lipoproteins (HDL), also called the 'good' cholesterol, may protect against cardiovascular diseases (38). Therefore, a decrease of the LDL or an increase of HDL cholesterol concentrations reduces cardiovascular disease risk. Calculated from the concentrations of total and HDL cholesterol, the ratio of total to HDL cholesterol is a strong indicator of cardiovascular disease risk. In the Netherlands, the total to HDL cholesterol ratio is used together with other major risk markers i.e. gender, age, smoking, the presence of diabetes mellitus, and the presence of hypertension to predict the risk to develop coronary heart disease in the following 10 y as summarised in the cholesterol consensus (39).

Thrombotic tendency

Several thrombogenic factors are involved in the development of atherosclerosis and/or in the initiation of thrombosis at the atherosclerotic surface. Since platelets are importantly involved in arterial thrombus formation (40), measurements of platelet aggregation are widely used to examine platelet function. The principle method in platelet studies is the *in vitro* platelet aggregation test, whereby platelet aggregation is initiated *in vitro* by an inducer such as adrenalin, thrombin, collagen or adenosine diphosphate (ADP). Either whole blood or plasma enriched in platelets, platelet-rich plasma (PRP), can be used for these analyses. Indeed, several studies confirmed the association between *in vitro* platelet aggregation and the risk to develop cardiovascular diseases (41,42) but the predictive value of a platelet aggregation test remains unclear (43,44). As it is difficult to assess platelet function *in vivo*, an *ex vivo* method has been developed to measure platelet aggregation using the filrtragometer (45).

With respect to the blood coagulation system, often activity of coagulation factor VII, and concentrations of fibrinogen or prothrombin fragments 1 and 2 have been measured in dietary studies. Several prospective epidemiologic studies have

associated these hemostatic markers to cardiovascular disease risk (46-48). Also a decreased fibrinolytic function, which can be due to an increased concentration or activity of plasminogen activator inhibitor-1 (PAI-1), or an impaired possibility to release tissue plasminogen activator (tPA) are correlated with the development of cardiovascular diseases (47,49). In addition, concentrations of the resulting tPA/PAI-1 complex are elevated in patients with cardiovascular diseases (50).

Lipid peroxidation

The atherogenicity of lipid particles is largely increased by lipid peroxidation (32). Initially, most dietary studies measured lipid peroxidation indirectly as susceptibility of LDL to modification by oxidizing agents such as metal ions in an *in vitro* situation. Recently, assays to measure lipid peroxidation *in vivo* have been developed. Isoprostanes are a complex family of prostaglandin isomers which are produced by the oxidative modification of polyunsaturated fatty acids and are therefore direct biomarkers of lipid peroxidation (51-53). Urinary concentrations of isoprostanes have been positively associated with coronary heart disease (54).

Inflammation

Over the past years, several inflammatory risk markers have been suggested to be involved in cardiovascular diseases (55-57). High sensitivity C-reactive protein (hsCRP) is a circulating acute phase reactant that reflects systemic inflammation. It has been associated with cardiovascular events (58) and the severity of atherosclerosis (59).

As response to injury, human peripheral blood mononuclear cells (PBMC) in particular monocytes and lymphocytes produce a wide range of immunomodulatory molecules. Although insights in their functions in inflammatory processes and the pathogenesis of atherosclerosis are accumulating, their use as prognostic inflammatory markers in cardiovascular diseases remains to be established (60).

FATTY ACIDS AND CARDIOVASCULAR DISEASES

Almost four decades ago, Keys and colleagues (61) already evaluated the differences in prevalence of coronary heart disease in 16 defined cohorts from seven countries (Finland, Greece, Italy, Japan, The Netherlands, the United States, and Yugoslavia). The Seven Countries Study has been the first study that showed a strong correlation between the dietary intake of saturated fatty acids and the incidence and mortality from cardiovascular diseases after 5 y of follow-up (61). Recently, the fatty acid composition of duplicate portions of the foods in the seven

countries was analysed accurately and the vital status of the participants was verified after 25 y of follow-up (62). Mortality rates from cardiovascular diseases were strongly associated with the average intakes of the four most common saturated fatty acids, lauric, myristic, palmitic, and stearic acids. For lauric and myristic acids, these relationships depended on serum total cholesterol concentrations, whereas palmitic and stearic acids were more strongly related to cardiovascular disease mortality rates than to serum total cholesterol concentrations (62). This may suggest that these latter two fatty acids also exert their effects on cardiovascular mortality by other metabolic pathways than lipid and lipoprotein metabolism. Several later studies supported the association between the dietary intake of saturated fatty acids and the mortality from cardiovascular diseases. In addition, monounsaturated and polyunsaturated fatty acids have been reported to be inversely associated with cardiovascular disease risk. A comprehensive review of epidemiological studies has been published recently (63).

In contrast, data of within population studies are less unequivocal. Moreover, the interpretation of these studies is complicated by small study sizes, inadequate dietary assessments and heterogeneity of the study designs (63,64). In a large prospective cohort study in the United States, the Nurses' Health study, the incidence of cardiovascular diseases after 14 y of follow-up was correlated to the dietary intake of saturated fatty acids in women (65). In this study, however, the relative risk to develop cardiovascular diseases was strongest associated with the intake of *trans* fatty acids. Dietary intakes of monounsaturated and polyunsaturated fatty acids were inversely associated. Total dietary fat intake was not significantly related to cardiovascular disease risk (65). Later analyses, after 20 y of follow-up, found similar relationships (66). Hence, the replacement of saturated and *trans* unsaturated fatty acids in the diet by monounsaturated and polyunsaturated fatty acids would be more effective in preventing cardiovascular diseases than reducing overall fat intake. Therefore, cardiovascular disease risk would depend on the quality rather than the quantity of dietary fat (67).

Because prospective epidemiologic studies are quite difficult to perform and susceptible to confounding which may reveal inconsistent results, in most human studies the effects of dietary fats on the risk of cardiovascular diseases have been estimated in randomised clinical trials mainly from their effects on cardiovascular disease risk markers, in particular serum lipid and lipoprotein concentrations.

Effects of fatty acids on lipids and lipoproteins

In the 1950s and 1960s Keys *et al* (68) and Hegsted *et al* (69) summarised the results of several well-controlled studies. The relationships between changes in

dietary fatty acid intake and changes in serum total cholesterol in men have been described in the following predictive equations:

$$\Delta \text{ serum total cholesterol (mmol/L)} = 0.03 \times (2 \times \Delta \text{Sat}_{12-16}' - \Delta \text{Poly}) \quad (68)$$

$$\Delta \text{ serum total cholesterol (mmol/L)} = 0.06 \times \Delta S - 0.04 \times \Delta P - 0.01 \quad (69)$$

Relative to an iso-energetic amount of carbohydrates, saturated fatty acids (S or Sat) increased serum total cholesterol concentrations. In contrast, polyunsaturated fatty acids (P or Poly) like linoleic acid reduced serum total cholesterol concentrations about half as much as saturated fatty acids raised it (68,69). The major cholesterol-raising saturated fatty acid appeared to be myristic acid. As exception to the other saturated fatty acids, stearic acid had little or no effect on serum total cholesterol concentrations. Also monounsaturated fatty acids did not change serum total cholesterol concentrations and were therefore assumed to be cholesterol-neutral (68,69). These earlier studies, however, did not differentiate between the effects on LDL and HDL cholesterol nor were gender effects examined.

Table 1.2 Predictive equations for mean changes in concentrations of serum total (TC), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol in mmol/L when 1% of energy of dietary carbohydrates is replaced iso-energetically by fatty acids.

| Reference | Equations |
|-----------------------------|---|
| Mensink and Katan, 1992 | (70) $\Delta \text{TC} = 0.039 \times \Delta S - 0.003 \times \Delta M - 0.015 \times \Delta P$ $\Delta \text{LDL} = 0.033 \times \Delta S - 0.006 \times \Delta M - 0.0014 \times \Delta P$ $\Delta \text{HDL} = 0.012 \times \Delta S + 0.009 \times \Delta M + 0.007 \times \Delta P$ |
| Hegsted <i>et al</i> , 1993 | (71) $\Delta \text{TC} = 0.0543 \times \Delta S - 0.0301 \times \Delta P + 0.00725 \times \Delta C$ $\Delta \text{LDL} = 0.0449 \times \Delta S - 0.0198 \times \Delta P + 0.00475 \times \Delta C$ $\Delta \text{HDL} = 0.0110 \times \Delta S + 0.0026 \times \Delta M + 0.0056 \times \Delta P + 0.0047 \times \Delta C$ |

In later studies regression equations were developed to predict dietary responsiveness of concentrations of total, LDL, and HDL cholesterol, and triacylglycerols to saturated, monounsaturated, and polyunsaturated fatty acids (70,71). Predictive equations of these two studies are summarised in **Table 1.2**. More recent meta-analyses even distinguished between the effects of the individual saturated fatty acids (72,73). Yu *et al* (72) provided new evidence that stearic acid would have unique effects on total, LDL, and HDL cholesterol concentrations. Unlike the other saturated fatty acids, stearic acid appeared to be cholesterol-neutral (72). In another meta-analysis of 60 clinical trials the effects of fatty acids were estimated on concentrations of total, LDL, and HDL cholesterol, and of triacylglycerols as well as on the ratio of total to HDL cholesterol. Three distinct models were used to

estimate effects of the major lipid classes (saturated, monounsaturated, and polyunsaturated fatty acids), the individual saturated fatty acids (lauric acid, myristic acid, palmitic acid, and stearic acid), and *trans* fatty acids (73). Interestingly, in this meta-analysis, effects of saturated and polyunsaturated fatty acids also were less than predicted by the equations of Keys *et al* (68) and Hegsted *et al* (69). Moreover, lauric acid had a more favourable effect on the total to HDL cholesterol ratio than any other saturated or unsaturated fatty acid.

While most dietary intervention studies focused on the effects of fatty acids on the lipoprotein profile, less data are available about the effects of dietary fatty acids on biomarkers of thrombotic tendency, lipid peroxidation and inflammation.

OUTLINE OF THIS THESIS

This thesis describes the effects of fatty acids, in particular stearic acid, oleic acid, and linoleic acid, and the conjugated linoleic acid (CLA) isomers, *c9,t11* and *t10,c12* CLA on risk markers of cardiovascular diseases and on fatty acid desaturation and elongation.

In **Chapter 2** the effects of fatty acids are described on atherosclerotic risk markers. We have critically reviewed the literature on this topic and focused on the influence of saturated, monounsaturated as well as polyunsaturated fatty acids on a variety of cardiovascular disease risk markers. In particular, effects on lipid and lipoprotein metabolism, lipid peroxidation, inflammation, and thrombotic tendency have been summarised.

In the following three chapters the results are discussed from a crossover study in which the effects of stearic acid, oleic acid, and linoleic acid on several cardiovascular disease risk markers have been compared. **Chapter 3** describes the effects of these three C18 fatty acids with increased degree of unsaturation on the serum lipoprotein profile. **Chapter 4** is focused on the effects on thrombotic tendency. Effects of stearic, oleic, and linoleic acids are reported on hematological variables, blood coagulation and fibrinolytic factors, and *in vitro* and *ex vivo* platelet aggregation. **Chapter 5** discusses the effects on markers of non-enzymatic and enzymatic lipid peroxidation, 8-iso-prostaglandin $F_{2\alpha}$ and 15-keto-dihydro-prostaglandin $F_{2\alpha}$, respectively, and inflammatory variables such as high-sensitivity C-reactive protein and the expression profile of immunomodulatory molecules.

In another human intervention study, presented in **Chapter 6**, the effects of the individual CLA isomers, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA were investigated on the fatty acid composition of phospholipids, triacylglycerols and cholesteryl esters. To relate these changes to the enzymes involved in desaturation and elongation, also mRNA expression of desaturases and elongases was measured in peripheral blood mononuclear cells.

Finally, in **Chapter 7** the main findings and implications of the studies described in this thesis are discussed.

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2

Fatty acids and atherosclerotic risk

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ABSTRACT

Most research concerning the effects of dietary fatty acids on atherosclerotic risk has focused on their effects on lipid and lipoprotein metabolism. However, it is known that fatty acids also influence a number of other relevant mechanisms involved in atherosclerosis such as lipid peroxidation, inflammation and hemostasis. The most favourable distribution of cholesterol over the various lipoproteins is achieved when a mixture of cis-unsaturated fatty acids replaces saturated and trans fatty acids. Furthermore, fatty acids from fish oil lower concentrations of triacylglycerols. Effects on other atherosclerotic risk markers are less evident. Monounsaturated fatty acids may be preferable above other fatty acids with respect to low-density lipoprotein (LDL) oxidation as measured by indirect in vitro assays. The relevance of these assays for the in vivo situation is, however, limited. With respect to inflammation, mainly the effects of n-3 polyunsaturated fatty acids from fish oil have been studied but results were inconsistent. Also results from studies evaluating the effects of fatty acids on hemostatic risk markers were inconsistent, which may be partly related to the use of different analytical methods. The most consistent finding, however, is the potential beneficial effect of moderate intakes of fish oil on platelet aggregation. Furthermore, reducing total fat intake rather than changing the fatty acid composition of the diet may beneficially affect the coagulation system.

In conclusion, while beneficial effects on atherosclerotic risk are mainly ascribed to cis-unsaturated fatty acids, it remains debatable whether trans and saturated fatty acids in the diet have to be replaced by cis-unsaturated fatty acids or by carbohydrates. To answer this question adequately more validated methods are needed that reflect in vivo lipid peroxidation, inflammation and hemostasis.

DIETARY FATTY ACIDS

Although dietary fats and oils always consist of a mixture of fatty acids, each fat and oil has its own characteristic fatty acid composition. Usually, one or two fatty acids are predominant (**Table 2.1**), each with its characteristics. Based on chain length, fatty acids can be classified as short-chain fatty acids (4 to 6 carbon atoms), medium-chain fatty acids (8 to 10 carbon atoms), long-chain fatty acids (12 to 18 carbon atoms) and very-long-chain fatty acids (more than 18 carbon atoms). In addition, fatty acids may vary in the number of double bonds. Major fatty acid classes are saturated fatty acids with no double bonds, monounsaturated fatty acids with one double bond, and polyunsaturated fatty acids with two or more double bonds. Based on the position of the double bond nearest to the methyl end of the carbon chain, fatty acids are divided into families. So palmitoleic acid, a metabolite of palmitic acid, belongs to the n-7 family, oleic acid to the n-9 family, linoleic acid to the n-6 family, and α -linolenic acid to the n-3 family. Finally, the configuration of the double bond can be *cis* or *trans*.

Table 2.1 Major fatty acids in some edible fats and oils.

| Common name | Formula | Source |
|------------------------------------|------------|---|
| <i>Saturated fatty acids</i> | | |
| Medium-chain fatty acids | C4:0-C10:0 | Dairy fat, coconut oil, palm kernel oil |
| Lauric acid | C12:0 | Dairy fat, coconut oil, palm kernel oil |
| Myristic acid | C14:0 | Dairy fat, coconut oil, palm kernel oil |
| Palmitic acid | C16:0 | Meat, palm oil |
| Stearic acid | C18:0 | Meat, cocoa butter |
| <i>Monounsaturated fatty acids</i> | | |
| Oleic acid | C18:1n-9 | Olive oil, rapeseed oil, avocado, nuts |
| <i>Polyunsaturated fatty acids</i> | | |
| Linoleic acid | C18:2n-6 | Sunflower oil, safflower oil, soybean oil |
| α -linolenic acid | C18:3n-3 | Soybean oil, rapeseed oil, flaxseed |
| EPA | C20:5n-3 | Fish |
| DHA | C22:5n-3 | Fish |

In most diets, about 30-40% of total dietary energy intake is provided by fat. Palmitic and stearic acids are the most prevailing saturated fatty acids, while the most widespread monounsaturated and polyunsaturated fatty acids are oleic acid and linoleic acid, respectively. Ultimately all fatty acids are degraded and oxidised for energy via β -oxidation in the mitochondria of cells. However, fatty acids not only provide energy but also are important structural components of cell membranes and

precursors of a wide range of eicosanoids (prostaglandins, thromboxanes and leukotrienes) involved in hemostasis and inflammation. Furthermore, fatty acids are ligands for transcription factors, thereby modulating gene expression. In this manner, dependent on their characteristics, fatty acids exert different effects on atherosclerotic risk markers. In this chapter, the effects of fatty acids on lipid and lipoprotein metabolism, lipid peroxidation, inflammation and hemostasis will be discussed (Table 2.2).

Table 2.2 Factors that are positively (\uparrow) or negatively (\downarrow) related to atherosclerotic risk.

| Risk marker | Atherosclerotic risk |
|---|----------------------|
| <i>Lipid and lipoprotein metabolism</i> | |
| Total cholesterol | \uparrow |
| LDL cholesterol | \uparrow |
| HDL cholesterol | \downarrow |
| Total to HDL cholesterol ratio | \uparrow |
| Triacylglycerols | \uparrow |
| <i>Oxidative stress</i> | |
| <i>In vitro</i> LDL susceptibility to oxidation | \uparrow |
| Oxidised LDL | \uparrow |
| F ₂ -isoprostanes | \uparrow |
| <i>Inflammation</i> | |
| Adhesion molecules | \uparrow |
| Pro-inflammatory cytokines | \uparrow |
| Anti-inflammatory cytokines | \downarrow |
| <i>Hemostatic function</i> | |
| Platelet aggregation | \uparrow |
| Coagulation | |
| Factor VII | \uparrow |
| Fibrinogen | \uparrow |
| Prothrombin fragment 1 and 2 | \uparrow |
| Fibrinolysis | |
| Tissue plasminogen activator (tPA) | \downarrow |
| Plasminogen activator inhibitor 1 (PAI-1) | \uparrow |
| D-dimers | \uparrow |

FATTY ACIDS AND LIPOPROTEIN METABOLISM

Lipoproteins are the major transporters of lipids in the blood. While the classical risk factor for coronary heart disease is an increased concentration of serum total cholesterol, later studies have demonstrated that the various lipoproteins each have

its specific effects on cardiovascular risk. Two major cholesterol-transporting lipoproteins are the low-density lipoproteins (LDL) and high-density lipoproteins (HDL), carrying respectively 60-70% and 20-30% of the total amount of cholesterol in the blood. While LDL is atherogenic, HDL may protect against atherosclerosis. Epidemiological studies now suggest that an increment of 0.1 mmol/L in LDL cholesterol results in an increase of 3.5-4.0% in cardiovascular risk, while an increase of 0.1 mmol/L in HDL cholesterol lowers cardiovascular risk with 8-12% (1). However, the total to HDL cholesterol ratio may be an even more specific marker to predict cardiovascular risk than total or lipoprotein cholesterol concentrations. A decrease of 0.1 unit in this ratio is associated with a 5.3% reduction in the risk of myocardial infarction (2). Another, less-validated, risk marker is the concentration of triacylglycerols. Triacylglycerols, which are mainly found in the very-low density lipoproteins (VLDL), are positively related to cardiovascular risk and a 0.1 mmol/L increase in triacylglycerols is associated with a 1.4% increase in cardiovascular risk for men and a 3.7% increase for women (3).

As outlined below, these cardiovascular risk markers are differently affected by the various fatty acids in the diet. However, the definition of a cholesterol-raising or cholesterol-lowering fatty acid is not straightforward. When fat is added to a diet, energy intake increases and as result body weight will increase also. As body weight is an important determinant of serum total cholesterol concentrations, it will not be possible to disentangle dietary effects from those of changes in body weight. Therefore, in dietary intervention studies or meta-analyses intended to compare the effects of fatty acids on the serum lipoprotein profile, any change in the saturated, monounsaturated or polyunsaturated fatty acid, or carbohydrate composition of the diet is balanced by opposite changes in one or more of the others. Therefore, the effects of specific fatty acids are generally expressed relative to those of an iso-caloric amount of carbohydrates or of another fatty acid.

Mixtures of saturated fatty acids

Well-controlled dietary studies carried out in the 1950s and 1960s by Keys *et al* and Hegsted *et al* found that - relative to an iso-energetic amount of carbohydrates - a mixture of saturated fatty acids increased serum total cholesterol concentrations (4,5). These earlier studies, however, did not examine the effects of fatty acids on specific lipoproteins. From a recent meta-analysis, it can be concluded that replacement of carbohydrates with saturated fatty acids not only increased serum total cholesterol concentrations but also those of LDL and HDL (**Figure 2.1**). The total to HDL cholesterol ratio, however, was not affected. In addition, effects on the

serum lipoprotein profile were dependent on the chain length of the saturated fatty acid (6).

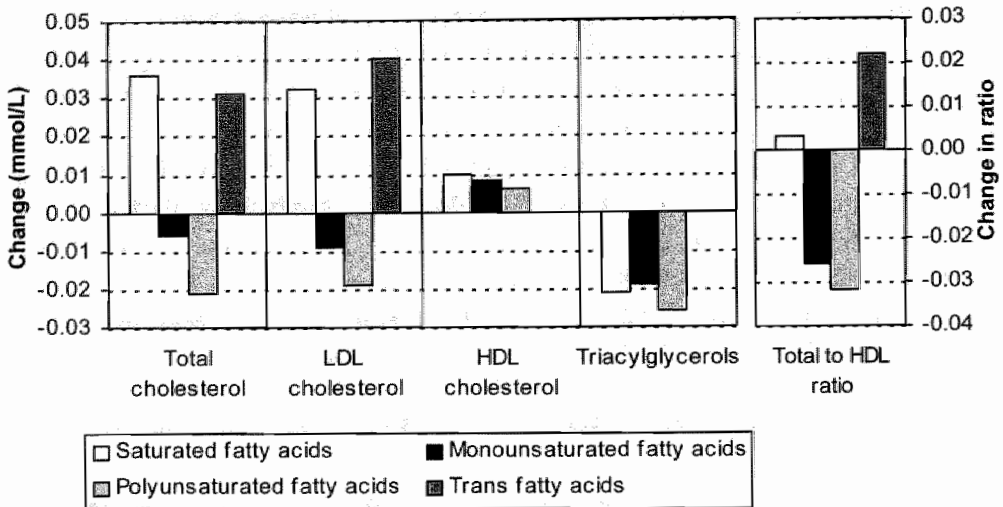


Figure 2.1 Effects of the major fatty acids on serum total, LDL, and HDL cholesterol, triacylglycerol concentrations, and total to HDL cholesterol ratio when 1% of dietary carbohydrates are replaced by fatty acids under iso-energetic conditions.

Medium-chain fatty acids

Although medium-chain fatty acids are found in significant quantities in several natural fats and oils, the total amount in regular diets is in general low. This may explain why effects of medium-chain fatty acids on lipoprotein metabolism have not been well studied. Initially, it was suggested that medium-chain fatty acids had a neutral effect on serum total cholesterol concentrations (4,5). Results from two recent studies, however, suggested that relative to oleic acid, medium-chain fatty acids slightly increased serum total and LDL cholesterol but did not affect HDL cholesterol concentrations. Triacylglycerol concentrations were slightly increased (7,8).

Lauric, myristic, and palmitic acids

Palmitic acid (C16:0) along with lauric (C12:0) and myristic (C14:0) acids, are the most potent cholesterol-raising saturated fatty acids. Their relative cholesterol elevating effects, however, are controversial. In fact, it is difficult to examine the effects of the individual saturated fatty acids, because in natural fats high levels of one fatty acid are associated with high levels of another fatty acid. Coconut oil, for example, contains high amounts of both myristic and lauric acids, while dairy fat is

rich in both myristic and palmitic acids. This makes it difficult to ascribe the observed effects to one single fatty acid. Therefore, more recent studies have used synthetic and semi-synthetic fats, specifically enriched in one of the saturated fatty acids.

Palmitic acid is the major saturated fatty acid in the diet. It is well accepted that palmitic acid raises total, LDL and HDL cholesterol and decreases triacylglycerol concentrations, relative to carbohydrates. Effects of palmitic acid relative to those of oleic acid are more controversial. In studies that compared the effects of palmolein oil (rich in palmitic acid) with olive oil (rich in oleic acid), palmitic and oleic acids had comparable effects on the serum lipoprotein profile (9,10). However, the majority of well-controlled studies have found that relative to oleic acid, palmitic acid increased total and LDL cholesterol concentrations (11-13).

Myristic acid has for long been suspected to be the most cholesterol-raising fatty acid (4). Several studies concluded that myristic acid increased total cholesterol concentrations relative to oleic acid, due to an increase in LDL as well as in HDL cholesterol (7,13). However, these effects of the semi-synthetic fats enriched in myristic acid were much less than suggested by two independent meta-analyses (4,6).

Lauric acid is the fourth most common saturated fatty acid in the diet after palmitic, stearic and myristic acids. Hegsted *et al* (4) already reported that lauric acid had only a mild cholesterol-raising effect relative to carbohydrates. The effects of lauric acid on lipoprotein concentrations were compared with those of oleic acid by Denke and Grundy (11). It was concluded that lauric acid elevated total and LDL cholesterol concentrations relative to oleic acid but did not have any effect on HDL cholesterol and triacylglycerol concentrations. Using mixtures of natural fats, these results were confirmed by Temme *et al* (12) but in that study also an additional, significant increase in HDL cholesterol concentrations was observed.

In a recent meta-analysis, the effects of the individual saturated fatty acids on the serum lipoprotein profile have been estimated (6). Iso-energetic replacement of carbohydrates with lauric, myristic, and palmitic acids all resulted in increased total, LDL and HDL cholesterol concentrations (**Figure 2.2**). With increasing chain length, these effects decreased. Because the cholesterol-raising effects of lauric acid were proportionally higher on HDL than on LDL cholesterol, replacement of carbohydrates by lauric acid resulted in a significantly lower total to HDL cholesterol ratio, which suggests a decrease in atherosclerotic risk. Compared with carbohydrates, myristic and palmitic acids did not affect the ratio of total to HDL cholesterol, whereas lauric, myristic, and palmitic acids lowered triacylglycerol concentrations to the same extent (6).

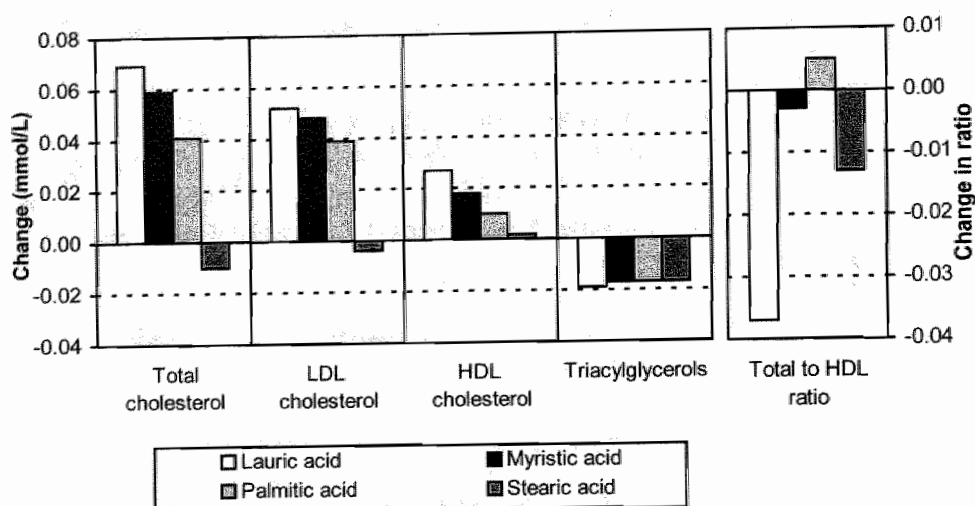


Figure 2.2 Effects of the individual saturated fatty acids lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) on serum total, LDL, and HDL cholesterol, triacylglycerol concentrations, and total to HDL cholesterol ratio when 1% of dietary carbohydrates is replaced by a specific fatty acid under iso-energetic conditions.

Stearic acid

Compared with the other long-chain saturated fatty acids, stearic acid significantly lowered total, LDL and HDL cholesterol concentrations (14,15). In fact, it has been found that stearic acid and oleic acid, the major monounsaturated fatty acid, had similar effects on serum lipoproteins (16). However, other studies found an HDL cholesterol-lowering effect of stearic acid relative to unsaturated fatty acids (17,18). Thus, stearic and oleic acids are equivalent in their effects on LDL cholesterol and triacylglycerols but may differ somewhat in their effects on HDL. These effects of stearic acid were confirmed by a meta-analysis, which furthermore reported that stearic acid did not change the total to HDL cholesterol ratio when compared with carbohydrates (6).

Monounsaturated fatty acids

Mortality rates of coronary heart disease in traditional Mediterranean populations consuming high-fat diets rich in olive oil, a major source of monounsaturated fatty acids, are low (19). Previous studies concluded that monounsaturated fatty acids had similar effects on serum total cholesterol concentrations as carbohydrates (4,5). Hence, many researchers compared the effects of monounsaturated fatty acids, in particular of oleic acid, and carbohydrates on the distribution of cholesterol over the

different lipoproteins (20,21). From these studies, it appeared that effects of oleic acid and carbohydrates on total cholesterol concentrations are indeed similar but that oleic acid increased HDL cholesterol and lowered VLDL cholesterol and triacylglycerol concentrations. As a result, a significant decrease in the total to HDL cholesterol ratio was observed. Similar conclusions were drawn based on results of a meta-analysis (6). Thus, monounsaturated fatty acids have a more favourable effect on atherosclerotic risk than carbohydrates, because of the increase in HDL and decrease in VLDL concentrations.

Polyunsaturated fatty acids

Polyunsaturated fatty acids belong to either the n-6 or n-3 families. Unlike saturated and monounsaturated fatty acids, the polyunsaturated fatty acids, linoleic acid and α -linolenic acid, cannot be synthesised *de novo* by humans. These fatty acids need to be provided by the diet and are therefore called essential fatty acids. The most abundant essential fatty acid in the diet is linoleic acid (C18:2n-6), whereas a small part of the dietary polyunsaturates is provided by α -linolenic acid (C18:3n-3). Linoleic acid, a member of the n-6 family of fatty acids, serves as the precursor of arachidonic acid (C20:4n-6), which has important biological effects in the body. α -Linolenic acid, an n-3 fatty acid, can be converted into eicosapentaenoic acid (C20:5n-3, EPA), which can be further elongated, desaturated and β -oxidised into docosahexaenoic acid (C22:6n-3, DHA). However, the major part of the very-long chain fatty acids in the human body are provided through the consumption of fatty fish, rich in EPA and DHA.

N-6 polyunsaturated fatty acids

Relative to carbohydrates, Keys *et al* (5) have estimated that the hypocholesterolemic effect of linoleic acid is half as much as the hypercholesterolemic effect of saturated fatty acids. However, more recent meta-analyses reported slightly less but still significant effects of linoleic acid, not only on serum total cholesterol but also on LDL cholesterol concentrations. Moreover, linoleic acid lowered triacylglycerols and increased HDL cholesterol concentrations compared with carbohydrates. Although linoleic acid may raise HDL cholesterol less than monounsaturated and saturated fatty acids, linoleic acid still has the most favourable effect on the total to HDL cholesterol ratio (6).

To investigate whether linoleic acid is more beneficial than oleic acid, several studies compared the effects of linoleic acid with those of oleic acid side-by-side. However, results are inconsistent, which may relate to differences in the intake of linoleic acid. When intake of linoleic acid exceeds 15% of energy, linoleic acid

lowered serum total, LDL, and HDL cholesterol, and serum triacylglycerol concentrations compared to oleic acid (22). At more realistic intakes of linoleic acid (less than 15% of energy), differences in effects on lipoprotein profile between linoleic and oleic acids are marginal (23-25).

N-3 polyunsaturated fatty acids

The principal n-3 fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are typically present in fatty fish and fish oils. Although normally dietary intakes of these long-chain, highly unsaturated fatty acids are very low, almost 30 times lower than that of linoleic acid (26), these fatty acids lower triacylglycerols and VLDL concentrations compared to carbohydrates and other fatty acids. Furthermore, fish fatty acids may slightly raise LDL cholesterol, especially in hypertriglyceridemic subjects but do not affect HDL cholesterol (27).

The metabolic precursor of the marine n-3 fatty acids is α -linolenic acid, a plant-derived fatty acid. The effects of α -linolenic acid are comparable to those of linoleic acid, an n-6 polyunsaturated fatty acid. In particular, the characteristic effects of EPA and DHA on serum triacylglycerol concentrations are not shared by α -linolenic acid (27).

Trans fatty acids

Trans and *cis* isomers of unsaturated fatty acids are produced during hydrogenation of vegetable oils, either by bacteria in the first stomach (rumen) of ruminant animals or by industrial hardening of oil. The main purpose of this latter process is to convert the liquid oil into a solid or semi-solid fat, which can be used for the production of certain types of margarines or shortenings for frying or baking. More than 80% of all *trans* fatty acids in the diet are *trans* isomers of oleic acid, and more than 10% are isomers of *trans* linoleic acid.

Many studies have shown unfavourable effects of *trans* fatty acids on serum lipids. Relative to *cis*-monounsaturated fatty acids, *trans*-monounsaturated fatty acids raise total and LDL cholesterol, and lower HDL cholesterol concentrations, resulting in an increased total to HDL cholesterol ratio. Furthermore, *trans* fatty acids elevate triacylglycerol concentrations (17,28,29). Effects of *trans* fatty acids and saturated fatty acids on total and LDL cholesterol concentrations are not very different. However, *trans* fatty acids lower HDL cholesterol as compared to saturated fatty acids (28,29). This means that the total to HDL cholesterol ratio is also unfavourably changed (6). Therefore, *trans* fatty acids have the worst effects on blood lipids among all dietary fatty acids.

Conclusions

Dietary fatty acid composition affects the distribution of cholesterol over the various lipoproteins. Under iso-energetic metabolic conditions, the most favourable lipoprotein profile to lower atherosclerotic risk is achieved when a mixture of *cis*-unsaturated fatty acids replaces saturated and *trans* fatty acids. However, which unsaturated fatty acid - oleic acid, linoleic acid or fish fatty acids - is the most beneficial, is hard to conclude, because fatty acids also affect other pathways involved in the development of atherosclerosis. Furthermore, it should be noted, that not much is known about the effects of diet on the composition and particle size distribution of LDL, HDL, and VLDL, which may also affect cardiovascular risk. For example, carbohydrates lower LDL cholesterol concentrations but at the same time unfavourably change LDL particle size (30). These effects are more difficult to translate into cardiovascular risk and are as yet not a solid basis for dietary recommendations. Therefore, more information is needed to elucidate how fatty acids affect lipid and lipoprotein metabolism at the molecular level.

FATTY ACIDS AND LIPID PEROXIDATION

Reactive oxygen species, as present *in vivo*, induce the oxidation of lipids. This may lead to oxidative modification of LDL, which is critical in the initiation and evolution of atherosclerosis. Increased uptake of oxidised LDL by macrophages via the scavenger pathway results in the formation of foam cells and, ultimately, atherosclerotic plaques (**Figure 2.3**). Oxidised LDL is cytotoxic and induces atherogenic mechanisms such as chemotaxis, and transmigration and transformation of monocytes into macrophages. In addition, oxidised LDL is a potent inducer for the production of inflammatory molecules (31).

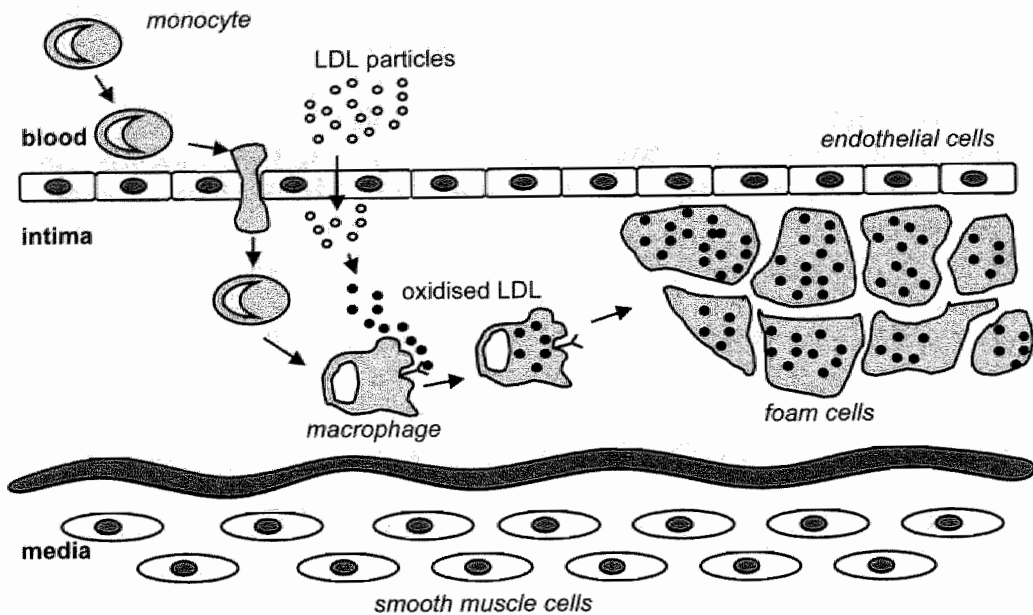


Figure 2.3 Involvement of lipid peroxides and inflammation in the development of atherosclerosis.¹

¹Endothelial dysfunction causes increased endothelial permeability to lipoproteins and up-regulation of leukocyte and endothelial adhesion molecules followed by the recruitment of monocytes and T lymphocytes into the arterial wall. LDL enters the intima layer of the vascular endothelium and is modified by reactive oxygen species into oxidised LDL. Oxidised LDL causes endothelial damage resulting in the release of cytokines. Monocytes recruited into the artery wall become macrophages and express scavenger receptors that bind oxidised LDL particles. Macrophages become lipid-loaded foam cells by engulfing oxidised LDL.

A variety of methods had been developed to assess LDL oxidation but no golden standard exists. In fact, there is a clear need for validated biomarkers to measure *in vivo* lipid peroxidation and LDL oxidation. Methods for evaluation of LDL oxidation include direct and indirect assays. Direct assays measure certain lipid peroxides, such as malondialdehydes (MDA), thiobarbituric acid reactive substances (TBARS), or conjugated dienes. However, these assays lack specificity in particular for body fluids and tissue samples. In this respect, measurement of isoprostanes is more promising. Isoprostanes are isomers of prostaglandin, which are primarily generated by free-radical mediated peroxidation of polyunsaturated fatty acids and are chemically stable. Urinary concentrations of F₂-isoprostanes were indeed increased in patients with hypercholesterolemia (32). Another assay measures the concentrations of MDA-modified LDL, a lipid peroxide decomposition product, which

is increased in patients with unstable atherosclerotic cardiovascular disease (33). Also the amount of circulating oxidised LDL can be measured and is associated with cardiovascular diseases (34). Furthermore, the presence of auto-antibodies against epitopes on oxidised LDL can be quantified and is an independent predictor of the progression of atherosclerosis (35). However, all of these assays need further validation. In the past, mainly indirect assays were used, which measured the *in vitro* susceptibility of LDL to oxidation induced by metal ions. Three parameters are usually measured in these assays. The lag time indicates the time until oxidation of the LDL particle starts, whereas the rate of oxidation denotes the amount of peroxidation products formed per unit of time. Finally, the total amount of lipid peroxidation products formed can be analysed. Frequently used methods are based on the spectrophotometric measurements of the cytotoxic aldehydes, conjugated dienes, lipid hydroperoxides or apo B-100 fluorescence after induction of LDL oxidation with copper (35). Although this has been the most frequently used assay to examine effects of fatty acids on LDL oxidation, the relevance of these outcome parameters for the *in vivo* situation is doubtful.

Due to the presence of double bonds, the susceptibility of fatty acids to oxidative modification increases with the degree of unsaturation. Because the fatty acid composition of the diet is reflected by the fatty acid composition of the LDL particle, dietary fat not only determines LDL cholesterol concentrations but also the *in vitro* susceptibility of LDL to oxidative modification. Particle size may also be important, as small dense LDL is more readily modified than larger LDL (36) but effects of fatty acid intake on LDL particle size as related to LDL modification has not been studied in detail.

Dietary fat and saturated fatty acids

Low-fat diets and high-fat diets rich in monounsaturated fatty acids do not differ in their effects on the susceptibility of LDL to oxidation (37). Effects of saturated fatty acids on LDL oxidation have not been well examined. In theory, saturated fatty acids should affect *in vitro* LDL susceptibility beneficially, because they do not have any double bonds. However, some studies surprisingly found unfavourable effects on the susceptibility of LDL to oxidation, when monounsaturated fatty acids were replaced by saturated fatty acids (38,39). In particular, the lag time was decreased. This suggests that minor dietary components from edible oils affect LDL oxidation as well.

Oleic acid versus linoleic acid

Several human studies have shown that enrichment of the diet with oleic acid at the expense of linoleic acid increased resistance of LDL to oxidative modification. When oleic acid is replaced with linoleic acid, lag time decreased, oxidation rate increased, and production of conjugated dienes was higher after copper-induced LDL oxidation (38,39). Whether or not these results also indicate that compared with oleic acid, linoleic acid elevates atherogenicity of lipoproteins *in vivo* has to be shown.

N-6 and n-3 polyunsaturated fatty acids

Replacement of n-6 with n-3 polyunsaturated fatty acids in the normal diet did not affect lag time and TBARS but conjugated diene production was significantly increased after the n-3 enriched diet (39). In another study, effects of fish oil and oils rich in n-6 polyunsaturated fatty acids were compared using indirect (*in vitro* LDL oxidation and TBARS) and direct (F_2 -isoprostanes and MDA-modified LDL) assays but a trend towards higher LDL susceptibility to *in vitro* oxidation was only found on the diet rich in n-3 fatty acids (40). Only one study evaluated the effects of α -linolenic acid, and found a beneficial effect of α -linolenic acid on susceptibility of LDL to oxidation above EPA and DHA (41).

The effects of diets supplemented with n-3 polyunsaturated fatty acids, mainly EPA and DHA from fish oils, on LDL oxidation are contradictory. Using fish oil supplements for 3 or 6 weeks, some studies observed an increased susceptibility of LDL to oxidation (42,43) but other studies did not see any effects when supplements were given for 2 or 4 months (44,45). Not only may the duration of supplementation but also differences in the doses of n-3 polyunsaturated fatty acids explain these apparent discrepancies.

Conclusions

While polyunsaturated fatty acids have a larger hypocholesterolemic effect, *in vitro* assays suggest that the effects of monounsaturated fatty acids on LDL oxidation are the most beneficial. However, the relevance of these indirect, *in vitro*, assays to measure LDL oxidation, for the *in vivo* situation is limited. Future studies should therefore use more direct measurements of lipid peroxidation products such as F_2 -isoprostanes and MDA-modified LDL.

FATTY ACIDS AND INFLAMMATION

During the early phases of plaque development, inflammatory processes already play an important role, starting with the interaction between the vascular

endothelium and circulating blood leukocytes. After recruitment and infiltration of mainly monocytes and T-lymphocytes into the arterial intima, monocytes are transformed into macrophages, which can take up oxidised LDL rapidly. This process results in the formation of foam cells and ultimately a fatty streak (46). Moreover, T-lymphocytes produce several pro- and anti-inflammatory cytokines, which play an important role in orchestrating the inflammatory process (47). More recently, C-reactive protein (CRP), an acute phase reactant produced by the liver during systemic inflammation, has also been identified as an important risk marker for cardiovascular disease (48).

Endothelial cell adhesion

Cellular adhesion molecules mediate the attachment of leukocytes to vascular endothelial cells and the subsequent trans-endothelial migration of monocytes and lymphocytes into the arterial wall. While selectins are involved in the initial rolling of the circulating leukocyte over the endothelium, other cellular adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), mediate the final firm attachment (46).

Because *in vivo* the accessibility of human endothelial cells is limited, effects of fatty acids on expression of adhesion molecules have been examined mainly *in vitro* using endothelial cell lines. From a series of experiments in which endothelial cells were incubated with various fatty acids, it was concluded that with increased degree of unsaturation of the fatty acid, VCAM-1 expression on the surface of endothelial cells decreased. These effects were observed when cells were stimulated with lipopolysaccharide or cytokines (interleukin-1 α (IL-1 α) or interleukin-1 β (IL-1 β) or tumour necrosis factor α (TNF α)). Inhibitory potencies of the fatty acids were not influenced by chain length, *cis/trans* configuration, or the position of the double bond. Results were confirmed by analysis of messenger RNA (mRNA) expression of VCAM-1. Without stimulation, however, none of the fatty acids affected VCAM-1 expression (49). In contrast, another study reported that the addition of linoleic acid and α -linolenic acid to the medium increased mRNA expression of ICAM-1 and VCAM-1 even in unstimulated endothelial cells. On the other hand, oleic acid inhibited mRNA expression of these adhesion molecules (50). Whether these changes in mRNA expression also resulted in changes in surface protein expression was not examined. These contrasting findings indicate that *in vitro* findings depend on the experimental conditions used and are difficult to extrapolate to the *in vivo* situation.

In contrast with endothelial cells, peripheral blood mononuclear cells (PBMC) are easily sampled which gives the opportunity to use PBMC to examine the effects of

dietary fatty acids on the expression of the ligands for the endothelial adhesion molecules. Furthermore, ICAM-1 is also present on PBMC. Until now, not many studies have made use of these possibilities. In one study, effects of fish oil supplementation for 3 weeks were studied on ICAM-1 and LFA-1 expression, the ligand for ICAM-1. After stimulation of monocytes with interferon- γ , the expression of these adhesion molecules was lowered in the fish oil group relative to baseline values and to those of control subjects (51). In a study with healthy volunteers, who consumed diets enriched with α -linolenic acid or fish oil for 12 weeks, no effect on ICAM-1 surface expression was found. In this latter study, PBMC were not stimulated (52). In another study with healthy men, the effects of consumption for 2 months of a diet rich in monounsaturated fatty acids were compared with those of a regular diet. Expression of ICAM-1 on PBMC was decreased in the subjects on the monounsaturated fatty acid enriched diet (53).

Alternatively, the soluble variants of the above mentioned adhesion molecules - sICAM-1, sVCAM-1 and sE-selectin - can be analysed in plasma. Increased concentrations of these soluble adhesion molecules in the blood, which may indicate increased expression of membrane-bound molecules and impaired endothelial function, are indeed associated with future cardiovascular events in apparently healthy individuals (54). It should be emphasised, however, that sVCAM-1 and sE-selectin are almost exclusively derived from endothelial cells, whereas ICAM-1 is expressed and shed from several cell types (55). In patients with increased atherosclerotic risk, the effects of supplementation with relatively high doses of n-3 fatty acids (4-5 g per day) have been investigated but results were inconsistent (56). In healthy subjects, α -linolenic acid and fish oil but not purified DHA, decreased sVCAM-1 expression. This suggests that possible favourable effects of fish oil should be attributed to EPA (57).

Cytokines

Inflammation is mediated by cytokines, which modulate infiltration and accumulation of immuno-competent cells (T-lymphocytes and macrophages) by increasing the expression of adhesion molecules by endothelial cells. Furthermore, cytokines mediate activation and proliferation of both smooth muscle cells and macrophages. With respect to atherosclerosis, cytokines can be divided into three major classes. The pro-inflammatory cytokines typically mediate pro-atherogenic processes, whereas anti-inflammatory cytokines are involved in anti-atherogenic pathways. Major pro-inflammatory cytokines are interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor α (TNF α), whereas interleukin-4 (IL-4) and interleukin-10 (IL-10) are examples of anti-inflammatory cytokines. In addition, some cytokines, such

as interferon- γ have pro- as well as anti-inflammatory effects (47). $\text{TNF}\alpha$, which is produced by endothelial cells, smooth muscle cells and macrophages, plays a pivotal role in the cytokine cascade as it stimulates the synthesis of other cytokines. Also IL-1 and IL-6 are versatile cytokines. For example IL-6 is a central mediator of the acute-phase response and the primary determinant of CRP production by the liver (58).

In one of the earlier studies, production of the pro-inflammatory cytokines IL-1 and $\text{TNF}\alpha$ by *in vitro* stimulated PBMC was suppressed after supplementation with a high dose (18 g fish oil per day) of n-3 polyunsaturated fatty acids (59). Because some (60) but not all (52,57) confirmed these results, it was suggested that *in vitro* production of pro-inflammatory cytokines is only decreased when EPA plus DHA was consumed for at least 4 weeks and daily intake exceeded 2.4 g (61). However, even long-term supplementation with 3.2 g of fish oil per day for 6 or 12 months did not decrease *in vitro* production of IL-1 and $\text{TNF}\alpha$ after whole-blood stimulation (62). Despite these inconsistent results, fish fatty acids are considered to exert anti-inflammatory properties.

In another study, it was found that replacement of the habitual fat from the diet of Dutch volunteers by palm oil reduced $\text{TNF}\alpha$ production, whereas IL-6 and IL-8 - two other pro-inflammatory cytokines - concentrations were not affected (63). Compared with soybean oil (linoleic acid with α -linolenic acid), hydrogenated fat rich in *trans* fatty acids increased production of IL-6 and $\text{TNF}\alpha$ but not of IL-1 in humans with moderately elevated LDL cholesterol levels. In this study, soybean oil and butter, the latter rich in saturated fatty acids, had similar effects (64). Finally, supplementation of arachidonic acid did not alter pro-inflammatory cytokine production (65).

Though most studies investigated *in vitro* or *ex vivo* cytokine production after exposing PBMC to an inflammatory stimulant, a few studies have evaluated the effects of in particular polyunsaturated fatty acids on circulating plasma cytokine concentrations. It was found that fish oil decreased circulating concentrations of several pro- as well as anti-inflammatory cytokines in patients with a wide variety of inflammatory diseases (61). In healthy volunteers, fish oil did not reduce plasma cytokine concentrations (62). Compared with linoleic acid, consumption of α -linolenic acid for 3 months decreased concentrations of the pro-inflammatory IL-6 in dyslipidemic patients (66).

C-reactive protein

With the newly developed high-sensitive assays, even slightly elevated C-reactive protein (CRP) concentrations can be detected in individuals with mild, non-overt inflammation that may result from the ongoing atherosclerotic process. However,

effects of fatty acids on serum CRP concentrations have not been studied very well. Some studies focused on the effects of n-3 fatty acids but results are equivocal. In studies with healthy volunteers no effects of fish oil supplements on CRP concentrations were found, despite reductions of several other acute-phase proteins (67,68). In dyslipidemic patients, however, replacement of linoleic acid for α -linolenic acid during 3 months reduced CRP concentrations, independently of lipid changes (66).

Conclusions

As atherosclerosis has a strong inflammatory component, it is important to examine the effects of fatty acids on inflammatory risk markers. Until now, many *in vivo* studies have focused on the effects of n-3 polyunsaturated fatty acids from fish oil. However, results are inconsistent. Effects of other fatty acids on *in vivo* inflammatory markers have been studied even less and this area of research clearly deserves further exploration. Furthermore, attention has to be paid to underlying mechanisms.

FATTY ACIDS AND THROMBOTIC TENDENCY

Under normal physiological conditions, hemostatic balance between thrombus formation and dissolution is regulated by the endothelial wall, blood platelets, and coagulation and fibrinolytic factors. Any disturbances of this delicate balance might result in activation of the hemostatic system and in increased thrombotic tendency. In this way, the most common complications of cardiovascular disease result from thrombus formation, caused by the exposure of blood platelets to the subendothelial matrix material or disruption of an atherosclerotic plaque (**Figure 2.4**). Thrombosis is initiated by platelet activation, adhesion and aggregation. Platelets become activated by compounds released from the endothelium, or exposed during rupture of an atherosclerotic plaque. After activation, platelets adhere on the place of injury and release their granules, which results in platelet aggregation. Activation of platelets also leads to the release of free arachidonic acid, which can be metabolised into eicosanoids. Following platelet activation, the coagulation cascade is initiated, resulting in the activation of several clotting factors. Ultimately fibrinogen is converted by activated thrombin into fibrin monomers, which polymerise into a fibrin network. In this fibrin network, blood cells and aggregated platelets are captured to form a thrombus, which can occlude the blood vessel. The dissolution of the blood thrombus is regulated by the fibrinolytic system (69).

Because of the findings from the 1960s showing a very low incidence of thrombosis among fish-eating Greenland Eskimos, considerable work has been

carried out in the past few decades to understand how dietary fat and fatty acids, especially long-chain n-3 fatty acids, affect hemostatic risk markers.

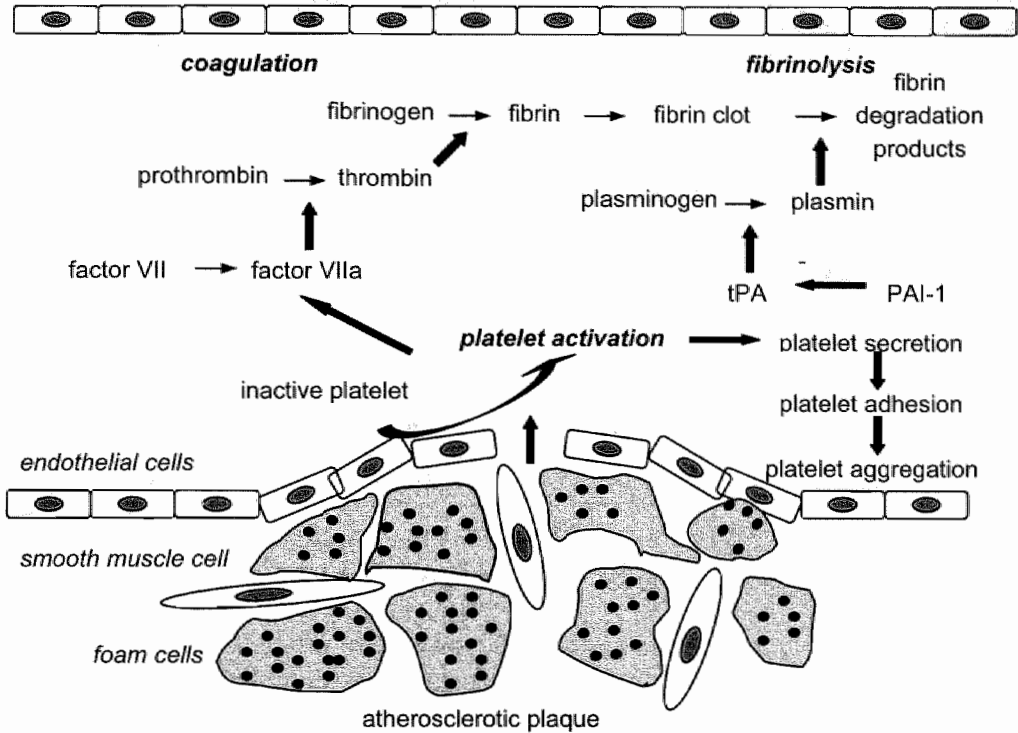


Figure 2.4 Schematic representation of the activation of the hemostatic system at the site of an atherosclerotic plaque.

Platelet aggregation

A frequently used method to assess platelet aggregability is the *in vitro* platelet aggregation test. However, this method measures the ability of platelets to react to a single external stimulus, a situation not comparable with platelet aggregation *in vivo*. Still, increased ADP-induced platelet aggregation is associated with increased atherosclerotic risk (70). Unfortunately, however, studies are difficult to compare due to the many different methods used to measure platelet aggregation. For example, blood can be anti-coagulated with citrate, heparin or hirudin, while platelet aggregation - in either whole blood or platelet-rich plasma - can be triggered with collagen, ADP, adrenalin or thrombin.

Many studies have focused on the effects of n-3 polyunsaturated fatty acids. In general, collagen-induced aggregation decreased, whereas results of ADP-induced

aggregation were very inconsistent (71). Effects of other fatty acids have also been examined but results are conflicting. When saturated fatty acids in the diet are replaced by oleic acid or linoleic acid, platelet aggregation was increased, decreased, or did not change. Comparable conflicting results have been found when oleic acid and linoleic acid were compared side-by-side (72-74). Some studies compared the effects of the different saturated fatty acids with each other. Relative to oleic acid, medium-chain fatty acids, lauric acid, myristic acid or palmitic acid did not affect collagen-induced aggregation in whole blood. Furthermore, ADP-induced aggregation was not changed by medium-chain fatty acids or myristic acid (75). Thus, dietary fatty acids can modulate platelet aggregation but the use of many different *in vitro* methods makes comparison and extrapolation to the *in vivo* situation difficult.

Eicosanoid production

Thromboxanes (TX) and prostaglandins (PG), two eicosanoids, play an important role in the hemostatic balance. Both types of eicosanoids are synthesised from the C20 fatty acids, arachidonic acid (C20:4n-6) and eicosapentaenoic acid (C20:5n-3, EPA) after release from membrane phospholipids (**Figure 2.5**). Eicosanoids of the 2-series such as thromboxane A₂ (TXA₂) are synthesised from the n-6 fatty acid arachidonic acid in platelets, whereas prostaglandin I₂ (PGI₂) is synthesised in the vascular endothelium. TXA₂ is a potent vasoconstrictor and a stimulus for platelet aggregation, whereas PGI₂ has opposite effects. Eicosanoids of the 3-series such as thromboxane A₃ (TXA₃) and prostacyclin (PGI₃) are principal metabolites of the n-3 fatty acid EPA. However, TXA₃ is biologically less active than TXA₂, while the anti-aggregatory effects of PGI₃ and PGI₂ are comparable. This may explain why fish oils lower platelet aggregation (76).

Eicosanoids have a short half-life time and are quickly catabolised into their stable metabolites, such as TXB₂ and 6-keto-PGF_{1α}. These metabolites can be converted into respectively 2,3 dinor-TXB₂ or 11-dehydro-TXB₂, and 2,3 dinor-6-keto-PGF_{1α}, which are excreted in the urine. In this way, analysis of these urinary metabolites represents a non-invasive surrogate to assess *in vivo* eicosanoid formation (77). In line with the anti-aggregatory effects of fish oils, several studies showed that n-3 fatty acids indeed decreased urinary excretion of TXA₂ metabolites. Prostaglandin excretion, however, was not always affected (74,76). With respect to saturated fatty acids, lauric, myristic, palmitic, and stearic acids had similar effects on urinary thromboxane and prostaglandin excretion (78,79). Also the effects of *trans* fatty acids were comparable with those of stearic acid (80). Furthermore, n-6 polyunsaturated fatty acids increased urinary 11-dehydro-TXB₂ excretion compared

experiments. Markedly, although n-3 fatty acids from fish oil affected platelet aggregation favourably, no effects on factor VII were observed (84).

Likewise, postprandial studies have indicated that the total fat content of the diet, rather than the fatty acid composition, increased factor VII concentrations (85,86). In one study, postprandial increases in activity of factor VII were less after consumption of meals rich in saturated fat, especially stearic acid, than after consumption of meals enriched with unsaturated fatty acids (87).

Fibrinogen

Although fibrinogen plays a crucial role in the clotting cascade to stabilise a loose thrombus, it is doubtful whether this functional role of fibrinogen explains the association between fibrinogen and atherosclerosis (81). Because fibrinogen is also an acute phase reactant, fibrinogen concentrations might also increase at least in part as a consequence of inflammatory reactions that occur in atherosclerosis. However, dietary fatty acid composition does not seem to regulate fibrinogen levels (15,83,84,88,89).

Prothrombin fragment 1 and 2

Fasting prothrombin fragment 1 and 2 concentrations are not changed by the fatty acid composition of the diet (83,84,88,89). Postprandially, concentrations increased after a high-fat meal independently of the fatty acid composition, which agrees with the finding that high-fat diets increase factor VII (90).

Fibrinolysis

Fibrinolysis is initiated by the conversion of plasminogen into plasmin through the action of tissue plasminogen activator (tPA). Plasmin catalyses the degradation of cross-linked fibrin of a thrombus. TPA activity is inhibited by plasminogen activator inhibitor-1 (PAI-1). Decreased concentrations of tPA and elevated concentrations of PAI-1 are associated with increased atherosclerotic risk. Furthermore, degradation products of cross-linked fibrin such as D-dimers reflect fibrinolytic activity and increased concentrations are surprisingly associated with an increased risk for atherosclerosis (91).

Tissue plasminogen activator

Several studies examined the effects of fatty acid composition of the diet on tissue plasminogen activator (tPA) activity but no different effects of specific fatty acids were found (14,88,89,92). Also reduction of dietary fat content did not affect tPA activity (93). Postprandial tPA levels also did not change significantly from fasting

levels after consumption of a high-fat meal (90,94). However, in one study diets rich in saturated fatty acids (stearic, palmitic or a mixture of myristic with palmitic acids) resulted in a greater rise in postprandial tPA concentrations than unsaturated test fats rich in oleic, linoleic, or *trans* fatty acids (87).

Plasminogen activator inhibitor-1

While high-fat diets do not affect plasminogen activator inhibitor-1 (PAI-1) concentrations, effects of the fatty acid composition of the diet on PAI-1 are not uniform. Although some studies concluded that n-3 polyunsaturated fatty acids increased PAI-1 activity, others found no effects. A recent meta-analysis, however, concluded that fish oil had no specific effects on PAI-1 (95). Effects of other fatty acids on PAI-1 are also marginal, although in one study an increase in PAI-1 activity was found when oleic acid in the diet was replaced by palmitic acid (83). Postprandially, however, PAI-1 concentrations decreased but these changes were not related to the fat content or fatty acid composition of the diet (90,96).

D-dimers

No changes in D-dimers concentrations after consumption of specific fatty acids have been detected (89,92,97).

Conclusions

Interpretation of the effects of fatty acids on hemostatic risk markers is difficult, partly due to the use of several methods and difficulties in extrapolating *in vitro* findings to the more complex *in vivo* situation. The most consistent finding is that n-3 fatty acids lower collagen-induced *in vitro* platelet aggregation. Furthermore, reducing total fat intake rather than changing the fatty acid composition of the diet may beneficially affect the coagulation system.

CONCLUDING REMARKS

To prevent the development of atherosclerosis, no doubt exists that the dietary intake of *trans* fatty acids should be as low as possible. Saturated fat intake should also decrease, although effects of the various saturated fatty acids differ. These conclusions are based mainly on effects of fats and oils on the lipoprotein profile, because effects on the other atherosclerotic risk markers discussed in this chapter are less evident. An exception, however, are the potential beneficial effects of moderate intakes of fish oil on inflammatory markers and on platelet aggregation, whereas total fat intake has unfavourable effects on both fasting and postprandial

factor VII concentrations. Furthermore, monounsaturated fatty acids may be preferable above polyunsaturated fatty acids with respect to oxidative processes measured by indirect *in vitro* assays. However, this latter conclusion has to be confirmed by direct methods measuring lipid peroxidation products.

The question can then be raised if saturated and *trans* fatty acids have to be replaced by unsaturated fatty acids or by carbohydrates. The favourable effects of unsaturated fatty acids on lipoprotein metabolism might be opposed by unfavourable effects of high-fat diets on thrombotic tendency, mainly due to effects on the coagulation system. Furthermore, high-fat diets might promote weight gain resulting in obesity. Also increased amounts of dietary fat have been related to changes in insulin sensitivity leading to diabetes and an increased risk to develop cancer. However, evidence for these latter associations is still a matter of debate. With respect to atherosclerosis, both prospective epidemiological and intervention studies have shown that high-unsaturated fat diets lower atherosclerotic risk (98). Therefore, fat is not necessarily bad, as long as body weight is not increased. However, when body weight is increased, it is advisable to reduce not only the intake of fat but also of the other macronutrients.

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3

Small differences in the effects of stearic acid, oleic acid, and linoleic acid on the serum lipoprotein profile of humans

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ABSTRACT

Background: Studies have suggested that oleic and stearic acids, as well as oleic and linoleic acids, have comparable effects on the serum lipoprotein profile. If so, then substituting these three C18 fatty acids for each other would result in similar effects on the serum lipoprotein profile.

Objective: The aim of this study was to compare simultaneously the effects of stearic, oleic, and linoleic acids on the serum lipoprotein profile of healthy subjects.

Design: Forty-five subjects (27 women and 18 men) consumed in random order 3 experimental diets, each for 5 weeks. The diets provided 38 percent of energy (% of energy) from fat, of which 60% was supplied by the experimental fats. The dietary compositions were the same, except for 7% of energy, which was provided by stearic, oleic, or linoleic acid. At the end of each intervention period, serum lipid and lipoprotein concentrations were measured. In addition, LDL, HDL, and VLDL particle sizes and particle concentrations of lipoprotein subclasses were analysed by nuclear magnetic resonance (NMR) spectroscopy.

Results: No significant diet-induced changes in serum lipids and lipoproteins were found. Mean (\pm standard deviations) serum LDL cholesterol concentrations were 3.79 ± 0.91 , 3.71 ± 0.79 , and 3.65 ± 0.91 mmol/L with the high-stearic acid, high-oleic acid, and high-linoleic acid diets, respectively ($P = 0.137$ for diet effects). Mean (\pm standard deviations) HDL cholesterol concentrations were 1.45 ± 0.43 , 1.46 ± 0.45 and 1.46 ± 0.44 mmol/L ($P = 0.866$). LDL, HDL, and VLDL particle sizes and lipoprotein subclass distributions also did not differ significantly between the 3 diets.

Conclusions: With realistic intakes of stearic, oleic, and linoleic acids, differences between their effects on the serum lipoprotein profile are small.

INTRODUCTION

It is well known that the various fatty acids in the diet exert different effects on serum lipid and lipoprotein concentrations. Saturated fatty acids are thought to increase cardiovascular risk because they elevate serum total and LDL cholesterol concentrations relative to monounsaturated and polyunsaturated fatty acids. These effects have been quantified by earlier well-controlled dietary studies (1,2). Relative to an iso-energetic amount of carbohydrates, a mixture of saturated fatty acids elevated serum total cholesterol concentrations, monounsaturated fatty acids had comparable effects, and polyunsaturated fatty acids were hypocholesterolemic. As an exception to the other saturated fatty acids, stearic acid - a saturated fatty acid with 18 carbon atoms - had no effects on serum total cholesterol concentrations (1,2). These earlier studies, however, did not examine the effects of fatty acids on specific lipoproteins, which is important because of the opposing effects of LDL and HDL cholesterol on cardiovascular disease risk.

More recently, several studies have compared the effects of stearic acid on lipid and lipoprotein concentrations with those of unsaturated fatty acids. When stearic acid was substituted for oleic acid, effects on serum LDL and HDL cholesterol concentrations did not differ (3). Similarly, the use of realistic intakes of linoleic acid (less than 13% of energy), oleic acid and linoleic acid had similar effects on the serum lipoprotein profile (4,5). If these findings are true (3-5), then the consequence is that the effects of stearic, oleic, and linoleic acids on serum lipid and lipoprotein concentrations would be comparable. To examine this hypothesis, we compared the effects of diets enriched in these three C18 fatty acids on serum total, LDL, and HDL cholesterol and triacylglycerol concentrations in a controlled crossover study in healthy subjects. In addition, we investigated the effects of these diets on LDL, HDL, and VLDL particle sizes and on the subclass distributions of these lipoprotein particles by nuclear magnetic resonance (NMR) spectroscopy.

SUBJECTS AND METHODS

Subjects

Healthy, male and female non-smoking volunteers were recruited via advertisements in local newspapers and in a university hospital newsletter and via posters in university buildings. Persons who were interested were informed about the purposes and requirements of the study and had to give their written informed consent before entering the screening phase. At screening, two fasting blood samples were taken for the measurement of serum lipid and lipoprotein

concentrations and hematological variables, and blood pressure and urinary glucose and protein from a morning urine specimen were measured. Subjects were included in the study if they were aged 18-65 y, were healthy on the basis of a medical questionnaire, were not pregnant, were weight stable and had a body mass index (BMI) below 32 kg/m^2 , had a diastolic blood pressure below 95 mmHg, had a systolic blood pressure below 160 mmHg, had a fasting serum total cholesterol concentration between 5.0 and 8.0 mmol/L, and had a serum triacylglycerol concentration below 4.0 mmol/L. Subjects with a history of atherosclerotic disease, glycosuria, proteinuria, or anemia, and who were taking medications known to affect blood lipids or hemostatic variables were excluded from the study. Fifty-eight persons met the eligibility criteria. Blood donation or participation in another biomedical trial was not allowed within 4 weeks before the start of the study or during the study. The study protocol was approved by the Medical Ethics Committee of the Maastricht University.

Subjects withdrew mainly in the first 2 weeks of the study, for reasons specifically related to the strict study protocol ($n = 4$ subjects), stressful personal or job circumstances ($n = 5$ subjects), and physical illness ($n = 2$ subjects in the first intervention period and 1 subject in the second intervention period). One subject was excluded after the first period, because he did not follow the protocol. Ultimately, 45 subjects (18 men and 27 women) aged 28-66 y (mean \pm standard deviation: 51 ± 10 y) completed the protocol. During the screening period, body mass indexes ranged from 18.0 to 29.8 kg/m^2 ($24.9 \pm 2.7 \text{ kg/m}^2$). The subjects' fasting serum lipid concentrations ranged from 4.97 to 7.76 mmol/L for total cholesterol ($6.04 \pm 0.75 \text{ mmol/L}$), from 0.83 to 3.60 mmol/L for HDL cholesterol ($1.48 \pm 0.54 \text{ mmol/L}$), and from 0.49 to 2.80 mmol/L for triacylglycerols ($1.15 \pm 0.55 \text{ mmol/L}$). Sixteen women were postmenopausal and five used oral contraceptives.

Experimental design and diets

The study had a randomised, multiple crossover design and consisted of 3 consecutive periods. Each participant consumed each of the 3 different diets during three 5-week periods. One diet was high in stearic acid (C18:0), another was high in oleic acid (C18:1), and the third was high in linoleic acid (C18:2). Before the study started, the subjects were categorised according to sex and were then randomly divided into 6 groups. Each group received the diets in 1 of the 6 possible treatment orders. Between each 5-week intervention period was a washout period of at least 1 week during which time the participants consumed their habitual diets (**Figure 3.1**).

The prescribed nutrient composition of the diets did not differ, except for a 7 percent difference in energy intake (% of energy) provided by stearic acid, oleic

acid, or linoleic acid. Before the participants started the study, their total energy intake was estimated with the Harris-Benedict equation (6). The diets were formulated to provide 16 different energy intakes ranging from 6 to 13.5 MJ per day. The experimental products supplied 60% of the total fat energy at a targeted total fat intake of 37% of energy. For the remaining 40% of the total daily fat intake, subjects were free to consume a certain amount of 'free-choice' fat-containing products. Therefore, participants received a list of fat-containing products to which points had been assigned on the basis of fat content (1 point equals 1 g fat). These products had to be recorded in a diary. Furthermore, alcohol consumption, medications used, signs of illness, menstruation, and any deviations from the study protocol were noted in this diary. The subjects were asked not to change their level of physical exercise or their use of alcohol, vitamins or oral contraceptives during the study.

| Group | Period | | | Subjects | |
|-------|---------|---------|---------|---------------|-----------------|
| | 1 | 2 | 3 | Men n = 18 | Women n = 27 |
| | Diet | Diet | Diet | | |
| 1 | C18:0 | C18:1 | C18:2 | 3 | 5 |
| 2 | C18:0 | C18:2 | C18:1 | 4 | 4 |
| 3 | C18:1 | C18:0 | C18:2 | 3 | 4 |
| 4 | C18:1 | C18:2 | C18:0 | 3 | 5 |
| 5 | C18:2 | C18:0 | C18:1 | 3 | 5 |
| 6 | C18:2 | C18:1 | C18:0 | 2 | 4 |
| | 5 weeks | 5 weeks | 5 weeks | | |

Figure 3.1 Experimental design of the study.

During the study periods, the subjects visited the university at least once every week to receive a new supply of products and to be weighed. Any leftover products had to be returned and were weighed. Individual allowances were adjusted if weight changed more than 1.5 kg from the initial weight during the first week or more than 2 kg during the following weeks. At each visit, the diary was checked by a dietician. In the last week of each intervention period, the subjects filled in a food-frequency questionnaire to estimate energy and nutrient intakes. These food-frequency questionnaires were immediately checked by a dietician. Items were coded, and the composition of the diets was calculated according to the Dutch food-composition table (7).

Experimental fats

Experimental fats were produced by NIZO Food Research (Ede, The Netherlands). The high-stearic acid fat was composed of 9.0% palm oil, 5.5% safflower oil, 5.0% olive oil, 33.5% cocoa butter, 18.0% high-oleic acid sunflower oil and 29.0% glycerol tristearate. The high-oleic acid fat consisted of 19.5% palm oil, 26.0% olive oil, 7.5% cocoa butter, and 47.0% high-oleic acid sunflower oil. The high-linoleic acid fat was a mixture of 20.0% palm oil, 52.0% safflower oil, 7.0% olive oil, 9.0% cocoa butter and 12.0% high-oleic acid sunflower oil. The fatty acid compositions of the experimental fats as determined by gas-liquid chromatography are shown in **Table 3.1**. From these fats, margarines were produced with a fat content (w/w) of 84%. The margarines were used to bake sponge cakes with a margarine content of 25% and bread with a margarine content of 10%. Products were labelled with a blue, orange, or yellow label to blind the subjects.

Table 3.1 Fatty acid composition of the 3 experimental fats.¹

| Fatty acid | Stearic acid fat | Oleic acid fat | Linoleic acid fat |
|------------------------------------|------------------------------|----------------|-------------------|
| | % of total fatty acids (w/w) | | |
| <i>Saturated fatty acids</i> | 57.0 | 22.6 | 23.0 |
| Lauric acid (C12:0) | 0.1 | 0.1 | 0.1 |
| Myristic acid (C14:0) | 0.2 | 0.3 | 0.3 |
| Palmitic acid (C16:0) | 16.2 | 15.3 | 15.8 |
| Stearic acid (C18:0) | 38.6 | 5.7 | 5.9 |
| <i>Monounsaturated fatty acids</i> | 33.9 | 66.5 | 32.6 |
| Palmitoleic acid (C16:1n-7) | 0.2 | 0.4 | 0.2 |
| Oleic acid (C18:1n-9) | 33.0 | 64.9 | 31.3 |
| <i>Polyunsaturated fatty acids</i> | 9.2 | 10.9 | 44.4 |
| n-6 polyunsaturated fatty acids | 9.0 | 10.6 | 44.0 |
| Linoleic acid (C18:2n-6) | 8.9 | 10.6 | 43.9 |
| n-3 polyunsaturated fatty acids | 0.1 | 0.2 | 0.3 |
| α-Linolenic acid (C18:3n-3) | 0.1 | 0.2 | 0.2 |

¹Values were determined by gas-liquid chromatography of triplicate samples of the margarines.

Blood sampling

Venous blood samples were obtained twice at the end of each period (weeks 4 and 5) while the subjects were in a recumbent position and after they had fasted overnight. Blood was collected with minimum stasis by using a 0.9 mm-needle (PrecisionGlide, Becton-Dickinson Vacutainer systems, Plymouth, United Kingdom) in week 4 or with a 1.0-mm infusion needle (Microflex, Vygon, Ecouen, France) in

week 5. All venipunctures were done by the same person, in the same room, and mostly at the same time of the day.

For lipid and lipoprotein analyses, 10 mL blood was collected into a serum tube (Corvac, Becton Dickinson Vacutainer Systems, Plymouth, UK). At least 1 hour after venipuncture, serum was obtained by centrifugation at 3500 x g for 30 min at 4°C and stored at -80°C.

Lipids and apolipoproteins

Serum total cholesterol (ABX Diagnostics, Montpellier, France), HDL cholesterol (precipitation method; Roche Diagnostics Corporation, Indianapolis, United States), and triacylglycerol (Sigma Aldrich Chemie, Steinheim, Germany) concentrations were analysed enzymatically. The within-run coefficients of variation (CV) were 1.3% for total cholesterol, 4.8% for HDL cholesterol and 3.7% for triacylglycerols. LDL cholesterol was calculated by using the equation of Friedewald (8).

Apolipoprotein A-I (apo A-I) and apolipoprotein B (apo B) were measured in serum by using an immunoturbidimetric method (ABX Diagnostics). The within-run CVs for apo A-I and apo B were 0.9% and 1.2%, respectively. All samples from one subject were analysed within one run.

Serum concentrations of lipoprotein particles and their subclasses and particle sizes of lipoproteins were analysed in a randomly chosen subset (stratified for sex) of 22 subjects (9 men and 13 women) by NMR spectroscopy (Liposcience, Raleigh, United States) as previously described (9). Before NMR analysis, serum samples from the end of each intervention period (weeks 4 and 5) were pooled.

Fatty acid composition

The fatty acid compositions of serum phospholipids in a pooled sample from weeks 4 and 5 and of the margarines were analysed as previously described (10). Briefly, total lipids were extracted from 100 µL serum or 10 mg margarine according to the method of Bligh and Dyer (11). Aminopropyl-bonded silica columns (Varian, Harbor City, CA, United States) were used to separate phospholipids from the total lipid extract of serum (12). The phospholipids from the serum and the triacylglycerols from the margarines were then saponified, and the resultant fatty acids were methylated into their corresponding fatty acid methyl esters (FAMES) (13). Fatty acids were separated on an Autosystem (Perkin Elmer, Norwalk, Connecticut, United States) gas chromatograph that was fitted with a silica-gel column (Cp-sil 88 for FAME, 50 m x 0.25 mm, 0.2-µm film thickness, Chrompack, Middelburg, The Netherlands) with helium gas (130 kPa) as the carrier gas (10). A comparable protocol was used to separate FAMES from the triacylglycerols. For triacylglycerols,

the injection and detection temperatures were both 300°C. The starting temperature of the column was 160°C. Ten minutes after injection, the temperature was increased up to 190°C at a rate of 2.5°C/min. After 20 min at 190°C, the temperature was increased up to 230°C at a rate of 4°C/min. The final temperature of 230°C was maintained for 10 min.

Data were analysed using Chromcard software (version 1.21, CE instruments, Milan, Italy). The fatty acid compositions of the margarines and serum phospholipids are expressed in relative amounts (% of total fatty acids identified, w/w).

Table 3.2 Mean nutrient composition of the 3 diets according to the food-frequency questionnaires.¹

| | Stearic acid diet | Oleic acid diet | Linoleic acid diet | P for diet effects ² |
|------------------------------------|-------------------------|-------------------------|-------------------------|---------------------------------|
| Energy | | | | |
| (MJ/day) | 8.4 ± 1.5 | 8.6 ± 1.7 | 8.6 ± 1.7 | 0.086 |
| (Kcal/day) | 1997 ± 348 | 2047 ± 400 | 2058 ± 406 | 0.086 |
| Protein (% of energy) | 14.0 ± 1.8 | 14.0 ± 2.1 | 13.8 ± 2.0 | 0.412 |
| Fat (% of energy) | 38.2 ± 5.1 | 37.7 ± 5.6 | 38.0 ± 5.3 | 0.701 |
| Saturated fatty acids | 18.0 ± 2.3 ^a | 11.0 ± 2.0 ^b | 11.2 ± 1.9 ^b | < 0.001 |
| Stearic acid (C18:0) ³ | 7.7 ± 1.1 ^a | 1.2 ± 0.2 ^b | 1.2 ± 0.2 ^b | < 0.001 |
| Monounsaturated fatty | 12.9 ± 2.0 ^a | 19.1 ± 2.9 ^b | 12.5 ± 1.8 ^a | < 0.001 |
| Oleic acid (C18:1) ³ | 6.8 ± 1.0 ^a | 13.1 ± 2.0 ^b | 6.6 ± 1.0 ^a | < 0.001 |
| Polyunsaturated fatty acids | 4.7 ± 1.2 ^a | 5.0 ± 1.1 ^a | 11.8 ± 1.8 ^b | < 0.001 |
| Linoleic acid (C18:2) ³ | 2.1 ± 0.3 ^a | 2.4 ± 0.3 ^a | 9.3 ± 1.3 ^b | < 0.001 |
| α-Linolenic acid (C18:3) | 0.2 ± 0.1 ^a | 0.2 ± 0.1 ^{ab} | 0.2 ± 0.1 ^b | 0.006 |
| Carbohydrates (% of energy) | 45.8 ± 5.6 | 46.3 ± 6.6 | 46.3 ± 6.2 | 0.624 |
| Alcohol (% of energy) | 2.3 ± 2.4 | 2.2 ± 2.3 | 2.1 ± 2.3 | 0.418 |
| Cholesterol (mg/MJ) | 17.7 ± 3.2 | 17.4 ± 4.2 | 17.9 ± 3.3 | 0.502 |
| Dietary fibre (g/MJ) | 3.1 ± 0.6 | 3.1 ± 0.7 | 3.1 ± 0.7 | 0.686 |

¹All values are means ± standard deviations, $n = 45$ (18 men and 27 women). Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni-corrected pairwise comparisons in general linear model).

²Calculated by using a general linear model with subject number as random factor and diet and period as fixed factors.

³As provided by the experimental fats only.

Statistics

For serum lipids and (apo)lipoproteins, the results of the 2 serum samples from weeks 4 and 5 were averaged before the statistical analyses. The statistical power to detect a true difference in total cholesterol of 0.21 mmol/L, in LDL cholesterol of

0.17 mmol/L, and in HDL cholesterol of 0.06 mmol/L was more than 80%. The data were analysed with the general linear model procedure of the SPSS 11 for Macintosh OS X package. A probability level (P-value) of less than 0.05 was considered statistically significant. Differences in effects on lipid and lipoprotein concentrations were examined with diet and period as fixed factors and subject number as random factor. To analyse whether the effects of diet were modified by sex or BMI, the diet x sex or diet x BMI interactions terms were added to the model as fixed factors. To examine effects of BMI, the subjects were divided into 2 groups. One group consisted of subjects with a BMI below 25 kg/m² ($n = 25$) and the other group of subjects with a BMI equal to or above 25 kg/m² ($n = 20$). When the analyses indicated a significant effect of diet, the diets were compared pairwise. When the interaction terms diet x sex or diet x BMI were significant, the diets were compared pairwise for the two sex or BMI groups separately. Between-diets comparisons were corrected for 3-group comparisons by the Bonferroni correction; confidence intervals (CI, 95%) were calculated for the differences between the diets. Values are presented as means \pm standard deviations. Pearson's correlations were determined to examine linear relationships between parameters.

RESULTS

Diets and dietary adherence

The mean daily energy intake and the composition of the 3 diets, as determined by the food-frequency questionnaires (**Table 3.2**), agreed well with the prescribed composition of the diets. Intakes of test products (bread, cake, and margarines) did not differ between diets. Total fat intake, on average, was 38% of energy, and did not differ between the 3 diets ($P = 0.701$). The nutrient composition of the diets also did not differ, except that 7% of energy was provided by different fats: stearic, oleic, or linoleic acid. Because of minor differences in the fatty acid composition of the experimental fats, the estimated intakes of α -linolenic acid were, respectively, 0.02% of energy ($P = 0.214$) higher with the oleic acid diet and 0.03% of energy ($P = 0.004$) with the linoleic acid diet than with the stearic acid diet. The mean amount of fat consumed as free-choice fat-containing products denoted in the subjects' diaries was 41.5% of total fat intake. This agreed well with the intended amount of 40%.

Mean body weights at the end of each dietary period did not differ significantly between the 3 diets ($P = 0.449$) and were 72.5 ± 13.0 kg with the stearic acid diet, 72.5 ± 13.2 kg with the oleic acid diet, and 72.7 ± 12.9 kg with the linoleic acid diet.

Dietary adherence was confirmed by the fatty acid compositions of serum phospholipids (**Table 3.3**). During the stearic acid diet, the proportion of stearic acid

was increased mainly at the expense of oleic acid. Likewise, the proportion of oleic acid increased after consumption of the oleic acid diet, mainly at the expense of stearic acid. During the diet rich in linoleic acid, the proportion of linoleic acid increased, whereas those of α -linolenic acid, eicosapentaenoic acid, oleic acid, and stearic acid decreased.

Table 3.3 Fatty acid composition of serum phospholipids during the 3 dietary periods.¹

| Fatty acid | Stearic acid diet | Oleic acid Diet | Linoleic acid diet | P for diet effects ² |
|-------------------------------------|-------------------------|--------------------------|-------------------------|---------------------------------|
| % of total fatty acids (w/w) | | | | |
| <i>Saturated fatty acids</i> | 46.5 ± 1.5 ^a | 45.6 ± 1.5 ^b | 46.2 ± 1.9 ^a | 0.001 |
| Palmitic acid (C16:0) | 26.5 ± 1.6 ^a | 26.8 ± 1.4 ^{ab} | 26.9 ± 1.6 ^b | 0.014 |
| Stearic acid (C18:0) | 14.3 ± 1.2 ^a | 13.1 ± 1.1 ^b | 13.7 ± 1.3 ^c | < 0.001 |
| <i>Monounsaturated fatty acids</i> | 13.6 ± 1.1 ^a | 15.0 ± 1.3 ^b | 12.2 ± 0.9 ^c | < 0.001 |
| Oleic acid (C18:1n-9) | 9.3 ± 1.1 ^a | 10.5 ± 1.2 ^b | 7.7 ± 0.8 ^c | < 0.001 |
| <i>Polyunsaturated fatty acids</i> | 39.1 ± 1.6 ^a | 38.6 ± 1.7 ^a | 40.6 ± 2.1 ^b | < 0.001 |
| N-6 polyunsaturated fatty acids | 33.7 ± 2.0 ^a | 33.3 ± 1.9 ^a | 35.8 ± 2.2 ^b | < 0.001 |
| Linoleic acid (C18:2n-6) | 20.7 ± 1.8 ^a | 20.5 ± 2.0 ^a | 23.2 ± 2.4 ^b | < 0.001 |
| Arachidonic acid (C20:4n-6) | 8.9 ± 1.5 | 8.7 ± 1.5 | 8.6 ± 1.7 | 0.103 |
| N-3 polyunsaturated fatty acids | 5.2 ± 1.2 ^a | 5.1 ± 1.0 ^a | 4.7 ± 1.0 ^b | < 0.001 |
| α -Linolenic acid (C18:3n-3) | 0.1 ± 0.0 ^a | 0.1 ± 0.0 ^a | 0.1 ± 0.0 ^b | < 0.001 |
| EPA (C20:5n-3) | 0.8 ± 0.4 ^a | 0.7 ± 0.3 ^a | 0.5 ± 0.3 ^b | < 0.001 |
| DHA (C22:6n-3) | 3.4 ± 0.9 | 3.3 ± 0.7 | 3.2 ± 0.7 | 0.063 |
| <i>Trans fatty acids</i> | 0.8 ± 0.3 | 0.8 ± 0.2 | 0.9 ± 0.2 | 0.060 |

¹All values are means ± standard deviations, $n = 45$ (18 men and 27 women). EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni-corrected pairwise comparisons in general linear model).

²Calculated by using a general linear model with subject number as random factor and diet and period as fixed factors.

Serum lipids and (apo)lipoproteins

The effects of the 3 different diets on the serum lipid and lipoproteins concentrations are given in **Table 3.4**. No statistically significant changes in serum concentrations of total ($P = 0.110$ for diet effects) and LDL ($P = 0.137$ for diet effects) cholesterol were found. Effects on HDL cholesterol ($P = 0.866$) and triacylglycerol ($P = 0.670$) concentrations also did not differ between the 3 diets. With respect to the total to HDL cholesterol ratio, no significant differences existed between the 3 diets ($P = 0.303$). Changes in concentrations of apo B ($P = 0.122$) and A-I ($P = 0.534$) were also not statistically significant between the 3 diets and they paralleled those of LDL and HDL cholesterol, respectively. A statistically significant diet x BMI interaction

effect ($P = 0.029$) was observed for apo B. In the high-BMI group ($P = 0.011$ for diet effects), the linoleic acid diet reduced apo B concentrations by 0.08 g/L relative to stearic acid ($P = 0.010$, 95% CI for the difference 0.02 to 0.15 g/L). In the low-BMI group, apo B concentrations did not differ between the three diets ($P = 0.689$). None of the dietary effects differed significantly between men and women (data not shown).

Table 3.4 Fasting serum lipids and lipoproteins concentrations and the ratio of total to HDL cholesterol during consumption of diets enriched in stearic, oleic and linoleic acids for 5 weeks by healthy men and women.¹

| | Stearic acid diet | Oleic acid diet | Linoleic acid diet | P for diet effects ² |
|--------------------------------|-------------------|-----------------|--------------------|---------------------------------|
| Total cholesterol (mmol/L) | 5.81 ± 0.94 | 5.73 ± 0.81 | 5.66 ± 0.91 | 0.110 |
| LDL cholesterol (mmol/L) | 3.79 ± 0.91 | 3.71 ± 0.79 | 3.65 ± 0.91 | 0.137 |
| HDL cholesterol (mmol/L) | 1.45 ± 0.43 | 1.46 ± 0.45 | 1.46 ± 0.44 | 0.866 |
| Triacylglycerols (mmol/L) | 1.24 ± 0.55 | 1.22 ± 0.52 | 1.21 ± 0.60 | 0.670 |
| Apolipoprotein A-I (g/L) | 1.39 ± 0.23 | 1.41 ± 0.25 | 1.40 ± 0.24 | 0.534 |
| Apolipoprotein B (g/L) | 1.08 ± 0.20 | 1.06 ± 0.19 | 1.04 ± 0.17 | 0.122 |
| Total to HDL cholesterol ratio | 4.31 ± 1.33 | 4.22 ± 1.23 | 4.19 ± 1.28 | 0.303 |

¹All values are means ± standard deviations, $n = 45$ (18 men and 27 women).

²There were no significant differences between the 3 diets (general linear model with subject number as random factor and diet and period as fixed factors).

Lipoprotein particle concentrations and sizes

Changes in VLDL, LDL, and HDL particle sizes and subclass concentrations did not differ significantly between the 3 diets (Table 3.5). No sex-dependent diet effects were observed (data not shown). The diet x BMI interaction was significant for small VLDL concentrations ($P = 0.030$). In the low-BMI group ($P = 0.043$ for diet effects), linoleic acid increased the small VLDL concentration by 9.7 nmol/L ($P = 0.042$, 95% CI for the difference 0.3 to 19.1 nmol/L) when compared with oleic acid. In the high-BMI group, diet effects were not statistically significant ($P = 0.189$). Concentrations of small VLDL particles were 19.9 nmol/L ($P = 0.002$, 95% CI -31.2 to -8.6 nmol/L), of intermediate-density lipoprotein (IDL) particles were 31.5 nmol/L ($P = 0.018$, 95% CI -57.0 to -5.9 nmol/L), of total LDL particles were 402 nmol/L ($P = 0.024$, 95% CI -745 to -59 nmol/L), of small LDL particles were 535 nmol/L ($P = 0.010$, 95% CI -927 to -143 nmol/L), of medium-small LDL particles were 108 nmol/L ($P = 0.016$, 95% CI -193 to -22 nmol/L), and of very small LDL particles were 427 nmol/L ($P = 0.009$, 95% CI -734 to -120 nmol/L) lower in women than in men. Large HDL particle concentrations were 3.9 μ mol/L ($P = 0.002$, 95% CI 1.7 to 6.1 μ mol/L) higher in women. LDL and HDL particle size were 1.0-nm ($P = 0.005$, 95% CI 0.3 to 1.6 nm)

and 0.6-nm ($P = 0.003$, 95% CI 0.2 to 0.9 nm) higher, respectively, in women than in men. With the high-oleic acid diet, BMI was significantly correlated with total LDL ($r = 0.491$, $P = 0.020$), IDL ($r = 0.431$, $P = 0.045$), small LDL ($r = 0.440$, $P = 0.040$), medium-small LDL ($r = 0.457$, $P = 0.032$), and very small LDL ($r = 0.435$, $P = 0.043$) particle concentrations and with HDL particle size ($r = -0.532$, $P = 0.011$). Age correlated with LDL ($r = 0.468$, $P = 0.028$) and IDL ($r = 0.486$, $P = 0.022$) particle concentrations. Comparable relations were observed when subjects consumed the high-stearic acid or high-linoleic acid diets.

Table 3.5 Particle concentrations of lipoprotein subclasses and lipoprotein particle sizes as measured by NMR spectroscopy during consumption of diets enriched in stearic, oleic, or linoleic acid for 5 weeks by healthy men and women.¹

| | Stearic acid diet | Oleic acid diet | Linoleic acid diet | P for diet effects ² |
|---------------------------------------|-------------------|-----------------|--------------------|---------------------------------|
| <i>Particle concentrations</i> | | | | |
| VLDL (nmol/L) | | | | |
| Total VLDL | 83.5 ± 29.1 | 82.1 ± 30.8 | 86.3 ± 33.2 | 0.560 |
| Large VLDL and chylomicrons | 2.6 ± 3.1 | 2.8 ± 3.2 | 2.1 ± 3.4 | 0.209 |
| Medium VLDL | 31.4 ± 15.7 | 32.8 ± 14.9 | 33.5 ± 20.7 | 0.716 |
| Small VLDL | 49.5 ± 16.3 | 46.6 ± 17.8 | 50.8 ± 18.4 | 0.332 |
| IDL (nmol/L) | 47.8 ± 43.6 | 44.5 ± 30.2 | 36.7 ± 33.0 | 0.215 |
| LDL (nmol/L) | | | | |
| Total LDL | 1305 ± 468 | 1244 ± 437 | 1262 ± 387 | 0.213 |
| Large LDL | 561 ± 204 | 551 ± 221 | 567 ± 223 | 0.875 |
| Small LDL | 696 ± 558 | 648 ± 542 | 658 ± 441 | 0.568 |
| Medium small LDL | 133 ± 118 | 124 ± 108 | 130 ± 101 | 0.595 |
| Very small LDL | 563 ± 441 | 524 ± 435 | 528 ± 342 | 0.550 |
| HDL (μmol/L) | | | | |
| Total HDL | 33.8 ± 4.3 | 33.4 ± 4.3 | 34.1 ± 4.5 | 0.545 |
| Large HDL | 8.4 ± 3.6 | 8.3 ± 3.0 | 8.8 ± 3.3 | 0.468 |
| Medium HDL | 3.2 ± 3.6 | 3.4 ± 3.9 | 3.4 ± 3.6 | 0.942 |
| Small HDL | 22.2 ± 4.4 | 21.7 ± 4.8 | 21.9 ± 3.6 | 0.759 |
| <i>Lipoprotein particle size (nm)</i> | | | | |
| VLDL particle size | 45.4 ± 4.1 | 45.0 ± 4.4 | 46.2 ± 5.9 | 0.277 |
| LDL particle size | 21.5 ± 0.9 | 21.5 ± 1.0 | 21.5 ± 0.8 | 0.985 |
| HDL particle size | 9.1 ± 0.5 | 9.2 ± 0.5 | 9.2 ± 0.5 | 0.907 |

¹All values are means ± standard deviations, $n = 22$ (9 men and 13 women).

²There were no significant differences between the 3 diets (general linear model with subject number as random factor and diet and period as fixed factors).

DISCUSSION

In this well-controlled crossover study of healthy subjects, we found that the differences in effects of stearic, oleic, and linoleic acids on the serum lipoprotein profile were less than expected. Although total and LDL cholesterol concentrations tended to decrease with the increasing degree of unsaturation, the changes between the 3 diets were not significant. Based on the classic equations derived by Keys *et al* (1), a decrease of 0.21 mmol/L in total cholesterol concentrations is expected when 7% of energy from stearic acid or oleic acid in the diet is exchanged for linoleic acid. However, we found decreases of 0.15 mmol/L and 0.07 mmol/L, respectively.

Until now, only a few studies have examined simultaneously the effects of stearic acid, oleic acid, and linoleic acid. Consistent with our results, Hunter *et al* (14) found no differences in the effects of these fatty acids on plasma total or LDL cholesterol concentrations. However, only 6 healthy male subjects participated in that study, and the statistical power may have been too low to detect any changes. Kris-Etherton *et al* (15) examined in 19 young men the effects of natural edible fats and oils rich in stearic acid (cocoa butter), oleic acid (olive oil) or linoleic acid (soybean oil) on the serum lipoprotein profile. It was found that the diet rich in linoleic acid significantly lowered serum total cholesterol concentrations relative to stearic acid or oleic acid. In addition, the LDL cholesterol concentration was lower with the diet rich in linoleic acid than with the diet rich in stearic acid. A possible explanation for these apparent discrepancies with our results might be that in their study approximately 10% of energy from stearic acid and 16% of energy from oleic acid was exchanged for linoleic acid. The expected decreases in total and LDL cholesterol concentrations were therefore greater. In that study (15), the high-oleic acid diet also decreased total and LDL cholesterol concentrations significantly more than did the high-stearic acid diet. The difference in response between these 2 diets can at least partly be explained by the higher intake of palmitic acid from the diet rich in stearic acid. Palmitic acid is known to increase serum total and LDL cholesterol levels relative to stearic or oleic acid (3,16).

Our results agree with the many studies that compared stearic acid with oleic acid (3) or oleic acid with linoleic acid (4,5,17,18) and also found no different effects on the serum lipoprotein profile. In one study, however, an exchange of 8% of energy from stearic acid for oleic acid significantly decreased serum LDL cholesterol by 0.15 mmol/L. Surprisingly, no effects on apo B concentrations were found (19). In addition, Zock and Katan (20) found that when 8% of energy from stearic acid was replaced by linoleic acid, the linoleic acid diet significantly decreased serum LDL

cholesterol by 0.17 mmol/L. When expressed as a percentage of energy, however, their effects did not differ from those in our study.

In a recent meta-analysis, equations were developed to describe the effects of individual fatty acids on serum lipids and lipoproteins (21). On the basis of these equations, replacement of 7% of energy from stearic acid by oleic acid may result in a decrease in LDL cholesterol concentrations of 0.04 mmol/L and a decrease of 0.11 mmol/L when replaced by linoleic acid. These estimates agree well with the observed differences in LDL cholesterol concentrations of -0.08 mmol/L between the diets enriched in stearic and oleic acids and of -0.14 mmol/L between the diets high in stearic acid and linoleic acid. The power of our study to pick up this latter difference was 60%. Taken together, evidence continues to accumulate to suggest that the earlier formulas (1,2) overestimate the effects of linoleic acid on serum total cholesterol concentrations.

On the basis of the earlier meta-analysis (21), decreases in HDL cholesterol concentrations of 0.04 mmol/L were predicted when oleic acid was replaced by stearic acid, and of 0.03 mmol/L when stearic acid was exchanged for linoleic acid. In our study, however, decreases were slightly but not significantly lower when stearic acid replaced either oleic or linoleic acid. Other studies also reported no differential effects of oleic and linoleic acids on HDL cholesterol concentrations (4,5,14,15). In contrast, some studies have reported that linoleic acid decreases HDL cholesterol concentrations relative to oleic acid (17,18). Zock and Katan (20) reported a decrease in HDL cholesterol when linoleic acid was exchanged for stearic acid, whereas Judd *et al* (19) reported a decrease when oleic acid was replaced for stearic acid. A non-significant decrease was also observed by Bonanome and Grundy (3). Thus, these 3 studies suggest that stearic acid may lower HDL relative to oleic and linoleic acids, which is not supported by our results or the studies that simultaneously compared stearic, oleic, and linoleic acids (14,15). In the 3 other studies, stearic acid was largely provided by interesterified and hydrogenated synthetic fats (3,19,20). In these fats, stearic acid was not only located at the *sn*-1 and *sn*-3 positions, as is the case in natural fats, but also at the *sn*-2 position (22). Because of these stereospecific distributions, it is possible that the effects of natural fats rich in stearic acid on the serum lipoprotein profile are different from those of synthetic fats. This suggestion, however, requires further investigation.

No differential effects of stearic acid, oleic acid, or linoleic acid were found on lipoprotein particle sizes and concentrations. As is true for small, dense LDL particles (23,24), small HDL particles (25,26) are positively associated with increased cardiovascular disease risk. Therefore, we also examined the effects of

stearic, oleic, and linoleic acids on LDL, HDL, and VLDL particle size and subclass particle concentrations by using NMR spectroscopy. Until now, only a few studies have examined the effects of quality of dietary fat on lipoprotein particle sizes or subclass distributions of lipoprotein particles. Relative to saturated fat, monounsaturated and n-6 and n-3 polyunsaturated fatty acids slightly but significantly decreased LDL particle size (27). In contrast, in another study no significant changes in LDL particle size were observed when saturated fatty acids were exchanged for monounsaturated fatty acids (28). Unfortunately, no details about the individual saturated fatty acid composition of the diets were given. Observed differences in particle sizes and particle concentrations between men and women in our study agreed well with those of The Framingham Offspring study, in which these variables were measured in a large group of 1574 men and 1692 women (9).

In summary, the effects of stearic acid, oleic acid, and linoleic acid on LDL cholesterol concentrations were less than expected. Effects on HDL cholesterol and triacylglycerol concentrations as well as the size and the concentration of the lipoprotein particles also did not differ significantly between diets. These findings, however, do not imply that these three C18 fatty acids can be exchanged without affecting cardiovascular disease risk, because other cardiovascular disease risk markers (e.g. hemostatic function, oxidative stress, and low-grade inflammation) are also influenced by the fatty acid composition of the diet.

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4

Stearic, oleic, and linoleic acids have comparable effects on markers of thrombotic tendency in healthy human subjects

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ABSTRACT

Because human studies concerning the effects of stearic acid on thrombotic tendency are inconsistent, we compared the effects of stearic acid with those of its unsaturated derivatives, oleic acid and linoleic acid. In this randomised, crossover study, 45 subjects (27 women and 18 men) consumed, in random order, 3 experimental diets, each for 5 weeks. Diets contained approximately 38% of energy as fat. Dietary compositions were the same except for 7% of energy from stearic, oleic, or linoleic acids. At the end of each period, ex vivo and in vitro platelet aggregation, and variables of coagulation, fibrinolysis, and hematology were evaluated. In men, ex vivo platelet aggregation time as measured by filragnetometry ($P = 0.036$ for diet effects) was favourably prolonged during consumption of the linoleic acid diet compared with the stearic acid diet ($P = 0.040$), but there was no difference with consumption of the oleic acid diet ($P = 0.198$). In vitro platelet aggregation induced by collagen and ADP, and variables of coagulation (factor VII amidolytic activity and concentrations of fibrinogen and prothrombin fragment 1 and 2) and fibrinolysis (plasminogen activator inhibitor (PAI) activity and concentrations of tissue plasminogen activator (tPA)/PAI-1 complexes) did not differ among the 3 diets. The mean platelet volume of the subjects decreased during consumption of the stearic acid diet by 0.32 fL compared with the oleic acid diet ($P < 0.001$) and by 0.35 fL compared with the linoleic acid diet ($P < 0.001$). In conclusion, our results do not suggest that stearic acid is highly thrombogenic compared with oleic and linoleic acids.

INTRODUCTION

The most common complications of cardiovascular disease result from the formation of an arterial thrombus (1,2), which is initiated by disturbances in the hemostatic balance. Key regulators of this delicate balance are the endothelial wall, blood platelets, and coagulation and fibrinolytic factors (3). Indeed, epidemiological studies showed that factors related to an enhanced thrombotic tendency such as increased blood platelet aggregation (4,5), increased concentrations of coagulation and decreased concentrations of fibrinolytic factors (6-8), are positively associated with cardiovascular risk. Furthermore, it was demonstrated that the thrombotic tendency of the blood is influenced by total fat intake as well as the fatty acid composition of the diet. Although the biochemical basis of the effects of dietary fatty acids on thrombotic tendency have not been fully elucidated, dietary fatty acids can modulate the fatty acid compositions of platelets and other cell membranes, thereby changing the availability of arachidonic acid. This fatty acid is a precursor for eicosanoid synthesis, which is involved in platelet aggregation (9).

The effects of individual fatty acids on thrombotic tendency were evaluated in animal models and in human studies. In rats, arterial thrombosis tendency as measured with the aortic loop technique, was decreased by n-6 and n-3 polyunsaturated fatty acids, whereas saturated fatty acids with 12 to 16 carbon atoms promoted arterial thrombus formation. The effects of oleic acid as a major monounsaturated fatty acid were neutral or even antithrombotic compared with saturated fatty acids (10). In these studies, stearic acid did not seem to affect arterial thrombosis tendency, whereas other studies in rats indicated that stearic acid was highly thrombogenic in a model of venous thrombosis (11). Studies in humans also suggested that stearic acid is prothrombotic. In French farmers, for example, dietary stearic acid intake was related to increased platelet activity (12). In another study, plasma concentrations of free stearic acid correlated positively with factor VII activation (13). In contrast, later studies reported beneficial effects of stearic acid on platelet aggregation (14) and coagulation variables (15,16) compared with other saturated fatty acids. Not only are the data for stearic acid inconsistent, but also the data for oleic acid and linoleic acid, two other fatty acids with 18 carbon atoms (17-19). The objective of the present study was therefore to compare the effects of stearic, oleic, and linoleic acids on platelet aggregation, coagulation, fibrinolysis, and hematological variables.

SUBJECTS AND METHODS

Experimental design

The study had a randomised, multiple crossover design. The effects of stearic, oleic, and linoleic acids on lipid and lipoprotein concentrations were investigated earlier in this study, and the study protocol was described in detail (20). Briefly, each participant consumed 3 different diets in random order over three 5-week periods. After each intervention period, there was a washout period of at least 1 week, when participants consumed their habitual diets. The protocol of the study was approved by the Medical Ethics Committee of Maastricht University.

Diets

The prescribed nutrient composition of the 3 diets did not differ, except for 7% of energy provided by stearic acid, oleic acid, or linoleic acid. The experimental products (margarines, bread, and sponge cakes) supplied 60% of total daily fat intake at a targeted total fat intake of 37% of energy. For the remaining 40% of the total fat intake, subjects had to consume a certain amount of 'free-choice' fat-containing products. These products had to be recorded in a diary, in which alcohol consumption, medication used, any sign of illness, menstruation, and any deviations from the study protocol also were noted. At least once every week, subjects visited a dietician at the university to receive a new supply of products and to be weighed. Individual allowances were adjusted when subjects' weight differed by > 1.5 kg from initial weight during week 1 or > 2 kg during the following weeks. The mean daily energy intake and the composition of the diets were determined from food-frequency questionnaires, which were filled out by the subjects in week 5 of each intervention period.

Subjects

The screening procedure and eligibility criteria of the subjects were described in detail earlier (20). Briefly, 45 healthy, non-smoking, slightly hypercholesterolemic subjects, 18 men and 27 women, completed the study protocol. All subjects were between 28 and 66 y old (mean age was 51 ± 10 y). The men had body mass indexes ranging from 21.8 to 29.8 kg/m² (mean 26.0 ± 2.2 kg/m²); those of the women ranged from 18.0 to 29.4 kg/m² (mean 24.1 ± 2.8 kg/m²). Among the women, 16 were postmenopausal and 5 used oral contraceptives. All subjects had given their written informed consent, before they entered the study protocol.

Blood sampling

At the end of each period (in weeks 4 and 5), blood samples were drawn after the subjects had fasted overnight. Blood was sampled by venipuncture with minimum stasis using a 0.9 mm-needle (PrecisionGlide, Becton-Dickinson Vacutainer systems, Plymouth, United Kingdom) in week 4 or with a 1.0 mm-infusion needle (Microflex, Vygon, Ecoen, France) in week 5. The first 6 mL was collected into EDTA tubes (Becton Dickinson Vacutainer Systems, Plymouth, United Kingdom) and used for analysis of hematological variables. Platelet, erythrocyte, and leukocyte counts, hemoglobin concentration, and hematocrit were measured with the Coulter Microdiff 18 (Beckman Coulter, Miami, United States). The next 9 mL was collected in sodium citrate tubes (Becton Dickinson Vacutainer Systems) and immediately placed on ice. Within 1 hour of collection, plasma was separated by centrifugation at $3500 \times g$ for 30 min. Plasma samples were snap-frozen in liquid nitrogen and stored at -80°C until analyses of coagulation and fibrinolytic factors. In week 5, an extra sodium citrate tube was drawn, which was kept at 37°C , for *in vitro* platelet aggregation measurements. Then, 10 mL of blood was collected into a serum tube (Corvac, Becton Dickinson Vacutainer Systems) for the analysis of the fatty acid composition of serum phospholipids. At least 1 hour after venipuncture, serum was obtained by centrifugation at $3500 \times g$ for 30 min at 4°C and stored at -80°C . Finally, after blood sampling in week 5, the tube of the infusion needle was connected to the filragnetometer for the measurement of *ex vivo* platelet aggregation. A pool of citrated plasma was obtained from healthy blood donors, and prepared by methods described above.

Fatty acid composition

Fatty acid compositions of serum phospholipids in a pooled sample of weeks 4 and 5 were analysed, as described previously (20). Values were expressed as a percentage of total fatty acids (w/w).

Ex vivo platelet aggregation

Ex vivo platelet aggregation was measured using filragnetometry. The principle of this method was described and validated by Hornstra and ten Hoor (21). Filragnetometry is based on the continuous measurement of the pressure difference (ΔP) across a filter with pores of $20 \mu\text{m}$ in diameter through which blood flows. Platelet aggregates, obstructing the filter, cause a change in the ΔP , which is proportional to the mass of the platelet aggregates that obstruct the filter. For measurements, blood from a forearm vein was drawn via an infusion needle by a motor-driven syringe at a flow rate of 2 mL/min. The infusion needle was connected by a tubing system with the

filtragometer. Initially, the blood was anticoagulated with heparin. When ΔP reached 5 mmHg, which corresponded to 25% of filter pore occlusion, the heparin was switched off and a citrate infusion was started, which may partially reverse the occlusion of the filter resulting in a transient decrease in ΔP . The change in ΔP was monitored continuously for 10 minutes after connecting the filtragometer, and registered as aggregation curves. Aggregation variables that were calculated from these aggregation curves were the aggregation time (T_a in s, the time necessary to reach a ΔP of 5 mmHg), the aggregation slope (T_s in mmHg/s, the slope of the tangent to the curve at $\Delta P = 5$ mmHg), the maximum aggregation (A_{max} in mmHg, the maximum height of the aggregation curve), and the desaggregation induction time (T_{di} in s, time between the termination of the heparin infusion and the beginning of the desaggregation). For 3 measurements, ΔP did not reach 5 mmHg within 10 minutes and the aggregation time (T_a) was then set at 600 s. For these measurements, other aggregation variables could not be calculated. Evaluation by scanning electron microscopy showed that filter occlusion is due mainly to platelet aggregates. In addition, it was demonstrated that treatment with acetylsalicylic acid prolonged *ex vivo* platelet aggregation (21).

***In vitro* platelet aggregation**

In vitro platelet aggregation was measured in whole blood with a dual-sample aggregometer (whole blood aggro-meter model 540, Chrono-log corporation, Havertown, PA, United States), immediately after blood sampling. For each measurement, 1 mL of anticoagulated blood was transferred into a pre-warmed plastic cuvette and electrodes were inserted into the cuvettes. The sample was kept at 37°C and stirred. After equilibration of the sample and calibration of the instrument, aggregation was induced by the addition of collagen (Chrono-PAR #385 Collagen Reagent, Chrono-log corporation) or ADP (Chrono-PAR #384 ADP Reagent, Chrono-log corporation). The change in impedance was monitored continuously for 15 minutes and registered as aggregation curves. Collagen-induced aggregation was measured at a final concentration of 2 mg/L collagen and ADP-induced aggregation was measured at final concentrations of 15 and 5 $\mu\text{mol/L}$ ADP. Whole-blood platelet aggregation was quantified by measuring the aggregation time (T_{ai}), the aggregation velocity (V_a) and the maximum aggregation (I_{max}).

Measurements of coagulation and fibrinolysis

Before analysis of coagulation and fibrinolytic factors, equal volumes of the citrated plasma samples from weeks 4 and 5 were pooled. A chromogenic assay (Coaset F.VII, Chromogenix Instrumentation Laboratory, Milano, Italy) was used to assess

factor VII amidolytic (factor VIIam) activity. Activities were expressed as the percentage of a plasma pool. Prothrombin fragment 1 and 2 (PTF1 + 2) concentrations were analysed with an enzyme immuno assay (Enzygnost F1 + 2 micro, Dade Behring, Marburg, Germany). Plasma fibrinogen concentrations were measured by a clotting assay (Dade Thrombin reagent, Dade Behring, Marburg, Germany) based upon the method of Clauss (22). Plasminogen activator inhibitor (PAI) activity was determined with a chromogenic assay (Spectrolyse/pL PAI, Trinity Biotech, Wicklow, Ireland). Concentrations of tPA/PAI-1 complexes were analysed by an enzyme-linked immunosorbent assay (tPA/PAI-1 Complex ELISA reagent kit, Technoclone, Vienna, Austria). All samples from one subject were performed in the same analytic run. The coefficients of variation within runs were 1.8% for fibrinogen, 4.2% for factor VIIam, 9.5% for PTF1 + 2, 8.3% for PAI activity, and 13.6% for tPA/PAI-1 complexes.

Statistics

For hematological variables, the mean of the 2 plasma samples from weeks 4 and 5 was calculated before statistical analyses. All data were analysed with the general linear model (GLM) procedure of the SPSS 11 for Mac OS X package. A probability level (P-value) of < 0.05 was considered significant. Differences in effects were examined with diet and period as fixed factors and subject number as random factor. Because previous studies showed gender-dependent effects of dietary fatty acid intake on the variables of interest or examined effects only in men, we also analysed data by gender. It should be noted, however, that the study was not specifically designed to look for gender-dependent effects and the statistical power may have been too limited to specifically address this question. To analyse whether effects of diet were modified by gender, the diet x gender interaction term was added to the model as a fixed factor. When the analyses indicated a significant effect of diet ($P < 0.05$), the diets were compared pair-wise. Between diets comparisons were corrected for 3-group comparisons by the Bonferroni correction, and 95% confidence intervals were calculated for the differences among the diets. Values are presented as means \pm standard deviations.

RESULTS

Dietary composition

The subjects' body weights at the end of each dietary period were 72.5 ± 13.0 kg after consumption of the stearic acid diet, 72.5 ± 13.2 kg after the oleic acid diet and 72.7 ± 12.9 kg after the linoleic acid diet. Weights did not differ among those

consuming the 3 diets ($P = 0.449$). Daily intakes of energy, protein, carbohydrates, fat, cholesterol, and fibre did also not differ among subjects consuming the 3 diets (**Table 4.1**). Fatty acid intakes were comparable, except for about 7% of energy, which was provided by stearic acid, oleic acid, or linoleic acid.

Table 4.1 Daily intakes of fat and fatty acids by healthy men and women during consumption of the 3 diets for 5 weeks.^{1,2}

| | Stearic acid diet | Oleic acid diet | Linoleic acid diet | P for diet effects ³ |
|---------------------------------------|-------------------------|-------------------------|-------------------------|---------------------------------|
| | % of energy | | | |
| Fat | 38.2 ± 5.1 | 37.7 ± 5.6 | 38.0 ± 5.3 | $P = 0.701$ |
| Saturated fatty acids | 18.0 ± 2.3 ^a | 11.0 ± 2.0 ^b | 11.2 ± 1.9 ^b | $P < 0.001$ |
| Stearic acid (C18:0) ⁴ | 7.7 ± 1.1 ^a | 1.2 ± 0.2 ^b | 1.2 ± 0.2 ^b | $P < 0.001$ |
| Monounsaturated fatty acids | 12.9 ± 2.0 ^b | 19.1 ± 2.9 ^a | 12.5 ± 1.8 ^b | $P < 0.001$ |
| Oleic acid (C18:1n-9) ⁴ | 6.8 ± 1.0 ^b | 13.1 ± 2.0 ^a | 6.6 ± 1.0 ^b | $P < 0.001$ |
| Polyunsaturated fatty acids | 4.7 ± 1.2 ^b | 5.0 ± 1.1 ^b | 11.8 ± 1.8 ^a | $P < 0.001$ |
| Linoleic acid (C18:2n-6) ⁴ | 2.1 ± 0.3 ^b | 2.4 ± 0.3 ^b | 9.3 ± 1.3 ^a | $P < 0.001$ |

¹Values are means ± standard deviations, $n = 45$ (18 men and 27 women) as calculated from the food frequency questionnaires. Means in a row with superscripts without a common letter differ, $P < 0.05$.

²Intakes of energy (8.5 ± 1.6 MJ), carbohydrates (46.1 ± 6.1 % of energy), proteins (13.9 ± 2.0 % of energy), alcohol (2.2 ± 2.3 % of energy), cholesterol (17.7 ± 3.6 mg/MJ) and dietary fibre (3.1 ± 0.7 g/MJ) did not differ significantly among the 3 diets.

³ P -values for diet effects were calculated by the general linear model with subject number as random factor and diet and period as fixed factors.

⁴As provided by the experimental fats only.

Dietary adherence of the subjects was confirmed by the fatty acid composition of serum phospholipids (**Table 4.2**). The 3 diets differed in their effects on the proportions of stearic acid, oleic acid, and linoleic acid ($P < 0.001$ for diet effects). The proportions of arachidonic acid ($P = 0.103$ for diet effects) and docosahexaenoic acid (DHA; $P = 0.063$ for diet effects) did not differ. The proportions of the n-3 polyunsaturated fatty acids, α -linolenic acid ($P < 0.001$ for diet effects) and eicosapentaenoic acid (EPA; $P < 0.001$ for diet effects) were lower during consumption of the linoleic acid diet compared with the other 2 diets.

Table 4.2 Fatty acid composition of serum phospholipids in healthy men and women during consumption of the 3 diets for 5 weeks.¹

| Fatty acid | Stearic acid diet | Oleic acid diet | Linoleic acid diet | P for diet effects ² |
|------------------------------------|------------------------------|--------------------------|--------------------------|---------------------------------|
| | % of total fatty acids (w/w) | | | |
| <i>Saturated fatty acids</i> | 46.5 ± 1.5 ^a | 45.6 ± 1.5 ^b | 46.2 ± 1.9 ^a | P = 0.001 |
| Stearic acid (C18:0) | 14.3 ± 1.2 ^a | 13.1 ± 1.1 ^b | 13.7 ± 1.3 ^c | P < 0.001 |
| <i>Monounsaturated fatty acids</i> | 13.6 ± 1.1 ^a | 15.0 ± 1.3 ^b | 12.2 ± 0.9 ^c | P < 0.001 |
| Oleic acid (C18:1n-9) | 9.3 ± 1.1 ^a | 10.5 ± 1.2 ^b | 7.7 ± 0.8 ^c | P < 0.001 |
| <i>Polyunsaturated fatty acids</i> | 39.1 ± 1.6 ^a | 38.6 ± 1.7 ^a | 40.6 ± 2.1 ^b | P < 0.001 |
| Linoleic acid (C18:2n-6) | 20.7 ± 1.8 ^a | 20.5 ± 2.0 ^a | 23.2 ± 2.4 ^b | P < 0.001 |
| Arachidonic acid (C20:4n-6) | 8.9 ± 1.5 | 8.7 ± 1.5 | 8.6 ± 1.7 | P = 0.103 |
| α-Linolenic acid (C18:3n-3) | 0.14 ± 0.04 ^a | 0.13 ± 0.04 ^a | 0.11 ± 0.04 ^b | P < 0.001 |
| EPA (C20:5n-3) | 0.8 ± 0.4 ^a | 0.7 ± 0.3 ^a | 0.5 ± 0.3 ^b | P < 0.001 |
| DHA (C22:6n-3) | 3.4 ± 0.9 | 3.3 ± 0.7 | 3.2 ± 0.7 | P = 0.063 |
| <i>Trans fatty acids</i> | 0.8 ± 0.3 | 0.8 ± 0.2 | 0.9 ± 0.2 | P = 0.060 |

¹Values are means ± standard deviations, n = 45 (18 men and 27 women). Means in a row with superscripts without a common letter differ, P < 0.05.

²P-values for diet effects were calculated by the general linear model with subject number as random factor and diet and period as fixed factors.

Hematological variables

When all subjects were analysed together, the 3 diets differed significantly in their effects on the number of erythrocytes and mean platelet volumes (**Table 4.3**). The number of erythrocytes tended to be lower when subjects consumed the diet high in linoleic acid rather than the stearic acid diet (P = 0.054, 95% CI for the difference -0.259 to -0.001 × 10¹²/L). In men, the numbers of erythrocytes during consumption of the linoleic acid diet were lower than during consumption of the oleic acid diet (P = 0.005, 95% CI for the difference -0.365 to -0.057 × 10¹²/L) and only slightly lower than during consumption of the stearic acid diet (P = 0.106, 95% CI for the difference -0.288 to 0.020 × 10¹²/L). Hematocrit values were slightly lower in men consuming the linoleic acid diet compared with the diets high in stearic acid (P = 0.107, 95% CI for the difference -0.026 to 0.002 L/L) and oleic acid (P = 0.021, 95% CI for the difference -0.030 to -0.002 L/L). No other gender-dependent effects were observed. When subjects consumed the stearic acid diet, the platelet volume decreased by 0.32 fL compared with the oleic acid diet (P < 0.001, 95% CI for the difference -0.495 to -0.144 fL) and by 0.35 fL compared with the linoleic acid diet (P < 0.001, 95% CI for the difference -0.526 to -0.175 fL). These effects were evident in both men and women.

Table 4.3 Hematological variables during consumption of diets enriched in stearic, oleic, and linoleic acids for 5 weeks by healthy men and women.¹

| | Stearic acid diet | Oleic acid diet | Linoleic acid diet | P for diet effects ² |
|----------------------|---------------------------|--------------------------|--------------------------|---------------------------------|
| Leukocytes | | $\times 10^9/L$ | | |
| All | 6.05 ± 1.28 | 6.23 ± 1.26 | 6.05 ± 1.29 | P = 0.211 |
| Men | 6.33 ± 1.20 | 6.41 ± 1.07 | 6.24 ± 1.15 | P = 0.540 |
| Women | 5.86 ± 1.32 | 6.12 ± 1.38 | 5.92 ± 1.38 | P = 0.244 |
| Erythrocytes | | $\times 10^{12}/L$ | | |
| All | 4.74 ± 0.55 | 4.72 ± 0.49 | 4.61 ± 0.44 | P = 0.046 |
| Men | 4.98 ± 0.53 ^{ab} | 5.06 ± 0.47 ^a | 4.84 ± 0.48 ^b | P = 0.006 |
| Women | 4.59 ± 0.51 | 4.48 ± 0.36 | 4.46 ± 0.34 | P = 0.254 |
| Hemoglobin | | g/L | | |
| All | 145 ± 127 | 143 ± 102 | 142 ± 111 | P = 0.133 |
| Men | 153 ± 95 | 151 ± 84 | 151 ± 93 | P = 0.504 |
| Women | 139 ± 116 | 138 ± 71 | 136 ± 77 | P = 0.255 |
| Hematocrit | | L/L | | |
| All | 0.41 ± 0.04 | 0.41 ± 0.03 | 0.40 ± 0.03 | P = 0.108 |
| Men | 0.43 ± 0.03 ^{ab} | 0.44 ± 0.03 ^a | 0.42 ± 0.03 ^b | P = 0.019 |
| Women | 0.40 ± 0.04 | 0.39 ± 0.02 | 0.39 ± 0.02 | P = 0.302 |
| Platelets | | $\times 10^9/L$ | | |
| All | 273 ± 64 | 265 ± 62 | 266 ± 60 | P = 0.457 |
| Men | 263 ± 57 | 240 ± 60 | 257 ± 64 | P = 0.088 |
| Women | 279 ± 69 | 281 ± 58 | 271 ± 57 | P = 0.458 |
| Mean platelet volume | | fL | | |
| All | 7.89 ± 0.76 ^a | 8.21 ± 0.66 ^b | 8.25 ± 0.72 ^b | P < 0.001 |
| Men | 7.86 ± 0.98 ^a | 8.19 ± 0.82 ^b | 8.23 ± 0.89 ^b | P = 0.003 |
| Women | 7.92 ± 0.59 ^a | 8.23 ± 0.54 ^b | 8.26 ± 0.62 ^b | P = 0.001 |

¹Values are means ± standard deviations at the end of each intervention period (mean of weeks 4 and 5), $n = 45$ (18 men and 27 women). Means in a row with superscripts without a common letter differ, $P < 0.05$.

²P-values for diet effects were calculated by the general linear model with subject number as random factor and diet and period as fixed factors.

Ex vivo and in vitro platelet aggregation

In all subjects, *ex vivo* platelet aggregation time (Ta) as measured by filtragometry did not differ among those consuming the 3 diets ($P = 0.200$ for diet effects, **Table 4.4**). In men only ($P = 0.036$ for diet effects), Ta increased by 69 s during consumption of the linoleic acid diet relative to the diet high in stearic acid ($P = 0.040$, 95% CI for the difference 2 to 136 s). The aggregation time during consumption of the linoleic acid diet tended to increase compared with that when the

oleic acid diet was consumed ($P = 0.198$, 95% CI for the difference -16 to 113 s). The diets high in stearic acid or oleic acid did not differ in their effects on aggregation time ($P = 1.00$, 95% CI for the difference -47 to 87 s). No diet effects were evident in women ($P = 0.713$ for diet effects).

Table 4.4 Effects on *ex vivo* platelet aggregation variables as measured by filtragometry during consumption of diets enriched in stearic, oleic, and linoleic acids for 5 weeks in healthy men and women.¹

| | Stearic acid diet | Oleic acid diet | Linoleic acid diet | P for diet effects ² |
|-------------------------------------|-----------------------|------------------------|------------------------|---------------------------------|
| Aggregation time (Ta) | s | | | |
| All | 113 ± 96 | 109 ± 62 | 142 ± 125 | P = 0.200 |
| Men | 109 ± 70 ^a | 117 ± 61 ^{ab} | 164 ± 127 ^b | P = 0.036 |
| Women | 115 ± 110 | 104 ± 62 | 126 ± 123 | P = 0.713 |
| Aggregation slope (Ts) | mmHg/s | | | |
| All | 0.61 ± 0.68 | 0.52 ± 0.58 | 0.71 ± 1.06 | P = 0.243 |
| Men | 0.48 ± 0.42 | 0.38 ± 0.37 | 0.40 ± 0.60 | P = 0.789 |
| Women | 0.68 ± 0.79 | 0.62 ± 0.68 | 0.94 ± 1.26 | P = 0.259 |
| Maximum aggregation (Amax) | mmHg | | | |
| All | 81 ± 110 | 97 ± 121 | 94 ± 123 | P = 0.795 |
| Men | 48 ± 82 | 74 ± 113 | 49 ± 95 | P = 0.657 |
| Women | 99 ± 121 | 113 ± 126 | 128 ± 133 | P = 0.786 |
| Desaggregation induction time (Tdi) | s | | | |
| All | 29.5 ± 24.1 | 24.6 ± 11.2 | 24.2 ± 12.7 | P = 0.400 |
| Men | 21.7 ± 12.0 | 22.2 ± 5.5 | 23.1 ± 13.5 | P = 0.723 |
| Women | 34.1 ± 28.4 | 26.5 ± 14.0 | 25.2 ± 12.2 | P = 0.521 |

¹Values are means ± standard deviations, $n = 18$ men and 27 women, at the end of each intervention period (week 5). Means in a row with superscripts without a common letter differ, $P < 0.05$.

²P-values for diet effects were calculated by the general linear model with subject number as random factor and diet and period as fixed factors.

There were no diet effects for collagen-induced platelet aggregation or ADP-induced platelet aggregation in whole blood *in vitro* (Table 4.5). Because effects on *in vitro* platelet aggregation induced by 5 $\mu\text{mol/L}$ ADP were comparable to those induced by 15 $\mu\text{mol/L}$ ADP, only results with a final concentration of 15 $\mu\text{mol/L}$ are reported.

Table 4.5 Effects on *in vitro* platelet aggregation in whole blood induced by either collagen or ADP during consumption of diets enriched in stearic, oleic, and linoleic acids for 5 weeks in healthy men and women.¹

| | Stearic acid diet | Oleic acid diet | Linoleic acid diet | P for diet effects ² |
|--|-------------------|-----------------|--------------------|---------------------------------|
| <i>Collagen-induced platelet aggregation (2 mg/L)</i> ³ | | | | |
| Aggregation time (Tai) | <i>Min</i> | | | |
| All | 0.93 ± 0.23 | 0.99 ± 0.30 | 0.90 ± 0.25 | P = 0.146 |
| Men | 1.04 ± 0.22 | 1.17 ± 0.29 | 0.97 ± 0.31 | P = 0.091 |
| Women | 0.86 ± 0.22 | 0.87 ± 0.25 | 0.85 ± 0.20 | P = 0.909 |
| Aggregation velocity (Va) | <i>Ω/min</i> | | | |
| All | 6.11 ± 2.84 | 6.28 ± 3.06 | 5.86 ± 2.75 | P = 0.776 |
| Men | 5.21 ± 2.00 | 5.49 ± 1.97 | 5.35 ± 2.17 | P = 0.805 |
| Women | 6.73 ± 3.18 | 6.80 ± 3.55 | 6.21 ± 3.08 | P = 0.717 |
| Maximum aggregation (Imax) | <i>Ω</i> | | | |
| All | 12.4 ± 5.5 | 13.3 ± 4.8 | 12.0 ± 5.1 | P = 0.449 |
| Men | 11.3 ± 4.5 | 13.7 ± 4.8 | 12.1 ± 4.3 | P = 0.232 |
| Women | 13.2 ± 6.1 | 12.9 ± 4.9 | 11.9 ± 5.6 | P = 0.513 |
| <i>ADP-induced platelet aggregation (15 μmol/L)</i> | | | | |
| Aggregation time (Tai) | <i>Min</i> | | | |
| All | 0.58 ± 0.23 | 0.57 ± 0.19 | 0.59 ± 0.25 | P = 0.705 |
| Men | 0.74 ± 0.20 | 0.65 ± 0.21 | 0.63 ± 0.23 | P = 0.316 |
| Women | 0.48 ± 0.18 | 0.52 ± 0.16 | 0.57 ± 0.26 | P = 0.380 |
| Aggregation velocity (Va) | <i>Ω/min</i> | | | |
| All | 4.65 ± 2.51 | 5.03 ± 2.42 | 4.41 ± 2.38 | P = 0.518 |
| Men | 3.53 ± 2.07 | 4.57 ± 1.92 | 4.23 ± 2.26 | P = 0.374 |
| Women | 5.42 ± 2.54 | 5.36 ± 2.72 | 4.51 ± 2.49 | P = 0.424 |
| Maximum aggregation (Imax) | <i>Ω</i> | | | |
| All | 8.8 ± 5.1 | 10.4 ± 4.5 | 8.9 ± 4.5 | P = 0.304 |
| Men | 7.7 ± 5.8 | 10.4 ± 4.1 | 8.9 ± 4.5 | P = 0.410 |
| Women | 9.6 ± 4.5 | 10.4 ± 4.9 | 8.9 ± 4.6 | P = 0.564 |

¹Values are means ± standard deviations, *n* = 18 men and 27 women at the end of each intervention period (week 5).

²*P*-values for diet effects were calculated by the general linear model with subject number as random factor and diet and period as fixed factors. The 3 diets did not differ.

³Values are means of 2 measurements.

Coagulation and fibrinolysis

Coagulation (factor VIIam activity, prothrombin fragment 1 and 2, and fibrinogen) and fibrinolytic (PAI activity and tPA/PAI-1 complexes) factors did not differ among the 3 diets (Table 4.6). These variables did also not differ between men and women.

Table 4.6 Coagulation and fibrinolytic variables during consumption of diets enriched in stearic, oleic, and linoleic acids for 5 weeks in healthy men and women.¹

| | Stearic acid diet | Oleic acid diet | Linoleic acid diet | P for diet effects ² |
|--------------------------|-------------------|-----------------|--------------------|---------------------------------|
| Factor VIIam activity | | % of standard | | |
| All | 98 ± 31 | 100 ± 32 | 97 ± 33 | P = 0.398 |
| Men | 99 ± 26 | 98 ± 22 | 99 ± 26 | P = 0.908 |
| Women | 98 ± 34 | 102 ± 37 | 96 ± 37 | P = 0.245 |
| Prothrombin fragment 1+2 | | nmol/L | | |
| All | 2.51 ± 2.98 | 2.51 ± 2.95 | 2.78 ± 3.14 | P = 0.360 |
| Men | 3.12 ± 4.01 | 2.67 ± 3.62 | 2.96 ± 3.74 | P = 0.482 |
| Women | 2.11 ± 2.05 | 2.40 ± 2.49 | 2.67 ± 2.75 | P = 0.115 |
| Fibrinogen | | g/L | | |
| All | 3.2 ± 0.5 | 3.2 ± 0.5 | 3.2 ± 0.7 | P = 0.940 |
| Men | 3.1 ± 0.5 | 3.0 ± 0.6 | 3.2 ± 0.9 | P = 0.599 |
| Women | 3.2 ± 0.6 | 3.3 ± 0.4 | 3.2 ± 0.5 | P = 0.389 |
| PAI activity | | kU/L | | |
| All | 10.47 ± 7.25 | 9.89 ± 7.15 | 9.79 ± 6.82 | P = 0.346 |
| Men | 12.76 ± 7.01 | 11.65 ± 6.63 | 10.85 ± 6.96 | P = 0.077 |
| Women | 8.94 ± 7.12 | 8.72 ± 7.37 | 9.09 ± 6.76 | P = 0.842 |
| TPA/PAI-1 complexes | | µg/L | | |
| All | 43.1 ± 33.6 | 41.2 ± 33.8 | 39.0 ± 23.7 | P = 0.533 |
| Men | 57.0 ± 42.1 | 49.9 ± 41.1 | 45.2 ± 22.9 | P = 0.293 |
| Women | 33.9 ± 23.1 | 35.4 ± 27.2 | 34.8 ± 23.7 | P = 0.894 |

¹Values are means ± standard deviations, n = 18 men and 27 women at the end of each intervention period (pooled weeks 4 and 5).

²P-values for diet effects were calculated by the general linear model with subject number as random factor and diet and period as fixed factors. The 3 diets did not differ.

DISCUSSION

The purpose of this well-controlled crossover study was to evaluate the effects of stearic, oleic, and linoleic acids on platelet aggregation, coagulation, and fibrinolytic factors, and hematological variables in healthy men and women. When 7% of energy of these fatty acids was exchanged among the diets, small differences on these markers of thrombotic tendency appeared. Compared with the stearic acid diet, the diet high in linoleic acid decreased *ex vivo* platelet aggregation in men only. Moreover, stearic acid decreased platelet volume relative to both other fatty acids in both men and women. Effects on coagulation and fibrinolytic variables did not differ

after consumption of the 3 fatty acids. Therefore, the finding in previous studies that stearic acid is highly thrombogenic (12,13) is not supported by the present study.

Ex vivo platelet aggregation time in men increased with consumption of linoleic acid relative to the stearic acid diet. In 2 earlier studies with the filrtragometer, linoleic acid prolonged aggregation time compared with a mixture of saturated fatty acids (23) or a mixture of saturated and monounsaturated fatty acids (24). A prolonged aggregation time as measured by filrtragometry indicates lower *in vivo* platelet aggregability and is negatively associated with mortality from coronary heart disease (21). In the study of Hornstra *et al* (23) only men participated, whereas in the study of Fleischman *et al* (24) both men and women participated. Unfortunately, in the latter study, results were not reported for men and women separately. In our study, *ex vivo* platelet aggregation time was significantly increased by linoleic acid in men, but not in women relative to stearic acid. Compared with oleic acid, this effect tended to be significant ($P = 0.198$). These findings indicate a gender-dependent effect of dietary fatty acid intake on *ex vivo* platelet aggregation. In contrast to the other 2 studies (23,24), we could specifically attribute the observed effects to stearic acid. Whether other saturated fatty acids would have given similar effects, remains to be clarified.

Stearic acid, oleic acid, and linoleic acid did not differ in their effects on *in vitro* whole blood platelet aggregation variables induced by either ADP or collagen, a technique used in many other studies. This extends the findings of Hunter *et al* (25), who reported the effects of stearic acid, oleic acid, and linoleic acid in a crossover study with only 6 young healthy men. When experimental diets were consumed for 2 weeks, ADP-induced platelet aggregation in platelet-rich plasma (PRP) was not affected (25). Several studies focused on the effects of oleic and linoleic acids, but results were inconsistent. As in our study, there was no difference in effect between oleic acid and linoleic acid in most of the studies (17,18,25-28). An exception is the study of Burri *et al* (19) with 7 healthy male subjects in which 7-8% of energy of oleic acid and linoleic acid were exchanged. Consumption of the diet high in linoleic acid decreased ADP- and collagen-induced platelet aggregation relative to oleic acid. In another study, consumption of oleic acid as well as linoleic acid decreased collagen-induced platelet aggregation compared with a diet high in saturated fatty acids (29). In the studies mentioned earlier, PRP was used to measure platelet aggregation, whereas in the last-mentioned study, platelet aggregation was analysed in whole blood. Finally, effects depend on the way in which platelet aggregation is induced. In the study of Lahoz *et al* (18), consumption of linoleic acid but not oleic acid enhanced platelet aggregation induced by ADP, relative to saturated fatty acids. This proaggregatory effect was not observed when collagen or adrenalin was used

as the inducer. In the other study (26), oleic acid and linoleic acid increased collagen- but not ADP-induced platelet aggregation compared with saturated fatty acids. In general, *in vitro* platelet aggregation studies are difficult to compare because of differences in methodologies. Different anticoagulants may be used or different inducers (collagen, ADP, adrenalin, or thrombin) at different concentrations; in some studies, PRP was used, and in others whole blood.

Stearic acid, oleic acid, and linoleic acid did not differ in their effects on factor VIIam activity. Hunter *et al* (25) also observed comparable effects of stearic acid and these 2 *cis*-unsaturated fatty acids using 3 different methods to assess factor VII activity. In addition, when the effects of dietary oleic acid and linoleic acid were compared in a study with subjects aged between 45 and 65 y old, these fatty acids did not differently affect factor VII coagulant activity (30). In contrast, in 2 studies with healthy young male and female students, oleic acid consumption reduced factor VII coagulant activity compared with linoleic acid (28,31). In one of these studies, the concentration of activated factor VII (FVIIa), however, did not differ between subjects consuming the 2 diets (28). FVIIa was not measured in the other study (31). These contrasting findings (28,30,31) cannot be attributed to differences in the age of the subjects, because in 2 other studies in young healthy human subjects, consumption of oleic acid and linoleic acid did not differently affect factor VII coagulant activity (32,33). Thus, despite the use of a wide variety of methods to measure factor VII activity, the effects of stearic, oleic, and linoleic acid consumption on this coagulation factor did not differ.

Although concentrations of fibrinogen (6,7) and prothrombin fragment 1 and 2 (7) are positively associated with the risk of coronary heart disease, only a few studies have investigated the effects of dietary fatty acids on these coagulation variables. Although some studies reported different effects of oleic acid and linoleic acid on factor VII, concentrations of prothrombin fragment 1 and 2 and fibrinogen did not differ (28,31). Similar to Hunter *et al* (25), we also found comparable effects of stearic, oleic, and linoleic acids on these coagulation variables. In contrast, when 8% of energy from stearic acid was replaced by oleic acid, fibrinogen concentrations increased by 0.15 g/L in a recent study by Baer *et al* (34). In addition, compared with a diet rich in myristic and lauric acids, stearic acid raised fibrinogen concentrations (16). When 7-8% of energy from stearic acid was exchanged for palmitic acid, however, fibrinogen was not affected (14). The effects of lauric and palmitic acid consumption on concentrations of fibrinogen and prothrombin fragment 1 and 2 did not differ from those of oleic acid (35). Thus, although the effects of oleic and linoleic acid consumption on fibrinogen concentrations seem to be comparable, the effects

of stearic acid relative to the other saturated fatty acids or unsaturated fatty acids were inconsistent.

As markers of fibrinolytic activity, concentrations of tPA and PAI-1 antigen, tPA and PAI activities (6), and concentrations of tPA/PAI-1 complexes (36) are related to the risk of coronary heart disease. Various earlier studies evaluated the effects of dietary fatty acid composition on variables of the fibrinolytic system. In our study, stearic, oleic, and linoleic acid consumption did not affect PAI activity and concentrations of tPA/PAI-1 complexes, which are strongly correlated with plasma levels of PAI activity or tPA antigen (36). These findings not only agree with the results of the study of Hunter *et al* (25), but also with the few studies that have compared oleic acid and linoleic acid. In one study, high-fat diets rich in oleic or linoleic acids did not differ in their effects on tPA activity and PAI-1 antigen concentrations (33). Dietary oleic and linoleic acids also had comparable effects on tPA and PAI activities (28,31). Therefore, our study does not provide evidence that these 3 fatty acids have differential effects on fibrinolytic variables.

In both men and women, platelet volume was decreased after consumption of the diet high in stearic acid. Stearic acid-enriched diets also decreased platelet volume compared with diets rich in palmitic acid (14,37,38). Larger platelets are associated with increased platelet reactivity and risk of myocardial infarction (39,40). Survivors, 6 months after a myocardial infarction, had a 0.43 fL lower mean platelet volume than nonsurvivors (39). In our study, platelet volumes of the subjects during consumption of the stearic acid diet were 0.32 and 0.35 fL lower than when they consumed diets high in oleic acid and linoleic acid, respectively.

To summarise, when 7% of dietary energy of stearic acid was replaced by linoleic acid, *ex vivo* platelet aggregation was beneficially affected in men. On the other hand, stearic acid reduced platelet volume relative to the other 2 fatty acids, whereas effects on coagulation and fibrinolytic variables did not differ among the 3 fatty acids. Overall, we therefore conclude that our results do not provide evidence that stearic acid is highly thrombogenic, as suggested by some earlier studies (12,13).

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Effects of stearic, oleic, and linoleic acids on biomarkers of lipid peroxidation and inflammation in healthy human subjects

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ABSTRACT

Background: Markers of lipid peroxidation and inflammation, which are related to cardiovascular disease risk, are affected by the dietary fatty acid composition.

Objective: To examine the effects of stearic, oleic, and linoleic acids on biomarkers of oxidative stress and inflammation.

Design: Forty-five healthy subjects (18 men and 27 women) consumed 3 experimental diets enriched in stearic acid, oleic acid, or linoleic acid (exchange of 7% of energy) for 5 weeks in a randomised, crossover study. Diets contained 38% of energy as fat. As biomarkers of non-enzymatic and enzymatic lipid peroxidation, urinary excretions of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$) and 15-keto-dihydro-prostaglandin $F_{2\alpha}$ (15-K-DH-PGF $_{2\alpha}$) were analysed. Concentrations of high-sensitivity CRP (hsCRP) were measured as biomarker of inflammation.

Results: No effects were observed on urinary excretion of 8-iso-PGF $_{2\alpha}$ ($P = 0.154$) and 15-K-DH-PGF $_{2\alpha}$ ($P = 0.492$). Plasma concentrations of hsCRP did not differ between the three diets ($P = 0.408$) and were 1.44 ± 1.59 mg/L (mean \pm standard deviation) during consumption of the stearic acid diet, 2.00 ± 2.64 mg/L on the oleic acid diet and 2.05 ± 3.57 mg/L on the linoleic acid diet. Serum concentrations of IL-10 were 7.00 ± 8.49 pg/mL, 7.08 ± 8.03 pg/mL and 6.29 ± 5.68 pg/mL on the diets rich in stearic, oleic, or linoleic acids ($P = 0.268$ for diet effects).

Conclusions: In healthy non-smoking human subjects, stearic, oleic, and linoleic acids - three dietary fatty acids with 18 carbon atoms, but with different degrees of unsaturation - do not have different effects on markers of lipid peroxidation or the inflammatory response.

INTRODUCTION

It is well established that the fatty acid composition of the diet affects cardiovascular disease risk (1). When compared with saturated fatty acids, monounsaturated and polyunsaturated fatty acids lower LDL cholesterol, while effects on HDL cholesterol are comparable (2). However, dietary fatty acids not only affect the serum lipoprotein profile but also other cardiovascular risk markers such as lipid peroxidation and inflammation.

Lipid peroxidation and inflammation are importantly involved in the pathogenesis of atherosclerosis and cardiovascular diseases (3,4). When lipids or lipoproteins become modified by oxidation, an inflammatory response is initiated. Especially polyunsaturated fatty acids are thought to be susceptible to oxidative modification because of the presence of multiple double bonds. Indeed, several human studies reported that dietary linoleic acid increased *in vitro* the susceptibility of LDL to copper-induced oxidation (5,6) but compelling evidence that this occurs *in vivo* is lacking. In fact, data from dietary studies using urinary isoprostane concentrations, a biomarker for *in vivo* oxidative stress, are contradictory (7-9). F₂-Isoprostanes are free radical catalysed products of arachidonic acid (10). 8-Iso-prostaglandin F_{2α} (8-iso-PGF_{2α}) is a major isoprostane that is considered to be a reliable indicator of lipid peroxidation and oxidative stress (11). In addition, 15-keto-dihydro-prostaglandin F_{2α} (15-K-DH-PGF_{2α}), a major metabolite of prostaglandin F_{2α} which is a cyclooxygenase mediated product of arachidonic acid, is regarded as a reliable indicator of enzymatic lipid peroxidation and inflammatory responses (12).

Although interest is growing, not many studies addressed the effects of individual fatty acids on inflammatory markers and markers of immune function. As parameter of inflammation, the acute-phase reactant high-sensitivity C-reactive protein (hsCRP), which is a strong predictor of cardiovascular events, is frequently used (13). Moreover, the accumulation of inflammatory cells in an atherosclerotic plaque is orchestrated by the production of immunomodulatory molecules such as chemoattractants, adhesion molecules, pro- and anti-inflammatory cytokines and growth factors (14). N-3 polyunsaturated fatty acids from fish oil may reduce C-reactive protein concentrations (15,16), although some studies also reported no effects (17). Data from human studies investigating the effects of fatty acids other than n-3 polyunsaturated fatty acids on inflammation and lipid peroxidation markers are, however, limited.

Recently, we have reported that at realistic intakes differences between the effects of stearic, oleic, and linoleic acids on the serum lipoprotein profile are small (18). Whether effects on lipid peroxidation and inflammatory markers are also

comparable has not been investigated before. Therefore, the purpose of the present study was to examine in humans the effects of these three C18 fatty acids with increased degree of unsaturation on biomarkers of oxidative stress and inflammation.

SUBJECTS AND METHODS

Subjects

Subjects were recruited by advertisements in local newspapers and a university hospital newsletter, and by posters in university buildings. The screening and eligibility criteria of the subjects were described in detail before (18). Fifty-eight healthy, non-smoking, slightly hypercholesterolemic subjects were selected for the study. None of the participants used medication or a prescribed diet known to affect serum lipids or thrombosis tendency. Thirteen subjects withdrew mainly in the first 2 weeks of the study because of reasons specifically related to the strict study protocol (4 subjects), stressful personal or job circumstances (5 subjects), and physical illness (2 subjects in the first intervention period and 1 subject in the second intervention period). One subject was excluded after the first intervention period because he did not follow the protocol. Ultimately, 45 subjects, 18 men and 27 women, completed the study protocol. The characteristics of these subjects are shown in **Table 5.1**. Sixteen women were postmenopausal and 5 used oral contraceptives. The subjects were asked not to change their level of physical exercise or their use of alcohol, vitamins or oral contraceptives during the study. Participants were fully informed of study requirements. All subjects had given their written informed consent, before they entered the study.

Table 5.1 Characteristics of the participants.¹

| | Men | Women | All |
|---------------------------------|---------------|---------------|---------------|
| | <i>n</i> = 18 | <i>n</i> = 27 | <i>n</i> = 45 |
| Age (y) | 54.0 ± 9.9 | 49.7 ± 10.4 | 51.4 ± 10.3 |
| BMI (kg/m ²) | 26.0 ± 2.2 | 24.1 ± 2.8 | 24.9 ± 2.7 |
| Systolic blood pressure (mmHg) | 127 ± 12 | 118 ± 14 | 122 ± 14 |
| Diastolic blood pressure (mmHg) | 80 ± 6 | 74 ± 9 | 77 ± 8 |
| Total cholesterol (mmol/L) | 6.19 ± 0.78 | 5.94 ± 0.73 | 6.04 ± 0.75 |
| LDL cholesterol (mmol/L) | 3.17 ± 0.57 | 3.29 ± 0.53 | 3.24 ± 0.54 |
| HDL cholesterol (mmol/L) | 1.17 ± 0.29 | 1.70 ± 0.56 | 1.48 ± 0.54 |
| Triacylglycerols (mmol/L) | 1.37 ± 0.61 | 0.99 ± 0.45 | 1.15 ± 0.55 |

¹Values are means ± standard deviations.

Experimental design and diets

The study had a randomised, multiple crossover design. Each participant consumed each of the 3 different diets in random order during three 5-week periods. Between each intervention period was a washout period of at least 1 week, during which the participants consumed their habitual diets. One diet was high in stearic acid (C18:0), another high in oleic acid (C18:1), and the third one high in linoleic acid (C18:2). The protocol of the study was approved by the Medical Ethics Committee of Maastricht University (18).

Before participants started the study, their total energy intake was estimated with the Harris-Benedict equation (19). The study diets were formulated at 16 different levels of energy intake ranging from 6 to 13.5 MJ per day. During the intervention periods, subjects received experimental products (bread, margarine, and cake) in which the normal fats were replaced by experimental fats.

Experimental fats were produced by NIZO Food Research (Ede, The Netherlands). The high-stearic acid fat was composed of 9.0% palm oil, 5.5% safflower oil, 5.0% olive oil, 33.5% cocoa butter, 18.0% high-oleic sunflower oil and 29.0% glycerol tri-stearate. The high-oleic acid fat consisted of 19.5% palm oil, 26.0% olive oil, 7.5% cocoa butter, and 47.0% high-oleic sunflower oil. The high-linoleic acid fat was a mixture of 20.0% palm oil, 52.0% safflower oil, 7.0% olive oil, 9.0% cocoa butter and 12.0% high-oleic sunflower oil.

The experimental products supplied 60% of total fat energy at a targeted total fat intake of 37% of energy. For the remaining 40% of the total fat intake, subjects consumed daily a certain amount of 'free-choice' fat-containing products. These products had to be recorded in a diary. Subjects visited the university at least once every week to receive a new supply of products. At each visit, subjects were weighed and diaries were checked by a dietician. Individual allowances were adjusted, when subjects' weight changed > 1.5 kg from initial weight during the first week of the study or > 2 kg during the following weeks. Mean daily intakes of energy, protein, carbohydrates, fat, cholesterol, and fibre as calculated by food-frequency questionnaires, which were filled in by the subjects at the end of each period, are summarised in **Figure 5.1**. The prescribed nutrient composition of the 3 diets did not differ, except for a difference of 7% in energy intake provided by stearic, oleic, or linoleic acids.

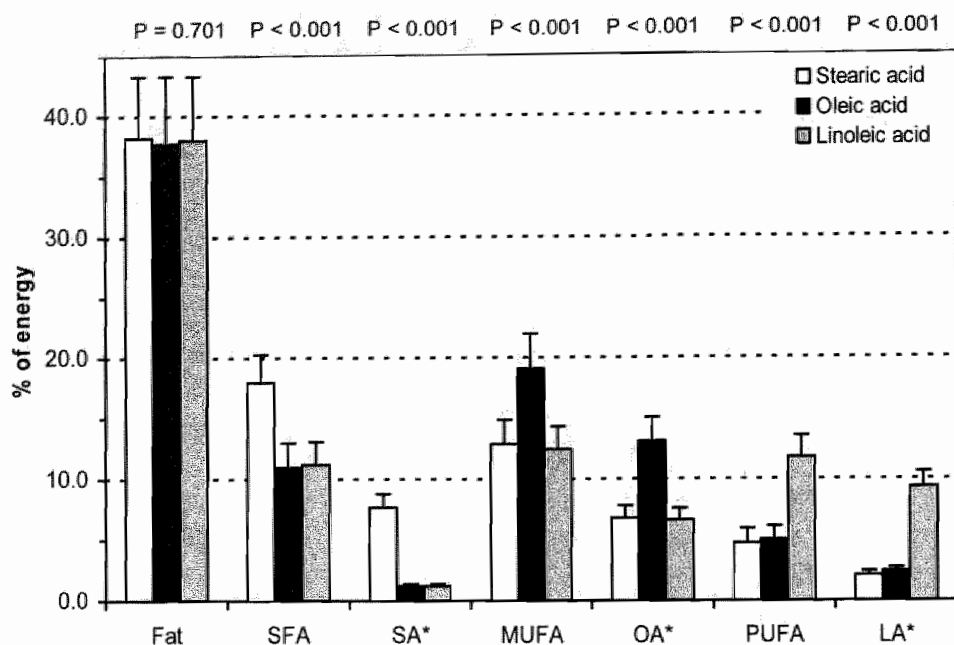


Figure 5.1 Mean nutrient composition of the 3 diets according to the food-frequency questionnaires.^{1,2,3}

¹Values are means \pm standard deviations, $n = 45$ (18 men and 27 women) as calculated from the food-frequency questionnaires. *P*-values for diet effects were calculated by general linear model with subject number as random factor and diet and period as fixed factors.

²Intakes of energy (8.5 ± 1.6 MJ), carbohydrates (46.1 ± 6.1 % of energy), proteins (13.9 ± 2.0 % of energy), alcohol (2.2 ± 2.3 % of energy), cholesterol (17.7 ± 3.6 mg/MJ) and dietary fibre (3.1 ± 0.7 g/MJ) did not differ significantly between the 3 diets.

³Abbreviations used: saturated fatty acids (SFA), stearic acid (SA), monounsaturated fatty acids (MUFA), oleic acid (OA), polyunsaturated fatty acids (PUFA), linoleic acid (LA).

*As provided by the experimental fats only.

Blood sampling and urine collection

Venous blood samples with the volunteer in a recumbent position were obtained after an overnight fast twice at the end of each period (weeks 4 and 5). Blood was sampled with minimum stasis by using a 0.9 mm-needle (PrecisionGlide, Becton-Dickinson Vacutainer systems, Plymouth, United Kingdom) in week 4 or with a 1.0 mm-infusion needle (Microflex, Vygon, Ecouen, France) in week 5. All venipunctures were done by the same person, in the same room, and mostly at the same time of the day. The first 6 mL of blood was collected in EDTA tubes (Becton Dickinson Vacutainer Systems) and used for the analysis of high-sensitivity C-reactive protein

(hsCRP). The following 9 mL was collected in sodium citrate tubes (Becton Dickinson Vacutainer Systems) and used for the antibody array analysis of immunomodulatory molecules. Finally, 10 mL blood was collected in a serum tube (Corvac, Becton Dickinson Vacutainer Systems). EDTA and citrate tubes were kept on ice and centrifuged 3500 x g for 30 min within 1 hour after venipuncture. Plasma samples were snap-frozen in liquid nitrogen and stored at -80°C. At least 1 hour after venipuncture, serum was obtained by centrifugation at 3500 x g for 30 min at 4°C and stored at -80°C.

At the end of each intervention period, subjects collected a 24-hour urine sample. The urine samples were frozen and stored at -20°C.

Non-enzymatic and enzymatic lipid peroxidation

Unextracted 24-hour urine samples were analysed for free 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$, non-enzymatic lipid peroxidation) and 15-keto-dihydro-prostaglandin $F_{2\alpha}$ (15-K-DH-PGF $_{2\alpha}$, enzymatic lipid peroxidation) by specific and validated RIA analyses as described earlier (12,20). Concentrations of 8-iso-PGF $_{2\alpha}$ and 15-K-DH-PGF $_{2\alpha}$ were adjusted for creatinine concentrations measured with a commercial kit (IL Test, Instrumentation Laboratories, Lexington, United States).

High-sensitivity C-reactive protein

High-sensitivity C-reactive protein (hsCRP) concentrations were measured in pooled EDTA plasma samples of weeks 4 and 5 by an immunoturbidimetric assay (K-ASSAY CRP-2, Kamiya Biomedical Company, Seattle, United States) as described previously (21).

Cytokine expression profiles

Before analysis of cytokine expression profiles, citrated plasma samples from weeks 4 and 5 of each diet were pooled, for men and women separately. To measure expression patterns of several cytokines, chemokines and growth factors, the human cytokine antibody array III (RayBiotech Inc., Norcross, United States) was used according to the manufacturer's instructions. Six arrays were analysed, namely 1 for the stearic acid diet, 1 for the oleic acid diet and 1 for the linoleic acid diet for respectively male and female subjects. Array spot intensity was detected by using a LAS-3000 Lite Image reader (Raytest GmbH, Straubenhart, Germany) on the basis of chemiluminescence imaging. Intensity of the spots was quantified by densitometry using AIDA software version 3.50 (Raytest GmbH, Straubenhart, Germany), correcting for differences in background staining of the gel. Comparison of the expression profiles, which consisted of 42 different immunomodulatory

molecules, was possible after normalisation of the individual arrays using the positive controls present on each membrane. Differences in responses were calculated and expressed relative to the oleic acid diet as percentage of change.

High-sensitivity interleukin-10

Concentrations of interleukin-10 (IL-10) were analysed in pooled serum samples of weeks 4 and 5 by high-sensitivity enzyme-linked immunosorbent assays (human (h)IL-10, Biotrak ELISA system, Amersham Biosciences, Buckinghamshire, United Kingdom).

Statistical analysis

All statistical analyses were performed using SPSS 11 for Macintosh OS X package. The data were analysed with the general linear model (GLM) procedure. A probability level (P-value) of < 0.05 was considered statistically significant. Differences in effects on IL-10, hsCRP and lipid peroxidation parameters were examined with diet and period as fixed factors and subject number as random factor. To analyse whether effects of diet were modified by gender, the diet x gender interaction term was added to the model as fixed factor. Between diets comparisons were corrected for 3-group comparisons by the Bonferroni correction. Confidence intervals (CI, 95%) were calculated for the differences between the diets. Pearson's correlations between concentrations of hsCRP and IL-10, and urinary excretion of 8-iso-PGF_{2α} and 15-K-DH-PGF_{2α} were determined to examine linear relationships between parameters.

RESULTS

Lipid peroxidation

Urinary excretion of 8-iso-PGF_{2α} (P = 0.154 for diet effects) did not differ significantly between the 3 diets (**Figure 5.2**). When oleic acid in the diet was substituted by stearic acid or linoleic acid, urinary excretion of 8-iso-PGF_{2α} increased by 0.03 nmol/mmol creatinine (P = 0.304, 95% CI for the difference: -0.01 to 0.07 nmol/mmol creatinine) and 0.03 nmol/mmol creatinine (P = 0.263, 95% CI for the difference: -0.01 to 0.07 nmol/mmol creatinine), respectively. Urinary excretion of 8-iso-PGF_{2α} did not differ between the diets rich in stearic acid and linoleic acid (P = 1.00, 95% CI for the difference: -0.04 to 0.04 nmol/mmol creatinine). Urinary excretion of 15-K-DH-PGF_{2α} was also not affected by the 3 diets (P = 0.492 for diet effects). Compared with the oleic acid diet, urinary excretion of 15-K-DH-PGF_{2α} increased by 0.01 nmol/mmol creatinine (P = 0.733, 95% CI for the difference: -0.01 to 0.03

nmol/mmol creatinine) on the stearic acid diet and by 0.01 nmol/mmol creatinine ($P = 1.00$, 95% CI for the difference: -0.01 to 0.03 nmol/mmol creatinine) on the linoleic acid diet. In addition, urinary excretion of 15-K-DH-PGF_{2α} did not differ on the stearic acid and linoleic acid diets ($P = 1.00$, 95% CI for the difference: -0.02 to 0.02 nmol/mmol creatinine). None of the dietary effects differed significantly between men and women.

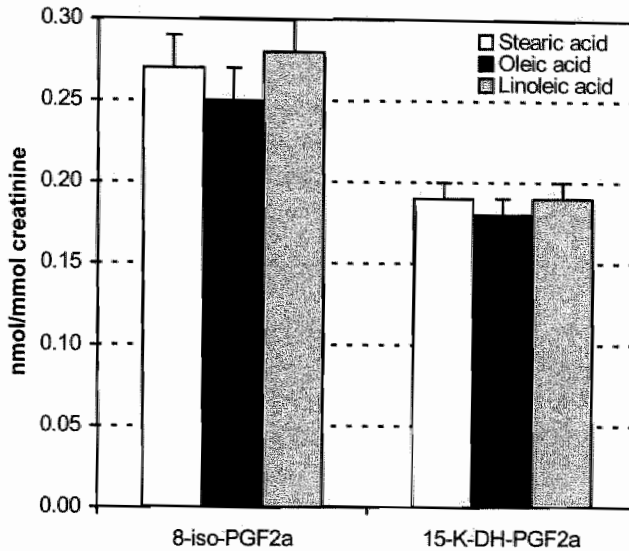


Figure 5.2 Urinary excretion of 8-iso-PGF_{2α} and 15-K-DH-PGF_{2α} as parameters of lipid peroxidation during consumption of diets enriched in stearic, oleic, and linoleic acids for 5 weeks by healthy humans, $n = 45$ (18 men and 27 women).^{1,2}

¹Values are means \pm standard error of the mean at the end of each intervention period (24-hour urine in week 5).

²There were no significant differences between the 3 diets, $P < 0.05$ (general linear model with subject number as random factor and diet and period as fixed factors).

High-sensitivity C-reactive protein (hsCRP)

Concentrations of hsCRP (Figure 5.3) did not differ significantly between the 3 diets ($P = 0.408$). When stearic acid in the diet was substituted by oleic acid or linoleic acid, hsCRP concentrations increased non-significantly by 0.56 mg/L ($P = 0.767$, 95% CI for the difference: -1.77 to 0.64 mg/L) and 0.59 mg/L ($P = 0.715$, 95% CI for the difference: -1.79 to 0.62 mg/L), respectively. Dietary effects did not depend on gender.

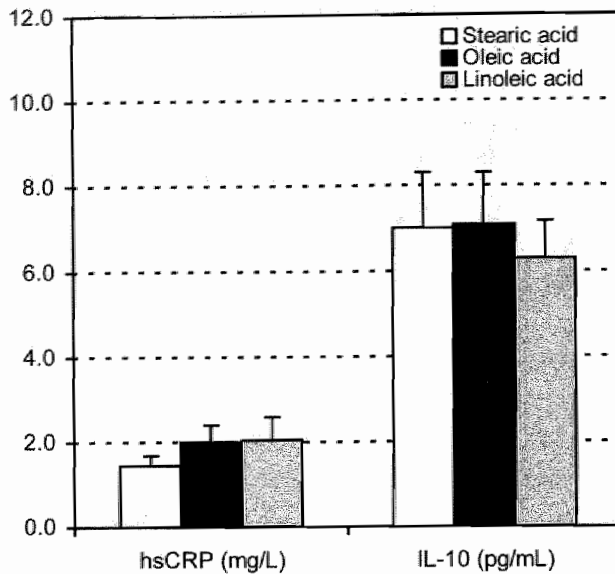


Figure 5.3 Inflammatory parameters during consumption of diets enriched in stearic, oleic, and linoleic acids for 5 weeks by healthy humans, $n = 45$ (18 men and 27 women).^{1,2}

¹Values are means \pm standard error of the mean at the end of each intervention period (pooled weeks 4 and 5).

²There were no significant differences between the 3 diets, $P < 0.05$ (general linear model with subject number as random factor and diet and period as fixed factors).

Expression profiles of immunomodulatory molecules

Twenty cytokines, chemokines or growth factors of the 42 different spots on the antibody arrays were detectable in plasma and were quantified separately for male and female subjects (**Figures 5.4 and 5.5**). During all 3 diets, relative expression of almost all 20 cytokines was higher in men than in women. Moreover, the observed changes of several immunomodulatory molecules differed between men and women during the diets. While in men opposite changes of stearic acid and linoleic acid were observed for interleukin-8 (IL-8), macrophage derived chemokine (MDC), thrombopoietin (TPO), epidermal growth factor (EGF), platelet-derived growth factor β (PDGF β) and vascular endothelial growth factor (VEGF), changes in women were opposite for RANTES, EGF, PDGF β , VEGF, tumour necrosis factor α (TNF α) and tumour necrosis factor β (TNF β). Compared with the oleic acid diet, relative expressions of monocyte chemotactic protein 1 (MCP-1), EGF, TPO and leptin was decreased by more than 5% by stearic acid in men and of MCP-1, EGF, TNF α and TNF β in women, whereas relative expressions of most other cytokines was

increased. Relative expressions of MCP-1, PDGF β and VEGF was decreased by more than 5% in men during the linoleic acid diet and of IL-8, RANTES, TPO, PDGF β , VEGF and leptin in women, compared with the oleic acid diet. Observed changes between stearic acid and oleic acid were less than 5% for IL-8, MDC, oncostatin M (OSM), angiogenin (Ang) in men and for IL-8, TPO and VEGF and leptin in women. Effects of linoleic acid were comparable to those of oleic acid for macrophage inflammatory protein-1 δ (MIP-1 δ), Ang and leptin in men and for MCP-1 and MIP-1 δ in women.

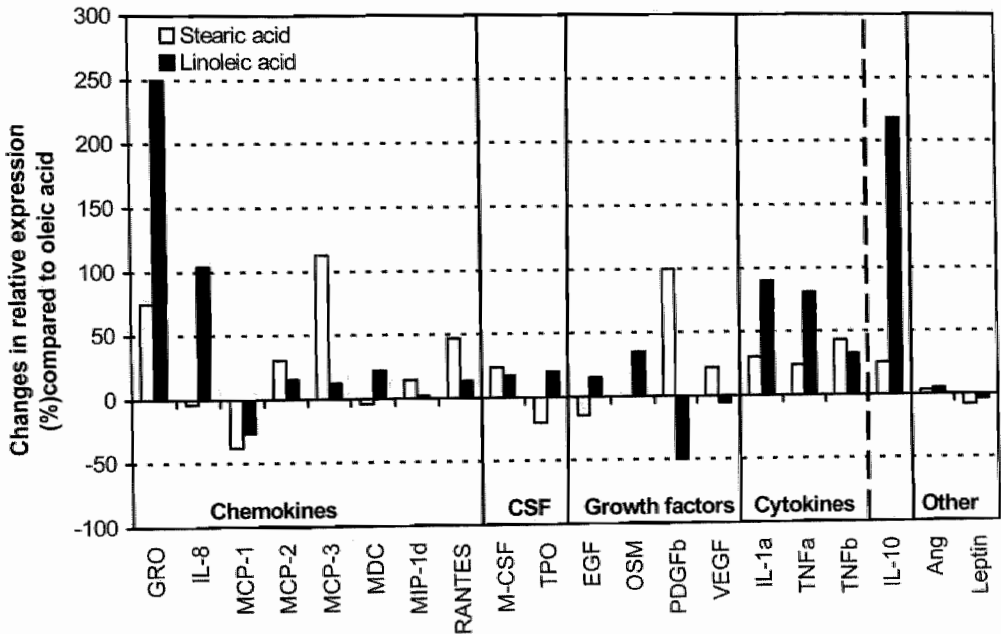


Figure 5.4 Changes in relative expression levels of different cytokines, chemokines and growth factors as measured by an antibody array in male subjects ($n = 18$).^{1,2}

¹Changes in effects of the stearic and linoleic acids diets are expressed relative to the oleic acid diet. The immunomodulatory molecules are divided into different classes i.e. chemokines, colony stimulating factors (CSF), growth factors, and pro- (IL-1 α , TNF α and TNF β) and anti- (IL-10) inflammatory cytokines.

²Abbreviations used: Growth regulated protein (GRO), interleukin-8 (IL-8), monocyte chemotactic protein 1 (MCP-1), monocyte chemotactic protein 2 (MCP-2), monocyte chemotactic protein 3 (MCP-3), macrophage derived chemokine (MDC), macrophage inflammatory protein-1 δ (MIP-1 δ), macrophage colony stimulating factor (M-CSF), thrombopoietin (TPO), epidermal growth factor (EGF), oncostatin M (OSM), platelet-derived growth factor β (PDGF β), vascular endothelial growth factor (VEGF), interleukin-1 α (IL-1 α), tumour necrosis factor α (TNF α), tumour necrosis factor β (TNF β), interleukin-10 (IL-10) and angiogenin (Ang).

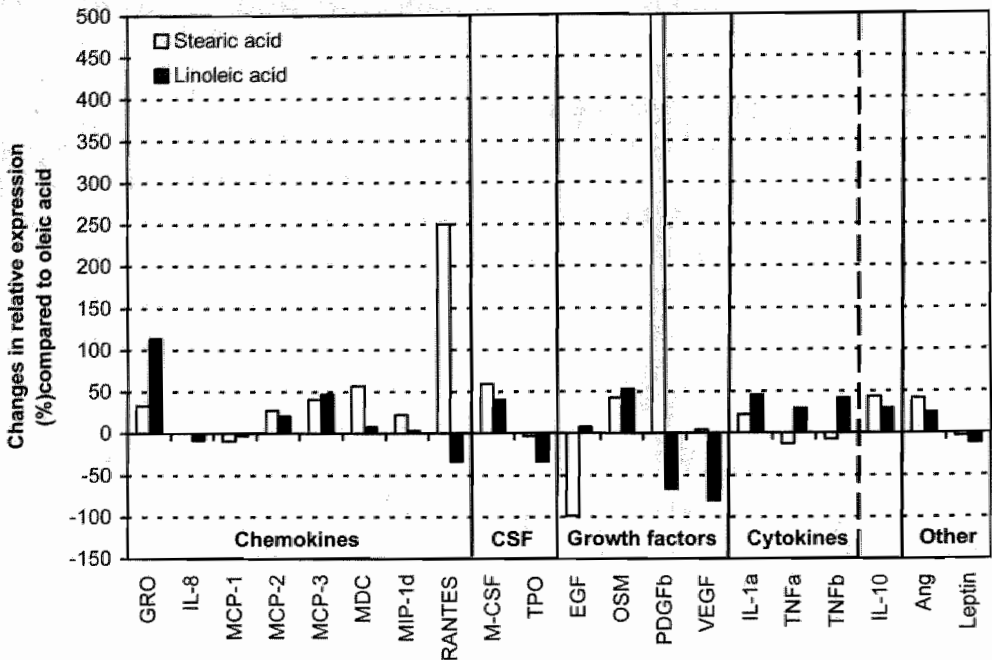


Figure 5.5 Changes in relative expression levels of different cytokines, chemokines and growth factors as measured by an antibody array in female subjects ($n = 27$).^{1,2}

¹Changes in effects of the stearic and linoleic acids diets are expressed relative to the oleic acid diet. The immunomodulatory molecules are divided into different classes i.e. chemokines, colony stimulating factors (CSF), growth factors, and pro- (IL-1 α , TNF α and TNF β) and anti- (IL-10) inflammatory cytokines.

²Abbreviations used: Growth regulated protein (GRO), interleukin-8 (IL-8), monocyte chemotactic protein 1 (MCP-1), monocyte chemotactic protein 2 (MCP-2), monocyte chemotactic protein 3 (MCP-3), macrophage derived chemokine (MDC), macrophage inflammatory protein-1 δ (MIP-1 δ), macrophage colony stimulating factor (M-CSF), thrombopoietin (TPO), epidermal growth factor (EGF), oncostatin M (OSM), platelet-derived growth factor β (PDGF β), vascular endothelial growth factor (VEGF), interleukin-1 α (IL-1 α), tumour necrosis factor α (TNF α), tumour necrosis factor β (TNF β), interleukin-10 (IL-10) and angiogenin (Ang).

High-sensitivity interleukin-10

To confirm the data of the cytokine antibody array, IL-10 was chosen to analyse by a high-sensitivity ELISA. Data are shown in **Figure 5.3**. No statistically significant changes in serum concentrations of IL-10 were observed ($P = 0.268$ for diet effects). The diet rich in linoleic acid reduced IL-10 concentrations by 0.66 pg/mL ($P = 0.183$, 95% CI for the difference: -1.64 to 0.32 pg/mL) and 0.73 pg/mL ($P = 0.143$, 95% CI for the difference: -1.71 to 0.25 pg/mL) relative to the diets rich in respectively stearic acid and oleic acid. When stearic acid was substituted by oleic acid, the

difference in IL-10 concentrations was 0.07 pg/mL ($P = 0.892$, 95% CI for the difference: -0.91 to 1.05 pg/mL). Dietary effects did not differ significantly between men and women. In men ($P = 0.248$ for diet effects), mean IL-10 concentrations were 5.91 ± 3.68 pg/mL on the stearic acid diet, 6.82 ± 5.74 pg/mL on the oleic acid diet, and 6.26 ± 4.51 pg/mL on the linoleic acid diet, while IL-10 concentrations were respectively 7.77 ± 10.73 pg/mL, 7.27 ± 9.45 pg/mL, and 6.30 ± 6.49 pg/mL in women ($P = 0.096$ for diet effects).

Correlations

Urinary excretion of 8-iso-PGF_{2 α} when expressed as nmol/mmol creatinine, was significantly correlated with that of 15-K-DH-PGF_{2 α} in the stearic acid group ($r = 0.582$, $P < 0.001$) as well as in the oleic acid group ($r = 0.469$, $P = 0.001$). The correlation coefficient nearly reached statistical significance when subjects consumed the linoleic acid diet ($r = 0.290$, $P = 0.053$). No significant correlations of hsCRP or IL-10 with urinary excretion of 8-iso-PGF_{2 α} or 15-K-DH-PGF_{2 α} were observed.

DISCUSSION

In the present study the effects of stearic acid, oleic acid, and linoleic acid were investigated on biomarkers of lipid peroxidation and inflammation of apparently healthy men and women. Urinary isoprostanes were measured as *in vivo* markers of lipid peroxidation. HsCRP was measured as marker of inflammation. Moreover, using an array containing antibodies against several chemokines, growth factors and cytokines, inflammatory responses were evaluated. No dietary effects were found.

Although various studies suggested that the *in vitro* susceptibility to lipid peroxidation of fatty acids is positively related to its number of double bonds, this concept is not confirmed by studies using *in vivo* biomarkers of lipid peroxidation. Isoprostanes represent a reliable and sensitive marker of *in vivo* lipid peroxidation and oxidative stress (10). Increased levels of isoprostanes have been reported in cardiovascular diseases (22). In our study, however, fatty acids with 18 carbon atoms and increased degree of unsaturation - stearic acid, oleic acid, and linoleic acid - did not differ in their effects on *in vivo* lipid peroxidation as measured by urinary excretion of 8-iso-PGF_{2 α} and 15-K-DH-PGF_{2 α} in healthy, middle-aged subjects. These observations agree with the results of other well-controlled dietary studies which compared the effects of oleic acid and linoleic acid on concentrations of isoprostanes in urine and plasma of humans (7,9,23). In post-menopausal

women, these 2 fatty acids did also not differ in their effects on the plasma concentrations of F_2 -isoprostanes, thiobarbituric acid reactive substances (TBARS) or malondialdehydes (MDA) (23). Furthermore, in young healthy subjects, oleic acid and linoleic acid had comparable effects on concentrations of plasma TBARS and urinary excretion of 8-iso-PGF_{2α} (7). In another study, high-oleic acid and high-linoleic acid diets did not differ in their effects on the urinary excretion of 8-iso-PGF_{2α}, although the excretion of this biomarker was increased during the high-linoleic acid diet relative to the baseline saturated fat diet (9). In this latter study, the mean age of the subjects was only 27 y. Hence, effects of fatty acids on *in vivo* parameters of oxidative stress may not depend on age. Relative to a diet rich in saturated fatty acids, a rapeseed oil diet rich in monounsaturated and polyunsaturated fatty acids did also not increase parameters of lipid peroxidation including hydroperoxides and malondialdehydes in plasma and 8-iso-PGF_{2α} in plasma and urine (24). Whether the presence of antioxidants in oils rich in monounsaturated or polyunsaturated fatty acids may compensate for the oxidative effects of fatty acids remains to be unravelled. However, fish oil which is poor in antioxidants did not differently affect urinary and plasma 8-iso-PGF_{2α} concentrations, when compared with saturated fatty acids (25). Although these findings may also suggest that these *in vivo* markers of lipid peroxidation are not diet-sensitive, this is contradicted by findings that supplements of the conjugated linoleic acid (CLA) isomer *trans*-10, *cis*-12 CLA increased urinary excretion of 8-iso-PGF_{2α} and of 15-K-DH-PGF_{2α} relative to supplements containing a mixture of CLA isomers or placebo oil (26). Taken together, these studies do not provide evidence that dietary fatty acids affect *in vivo* biomarkers of lipid peroxidation.

In contrast, when effects of diets rich in oleic and linoleic acids were compared using *in vitro* methods to measure lipid peroxidation, several earlier studies have shown that diets rich in oleic acid lowered the susceptibility of LDL to oxidative modification when compared with diets rich in linoleic acid (5,6,8,27-30). When *in vitro* oxidation of LDL was induced by metal ions, lag time decreased, oxidation rate increased and formation of conjugated dienes or lipid peroxides increased on the diets rich in linoleic acid. When *in vitro* as well as *in vivo* methods were used, results were inconsistent. In two human studies *in vitro* susceptibility of LDL to oxidation increased by linoleic acid relative to oleic acid, whereas no or slightly decreased concentrations of conjugated dienes or malondialdehyde in plasma and lipid hydroperoxides or thiobarbituric acid reactive substances (TBARS) in plasma lipoprotein fractions were measured (8). Thus, it is doubtful whether *in vitro* methods evaluating LDL susceptibility to oxidation truly reflect the *in vivo* situation (31).

Data about the effects of individual dietary fatty acids on hsCRP concentrations are scarce. Some studies focused on the effects of n-3 polyunsaturated fatty acids but results were inconsistent. In two studies, hsCRP concentrations were reduced by the n-3 polyunsaturated fatty acids, α -linolenic acid (16), or EPA and DHA (15), while in two other studies no effects of the marine n-3 polyunsaturated fatty acids, EPA and DHA, were found (17,32). In another recent study, effects of diets enriched with *trans* fatty acids, C12:0-C16:0 saturated fatty acids, stearic acid, oleic acid, or carbohydrates were compared. Although *trans* fatty acids increased hsCRP concentrations relative to the other diets, no significant different effects of stearic acid and oleic acid were found after 5 weeks of consumption of each diet (33), which agrees with our findings. While in that study only male subjects participated, we also found no different effects of stearic, oleic, and linoleic acids in female healthy subjects. This may indicate that effects on hsCRP concentration are independent of gender. However, more research in human subjects is necessary to clarify the effects of the dietary fatty acid composition on hsCRP concentrations.

To generate new leads for the potential inflammatory effects of stearic, oleic, and linoleic acids, expression profiles of multiple cytokines were measured by an antibody array. The measurement of expression signatures of immunomodulatory molecules such as cytokines, chemokines and growth factors by an antibody array is an interesting new tool to detect multiple cytokines simultaneously and to evaluate inflammatory effects of fatty acids (34). In our study, women not only had higher levels of many immunomodulatory molecules than men, but expression profiles induced by dietary stearic, oleic, and linoleic acids differed also between men and women. However, for most cytokines no consistent patterns emerged. IL-10 levels, however, were increased in both men and women on the stearic and linoleic acid diets. Samples were therefore also analysed at the individual level by ELISA but results were not in agreement. Although analytical sensitivity for IL-10 differed between ELISA (0.1 pg/mL) and antibody array (10 pg/mL), we do not have a clear explanation for these discrepant findings. Thus, the effects of fatty acids on the cytokine profile need to be addressed in future studies into more detail.

In summary, although dietary fatty acid composition may influence lipid peroxidation, stearic, oleic, and linoleic acids did not differ in their effects on biomarkers of *in vivo* non-enzymatic and enzymatic lipid peroxidation of healthy non-smoking human subjects. Inflammatory response as measured by hsCRP was also not affected by one of these 3 fatty acids.

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Effects of specific CLA isomers on plasma fatty acid profile and expression of desaturases in humans

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ABSTRACT

Human studies suggest that conjugated linoleic acid changes fatty acid metabolism, possibly through effects on mRNA expression of desaturase and elongase enzymes. In this respect, differential effects of the two most common dietary CLA isomers, cis-9,trans-11 (c9,t11) and trans-10,cis-12 (t10,c12) CLA, have hardly been studied. We therefore gave 25 healthy, overweight men and women daily for 6 weeks a drinkable dairy product containing 3 g of oil that was rich in oleic acid. For the next 18 weeks, the control group (n = 7) continued to use this product, whereas the second (n = 9) and third groups (n = 9) received products with 3 g of purified c9,t11 CLA or t10,c12 CLA. For each g of c9,t11 CLA consumed, its proportion in plasma phospholipids increased by 0.26%. For t10,c12 CLA, this value was 0.20%. The t10,c12 CLA isomer increased plasma triacylglycerol levels of conjugated C18:3, whereas c9,t11 CLA increased those of both conjugated C18:3 and C20:3. In plasma phospholipids, the $\Delta 9$ desaturation index of C18:0 ($C18:1n-9/C18:0$) was decreased by t10,c12 CLA ($P = 0.03$ for diet effects), and the $\Delta 6$ desaturation index ($(C18:3n-6+C20:3n-6)/C18:2n-6$) was decreased by both CLA isomers ($P < 0.01$ for diet effects). The $\Delta 5$ desaturation index ($C20:4n-6/C20:3n-6$) and the $\Delta 9$ desaturation index of C16:0 ($C16:1n-7/C16:0$) were not affected. No effects were seen on mRNA expression of desaturases and elongase in peripheral blood mononuclear cells (PBMC). We therefore conclude that incorporation of c9,t11 and t10,c12 CLA into plasma lipids reflects dietary intakes. Compared with oleic acid, $\Delta 9$ and $\Delta 6$ desaturation indices in plasma phospholipids are decreased after consumption of c9,t11 or t10,c12 CLA. Effects on desaturation indices were, however, not reflected by changes at the transcriptional level for the various desaturases and elongase enzymes in PBMC.

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of positional and geometrical isomers of linoleic acid containing conjugated double bonds. *Cis-9, trans-11* (c9,t11) CLA is the most common CLA isomer in nature and is present in ruminant products, whereas commercially available capsules enriched with CLA also contain other isomers like *trans-10, cis-12* (t10,c12) CLA (1). Because of their postulated health benefits (2,3), dietary supplements with CLA are now widely available. To explain these health effects, several mechanisms have been proposed, including regulation of genes coding for enzymes known to modulate lipid and fatty acid metabolism such as desaturases (2,4).

Desaturases are involved in the formation of long-chain metabolites from precursor fatty acids like stearic, linoleic, and α -linolenic acids. Studies in mice and rats have indeed reported that CLA, mainly the t10,c12 isomer, suppressed $\Delta 9$ desaturase mRNA expression or enzyme activity (5-7). Moreover, the c9,t11 isomer decreased $\Delta 6$ desaturase activity in liver microsomes (5). In cell culture studies, effects of t10,c12 CLA on $\Delta 9$ desaturase activity were confirmed in a human hepatoma-derived cell line (HepG2) (8,9). However, effects of c9,t11 CLA on desaturase activities are inconsistent (8,9). Furthermore, effects of both CLA isomers at the mRNA expression level of desaturases have hardly been studied. As desaturation products play an important role in many processes including eicosanoid production, lipid metabolism, and immune function (2,3), inhibition of desaturation may influence health. Most human studies, however, have been carried out using a mixture of CLA isomers and have not focused on specific effects of the major CLA isomers (10-12). Isomer-specific effects of CLA, as observed in animal studies, may explain conflicting results in humans (4,13). Therefore, to examine whether c9,t11 CLA and t10,c12 CLA affect desaturation differently in humans, we investigated the effects of these two CLA isomers on the fatty acid compositions of plasma phospholipids (PL), cholesteryl esters (CE), and triacylglycerols (TAG). In addition, effects on mRNA expression of $\Delta 9$, $\Delta 6$, and $\Delta 5$ desaturases and of elongase by peripheral blood mononuclear cells (PBMC) were studied. Recently, the human $\Delta 5$ (14), $\Delta 6$ (15), and $\Delta 9$ (16) desaturase, and elongase (17) genes have been characterised and reverse transcription quantitative PCR (RT-qPCR) protocols were developed based upon the desaturase and elongase gene sequences.

EXPERIMENTAL PROCEDURES

Subjects

Healthy male and female volunteers were recruited via advertisements in local newspapers. Interested people were informed about the purposes and requirements of the study and had to give their written informed consent before they entered the screening procedure. This procedure consisted of two fasting blood samples for analysis of serum lipids and lipoproteins, hematological parameters, C-reactive protein, and liver and kidney functions, measurement of blood pressure, and collection of a morning urine specimen for analysis of glucose. Participants had to meet all of the eligibility criteria: aged 35-65 y, body mass index between 25 and 30 kg/m², diastolic blood pressure below 95 mmHg and systolic blood pressure below 160 mmHg, fasting serum total cholesterol concentrations less than 7.0 mmol/L, serum triacylglycerol concentrations below 3.0 mmol/L, and plasma glucose below 6.0 mmol/L. Furthermore, subjects were apparently healthy as indicated by a medical questionnaire, not pregnant, and weight stable during the past 3 months; had no history of atherosclerotic disease or malignancy within the past 5 y, normal liver and kidney function, no glycosuria or anemia, no abuse of drugs or alcohol, and no use of any medication known to affect lipid or glucose metabolism. Blood donation or participation in another biomedical trial was not allowed within 4 weeks before the start of the study or during the study.

The Medical Ethics Committee of the Maastricht University had approved the study protocol. This study was part of a multicenter study as reported elsewhere (18). For 25 subjects of the placebo group and the high-dose CLA groups of the Maastricht cohort, desaturase expression in PBMC and fatty acid compositions of plasma lipids were analysed. During the screening period, their body mass indexes ranged from 24.4 to 30.5 kg/m² (mean 27.4 kg/m²). The subjects' fasting serum lipid levels ranged from 3.2 to 6.8 mmol/L for total cholesterol (mean 5.0 mmol/L), from 0.8 to 2.8 mmol/L for HDL cholesterol (mean 1.2 mmol/L), and from 0.4 to 2.4 mmol/L for triacylglycerols (mean 1.2 mmol/L).

Study design

The study had a randomised, double-blind, placebo-controlled, parallel design. During the first 6 weeks of the trial (run-in period), all volunteers consumed daily a dietary supplement providing 3 g of high-oleic sunflower oil (placebo). After the run-in period, subjects were randomly allocated to one of the three treatment groups. For the next 18 weeks of the study (intervention period), one group ($n = 7$) continued to consume the placebo dairy product daily. The second ($n = 9$) and third ($n = 9$)

groups consumed the dairy product with 3 g of purified c9,t11 CLA or 3 g of t10,c12 CLA, respectively.

Dietary supplements containing CLA were provided as an acidified drinkable dairy product produced by Danone (Palaiseau, France) as described earlier (18). The two different isomers of CLA, c9,t11 CLA and t10,c12 CLA, were incorporated as a triacylglycerol in the dairy drinks and were produced by Natural Lipids Ltd (Hovdebygda, Norway). Different flavours were added to the products. The placebo product used during the run-in period had a different flavour from the one used during the intervention period. Bottles were packaged in boxes of 14.

Participants consumed one bottle daily (100 mL) of this dietary supplement between lunch and dinner. In a diary, participants had to record the daily time of consumption of the supplements and to note any signs of illness, medication used, alcohol consumption and any deviations from the study protocol. Subjects were urged not to change their habitual diet, level of physical exercise, smoking habits, or use of alcohol during the study. Volunteers visited the university at least once every two weeks to receive a new supply of supplements. Supplements that were left over had to be returned and were counted as a measure of compliance. Compliance was also checked by analysis of the fatty acid composition of plasma phospholipids. Furthermore, at each visit a dietician checked the diary.

Blood sampling

Blood samples were obtained at the end of the run-in period (week 6) and at the end of the intervention period (week 24) after an overnight fast. Moreover, participants were instructed not to use alcohol during the previous day and not to smoke on the morning before blood sampling. Venous blood was sampled using a vacutainer system with the volunteer in recumbent position. For RNA isolation from PBMC, blood was collected into two EDTA tubes and kept on ice until leukocyte isolation. In addition, one EDTA tube was collected to obtain plasma for analysis of the fatty acid composition of plasma lipids. This tube was centrifuged within 1 hour after venipuncture at 3500 x g for 30 min at 4°C. Plasma samples were snap-frozen in liquid nitrogen and stored at -80°C.

Leukocyte isolation

PBMC were isolated from blood by gradient centrifugation using Lymphoprep (Nycomed Ltd, Birmingham, United Kingdom). Before isolation of PBMC, blood was diluted by addition of an equal volume of Hanks' balanced salt solution (HBSS, Gibco BRL, Life Technologies, Breda, The Netherlands). Then 2 volumes of diluted blood were layered over 1 volume of Lymphoprep in a 50-mL centrifuge tube

(Greiner Bio-One, Alphen a/d Rijn, The Netherlands). After centrifugation at 800 x g for 30 min at 4°C, the interphase containing PBMC was transferred into a new 50-mL centrifuge tube. PBMC were washed with an equal volume of HBSS and centrifuged for 10 minutes at 250 x g at 4°C. The cell pellet was resuspended in 1.5 mL Trizol reagent (Gibco BRL, Gaithersburg, MD, United States) and transferred into an RNase-free 2 mL tube (Eppendorf, Hamburg, Germany).

RNA preparation

Total RNA from PBMC was prepared according to the manufacturer's instructions of the Trizol reagent (Gibco BRL). Briefly, 300 µL chloroform was added to separate phases. After incubation for 2-3 min at room temperature and centrifugation for 15 min at 12000 x g (4°C), the water phase, containing the RNA, was pipetted into a clean RNase-free tube. Then, the RNA was precipitated by addition of 750 µL isopropanol. After incubation for 20 min at -80°C and centrifugation for 10 min at 12000 x g (4°C), the RNA pellet was washed twice with 75% ethanol. In each washing step, the samples were centrifuged for 5 min at 7500 x g (4°C). Finally, RNA was dried and dissolved in 50 µL RNase-free water by incubation at 55-60°C for 10 min. Subsequently both RNA samples were pooled and further purified using the RNeasy mini kit (Qiagen, Leusden, The Netherlands). During this purification procedure, RNA was treated on-column with DNase (RNase-free DNase set, Qiagen) as described in the manufacturer's protocol. Before storage at -80°C, the RNA concentrations were measured spectrophotometrically at 260 nm. All RNA preparations must have an A260/A280 ratio of >1.8 to ensure RNA purity.

cDNA synthesis

Aliquots of 350 ng of total cellular RNA were reverse-transcribed. All samples were denatured for 5 min at 85°C. Reverse transcription of RNA was performed in 40 µL RT mixture containing first strand buffer, 10 mmol/L DTT, 400 U M-MLV reverse transcriptase, 20 U ribonuclease inhibitor (all provided by Gibco BRL), 5 µmol/L random hexamers (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and 375 µmol/L deoxynucleotides (Amersham Pharmacia Biotech, Woerden, The Netherlands). After hexanucleotides had been annealed for 10 min at 22°C, cDNA synthesis was performed for 90 min at 37°C, followed by an enzyme inactivation step for 3 min at 95°C. Finally, cDNA was rapidly chilled to 4°C and stored at -20°C until use.

Reverse transcription quantitative PCR (RT-qPCR)

A RT-qPCR protocol was developed for the quantification of the human housekeeping gene β -actin and desaturase and elongase enzymes. Primers and probe for the desaturase and elongase assays were derived from Genbank sequence NM_013402 for $\Delta 5$ desaturase (fatty acid desaturase 1), NM_004265 for $\Delta 6$ desaturase (fatty acid desaturase 2), NM_005063 for $\Delta 9$ desaturase (stearoyl-CoA desaturase) and NM_021814 for elongase (ELOVL5) using Primer Express 1.5 software (Applied Biosystems). To prevent pseudogene amplification of $\Delta 9$ desaturase, a minor groove binder (MGB) probe was developed to anneal specifically to the cDNA strand of the functional gene. Primer and probe sequences for β -actin were identical to those described by Kreuzer *et al* (19) and those for $\Delta 6$ desaturase similar as described by Leonard *et al* (20). All probes were dual-labelled with a fluorescent FAM reporter dye at the 5' end and a TAMRA quencher dye (or a non-fluorescent quencher and MGB for $\Delta 9$ desaturase) at the 3' end. Primer and probe sequences are listed in **Table 6.1**.

Table 6.1 Primer and probe sequences for reverse transcription quantitative PCR.

| Target sequence | Primer or probe | Sequence (5'-3') | Amplicon length (bp) |
|-----------------------|-------------------------------|---|----------------------|
| β -Actin | β -Actin forward | agc ctc gcc ttg gcc ga | 176 |
| | β -Actin reverse | cag cag ccc gtc cac acc cgc c | |
| | β -Actin probe | ctg gtg cct ggg gcg | |
| $\Delta 5$ Desaturase | $\Delta 5$ Desaturase forward | ggt gta caa cat cag cga gtt ca | 148 |
| | $\Delta 5$ Desaturase reverse | aat cag gag aga gtt cat ata ctt ctt cac | |
| | $\Delta 5$ Desaturase probe | cag gat gcc acg gat ccc ttg gtg | |
| $\Delta 6$ Desaturase | $\Delta 6$ Desaturase forward | tgg caa tgg ctg gat tcc ta | 65 |
| | $\Delta 6$ Desaturase reverse | cag ctt ggg cct gag agg t | |
| | $\Delta 6$ Desaturase probe | cct cat cac ggc ctt tgt cct tgc | |
| $\Delta 9$ Desaturase | $\Delta 9$ Desaturase forward | gcc ctg tat ggg atc act ttg a | 108 |
| | $\Delta 9$ Desaturase reverse | acg atg agc tcc tgc tgt tat g | |
| | $\Delta 9$ Desaturase probe | cta cct gca agt tct aca | |
| Elongase | Elongase forward | aac agg agt atg gga agg caa a | 86 |
| | Elongase reverse | gga cac gga taa tct tca tat ctg att | |
| | Elongase probe | ctt ctg tca ggg cac acg cac cg | |

Thirty-five ng of cDNA was amplified in a total reaction volume of 25 μ L containing 12.5 μ L universal master mix (Applied Biosystems), 7.5 pmol of each primer and 5 pmol probe. PCR reactions were performed in an optical 96-well reaction plate (Applied Biosystems) on an ABI PRISM 7000 Sequence Detection System (Applied

Table 6.2 Incorporation of CLA isomers and its metabolites into plasma phospholipids at the end of the run-in period and at the end of the experimental period.

| Fatty acid | Run-in period ^{1,2} | | | Experimental period ^{1,2} | | |
|------------------|------------------------------|-----------|-----------|------------------------------------|------------------------|------------------------|
| | Placebo | C9,t11 | T10,c12 | Placebo | C9,t11 | T10,c12 |
| | % of total fatty acids (w/w) | | | | | |
| | n = 6 | n = 8 | n = 6 | n = 6 | n = 8 | n = 6 |
| C9,t11 | 0.15±0.06 | 0.11±0.05 | 0.11±0.04 | 0.16±0.05 ^a | 0.88±0.40 ^b | 0.22±0.06 ^a |
| T10,c12 | ND | ND | ND | ND ^a | 0.03±0.05 ^a | 0.61±0.27 ^b |
| C,c CLA | ND | ND | ND | ND ^a | 0.01±0.00 ^b | ND ^a |
| T,t CLA | ND | ND | ND | ND ^a | 0.02±0.01 ^b | 0.02±0.01 ^b |
| t11 C18:1 | 0.34±0.11 | 0.34±0.13 | 0.27±0.10 | 0.31±0.11 | 0.25±0.07 | 0.23±0.02 |
| Conjugated C18:3 | ND | ND | ND | ND | ND | ND |
| Conjugated C20:3 | ND | ND | ND | ND | ND | ND |

¹Values are means ± standard deviations. ND, not detectable (< 0.01%). For statistical analysis, not detectable values were set at zero.

²Values in a row without a common superscript differ from each other ($P < 0.017$).

Table 6.3 Incorporation of CLA isomers and its metabolites into plasma cholesteryl esters and triacylglycerols at the end of the experimental period.

| Fatty acid | Cholesteryl esters ^{1,2} | | | Triacylglycerols ^{1,2} | | |
|------------------|-----------------------------------|------------------------|------------------------|---------------------------------|------------------------|-------------------------|
| | Placebo | C9,t11 | T10,c12 | Placebo | C9,t11 | T10,c12 |
| | % of total fatty acids (w/w) | | | | | |
| | n = 6 | n = 7 | n = 6 | n = 6 | n = 7 | n = 6 |
| C9,t11 | 0.12±0.05 ^a | 0.61±0.24 ^b | 0.16±0.05 ^a | 0.34±0.09 ^a | 1.16±0.33 ^b | 0.42±0.13 ^a |
| T10,c12 | ND ^a | 0.04±0.02 ^a | 0.42±0.18 ^b | ND ^a | 0.06±0.02 ^a | 0.53±0.27 ^b |
| C,c CLA | ND ^a | 0.02±0.01 ^b | ND ^a | ND ^a | 0.02±0.02 ^b | 0.01±0.01 ^a |
| T,t CLA | ND ^a | 0.03±0.01 ^b | 0.02±0.01 ^b | ND ^a | 0.04±0.02 ^b | 0.03±0.01 ^b |
| t11 C18:1 | ND | ND | ND | 1.55±0.83 ^a | 0.81±0.29 ^b | 0.97±0.05 ^{ab} |
| Conjugated C18:3 | ND ^a | ND ^a | 0.03±0.01 ^b | ND ^a | 0.05±0.04 ^a | 0.20±0.12 ^b |
| Conjugated C20:3 | ND | ND | ND | ND ^a | 0.03±0.03 ^b | 0.00±0.00 ^a |

¹Values are means ± standard deviations. ND, not detectable (< 0.01%). For statistical analysis, not detectable values were set at zero.

²For each lipid class, values in a row without a common superscript differ from each other ($P < 0.017$).

Fatty acid compositions of plasma lipid classes

At baseline, only the proportion of Mead acid (C20:3n-9) in PL was significantly different between the three diet groups ($P = 0.048$ for differences between the three groups). Supplementation of CLA isomers resulted in decreases in the proportions of oleic acid (C18:1n-9), C16:1n-9, C20:3n-9 and dihomo- γ -linolenic acid (C20:3n-6) in one or more plasma lipid classes (**Tables 6.4 and 6.5**). Although these decreases were more pronounced in the t10,c12 group, differences between the two CLA

groups never reached statistical significance. The decreases were compensated for by increases in linoleic acid (C18:2n-6) and CLA. In CE ($P = 0.029$ for diet effects), the proportions of C18:2n-6 were higher in the t10,c12 CLA group than in the c9,t11 CLA ($P = 0.044$) and the placebo group ($P = 0.011$). Moreover, in TAG ($P = 0.017$ for diet effects), proportions of C18:2n-6 were higher in both CLA intervention groups ($P = 0.038$ for c9,t11 and $P < 0.01$ for t10,c12) than in the placebo group.

Table 6.4 Fatty acid composition of plasma phospholipids at the end of the run-in period and at the end of the experimental period.

| Fatty acid | Run-in period ^{1,2} | | | Experimental period ^{1,2} | | |
|------------|------------------------------|------------------------|------------------------|------------------------------------|------------------------|------------------------|
| | Placebo | C9,t11 | T10,c12 | Placebo | C9,t11 | T10,c12 |
| | % of total fatty acids (w/w) | | | | | |
| | <i>n</i> = 6 | <i>n</i> = 8 | <i>n</i> = 6 | <i>n</i> = 6 | <i>n</i> = 8 | <i>n</i> = 6 |
| C16:0 | 28.73±1.24 | 28.53±1.67 | 27.98±0.95 | 28.51±1.71 | 28.05±1.68 | 28.32±1.38 |
| C16:1n-9 | 0.24±0.04 | 0.22±0.04 | 0.20±0.01 | 0.24±0.03 ^a | 0.20±0.03 ^b | 0.18±0.03 ^b |
| C16:1n-7 | 0.64±0.19 | 0.67±0.27 | 0.54±0.13 | 0.66±0.19 | 0.58±0.21 | 0.45±0.05 |
| C18:0 | 15.42±0.50 | 15.20±1.11 | 14.62±1.57 | 14.99±0.74 | 15.11±0.72 | 15.21±0.84 |
| C18:1n-9 | 10.80±1.13 | 11.24±1.58 | 9.51±1.22 | 10.75±1.62 | 9.77±0.69 | 9.01±1.09 |
| C18:1n-7 | 1.53±0.24 | 1.45±0.17 | 1.38±0.16 | 1.40±0.17 | 1.44±0.25 | 1.29±0.16 |
| C18:2n-6 | 21.12±2.25 | 22.12±2.58 | 22.31±1.61 | 20.97±2.03 | 22.21±2.49 | 23.50±1.96 |
| C18:3n-6 | 0.09±0.05 | 0.11±0.05 | 0.09±0.05 | 0.10±0.03 | 0.08±0.06 | 0.03±0.04 |
| C18:3n-3 | 0.19±0.04 | 0.21±0.07 | 0.14±0.03 | 0.22±0.02 | 0.23±0.07 | 0.22±0.07 |
| C20:3n-9 | 0.25±0.14 ^a | 0.14±0.03 ^b | 0.14±0.04 ^b | 0.22±0.09 ^a | 0.12±0.04 ^b | 0.10±0.03 ^b |
| C20:3n-6 | 3.81±0.85 | 3.10±0.91 | 3.66±0.71 | 3.93±0.67 ^a | 2.91±0.96 ^b | 2.65±0.40 ^b |
| C20:4n-6 | 9.98±1.35 | 9.61±1.95 | 11.37±0.51 | 10.35±2.15 | 10.51±1.54 | 10.50±1.60 |
| C20:5n-3 | 0.89±0.42 | 0.95±0.38 | 0.99±0.41 | 0.94±0.34 | 0.96±0.50 | 0.91±0.46 |
| C22:4n-6 | 0.38±0.05 | 0.33±0.09 | 0.38±0.12 | 0.41±0.02 | 0.40±0.07 | 0.42±0.16 |
| C22:5n-6 | 0.18±0.03 | 0.13±0.04 | 0.16±0.05 | 0.20±0.03 | 0.16±0.03 | 0.19±0.08 |
| C22:5n-3 | 0.90±0.16 | 0.82±0.18 | 0.95±0.09 | 0.96±0.15 | 1.01±0.17 | 1.01±0.19 |
| C22:6n-3 | 2.40±0.48 | 2.85±0.59 | 3.42±0.90 | 2.79±0.93 | 3.34±0.95 | 3.10±0.90 |

¹Values are means ± standard deviations.

²Values in a row without a common superscript differ from each other ($P < 0.017$).

Table 6.5 Fatty acid composition of plasma cholesteryl esters and triacylglycerols at the end of the experimental period.

| Fatty | Cholesteryl esters ^{1,2} | | | Triacylglycerols ^{1,2} | | |
|----------|-----------------------------------|--------------------------|-------------------------|---------------------------------|-------------------------|-------------------------|
| | Placebo | C9,t11 | T10,c12 | Placebo | C9,t11 | T10,c12 |
| | <i>n</i> = 6 | <i>n</i> = 7 | <i>n</i> = 6 | <i>n</i> = 6 | <i>n</i> = 7 | <i>n</i> = 6 |
| | % of total fatty acids (w/w) | | | | | |
| C16:0 | 10.82±1.02 | 10.81±0.98 | 9.89±0.79 | 29.00±4.14 | 28.52±4.49 | 25.84±2.54 |
| C16:1n-9 | 0.50±0.12 ^a | 0.40±0.11 ^{ab} | 0.32±0.04 ^b | 0.96±0.14 | 0.81±0.22 | 0.83±0.09 |
| C16:1n-7 | 3.30±1.20 | 2.74±1.12 | 2.12±0.34 | 4.26±0.87 | 3.68±1.20 | 3.28±0.62 |
| C18:0 | 0.60±0.36 | 0.78±0.14 | 0.75±0.16 | 4.12±0.96 | 4.26±0.99 | 3.72±0.57 |
| C18:1n-9 | 20.07±2.14 ^a | 18.21±1.53 ^{ab} | 16.98±2.19 ^b | 36.76±2.48 | 33.18±4.27 | 34.51±4.30 |
| C18:1n-7 | 1.10±0.39 | 1.49±0.43 | 1.11±0.33 | 3.17±0.79 | 2.74±0.48 | 3.00±0.27 |
| C18:2n-6 | 51.29±4.83 ^a | 52.84±2.96 ^a | 57.18±2.62 ^b | 12.37±4.39 ^a | 16.67±3.52 ^b | 18.68±1.85 ^b |
| C18:3n-6 | 1.23±0.45 | 1.06±0.40 | 0.83±0.34 | 0.30±0.15 | 0.32±0.13 | 0.34±0.24 |
| C18:3n-3 | 0.63±0.07 | 0.64±0.10 | 0.61±0.15 | 0.98±0.44 | 0.96±0.25 | 1.30±0.49 |
| C20:3n-9 | 0.07±0.05 | 0.05±0.01 | 0.03±0.01 | 0.15±0.07 | 0.13±0.04 | 0.12±0.07 |
| C20:3n-6 | 0.92±0.15 ^a | 0.67±0.21 ^b | 0.61±0.09 ^b | 0.23±0.04 | 0.25±0.08 | 0.24±0.07 |
| C20:4n-6 | 7.09±2.19 | 7.30±0.82 | 7.01±1.10 | 1.04±0.38 | 1.54±0.56 | 1.37±0.41 |
| C20:5n-3 | 0.98±0.57 | 1.11±0.58 | 0.81±0.38 | 0.17±0.07 | 0.27±0.18 | 0.24±0.11 |
| C22:4n-6 | ND | ND | ND | 0.13±0.04 | 0.15±0.03 | 0.18±0.07 |
| C22:5n-6 | ND | ND | ND | 0.08±0.02 | 0.07±0.02 | 0.10±0.05 |
| C22:5n-3 | ND | ND | ND | 0.29±0.09 | 0.38±0.21 | 0.43±0.10 |
| C22:6n-3 | 0.45±0.19 | 0.55±0.21 | 0.51±0.16 | 0.38±0.16 | 0.67±0.37 | 0.69±0.28 |

¹Values are means ± standard deviations. ND, not detectable (< 0.01%).

²For each lipid class, values in a row without a common superscript differ from each other (*P* < 0.017).

Effects of individual CLA isomers on desaturation indices and mRNA expressions

From the fatty acid compositions of PL, CE and TAG the ratio of C20:4n-6/C20:3n-6 was calculated as an index for $\Delta 5$ desaturation, the ratio of (C18:3n-6+C20:3n-6)/C18:2n-6 for $\Delta 6$ desaturation, and the ratios of C16:1n-7/C16:0 and of C18:1n-9/C18:0 for $\Delta 9$ desaturation (**Figure 6.1**). With respect to the $\Delta 5$ and $\Delta 6$ desaturation indices, comparable patterns were seen in all three lipid classes. The $\Delta 5$ desaturation index was slightly higher in both CLA groups than in the placebo group, but differences between groups did not reach statistical significance ($P = 0.249$ in PL, $P = 0.148$ in CE, $P = 0.444$ in TAG for diet effects). The $\Delta 6$ desaturation index in PL was significantly decreased in both CLA groups ($P < 0.01$ for diet effects). Compared with the placebo group, the decrease in the c9,t11 group was 0.06 ($P = 0.013$, 95% CI for the difference: -0.102 to -0.014) and in the t10,c12 group 0.08 ($P < 0.01$, 95% CI for the difference: -0.125 to -0.031). This change nearly reached statistical significance in CE ($P = 0.054$ for diet effects), but not in TAG ($P = 0.328$ for diet effects). Also for the $\Delta 9$ desaturation index, dietary effects were observed. Compared with the placebo group, the C18:1n-9/C18:0 ratio in PL ($P = 0.032$ for diet effects) was significantly decreased in the t10,c12 CLA supplemented group ($P = 0.010$, 95% CI for the difference: -0.232 to -0.010) and tended to decrease in the c9,t11 group ($P = 0.102$, 95% CI for the difference: -0.172 to 0.036). In CE, effects on the same index nearly reached statistical significance ($P = 0.079$ for diet effects) and both CLA isomers tended to decrease the $\Delta 9$ desaturation index relative to the placebo ($P = 0.048$ for c9,t11 CLA, 95% CI for the difference: -68.85 to 7.65 and $P = 0.050$ for t10,c12 CLA, 95% CI for the difference: -71.17 to 8.22). In contrast, intervention groups did not differ significantly in their effects on the $\Delta 9$ desaturation index when calculated as C16:1n-7/C16:0 ($P = 0.116$ in PL, $P = 0.223$ in CE, $P = 0.296$ in TAG).

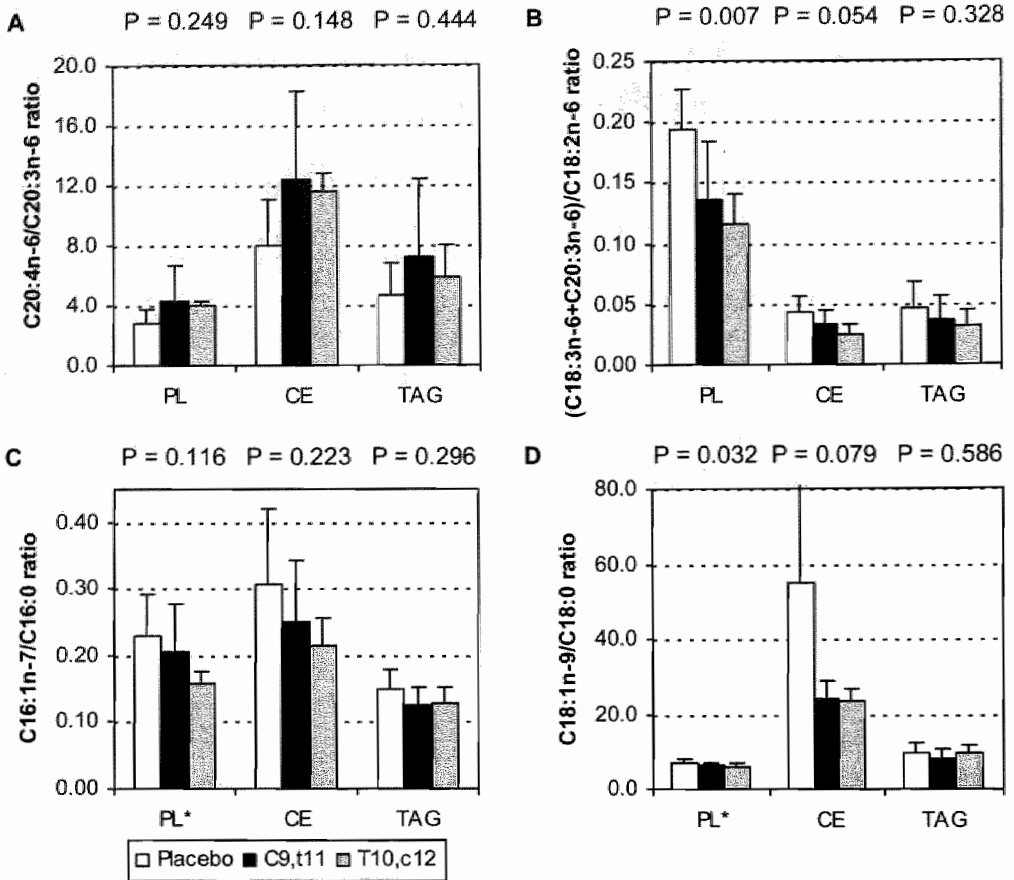


Figure 6.1 Effects of placebo, c9,t11 and t10,c12 CLA on (a) $\Delta 5$ (C20:4n-6/C20:3n-6), (b) $\Delta 6$ ((C18:3n-6+C20:3n-6)/C18:2n-6), and (c,d) $\Delta 9$ (C16:1n-7/C16:0 and C18:1n-9/C18:0) desaturation indices.¹

¹Values are means \pm standard deviations. $N = 6$ in the placebo group, $n = 7$ in the c9,t11 group for CE and TAG and $n = 8$ for PL and $n = 6$ in the t10,c12.

* $\Delta 9$ desaturation indices in phospholipids are multiplied by 10, because indices are very low owing to the high abundance of saturated fatty acids in phospholipids.

MRNA expression of $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases and elongase did not differ between the three intervention groups (**Figure 6.2**). P-values for diet effects were respectively 0.914 for $\Delta 5$ desaturase, 0.563 for $\Delta 6$ desaturase, 0.234 for $\Delta 9$ desaturase and 0.807 for elongase. The various desaturation indices in PL, CE and TAG were not correlated with their respective mRNA levels.

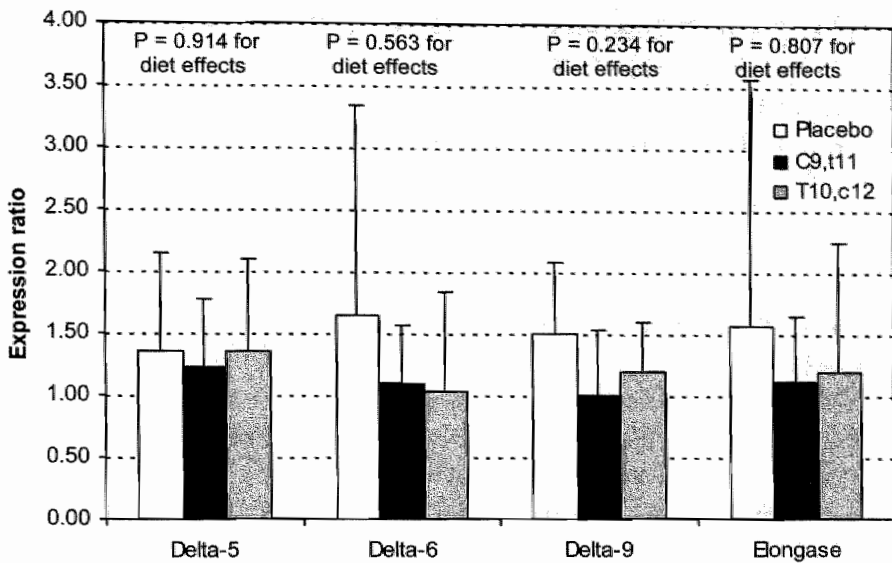


Figure 6.2 Effects of placebo, c9,t11 and t10,c12 CLA on $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturase and elongase expression ratios.^{1,2,3}

¹Values (means \pm standard deviations) are obtained using TaqMan procedures (comparative cycle threshold method) and are normalised to the housekeeping gene β -actin and relative to the run-in period.

²No statistically significant differences were observed between treatments.

³All samples were analysed in duplicate. N = 6 in the placebo group, n = 7 in the c9,t11 group and n = 6 in the t10,c12 group.

DISCUSSION

In this study we evaluated the effects of c9,t11 CLA and t10,c12 CLA, the two most common CLA isomers in the diet, on the fatty acid compositions of plasma PL, CE, and TAG. As these effects may be mediated through effects of CLA on desaturase activity, we also studied effects on mRNA expression of $\Delta 5$, $\Delta 6$ and $\Delta 9$ desaturases and elongase by PBMC. Our results suggest that c9,t11 CLA and t10,c12 CLA affect the fatty acid composition of plasma lipid classes differently but do not change mRNA expression of desaturases or elongase in PBMC.

Both c9,t11 CLA and t10,c12 CLA were incorporated into plasma PL, CE and TAG. Based on the proportional increases in PL, it can be estimated that each g of c9,t11 CLA in the diet increases its proportion in PL with 0.26%. For t10,c12 CLA, this estimate is 0.20%. These values are in good agreement with those of a recent study in which healthy men received each CLA isomer in increasing doses from 0 to 0.6 to 1.2 to 2.4 g per day (26). In that study, both isomers were incorporated in a

dose-dependent manner into plasma phosphatidylcholine (PC) and CE, and into total lipids of PBMC. In plasma PC, the proportions of *c9,t11* CLA increased from 0.21 to 0.32 to 0.52 to 0.79% in PC, or on average 0.25% per g of *c9,t11* CLA. Similarly, for *t10,c12* CLA it can be calculated that for each g in the diet, the increase in PC was 0.17%. These estimates agree very well with our values. Moreover, these findings are in concordance with studies in rats and mice, in which incorporation of *c9,t11* CLA into lipid fractions was also slightly higher than that of *t10,c12* CLA (27,28).

Because *c9,t11* CLA - not the *t10,c12* CLA isomer - was already present in plasma PL after the run-in period, this CLA isomer should have been provided by the habitual diet. By assuming a linear relationship between the intake of *c9,t11* CLA and its incorporation into plasma PL (26), it can be calculated that the habitual diet of our subjects should have provided daily about 430 mg of *c9,t11* CLA. This is much higher than the mean estimated intake of 200 mg in the Netherlands Cohort Study (29). In other western countries comparable dietary CLA intakes were observed (30). Foods that provide the *c9,t11* CLA isomer are ruminant products such as meat, cheese, milk, and yogurt, in which *c9,t11* CLA accounts for 75-90% of total CLA (1). Part of the discrepancy between our estimates and these studies can be explained by the fact that methods to assess dietary intakes underestimate real intakes (30). However, the largest part may be explained by the conversion of vaccenic acid (*t11* C18:1) into *c9,t11* CLA *in vivo*, as observed in several human studies (31,32). If one assumes a conversion rate of 19% of *t11* C18:1 into *c9,t11* CLA (32), a habitual daily intake of approximately 1200 mg of *t11* C18:1 is necessary to explain a difference of 230 mg in *c9,t11* CLA intake. In the Netherlands Cohort Study, daily intake of *t11* C18:1 was 700-800 mg (29). Thus, our data on *c9,t11* CLA levels in plasma PL after the run-in period can be explained by habitual intakes of this isomer and by the conversion of *t11* C18:1 into *c9,t11* CLA.

The fatty acid composition of plasma TAG suggested that the *t10,c12* CLA isomer was mainly converted into the conjugated C18:3 metabolite, whereas supplementation with the *c9,t11* CLA isomer increased both C18:3 and C20:3 CLA metabolites, though to a lesser extent. Similar results have been observed in rats (28). This might suggest a blockade of the elongation step by *t10,c12* CLA, which we could not, however, demonstrate at the mRNA expression level in PBMC. It is also possible that the conjugated C18:3 metabolite of *t10,c12* CLA has a lower affinity for elongase than the conjugated C18:3 metabolite of *c9,t11* CLA.

In contrast to several other studies (10,11), CLA supplementation significantly changed the fatty acid compositions of plasma PL, TAG and CE. In general, proportions of C18:1n-9, C16:1n-9, C20:3n-9 and C20:3n-6 acids decreased at the

expense of CLA and C18:2n-6. These effects were more pronounced in the *t*10,*c*12 CLA group than in the *c*9,*t*11 CLA-supplemented group, although differences between the two CLA groups did not reach statistical significance. Smedman and Vessby (12) found comparable effects on the fatty acid composition of serum PL in humans consuming 4.2 g of CLA isomers (*c*9,*t*11:*t*10,*c*12 as 50:50) per day for 12 weeks. In addition, in that study also proportions of stearic (C18:0), γ -linoleic (C18:3n-6), docosatetraenoic (C22:4n-6), and docosapentaenoic (C22:5n-3) acids increased and that of palmitic acid (C16:0) decreased. It is possible that the intervention periods of 4 weeks (11) or 45 days (10) were too short to change the composition of plasma lipids. Surprisingly, in our study proportions of linoleic acid increased. Thus, linoleic acid was not displaced by conjugated linoleic acids in plasma lipids as suggested earlier (12). Because linoleic acid is an essential fatty acid and is not produced endogenously, this effect might suggest a decrease in Δ 6 desaturation. However, unlike some other studies (5,9,11), we did not find decreased proportions of longer-chain metabolites of linoleic acid including arachidonic acid, which is an important precursor for eicosanoids.

With respect to the Δ 9 desaturation index, our data confirmed those of Smedman and Vessby (12). In their study, a daily supplement of 4.2 g of a mixture of CLA isomers resulted in a decreased Δ 9 desaturation index (12). Although the Δ 9 desaturation index in that study was only calculated from the conversion of C18:0 into C18:1n-9, we calculated it also from the conversion of C16:0 into C16:1n-7. However, only the C18:1n-9/C18:0 ratio was significantly reduced in PL by the *t*10,*c*12 CLA isomer. This may indicate that the preferred substrate of Δ 9 desaturase is C18:0 rather than C16:0, as also is suggested by stable-isotope studies in humans (33,34). In addition, C16:0 is preferentially elongated instead of Δ 9-desaturated (34). Also in cell culture (8,9) and animal studies (5-7), the *t*10,*c*12 CLA isomer inhibited Δ 9 desaturase activity. In contrast, the *c*9,*t*11 CLA isomer did not affect Δ 9 desaturase activity in all these studies, except for one. In a human hepatoma-derived cell line (HepG2 cells), the *c*9,*t*11 CLA isomer slightly decreased Δ 9 desaturase activity when added at nonphysiological concentrations (9).

As for a mixture of CLA isomers (12), the Δ 6 desaturation index was decreased by *c*9,*t*11 as well as by *t*10,*c*12 CLA. In contrast, in HepG2 cells *t*10,*c*12 CLA, but not *c*9,*t*11 CLA, suppressed Δ 6 desaturation (9). Furthermore, in hepatic rat microsomes *c*9,*t*11 CLA decreased Δ 6 desaturation of linoleic acid but not that of α -linolenic acid. In that study, *t*10,*c*12 CLA decreased the Δ 6 desaturation of linoleic acid only at the highest concentration (5). Taken together, whereas *in vitro* effects of the individual CLA isomers on Δ 6 desaturation are rather inconsistent, *in vivo* both

CLA isomers seem to decrease $\Delta 6$ desaturation activity in humans. Unlike Smedman and Vessby (12), who reported an increase in the $\Delta 5$ desaturation index, we found no effect on this index. In contrast, in HepG2 cells, *t10,c12* CLA even suppressed $\Delta 5$ desaturation (9). Therefore, effects of the individual CLA isomers on $\Delta 5$ desaturase activities are until now rather inconsistent and clearly deserve further exploration.

No effects of the *t10,c12* and *c9,t11* CLA isomers on mRNA expression in PBMC were evident. Only a few studies have evaluated the effects of CLA on mRNA expression of desaturases before (7,8). In mice that were fed a mixture of CLA isomers, other isomers than *c9,t11* CLA were held responsible for the inhibitory effect on stearoyl-CoA desaturase gene 1 mRNA expression (7). However, in cell culture studies, Choi *et al* (8) found that the *t10,c12* and *c9,t11* CLA isomers did not influence mRNA expression in human-derived HepG2 cells. In that study, *t10,c12* CLA decreased $\Delta 9$ desaturase activity but did not change desaturase protein levels. Hence, *t10,c12* CLA might regulate the activity of $\Delta 9$ desaturase without any effect at the transcriptional level.

To quantify mRNA expression ratios of the $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases, and elongase we developed RT-qPCR assays. With this method, the PCR product is already accurately detected during the PCR amplification, which is less time-consuming and laborious than conventional PCR techniques, because no post-PCR handling is required. Although assays for $\Delta 5$ and $\Delta 6$ desaturase, and elongase were easily developed, for $\Delta 9$ desaturase or stearoyl-CoA desaturase a pseudogene has been reported (16), which asked for additional precautions. Instead of the more regular dual-labelled probes, we therefore used an MGB probe, which is more specific to the target sequence and does not anneal to the pseudogene sequence. These assays can easily be used in large-scale human studies. Tissue accessibility, however, remains a problem. We chose to analyse desaturase expression in PBMC, as liver tissue is for obvious reasons difficult to obtain. Although for some genes a correlation between liver and PBMC mRNA levels has been demonstrated (35), it is still unknown whether mRNA expression ratios of $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases and elongase in PBMC are representative for those in the liver. This may also explain the lack of effects of the two CLA isomers on mRNA expression. Future studies should therefore concentrate to validate molecular signatures (e.g. mRNA, protein and metabolic profiles) in PBMC as a marker for other, less accessible tissues and organs.

In conclusion, incorporation of the individual CLA isomers, *c9,t11* and *t10,c12*, into plasma lipids (PL, CE and TAG) reflects dietary intakes. Compared with oleic acid, $\Delta 9$ and $\Delta 6$ desaturation indices in plasma phospholipids are decreased after

consumption of c9,t11 or t10,c12 CLA. Effects on desaturation indices, however, were not reflected by changes at the transcriptional level for the various desaturases and elongase enzymes in peripheral blood mononuclear cells.

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7

General Discussion

GENERAL DISCUSSION

The major objectives of the studies described in this thesis were to evaluate the effects of stearic acid, oleic acid, and linoleic acid on the serum lipoprotein profile, on the thrombotic tendency, and on biomarkers of lipid peroxidation and inflammation of healthy human subjects. In another human study, the effects of the conjugated linoleic acid (CLA) isomers *c9,t11* and *t10,c12* CLA were investigated on the fatty acid profile of serum lipids and the expression of desaturases and elongases in peripheral blood mononuclear cells (PBMC).

STEARIC, OLEIC, AND LINOLEIC ACIDS AND CARDIOVASCULAR DISEASE RISK

The effects of stearic acid, oleic acid, and linoleic acid were studied in a crossover designed study in healthy, non-smoking human subjects ($n = 45$) as described in **Chapters 3, 4 and 5**. Both male ($n = 18$) and female ($n = 27$) subjects participated. Each participant consumed in random order each of the 3 different diets during three 5-week periods. Diets provided 38% of energy from fat of which 60% was supplied by the experimental fats of which bread, margarines and cakes were produced. The prescribed nutrient composition of the diets did not differ, except for a 7% difference in energy intake provided by stearic, oleic, or linoleic acids. The mean daily energy intake and the composition of the diets were estimated by food frequency questionnaires filled in at the end of each intervention period. Dietary adherence was confirmed by the fatty acid composition of serum phospholipids.

Lipoprotein profile

Total, LDL, and HDL cholesterol

Because dairy fat is rich in saturated fatty acids, milk and milk-derived products have for long had a negative image concerning the risk to develop cardiovascular diseases. Lauric, myristic, palmitic, and stearic acids account approximately for 3%, 11%, 28%, and 10% of total fatty acids in milk fat, respectively (**Table 7.1**). Furthermore, a considerable part of the saturated fatty acids in milk fat, about 10% of total fatty acids, are short- and medium-chain fatty acids (MCFA). Earlier studies at our department already reported the effects of medium-chain fatty acids, lauric acid, myristic acid, and palmitic acid on concentrations of serum lipids and lipoproteins relative to those of oleic acid. Because study designs were largely comparable to that of our study, in **Figure 7.1** data of our study and those of Temme *et al* (1,2) were combined.

Table 7.1 Fatty acid composition of milk fat.

| Fatty acid | Notation | % of total fatty acids (w/w) |
|--|----------------|------------------------------|
| <i>Saturated fatty acids</i> | | 64.0 |
| Short-chain fatty acids | C4:0 and C6:0 | 5.9 |
| Medium-chain fatty acids | C8:0 and C10:0 | 4.0 |
| Lauric acid | C12:0 | 3.3 |
| Myristic acid | C14:0 | 10.7 |
| Palmitic acid | C16:0 | 27.6 |
| Stearic acid | C18:0 | 10.1 |
| <i>Monounsaturated fatty acids</i> | | 30.0 |
| Palmitoleic acid | C16:1n-7 | 2.6 |
| Oleic acid | C18:1n-9 | 26.0 |
| <i>Polyunsaturated fatty acids</i> | | 4.0 |
| Linoleic acid | C18:2n-6 | 2.5 |
| α -Linolenic acid | C18:3n-3 | 1.4 |
| <i>Trans fatty acids</i> | | 1.5-6.5 |
| Vaccenic acid | T11 C18:1 | 1.0 |
| Elaidic acid | T9 C18:1 | 0.2 |
| <i>Conjugated linoleic acids (CLA)</i> | | < 0.5 |
| <i>Cis-9, trans-11 CLA</i> | C9,t11 C18:2 | 0.4 |
| <i>Trans-10, cis-12 CLA</i> | T10,c12 C18:2 | < 0.01 |

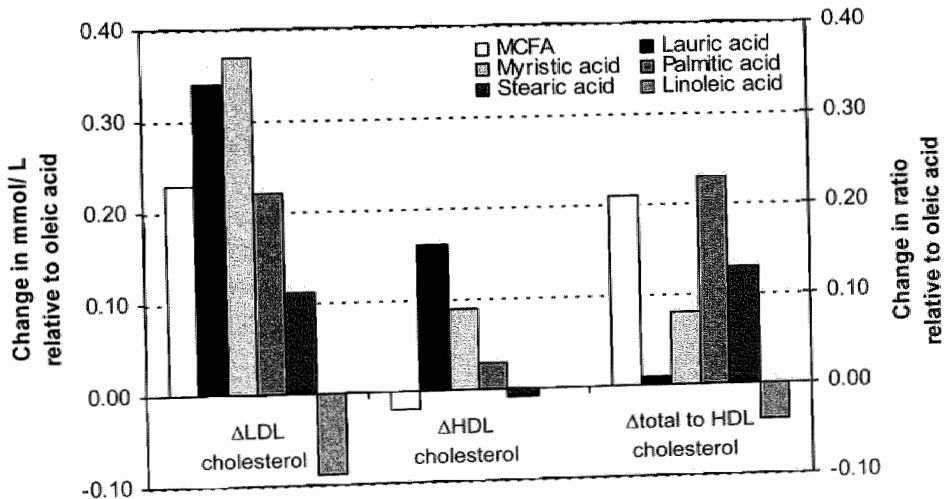


Figure 7.1 Mean changes in LDL and HDL cholesterol concentrations, and total to HDL cholesterol ratio, when 10% of energy from oleic acid is exchanged for medium-chain fatty acids (MCFA), lauric acid, myristic acid, or palmitic acid (1,2), or stearic acid, or linoleic acid (Chapter 3).

When 10% of dietary energy from oleic acid was replaced by lauric acid (C12:0), myristic acid (C14:0), or palmitic acid (C16:0), total, LDL, and HDL cholesterol concentrations increased (1,2). Strikingly, stearic acid (C18:0) increased total and LDL cholesterol concentrations less than the other saturated fatty acids. With increasing chain length from lauric acid to stearic acid, the HDL cholesterol elevating effects of the saturated fatty acids decreased. As a consequence, palmitic acid increased the total to HDL cholesterol ratio and thereby cardiovascular disease risk more than the other saturated fatty acids did. Markedly, because of its effects on HDL cholesterol, lauric acid affected the total to HDL cholesterol ratio even more beneficially than stearic acid, which increased the total and LDL cholesterol concentrations less than the other saturated fatty acids. Hence, saturated fatty acids differ in their effects on the serum lipoprotein profile. Because linoleic acid did not differ from oleic acid in its effects on HDL cholesterol and lowered concentrations of total and LDL cholesterol, linoleic acid slightly decreased the total to HDL cholesterol ratio relative to oleic acid.

Several dietary intervention studies compared the effects of stearic acid to those of the other long-chain saturated fatty acids. Natural as well as synthetic fats high in stearic acid resulted in lower concentrations of total or LDL cholesterol than diets high in lauric and myristic acids, myristic and palmitic acids, myristic acid or palmitic acid (3-8). Therefore, stearic acid has been suggested to differ from the other saturated fatty acids and would affect cardiovascular disease risk more beneficially than the other saturated fatty acids. In this respect, effects of stearic acid relative to lauric acid are not yet clear. When data from our studies were summarised, lauric acid had more beneficial effects on the concentration of HDL cholesterol and on the total to HDL cholesterol ratio whereas stearic acid had less harmful effects on the total and LDL cholesterol concentrations than the other saturated fatty acids. Relative to *trans* fatty acids, the effects of stearic acid are rather inconsistent. In the study of Zock *et al* (9), stearic acid and *trans* fatty acids did not differ in their effects on total, LDL, and HDL cholesterol concentrations. However, in another study stearic acid decreased the concentrations of total and LDL cholesterol compared with *trans* fatty acids (10).

With increasing degree of unsaturation, the differences in effects on serum lipid and lipoprotein concentrations between the three C18 fatty acids - stearic acid, oleic acid, and linoleic acid - did not reach statistical significance in our study. Also most other studies observed no different effects on the serum lipoprotein profile when stearic acid was exchanged by oleic acid (6) or oleic acid by linoleic acid (11-14). If the effects of stearic acid are compared to those of linoleic acid, linoleic acid significantly decreased total and LDL cholesterol or increased HDL cholesterol

concentrations (5,9). Dietary exchange of fatty acids in the earlier mentioned studies was, however, larger than in our study and sometimes dietary intake of linoleic acid was even unrealistically high. Other possible explanations for the differences in effects between studies will be discussed later in this chapter.

In a recent meta-analysis the effects of the individual saturated, monounsaturated, polyunsaturated, and *trans* fatty acids were summarised using data of 60 clinical trials (15). The results of our study are compared to the predicted changes from the equations of this meta-analysis in **Figure 7.2**. As already predicted by the equations of Mensink *et al* (15), the effects of linoleic acid relative to the other fatty acids were overestimated by the equations of Keys *et al* (16) and Hegsted *et al* (17). Despite these comparable smaller changes of total cholesterol concentrations, also some discrepancies exist between the observed changes in our dietary intervention study and the predicted changes (15). While observed changes of LDL cholesterol concentrations largely reflected predicted changes, mainly changes of HDL cholesterol concentrations were smaller than in the meta-analysis. Observed changes in the effects of stearic, oleic, and linoleic acids on the major cardiovascular disease risk marker, the total to HDL cholesterol ratio were, however, comparable to those predicted.

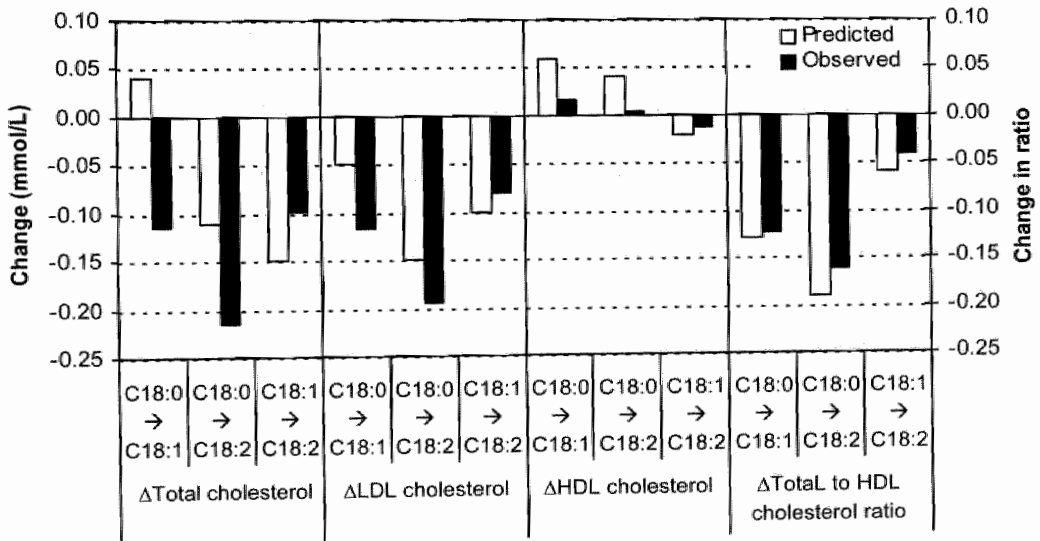


Figure 7.2 Mean changes in total, LDL and HDL cholesterol concentrations and total to HDL cholesterol ratio, when 10% of energy from stearic acid (C18:0) or oleic acid (C18:1) is exchanged by oleic acid (C18:1) or linoleic acid (C18:2) as predicted (white bars) by the equations of Mensink *et al* (15) and observed (black bars) in our study.

Lipid and lipoprotein subclasses and particle sizes

A relatively new technique to analyse concentrations of lipoprotein particles and their subclasses, and to measure the particle size of lipoproteins is NMR spectroscopy (18). In our study, particle concentrations of VLDL, LDL, and HDL subclasses and particle sizes of VLDL, LDL, and HDL did not differ between healthy subjects when they consumed diets rich in stearic, oleic, or linoleic acids. These results are in agreement with the effects on the more traditional cardiovascular disease risk markers. As some lipoprotein subclasses such as small, dense LDL are positively associated with cardiovascular disease risk (19,20), this new technique is a promising new tool to analyse the effects of fatty acids on the subclass distribution of lipoproteins.

Thrombotic tendency

Dietary fatty acid composition not only affects the lipoprotein profile but also thrombotic tendency might be influenced by the exchange of fatty acids in the diet. In our study the effects of stearic, oleic, and linoleic acids were investigated on platelet aggregation, and variables of coagulation and fibrinolysis. In the past, stearic acid has been assumed to exert prothrombotic effects but more recent studies did not confirm the unbeneficial effects of stearic acid on platelet aggregation, coagulation as well as fibrinolysis, relative to other fatty acids (**Chapter 4**).

Platelet aggregation

Various earlier studies reported in particular thrombogenic effects of saturated fatty acids in animal models and in humans. In rats, n-6 and n-3 polyunsaturated fatty acids decreased arterial thrombosis tendency as measured with the aortic loop technique, whereas the saturated fatty acids with 12 to 16 carbon atoms had opposing effects (21). Myristic acid was the strongest prothrombotic fatty acid, whereas linoleic acid was the strongest antithrombotic fatty acid. In another study with rats, diets high in butter or stearic acid enhanced thrombin-induced aggregation, which is related to changes in the fatty acid composition of platelet phospholipids. Furthermore, the severity of thrombotic lesions was increased relative to oleic and linoleic acids (22). Also in human studies prothrombotic effects of stearic acid have been suggested. In French farmers, for example, dietary stearic acid intake changed the fatty acid composition of platelet phospholipids and was positively correlated with an increased platelet activity (23).

In our study, two different methods were used to evaluate the effects of stearic, oleic, and linoleic acids on platelet aggregation. Using filrtagometry, a method to

measure *ex vivo* platelet aggregation, gender-dependent effects were found. In men, *ex vivo* platelet aggregation time was favourably prolonged by linoleic acid relative to the diet rich in stearic acid. Already in two other studies (24,25) a prolonged platelet aggregation time was reported when mixtures of saturated fatty acids or saturated and monounsaturated fatty acids were substituted by linoleic acid. We could now specifically attribute the observed effects to stearic acid. Most other studies used *in vitro* techniques to examine platelet aggregation. With this approach, stearic, oleic, and linoleic acids did not differ in their effects on *in vitro* whole blood platelet aggregation parameters either induced by ADP or collagen. Others also found comparable effects of stearic, oleic, and linoleic acids on ADP-induced platelet aggregation (26) or of oleic and linoleic acids using different methodologies (26-31). Compared with other fatty acids, effects of stearic acid on *in vitro* platelet aggregation did not differ from those of *trans* fatty acids (32) and palmitic acid (33). However, in one study stearic acid improved *in vitro* platelet aggregation relative to palmitic acid (7). In the earlier mentioned studies of Temme *et al* (1,2) also medium-chain fatty acids, lauric, myristic, and palmitic acids did not differ in their effects on *in vitro* whole blood platelet aggregation as compared with oleic acid (34). Hence, in recent studies no thrombotic effects of saturated fatty acids in particular of stearic acid were reported on *in vitro* platelet aggregation relative to unsaturated fatty acids. Thus, stearic acid may affect *in vitro* platelet aggregation similarly when compared with oleic and linoleic acids.

Coagulation

Stearic, oleic, and linoleic acids did not differ in their effects on the coagulation variables measured in our study - factor VIIam activity and concentrations of prothrombin fragment 1 and 2, and of fibrinogen. In the past, stearic acid was suggested to increase concentrations of coagulation factor VII (35,36), which is associated with an increased cardiovascular disease risk (37). These thrombogenic effects on coagulation factor VII were, however, not confirmed in more recent, well-controlled dietary intervention studies. As in our study, Hunter *et al* (26) observed comparable effects of stearic, oleic, and linoleic acids using 3 different methods to measure factor VII activity. When the effects of dietary oleic acid and linoleic acid were compared, most studies reported also no different effects of these two unsaturated fatty acids on factor VII coagulant activity in young healthy subjects (38,39) and middle-aged subjects (40). In contrast, in two studies with healthy young male and female students, oleic acid reduced factor VII coagulant activity compared with linoleic acid (31,41). No explanation exists for the effects observed in the latter two studies. Despite the use of a wide variety of methods to measure factor VII

activity, no distinct effects of stearic, oleic, and linoleic acids were found in middle-aged human subjects.

Although only a few studies have examined the effects of stearic acid and unsaturated fatty acids on factor VII activity simultaneously, several earlier studies have compared the effects of stearic acid to those of the other saturated fatty acids. Fats high in stearic acid decreased factor VII activity relative to fats high in lauric and myristic acids, or palmitic acid (7,8). In another study, a stearic acid diet decreased concentrations of factor VII antigen as well as of factor VII coagulant activity relative to a diet rich in lauric, myristic, and palmitic acids (42). In the studies of Temme *et al*, diets rich in lauric, myristic, and palmitic acids increased factor VIIam activity relative to the oleic acid diet (43,44). These effects were more pronounced in women than in men. In our study no gender-dependent effects were apparent. Thus, the effects of stearic acid on factor VII activity may be less harmful than those of the other saturated fatty acids with 12 to 16 carbon atoms.

With respect to the effects on concentrations of fibrinogen and prothrombin fragment 1 and 2, no differences were observed between the experimental diets. Prothrombin fragment 1 and 2 is a marker for thrombin formation *in vivo*, while fibrinogen is both the precursor for fibrin and an acute phase reactant which is increased in inflammation. As in other studies, the fatty acid composition of the diet did not change fasting concentrations of prothrombin fragment 1 and 2 (26,31,44,45). In one study, however, stearic acid decreased concentrations of prothrombin fragment 1 and 2 compared to a diet rich in myristic and lauric acids but in the same study fibrinogen concentrations were raised by stearic acid (42). In another study, an increase in fibrinogen concentrations was also observed when dietary saturated fatty acids with 12 to 16 carbon atoms, oleic acid or *trans* fatty acids were substituted by stearic acid (46). Oleic and linoleic acids did not differ in their effects on fibrinogen concentrations (26,31,41). Therefore, the effects of oleic and linoleic acids seem to be comparable on fibrinogen concentrations whereas the effects of stearic acid relative to the other saturated or unsaturated fatty acids are inconsistent.

Fibrinolysis

Major regulators of fibrinolysis are tissue plasminogen activator (tPA) and its inhibitor plasminogen activator inhibitor-1 (PAI-1). In most studies no different postprandial responses to meals rich in myristic and palmitic acids, palmitic acid, stearic acid, oleic acid, or linoleic acid were observed on fibrinolytic variables (47-49). Myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, α -linolenic acid, and *trans* fatty acids did also not differ in their effects on fasting levels or activities in

human intervention studies (8,26,31,50). In concordance, in our study stearic, oleic, and linoleic acids did also not differ in their effects on PAI activity and concentrations of tPA/PAI-1 complexes. Hence, fibrinolytic variables may not be influenced by the fatty acid composition of the diet.

Lipid peroxidation

To investigate the effects of stearic, oleic, and linoleic acids on lipid peroxidation, urinary 24-hour excretions of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$) and 15-keto-dihydro-prostaglandin $F_{2\alpha}$ (15-K-DH-PGF $_{2\alpha}$) were measured as described in **Chapter 5**. Elevated concentrations of F_2 -isoprostanes have been associated with increased risk to develop cardiovascular diseases (51). Stearic, oleic, and linoleic acids did not differ in their effects on these biomarkers of non-enzymatic and enzymatic lipid peroxidation, respectively. This agrees with earlier dietary studies, in which comparable effects of oleic and linoleic acids were observed on concentrations of isoprostanes in urine or plasma of humans (52-54). Although these findings may suggest that these *in vivo* markers are not diet-sensitive, this is contradicted by results from a study investigating the effects of CLA isomers. Relative to supplements containing a mixture of CLA isomers or placebo oil, the urinary excretion of 8-iso-PGF $_{2\alpha}$ and 15-K-DH-PGF $_{2\alpha}$ was significantly increased after consumption of a supplement containing the CLA isomer *trans*-10, *cis*-12 CLA in men with metabolic syndrome (55). Thus, the urinary excretion of isoprostanes is a useful marker to evaluate dietary effects on *in vivo* lipid peroxidation.

Discrepancies exist between dietary studies, which investigated effects on *in vivo* methods and of those using *in vitro* methods. Earlier studies showed that diets rich in oleic acid lowered the *in vitro* susceptibility of LDL to oxidative modification induced by metal ions when compared with diets rich in linoleic acid (56-62). In these studies, lag time increased, rate of oxidation decreased and formation of conjugated dienes or lipid peroxides decreased on the oleic-acid diet. Hence, data about the effects of oleic and linoleic acids on lipid peroxidation are inconsistent using different methods. This could be explained by the difficulties of *in vitro* methods evaluating LDL susceptibility to oxidative modification to reflect the *in vivo* situation but also the compounds measured differ from each other.

Inflammation

Since atherosclerosis is considered as a chronic inflammatory disease, a broad range of inflammation markers has been investigated in patients with cardiovascular diseases (63). Increased concentrations of (soluble) cellular adhesion molecules or pro-inflammatory cytokines and decreased concentrations of anti-inflammatory

cytokines have been associated with an increased risk of cardiovascular diseases (64,65). Although most studies focused on the *in vitro* effects of fatty acids on endothelial and immune cell function (66), some studies have now reported effects on circulating *in vivo* markers of inflammation. Literature about the effects of fatty acids on endothelial cell adhesion molecules and cytokines has been reviewed extensively in **Chapter 2**.

In our study, the effects of stearic, oleic, and linoleic acids did not differ on concentrations of high-sensitivity C-reactive protein (hsCRP) as described in **Chapter 5**. Previous studies investigated mainly the effects of n-3 polyunsaturated fatty acids on concentrations of hsCRP. Some of these studies (67,68) but not all (69,70) reported decreased concentrations of hsCRP by the consumption of n-3 polyunsaturated fatty acids. Dietary *trans* fatty acids increased hsCRP relative to diets rich in saturated fatty acids with 12 to 16 carbon atoms, stearic, and oleic acids (46). Therefore, changes in the dietary fatty acid composition may result in different effects on hsCRP concentrations.

An interesting new tool to detect multiple inflammatory molecules simultaneously and to evaluate inflammatory effects of fatty acids is by antibody arrays. The effects of stearic, oleic, and linoleic acids on the expression profile of 42 immunomodulatory molecules including cytokines, chemokines and growth factors were evaluated but results were inconsistent. A high-sensitivity ELISA of IL-10 of the individual samples could not confirm the data obtained by the antibody arrays. Although with the use of antibody arrays easily insights of the total profile of immunomodulatory molecules could be obtained, inflammatory effects in the individual subjects are more easily distinguished using quantitative assays of the different cytokines. However, it remains to be recommended to analyse expression profiles using one antibody array per subject on each diet. Future studies are necessary to address the effects of fatty acids on the expression profile of immunomodulatory molecules in healthy human subjects.

Some other new biomarkers of inflammation are the acute phase reactants serum amyloid A (SAA) and fibrinogen and the messenger cytokine interleukin-6 (71,72). Also increased concentrations of CD40 ligand, a transmembrane protein related to tumour necrosis factor α (TNF α), have been associated with cardiovascular events (73). Because of the huge amount of possible markers of inflammation, it remains to be established which biomarker - or set of biomarkers - of inflammation is most appropriate in the risk assessment of cardiovascular diseases.

Potential mechanisms

Several mechanisms have been proposed to explain why stearic acid is less hypercholesterolemic than the other saturated fatty acids. One hypothesis is that stearic acid is poorly absorbed, which may be related to the dietary source and the stereospecific distribution of the stearic acid over the glycerol backbone of the triacylglycerol molecule.

Earlier studies have compared the absorption, digestibility, or faecal excretion of stearic acid with those of other fatty acids in animals as reviewed by Kritchevsky (74). From these studies, it was concluded that stearic acid is less well absorbed than lauric, myristic, palmitic, oleic, or linoleic acids. In humans, however, considerable variations have been reported and absorption rates of stearic acid varied between 68 to 98% (6,75,76). In a more recent study, absorption of stearic acid (94%) was lower than that of palmitic acid (97%) and other fatty acids including lauric, myristic, oleic, and linoleic acids which were absorbed for more than 99% (77). However, these differences in absorption could not explain the differential effects of these fatty acids on the serum lipoprotein profile (10,77).

It also has been suggested that the effects of stearic acid depend on its dietary source and the stereospecific distribution of stearic acid over the glycerol backbone of triacylglycerols. Of the natural fats containing stearic acid such as cocoa butter and shea butter, it is known that stearic acid is predominantly located at the *sn-1* and *sn-3* positions of glycerol in triacylglycerols, whereas in synthetic fats stearic acid is randomly distributed over all three *sn-1*, *sn-2* and *sn-3* positions due to interesterification (78). Although animal studies have shown enhanced absorption of fatty acids at the *sn-2* position of dietary triacylglycerols, in humans little evidence exists that the position of stearic acid on the triacylglycerol affects biological functions (78). Stearic acid present in synthetic fats as tristearins is less readily absorbed than mono- or distearins from natural sources (79) but the magnitude of the difference turned out to be too small to result in different effects on serum lipids and lipoproteins. Grande *et al* (3) showed that cocoa butter rich in stearic acid and an interesterified fat with an identical fatty acid composition but made by hydrogenation and randomisation of different fats and oils, had identical effects on concentrations of serum total cholesterol. Several other studies could also not demonstrate a different response of natural and synthetic fats rich in palmitic, stearic, oleic, or α -linolenic acids on fasting lipid and lipoprotein concentrations (80-82).

When changes in concentrations of HDL cholesterol between diets enriched in stearic, oleic, or linoleic acids were related to the dietary source of stearic acid, no consistent results were obtained. In our study, dietary stearic acid as provided by a

combination of cocoa butter and tri-stearate, did not decrease HDL cholesterol concentrations when compared with oleic or linoleic acids. Also no different effects of stearic, oleic, and linoleic acids were observed on HDL cholesterol concentrations when either a synthetic fat was used in which the stearic acid was predominantly located at the *sn-1* and *sn-3* positions of the glycerol backbone (26) or natural fats such as cocoa butter (5). In another human study, stearic acid as provided by a synthetic fat did not differ in its effects on HDL cholesterol compared with oleic acid (6). However, in the study of Zock and Katan (9), stearic acid decreased concentrations of HDL cholesterol by 0.06 mmol/L, when 8% of dietary energy of linoleic acid was substituted by an interesterified fat providing stearic acid. Judd *et al* (10) also found that substituting oleic acid by a synthetic fat providing stearic acid decreased HDL cholesterol concentrations.

Metabolic differences between (saturated) fatty acids may partly explain differences in hypercholesterolemic effects. Stable-isotope studies in healthy human subjects indicated that stearic acid is more rapidly converted by $\Delta 9$ desaturase than palmitic acid (83,84). As a result, the fatty acid profile of lipids and lipoproteins changed (83). Dietary consumption of stearic acid increased concentrations of oleic acid in triacylglycerols and cholesteryl esters compared with dietary palmitic acid (6). Therefore, the biological effects of stearic acid might be more comparable to those of oleic acid than to those of the other saturated fatty acids.

Thus, although several possible mechanisms have been discussed, it is not possible to explain the specific effects of stearic acid compared with the other saturated fatty acids from a metabolic perspective. The cholesterol-neutral effects of stearic acid could not be explained by a reduced absorption of stearic acid but positional effects of stearic acid cannot be excluded. Also, effects on gene expression have never been studied into detail. Probably more than one or still unknown mechanisms might be responsible for the specific effects of stearic acid on the serum lipoprotein profile, thrombotic tendency, lipid peroxidation, and inflammation.

FATTY ACID DESATURATION AND ELONGATION

The hepatic desaturase and elongase enzymes are importantly involved in fatty acid metabolism and lipid synthesis (85,86). Several factors including dietary fatty acids and hormones such as leptin are known to regulate expression of desaturases and elongases (87). To investigate the mechanisms by which fatty acids regulate fatty acid desaturation and elongation, the effects of the two individual conjugated linoleic acid (CLA) isomers, *c9,t11* and *t10,c12* CLA, were evaluated in a human

intervention study. Moreover, in cell culture experiments the effects of stearic, oleic, and linoleic acids were investigated on the transcriptional and translational regulation of desaturases and elongases in a human hepatoma-derived cell line (HepG2 cells). To measure mRNA expression by real-time reverse transcription quantitative polymerase chain reactions (RT-qPCRs), specific assays were developed based upon the gene sequences of $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases and of elongase (ELOVL5). As the expression of β actin would not be influenced by the different interventions, β actin was used as a housekeeping gene.

Conjugated linoleic acids

In contrast to most human studies that have investigated the effects of a mixture of CLA isomers, we have evaluated isomer-specific effects of the pure isomers, *c9,t11* and *t10,c12* CLA on the plasma fatty acid profile and expression of desaturases of healthy humans (**Chapter 6**). The study had a parallel design and 20 healthy, slightly overweight men ($n = 10$) and women ($n = 10$) participated. A run-in period of 6 weeks, in which all participants daily consumed a dairy product containing 3 g of high-oleic sunflower oil (placebo), was followed by an intervention period of 18 weeks. During this intervention period, the control group continued to use this product, whereas the second and third groups received products with 3 g of purified *c9,t11* or *t10,c12* CLA. At the end of the intervention period, the fatty acid composition of plasma lipids - phospholipids, cholesteryl esters, and triacylglycerols - was analysed. Also mRNA expressions of desaturases and elongase were measured in peripheral blood mononuclear cells (PBMC).

Dietary CLA isomers changed the fatty acid composition of lipid fractions in three different ways. Firstly, like most other fatty acids, the two isomers of CLA were incorporated into lipid fractions. In agreement with another human study (88), *c9,t11* and *t10,c12* CLA were incorporated in a dose-dependent manner into plasma phospholipids, and also in cholesteryl esters and triacylglycerols. As observed in animal studies, the incorporation of *c9,t11* into lipid fractions was slightly higher than that of *t10,c12* CLA (89,90). Secondly, the *t10,c12* isomer was mainly converted into the C18:3 metabolite, whereas supplementation with *c9,t11* CLA increased both C18:3 and C20:3 conjugated isomers. Those findings agree with previous studies in rats (90). In the third place, except for the CLA isomers, also the percentage of other fatty acids changed in the plasma lipid fractions. Surprisingly, in our study the proportions of linoleic acid increased in plasma phospholipids, cholesteryl esters and triacylglycerols, which suggest a decrease in $\Delta 6$ desaturation. However, proportions of long-chain metabolites of linoleic acid did not decrease in contrast to other studies (91-93).

Compared with *c9,t11* CLA, *t10,c12* CLA decreased the $\Delta 9$ desaturation index of C18:0, but not that of C16:0. In another human study, a mixture of CLA isomers resulted in a decreased $\Delta 9$ desaturation index (94). Also in cell culture studies (91,95) and animal studies (93,96,97), the *t10,c12* isomer inhibited $\Delta 9$ desaturation activity. Moreover, as in the study of Smedman and Vessby (94), both CLA isomers decreased the $\Delta 6$ desaturation index as calculated from the ratio of C18:3n-6 plus C20:3n-6 to C18:2n-6. With respect to the $\Delta 5$ desaturation index, results are rather inconsistent. In our study, no different effects of *c9,t11*, *t10,c12* CLA or placebo (high-oleic acid sunflower oil) were observed. In another human study, the $\Delta 5$ desaturation index increased when a mixture of CLA isomers was consumed at the expense of oleic acid (94). In contrast, in HepG2 cells *t10,c12* CLA inhibited $\Delta 5$ desaturation (91). Hence, particularly *t10,c12* CLA decreased desaturation indices, which may result in less desirable physiological effects of this CLA isomer.

CLA may modulate gene expression of enzymes involved in fatty acid metabolism like desaturases and elongases. However, although *c9,t11* and *t10,c12* CLA isomers differed in their effects on desaturation indices, in our study no different effects of the individual CLA isomers were found on the mRNA expression of $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases and elongase (ELOVL5) in PBMC relative to placebo. In mouse hepatocytes, mRNA expression of stearoyl-CoA desaturase (SCD) gene 1 or $\Delta 9$ desaturase was inhibited by *t10,c12* CLA but not by *c9,t11* CLA (97). Moreover, in HepG2 cells *t10,c12* CLA decreased $\Delta 9$ desaturation of palmitic acid but mRNA expression and protein levels of $\Delta 9$ desaturase were not influenced by *t10,c12* or *c9,t11* CLA isomers (95). Hence, *t10,c12* CLA might regulate the activity of $\Delta 9$ desaturase without any effect at the transcriptional or translational levels.

Health benefits of CLA isomers have been reported mainly in animal studies. Until recently, only mixtures of CLA isomers were used in humans but now some recent studies have reported the individual effects of the pure *c9,t11* and *t10,c12* CLA isomers. *C9,t11* and *t10,c12* CLA did not affect body fat composition in our study (98). In another human study, the *c9,t11* and *t10,c12* CLA isomers did also not change body weight, body fat or fat-free mass and parameters of immune function such as lymphocyte subpopulations, *ex vivo* cytokine production, or CRP concentrations. In a dose-dependent manner, both *c9,t11* and *t10,c12* CLA decreased mitogen-induced lymphocyte activation beneficially (99). On the blood lipid profile, however, opposing effects of the two isomers were observed (100). *C9,t11* CLA decreased the total to HDL cholesterol ratio, when compared with *t10,c12* CLA. Beneficial health effects of one or both of these CLA isomers are therefore not proved yet. In a recent overview the results of studies using mixtures of CLA isomers in humans have been summarised (101). In general, the effects of

both CLA isomers on body fat and plasma lipids in humans were considerably less than expected from animal studies. The t10,c12 CLA isomer may even have some undesirable side effects.

In conclusion, the c9,t11 and t10,c12 CLA isomers differ in their effects on the plasma fatty acid profile. Health effects of CLA, if any, are less than anticipated from animal studies.

Regulation of desaturases and elongases

Other fatty acids are also known to modulate the expression of desaturases and elongases. Most attention has been paid to the regulation of $\Delta 9$ desaturase. Diets containing linoleic acid, arachidonic acid and α -linolenic acid repressed the mRNA expression of the mouse SCD1 gene, relative to a fat-free diet and diets rich in saturated or monounsaturated fatty acids (102). The effects of n-6 and n-3 polyunsaturated fatty acids on $\Delta 9$ desaturase expression were confirmed by studies in adipocytes (103), and in mouse liver cells (104,105). Suppressive effects of polyunsaturated fatty acids increased with the degree of unsaturation (102-104). Hence, it is generally accepted that polyunsaturated fatty acids inhibit mRNA expression of $\Delta 9$ desaturase in animal models.

Also expression of $\Delta 5$ and $\Delta 6$ desaturases in rat and mice hepatocytes is inhibited by diets containing n-6 or n-3 polyunsaturated fatty acids such as linoleic acid or fish fatty acids relative to a diet rich in oleic acid or a fat-free diet (106-109). More recently, the effects of fatty acids were reported on the expression of the different elongases. In rats fed diets supplemented with fish oil hepatic mRNA expression of $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases and some but not all elongases decreased relative to rats fed a diet rich in oleic acid (110). Thus, the $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases, and elongases might be regulated coordinately.

Through interaction with nuclear receptors, fatty acids regulate expression of desaturase and elongase genes. Nuclear receptors such as peroxisome proliferator-activated receptors (PPARs), sterol regulatory element-binding proteins (SREBPs), and the liver X receptors (LXRs) play a pivotal role in the expression of lipogenic genes (85,111,112). PPARs are ligand-activated transcription factors that regulate gene expression through binding to specific DNA sequences called peroxisome proliferator response elements (PPREs). Indeed, PPAR α agonists like fibrates and WY14,643 have been shown to activate mRNA expression of desaturase and elongase enzymes in rats (113). A wide variety of saturated and unsaturated fatty acids are known as ligands of PPAR α (114,115). Not only PPAR α but also other nuclear receptors or transcription factors like sterol regulatory element-binding proteins (SREBPs) and the liver X receptors (LXRs) might be involved in the

regulation of desaturases and elongases by fatty acids (85,111,112). Especially the SREBP-1a and SREBP-1c isoforms were reported to activate the human promoter of $\Delta 9$ desaturase (116). Also $LXR\alpha$, the oxysterol receptor that mediates regulation of gene expression by cholesterol or its metabolites, has been associated with the regulation of desaturases and elongases in rats (113,117).

To investigate the effects of fatty acids on desaturation and elongation, we performed some cell culture experiments in a human hepatoma-derived cell line (HepG2 cells). In these experiments, we observed that linoleic acid decreased $\Delta 6$ and $\Delta 9$ desaturation indices of fatty acids in membrane phospholipids relative to oleic and linoleic acids but the suppressive effects of linoleic acid could not be confirmed at the transcriptional level by the mRNA expression of $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases and elongase (**Figure 7.3**). The $PPAR\alpha$ agonists fenofibric acid and ciprofibrate induced mRNA expression of desaturases and elongase mainly of $\Delta 5$ and $\Delta 9$ desaturase. Previous studies already reported that $PPAR\alpha$ agonists induced mRNA expression of $\Delta 9$ desaturase. Expression of mouse liver stearoyl-CoA desaturase expression was induced by the addition of clofibrate or gemfibrozil to the diet of mice (105). In HepG2 cells, ciprofibrate increased expression of stearoyl-CoA desaturase (118). Moreover, adult rats fed the $PPAR\alpha$ agonist WY14,643 had elevated elongase activity and mRNA abundance of elongases (ELOVL1, ELOVL5 and ELOVL6) and $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases (110). Hence, $PPAR\alpha$ is involved in the regulation of desaturases and elongases. To investigate whether desaturases and elongase are regulated at the transcriptional or translational level, HepG2 cells were incubated with or without a transcription inhibitor (actinomycin D) or translation inhibitor (cycloheximide). Results were rather inconsistent (**Figure 7.3**). Because we could not investigate extensively the regulation of desaturases and elongases by fatty acids and the involvement of nuclear factors herein in HepG2 cells, our results should be considered as preliminary. To draw more definite conclusions, these cell culture experiments should be extended.

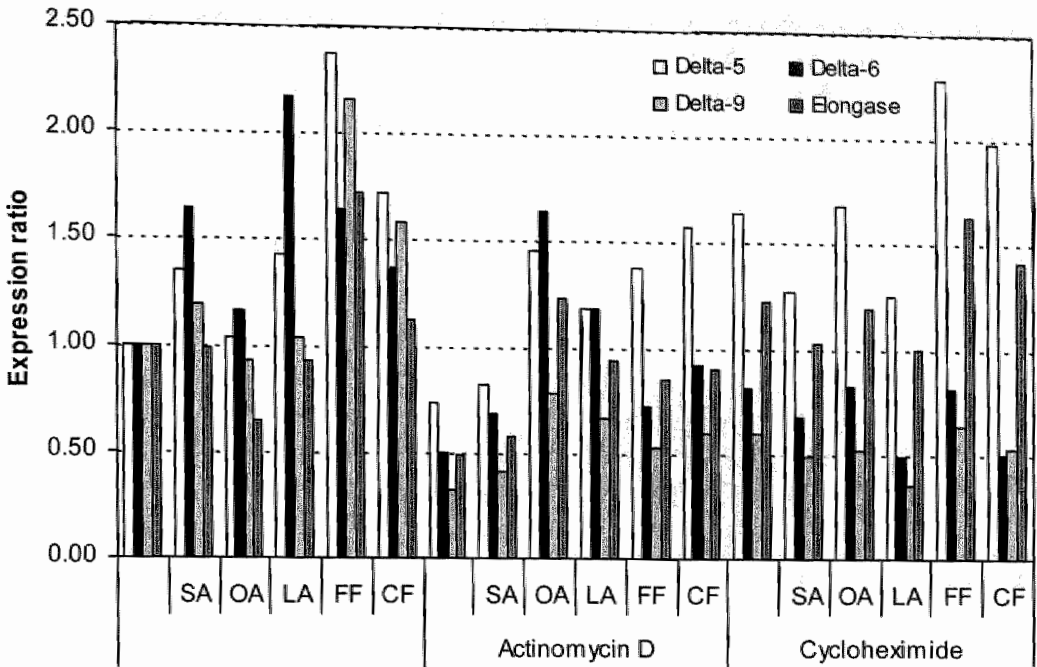


Figure 7.3 Differential induction of mRNA expression of $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases and elongase (ELOVL5) by incubation of HepG2 cells with 200 μM of stearic (SA), oleic (OA) or linoleic acid (LA) or 0.3 mM of fenofibrate (FF) or ciprofibrate (CF) for 24 hours.^{1,2}

¹Values were obtained using TaqMan procedures (comparative cycle threshold method) and are normalised to the housekeeping gene β -actin and relative to control HepG2 cells incubated with medium containing 0.1% BSA, 0.1% DMSO and 0.2% ethanol. All samples were analysed in duplicate.

²Transcription was inhibited by the addition of actinomycin D (5 $\mu\text{g}/\text{mL}$) to the culture medium for 6 hours or translation by the addition of cycloheximide (10 $\mu\text{g}/\text{mL}$) for 24 hours.

Regulation of desaturases and elongases by fatty acids or fibrates has mostly been investigated in animal studies. Whether animal or cell models reflect the *in vivo* situation in humans is still unknown. In human intervention studies, the accessibility of liver tissue is difficult, as not almost impossible. Therefore, in our human intervention study with the pure CLA isomers, mRNA was isolated from PBMC which are easily obtained by venipuncture. However, it is unknown whether mRNA expression or activity of desaturases and elongase in PBMC would reflect desaturation and elongation of fatty acids in liver cells. In rats, a tissue-specific expression of fatty acid elongases was reported (113). It remains to be established whether the effects of fatty acids on elongation are tissue- or cell-specific or that elongases with comparable functions in different tissues are involved in fatty acid

metabolism. The RT-qPCRs of $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases, and of elongase (ELOVL5) in humans would be valuable to investigate the effects of fatty acids in different tissues. Moreover, new technologies like microarray analysis and bioinformatics will make it possible to study the regulation of a lot of genes involved in fatty acid metabolism simultaneously. In this way, new insights could be gathered about the molecular mechanisms underlying the regulatory effects of fatty acids on fatty acid metabolism including desaturation and elongation in different tissues.

CONCLUSIONS

In the past, reduction of dietary fat intake has been the major focus of dietary recommendations to lower cardiovascular risk. In the last decades, evidence is accumulating that an exchange of harmful fatty acids by fatty acids with beneficial effects on lipid and lipoprotein metabolism, thrombotic tendency, lipid peroxidation and inflammation rather than a reduction of the total dietary fatty acid intake would decrease cardiovascular disease risk. In our study, only small differences in the effects of stearic, oleic, and linoleic acids have been observed on lipid and lipoprotein concentrations, thrombotic tendency, and risk markers of lipid peroxidation and inflammation. When 7% of energy of stearic acid in the diet was exchanged by oleic or linoleic acids, changes in the concentrations of total and LDL cholesterol were less than expected from earlier studies. Stearic, oleic, and linoleic acids did not differ in their effects on other parameters of the serum lipoprotein profile. Relative to the other long-chain saturated fatty acids - in particular myristic acid and palmitic acid - stearic acid had more beneficial effects on serum total and LDL cholesterol concentrations.

With respect to thrombotic tendency, no different effects of stearic, oleic, and linoleic acids on coagulation and fibrinolytic factors were found but stearic acid may affect these variables beneficially relative to the other saturated fatty acids. Compared with diets rich in oleic or linoleic acids, stearic acid decreased platelet volumes. However, in men *ex vivo* platelet aggregation was beneficially affected by linoleic acid relative to stearic acid. Moreover, in recent studies no thrombotic effects of saturated fatty acids in particular of stearic acid were reported on *in vitro* platelet aggregation relative to unsaturated fatty acids. Although the effects of stearic acid on lipid peroxidation and inflammation have not been studied yet relative to lauric, myristic, and palmitic acids, no different effects of stearic, oleic, and linoleic acids were observed on the urinary excretion of isoprostanes and concentrations of hsCRP.

Mainly due to its effects on the lipoprotein profile, stearic acid is a unique saturated fatty acid. Whether its effects are indeed comparable to those of monounsaturated and polyunsaturated fatty acids has to be considered carefully. It is unknown whether cardiovascular disease risk itself is reduced, when other saturated fatty acids in the diet are substituted by stearic acid. For food applications, however, the incorporation of stearic acid is preferred to that of palmitic acid, the most common saturated fatty acid in the diet, and of *trans* fatty acids from hydrogenated oils.

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Fatty acid nomenclature and abbreviations

FATTY ACID NOMENCLATURE

| Notation | Common name |
|--|---|
| <i>Saturated fatty acids</i> | |
| C12:0 | Lauric acid |
| C14:0 | Myristic acid |
| C16:0 | Palmitic acid |
| C18:0 | Stearic acid |
| <i>Monounsaturated fatty acids</i> | |
| C16:1n-7 | Palmitoleic acid |
| C18:1n-9 | Oleic acid |
| <i>N-9 polyunsaturated fatty acids</i> | |
| C20:3n-9 | Mead acid |
| C22:3n-9 | Dihomo-Mead acid |
| <i>N-6 polyunsaturated fatty acids</i> | |
| C18:2n-6 | Linoleic acid |
| C18:3n-6 | γ -Linolenic acid |
| C20:3n-6 | Dihomo- γ -linolenic acid |
| C20:4n-6 | Arachidonic acid |
| C22:4n-6 | Adrenic acid |
| C22:5n-6 | Osbond acid |
| <i>N-3 polyunsaturated fatty acids</i> | |
| C18:3n-3 | α -Linolenic acid |
| C18:4n-3 | Stearidonic acid |
| C20:4n-3 | Eicosatetraenoic acid |
| C20:5n-3 | Eicosapentaenoic acid (EPA) |
| C22:5n-3 | ω 3-Docosapentaenoic acid |
| C22:6n-3 | Docosahexaenoic acid (DHA) |
| <i>Conjugated linoleic acids (CLA)</i> | |
| C9,t11 CLA | <i>Cis</i> -9, <i>trans</i> -11 CLA, rumenic acid |
| T10,c12 CLA | <i>Trans</i> -10, <i>cis</i> -12 CLA |
| T11 C18:1 | Vaccenic acid |

ABBREVIATIONS

| | |
|---|---|
| ΔP | pressure difference |
| 15-K-DH-PGF _{2α} | 15-keto-dihydro-prostaglandin F _{2α} |
| 8-iso-PGF _{2α} | 8-iso-prostaglandin F _{2α} |
| ADP | adenosine di-phosphate |
| Amax | <i>ex vivo</i> maximum platelet aggregation |
| Ang | angiogenin |
| Apo A-I | apolipoprotein A-I |
| Apo B | apolipoprotein B |
| BMI | body mass index |
| C | carbon |
| CE | cholesteryl esters |
| CF | ciprofibrate |
| CI | confidence interval |
| CH ₃ | methyl |
| CLA | conjugated linoleic acids |
| CoA | coenzyme A |
| COOH | carboxyl |
| CRP | C-reactive protein |
| CSF | colony stimulating factor |
| Ct | cycle threshold |
| CV | coefficient of variation |
| D-dimers | fibrin degradation products |
| DHA | docosahexaenoic acid |
| EGF | epidermal growth factor |
| ELISA | enzyme-linked immunosorbent assay |
| ELOVL | gene family of elongases |
| EPA | eicosapentaenoic acid |
| E-selectin | selectin derived from endothelial cells |
| factor VIIam | factor VII amidolytic |
| FAME | fatty acid methyl ester |
| FF | fenofibric acid |
| FVIIa | activated factor VII |
| GLM | general linear model |
| GRO | growth regulated protein |
| HDL | high-density lipoprotein |
| HELO | human fatty acid elongase |
| HepG2 | human hepatoma-derived cell line |
| hsCRP | high sensitivity C-reactive protein |

| | |
|----------------|--|
| ICAM-1 | intercellular adhesion molecule-1 |
| IFN γ | interferon γ |
| IL | interleukin |
| Imax | <i>in vitro</i> maximum platelet aggregation |
| LA | linoleic acid |
| LDL | low-density lipoprotein |
| LFA-1 | leukocyte-function-associated antigen-1 |
| LPS | lipopolysaccharide |
| LXR | liver X receptor |
| MCFA | medium-chain fatty acids |
| MCP | monocyte chemotactic protein |
| M-CSF | macrophage colony stimulating factor |
| MDA | malondialdehydes |
| MDC | macrophage derived chemokine |
| MGB | minor groove binder |
| MIP-1 δ | macrophage inflammatory protein-1 δ |
| mRNA | messenger RNA |
| MUFA | monounsaturated fatty acids |
| NMR | nuclear magnetic resonance |
| OA | oleic acid |
| OSM | oncostatin M |
| P-value | probability level |
| PAI | plasminogen activator inhibitor |
| PBMC | peripheral blood mononuclear cells |
| PDGF β | platelet-derived growth factor β |
| PG | prostaglandin |
| PGI $_2$ | prostaglandin I $_2$ |
| PGI $_3$ | prostacyclin |
| PL | phospholipids |
| PPAR | peroxisome proliferator-activated receptor |
| PPRE | peroxisome proliferator response element |
| PRP | platelet-rich plasma |
| PTF1 + 2 | prothrombin fragment 1 and 2 |
| PUFA | polyunsaturated fatty acids |
| Rn | fluorescence threshold |
| RT-qPCR | reverse transcription quantitative PCR |
| SA | stearic acid |
| SAA | serum amyloid A |
| SCD | stearoyl-CoA desaturase |
| SFA | saturated fatty acids |

| | |
|--------------|---|
| SREBP | sterol regulatory element-binding protein |
| Ta | <i>ex vivo</i> platelet aggregation time |
| TAG | triacylglycerols |
| Tai | <i>in vitro</i> platelet aggregation time |
| TBARS | thiobarbituric acid reactive substances |
| TC | total cholesterol |
| Tdi | <i>ex vivo</i> platelet desaggregation induction time |
| TNF | tumour necrosis factor |
| tPA | tissue plasminogen activator |
| TPO | thrombopoietin |
| <i>trans</i> | <i>trans</i> fatty acids |
| Ts | <i>ex vivo</i> platelet aggregation slope |
| TX | thromboxane |
| Va | <i>in vitro</i> platelet aggregation velocity |
| VCAM-1 | vascular cell adhesion molecule-1 |
| VEGF | vascular endothelial growth factor |
| VLDL | very low-density lipoprotein |

Summary

SUMMARY

Cardiovascular diseases are a major cause of morbidity and mortality in western and developed countries. Because a diversity of risk markers is related with its prevalence, cardiovascular diseases have a multifactorial aetiology. Some of these risk markers such as an unfavourable lipoprotein profile, increased thrombotic tendency, increased oxidative stress and low-grade inflammation can be affected by changing total fat or fatty acid intake. In the past, reduction of the dietary fat intake has been the major focus of dietary recommendations to lower cardiovascular disease risk. In the last decades, evidence is accumulating that an exchange of harmful fatty acids by fatty acids with more beneficial effects rather than a reduction in total fat intake decreases cardiovascular disease risk more effectively.

Dependent on their structure and function, fatty acids exert differential effects on cardiovascular risk markers. In this thesis, the effects of fatty acids - in particular of the C18 fatty acids stearic acid, oleic acid, and linoleic acid and of conjugated linoleic acids (CLA) - on cardiovascular disease risk markers have been described. Also effects on fatty acid desaturation and elongation were investigated.

Earlier studies have suggested comparable effects of stearic acid - a saturated fatty acid - and oleic acid - a *cis*-monounsaturated fatty acid - on the distribution of cholesterol over the various lipoproteins, which are the major transporters of lipids in the blood and differentially affect cardiovascular risk. In addition, other studies have found similar effects of oleic acid and linoleic acid - a *cis*-polyunsaturated fatty acid. If true, than these three C18 fatty acids should have comparable effects on the serum lipoprotein profile. We therefore initiated a crossover study with 45 healthy non-smoking human subjects. Each participant consumed in random order each of the 3 different diets during three 5-week periods. Diets provided 38% of energy from fat of which 60% was supplied by the experimental fats. These fats were incorporated into bread, margarines, and cakes. The nutrient composition of the diets did not differ, except for a 7% difference in energy intake provided by stearic, oleic, or linoleic acids. Except for the effects on the serum lipoprotein profile, also other cardiovascular risk markers (thrombotic tendency, lipid peroxidation, and inflammation) were studied.

Only small differences in the effects of stearic, oleic, and linoleic acids were observed on lipid and lipoprotein concentrations. Changes in the concentrations of the atherogenic LDL and anti-atherogenic HDL cholesterol concentrations were less than expected from earlier studies. Stearic, oleic, and linoleic acids did also not differ in their effects on lipoprotein particle sizes, another risk marker of cardiovascular diseases. Based on these findings and other studies from the

literature, the most favourable lipoprotein profile is achieved when a mixture of *cis*-unsaturated fatty acids replaces in particular palmitic and myristic acids - two saturated fatty acids - and *trans* fatty acids in the diet.

With respect to thrombotic tendency, earlier studies have suggested prothrombotic effects of stearic acid. More recent studies, however, could not confirm these unbeneficial effects of stearic acid on platelet aggregation, coagulation, and fibrinolysis. We also found no different effects of stearic, oleic, and linoleic acids on coagulation and fibrinolytic factors. Stearic acid decreased platelet volumes compared with oleic or linoleic acids. Larger platelets are associated with increased cardiovascular risk. On the other hand, *ex vivo* platelet aggregation in men was affected unbeneficially by stearic acid relative to linoleic acid. Therefore, our results do not indicate that stearic acid is thrombogenic compared with oleic and linoleic acids.

The atherogenicity of lipid particles is largely increased by lipid peroxidation. With increasing degree of unsaturation of fatty acids, lipid peroxidation is expected to increase. In our study, no different effects of stearic, oleic, and linoleic acids were observed on the urinary excretion of the F_2 -isoprostanes 8-iso-prostaglandin $F_{2\alpha}$ and 15-keto-dihydro-prostaglandin $F_{2\alpha}$, which are biomarkers of respectively non-enzymatic and enzymatic lipid peroxidation. In contrast, results from studies using *in vitro* methods to measure LDL susceptibility to oxidation suggest that linoleic acid is more readily oxidised than *cis*-monounsaturated or saturated fatty acids. The *in vivo* relevance of this latter method is, however, limited.

Several inflammatory risk markers may be involved in the development of cardiovascular diseases. High sensitivity C-reactive protein (hsCRP) is a circulating acute phase reactant that reflects systemic inflammation. Stearic, oleic, and linoleic acids did not differ in their effects on this marker. The effects of these C18 fatty acids on multiple immunomodulatory molecules were simultaneously detected by the use of antibody arrays. However, results were not consistent and future studies are needed to address the effects of fatty acids on the cytokine profile into more detail.

In conclusion, differences between the effects of the C18 fatty acids stearic, oleic, and linoleic acids on the serum lipoprotein profile are less than was predicted based on earlier studies. Effects on other cardiovascular risk markers were also marginal. Whether the effects on cardiovascular disease risk itself are comparable, is unknown. For food applications, the use of stearic acid may be preferred over that of myristic and palmitic acids - two saturated fatty acids in the diet - and of *trans* fatty acids from hydrogenated oils.

Desaturation and elongation enzymes might be regulated differently by the individual fatty acids. As almost all human studies reported the effects of a mixture of CLA isomers, we have investigated the isomer-specific effects of the two most common CLA isomers - *cis*-9,*trans*-11 (*c9,t11*) and *trans*-10,*cis*-12 (*t10,c12*) CLA - on fatty acid desaturation and elongation. In a double-blind, placebo-controlled study with parallel design, 25 healthy overweight men and women consumed daily a dietary supplement providing 3 g of high-oleic sunflower oil (placebo) during the first 6 weeks of the trial (run-in period). After randomisation, one group ($n = 7$) continued to consume the placebo dairy product daily for the next 18 weeks of the study (intervention period). The second ($n = 9$) and third ($n = 9$) groups consumed the dairy product with 3 g of purified *c9,t11* CLA or 3 g of *t10,c12* CLA, respectively. Dietary supplements containing CLA were provided as an acidified drinkable dairy product.

Compared with *c9,t11* CLA, *t10,c12* CLA lowered the ratio of C18:1n-9 to C18:0 in plasma phospholipids, suggesting a decreased $\Delta 9$ desaturation index but the C16:1n-7 to C16:0 ratio was not affected. Both CLA isomers decreased the $\Delta 6$ desaturation index as calculated from the ratio of C18:3n-6 plus C20:3n-6 to C18:2n-6. mRNA expressions of desaturases and elongase in peripheral blood mononuclear cells (PBMC) were, however, not affected. Hence, effects on desaturation indices were not reflected by changes at the transcriptional level. It is unknown, however, whether mRNA expressions or activities of desaturases and elongases in PBMC reflect those in the liver, the major organ for desaturation and elongation of fatty acids. Therefore, it is interesting to identify the similarities and differences in the molecular effects of fatty acids on fatty acid metabolism in PBMC, in the liver or other tissues.

Samenvatting

SAMENVATTING

Hart- en vaatziekten vormen een belangrijke oorzaak van ziekte en sterfte in westerse en ontwikkelde landen. Omdat vele risicomerkers gerelateerd zijn aan de prevalentie, hebben hart- en vaatziekten een multifactoriële etiologie. Sommige risicomerkers zoals een ongunstig lipoproteïnenprofiel, verhoogde thromboseneiging, verhoogde oxidatieve stress en een laag-gradige chronische ontsteking worden beïnvloed door een verandering in de totale vet- of vetzuuriname. In het verleden werd in voedingsadviezen om het risico op hart- en vaatziekten te verlagen vooral de nadruk gelegd op het reduceren van de vetinname. In de laatste decennia bestaan er echter steeds meer bewijzen dat een uitwisseling van ongezonde vetzuren door vetzuren met gunstige effecten tot een lager risico op hart- en vaatziekten leidt dan het verlagen van de totale vetinname.

Vetzuren hebben afhankelijk van structuur en functie verschillende effecten op risicomerkers voor hart- en vaatziekten. In dit proefschrift zijn vooral de effecten van de C18 vetzuren - stearinezuur, oliezuur, linolzuur en geconjugeerd linolzuur (CLA) - op risicomerkers voor hart- en vaatziekten beschreven. Ook zijn de effecten op de desaturatie en elongatie van vetzuren onderzocht.

Eerdere studies suggereerden vergelijkbare effecten van stearinezuur - een verzadigd vetzuur - en oliezuur - een *cis* enkelvoudig onverzadigd vetzuur - op de verdeling van cholesterol over de verschillende lipoproteïnen. Dit zijn de belangrijkste transporters van cholesterol in het bloed en ze beïnvloeden het risico op hart- en vaatziekten verschillend. Bovendien hebben andere studies vergelijkbare effecten van oliezuur en linolzuur - een *cis* meervoudig onverzadigd vetzuur - gevonden. Indien dit klopt, dan zouden deze drie C18 vetzuren vergelijkbare effecten op het serum lipoproteïnenprofiel hebben. De effecten van stearinezuur, oliezuur en linolzuur werden onderzocht in een cross-over studie met 45 gezonde, niet-rokende proefpersonen. Elke deelnemer gebruikte de 3 verschillende voedingen in willekeurige volgorde gedurende drie perioden van 5 weken. De voedingen bevatten 38 energieprocenten vetten, waarvan 60% werd geleverd door experimentele vetten. Deze vetten werden verwerkt in brood, margarine en cake. Behalve een verschil van 7% in de energie inname van stearinezuur, oliezuur of linolzuur, was de voorgeschreven voedingssamenstelling van de drie voedingen gelijk. Naast de effecten op het serum lipoproteïnenprofiel, werden ook andere risicomerkers van hart- en vaatziekten (thromboseneiging, lipidenperoxidatie en inflammatie) bestudeerd.

Slechts kleine verschillen werden waargenomen in de effecten van stearinezuur, oliezuur en linolzuur op de lipiden- en lipoproteïnenconcentraties. De veranderingen

in de concentraties van de atherogene LDL en niet-atherogene HDL cholesterolconcentraties waren kleiner dan werd verwacht op basis van de eerdere studies. Stearinezuur, oliezuur en linolzuur verschilden ook niet in de effecten op de deeltjesgrootte van de lipoproteïnen, een andere risicomarker van hart- en vaatziekten. Gebaseerd op deze bevindingen en andere studies in de literatuur wordt het meest gunstige lipoproteïnenprofiel bereikt wanneer palmitinezuur en myristinezuur - 2 verzadigde vetzuren - en *trans* vetzuren in de voeding worden vervangen door een mengsel van *cis*-onverzadigde vetzuren.

Met betrekking tot de thrombose-eigiging hebben eerdere studies gesuggereerd dat stearinezuur prothrombotische effecten heeft. Meer recentere studies hebben deze nadelige effecten van stearinezuur op de bloedplaatjesaggregatie, stolling en fibrinolyse niet bevestigd. In onze studie verschilden de effecten van stearinezuur, oliezuur en linolzuur op factoren betrokken bij de stolling en fibrinolyse ook niet. In vergelijking met voedingen rijk aan oliezuur en linolzuur, verlaagde stearinezuur het volume van de bloedplaatjes. Grotere bloedplaatjes zijn geassocieerd met een hoger risico op hart- en vaatziekten. Aan de andere kant werd de *ex vivo* bloedplaatjesaggregatie in mannen nadelig beïnvloed door stearinezuur ten opzichte van linolzuur. Daarom duiden onze resultaten er niet op dat stearinezuur thrombogener is dan oliezuur of linolzuur.

De atherogeniteit van lipidendeeltjes wordt sterk vergroot door lipidenperoxidatie. De gedachte is dat onverzadigde vetzuren de kans op lipidenperoxidatie vergroten. Echter, de effecten van stearinezuur, oliezuur en linolzuur op de excretie in de urine van de F_2 -isoprostanen, 8-iso-prostaglandine $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$) en 15-keto-dihydroprostaglandine $F_{2\alpha}$ (15-K-DH-PGF $_{2\alpha}$), die biomerkers zijn voor respectievelijk niet-enzymatische en enzymatische lipidenperoxidatie, waren niet verschillend. Dit is in tegenspraak met de resultaten van studies waarin *in vitro* methoden werden gebruikt om de oxideerbaarheid van LDL door metaalionen te meten. In deze studies is gevonden dat linolzuur eerder geoxideerd wordt dan *cis*-enkelvoudige onverzadigde of verzadigde vetzuren. De *in vivo* relevantie van deze laatstgenoemde methoden is echter beperkt.

Verschillende ontstekingsmerkers kunnen betrokken zijn bij de ontwikkeling van hart- en vaatziekten. Het C-reactieve proteïne (CRP) is een circulerend acute fase eiwit, dat bij ontstekingen sterk verhoogd is. Stearinezuur, oliezuur en linolzuur verschilden niet in hun effecten op deze risicomarker. De effecten van deze C18 vetzuren op andere eiwitten betrokken bij een immuunreactie werden gedetecteerd met behulp van antilichaam arrays. De resultaten waren echter moeilijk te interpreteren en toekomstige studies zijn noodzakelijk om de effecten van vetzuren op het cytokinenprofiel in detail te onderzoeken.

Op basis van deze bevindingen concluderen wij dat de verschillen in de effecten van de C18 vetzuren stearinezuur, oliezuur en linolzuur op het lipoproteïnenprofiel kleiner zijn dan verwacht uit eerdere studies. De effecten op andere risicomerkers voor hart- en vaatziekten zijn ook gering. Of de effecten op het risico op hart- en vaatziekten zelf ook vergelijkbaar zijn, is nog onbekend. Voor voedingstoepassingen kan echter worden gesteld dat het toepassen van stearinezuur de voorkeur heeft boven het toepassen van myristine- en palmitinezuur - twee verzadigde vetzuren in de voeding - en die van *trans* vetzuren uit gehydrogeneerde oliën.

Desaturatie en elongatie enzymen worden verschillend gereguleerd door de individuele vetzuren. Omdat bijna alle humane studies de effecten van een mengsel van CLA isomeren hebben gerapporteerd, hebben wij de isomeer-specifieke effecten van de twee meest voorkomende CLA isomeren - *cis*-9,*trans*-11 (*c9,t11*) en *trans*-10,*cis*-12 (*t10,c12*) CLA - onderzocht op de desaturatie en elongatie van vetzuren. In een dubbelblinde, placebo-gecontroleerde studie met een parallel design, gebruikten 25 gezonde mannen en vrouwen met overgewicht gedurende de eerste 6 weken van de studie (run-in periode) dagelijks een voedingssupplement dat 3 gram zonnebloemolie met een hoog gehalte oliezuur (placebo) bevatte. Na randomisatie bleef één groep ($n = 7$) het placebo zuivelproduct dagelijks gebruiken voor de daaropvolgende 18 weken van de studie (interventieperiode). De tweede ($n = 9$) en derde ($n = 9$) groep consumeerden een zuivelproduct met respectievelijk 3 gram *c9,t11* CLA of *t10,c12* CLA.

In vergelijking met *c9,t11* CLA verlaagde de *t10,c12* CLA isomeer de C18:1n-9/C18:0 ratio in plasma fosfolipiden, duidend op een verminderde $\Delta 9$ desaturatie activiteit. De C16:1n-7/C16:0 ratio werd echter niet beïnvloed. Beide CLA isomeren verlaagden de $\Delta 6$ desaturatie index, die werd berekend uit de ratio van C18:3n-6 en C20:3n-6 ten opzichte van C18:2n-6. De mRNA expressie van desaturases en elongase in perifere bloed mononucleaire cellen (PBMC) werd echter niet beïnvloed. Hieruit volgt dat de effecten op de desaturatie indices niet werden weerspiegeld door veranderingen op transcriptioneel nivo. Het is echter onbekend of de mRNA expressies van desaturases en elongases in PBMC een weerspiegeling zijn van desaturatie en elongatie in de lever, dat het belangrijkste orgaan is voor de desaturatie en elongatie van vetzuren. Daarom is het van belang om verschillen en overeenkomsten te identificeren in de moleculaire effecten van vetzuren op het metabolisme in PBMC en in de lever of andere weefsels.

Dankwoord

DANKWOORD

Ergens aan beginnen is niet altijd eenvoudig (zo ook het begin van dit dankwoord), maar wat je doet moet je goed doen en waar je aan begint ook afmaken. Gelukkig is dankzij de steun van velen dit proefschrift tot stand gekomen en dat is iets om trots op te zijn. Dank je wel voor alle steentjes die er door een ieder aan zijn bijgedragen.

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Myriam

Curriculum vitae

CURRICULUM VITAE

Myriam Anna Maria Anthonia Thijssen was born on September 10, 1976 in Beers. After graduating secondary school at the Elshofcollege in Nijmegen, she started in 1993 with her study to become a medical laboratory technician (graduation direction clinical chemistry) at the Higher Laboratory School in Nijmegen. As part of this study, a clinical and research training period was performed at the laboratory of the Maasziekenhuis in Boxmeer. She graduated in July 1997. Afterwards, from 1997 to 2001, she studied Biomedical Sciences at the Faculty of Medicine of the University of Leiden. During training periods, she participated in different research projects at the department of Immunohematology and Blood Transfusion in the Leiden University Medical Centre (LUMC) with prof.dr. M. Giphart as supervisor and at the immunological laboratory of the department of Pediatrics, also at the LUMC with dr. M. van Tol and dr. M. Schilham as supervisors. The final training period was spent at the Central Clinical Chemical Laboratory at the University Medical Centre St. Radboud in Nijmegen and was supervised by dr. D. Swinkels and dr. J. de Kok. Finally she graduated in April 2001. At the same time she started her PhD project at the department of Human Biology of the Faculty of Health Sciences at Maastricht University. From 2001 to 2005 she performed the studies, which are described in this thesis. A dietary intervention study with healthy subjects was conducted to investigate the effects of stearic, oleic, and linoleic acids on risk markers for cardiovascular diseases. The effects of these fatty acids were also investigated in cell culture studies. Furthermore, effects of CLA isomers on desaturation and elongation of fatty acids were studied. Since June 2005 she is employed as clinical chemist trainee at the Ziekenhuisgroep Twente in Almelo/Hengelo.

Myriam Anna Maria Anthonia Thijssen werd geboren op 10 september 1976 in Beers. Na het behalen van haar VWO diploma aan het Elshofcollege in Nijmegen, startte ze in 1993 met haar studie tot medisch analist (afstudeerrichting klinische chemie) aan de Hogere Laboratorium Opleiding in Nijmegen. Als onderdeel van deze studie werd een klinische en onderzoeksstage gedaan in het laboratorium van het Maasziekenhuis in Boxmeer. Ze studeerde in juli 1997 af. Vervolgens studeerde ze van 1997 tot 2001 Biomedische Wetenschappen aan de Faculteit Geneeskunde van de Universiteit Leiden. Gedurende stageperiodes werkte ze mee aan verschillende onderzoeksprojecten bij de afdeling Immunohematologie en Bloedbank in het Leids Universitair Medisch Centrum (LUMC) onder begeleiding van prof.dr. M. Giphart en bij het laboratorium immunologie van de afdeling Kindergeneeskunde eveneens in het LUMC onder begeleiding van dr. M. van Tol en dr. M. Schilham. Het afsluitende onderzoeksproject werd uitgevoerd bij het Centraal Klinisch Chemisch Laboratorium van het Universitair Medisch Centrum St. Radboud in Nijmegen en was onder begeleiding van dr. D. Swinkels en dr. J. de Kok. Uiteindelijk studeerde ze in april 2001 af. Tegelijkertijd startte ze ook haar promotieonderzoek bij de capaciteitsgroep Humane Biologie van de Faculteit Gezondheidswetenschappen van de Universiteit Maastricht. Van 2001 tot 2005 werden de studies uitgevoerd die in dit proefschrift zijn beschreven. Een voedingsinterventiestudie in gezonde proefpersonen werd gedaan om de effecten van stearinezuur, oliezuur en linolzuur te onderzoeken op risicomerkers voor hart- en vaatziekten. De effecten van deze vetzuren werden ook onderzocht in celkweekstudies. Verder werden de effecten van CLA isomeren op desaturatie en elongatie van vetzuren onderzocht. Sinds juni 2005 is ze als klinisch chemicus in opleiding bij de Ziekenhuisgroep Twente te Almelo/Hengelo.

List of publications

LIST OF PUBLICATIONS

Full papers

Thijssen MA, Swinkels DW, Ruers TJ and de Kok JB. Difference between free circulating plasma and serum DNA in patients with colorectal liver metastases. *Anticancer Research* 2002; 22: 421-5.

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