DIETARY FATTY ACIDS AND RISK FACTORS FOR CORONARY HEART DISEASE: CONTROLLED STUDIES IN HEALTHY VOLUNTEERS

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Stellingen

1. Enkelvoudig onverzadigde *trans*-vetzuren hebben vergeleken met *cis*-onverzadigde vetzuren een ongunstige uitwerking op de serumlipoproteïnen.
   Dit proefschrift

2. De vetzuursamenstelling van voedingsvet heeft geen grote invloed op de bloeddruk.
   Dit proefschrift; proefschrift Ronald Mensink, Landbouwuniversiteit, 1990

3. *Trans*-vetzuren zijn een van de weinige voedingsdeterminanten van het lipoproteïne(a)-gehalte in het bloed.
   Dit proefschrift

4. Of het gebruik van stearinezuur als alternatief voor *trans*-vetzuren bij het harder maken van vetten gezondheidsvoordelen oplevert, is nog onvoldoende onderzocht.
   Dit proefschrift

5. Doordat myristinezuur ten opzichte van palmitinezuur zowel het HDL als het LDL cholesterolgehalte verhoogt, is het niet duidelijk welk van deze vetzuren het meeste risico voor coronaire hartziekte met zich meebrengt.
   Dit proefschrift

6. Het veranderen van de positie van een vetzuur binnen het triglyceridenmolecuul maakt weinig verschil uit voor de uitwerking van voedingsvetten op de bloedlipiden bij de mens.
   Dit proefschrift

7. Ondanks het algemeen heersende idee dat het drinken van veel koffie ongezond is, is consumptie van papier-gefilterde koffie onschadelijk.

8. Het is een wijdverbreide misvatting dat Darwins evolutietheorie gaat over een gericht proces dat leidt tot grotere complexiteit en uiteindelijk tot zelfbewuste intelligentie.
   Stephen J Gould, Interview door Wim Kayzer, VPRO TV, 1993

9. Het aanprijzen en verkopen van zogenaamde 'natuurprodukten' met behulp van ongegronde gezondheidsclaims en het verzwijgen van mogelijke bijwerkingen, zoals dat door sommige zaken voor alternatieve voeding wordt gedaan, is een zuivere vorm van kwakzalverij.

10. *P*-waardes worden vaak misbruikt om het denkproces bij het accepteren of verwerpen van hypotheses over te slaan.

11. Succes bij het volgen van een hogere opleiding wordt slechts in geringe mate door het IQ (datgene wat een intelligentietest meet) voorspeld.

12. Boven de 80 jaar is een matig verhoogd serumcholesterolgehalte eerder een teken van goede gezondheid dan een risico-indicator voor coronaire hartziekte.
Bernard Forette et al, Lancet 1989; i:868-870.

13. De hoeveelheid onzin die commentatoren bij het verslaan van sportwedstrijden uitkramen, is omgekeerd evenredig met hun taalvaardigheid.

14. De voedselschaarste die tot ondervoeding en hoge kindersterfte in sommige delen van de derde wereld leidt, is meestal een direct gevolg van oorlog.

15. Het feit dat mensen door te eten dagelijks ongeveer 600 m^2 darmepitheel aan 1,5 kg produkten van agrarische oorsprong blootstellen, onderstreept het belang van de Vakgroep Humane Voeding binnen de Faculteit der Landbouw- en Milieuwetenschappen.

Stellingen behorend bij het proefschrift

Dietary fatty acids and risk factors for coronary heart disease: controlled studies in healthy volunteers

Peter L. Zock
Wageningen, 11 januari 1995

Aan mijn ouders

The unique design of these studies was based on the belief that nutritional experiments can be carried out as precisely in man as in animals.

Edward H. Ahrens, Jr., et al. *The Lancet* 1957; **i:** p.6976.

Abstract

Dietary fatty acids and risk factors for coronary heart disease: controlled studies in healthy volunteers

PhD Thesis by Peter L. Zock, Department of Human Nutrition, Wageningen Agricultural University, Wageningen, The Netherlands. January 11, 1995.

High levels of LDL cholesterol, blood pressure and Lp(a), and low levels of HDL cholesterol increase the risk for coronary heart disease (CHD). This thesis describes the effects of dietary fatty acids on these risk factors. In each of three trials we fed diets with tailored fatty acid composition to about 60 healthy men and women. Each diet within a trial was supplied to every volunteer for 3 weeks.

In the ***first study*** we compared the effects of monounsaturated *trans* fatty acids with those of linoleic acid, the fatty acid from which *trans* fatty acids are formed upon partial hydrogenation, and with those of stearic acid, a product of complete hydrogenation of linoleic acid. Relative to linoleic acid, both *trans* fatty acids and stearic acid raised LDL and lowered HDL cholesterol. Thus, partial as well as complete hydrogenation of linoleic acid produces fatty acids that unfavorably affect serum lipids relative to linoleic acid itself. *Trans* fatty acids and stearic acid did not influence blood pressure, but *trans* fatty acids modestly raised Lp(a).

The ***second study*** addressed the relative cholesterol-raising potentials of two specific saturates, myristic and palmitic acid. Relative to oleic acid, myristic acid was about 1.5 times as cholesterol-raising as palmitic acid, due to increases in both LDL and HDL cholesterol. The differences between myristic and palmitic acid were statistically significant. However, both saturates caused high LDL cholesterol levels and raise the LDL to HDL cholesterol ratio.

In the ***third study*** we examined the effect of the positional distribution of fatty acids within dietary triglycerides. Two diets had identical total fatty acid composition, but a major contrast in fatty acid configuration. Total, LDL, and HDL cholesterol levels were the same on both diets. The position of the dietary fatty acids was partly reflected in fasting plasma lipids, but the fatty acid configuration had no important effect on lipoprotein levels.

In conclusion, monounsaturated *trans* fatty acids and the saturates myristic and palmitic acid have adverse effects on the serum lipoprotein risk profile for CHD. People at high risk for CHD should replace the hard fats in their diets by carbohydrates or unsaturated oils.

Contents

CHAPTER 1

General introduction

Background and scope

Coronary heart disease (CHD) is a major health problem in Western societies. In 1992, about 85,000 people in the Netherlands were hospitalized because of the disease, and it was the primary cause of more than 20,000 deaths [1]. The predominant clinical symptoms of CHD are angina, myocardial infarction, and sudden cardiac death [2]. The disease is characterized by an insufficient blood supply to the heart muscle. Its main cause is atherosclerosis, a combination of changes in the endothelium and smooth muscle of coronary arteries involving accumulation of lipids, fibrous lesions and ultimately impaired blood flow [3]. Several risk factors for CHD have been defined, including high blood cholesterol levels and blood pressure, smoking, obesity, diabetes and physical inactivity. The studies described in this thesis focus primarily on the influence of dietary fatty acids on the serum cholesterol concentration and its distribution over low density (LDL) and high density lipoproteins (HDL) in healthy men and women. In addition, effects of certain fatty acids on blood pressure and on the plasma lipoprotein(a) concentration, another risk factor for CHD, are addressed.

This introduction gives a brief overview of epidemiologic studies on the relation of serum lipoprotein cholesterol, lipoprotein(a), and blood pressure with CHD, and the involvement of diet. In addition, the possible adverse effects of low or lowered cholesterol levels are discussed. The rationale of the studies and an outline of the thesis are given at the end of this chapter.

Serum total or LDL cholesterol and coronary heart disease

A large body of epidemiologic data indicates a direct relation between the level of total or LDL cholesterol and the incidence of CHD. The evidence includes within-population studies [4-7] as well as cross-cultural comparisons [8,9]. Cohort studies within populations consistently show an association between blood cholesterol levels and CHD rates, this association is continuous throughout the entire range of cholesterol levels in the population [4]. An analysis of surveys which measured cholesterol levels in 17 different countries indicates that the variation in serum total and LDL cholesterol may explain four-fifths of the cross-cultural variation in CHD mortality [9]. Also, differences between populations in saturated fat and cholesterol intake are related with differences in CHD mortality rates [10,11]. It

should be mentioned, however, that the strength of associations observed in cross-cultural studies depends on the populations included in the analysis, e.g [12], and that comparisons can easily be confounded by other life-style factors that accompany differences in fat consumption. Nevertheless, the reductions in cholesterol that have been achieved in the past years in some Western countries, mainly through changes in the diet, are associated with a decrease in CHD mortality [9].

The strongest evidence for a link between total or LDL cholesterol levels and CHD comes from randomized clinical trials. Such studies have shown that lowering cholesterol levels can reduce CHD incidence in persons without evidence of prior CHD (primary prevention trials) [13,14]. Trials in patients with angiographically defined symptoms (secondary prevention trials) have revealed that cholesterol lowering by diet [15-20] or drug therapy [21-23] can slow progression of atherosclerosis in both men and women and that even regression of the atherosclerotic process may be accomplished. Meta-analyses that pool the results of prevention trials also demonstrate that dietary therapy alone is effective in reducing CHD rates [24-29].

Clinical trials of relatively short-term duration indicate that each 1% reduction in the total cholesterol level results in about a 2% reduction in CHD incidence [25,29,30]. Observational studies suggest that the reduction in CHD risk with long-term cholesterol lowering may be substantially greater [29,31], especially when underestimation of the association due to individual variability in cholesterol levels (regression dilution bias) is taken into account [32]. In summary, high LDL or total cholesterol levels have proven to be powerful risk factors for CHD.

Low cholesterol levels and total mortality

The evidence that cholesterol lowering prevents CHD is the corner stone of the public health policy to controlling high blood cholesterol. However, doubts have been voiced as to whether cholesterol lowering yields benefit in terms of reduced total mortality, and it has been proposed that low or lowered cholesterol levels may be a significant risk factor for non-CHD mortality [26,33-36]. Different types of epidemiologic studies provide evidence on the total mortality issue.

Observational studies

Ecological comparisons show that populations with lower cholesterol levels generally have lower age-adjusted mortality, mainly the results of less CHD, than populations with high cholesterol levels [9,10,37]. These population studies are suggestive, but confounding due to certain differences in life-style cannot be excluded.

Other observational information is derived from within-population cohort studies. In the 30-year follow-up of the Framingham Heart Study [38], young persons with the lowest serum cholesterol concentrations at entry were found to have lower total mortality rates at midlife than those with high initial concentrations. Similar findings were reported for the men in the Johns Hopkins Precursors Study [39], who were followed for 30.5 years. Jacobs et al. [40] recently reviewed a large number of cohort studies. They reported that men in the range of 4.14 to 5.15 mmol/L had a lower total mortality rate than men with higher cholesterol levels. However, the relation between cholesterol level and total mortality appeared to be J-shaped; higher total mortality occurred with elevated levels of cholesterol, but was also noted in men with very low total cholesterol levels [40]. The Multiple Risk Factor Intervention Trial (MRFIT) constituted two thirds of the total number of men in this review. A more detailed analysis of the large MRFIT follow-up study revealed that there was no trend for an increase in total mortality until the cholesterol concentration fell below 3.62 mmol/L, and a significant increase was noted only in the very small portion of men with levels lower than 3.10 mmol/L [41]. The pooled cohort data in the review by Jacobs and others [40] showed a statistical significant increase in total deaths at very low cholesterol levels for a variety of conditions, including some cancers (e.g. lung but not colon), respiratory disease, liver disease, hemorrhagic stroke, and trauma. It is conceivable is that some of these disorders lower serum cholesterol; in that case a low level at entry may be only a marker of increased risk and not the cause of excess mortality. A systematic review of the 10 largest cohort studies also reports increased mortality from various causes at low cholesterol levels [42]. Only hemorrhagic stroke was significantly associated with low cholesterol, however, increased hemorrhagic risk was clearly outweighed by a lower CHD mortality. Moreover, a higher non-CHD death rate at low cholesterol levels was limited to community cohorts; the association was absent in the cohorts of employed men. The chance of including subjects with pre-existing disease is higher in community cohorts than in cohorts of employed, generally healthy persons. This supports that

underlying illness may decrease serum cholesterol levels and confound the relation between low cholesterol and excess mortality [42]. Low cholesterol levels could also be associated with other characteristics that place people at a higher risk, such as heavy alcohol use or socioeconomic status [7,43].

Clinical trials

Randomized prevention trials have been designed to detect effects of cholesterol lowering on CHD incidence or recurrence, and because of their relatively small size and duration they are of limited value in assessing non-CHD or total mortality [44]. In addition, the treated subjects in the secondary prevention trials do not have low enough cholesterol levels to confirm or refute the excess non-CHD deaths at very low levels. Nevertheless, several meta-analysis of cholesterol lowering trials [26,33,35] show an increase in combined non-CHD deaths in the treated subjects, comprising small, non-significant increases in mortality from a variety of diseases. The diverse causes of death make it difficult to postulate a biological mechanism by which cholesterol lowering in itself produces specific adverse effects that increase risk. The lack of a plausible mechanism weakens the notion that low cholesterol levels may cause higher total mortality.

Meta-analyses of prevention trials have found increased non-CHD mortality in studies using drugs but not in those using dietary therapy [26,27,42]. The exact nature of any adverse side-effects of drugs and, if they are real, whether they are confined to one class of drugs or extend to all cholesterol lowering drugs remains to be established. A recent review of non-drug prevention trials shows that dietary interventions, with or without additional advice to quit smoking and/or to increase physical exercise, reduce CHD risk as well as total death rates [28]. Thus, there is no indication that lowering cholesterol by diet increases non-CHD or total mortality.

Conclusion

At present, the evidence that blood cholesterol lowering reduces mortality and morbidity is much more consistent and reliable than the evidence that certain cholesterol lowering drugs may increase non-CHD death rate, which may be an association based on confounding. Even if total mortality accompanying cholesterol reduction in is unchanged, substantial benefit will still be gained by a reduction in CHD morbidity. In any case, the available evidence suggests that dietary treatment of elevated total and LDL cholesterol levels is totally safe.

Other risk factors for coronary heart disease

HDL cholesterol and triglycerides

Studies in high-risk populations consistently show that low HDL cholesterol levels are associated with an increased CHD risk, independent of LDL cholesterol and other risk factors [31,45]. Data from the largest prospective studies in the United States indicate that for every 0.026 mmol/L (1 mg/dL) decrease in HDL cholesterol, the risk for CHD is increased by 2% to 3% [46,47], after adjustment for other risk factors. Similar results were found for men and women. None of the large-scale primary and secondary prevention trials was designed specifically to test the hypothesis that raising HDL cholesterol concentrations reduces risk. Thus it may be difficult to discriminate the relative contributions of LDL lowering and HDL raising to CHD risk reduction. Nevertheless, several trials show an independent contribution of increased HDL cholesterol to the overall reduction in CHD incidence [22,47,48]. Also, people with genetically deficient HDL have a high risk of premature CHD, in spite of low LDL cholesterol levels [49]. Taken together, the available information on HDL cholesterol and CHD provides considerable support for a causal relationship. However, to date there is no definitive evidence that manipulation of HDL levels modifies susceptibility for CHD. Although plausible, the beneficial effect of raising HDL by diet remains to be established.

Observational studies show that people with high fasting triglyceride levels have an enhanced CHD-risk [31,50]. However, high triglyceride concentrations go together with other risk factors such as obesity, diabetes, low HDL levels, and lack of physical activity. In multivariate analyses of cohort studies, triglycerides often lose their ability to predict CHD when these factors are taken into account [50,51]. Therefore, it is unclear whether a high level of triglycerides causes CHD by itself or serves mainly as an indicator of other risk factors, such as low HDL cholesterol levels. Some investigators suggest that postprandial rather than fasting triglycerides may be atherogenic [52,53], but the evidence on this point is still limited.

Lipoprotein(a)

Lipoprotein(a) (Lp(a)) is a plasma lipoprotein whose structure and composition closely resembles that of LDL, but with an additional molecule of apolipoprotein(a), a large glycoprotein showing sequence homology to plasminogen [54]. Levels of Lp(a) in the blood are largely genetically determined [54,55]. The interest in this

lipoprotein has recently been increased because of accumulating evidence that elevated Lp(a) concentrations are an independent risk factor for CHD, e.g. [56-60]. However, most of the studies are based on cross-sectional observations. The few prospective studies that have been published show either a higher risk of CHD in subjects with high Lp(a) levels [58,61] or no association [62-64]. These latter studies do not negate a role of Lp(a) in atherogenesis, but it is obvious that more large prospective studies are needed to confirm that high Lp(a) levels are causally related to CHD. Despite structural resemblance, the influence of diet on Lp(a) concentrations seems different from that on LDL cholesterol. Unlike LDL, Lp(a) levels are generally insensitive to dietary changes [60]. However, relatively little study on dietary determinants of Lp(a) have been done.

Blood pressure

High blood pressure is an established risk factor for CHD [65]; the higher the blood pressure, the greater the risk for CHD [66-68]. In several trials of relatively short-term duration, treatment of hypertension could not be shown to reduce CHD [69]. A recent overview of randomized trials, however, shows that lowering blood pressure significantly reduces the CHD incidence [70]. Thus, the relation between blood pressure and CHD appears to be causal. The majority of epidemiologic studies used diastolic blood pressure to characterize the blood pressure of an individual, irrespective of other parameters, such as systolic pressure. Therefore, diastolic rather than systolic blood pressure is generally regarded as an independent risk factor of CHD. In both observational studies and trials, however, differences between groups in diastolic pressure are much the same as differences in systolic pressure [68,70]. Thus, it is conceivable that high levels of both systolic and diastolic blood pressure are indicators of increased CHD risk. Several [71-74], but not all [75-78] studies on dietary fat and blood pressure suggest that the quantity and quality of fat consumption may modulate systolic and diastolic blood pressure.

Dietary fat and serum lipoprotein cholesterol

A host of metabolic studies conducted in the past 35 years shows that total and lipoprotein cholesterol levels can be influenced by fat intake [79-81]. Dietary fat largely consists of a mixture of triglycerides. The constituent fatty acids are usually classified according to their number of double bonds. We distinguish

saturated (zero double bonds), monounsaturated (one double bond), and polyunsaturated fatty acids (more than one double bond). The main monounsaturate in the diet is oleic acid (*cis*-C18:1n-9) and the main polyunsaturate is linoleic acid (*cis,cis*-C18:2n-6). A recent meta-analysis of 27 well-controlled dietary trials summarizes the effect of the 3 classes of fatty acids on serum lipoprotein cholesterol [81]. Replacement of carbohydrates by fat raises HDL cholesterol; saturated fatty acids being the most potent and linoleic acid the least. Increasing dietary fat intake at the expense of carbohydrates also lowers the fasting triglyceride levels; saturates, oleic acid, and linoleic acid all produce this effect to about the same extent. Effects on LDL cholesterol are markedly different, with saturated fatty acids strongly raising LDL, and linoleic acid modestly lowering it. Oleic acid also has a slight LDL cholesterol lowering effect relative to carbohydrates. The influence of the different fatty acids on total cholesterol largely resemble those on LDL [81]. Thus, changing the fatty acid composition of the diet provides a useful tool to favorably modify the lipoprotein risk profile for CHD.

Rationale of the studies and outline of the thesis

Dietary fatty acids may differ from each other in three aspects other than the number of double bonds, namely, 1. the configuration of the double bonds (*cis* or *trans*), 2. the number of carbon atoms (chain length), and 3. the position of the fatty acid on the glycerol molecule (triglyceride configuration). The present thesis addresses the influence of these aspects on serum lipoprotein cholesterol and other CHD risk factors. The effects of various fatty acids were investigated by means of strictly controlled trials, in which we supplied conventional solid food diets with specific fatty acid compositions to large groups of healthy men and women. Our studies focussed on the most common fatty acids in human diets. The medium chain fatty acids (C6:0, C8:0, and C10:0) and the n-3 polyunsaturated fatty acids (C18:3n-3, C20:5n-3, and C22:6n-3) are beyond the scope of this thesis.

1. Configuration of double bonds: monounsaturated trans fatty acids

Most natural fats and oils have their double bonds in the *cis* configuration. Monounsaturated *trans* fatty acids (positional isomers of *trans*-C18:1) are formed during hydrogenation of linoleic acid, either by bacteria in ruminant animals or by processing in oil-hardening factories. High-linoleic-acid vegetable oils are partially

hydrogenated by the edible oil industry to produce solid and stable fats. Certain types of margarine and shortenings are especially high in *trans* fatty acids. Interest in the health effects of these fatty acids has been increased since Mensink and Katan [82] reported that monounsaturated *trans* fatty acids raise LDL and lower HDL cholesterol relative to their *cis* isomer oleic acid. It has been proposed that stearic acid (C18:0), a saturate known to be 'neutral' in its effect on lipoprotein levels, might offer a favorable alternative to *trans* fatty acids for the production of solid and semi-solid fats.

In the first study we compared monounsaturated *trans* fatty acids with linoleic acid, the parent fatty acid from which *trans* fatty acids are formed upon partial hydrogenation, and with stearic acid, a product of complete hydrogenation of linoleic acid. ***Chapter 2*** addresses the effects on HDL and LDL cholesterol, and ***Chapter 3*** deals with systolic and diastolic blood pressure. The data on Lp(a) were combined with those from two previous dietary trials conducted at our department and are described in ***Chapter 4***.

2. Chain length: specific saturated fatty acids

It is common knowledge that saturated fatty acids with a chain length of 12 to 16 carbon atoms powerfully raise total and LDL cholesterol. Palmitic acid (C16:0) is by far the most abundant saturate in our diet. It occurs in all kinds of animal and in most vegetable fats. Palm oil, an important raw material for the edible oil industry, has a very high palmitic acid content. A major source of myristic acid (C14:0) in Western diets is dairy fat, which is also rich in palmitic acid. Certain tropical oils, such as coconut oil and palm kernel oil, are rich in both myristic acid and lauric acid (C12:0). Little information is available on the relative cholesterolemic effects of these specific saturates. Meta-analyses [80,81] indicate that myristic acid might be much more potent than palmitic acid. It has been suggested that myristic acid could be largely responsible for the cholesterol-raising effect of C12-C16 saturates, and that palmitic acid may be neutral [83-85]. However, other studies clearly show that palmitic acid has a cholesterol-raising potential [86-90].

The second study, reported in ***Chapter 5***, addresses the issue of myristic versus palmitic acid. In order to quantify their relative potencies, we compared the effects of myristic and palmitic acid on LDL and HDL cholesterol with each other and with those of oleic acid.

3. Triglyceride configuration: positional distribution of fatty acids

Natural dietary fats and oils differ in the distribution of their constituent fatty acid over the three possible attachment sites of the triglyceride molecule. For example, palmitic acid in lard is mainly esterified to the *sn*-2 position of glycerol, whereas palm oil contains its palmitic acid predominantly in the *sn*-1 and *sn*-3 position. In addition, the fatty acid configuration of food fats can be altered to produce confectionary and other fats with better texture or certain desired physical properties. Animal experiments have suggested that changes in the fatty acid distribution can alter their atherogenicity and cholesterolemic effects [91-93]. McGandy and co-workers [94] speculated that the stereospecific position of a fatty acid might modulate its effect on cholesterol levels in humans. The influence of triglyceride structure on lipid metabolism has been extensively studied in animal models (see for review [95]), but at present few human data are available.

In the third study we compared two dietary fats with equal amounts of individual fatty acids but different positional distribution. ***Chapter 6*** describes the effects on lipoprotein cholesterol levels and in ***Chapter 7*** we report the influence of triglyceride structure on the fatty acid composition and configuration of fasting plasma lipids.

Finally, ***Chapter 8*** summarizes the main results. Our findings are put into perspective with studies that have been reported since some chapters of this thesis were published. Furthermore, methodologic aspects of our diet studies are reviewed. Chapter 8 ends with a concluding paragraph.

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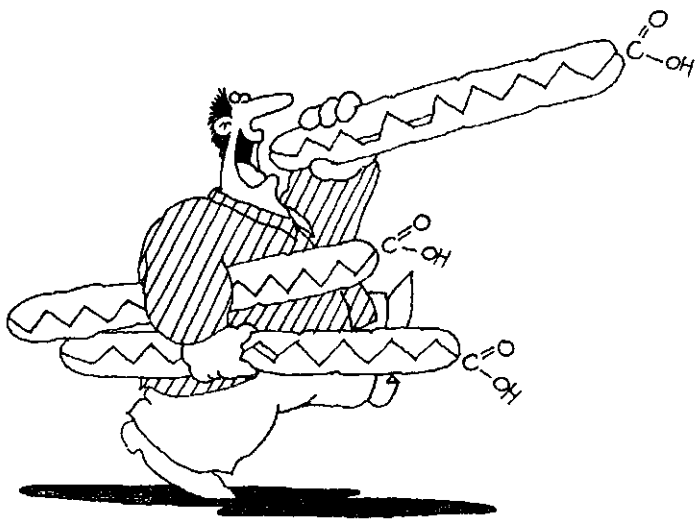
CHAPTER 2

Hydrogenation alternatives: effects of *trans* fatty acids and stearic acid versus linoleic acid on serum lipids and lipoproteins in humans



Zock PL, Katan MB.
Journal of Lipid Research 1992; **33:** 399-410.

ABSTRACT

Aim. The objective of this study was to compare the effects of linoleic acid (*cis,cis*-C18:2n-6,n-9) and its hydrogenation products elaidic (*trans*-C18:1n-9) and stearic acid (C18:0) on serum lipoprotein levels in man.

Methods. Twenty-six men and 30 women, all normolipemic and apparently healthy, completed the trial. Three experimental diets were supplied to every subject for three weeks each, in random order (multiple cross-over). The Linoleate-diet provided 12.0% of total energy intake as linoleic acid, 2.8% as stearic acid, and 0.1% as *trans* fatty acids. The Stearate-diet supplied 3.9 energy % as linoleic acid, 11.8% stearic acid, and 0.3% *trans* fatty acids. The *Trans*-diet provided 3.8 energy % as linoleic acid, 3.0% stearic acid, and 7.7% as monounsaturated *trans* fatty acids, largely elaidic acid (*trans*-C18:1n-9). Other nutrients were constant. Fasting blood was sampled at the end of each dietary period.

Results. Mean (±SD) serum LDL cholesterol was 109 ± 24 mg/dL (2.83 ± 0.63 mmol/L) on the Linoleate-diet. It rose to 116 ± 27 mg/dL (3.00 ± 0.71 mmol/L) on the Stearate-diet (change, 7 mg/dL or 0.17 mmol/L, $P=0.0008$) and to 119 ± 25 mg/dL (3.07 ± 0.65 mmol/L) on the *Trans*-diet (change, 9 mg/dL or 0.24 mmol/L, $P<0.0001$). HDL cholesterol decreased by 2 mg/dL (0.06 mmol/L, $P<0.0001$) on the Stearate-diet and by 4 mg/dL (0.10 mmol/L, $P<0.0001$) on the *Trans*-diet, both relative to linoleic acid.

Conclusion. Our findings show that 7.7% of energy (mean, 24 g/day) of *trans* fatty acids in the diet significantly lowered HDL cholesterol and raised LDL cholesterol relative to linoleic acid. Combination with earlier results (Mensink RP, Katan MB. *N Engl J Med* 1990; **323:** 439-445.) suggests a linear dose-response relation. Replacement of linoleic acid by stearic acid also caused somewhat lower HDL cholesterol and higher LDL cholesterol levels. Hydrogenation of linoleic acid to either stearic or *trans* fatty acids produces fatty acids that may increase LDL and decrease HDL cholesterol relative to linoleic acid itself.

INTRODUCTION

Monounsaturated *trans* fatty acids with a length of 18 carbon atoms are formed when vegetable oils, rich in linoleic acid, are partially hydrogenated so as to produce fats with better texture and stability [1]. Although the intake of these *trans* fatty acids, mainly elaidic acid and its isomers (**Fig. 1**), is much lower than that of saturated fatty acids, it is still considerable in industrialized countries. Their estimated average consumption is 3 to 4% of daily energy intake in the United States [2].

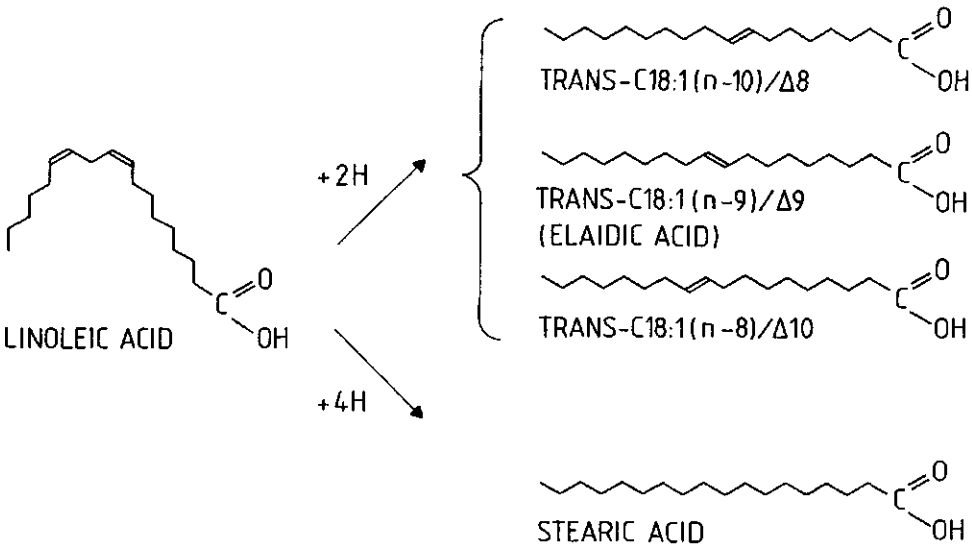


Fig 1. Structure of linoleic acid and of some products of hydrogenation. Partially hydrogenated soybean oil usually contains a spectrum of positional *trans*-C18:1 isomers with C18:1n-8 (Δ10) being the most abundant [3].

Early studies on the effects of *trans* fatty acids on blood lipids produced conflicting results. Some studies [4-6] suggested that *trans* fatty acids, as compared to their *cis* isomer oleic acid, elevate serum total cholesterol levels, while others [7-9] could not confirm this. In a previous study conducted at our

Department it was found that *trans* monounsaturated fatty acids were hypercholesterolemic as compared with oleic acid, and that their effect was about half of that of a mixture of saturated fatty acids (C12:0, C14:0, and C16:0) [10]. In this study *trans* fatty acids not only raised the serum level of atherogenic low density lipoprotein (LDL) cholesterol, but also lowered high density lipoprotein (HDL) cholesterol [10]. However, the amount of *trans* fatty acids given was quite high (11% of daily energy intake), and doubts have been voiced as to whether these findings could be extrapolated to lower levels of intake [11].

A product of further hydrogenation of linoleic acid (Fig. 1) is stearic acid, the completely saturated analogue of elaidic acid. Stearic acid has been found not to raise the serum cholesterol level relative to carbohydrates and to exert effects on serum lipids similar to those of oleic acid [12-15]. Like *trans* fatty acids and other saturated fatty acids, stearic acid is a rigid molecule whose presence adds firmness to foods. Although stearic acid has a higher melting point than *trans* fatty acids or palmitic acid (C16:0) and has a waxy nature, a limited increase in the stearic acid content at the expense of other saturated fatty acids or *trans* fatty acids has been suggested as a way for the edible-fats industry to produce semi-solid and solid fats with less of a cholesterol-raising potential [16].

Therefore we compared the effects of *trans* fatty acids on serum lipoprotein and apolipoprotein levels in healthy men and women with those of stearic acid and of linoleic acid, the parent fatty acid from which *trans* fatty acids and stearic acid are derived during the hydrogenation of soybean and other linoleic acid-rich oils.

SUBJECTS AND METHODS

Subjects

Out of 110 persons who responded to calls via local newspapers 45 women and 42 men were randomly selected for a screening procedure. Two women and one man were excluded for medical reasons and two men and two women withdrew during the screening. The remaining 80 volunteers, 39 men and 41 women, had no history of atherosclerotic disease, and all were apparently healthy, as indicated by a medical questionnaire. None had anemia, glycosuria, proteinuria, or hypertension, and none were taking medications known to affect blood lipids. Because only 62 subjects could be enrolled in the trial, 31 men and 31 women

were randomly selected to take part. Three men withdrew before the study began. The protocol and aims of the study were fully explained to the subjects, who gave their written consent. No reward was given, except for the free food in the study diets. Approval for the study had been obtained from the Ethics Committee of the Department. During the study two men withdrew, one because of personal reasons and one because of illness. One woman withdrew because of pregnancy. All analyses are based on the 26 men and 30 women who completed the trial. Before the beginning of the study diets, the subjects' fasting serum lipid levels ranged from 3.31 to 6.66 mmol/L for total cholesterol (mean, 4.84 mmol/L or 187.2 mg/dL), from 0.87 to 2.04 mmol/L for HDL cholesterol (mean, 1.38 mmol/L or 53.4 mg/dL), and from 0.39 to 1.58 mmol/L (mean, 0.94 mmol/L or 83.2 mg/dL) for triglycerides. The men's age ranged from 19 to 48 years (mean, 25 years), and they weighed between 60 and 84 kg (mean, 73 kg). Their body mass index ranged from 18.5 to 26.0 kg/m^2 (mean, 21.5 kg/m^2). The women were between 18 and 49 years old (mean, 24 years), and weighed between 51 and 80 kg (mean, 63 kg); their body mass index ranged from 17.9 to 27.5 kg/m^2 (mean, 21.5 kg/m^2). Thirteen women used oral contraceptives, and 4 men and 4 women smoked.

Design and statistical analyses.

The trial ran from January 29 until April 2, 1990. The study had a multiple crossover design and consisted of three consecutive periods. Each subject followed the three study diets for three weeks each. Our experience agrees with that of earlier workers [12,17] in that serum lipid and lipoprotein levels stabilize within two weeks after a dietary change [18,19]. One diet was high in linoleic acid (Linoleate-diet), one was high in stearic acid (Stearate-diet), and the third was high in elaidic acid (*Trans*-diet). Before the trial, the participants were categorized according to sex. Male and female subjects were then divided into six groups so that each group had a nearly identical number of subjects from both sexes. Women who used oral contraceptives were equally distributed over the six treatment sequences. Each group received the diets in different order (**Fig. 2**). In this way, variation due to residual effects of the previous diet or to drift of variables over time were eliminated [20]. We attempted to blind the participants as to the sequence of their diets, but at the end of the trial it turned out that all subjects had recognized the linoleic acid-rich diet, and most of them had also correctly identified

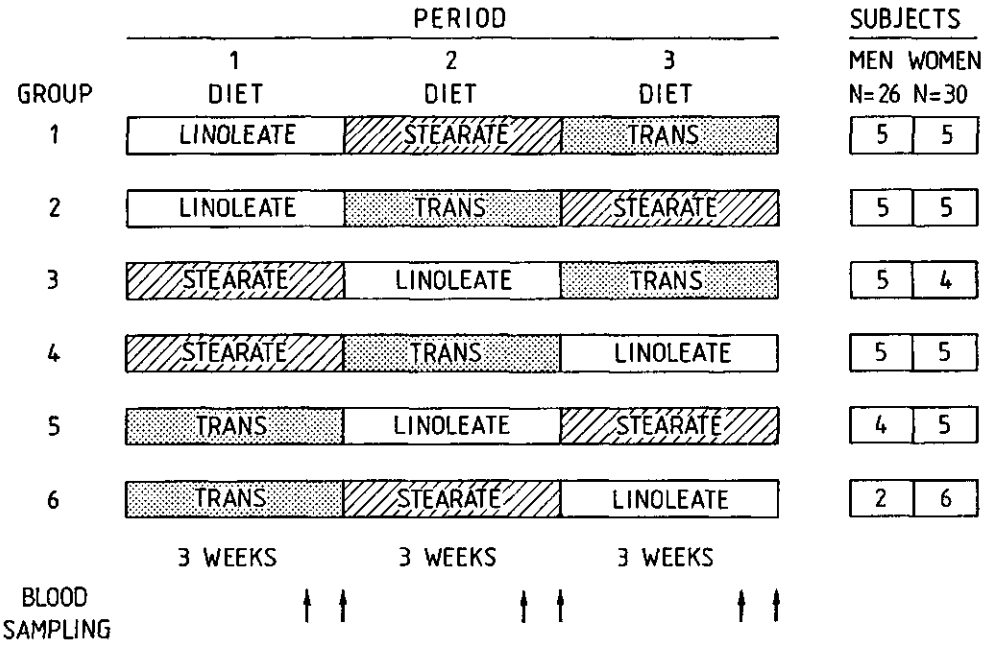


Fig 2. Experimental design.

the *trans* and stearic acid-rich diets. However, it is highly unlikely that awareness of the nature of the treatment could have affected the outcome of the study.

The data were analyzed with the General Linear Models (GLM) of the Statistical Analyses System [21]. When the analyses indicated a significant effect of diet ($P<0.05$), the Tukey method was used for pairwise comparisons of the diets and for calculation of 95 % confidence limits for the differences between two diets [21]. This method encompasses a downward adjustment of significance limits for multiple testing and, as a result, only *P*-values of less than 0.02 were considered significant.

Diets

Before the trial started, the participants recorded their habitual diet for two working days and one weekend day to allow us to estimate their energy and nutrient intake. The food records were coded and the nutrient composition and

energy content of the diets were calculated with use of the Netherlands Nutrient Data Base [22]. The study diets consisted of conventional solid foods, and menus were changed daily during each 3-week cycle. The planned nutrient content of the three diets was similar, except for 8% of total energy, which was provided by either linoleic acid, stearic acid, or elaidic acid. The linoleate group received a commercially available margarine high in linoleic acid (Becel, Van den Bergh foods, Rotterdam, The Netherlands). In addition, high-linoleic acid sunflower oil was used in the preparation of various dishes. For the *Trans*-diet, high-oleic acid sunflower oil (Trisun, SVO Enterprises, Wickliffe, OH) was hydrogenated with a sulferized nickel catalyst (Pricat 9908, Unichema, Emmerich, Germany) under conditions that favored formation of *trans* rather than saturated fatty acids. This hydrogenation procedure was largely similar to that applied for our previous study [10,23]. We used this procedure rather than hydrogenation of a linoleic acid-rich oil so as to avoid the formation of *trans*-isomers of C18:2 and to maximize the yield of *trans*-C18:1. Seventy-five parts of the hydrogenated fat were mixed with 25 parts of the unaltered oleic acid-rich oil, and used to produce a margarine and a shortening high in elaidic acid. Fats for the Stearate-diet were produced by interesterification of a mixture of 41 parts of fully hydrogenated high-linoleic acid sunflower oil (Chempri, Raamsdonksveer, The Netherlands), 50 parts of high-oleic acid sunflower oil, and 9 parts of unmodified high-linoleic acid sunflower oil.

Thus, the basic source of all fatty acids in which the three diets differed was sunflower oil. The special margarine and shortenings were developed and manufactured by the Unilever Research Laboratory (Vlaardingen, The Netherlands). **Table 1** gives the fatty acid composition of margarine and oil used in the three diets. Positional isomers of monounsaturated C18:1-fatty acids were identified by gas-liquid chromatography using a capillary Sil-88 column.

The diets were formulated at 24 levels of energy intake ranging from 5.5 to 17.5 MJ (1315 to 4185 kcal) per day. All foodstuffs were weighed out for each individual subject. On week days at noon, hot meals were served and eaten at the department. All other food was supplied daily as a package. Food for the weekend and guidelines for its preparation were provided on Fridays. In addition to the food supplied, subjects were allowed a limited number of items free from fat and cholesterol. The energy intake from these free-choice items was fixed for each level of energy intake and ranged from 7 to 13% of total energy intake. Subjects were urged not to change their selection of free-choice items throughout the trial. They were asked to maintain their usual pattern of physical activity and not to

Table 1. Fatty acid composition of the margarine and oil used in the three study diets.

Fatty Acid	Linoleate-Diet	Stearate-Diet	*Trans*-Diet
		g per 100 g of fatty acid	
Saturated	18.1	52.9	12.1
Lauric acid (C12:0)	1.5	0.1	0.0
Myristic acid (C14:0)	0.6	0.1	0.0
Palmitic acid (C16:0)	7.6	5.6	3.6
Stearic acid (C16:0)	7.0	45.5	6.6
Monounsaturated	18.8	37.4	84.2
Cis-C18:1	18.4	37.0	43.6[a]
Trans-C18:1	0.1	0.1	40.2[b]
Polyunsaturated	62.5	9.5	2.1
Linoleic acid (*cis,cis*-C18:2)	61.9	9.4	1.9
Trans-C18:2	0.5	0.0	0.2
Others	0.5	0.2	1.6

Commercially available linoleic acid-rich margarine and regular sunflower oil provided 37% of the total fat content of the Linoleate-diet, high-stearic acid margarine and shortening provided 48% of the total fat content of the Stearate-diet, and high-elaidic acid margarine and shortening provided 44% of the total fat content of the *Trans*-diet.

[a] Positional *cis* isomers: C18:1n-4, 0.1 g; C18:1n-5, 0.2 g; C18:1n-6, 0.4 g; C18:1n-7, 1.3 g; C18:1n-9, 38.5 g; C18:1n-12, 3.1 g.

[b] GLC showed three positional isomers, predominantly C18:1n-9 (Δ9) (elaidic acid) plus traces of C18:1n-8 (Δ10) and C18:1n-10 (Δ8).

change their smoking habits, consumption of coffee, or use of oral contraceptives. The participants recorded in diaries any sign of illness, medication used, the selection and use of free-choice items, the amount and type of coffee consumed, and any deviations from their diets. Inspection of the diaries did not reveal any deviations from the protocol which might have affected the results. Duplicate portions of each study diet were collected on each of the 63 trial days for an imaginary participant with a daily energy intake of 11 MJ (2630 Kcal), stored at −20 °C, and pooled and analyzed after the study. These analyzed values of the food supplied were combined with the values for the free-choice items (Table 2).

Body weights without shoes, jackets, and heavy sweaters were recorded twice weekly. The level of energy intake was adjusted when necessary, as indicated by weight changes. Over the 63 days of the trial average body weight fell by 0.2 ± 1.1 kg (range, −2.5 to 2.0 kg). The mean difference in body weight at the end of

the dietary periods was 0.1 ± 0.7 kg (range −1.7 to 1.4 kg) between the Linoleate-diet and the Stearate-diet, 0.0 ± 0.8 kg (range −2.1 to 1.8 kg) between the Linoleate-diet and the Trans-diet, and 0.1 ± 0.7 kg (range −1.8 to 2.4 kg) between the Trans-diet and the Stearate-diet.

Blood sampling and analyses

Before the trial each participant had been assigned a random number that was then used for labeling blood and serum tubes. In this way the laboratory technicians were kept unaware of the subjects' diet sequence. Blood samples were taken after a 12 hour fast on days 1, 18 and 21 (period 1), days 39 and 42 (period 2), and on days 60 and 63 (period 3). All venipunctures of each subject were performed by the same technician, in the same location, and at the same time of the same day of the week. Serum was obtained by low-speed centrifugation within 1 h of venepuncture, stored at −80 °C, and analyzed enzymatically for total and HDL cholesterol and triglycerides [24,25]. All samples from a particular subject were analyzed within the same run. The coefficient of variation within runs was 1.5% for total cholesterol, 1.6% for HDL cholesterol, and 2.9% for triglycerides. Mean bias with regard to the target values of 3 serum pools provided by the Centers for Disease Control (Atlanta, GA) [26] was 0.14 mmol/L for total cholesterol and 0.04 mmol/L for triglycerides. The mean bias with regard to target values of 4 serum pools obtained from the Solomon Park Research Laboratories (Kirkland, WA) was 0.09 mmol/L for HDL cholesterol. LDL cholesterol was calculated using the Friedewald equation [27]. Apolipoproteins were assayed by Dr. M. Sandkamp at the Institut für Klinische Chemie und Laboratoriumsmedizin, Münster, Germany (Head: Prof. Dr. G. Assmann), using a turbidimetric method on microtitre plates, as described [28]. All samples from the same proband were measured on the same day within one series. The coefficient of variation within the series was 3.5% for apolipoprotein A-I and 3.6% for apolipoprotein B. The two lipoprotein and apolipoprotein values obtained at the end of each dietary period were averaged for data analyses.

For each subject, the fatty acid composition of serum cholesterol esters was determined in samples obtained at the end of each dietary period (days 21, 42, and 63) as described earlier [29], with the following modifications: cholesteryl esters and triglycerides were separated with Bond Elute solid phase extraction columns

(Analytchem International, Harbor City, CA), the component fatty acids were methylated with 4% (v/v) H_2SO_4 in methanol, and a capillary Sil-88 column was used for gas chromatographic analysis. The results are expressed as a proportion by weight of all fatty acids detected.

Table 2. Mean daily intake of nutrients of subjects while on the high-linoleic acid diet, the high-stearic acid diet, and the high-*trans* fatty acid diet.

Energy/Nutrient	Linoleate-Diet	Stearate-Diet	*Trans*-Diet
Energy			
MJ/day	12.0 ± 2.8	11.0 ± 2.7	12.0 ± 2.7
kcal/day	2869 ± 670	2845 ± 646	2869 ± 646
Protein (% of energy)	12.3	12.3	12.8
Fat (% of energy)	41.1	43.5	39.7
Saturated fatty acids	11.0	20.1	3.0
Lauric acid (C12:0)	0.7	0.5	0.5
Myristic acid (C14:0)	0.9	1.0	1.0
Palmitic acid (C16:0)	5.8	5.7	4.8
Stearic acid (C16:0)	2.8	11.8	3.0
Monounsaturated fatty acids	15.8	16.6	23.3
Cis-C18:1	14.7	15.4	14.6
Trans-C18:1	0.1	0.3	7.7[a]
Polyunsaturated fatty acids	12.5	4.3	4.2
Linoleic acid (*cis,cis*-C18:2)	12.0	3.9	3.8
Trans-C18:2	0.0	0.0	0.0
Carbohydrates (% of energy)	46.0	43.5	46.6
Alcohol (% of energy)	0.9	0.9	1.0
Cholesterol (mg/MJ)	33.5	32.6	33.5
β-Sitosterol (mg/MJ)	23.8	25.1	22.6
Other plant sterols (mg/MJ)	3.0	3.9	3.0
Dietary fiber (g/MJ)	3.6	3.9	4.0

Values are based on chemical analyses of duplicate diets plus the calculated contribution of free-choice items (see Methods). Each value represents the mean of three independent duplicates collected in three different periods during which each diet was consumed by one-third of the subjects. Variations between periods were negligible.

[a] Values for total *trans* fatty acid content as determined by gas-liquid chromatography (GLC) and by infrared spectroscopy (IR) were similar: both GLC and IR indicated 20.2 g of *trans* fatty acids per 100 g of total fatty acids in the *trans* fatty acid diet.

RESULTS

Diets and dietary adherence

The mean daily intakes of energy and the composition of the three experimental diets as determined by chemical analyses of duplicate diets plus calculated contribution of free-choice items are given in **Table 2.** Energy and nutrients supplied by the free-choice items (mainly carbohydrates and some alcohol and protein) did not differ between the dietary regimes. The intake of protein, carbohydrates, alcohol, cholesterol, phytosterols, and fiber did not change throughout the study. Total fat intake differed somewhat between the *Trans*-diet and the Stearate-diet (3.8 energy %) due to small differences in the intake of oleic acid (*cis*-C18:1) and saturated fatty acids (C12:0, C14:0, C16:0), and also due to the fact that the difference in stearic acid (C18:0) intake turned out to be 8.8% of energy instead of the planned 8%. The percentage of total daily energy from linoleic acid decreased from 12.0% on the Linoleate-diet to 3.9% and 3.8% on the Stearate and *Trans*-diet, respectively. It was replaced by 9.0% stearic acid on the Stearate-diet and by 7.6% monounsaturated *trans* fatty acids on the *Trans*-diet. At an energy intake of 11 MJ (2630 kcal) per day the *Trans*-diet provided 8.7 g of stearic and 22.2 g of *trans* fatty acids (*trans*-C18:1) per day, while the Stearate-diet supplied 34.2 g of stearic acid and 0.8 grams of *trans* fatty acids.

Gas-liquid chromatography revealed 3 positional isomers of *trans*-C18:1 in the *trans* fatty acid-rich margarine and shortening; predominantly *trans*-C18:1n-9 (Δ9, elaidic acid) and minor amounts of *trans*-C18:1n-10 (Δ8) and *trans*-C18:1n-8 (Δ10).

The fatty acid composition of the serum cholesteryl esters at the end of the three dietary periods confirmed the subjects' adherence to the diets (**Table 3**). In 54 out of the 56 subjects the proportion of linoleic acid in the cholesteryl esters was higher on the Linoleate-diet than on either the Stearate-diet (average change, 6.60 g/100 g; 95% confidence interval, 5.87 to 7.33) or the *Trans*-diet (average change, 8.17 g/100 g; 95% confidence interval, 7.44 to 8.99). The percentage of stearic acid was increased on the Stearate-diet in 55 out of 56 subjects relative to the Linoleate-diet (change 0.97 g/100 g; 95% confidence interval, 0.86 to 1.09) or to the *Trans*-diet (change 0.88 g/100 g; 95% confidence interval, 0.76 to 0.99). Fifty-four out of the 56 subjects showed a larger proportion of elaidic acid in their

Table 3. Fatty acid composition of serum cholesteryl esters at the end of the three dietary periods.

Fatty Acid	Linoleate-Diet	Stearate-Diet	*Trans*-Diet
		g per 100 g of fatty acid	
C14:0	2.71 ± 0.85	2.29 ± 1.18	2.52 ± 1.05
C16:0[a]	8.75 ± 0.55	8.37 ± 0.64	9.10 ± 0.64
C16:1[a]	1.72 ± 0.77	1.97 ± 0.72	2.52 ± 0.62
C18:0	0.77 ± 0.11	1.74 ± 0.38[b]	0.87 ± 0.29
Cis-C18:1[a]	15.06 ± 1.57[b]	20.16 ± 1.44	20.62 ± 1.40
Trans-C18:1	0.13 ± 0.18	0.12 ± 0.19	0.94 ± 0.30[b]
Cis,cis-C18:2[a]	61.01 ± 3.92[b]	54.41 ± 3.23	52.84 ± 3.35
C20:4	6.36 ± 1.36	6.79 ± 1.32	6.42 ± 1.05
Other	3.51 ± 0.98	4.16 ± 0.81	4.17 ± 0.81

Values are means ± SD. The 26 men and 30 women consumed each diet for 3 weeks each, in random order.
[a] Significantly different between each of the diets, $P<0.02$.
[b] Significantly different from both other diets, $P<0.0001$.

cholesteryl esters when on the *Trans*-diet than when on the Linoleate-diet (change 0.81 g/100 g; 95% confidence interval, 0.70 to 0.92) or the Stearate-diet (change 0.83 g/100 g; 95% confidence interval, 0.72 to 0.94). All these differences are highly significant ($P<0.0001$).

Serum lipids and lipoproteins

Total and lipoprotein cholesterol and triglycerides

Table 4 gives the serum lipid and lipoprotein levels at the end of each experimental diet period. Compared with levels on the linoleic acid diet serum total cholesterol increased by 0.15 mmol/L or 5.8 mg/dL ($P=0.0081$; 95% confidence interval, 0.02 to 0.27 mmol/L) on the Stearate-diet and by 0.16 mmol/L or 6.2 mg/dL ($P=0.0041$; 95% confidence interval, 0.04 to 0.29 mmol/L) on the *Trans*-diet. LDL cholesterol rose by 0.17 mmol/L or 6.6 mg/dL ($P=0.0008$; 95% confidence interval, 0.05 to 0.28 mmol/L) on the Stearate-diet and by 0.24 mmol/L or 9.3 mg/dL ($P<0.0001$; 95% confidence interval, 0.12 to 0.35 mmol/L) on the *Trans*-diet; this latter value was not significantly greater than the increase on the Stearate-diet.

Table 4. Serum lipid and lipoprotein cholesterol levels at the end of the three dietary periods.

	Linoleate-Diet	Stearate-Diet	*Trans*-Diet
		mmol per liter	
Total cholesterol			
Men	4.66 ± 0.73	4.93 ± 0.82[a]	4.85 ± 0.65[a]
Women	4.81 ± 0.71	4.85 ± 0.74	4.95 ± 0.78
All	4.74 ± 0.72	4.89 ± 0.77[a]	4.90 ± 0.72[a]
HDL-cholesterol			
Men	1.34 ± 0.18	1.28 ± 0.17[a]	1.25 ± 0.17[a]
Women	1.58 ± 0.28	1.52 ± 0.26[a]	1.48 ± 0.25[a]
All	1.47 ± 0.27	1.41 ± 0.25[a]	1.37 ± 0.24[a]
LDL-cholesterol			
Men	2.90 ± 0.71	3.16 ± 0.74[a]	3.14 ± 0.65[a]
Women	2.78 ± 0.55	2.87 ± 0.66	3.01 ± 0.66[a]
All	2.83 ± 0.63	3.00 ± 0.71[a]	3.07 ± 0.65[a]
HDL/LDL ratio			
Men	0.50 ± 0.16	0.44 ± 0.14[a]	0.42 ± 0.11[a]
Women	0.59 ± 0.16	0.56 ± 0.16[a]	0.51 ± 0.15[a,b]
All	0.55 ± 0.16	0.50 ± 0.16[a]	0.47 ± 0.14[a,b]
Triglycerides			
Men	0.92 ± 0.31	1.07 ± 0.41[a]	1.00 ± 0.35
Women	0.96 ± 0.40	1.01 ± 0.36	1.00 ± 0.31
All	0.95 ± 0.36	1.04 ± 0.38[a]	1.00 ± 0.32

Values are means ± SD. The 26 men and 30 women consumed each diet for 3 weeks each, in random order. To convert values for total, HDL, and LDL cholesterol to mg/dL, multiply by 38.67. To convert values for triglycerides to mg/dL, multiply by 88.54.

[a] Significantly different from the Linoleate-diet, $P<0.02$.

[b] Significantly different from the Stearate-diet, $P<0.02$.

HDL cholesterol decreased by 0.06 mmol/L or 2.3 mg/dL ($P<0.0001$; 95% confidence interval, 0.03 to 0.10 mmol/L) on the Stearate-diet and by 0.10 mmol/L or 3.9 mg/dL ($P<0.0001$; 95% confidence interval, 0.06 to 0.13 mmol/L) on the *Trans*-diet. Lower HDL cholesterol levels on the *Trans*-diet than on the Linoleate-diet were seen in 46 of the 56 subjects (**Fig. 3**). The difference of 0.034 mmol/L in HDL cholesterol between the Stearate-diet and the *Trans*-diet just failed to reach significance ($P=0.0210$; 95% confidence interval, -0.00 to 0.07 mmol/L).

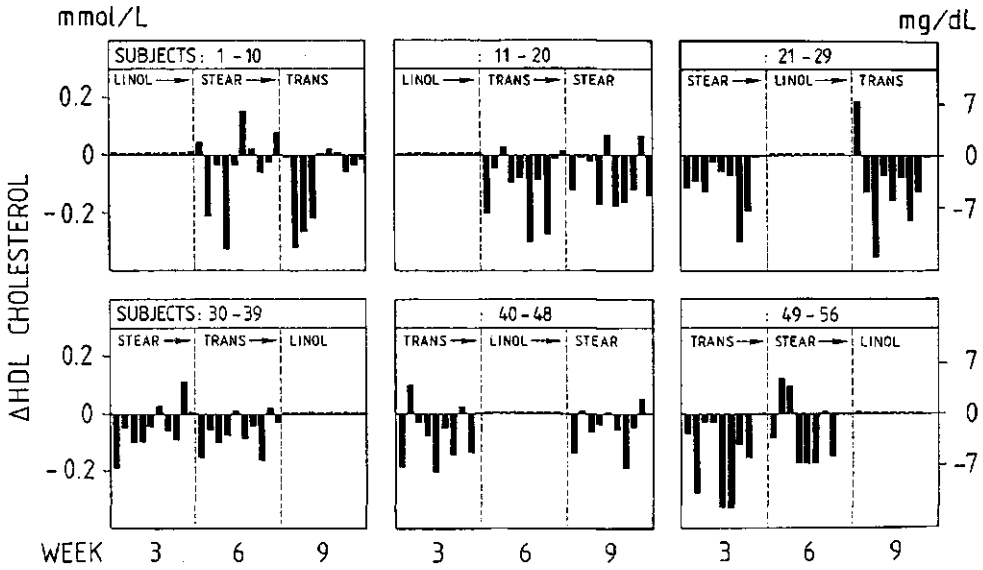


Fig 3. Individual changes in HDL cholesterol levels on diets high in *trans* fatty acids or stearic acid, relative to a diet high in linoleic acid. Bars indicate the level of each individual subject when on a particular diet minus his or her level when on the Linoleate-diet.

The responses of HDL and LDL cholesterol to the different diets were correlated neither with their initial levels (day 1) nor with the differences in body weight between the dietary regimes. Out of the 12 correlation coefficients calculated the largest observed was -0.24 ($P=0.08$) for the response in HDL cholesterol versus the difference in body weight between the *Trans*-diet and the Stearate-diet.

The HDL to LDL cholesterol ratio was 0.55 on the Linoleate-diet, 0.50 on the Stearate-diet, and 0.47 on the *Trans*-diet; all three values were significantly different from each other ($P<0.0037$ for each comparison). The level of serum triglycerides was 0.09 mmol/L or 8.0 mg/dL higher on the Stearate-diet than on the Linoleate-diet ($P=0.0074$; 95% confidence interval, 0.01 to 0.17 mmol/L). Triglyceride values did not differ significantly between the *Trans*-diet and the Stearate-diet ($P=0.22$; 95% confidence interval, 0.04 to -0.12) or between the *Trans*-diet and the Linoleate-diet ($P=0.14$; 95% confidence interval, -0.03 to 0.13 mmol/L).

Apolipoproteins

Table 5 shows the mean apolipoprotein levels and their ratios at the end of each dietary period. Relative to levels on the Linoleate-diet, serum apolipoprotein B rose by 3.4 mg/dL on the Stearate-diet (95% confidence interval, 1.7 to 5.0 mg/dL), and by 5.0 mg/dL (95% confidence interval, 3.4 to 6.6 mg/dL) on the *Trans*-diet. All three values are significantly different from each other ($P<0.0184$ for each comparison). The apoB to LDL cholesterol ratio was essentially the same for each diet. Mean apoA-I level was 2.0 mg/dL higher on the Linoleate-diet than on the *Trans*-diet ($P=0.0119$; 95% confidence interval, 0.1 to 4.0 mg/dL). The difference in serum apoA-I between the Linoleate- and the Stearate-diet periods of 1.3 mg/dL ($P=0.1044$; 95% confidence interval, −0.6 to 3.3 mg/dL), and between the Stearate- and the *Trans*-diet period of 0.7 mg/dL ($P=0.3586$; 95% confidence interval, −1.3 to 2.7 mg/dL) were not significant. The mean apoA-I to HDL cholesterol ratio (mg/mmol) increased from 856 on the Linoleate-diet to 887 on the Stearate-, and to 904 on the *Trans*-diet ($P<0.0081$ for each comparison).

Table 5. Serum apoA-I and apoB levels and their ratios at the end of the three dietary periods.

	Linoleate-Diet	Stearate-Diet	*Trans*-Diet
		mg/dL	
Apolipoprotein A-I			
Men	115.7 ± 7.8	115.6 ± 8.5	114.9 ± 8.4
Women	131.5 ± 16.8	129.1 ± 13.9	128.4 ± 14.6
All	124.1 ± 15.5	122.8 ± 13.5	122.1 ± 13.8[a]
Apolipoprotein B			
Men	69.5 ± 13.3	74.9 ± 14.4[a]	75.4 ± 12.7[a]
Women	68.8 ± 11.5	70.4 ± 12.2	73.1 ± 12.6[a,b]
All	69.1 ± 12.2	72.5 ± 13.4[a]	74.1 ± 12.6[a,b]
Apolipoprotein A-I/B ratio			
Men	1.73 ± 0.38	1.61 ± 0.37[a]	1.57 ± 0.32[a]
Women	1.95 ± 0.31	1.88 ± 0.33[a]	1.80 ± 0.31[a,b]
All	1.85 ± 0.36	1.75 ± 0.37[a]	1.69 ± 0.33[a,b]

Values are means ± SD. The 26 men and 30 women consumed each diet for 3 weeks each, in random order.

[a] Significantly different from the Linoleate-diet, $P<0.02$.

[b] Significantly different from the Stearate-diet, $P<0.02$.

The ratio of apoA-I to apoB was 1.85 ± 0.36 on the Linoleate-diet, 1.75 ± 0.37 on the Stearate-diet, and 1.69 ± 0.33 on the *Trans*-diet. These values are also significantly different from each other ($P<0.0012$ for each comparison).

Gender effects

As compared with the Linoleate-diet, the mean response of the men on the Stearate-diet was significantly greater than that of the women for total cholesterol (difference in change, 0.22 mmol/L or 8.5 mg/dL; $P=0.0438$) and for apoB (difference in change, 3.7 mg/dL; $P=0.0155$). The statistical analyses did not reveal any other significant differences between the sexes in the responses of their lipid, lipoprotein, or apolipoprotein levels to the diets.

DISCUSSION

The three fatty acids under study all had 18 carbon atoms. Thus effects of the chain length of the fatty acids on serum lipid and lipoproteins can be excluded. Since the experimental diets did not materially differ in nutrients other than *trans* fatty acids, stearic acid, and linoleic acid, the changes observed between the diets must be due to differences in the number and/or geometry of the double bonds in these fatty acids.

Trans fatty acids

Effects on lipids and lipoproteins

In a previous study conducted at our department it was found that monounsaturated *trans* fatty acids at a dose of 11% of energy (on average 33 g/day) increased LDL cholesterol by 14.3 mg/dL (0.37 mmol/L) and decreased HDL cholesterol by 6.6 mg/dL (0.17 mmol/L) as compared with their *cis* isomer oleic acid [10]. However, the dose of *trans* fatty acids consumed probably exceeded the range of intakes in free-living subjects. By contrast, the 7.7 energy % of total daily energy intake that was provided by the *trans* fatty acids in the present trial, although still higher than average consumption, may be reached by persons who eat large amounts of hardened vegetable fats and margarine and of products prepared with or fried in such fats [30,31]. Another difference with the previous trial was that we now studied the effects of *trans* fatty acids relative to linoleic

acid. If one assumes that linoleic acid and oleic acid differ little in their effects on serum lipids when consumed in moderate amounts [32-35], then the magnitude of changes observed here are compatible with a linear relation between the dose of *trans* monounsaturates consumed and the response of serum lipoprotein concentrations. **Fig. 4** shows, side-by-side, the effects of monounsaturated *trans*-C18:1 fatty acids as observed relative to linoleic acid in the present and relative to oleic acid in the previous study. Although more studies are needed to determine the true relationship between the dose of various types of *trans* fatty acids and their effects on serum lipoproteins, comparison of the results of the two studies

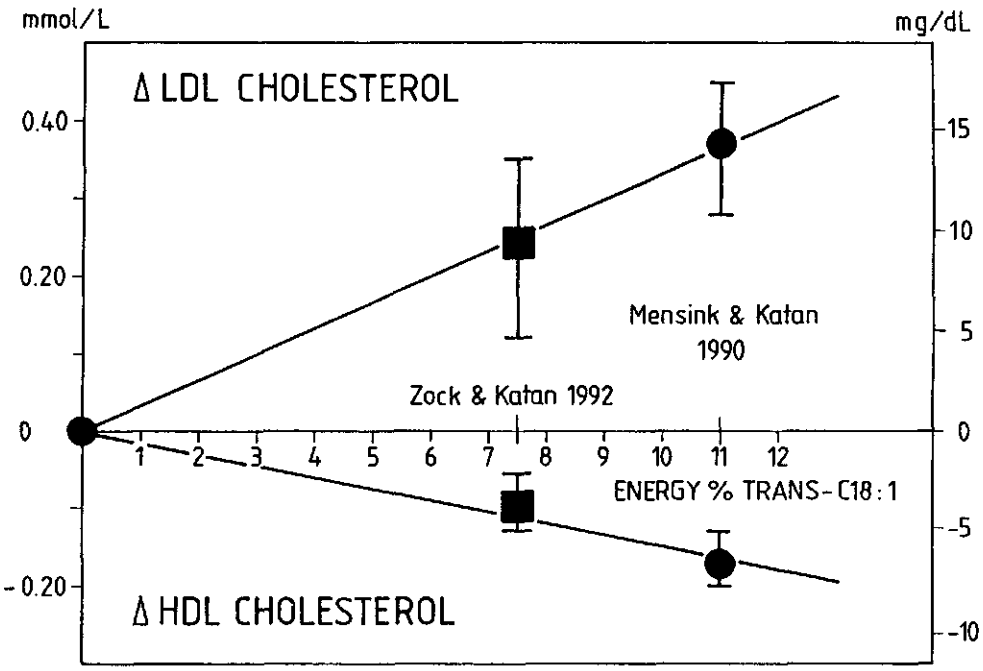


Fig. 4. Effects of *trans* fatty acids on LDL and HDL cholesterol in the present trial compared with those in a previous trial. Circles represent data from a comparison of *trans*-C18:1 with oleic acid [10]. Squares are based on the present comparison between *trans*-C18:1 and linoleic acid. Bars denote 95% confidence intervals. In addition to the two experiments, the origin provides a third point, because a zero change in intake will produce zero change in lipoprotein levels.

suggests that in our hands the effects of *trans* fatty acids on LDL and HDL cholesterol are proportional to the amounts consumed.

The *trans* fatty acids supplied to our subjects consisted largely of C18:1n-9 (elaidic acid), with traces of C18:1n-8 and C18:1n-10. These are also the predominant *trans* fatty acids produced by hydrogenation of vegetable oil in food manufacturing [2,3]. The spectrum of *trans*-C18:1 positional isomers consumed by the subjects in our previous trial was much broader [10,23]. Nevertheless, the observed effects in both trials were largely congruent if the differences in dose are taken into account. We therefore think that our findings will apply to most monounsaturated *trans*-C18:1 fatty acid isomers as present in commercial fats and foods.

Public-health implications

Based on our two studies as summarized in Fig. 4, it can be speculated that one dietary energy % of *trans* fatty acids raises LDL cholesterol by about 1.2 mg/dL (0.03 mmol/L) and lowers HDL cholesterol by about 0.6 mg/dL (0.015 mmol/L) relative to oleic or linoleic acid. The current average *trans* intake of 3 to 4% of energy in the United States might thus cause an elevation of LDL cholesterol by about 4 mg/dL and a depression of HDL cholesterol by about 2 mg/dL relative to *cis*-unsaturates. Although these figures appear modest, the predicted effect on HDL cholesterol is of the same magnitude as the difference in HDL cholesterol between physical active and sedentary people [36] or between individuals at the 10th percentile of body mass index versus those at the 50th percentile [37]. For comparison, smoking 10 cigarettes per day will on average lower HDL cholesterol by about 2 to 4 mg/dL [38].

What would be the predicted effect on coronary heart disease risk if the average *trans* fatty acid consumption of 7 to 10 g/day in the United States population were to be completely replaced by oleic and linoleic acid? Both epidemiological data and results of controlled trials suggest that the predicted decrease of 4 mg/dL in LDL cholesterol should lead to a decrease in the average risk for coronary heart disease of 3%. The increase of 2 mg/dL in HDL cholesterol should add another 4 to 5% reduction in risk [39,40]. Total risk reduction should thus average 7 to 8%, provided that raising HDL indeed reduces risk, which is a plausible but still unproven assumption [39]. A reduction in risk of this magnitude does not appear to justify total elimination of partially hydrogenated oils from the United States food supply, especially since a sizeable part of current *trans* fatty

acid intake is supplied by dairy and beef fat. However, it is possible that selected individuals consume considerably higher amounts of *trans* fatty acids than the average of 3 to 4 percent [41], and for them the effects of *trans* fatty acids can be of clinical importance. Obviously, the *trans* fatty acid intake of various segments of the population needs to be assessed, and groups with high intake need to be identified. Even the intake in the United States is controversial, with quoted figures ranging from 8 to 15 g/day [31]. The estimate of 17 g/day for the Netherlands [42] is similarly based on incomplete data and a host of assumptions. Better intake data are thus urgently needed before any public-health measures at curbing *trans* intake can even be considered.

Stearic acid

Recent studies [32-35] suggest that the effects of linoleic acid and oleic acid on serum total and LDL cholesterol levels are more similar than previously observed [12,13]. Stearic acid has also repeatedly been reported to produce serum lipid levels similar to those seen on oleic acid [12-15]. Thus one would expect stearic acid and linoleic to be more or less equivalent with respect to their cholesterolemic effect. This was indeed observed for the women in our study, but nor for the men, who showed a small but significant rise in total and LDL cholesterol and triglycerides on the Stearate- versus the Linoleate-diet. The increase of 10.4 mg/dL (0.27 mmol/L) in total cholesterol is in line with the equations of Keys, Anderson, and Grande [12] and Hegsted [13], which predict an increase of 9.7 to 11.6 mg/dL in men when 8 dietary energy % of linoleic acid is replaced by stearic or oleic acid. In the absence of an oleic acid diet period in the present trial it is impossible to tell whether the difference in cholesterol levels in males of 10.4 mg/dL between the Stearate and Linoleate diet periods was due to stearate being somewhat cholesterol-raising or linoleate being somewhat cholesterol-lowering relative to oleic acid. Whatever the relative effects of stearate, oleate, and linoleate, they are much smaller than the 28.6 mg/dL (0.74 mmol/L) increase that can be predicted for a replacement of 8 dietary energy % of linoleic acid by a mixture of lauric (C12:0), myristic (C14:0), and palmitic (C16:0) acids [12]. Obviously, stearic acid raises total and LDL cholesterol less than other saturated fatty acids, and it will probably take a number of very large trials to tell whether the effects of stearic and oleic acid differ at all.

Our data do suggest that stearic acid might have some HDL cholesterol-lowering potential relative to linoleic acid, 0.06 mmol/L or 2 mg/dL in the present study. In the study of Bonanome and Grundy [15] a diet enriched with 16 energy % from stearic acid resulted in HDL levels that were 3.9 mg/dL (0.10 mmol/L) lower than those on a diet enriched with oleic acid. This difference was not statistically significant, but at the very least the observations of Bonanome and Grundy [15] on stearic acid and HDL do not contradict ours. Becker et al. [43] compared the effects of saturated fat versus monounsaturated and polyunsaturated fat. Their saturated fat diet supplied 12 to 13 energy % from stearic acid as opposed to 1 energy % in the two other diets. The stearate-rich saturated fat diet decreased HDL cholesterol by 0.10 mmol/L compared with the monounsaturated and by 0.14 mmol/L compared with the polyunsaturated fat diet. As in the study of Bonanome and Grundy [15], these differences were not statistically significant. Nevertheless, data from both studies are in line with our present observation, and they suggest that stearic acid may be neutral as regards total but not as regards HDL cholesterol. The potential HDL cholesterol-depressing effect of stearic acid obviously needs more research.

Effect of gender on responses of lipid and lipoproteins to diet

Suitability of females for studies on diet and lipids

Most trials on the effects of diet on serum lipid and lipoprotein levels have employed men only, partly because coronary heart disease was considered primarily a male disease, but partly also because women were considered less suitable because of confounding effects of the menstrual cycle. In fact, different studies on the influence of the natural menstrual cycle on serum lipids show different effects [44,45] or only minor if any effects [46], and a recent textbook concluded "the degree of variation observed is relatively small and results of separate studies have sometimes been inconsistent" [47]. However, oral contraceptives do influence the concentrations of total and HDL cholesterol in a cyclical manner [46]. At first sight one might think that this might confound our findings in the 13 women who used oral contraceptives. This, however, is not the case. Different women entered the study at different points in their cycle, and in addition, diets were fed in random sequence, so that the effects of the contraceptive cycle cancelled each other and averaged out. Thus, any woman who

had an unusually low HDL cholesterol during week 3 of the Linoleate diet because she was at the end of her cycle was counterbalanced on average by another woman whose HDL cholesterol was high when she was at week 3 of the Linoleate-diet because she happened to be at the pill-free days of her cycle.

Unlike the means, one would expect the standard deviations of the responses to be biased upwards because cyclic effects add a random positive or negative term to each lipid data point. The standard deviations of the responses to dietary changes of the women using oral contraceptives were indeed somewhat larger than those of the women not using contraceptives (on average 0.43 mmol/L vs. 0.28 mmol/L for LDL cholesterol, and 0.15 mmol/L vs. 0.10 mmol/L for HDL cholesterol). However, standard deviations of the mean responses of all 30 women combined (13 users plus 17 non-users) were very similar to those of the 26 men (on average 0.38 vs. 0.34 mmol/L for LDL and 0.13 vs. 0.09 mmol/L for HDL cholesterol). This is in accord with our previous experience [19,33]. This would seem to validate the use of female volunteers as experimental subjects in studies of diet and lipids; at the very most, the number of females required to reach a certain statistical power should be adjusted upward when the majority is using oral contraceptives.

Gender effects on response

We found no differences between men and women in the response of serum lipoprotein or apolipoprotein levels to the *trans* fatty acid diet relative to linoleic acid (Tables 4 and 5). In a previous study the effects of replacing oleic acid by *trans* fatty acids were also similar in men and women [10]. It therefore appears that the lipoprotein levels of men and women respond similarly to dietary *trans* fatty acids.

The increase in total and LDL cholesterol and triglycerides in men on the Stearate-diet was not observed for the women in our study (Table 4). Compared with the Linoleate-diet, the changes of the men's total cholesterol and apoB levels on the Stearate-diet were significantly larger than those of the women (Tables 4 and 5). HDL cholesterol was decreased to a similar extent in men and women. The HDL/LDL ratio was significantly lowered on the Stearate-diet versus the Linoleate-diet in both men and women, although the effect was very small in the women (Table 4). Whether stearic acid is cholesterol-raising in men but not in women could not be definitively addressed here. Obviously this point needs further investigation.

Consequences for the production of hardened fats

Replacement of 8 dietary energy % of monounsaturated *trans* fatty acids by stearic acid resulted in only minor changes in serum lipid and lipoprotein values. Our data thus do not support the idea that increasing the stearic acid content of hardened fats at the expense of *trans* fatty acids is beneficial for the serum lipid profile; either mode of hydrogenation produced fatty acids that increased LDL and decreased HDL cholesterol relative to linoleic acid itself.

However, *trans* fatty acids and stearic acid probably raise total and LDL cholesterol to a lesser extent than C12-16 saturated fatty acids. Even if the *trans* fatty acids were to be equated with C12-16 saturated acids because of their added unfavorable effect on HDL, the content of *trans* plus cholesterol-raising saturates in butter still far exceeds that in soft margarine. Butter is also very high in cholesterol. Therefore, replacement of butter by soft margarine remains of value in the dietary treatment of hypercholesterolemia. However, manufacturers should consider the option of producing soft margarine free of *trans* fatty acids. Diet margarine with zero *trans* fatty acids made from unmodified sunflower oil and a small amount of a stearate-rich hard stock [48] have been marketed successfully for many years in Europe and are also available in North America.

Although data on the *trans* fatty acid content of brick margarine, vegetable shortenings, and solid vegetable frying fats are incomplete, it is probably much higher than in soft margarine, and the sum of *trans* fatty acids and cholesterol-raising saturates in such fats may approach that in beef tallow or lard. If the unfavorable effects of *trans* fatty acids on serum lipoproteins are borne out by future studies, then the use of so-called "cholesterol-free" solid vegetable fats for cooking or (deep-fat) frying may offer few health benefits over beef tallow or lard, and at that time replacement of such fats by unmodified or very lightly hydrogenated oils should be considered. Use of high-*trans* fats could then be limited to baked goods and other products where suitable replacements are much harder to find.

However, any large-scale displacement of partially hydrogenated vegetable oils from the market should await the outcome of further experiments, which in addition to lipids should also attempt to gauge effects on fibrinogen and on platelet function and aggregation tendency.

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CHAPTER 3

Effects of stearic acid and *trans* fatty acids versus linoleic acid on blood pressure in normotensive women and men

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ABSTRACT

Aim. The objective of this study was to compare the effect of linoleic acid (*cis,cis*-C18:2) with that of its hydrogenation products stearic acid (C18:0) and elaidic acid (*trans*-C18:1) on blood pressure levels in normotensive humans.

Methods. We therefore measured the effects of these fatty acids on systolic and diastolic blood pressure in 30 women and 25 men. Three strictly controlled experimental diets were supplied to every subject for three weeks each, in different order (multiple cross-over). The composition of the three diets was constant, except for 8% of daily energy, which was provided by either linoleic acid, stearic acid, or monounsaturated *trans* fatty acids. The statistical power for detecting a true difference between two diets of 3 mmHg in systolic and diastolic blood pressure was over 90%.

Results. Mean systolic/diastolic blood pressure at the end of the dietary periods was 114/69 mmHg on the linoleic-acid diet, 113/70 on the stearic-acid diet, and 113/69 on the *trans*-fatty-acid diet. No significant differences were observed in blood pressure levels after 3 weeks on each diet.

Conclusion. We conclude that a major increase in the intake of linoleic acid at the expense of stearic acid or *trans* fatty acids has no effect on blood pressure in normotensive young women and men.

INTRODUCTION

A considerable part of saturated fat in Western diets is supplied by stearic acid (C18:0). Cocoa fat, beef fat, and other animal fats are natural sources of stearic acid, but it can also been formed when vegetable oils, rich in linoleic acid, are hydrogenated to produce fats with better texture and stability. In addition, partial hydrogenation of linoleic-acid-rich oils yields monounsaturated *trans* fatty acids with a length of 18 carbon atoms. Some studies suggested that diets high in linoleic acid may lower blood pressure [1-3], possibly through an effect on prostaglandin formation [2]. It has also been reported that an increase in saturated fat content of the diet can raise blood pressure at a nearly constant intake of linoleic acid [4,5]. *Trans* fatty acids are consumed in considerable amounts in Western societies [6], but little is known about their effect on blood pressure in humans.

In this controlled trial we examined the effects of linoleic acid and its hydrogenation products stearic acid and *trans* fatty acids on systolic and diastolic blood pressure in normotensive subjects. Previously we have reported the effects of these diets on serum lipid and lipoprotein levels (Chapter 2). Linoleic acid in the diet was iso-energetically replaced by either stearic acid, or by monounsaturated *trans* fatty acids. The three experimental diets did not materially differ in other nutrients and minerals.

METHODS

Subjects

Subjects were recruited through advertisements in local newspapers. Thirty-one women and 28 men participated in the study, of which one purpose was to investigate the effects of *trans* fatty acids and stearic acid versus linoleic acid on serum lipids (Chapter 2). During the study 2 men withdrew, one because of personal reasons and one because of illness. One woman withdrew because of pregnancy and for one man the blood pressure measurements were discontinued halfway through the trial because he felt them to be too constraining. All analyses were therefore based on the 25 men and the 30 women in whom all blood pressure measurements were conducted successfully. The subjects were healthy,

as indicated by a medical questionnaire and by the absence of protein and glucose in the urine. None was hypertensive (systolic blood pressure >150 mmHg, diastolic pressure >90 mmHg) and none were taking blood-pressure-lowering medications. The men were between 19 and 48 years of age (mean, 26). They weighed 60 to 83 kg (mean, 73) and their body mass index ranged from 18.5 to 26.0 kg/m^2 (mean, 21.5). The women were between 19 and 49 years of age (mean, 24). They weighed 51 to 80 kg (mean, 63), and their body mass index ranged from 17.9 to 27.5 kg/m^2 (mean, 21.5). Thirteen women used oral contraceptives, and 4 men and 4 women smoked.

The protocol and aim of the study were fully explained, and subjects gave their written consent. Approval for the study had been obtained from the Ethics Committee of the Department.

Design and diets

The study was conducted using a multiple crossover design with each subject consuming the three experimental diets for three weeks each, in different order. In this way, any systematic bias due to the order in which the diets were supplied or to drift of variables over time were eliminated [7]. One diet was high in linoleic acid, another was high in stearic acid, and the third was high in monounsaturated *trans* fatty acids. Before the trial, the participants were stratified according to sex, and females also according to use of oral contraceptives. From each category about one sixth of the subjects were randomly allocated to one of the six possible treatment sequences. **Table 1** shows the mean baseline characteristics of each treatment group. All subjects were studied simultaneously from January 29 until April 2, 1990.

The study diets consisted of conventional solid foods, and menus were changed daily during each 3-week cycle. The planned composition of the three diets was similar, except for about 8% of daily energy, which was provided by either linoleic acid, stearic acid, or monounsaturated *trans* fatty acids (**Table 2**). For the linoleic acid group a commercially available margarine high in linoleic acid was used (Becel, Van den Bergh foods, Rotterdam, The Netherlands). Fats for the stearic-acid-rich diet were produced by interesterification of a mixture of 41 parts of fully hydrogenated high-linoleic-acid sunflower oil (Chempri, Raamsdonksveer, The Netherlands), 9 parts of unmodified high-linoleic-acid sunflower oil, and 50

Table 1. Baseline values (mean ± SD) of the age, body mass index, and systolic and diastolic blood pressure (BP) of male and female subjects per treatment sequence.

Sequence	Number of men/women	Age (years)	Body mass index (kg/m^2)	Systolic BP (mmHg)	Diastolic BP (mmHg)
L–S–T[a]	5 men	29 ± 11	21.7 ± 1.3	123.6 ± 6.7	76.4 ± 7.7
	5 women (3)[b]	24 ± 2	21.5 ± 0.5	101.2 ± 6.6	65.2 ± 4.5
L–T–S	5 men	24 ± 5	19.7 ± 0.6	128.0 ± 11.4	77.7 ± 8.5
	5 women (3)	22 ± 2	20.7 ± 1.9	116.4 ± 11.7	72.5 ± 10.1
S–L–T	4 men	24 ± 3	20.6 ± 2.3	124.7 ± 8.4	72.1 ± 0.7
	4 women (2)	21 ± 1	21.2 ± 3.6	114.1 ± 10.0	67.9 ± 5.4
S–T–L	5 men	24 ± 2	23.2 ± 1.4	121.3 ± 3.9	69.6 ± 9.0
	5 women (1)	28 ± 11	21.5 ± 1.9	114.2 ± 8.2	72.8 ± 7.9
T–L–S	4 men	21 ± 2	21.1 ± 1.1	127.4 ± 12.1	67.4 ± 8.6
	5 women (3)	28 ± 13	21.7 ± 3.8	116.8 ± 12.2	71.4 ± 8.5
T–S–L	2 men	36 ± 17	24.3 ± 2.4	112.6 ± 2.7	76.4 ± 4.4
	6 women (1)	21.1 ± 1	22.3 ± 1.5	115.4 ± 12.7	64.8 ± 10.0

[a] L, linoleic acid diet; S, stearic acid diet; T, *trans* fatty acid diet.

[b] Numbers in parentheses indicate the number of women using oral contraceptives in each treatment group.

parts of high-oleic-acid sunflower oil (Trisun, SVO Enterprises, Wickliffe, OH). To produce margarine and shortening for the *trans* fatty acid diet, the same high-oleic-acid sunflower oil (Trisun) was hydrogenated with a sulfurized nickel catalyst (Pricat 9908, Unichema, Emmerich, Germany) under conditions that favored formation of *trans* rather than saturated fatty acids. Seventy-five parts of this hydrogenated fat were mixed with 25 parts of the unaltered oleic-acid-rich oil. The special margarines and shortenings were developed and produced by the Unilever Research Laboratory, Vlaardingen, The Netherlands.

Diets were formulated at 24 levels of energy intake ranging from 5.5 to 17.5 MJ per day. All foodstuffs were weighed out for each individual subject. In addition to the food supplied, subjects were allowed a limited number of items free from fat and cholesterol. The energy intake of these free-choice items was fixed for each level of energy intake and ranged from 7 to 13% of total energy intake. The subjects were urged to maintain their usual smoking habits, physical activity pattern, and use of oral contraceptives during the trial, and they were repeatedly asked not to change their selection of free-choice items between periods. Subjects

recorded in diaries their free-choice items, the amount and type of coffee used, and any deviations from the protocol.

Duplicate portions of each study diet were collected on each of the 63 trial days for an imaginary participant with a daily energy intake of 11 MJ (2630 kcal), stored at −20 °C, and pooled and analyzed after the study.

Other details are described elsewhere (Chapter 2).

Measurements

Before the experiment, in October and November 1989, the subjects were asked to record their food intake on three separate days, including one weekend day. Foods were coded and the composition and the energy content of the habitual diets were calculated with use of the Netherlands Nutrient Data Base [8]. As it was known from previous experiments conducted at our department that such food records tend to underestimate actual energy needs by some 10 to 15%, levels of energy intake at the start of the study were set at 10% over stated habitual intake.

Blood pressure was measured at the left arm with an automatic sphygmomanometer with recorder (Takeda Medical UA-751, Adquipment Medical BV, Rotterdam, The Netherlands) on one occasion before and once a week during the experiment. The cuff used was 14 cm wide and had a greatest length of 40 cm. Systolic pressure was recorded at Korotkoff phase I and diastolic pressure at Korotkoff phase V. Subjects were asked not to perform any physical activity or to eat or smoke for 1 hour prior to the blood pressure measurements. Four measurements were made at each session with the subjects sitting upright. During the measurement the subject wore loosely fitting sleeves and they rested their left forearm on a table. One trained investigator (R.B.) performed all the measurements, using the same sphygmomanometer throughout the study. The first blood pressure measurement of each session was discarded and the three other measurements were averaged per subject. All measurements on a particular subject were generally made at the same time of the day to eliminate effects of diurnal variations in blood pressure. Subjects were never told their blood pressure readings while the study was still on.

The duplicate portions of each dietary period were mixed thoroughly, and then freeze-dried. The ash content and the moisture levels [9] were determined and then the material was stored at −20 °C. Aliquots were analyzed for protein [10], total

Table 2. Mean daily intake of energy and nutrients of the 55 subjects before the experiment, and during the different dietary regimes.

		Diet		
	Habitual[a]	Linoleic acid[b]	Stearic acid[b]	Trans fatty acids[b]
Energy				
(MJ/d)	10.3 ± 2.7	12.0	11.9	12.0
(kcal/d)	2464 ± 645	2869	2845	2869
Protein (% of energy)	14.1 ± 2.4	12.3	12.3	12.8
Fat (% of energy)	36.2 ± 4.8	41.1	43.5	39.7
Saturated fatty acids	14.6 ± 2.4	11.0	20.1	10.3
Lauric acid (C12:0)		0.7	0.5	0.5
Myristic acid (C14:0)		0.9	1.0	1.0
Palmitic acid (C16:0)		5.8	5.7	4.8
Stearic acid (C18:0)		2.8	11.8	3.0
Monounsaturated fatty acids	12.7 ± 2.3	15.8	16.6	23.3
Oleic acid (*cis*-C18:1)		14.7	15.4	14.6
Trans-C18:1		0.1	0.3	7.7
Polyunsaturated fatty acids	6.6 ± 1.8	12.5	4.3	4.2
Linoleic acid (*cis,cis*-C18:2)	5.6 ± 1.7	12.0	3.9	3.8
Trans-C18:2		0.0	0.0	0.0
Carbohydrates (% of energy)	48.6 ± 5.7	46.0	43.5	46.6
Alcohol (% of energy)	1.4 ± 2.1	0.9	0.9	1.0
Cholesterol (mg/MJ)	26.8 ± 11.0	33.5	32.6	33.5
Dietary fibre (g/MJ)	3.3 ± 9.9	3.6	3.9	4.0
Sodium (mg/d[c])	3117 ± 967	2918	3065	3311
Potassium (mg/d[c])	4052 ± 939	3893	4111	4149
Calcium (mg/d[c])	1387 ± 416	1283	1246	1384
Magnesium (mg/d[c])		359	378	355

[a] Estimated from three-day food records, which tend to underestimate energy needs (cf. Methods). No values for magnesium, *cis* and *trans* isomers, and specific saturated fatty acids are available in the food composition table used.

[b] Determined by chemical analysis plus the calculated contribution of free-choice items (cf. Methods). each value represents the mean of three independent duplicates collected in three different periods during which each diet was consumed by one-third of the subjects. Variations between periods were negligible and standard deviations are therefore not given.

[c] At an energy intake of 12 MJ (2869 kcal) per day.

fat [11], the proportion of individual fatty acids [12], dietary fibre [13] and cholesterol [14]. Available carbohydrate was calculated by difference. Sodium, potassium, calcium and magnesium were determined by atomic absorption spectrophotometry [15] after the sample had been wet-ashed and neutralized. The mean composition of the diets was calculated from the duplicate portion analysis plus the contribution of the free-choice items.

In order to estimate dietary adherence, the fatty acid composition of serum cholesteryl esters of each subject was determined in blood samples obtained at the end of each dietary period.

Body weights without shoes, jackets, and heavy clothing were recorded twice weekly. The level of energy intake was adjusted when necessary, as indicated by weight changes. Over the 63 days of the trial average body weight fell by 0.2 ± 1.1 kg (range, −2.5 to +2.0 kg). The mean difference in body weight at the end of the dietary periods was 0.1 ± 0.7 kg between the linoleic acid diet and the stearic acid diet, 0.0 ± 0.8 kg between the linoleic and the *trans* fatty acid diet, and 0.1 ± 0.7 kg between the *trans* fatty acid and the stearic acid diet.

Statistical methods

The null hypothesis to be tested was that blood pressures measured at the end of each dietary period would not differ between diets. This hypothesis was tested by analysis of variance of the systolic and diastolic pressure values obtained in the third week of each dietary period, with use of the General Linear Model (GLM) procedure of the Statistical Analysis System [16]. The Tukey method was used for pairwise comparison of the diets and for calculation of 95% confidence limits for the differences between two diets. The statistical power for detecting a true difference of 3 mmHg between two diets in systolic and diastolic blood pressure was over 90%. Effects of gender, use of oral contraceptives by women, and the sequence of the diets were checked by entering these class variables as independent factors in the model.

In secondary analyses, blood pressure values obtained in weeks 1 and 2 of the dietary periods were also tested.

RESULTS

Diets and dietary adherence

The habitual diets of the subjects, estimated from three-day food records, and the mean daily intakes of energy and nutrients of the three experimental diets as determined by chemical analyses of duplicate diets plus calculated contribution of free-choice items are given in Table 2. The estimate of habitual energy intake was 14% lower than energy intake determined during the trial. This confirmed our previous experience that food records underestimate actual energy needs by some 10 – 15% (cf. Methods). The intakes of total energy, protein, carbohydrates, alcohol, cholesterol, dietary fibre, sodium potassium, calcium and magnesium were similar on all three experimental diets (Table 2). Total fat intake differed somewhat between the *trans* fatty acid diet and the stearic acid diet (3.8% of energy) due to small differences in the intake of oleic acid and C12-C16 saturates, and due to the fact that the difference in stearic acid turned out to be 8.8% of daily energy instead of the planned 8%. The percentage of total daily energy from linoleic acid decreased from 12.0% on the linoleic acid diet to 3.9% and 3.8% on the stearic acid and *trans* fatty acid diets, respectively. It was replaced by 9.0% stearic acid on the stearic acid diet and by 7.6% monounsaturated *trans* fatty acids on the *trans* fatty acid diet.

Inspection of the subjects' diaries did not reveal any changes in smoking habits, physical activity pattern, use of oral contraceptives or other deviations from the protocol that might have affected the results. The proportion of linoleic acid in the serum cholesteryl esters was higher on the linoleic acid diet than on both other diets in 54 out of the 55 subjects. The average increase was 7.4 g linoleic acid per 100 g of all fatty acids detected. This confirmed dietary adherence of the subjects.

Blood pressure

Mean systolic and diastolic blood pressure levels did not significantly change over the 9 weeks of the study.

Blood pressure values averaged per dietary regime are shown in **Fig. 1**. In the third week on the linoleic acid diet, the mean systolic pressure was 114 ± 10.7 mmHg and the mean diastolic pressure was 69 ± 8.7 mmHg. On the stearic acid

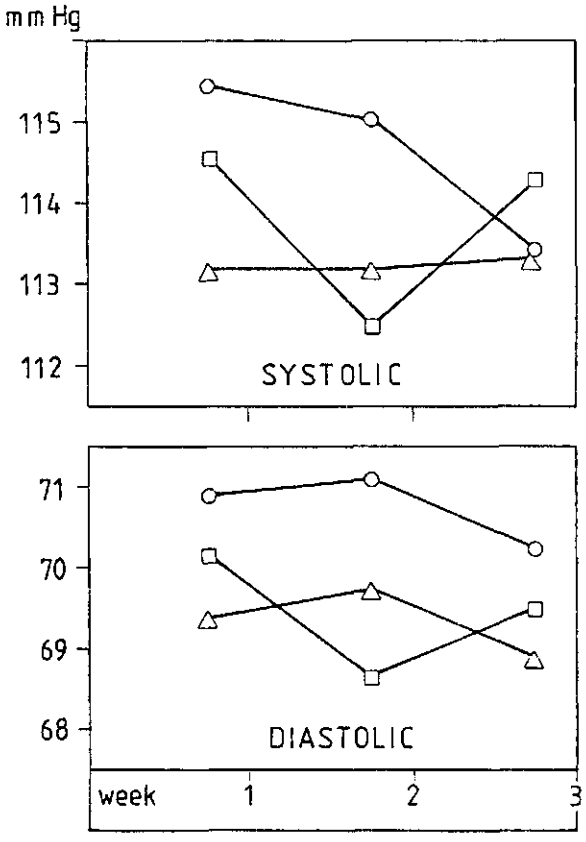

Fig. 1. Mean systolic and diastolic blood pressure during the experiment, averaged per diet. Thirty women and 25 men consumed the linoleic acid diet (□——□), the stearic acid diet (○——○), and the *trans* fatty acid diet (Δ——Δ) for 3 weeks each, in random order.

diet these values were 113 ± 10.4 (95% CI for difference with linoleic acid diet, −2.8 to 1.1 mmHg) and 70 ± 8.5 (95% CI, −1.1 to 2.7 mmHg). Levels on the *trans* fatty acid diet were 113 ± 10.8 mmHg for systolic (95% CI for difference with linoleic-acid diet, −2.9 to 1.0 mmHg) and 69 ± 7.5 mmHg for diastolic blood pressure (95% CI, −2.5 to 1.3 mmHg). The analysis of variance showed no significant effect of the order in which diets were supplied on the differences in blood pressure between the diets. Gender and the use of oral contraceptives by women also did not affect the responses of blood pressure to the diets.

Table 3 shows the mean blood pressure levels in the third week of the three dietary regimes for all subjects and for men and women separately. No significant differences were found between the different diets.

Table 3. Mean (± SD) systolic and diastolic blood pressure of the subjects after 3 weeks on the high-linoleic-acid diet, the high-stearic-acid diet, and the high-*trans*-fatty-acid diet.

	Linoleic acid diet	Stearic acid diet	*Trans* fatty acid diet
Systolic pressure (mmHg)			
Men	120 ± 8.4	119 ± 7.7	118 ± 9.6
Women	110 ± 10.5	109 ± 9.9	109 ± 10.1
All	114 ± 10.7	113 ± 10.4	113 ± 10.8
Diastolic pressure (mmHg)			
Men	71 ± 8.7	73 ± 8.6	71 ± 8.0
Women	68 ± 8.5	68 ± 7.7	67 ± 6.7
All	68 ± 8.7	70 ± 8.5	69 ± 7.5

In the 28 subjects with the highest systolic blood pressure before the study, levels were 119 ± 9.6 mmHg on the linoleic acid diet, 119 ± 8.4 mmHg on the stearic acid diet, and 118 ± 8.8 mmHg on the *trans* fatty acid diet. These values were not significantly different from each other. The mean diastolic pressure of the 28 subjects with the highest diastolic blood pressure levels before the study was 73 ± 9.2 mmHg on the linoleic acid diet, 73 ± 9.7 mmHg on the stearic acid diet, and 71 ± 8.6 mmHg on the *trans* fatty acid diet. Again, no significant differences were found between the three experimental diets. In a secondary statistical analysis, blood pressure measurements obtained in weeks 1 and 2 of each dietary period were added as dependent variables. The analysis of variance now showed a minor blood pressure-raising effect of stearic acid as compared with *trans* fatty acid; 1.4 mmHg (95% CI, 0.1 to 2.6 mmHg; $P=0.0167$) for systolic, and 1.4 mmHg (95% CI, 0.4 to 2.5; $P=0.0020$) for diastolic pressure. There was also a small effect on diastolic blood pressure of stearic vs. linoleic acid (95% CI, 0.3 to 2.4 mmHg, $P=0.0020$).

DISCUSSION

In humans, the essential fatty acid linoleic acid might lower blood pressure [1-3,17], while some studies reported that saturated fatty acids can raise blood pressure [4,5]. However, studies in normotensives have not produced consistent results. Two groups of investigators [2,18] did not observe an effect on blood pressure when the intake of saturated fat was decreased and replaced by carbohydrates. In other trials, the exchange of saturated fatty acids for linoleic acid at equal levels of total fat intake had no significant effects [19,20]. In the present study, no effects on blood pressure levels could be demonstrated after 3 weeks on a strictly controlled diet high in stearic acid, when compared with diets high in linoleic acid or in *trans* fatty acids; thus the null hypothesis had to be accepted.

Blood pressures measured during the first two weeks were marginally higher on the stearic acid diet than on the other diets. However, these small differences were not seen after 3 weeks on the experimental diets. Also, these tests were performed after inspection of the data, and they are thus much less convincing than the test of the original null hypothesis. The statistical power in this experiment for detecting a true difference between two diets of 3 mmHg in systolic and diastolic blood pressure in the third week of the diets was over 90%. We therefore conclude that stearic acid has no important effect on blood pressure in young normotensive women and men. At the very most, stearic acid in a dose of 8% of daily energy raises blood pressure by $1-2$ mmHg, which is of little biological significance.

Previous studies from our department showed that linoleic acid and oleic acid have similar effects on blood pressure [21], and that *trans* fatty acids do not affect blood pressure relative to oleic acid or saturated fatty acids [22]. The present observation that *trans* fatty acids have the same effects on blood pressure as linoleic acid and stearic acid is in line with these findings. Our study confirms that monounsaturated *trans* fatty acids have no effects on blood pressure.

It might be possible that the 3-week dietary periods in our trial were too short to induce a change in blood pressure. However, other studies suggested that blood pressure levels were already stabilized within 3 weeks after a dietary change [2,23].

We did not find a significant decrease in blood pressure during the 9 weeks of the trial due to a habituation effect [24]. Evidently the subjects had become habituated to the measurement procedures during the pre-experimental period.

Also, we did not observe an effect of the order in which the diets were supplied. Even if any of these effects would have been present, systematic bias in the comparison of the mean blood pressure levels between the different diet was eliminated by the balanced design of the study [7].

Puska and co-workers [25] found a larger decrease in systolic and diastolic blood pressure after dietary fat modification in subjects with mild hypertension than in normotensive subjects. The subjects in our study were normotensive, and it could be argued that their initial blood pressure values were too low to induce a change by diet. However, we think this is unlikely since blood pressure levels of subjects in two studies which did find effects of decreasing saturated fat intake [4,5] were even lower than those of our subjects. Moreover, we did not find differences in responses between subjects from the upper and the lower half of the blood pressure distribution.

Body weights were kept constant during the trial. Also, the three experimental diets did not differ in their content of sodium, potassium, calcium, magnesium, alcohol, and dietary fibre. Thus, blood pressure could not have been affected by these factors.

We conclude that a major increase in the intake of linoleic acid at the expense of stearic acid or monounsaturated *trans* fatty acids has no effect on blood pressure in normotensive young women and men.

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CHAPTER 4

Effect of dietary *cis* and *trans* fatty acids on serum lipoprotein(a) levels in humans

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ABSTRACT

Aim. Serum lipoprotein(a) (Lp(a)) is a strong risk factor for coronary heart disease. We therefore examined the effect of dietary fatty-acid composition on serum Lp(a) levels in three strictly-controlled experiments with healthy normocholesterolemic men and women.

Methods and Results.

In the first experiment (**Expt. I**), 58 subjects consumed for 17 days a control diet high in saturated fatty acids. For the next 36 days 6.5% of total energy intake from saturated fatty acids was replaced by monounsaturates plus polyunsaturates (monounsaturated fatty acid diet; $n = 29$) or by polyunsaturates alone (polyunsaturated fatty acid diet; $n = 29$). Both diets caused a slight, nonsignificant, increase in median Lp(a) levels, with no difference between diets.

In the second experiment (**Expt. II**), 10% of energy as the cholesterol-raising saturated fatty acids (lauric, myristic, and palmitic acid) was replaced by oleic acid or by *trans*-monounsaturated fatty acids. Each of the 59 participants received each diet for 3 weeks in random order. The median level of Lp(a) was 26 mg/L on the saturated fatty acid diet; it increased to 32 mg/L ($P < 0.020$) on the oleic acid diet and to 45 mg/L ($P < 0.001$) on the *trans* fatty acid diet. The difference in Lp(a) between the *trans*-fatty acid and the oleic acid diets was also highly significant ($P < 0.001$).

The third experiment (**Expt. III**) involved 56 subjects; all received 8% of energy from the stearic acid, from linoleic acid, or from *trans*-monounsaturates, for 3 weeks each. All other nutrients were equal. Median Lp(a) levels were 69 mg/L on both the stearate diet and linoleate diet, and rose to 85 mg/L ($P < 0.01$) on the *trans* diet. Changes in Lp(a) were positively related to initial levels.

Samples from Expt. I had been stored for 43 months, those from Expt. II for 31 months, and samples from Expt. III for 14 months at -40 °C or lower. Comparison of nineteen paired samples suggested that storage may have caused an overall decrease of 5 – 12% in Lp(a) levels, with no effect on the order of ranking of Lp(a) within studies.

Conclusion. These short term experiments suggest that diets high in *trans*-monounsaturated fatty acids may increase serum levels of Lp(a).

INTRODUCTION

Lipoprotein(a) (Lp(a)) is a macromolecular complex, made up of apoprotein B, cholesterol, and other lipids, and a protein called apo(a). Apo(a) shows sequence homology to plasminogen [1]. The Lp(a) concentration in the blood is largely under genetic control and does not change much with age [2,3]. Most subjects have Lp(a) levels below 150 mg/L, but levels in some may well exceed 400 mg/L [2,3]. Such subjects have a markedly increased risk for coronary heart disease, a relationship that is not confounded by serum low-density or high-density lipoprotein (LDL or HDL) cholesterol levels [3,4].

In spite of the structural resemblance between Lp(a) and LDL [5], determinants of serum Lp(a) levels are distinctly different from those of LDL. Although niacin used with neomycin has been reported to decrease Lp(a) levels [6], attempts to modify Lp(a) by drug [7] or diet [8,9] have not been very successful. Recently, however, we have shown that dietary fatty acid composition may affect Lp(a): replacement of the habitual fat in the Dutch diet by palm oil resulted in a significant decrease in serum Lp(a) levels [10]. We therefore decided to analyze Lp(a) levels in serum samples from three controlled studies on diet and lipoproteins. Expt. I [11] involved replacement of saturated fatty acids by monounsaturated (oleic acid) or polyunsaturated fatty acids (linoleic acid). Expt. II [12] compared the cholesterol-raising saturated fatty acids with *cis*-monounsaturated fatty acids (oleic acid) and with *trans*-monounsaturated fatty acids, and Expt. III (Chapters 2 and 3) dealt with stearic acid, linoleic acid, and the *trans* fatty acid elaidic acid.

METHODS

Subjects and methods

Most subjects were young, normolipidemic, nonobese students. They were all apparently healthy as indicated by a medical questionnaire, and by the absence of anaemia, glucosuria, and proteinuria. The experimental protocols, which had been approved by the Ethical Committee of the Department of Human Nutrition, Agricultural University, Wageningen, were fully explained to the subjects and their informed consent was obtained. No monetary inducement was offered, except for the food, which was free.

Diets consisted of conventional mixed solid foods. All foodstuffs were supplied individually according to each person's energy requirement. On weekdays at noon, hot meals were served at the Department of Human Nutrition in Wageningen. All other food was provided daily as a package. Food for the weekend and instructions for its preparation were provided on Fridays. In addition to the food supplied, each subject had to choose from a list food items that were free from fat and cholesterol. These items provided 9% of total daily energy intake. Body weight was recorded twice weekly and energy intake was adjusted when necessary to prevent changes in weight.

Subjects were asked to maintain their usual pattern of activity and not to change their smoking habits or use of oral contraceptives. They recorded in diaries any sign of illness, medication used, the free-choice items selected, and any deviations from their diets. Inspection of the diaries did not reveal any deviations from the protocol which might have affected the results.

Complete duplicate portions for one imaginary participant with a daily energy intake of 10 MJ or 11 MJ (2390 kcal or 2630 kcal) were collected daily for each diet. The duplicates were stored at -20 °C, and were pooled and analyzed after the study. The free-choice items consumed were coded and their composition calculated using the Netherlands Nutrient Data Base [13]. The analyzed values of the duplicate diets were combined with the calculated values for the free-choice items.

Fasting blood samples were collected between 7:15 AM and 10:00 AM. Blood was allowed to clot for 1 h and serum for lipid and lipoprotein measurements was obtained by low-speed centrifugation and stored at -40 °C or lower until analysis.

Lp(a) measurements

Serum Lp(a) levels were analyzed by enzyme linked immunosorbent assay (ELISA) using a commercial kit (TintElize® Lp(a), Biopool, Umea, Sweden). The assay utilized polyclonal antibodies raised against purified Lp(a). It employs micro-test wells coated with affinity purified goat anti-apo(a)-antibodies, which bind the Lp(a) particles present in the test sample. After incubation, anti-apo(a) antibodies conjugated to a peroxidase are added. These antibodies bind to the apo(a) of the immobilized Lp(a) from the test sample. Unbound conjugated antibodies are

washed off, and 1,2-phenylenediamine dihydrochloride is added. This is converted by peroxidase into a compound with a specific absorbance at 492 nm. A calibration curve is obtained from human standard plasma samples that are provided with the kit and represent eight different Lp(a) concentrations between 0 and 600 mg/L, expressed in terms of particle mass. In all assays the correlation coefficient for the calibration curve was over 0.990. Addition of 3 to 1300 mg/L of human plasminogen to an Lp(a)-free medium or to a human plasma containing 140 mg/L of Lp(a) did not change the assay response (Biopool Ltd, personal communication, 1991). A control serum pool was analyzed in each run. The average Lp(a) level for this pool was 245 mg/L and the inter-assay and intra-assay coefficient of variation were both 5.3%. However, due to a lack of international standardization, absolute levels of Lp(a) may not be directly comparable with those in other studies [14]. For each study, all samples of one subject were analyzed on the same plate. Samples had been stored at −40 °C or lower for 14 − 43 months.

In a separate run 29 pairs of serum and of citrated plasma, that had been stored for 1 − 3 weeks at −80 °C, were analyzed. Lp(a) levels ranged between 6 and 670 mg/L for the serum and from 6 to 637 mg/L for the plasma. Plasma samples were on average 11 mg/L lower than serum samples. The correlation coefficient was 0.99. These results suggest that the assay response is not materially changed when the clotting cascade has been activated, and that Lp(a) can be validly assayed in serum.

Design and diets

Expt. I was carried out from 4 October until 27 November 1987 [11]. Fifty-eight healthy men and women first consumed for 17 days a control diet high in saturated fat. For the next 36 days, 14 of the men and 15 of the women received a diet enriched with olive oil and sunflower oil (monounsaturated fatty acid diet). The other 13 men and 16 women received a diet enriched with sunflower oil alone (polyunsaturated fatty acid diet). For the monounsaturated fatty acid group, 6.5% of total energy intake provided by saturated fatty acids on the control diet was replaced by a mixture of oleic acid (*cis*-C18:1n-9) plus linoleic acid (*cis,cis*-C18:2n-6) and for the polyunsaturated fatty acid group by linoleic acid alone. The intake of other nutrients was kept constant.

At the start of the study the mean (± SD) serum total cholesterol level of the subjects was 5.00 ± 0.79 mmol/L, and their mean body mass index 21.6 ± 2.0 kg/m^2. During the study body weight increased by 0.2 ± 1.3 kg in the monounsaturated fatty acid group and by 0.2 ± 1.0 kg in the polyunsaturated fatty acid group. Three men and four women smoked. Nine women used oral contraceptive agents.

Blood was sampled on days 14 and 17 (control period) and on days 50 and 53 (test period). For Lp(a) analyses equal volumes were pooled per subject per diet period. The response to the monounsaturated fatty acid or the polyunsaturated fatty acid diet was calculated as the change from the end of the control period to the end of the test period. This design only allows comparison of differences between the effect of monounsaturated and polyunsaturated fatty acids; the absolute changes relative to the control diet high in saturated fatty acids may have been biased by unknown drifts with time.

Expt. II was performed from 26 September until 28 November 1988 [12]. Twenty-five men and 34 women consumed three different diets for 3 weeks each, in random order. The composition of the diets was identical, except for 10% of daily energy intake which was provided by either a mixture of the cholesterol-raising saturated fatty acids lauric, myristic, and palmitic acid, by oleic acid, or by *trans* isomers of oleic acid. The fat blend high in saturated fatty acids was composed of 55 parts of an interesterified mixture containing 40% of palm oil and 60% of palm kernel oil, 5 parts of an interesterified mixture containing equal volumes of fully hardened palm oil and palm kernel oil, and 40 parts of high oleic acid sunflower oil (TRISUN). The oleic acid group received olive oil and a margarine made with 85 parts of sunflower oil high in oleic acid and 15 parts of a mixture of equal volumes of interesterified fully hydrogenated palm oil and palm kernel oil. For the *trans*-fatty acid diet, the high oleic acid sunflower oil was hydrogenated under conditions promoting isomerization, and 78 parts were then mixed with 10 parts of the unaltered oleic acid-rich sunflower oil, 10 parts of regular sunflower oil and 2 parts of low-erucic acid rapeseed oil.

At the start of the study the mean (± SD) serum total cholesterol level was 4.75 ± 0.64 mmol/L, and the mean body mass index 22.0 ± 2.3 kg/m^2. Body weight increased by 0.1 ± 1.0 kg over the 63 days of the study. Two women, but none of the men, smoked. Eight women used oral contraceptive agents.

Blood was sampled on the 18th and 21st day of each diet period and equal volumes of the two sera samples were pooled per subject.

Expt. III (Chapter 2) had the same design as experiment II. Between 29 January and 2 April 1990, 26 men and 30 women participated. The difference between the three diets consisted of 8% of energy which was provided by either stearic acid, linoleic acid, or the *trans*-monounsaturated fatty acid, elaidic acid. A fat high in stearic acid was made by interesterification of 41 parts of fully hydrogenated high linoleic acid sunflower oil, 50 parts of high oleic acid sunflower oil and 9 parts of regular high linoleic acid sunflower oil. For the *trans*-fatty acid diet, high oleic acid sunflower oil was hydrogenated so as to favor formation of *trans* fatty acids (Chapter 2). Seventy-five parts of this fat were mixed with 25 parts of the unmodified oleic-acid rich sunflower oil. For the linoleate diet regular sunflower oil was the major source of linoleic acid.

The mean initial serum total cholesterol level was 5.05 ± 0.79 mmol/L, and the mean body mass index 21.5 ± 2.1 kg/m^2. During the 9 weeks of the study body weight decreased by 0.2 ± 1.1 kg. Four men and four women smoked, and thirteen women used oral contraceptives.

Statistical analyses

In all three studies the distribution of serum Lp(a) levels on the diets as well as that of the individual responses of Lp(a) to the diets was highly skewed. A square root-transformation rather than a log-transformation proved optimal for normalizing the distribution of Lp(a) levels and responses. In order to check the robustness of the statistical analyses we analyzed both the original and the normalized data. Absolute Lp(a) concentrations are presented as median levels and square root-transformed values as means. The untransformed individual changes were analyzed with nonparametric tests: the Mann-Whitney rank-sum test for Expt. I was used to examine the difference in change between the monounsaturated fatty acid and the polyunsaturated fatty acid diet, while the Friedman test for Expts. II and III was used to evaluate diet effects. Responses calculated from the square root-transformed Lp(a) levels were analyzed using an unpaired *t*-test for Expt. I and analysis of variance (ANOVA) for Expts. II and III. When a significant diet effect was observed ($P<0.05$) in Expts. II and III, the three diets were compared pairwise; to correct for multiple comparisons only *P* values of less than 0.020 were then considered significant [15,16].

RESULTS

Effect of storage time on Lp(a) levels

Nine subjects had participated in both Expt. I and Expt. II, and ten subjects in both Expt. II and Expt. III. To examine the effect of storage, Lp(a) levels of the nine subjects when they were eating the monounsaturated or polyunsaturated diet in Expt. I were compared with their values on the oleic acid diet in Expt. II. For the subjects who participated in both Expt. II and Expt. III, Lp(a) concentrations on the oleic acid diet in Expt. II were compared with levels on the linoleate diet in Expt. III. These diets were chosen, because they had similar effects on Lp(a) (see below). Samples from Expt. I had been stored for 43 months, those from Expt. II for 31 months, and samples from Expt. III for 14 months. The nine samples of Expt. I were on average 5.4% lower than the corresponding samples of Expt. II. The ten samples of Expt. II were 12.2% lower than those of Expt. III. Thus, Lp(a) levels had decayed slightly with storage, but the order of ranking remained nearly unchanged (**Fig. 1**). For all 19 pairs combined the correlation coefficient was 0.96.

Expt. I: Monounsaturates versus polyunsaturates

Total fat intake of the participants was similar on all three diets (**Table 1**). The proportion of energy from the cholesterol raising saturated fatty acids (lauric acid, myristic acid and palmitic acid) decreased by 4.3% on the monounsaturated fatty acid diet and by 5.3% on the polyunsaturated fatty acid diet. The intake of stearic acid decreased by slightly less than 1% on both test diets. These decreases were compensated for by an increased intake of oleic acid (4.3%) and linoleic acid (3.6%) on the monounsaturated fatty acid diet and by linoleic acid alone (8.5%) on the polyunsaturated fatty acid diet.

In the group that switched from the control diet high in saturated fatty acids to the monounsaturated fatty acid diet the median Lp(a) concentration increased by 7 mg/L, from 84 to 91 mg/L (range of individual changes: −34 to 89 mg/L; **Table 2**). The median value of the polyunsaturated fatty acid group rose by 3 mg/L, from an initial 37 to 40 mg/L (range of changes: −32 to 46 mg/L). The difference in response between the two diet groups was not significant (Mann-Whitney test: $P=0.852$, t-test: $P=0.815$).

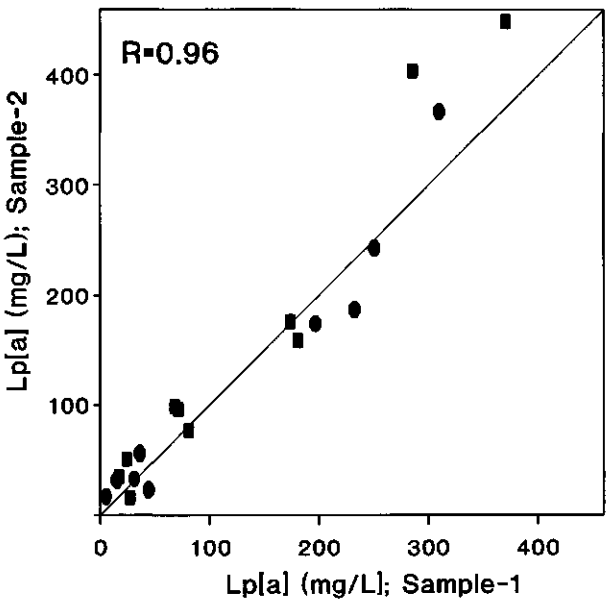

Fig. 1. Effect of storage on Lp(a) levels. Nine subjects had participated in both Expt. I and Expt. II (●) and 10 subjects in both Expt. II and Expt. III (■). To examine the effect of storage, Lp(a) levels of the 9 subjects when on the monounsaturated or polyunsaturated fatty acid diet in Expt. I (sample-1) were compared with their values on the oleic acid diet in Expt. II (sample-2). For the 10 subjects who participated in both Expt. II and Expt. III, Lp(a) concentrations on the oleic acid diet in Expt. II (sample-1) were compared with those on the linoleate diet in Expt. III (sample-2). Samples from Expt. I had been stored for 43 months, those from Expt. II for 31 months, and samples from Expt. II for 14 months.

Initial Lp(a) levels on the control diet high in saturated fatty acids were markedly though not significantly (Mann-Whitney test: $P = 0.224$, t-test: $P = 0.219$) higher in the monounsaturated-fatty acid than in the polyunsaturated-fatty acid group. As changes might occur only at relatively high levels, subjects were divided into two groups of 29 each, according to their Lp(a) concentration on the control diet high in saturated fatty acids. Of the 29 subjects with the highest initial Lp(a) levels, 17 were later to receive the monounsaturated fatty acid diet, and the other 12 the polyunsaturated fatty acid diet. For the monounsaturated fatty acid group initial median Lp(a) levels on the control diet were 151 mg/L (range: 45 – 340 mg/L)

Table 1. Dietary fatty acids in experiment I.

	Control Diet	Monounsaturated Fatty Acid Diet	Polyunsaturated Fatty Acid Diet
		% of daily energy intake	
Total fat	36.7	37.4	37.6
Saturated fatty acids	19.3	12.9	12.6
Lauric acid (C12:0)	1.2	0.7	0.9
Myristic acid (C14:0)	3.2	1.4	1.3
Palmitic acid (C16:0)	9.3	6.8	6.2
Stearic acid (C18:0)	4.1	3.2	3.4
Monounsaturated fatty acids	11.5	15.1	10.8
Cis-C18:1	10.2	14.5	10.2
Polyunsaturated fatty acids	4.6	7.9	12.7
Linoleic acid (cis,cis-C18:2n-6)	4.1	7.7	12.6

Values are based on chemical analyses of duplicate diets. All 58 subjects first consumed the control diet high in saturated fatty acids for 17 days; for the next 36 days 14 men and 15 women received the monounsaturated fatty acid diet, and 13 men and 16 women the polyunsaturated fatty acid diet. The intake of protein (13% of daily energy intake), carbohydrates (48 – 49%), alcohol (1 – 2%), cholesterol (33 – 66 mg/MJ), and other nutrients was virtually identical on all three diets.

and decreased by 1 mg/L to 150 mg/L (range of changes: −34 to 89 mg/L). In those who switched to the polyunsaturated fatty acid diet, Lp(a) declined by 8 mg/L, from 199 mg/L (range: 47 – 235 mg/L) to 191 mg/L (range of changes: -32 to 46 mg/L). Again, the difference in changes between the two groups did not reach statistical significance (Mann-Whitney test: $P = 0.929$, t-test: $P = 0.647$).

LDL cholesterol levels (Table 2) decreased by 0.59 mmol/L (23 mg/dL) on the monounsaturated and by 0.46 mmol/L (18 mg/dL) on the polyunsaturated fatty acid diet ($P = 0.05$ for difference between diets). Adherence to the diets was confirmed by changes in the fatty acid composition of serum cholesteryl esters. The proportion of oleic acid in serum cholesteryl esters decreased by 0.8% on the monounsaturated fatty acid group and by 4.6% on the polyunsaturated fat acid diet ($P < 0.001$). The level of linoleic acid increased by 4.2% and by 8.7%, respectively ($P < 0.001$).

Changes in Lp(a) levels, or the lack of them, were similar in man and women (Table 2), and in the five subjects who had suffered intercurrent illness [11] when compared with the other 54 subjects.

Table 2. Serum lipoprotein (a) and LDL-cholesterol levels in Experiment I.

Group	Control Diet	Mono- or Polyunsaturated Fatty Acid Diet
	mg/liter	
Lipoprotein(a)[a]		
Monounsaturated fatty acid group		
Men	45 (0-77)	52 (9-160)
Women	142 (0-340)	129 (0-336)
All	84 (0-340)	91 (0-336)
Polyunsaturated fatty acid group		
Men	37 (0-235)	36 (0-240)
Women	43 (0-220)	44 (0.237)
All	37 (0-235)	40 (0-240)
$\sqrt{}$ [Lipoprotein(a)][b]		
Monounsaturated fatty acid group		
Men	6.6 ± 5.0	6.7 ± 4.8
Women	10.9 ± 5.8	11.3 ± 5.8
All	8.8 ± 5.8	8.9 ± 5.7
Polyunsaturated fatty acid group		
Men	6.6 ± 5.1	6.5 ± 5.4
Women	7.3 ± 5.7	7.5 ± 5.7
All	7.0 ± 5.4	7.0 ± 5.5
LDL-cholesterol	*mmol/liter*	
Monounsaturated fatty acid group		
Men	3.05 ± 0.79	2.52 ± 0.73
Women	3.53 ± 0.65	2.89 ± 0.58
All[c]	3.30 ± 0.75	2.71 ± 0.67
Polyunsaturated fatty acid group		
Men	3.62 ± 0.68	3.06 ± 0.68
Women	3.09 ± 0.61	2.73 ± 0.52
All[c]	3.33 ± 0.68	2.87 ± 0.60

Fifty eight subjects first received the control diet high in saturated fatty acids for 17 days; for the next 36 days, 14 men and 15 women received a diet high in monounsaturated fatty acids, and 13 men and 16 women received a diet high in the polyunsaturated fatty acids.

[a] Values are median levels (ranges).

[b] Values are square root-transformed means ± SD.

[c] Denotes a significant difference in change between the two diet groups: $P<0.05$.

Expt. II: Saturates versus *cis*-monounsaturates versus *trans*-monounsaturates

The intake of the cholesterol-raising saturated fatty acids decreased by 8.5% of energy intake on the oleic acid diet and by 8.8% on the *trans*-fatty acid diet compared with the saturated fatty acid diet (**Table 3**). Stearic acid and total fat intake were similar on all three diets.

Table 3. Dietary fatty acids in experiment II.

	Saturated Fatty Acid Diet	Oleic Acid Diet	*Trans*-Fatty Acid Diet
		% of daily energy intake	
Total fat	38.8	39.6	40.2
Saturated fatty acids	19.4	9.5	10.0
Lauric acid (C12:0)	3.4	0.5	0.4
Myristic acid (C14:0)	2.7	0.5	0.7
Palmitic acid (C16:0)	8.1	4.7	4.3
Stearic acid (C18:0)	3.5	3.0	3.6
Monounsaturated fatty acids	14.7	24.1	24.2
Cis-C18:1	12.8	23.0	12.6
Trans-C18:1	1.8	0.0	10.9
Polyunsaturated fatty acids	3.4	4.6	4.6
Linoleic acid (cis,cis-C18:2n-6)	2.9	4.0	4.2

Values are based on chemical analyses of duplicate diets. Fifty-nine subjects received each diet for three weeks each in random order. The intake of protein (13 – 14% of daily energy intake), carbohydrates (46%), alcohol (1%), cholesterol (32 – 35 mg/MJ), and other nutrients was virtually identical on all three diets.

Table 4 shows that the median serum Lp(a) level was 26 mg/L on the saturated fatty acid diet and rose to 32 mg/L on the oleic acid diet (range of individual changes: −77 to +110 mg/L; Friedman test: $P=0.020$, ANOVA: $P=0.0024$) and to 45 mg/L on the *trans*-fatty acid diet (range of changes: −58 to +254 mg/L; Friedman test and ANOVA: $P<0.001$). The difference between the median Lp(a) levels on the *trans*-fatty acid and the oleic acid diet was also highly significant (range of changes: −32 to 144 mg/L; Friedman and ANOVA: $P<0.001$). The effects were observed to a similar extent in men and women.

Table 4. Serum lipoprotein(a) and LDL-cholesterol levels in Experiment II.

Group	Saturated Fatty Acid Diet	Oleic Acid Diet	*Trans*-Fatty Acid Diet
		mg/liter	
Lipoprotein(a)[a]			
Men	22 (0-220)	27 (0-200)	44 (0-297) [c,d]
Women	31 (0-447)	34 (0-484)[c]	48 (0-510) [c,d]
All	26 (0-447)	32 (0-484)[c]	45 (0-510) [c,d]
√[lipoprotein(a)][b]			
Men	5.6 ± 4.6	5.9 ± 4.6	6.8 ± 4.9 [c,d]
Women	5.5 ± 5.9	7.8 ± 5.9[c]	8.6 ± 6.4 [c,d]
All	6.5 ± 5.4	7.0 ± 5.4[c]	7.8 ± 5.8 [c,d]
LDL-cholesterol		*mmol/liter*	
Men	3.05 ± 0.66	2.59 ± 0.61[c]	2.93 ± 0.65 [c,d]
Women	3.20 ± 0.50	2.73 ± 0.48[c]	3.12 ± 0.58 [d]
All	3.14 ± 0.57	2.67 ± 0.54[c]	3.04 ± 0.61 [c,d]

Twenty five men and 34 women received each diet for 3 weeks each in random order.

[a] Values are median levels (ranges).

[b] Values are square root-transformed means ± SD.

[c] Significantly different from levels on the diet high in saturated fatty acids: $P<0.020$.

[d] Significantly different from levels on the diet high in oleic acid: $P<0.020$.

To examine the effect of intrinsic serum Lp(a) levels subjects were ranked according to their levels on the saturated fatty acid diet. **Fig. 2** shows that subjects with the highest innate Lp(a) levels had the greatest changes on both the oleic acid and the *trans*-fatty acid diet. Higher Lp(a) levels on the *trans*-fatty acid diet than on the saturated fatty acid diet were seen in 47 of the 59 subjects, while 40 subjects had higher Lp(a) levels on the oleic acid diet than on the saturated fatty-acid diet. Four subjects had no detectable amounts of Lp(a) on any diet.

Serum LDL cholesterol decreased by 0.47 mmol/L (18 mg/dL) on the oleic-acid diet ($P<0.001$) and by 0.10 mmol/L (4 mg/dL) on the *trans*-fatty acid diet ($P<0.001$) as compared with the saturated fatty acid diet (Table 4).

Relative to levels on the oleic acid diet, the proportion of oleic acid in serum cholesteryl esters decreased by 3.9% on the saturated fatty acid diet ($P<0.02$), and by 7.1% on the *trans*-fatty-acid diet ($P<0.02$). The percentage of *trans*-

monounsaturated fatty acids in serum cholesteryl esters was 0.13% on the oleic acid diet, 0.21% on the saturated fatty acid diet ($P<0.020$), and 1.34% on the *trans*-fatty acid diet ($P<0.020$).

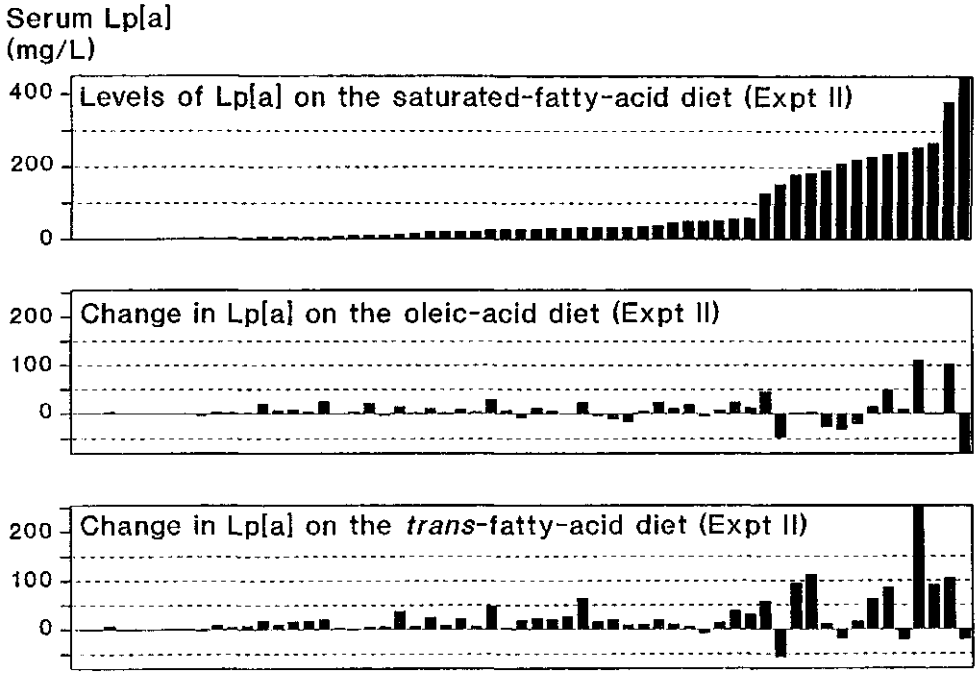

Fig. 2. Individual levels of serum Lp(a) in Expt. II (n = 59) on the diet high in the cholesterol-raising saturated fatty acids, and responses to the diet when 10% of energy from the cholesterol-raising saturated fatty acids was replaced by either oleic acid or *trans*-monounsaturated fatty acids.

Expt. III: Stearic acid versus linoleic acid versus *trans*-monounsaturates

Intake of the cholesterol-raising saturated fatty acids and of oleic acid differed by less than 1% between the three diets (**Table 5**). Nine percent of energy from stearic acid was replaced by 8.1% of linoleic acid on the linoleate diet and by 7.4% of *trans*-monounsaturated fatty acids on the *trans*-diet.

Table 5. Dietary fatty acids in experiment III.

	Stearate Diet	Linoleate Diet	*Trans*-Fatty Acid Diet
	% of daily energy intake		
Total fat	43.5	41.1	39.7
Saturated fatty acids	20.1	11.0	10.3
Lauric acid (C12:0)	0.5	0.7	0.5
Myristic acid (C14:0)	1.0	0.9	1.0
Palmitic acid (C16:0)	5.7	5.8	4.8
Stearic acid (C18:0)	11.8	2.8	3.0
Monounsaturated fatty acids	16.6	15.8	23.3
Cis-C18:1	15.4	14.7	14.6
Trans-C18:1	0.3	0.1	7.7
Polyunsaturated fatty acids	4.3	12.5	4.2
Linoleic acid (cis,cis-C18:2n-6)	3.9	12.0	3.8

Values are based on chemical analyses of duplicate diets. Fifty-six subjects received each diet for 3 weeks each in random order. The intake of protein (12 – 13% of daily energy intake), carbohydrates (44 – 47%), alcohol (1%), cholesterol (33 – 34 mg/MJ), and other nutrients was virtually identical on all three diets.

In this set of subjects the median Lp(a) level was 69 mg/L on both the stearate and the linoleate diets (**Table 6**). It rose to 85 mg/L on the *trans*-fatty acid diet (range of individual changes relative to stearic acid diet: −99 to 230 mg/L; Friedman test: $P=0.006$, ANOVA: $P=0.018$). The difference between the *trans*-fatty acid diet and the linoleate diet was also highly significant (Friedman test: $P<0.001$, ANOVA: $P=0.018$). Gender did not affect the changes observed.

As shown in **Fig. 3**, changes in Lp(a) on the *trans*-diet relative to the stearate diet were related to initial levels. Lp(a) increased on *trans*-fatty acids in 34 out of the 56 subjects, it decreased in 17 subjects, and 5 subjects showed no change.

The mean LDL cholesterol level was 3.00 mmol/L (116 mg/dL) on the stearate diet, 2.83 mmol/L (109 mg/dL) on the linoleate diet ($P<0.001$), and 3.07 mmol/L (119 mg/dL) on the *trans*-fatty acid diet (Table 6).

Table 6. Serum lipoprotein(a) and LDL-cholesterol levels in Experiment III.

Group	Stearate Diet	Linoleate Diet	*Trans*-Fatty Acid Diet
		mg/liter	
Lipoprotein(a)[a]			
Men	78 (0-298)	84 (1-310)	103 (0-351) [d]
Women	63 (0-749)	63 (0-782)	69 (0-891)
All	69 (0-749)	69 (0-782)	85 (0-891) [c,d]
√[lipoprotein(a)][b]			
Men	8.8 ± 5.5	8.8 ± 5.5	9.4 ± 5.9 [c,d]
Women	10.9 ± 7.8	11.0 ± 8.0	11.2 ± 8.2
All	9.9 ± 6.9	9.9 ± 7.0	10.3 ± 7.2 [c,d]
		mmol/liter	
LDL-cholesterol			
Men	3.16 ± 0.74	2.90 ± 0.71[c]	3.14 ± 0.65 [d]
Women	2.87 ± 0.66	2.78 ± 0.55	3.01 ± 0.66 [d]
All	3.00 ± 0.71	2.83 ± 0.63[c]	3.07 ± 0.65 [d]

Twenty six men and 30 women received each diet for 3 weeks each in random order.

[a] Values are median levels (ranges).

[b] Values are square root-transformed means ± SD.

[c] Significantly different from levels on the stearate diet: $P<0.020$.

[d] Significantly different from levels on the linoleate diet: $P<0.020$.

The proportion of linoleate in serum cholesteryl esters increased by 6.6% on the linoleate diet ($P<0.020$), but decreased by 1.6% on the *trans*-fatty acid diet ($P<0.020$) compared with the stearate diet. The proportion of *trans*-monounsaturated fatty acids in serum cholesteryl esters was 0.12% on the stearate diet, 0.13% on the linoleate diet, and 0.94% on the *trans*-fatty acid diet ($P<0.020$).

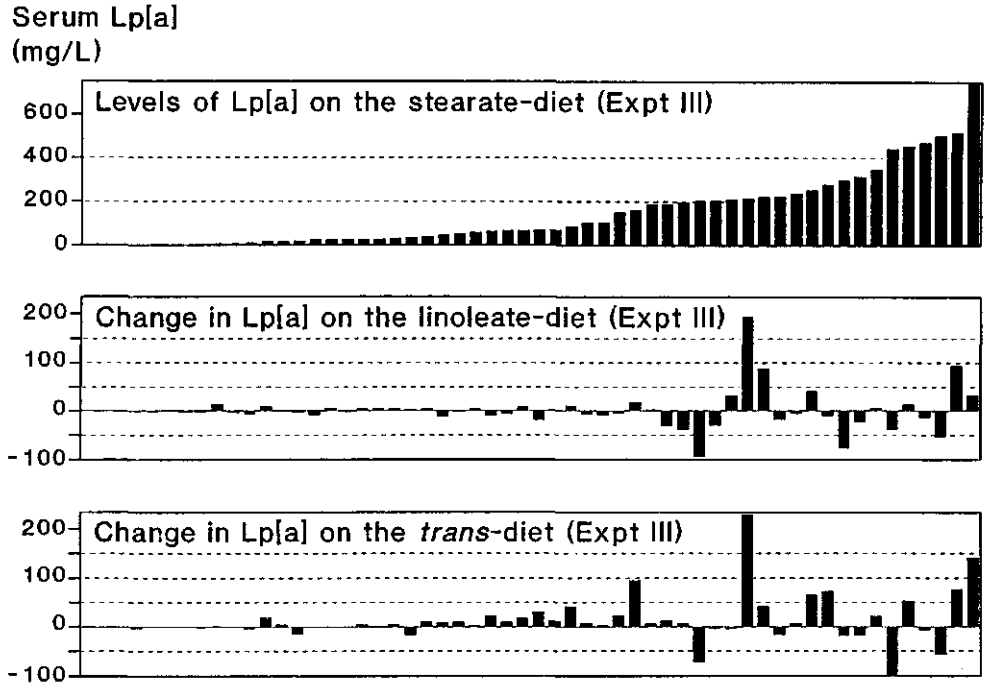

Fig. 3. Individual levels of serum Lp(a) in Expt. III (n = 56) on the diet high in the saturated fatty acid stearic acid, and responses to the diet when 8% of energy from stearic acid was replaced by either linoleic or *trans*-monounsaturated fatty acids.

Summary of results

These three studies together strongly suggest that *trans*-monounsaturated fatty acids elevate serum Lp(a) levels as compared with oleic acid, linoleic acid or stearic acid (Expts. II and III). Oleic acid and linoleic acid had similar effects on serum Lp(a) (Expt. I). The cholesterol-raising saturated fatty acids caused marginally lower Lp(a) levels than oleic acid in Expt. II. Changes were related to initial levels, and were similar for men and women.

DISCUSSION

The level of Lp(a) is largely under genetic control, and initial studies suggested that, unlike LDL, Lp(a) levels are singularly insensitive to diet. Although this suggestion appears to be correct as far as dietary cholesterol is concerned [9,17], dietary fat composition did affect Lp(a) concentrations in some [10,18], but not all [19,20], studies. Our results suggest that *trans*-monounsaturated fatty acids (*trans*-C18:1) increase Lp(a) levels relative to three other fatty acids with 18 carbon atoms: stearic acid (C18:0), oleic acid (*cis*-C18:1), and linoleic acid (*cis,cis*-C18:2). Compliance with the diets was very good, as indicated by changes in cholesteryl ester fatty acid composition.

We have shown recently in a 2 x 6 week double-blind cross-over trial that replacement of the habitual fat by palm oil lowered Lp(a) in healthy normolipidemic men [10]. We suggested that the effect observed was due either to a component in palm oil or to displacement of a component present in the habitual dietary fat. Our present findings are in agreement with the latter suggestion, as replacement of the habitual fat by palm oil decreased the intake of *trans* fatty acids by more than 50% [10]. Thus, the apparent Lp(a)-lowering effect of palm oil may have been due to displacement of *trans* fatty acids.

In Expt. II, Lp(a) levels were slightly, though significantly, lower on the diet high in the cholesterol-raising saturated fatty acids than on the oleic acid diet. In Expt. I, however, there was a only nonsignificant increase in median Lp(a) when subjects were switched from saturated fatty acids to oleic or linoleic acid. Apart from not being statistically significant, this slight change could also have been caused by an nonspecific drift with time, as a parallel design was used in Expt. I. Recently, Brown et al. [17] also reported similar Lp(a) levels on a saturated fatty acid diet and a diet high in polyunsaturated fatty acids. Although the level of the cholesterol-raising saturated fatty acids differed by less than 3% of total energy intake between the two diets [17] – the major exchange being between monounsaturated and polyunsaturated fatty acids, which, according to our results (Expt. I), have similar effects on Lp(a) – these findings do not support the evidence for an Lp(a)-lowering effect of saturated fatty acids.

Other studies have suggested that serum Lp(a) levels are not related to serum LDL cholesterol or apolipoprotein B levels [3,17]. This is confirmed by our present findings: depending on the type of fatty acid studied, diet-induced changes in Lp(a) and in LDL cholesterol or apolipoprotein B levels could be either in the opposite

direction, as in Expt. II where oleic acid lowered LDL cholesterol but raised Lp(a) relative to saturates, or in the same direction, as in Expt. III where *trans* fatty acids raised both LDL cholesterol and Lp(a) relative to linoleic acid. These observations make it less likely that dietary effects on LDL cholesterol and Lp(a) levels are mediated by the same pathway.

Changes were positively related to intrinsic Lp(a) levels as has also been observed by others [6,10].

It has been reported that storage of serum samples at −20 °C for 6 months [21] or even for up to seven years does not affect serum Lp(a) levels [22], when ELISA-kits were used from the same manufacturer as ours. Our results, however, do suggest that Lp(a) levels decay slightly with storage. However, in each experiment each subject was sampled on two (Expt. I) or three (Expts. II and III) different diets, and only changes in Lp(a) between diets within one experiment were compared. Thus, a slight overall decay in Lp(a) might underestimate slightly the extent of response of Lp(a) to diet, but is highly unlikely to affect the direction of this response. Also, the significance of responses was similar when tested by two different statistical approaches – the conventional *t*-test and ANOVA versus nonparametric tests – which supports the robustness and consistency of the effects observed.

The experimental diets were consumed for 3 – 5 weeks. Although 3 weeks is long enough for serum total or LDL lipoprotein cholesterol levels to stabilize [11], we cannot exclude the possibility that the observed changes in Lp(a) by us are transient or, alternatively, underestimated and not yet at their maximum level. Our results do suggest that the observed effects are proportional to the amounts of fatty acids consumed. The intake of *trans*-monounsaturated fatty acids in Expt III was about 4% of daily energy intake lower than in Expt. II, while the changes in Lp(a) were also less pronounced (Tables 4 and 6).

In conclusion, these short-term dietary experiments suggest that diets high in *trans*-monounsaturated fatty acids may increase serum levels of Lp(a).

Additional note

Recently, Nestel et al. [23] reported that a diet rich in the *trans* fatty acid elaidic acid elevated the levels of Lp(a) in mildly hypercholesterolemic men. Our findings our in good agreement with this observation.

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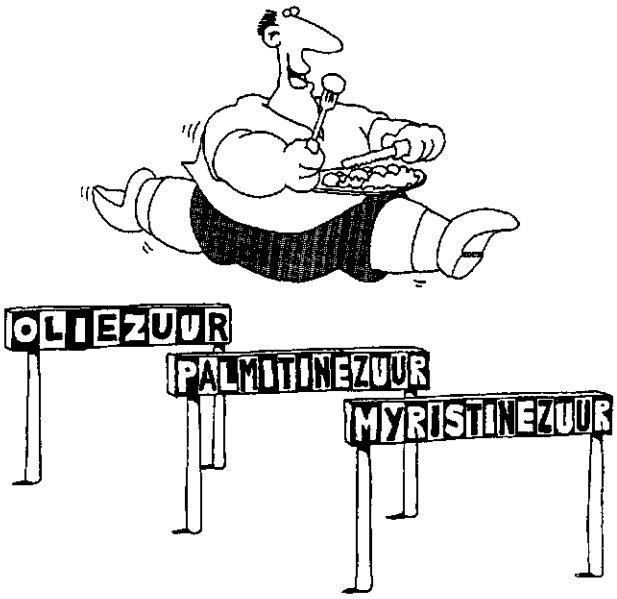
CHAPTER 5

Impact of myristic versus palmitic acid on serum lipid and lipoprotein levels in healthy women and men



Zock PL, de Vries JHM, Katan MB.
Arteriosclerosis and Thrombosis 1994; **14**: 567-575.

ABSTRACT

Aim. The cholesterol-raising effect of dietary saturated fatty acids is largely accounted for by lauric, myristic, and palmitic acid. Dairy fat is a major source of myristic acid, while palm oil is especially rich in palmitic acid. Myristic acid is suspected of being much more cholesterolemic than palmitic acid, but direct comparisons were lacking.

Methods. We therefore fed 36 women and 23 men three diets which differed from each other in palmitic, oleic and myristic acid content by about 10% of total energy. We used palm oil, high-oleic acid sunflower oil, and a specially produced high-myristic acid fat to achieve these differences. Each diet was supplied for 3 weeks, in random order.

Results. Mean serum cholesterol was 4.53 mmol/L on the high oleic acid diet, 4.96 mmol/L on the palmitic acid diet, and 5.19 mmol/L on the myristic acid diet ($P<0.0001$ for all comparisons). Myristic acid raised low-density (LDL) cholesterol level by 0.11 mmol/L, high-density (HDL) cholesterol by 0.12 mmol/L, and apolipoprotein (apo) A-I by 7.2 mg/dL relative to palmitic acid; increases relative to oleic acid were 0.50 mmol/L for LDL, 0.15 mmol/L for HDL cholesterol, 6.0 mg/dL for apoB, and 8.9 mg/dl for apoA-I ($P<0.01$ for all comparisons). The HDL cholesterol and apoA-I levels on the palmitic and the oleic acid diets were the same. None of the responses differed significantly between women and men.

Conclusion. Myristic and palmitic acid both caused high LDL cholesterol and apoB levels and low HDL to LDL ratios. Thus, diets for the treatment of hypercholesterolemia should be low in myristic and palmitic acids.

INTRODUCTION

Reducing saturated fat (and cholesterol) intake is the therapy of choice in the treatment of moderate hypercholesterolemia, and an important adjunct to drug therapy in the treatment of severe hypercholesterolemia [1]. Restriction of saturated fatty acids in the diet is more effective than limiting total fat consumption [2]. However, saturates with different chain lengths may differ in their cholesterol-raising effect. Lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0) are thought to increase cholesterol levels, while stearic acid (C18:0) has little or no effect [3-7]. In experimental animals, saturated fatty acids raise plasma cholesterol through down-regulation of LDL receptor activity, and subsequent accumulation of LDL cholesterol in plasma and increased LDL production from its precursor VLDL [8,9]. These effect are seen with lauric, myristic, and palmitic acid, but not with medium-chain saturates (C6:0, C8:0, and C10:0) or stearic acid [10]. In humans lauric acid has been found to be less potent than palmitic acid in raising total and LDL cholesterol [4,11,12]. Myristic and palmitic acid together make up about 25–30% of the fat in Western diets (D. Kromhout, *unpublished data*, 1988), but their relative cholesterol-raising potencies have not been clearly defined in man. Meta-analyses of dietary trials that employed commercially available fats indicated that myristic acid might be four to six times more cholesterolemic than palmitic acid [4,13]. Some recent studies also suggest that palmitic acid lowers cholesterol relative to a mixture of lauric and myristic acid [14-16]. However, others have found that palmitic acid strongly increases cholesterol levels [5,7,12,17]. A problem in studies with natural fats [4,13-16] is that the amount of myristic acid is linked with either the amount of lauric acid, such as in coconut oil, or with that of palmitic acid, such as in dairy fat. The effects of different saturates are therefore hard to examine. An early study with specially synthesized fats by McGandy et al. [11] did not resolve the issue. Synthetic fats are expensive, and the effort has not been repeated until now.

Myristic acid is the third common saturate in the diet. Average intake levels are about 1 g/d in Japan, 6 g/d in the United States, 8 g/d in the Netherlands, and 14 g/d in eastern Finland (Kromhout et al., unpublished data, 1988). Major sources are butter fat, which is also rich in palmitic acid, and two vegetable oils, coconut oil and palm kernel oil; the latter two also contain large amounts of lauric acid. Palm oil, another vegetable oil that is high in saturated fatty acids, is low in

myristic acid and high in palmitic acid. Palm oil is the number one edible oil worldwide, and its consumption is rising. If much of the cholesterol-raising effect of saturated fatty acids is indeed specifically due to myristic acid, then palm oil would be a suitable substitute for animal fats and hydrogenated vegetable oils [18] in a wide range of products for cholesterol-lowering diets. Also, modern biotechnology could be applied to replace myristic with palmitic acid in other fats.

To investigate the relative potencies of myristic and palmitic acids, we produced a fat high in myristic acid and compared its effects with those of palmitic and oleic acids on lipoprotein levels in humans. Serum lipids have an important role in the atherosclerotic process in both women and men [19], but data on the differences in dietary effects on serum lipids between women and men are scarce [20]. To determine whether women and men have similar lipoprotein responses to dietary saturated fat, we included enough female volunteers to allow detection of gender-specific responses.

SUBJECTS AND METHODS

Hypotheses, design, and statistical analysis

The objective of the trial was to estimate the effects of myristic and palmitic acids relative to each other and to oleic acid on serum lipids and lipoproteins in healthy women and men. The study was designed to detect a significant ($P = 0.05$) effect of myristic acid versus palmitic acid on total and LDL cholesterol with a power of 80% if the real population effect exceeded 0.13 mmol/L. The a priori power was also 80% for detecting a difference in response of total and LDL cholesterol of 0.30 mmol/L between women and men.

The trial consisted of three consecutive 3-week periods, during which each participant consumed each of the three diets. Our experience agrees with that of earlier workers [3,21] in that serum lipid and lipoprotein levels stabilize within 2 weeks after a dietary change [22,23]. One diet was high in palmitic acid, another high in myristic acid, and the third high in oleic acid. Thus, there were six possible treatment sequences. Each of the six diet sequence groups had the same ratio of men to women and of women using and not using oral contraceptives. In this way,

bias due to the order in which the diets were consumed or to drift of variables over time was eliminated [24]. All subjects participated simultaneously, from January 27 until March 30, 1992.

The data were analyzed with the General Linear Models (GLM) procedure of the SAS Program [25]. When the ANOVA indicated a significant effect of diet ($P<0.05$), the Tukey correction was used for pairwise comparisons of the diets and for calculation of 95% confidence limits for the differences between two diets.

Subjects

Seventy-nine persons responded to calls via local newspapers and posters in university buildings. Five women and 2 men withdrew before or during the screening procedure. Of the remaining 42 women and 30 men, 2 men were excluded because they consumed over 10% of daily energy intake as alcohol, 3 men and 3 women because their serum cholesterol levels exceeded 7.1 mmol/l or their blood pressure was over 140/90 mmHg, and 2 women for medical reasons as judged by an independent physician. One man and 1 woman withdrew after the screening because their partner was excluded for one of the reasons mentioned above, and one man for unstated reasons. The 36 women and 23 men who entered the study were all apparently healthy, as indicated by a medical questionnaire. None had anemia, glycosuria, proteinuria, and none were taking medications known to affect blood lipids. Eleven women used oral contraceptives, and 6 women and 2 men smoked cigarettes. Pre-experimental fasting serum cholesterol levels ranged from 3.67 to 7.10 mmol/L (mean, 5.06 mmol/L). The women were between 18 and 55 years old (mean, 29 years) and weighed between 53 and 83 kg (mean, 65 kg); their body mass index ranged from 19.0 to 26.8 kg/m^2 (mean, 22.4 kg/m^2). The men's ages ranged from 18 to 62 years (mean, 28 years), they weighed between 59 and 97 kg (mean, 76 kg), and their body mass index was 17.9 to 32.4 kg/m^2 (mean, 22.3 kg/m^2).

Approval for the study had been obtained from the Ethics Committee of the department, and the protocol and aims of the study were fully explained to the subjects, who gave their written informed consent. No reward was given, except for the food, which was free.

Diets

The diets consisted of conventional solid foods, and 21 different menus were provided over the course of each 3-week cycle. The nutrient composition of each diet was similar, except that approximately 10% of total energy intake was provided by either myristic acid, palmitic acid, or oleic acid. These differences were achieved by the use of special margarines (**Table 1**). To prepare a fat high in myristic acid, 46 parts of myristic acid were mixed with 10.8 parts of glycerol, 10.8 parts of stearic acid, 29 parts of oleic acid, and 3.5 parts of linoleic acid. This mixture was then heated under reduced pressure with 0.2% by weight tetrabutyl titanate as a catalyst until the reaction was complete, cooled, refined with diluted sodium hydroxide, dried, and filtered. The resulting product was free of titanium (detection limit, 5 mg/kg). Subsequently the triglycerides were dissolved at 45 °C in acetone, cooled and partly crystallized in a surface-scraped heat exchanger. After filtering off a fraction consisting mainly of trimyristate, the second crystallization yielded the desired fraction. The final fat had a myristic acid content

Table 1. Fatty acid composition of margarines used in the three study diets.

Fatty Acid	Margarine, g/100 g Fatty Acid		
	Myristic Acid Rich	Palmitic Acid Rich	Oleic Acid Rich
Saturated	65.5	58.5	19.2
Lauric acid (C12:0)	0.3	0.1	0.0
Myristic acid (C14:0)	50.7	1.1	0.1
Palmitic acid (C16:0)	1.0	45.6	6.5
Stearic acid (C18:0)	13.4	11.2	12.0
Monounsaturated	28.4	30.2	71.4
Oleic acid (*cis*-C18:1)	25.4	29.6	69.8
Elaidic acid + isomers (*trans*-C18:1)	3.0	0.3	0.5
Polyunsaturated	4.6	11.2	9.4
Linoleic acid (*cis,cis*-C18:2)	3.8	10.9	9.3
Trans isomers of C18:2	0.7	0.2	0.0
Others	1.5	0.1	0.1

The special margarine supplied 53% of the total fat in the myristic acid, 66% in the palmitic acid, and 54% in the oleic acid diets.

of 50.7 g/110 g fatty acids, with 10.2% of the triglycerides being trimyristate. A high-palmitic acid margarine was produced by blending 80.5 parts of fractionated palm oil, 12.5 parts cotton seed oil, and 7 parts fully hydrogenated sunflower oil free of *trans* fatty acids. It contained 45.6 g palmitic acid/100 g fatty acids, and 5.0% of all triglycerides was tripalmitate. Fat for the oleic acid diet was manufactured from a blend of 77 parts of high-oleic acid sunflower oil (Trisun, SVO Enterprises), 3.5 parts fully hydrogenated sunflower oil, 3.5 parts unmodified high-linoleic acid sunflower oil, 3 parts fractionated palm oil, and 13 parts of an interesterified mixture of sunflower oils (47% high-oleic, 15% unmodified high-linoleic, and 38% fully hydrogenated sunflower oil). The fats were developed by the Unilever Research Laboratory, Vlaardingen, The Netherlands, and manufactured in collaboration with Unichema Chemie, Gouda, The Netherlands, and the Unilever Research Laboratory, Colworth, England. The respective margarines were developed by the Unilever Research Laboratory, Vlaardingen. These margarines were used as spreads with the bread meals, in sauces and gravies, and for the preparation of a special bread containing 7% fat. Cookies prepared with palmitic acid-rich margarine and small amounts of olive oil and sunflower oil were used to fine-tune the fatty acid composition of the experimental diets.

Before the trial, participants recorded their usual diet for 1 weekend day and 2 working days as described (Chapter 2). The study diets were formulated at 30 levels of energy intake, ranging from 5.5 to 20 MJ per day, so that each subject received a diet that met his or her energy needs. Body weights were recorded twice weekly, and energy intake was adjusted when necessary as to maintain a stable weight. Over the 63 days of the trial average body weight fell by 0.4 ± 1.2 kg (range, −2.2 to 2.3 kg). The mean difference in body weight at the end of the dietary treatments ranged from −0.1 ± 0.7 kg (individual range, −2.2 to 1.6 kg) between the palmitic acid and the oleic acid diets to 0.0 ± 0.7 kg (range, −1.6 to 1.5 kg) between the myristic acid and the palmitic acid diets.

Each study diet was assigned a color code that was used for labeling all packages and foods supplied during the trial. In this way, the subjects were blinded as to the nature and the sequence of the diets. All foodstuffs were weighed out for each subject. On week days at noon, hot meals were served and eaten at the department. All other food was supplied daily as a package. Food for the weekend and guidelines for its preparation were provided on Fridays. In addition to the food supplied, subjects were allowed a limited number of items free from fat and

cholesterol. The energy intake from these free-choice items was fixed for each level of daily energy intake and ranged from 6% to 10% of total energy. Subjects were urged not to change their selection of free-choice items throughout the study. They were also repeatedly asked to maintain their usual pattern of physical activity and not to change their smoking habits, consumption of coffee, or use of oral contraceptives. The participants kept diaries in which they recorded any sign of illness, medication used, the consumption of free-choice items, and any deviations from their diets. At the end of the trial the subjects were asked to complete an anonymous questionnaire regarding the blinding of the diets, problems, and non-compliance during the study.

Duplicate portions of each study diet were collected on each of the 63 trial days for an imaginary participant with a daily energy intake of 11 MJ (2630 kcal), stored at −20 °C, and pooled and analyzed after the study. Records of the free-choice items were coded, and their energy and nutrient content [26] were combined with the analyzed values of the food supplied (**Table 2**).

Blood sampling and analysis

All participants were assigned a random number that was used for labeling blood and serum tubes. In this way the laboratory technicians were unaware of the subjects' diet sequence. Blood samples were taken after a 12-hour fast on days 1, 17, and 21 (period 1), days 38 and 42 (period 2), and days 59 and 63 (period 3). All venipunctures were performed by the same technician, in the same location, and at the same time of the same days of the week. Serum was obtained by low speed centrifugation within 1 hour of venipuncture, stored at −80 °C, and analyzed enzymatically for total cholesterol, HDL cholesterol, and triglycerides [27-29]. All samples from a particular subject were analyzed within one run. The coefficient of variation within runs was 1.1% for total cholesterol, 1.3% for HDL cholesterol, and 1.9% for triglycerides. Mean bias with regard to the target values of serum pools provided by the Centers for Disease Control and Prevention, Atlanta, GA, was −0.10 mmol/L for total cholesterol, 0.0 mmol/L for HDL cholesterol, and 0.07 mmol/L for triglycerides. LDL cholesterol was calculated using the Friedewald equation [30].

Apolipoproteins were measured by Dr. A. von Eckardstein at the 'Institut für Klinische Chemie und Laboratoriumsmedizin', Münster, Germany, by using a

Table 2. Mean daily intake of energy and nutrients of the subjects while on the three study diets.

Energy/Nutrient	Diet		
	Myristic Acid	Palmitic Acid	Oleic Acid
Energy			
MJ/d	11.9 ± 2.6	11.7 ± 2.6	11.7 ± 2.7
kcal/d	2841 ± 627	2807 ± 624	2806 ± 634
Protein, % of energy	13.2	12.6	12.4
Fat, % of energy	39.2	39.6	38.5
Saturated fatty acids	21.3	21.0	10.8
Lauric acid (C12:0)	0.4	0.3	0.3
Myristic acid (C14:0)	11.3	1.1	0.8
Palmitic acid (C16:0)	4.7	14.9	5.0
Stearic acid (C18:0)	4.3	4.1	3.8
Monounsaturated fatty acids	11.9	12.1	21.6
Oleic acid (*cis*-C18:1)	10.9	11.6	20.9
Elaidic acid (*trans*-C18:1)	0.7	0.2	0.3
Carbohydrates, % of energy	46.9	47.2	48.3
Alcohol, % of energy	0.7	0.6	0.8
Cholesterol			
mg/d	344.9	358.9	351.8
mg/MJ	29.0	30.6	30.0
Dietary fiber			
g/d	42.0	43.0	39.9
g/MJ	3.5	3.7	3.4

Each subject consumed each diet for 3 weeks in random order. Values are based on chemical analyses of duplicate diets plus the calculated contribution of free-choice items (see Methods). Each value represents the mean of three independent duplicates collected in three different periods during which each diet was consumed by one third of the subjects. Variations between periods were negligible.

turbidimetric method on microtiter plates as described [31]. Lipoprotein and apolipoprotein values obtained at the two sampling days at the end of each dietary period were averaged for data analyses.

The fatty acid composition of serum cholesteryl esters was determined in samples obtained at the end of each dietary period (days 21, 42, and 63) as described [32](Chapter 2). Results are expressed as proportion by weight of all fatty acids detected.

RESULTS

Diets and dietary adherence

The analyzed composition of the diets agreed with our objectives: about 10% of daily energy from oleic acid was replaced by palmitic acid or myristic acid, and other dietary constituents, notably lauric acid and trans fatty acids, were virtually unchanged (Table 2). Fifty-seven of the 59 subjects returned the anonymous questionnaire that was handed out at the end of the trial. The blinding was successful with respect to the myristic and palmitic acid diets, but most subjects had recognized the oleic acid diet. Neither the questionnaires nor the diaries revealed any deviations from the protocol that might have affected the results. The largest deviation reported in the questionnaire was the consumption by one subject of two herrings over the full 9-week study period. Adherence to the study diets was confirmed by the fatty acid composition of the serum cholesteryl esters, which uniformly followed the dietary composition (**Table 3**). In all 59 subjects the proportion of myristic acid in the cholesteryl esters was higher on the myristic acid diet than on either the palmitic or the oleic acid diet. The proportion of palmitic acid was increased on the palmitic acid diet in 58 out of 59 subjects relative to the myristic or the oleic acid diet, and all 59 subjects showed the highest proportion

Table 3. Fatty acid composition of serum cholesteryl esters on the three study diets.

Fatty Acid	Diet, g/100 g Fatty Acid		
	Myristic Acid	Palmitic Acid	Oleic Acid
C14:0	2.83 ± 0.62[a,b]	0.65 ± 0.17	0.66 ± 0.16
C16:0	9.25 ± 0.64 [b]	10.87 ± 0.54[a]	9.18 ± 0.65
C16:1n-7	2.67 ± 0.57 [a]	2.71 ± 0.56[a]	2.24 ± 0.86
C18:0	0.95 ± 0.15[a,b]	0.91 ± 0.16[a]	0.83 ± 0.12
C18:1n-9	16.76 ± 1.11 [a]	16.89 ± 1.01[a]	23.15 ± 1.83
C18:2n-6	54.56 ± 2.95[a,b]	56.12 ± 2.77[a]	51.34 ± 3.14
C20:4n-6	6.53 ± 1.20[a,b]	6.03 ± 1.07[a]	6.64 ± 1.20
Other	6.63 ± 0.83	5.83 ± 0.76	5.96 ± 1.00

Values are mean ± SD. The 59 subjects consumed each diet for 3 weeks in random order.

[a] Significantly different from the oleic acid diet, $P<0.02$.

[b] Significantly different from the palmitic acid diet, $P<0.02$.

of oleic acid in their cholesteryl esters when on the oleic acid diet. All these differences are highly significant ($P<0.0001$).

Serum lipids and lipoproteins

The mean levels of serum total and lipoprotein cholesterol and triglycerides on the three diets are shown in **Table 4.**

Table 4. Serum lipid and lipoprotein cholesterol levels on the three study diets.

Fatty Acid	Diet		
	Myristic Acid	Palmitic Acid	Oleic Acid
Total cholesterol			
All	5.19 ± 0.90[a,b]	4.96 ± 0.85[a]	4.53 ± 0.81
Women	5.27 ± 0.79[a,b]	5.03 ± 0.78[a]	4.67 ± 0.75
Men	5.08 ± 1.04[a,b]	4.85 ± 0.96[a]	4.32 ± 0.87
HDL cholesterol			
All	1.65 ± 0.37[a,b]	1.52 ± 0.33	1.50 ± 0.30
Women	1.76 ± 0.31[a,b]	1.63 ± 0.29	1.62 ± 0.25
Men	1.46 ± 0.38[a,b]	1.34 ± 0.32	1.32 ± 0.28
LDL cholesterol			
All	3.09 ± 0.78[a,b]	2.98 ± 0.72[a]	2.60 ± 0.71
Women	3.07 ± 0.75[a]	2.96 ± 0.71[a]	2.63 ± 0.71
Men	3.14 ± 0.83[a]	3.01 ± 0.75[a]	2.55 ± 0.73
HDL to LDL ratio			
All	0.56 ± 0.17[a,b]	0.54 ± 0.17[a]	0.62 ± 0.20
Women	0.61 ± 0.17[a]	0.58 ± 0.16[a]	0.66 ± 0.19
Men	0.49 ± 0.15[a]	0.47 ± 0.15[a]	0.56 ± 0.20
Triglycerides			
All	0.99 ± 0.52	1.00 ± 0.55	0.95 ± 0.43
Women	0.96 ± 0.42	0.95 ± 0.39	0.93 ± 0.36
Men	1.05 ± 0.65	1.10 ± 0.72	1.00 ± 0.54

Values are mean ± SD. The 23 men and 36 women consumed each diet for 3 weeks in random order. To convert values for total, HDL, and LDL cholesterol to milligrams per deciliter, multiply by 38.67. To convert values for triglycerides to milligrams per deciliter, multiply by 88.54.

[a] Significantly different from the oleic acid diet, $P<0.02$.

[b] Significantly different from the palmitic acid diet, $P<0.02$.

Fig. 1 depicts the changes in serum cholesterol levels on myristic acid and palmitic acid diets relative to the oleic acid diet. Compared with the level on the oleic acid diet, total cholesterol increased by 0.66 mmol/L ($P<0.0001$; 95% CI, 0.54 to 0.79 mmol/L) on the myristic acid diet and by 0.43 mmol/L ($P<0.0001$; 95% CI, 0.30 to 0.56 mmol/L) on the palmitic acid diet. The difference in total cholesterol between the myristic and the palmitic acid diet was 0.23 mmol/L ($P<0.0001$; 95% CI, 0.11 to 0.36 mmol/L). LDL cholesterol rose by 0.50 mmol/L (95% CI, 0.40 to 0.60 mmol/L) on the myristic acid diet, and by 0.38 mmol/L (95% CI, 0.28 to 0.48 mmol/L) on the palmitic acid diet compared with the level on the oleic acid diet ($P<0.0001$ for both comparisons). The difference in LDL cholesterol of 0.11 mmol/L between the two saturated fat diets was also statistically significant ($P=0.0086$; 95% CI, 0.01 to 0.22 mmol/L). The mean HDL cholesterol level on the myristic acid diet was higher than on the two other diets

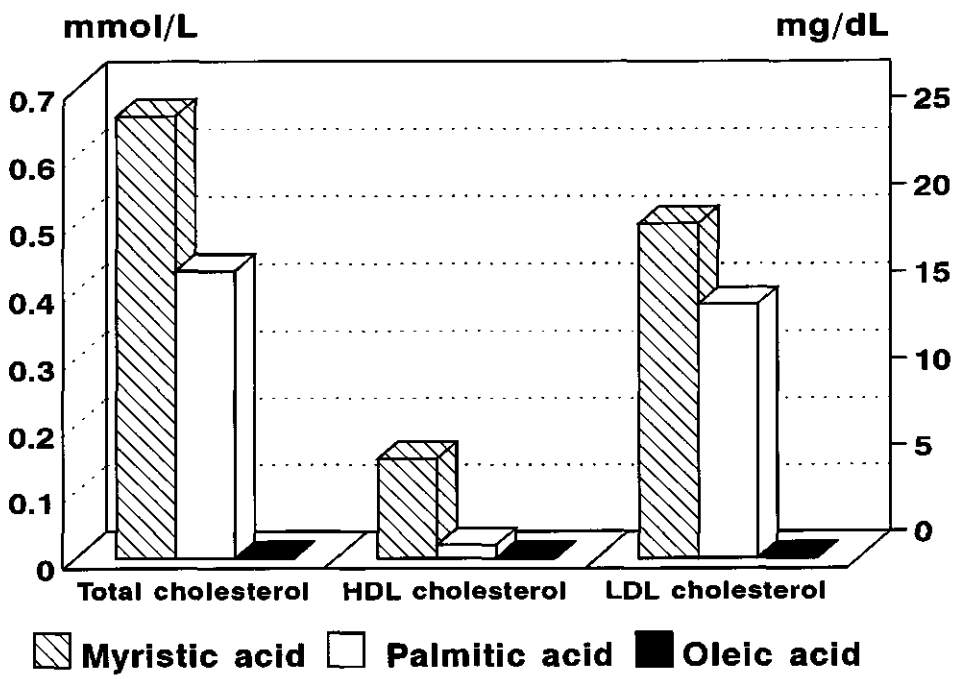


Fig. 1. Bar graph showing mean responses of total, HDL, and LDL cholesterol to myristic and palmitic acid diets relative to the level on the oleic acid diet. Differences between the myristic and palmitic acid diets were all statistically significant ($P<0.01$).

(Table 4 and Fig. 1), increasing by 0.12 mmol/L (95% CI, 0.05 to 0.18 mmol/L) compared with the palmitic acid diet and by 0.15 mmol/L (95% CI, 0.10 to 0.19 mmol/L) compared with the oleic acid diet ($P<0.0001$ for both comparisons). The HDL cholesterol- raising effect of myristic acid was evident in 51 of the 59 subject. Mean HDL cholesterol levels on the palmitic and the oleic acid diets were the same. The HDL to LDL cholesterol ratio on the myristic acid diet was significantly lower than that on oleic acid ($P<0.0001$) but slightly higher than that on the palmitic acid diet ($P=0.0133$). Serum triglyceride levels did not differ significantly between any of the diets (Table 4).

The rise of apolipoprotein A-I (**Table 5**) paralleled the change observed in HDL cholesterol (Table 4), although not to the same extent; the mean ratio of apoA-I to HDL cholesterol was 994 mg/mmol on both the palmitic acid and oleic acid diets, but it decreased significantly to 966 mg/mmol on the myristic acid diet ($P<0.0006$). ApoB levels on the myristic acid and palmitic acid diets were both higher than on the oleic acid diet ($P<0.0001$), but unlike LDL cholesterol, apoB levels did not differ between the two saturated fat diets (Table 5).

The responses of lipid, lipoprotein, or apolipoprotein levels to the diets did not differ significantly between men and women, or between women using and not using oral contraceptives.

Table 5. Serum apoA-I and apoB levels on the three study diets.

Fatty Acid	Diet		
	Myristic Acid	Palmitic Acid	Oleic Acid
ApoA-I, mg/dL			
All	154.6 ± 21.0[a,b]	147.4 ± 21.1	145.8 ± 18.9
Women	160.8 ± 19.7[a,b]	154.9 ± 20.6	153.0 ± 17.8
Men	145.0 ± 19.7[a,b]	135.7 ± 16.1	134.5 ± 14.8
ApoB, mg/dL			
All	74.6 ± 16.6[a]	74.3 ± 15.9[a]	68.6 ± 16.6
Women	73.6 ± 15.6[a]	74.0 ± 15.5[a]	68.5 ± 16.3
Men	76.1 ± 18.3[a]	74.7 ± 16.7[a]	68.6 ± 17.6

Apo indicates apolipoprotein. Values are mean ± SD. The 23 men and 36 women consumed each diet for 3 weeks in random order.

[a] Significantly different from the oleic acid diet, $P<0.02$.

[b] Significantly different from the palmitic acid diet, $P<0.02$

DISCUSSION

Myristic acid versus palmitic acid

The relative cholesterol-raising potential of the various saturated fatty acids has been controversial for many years. Several reports [4,11-16,33] indicate that the cholesterol-raising saturated fatty acids (lauric, myristic, and palmitic acids) differ in their effects on cholesterol levels. No study has directly examined myristic acid except that of McGandy et al. [11], who conclude that both myristic and palmitic acid raise total cholesterol. However, a consistent difference between the two could not be detected, in part because the number of subjects was small. In the present trial a large group of healthy volunteers consumed three strictly controlled diets in which either myristic acid or palmitic acid was exclusively substituted for oleic acid. Our data show that myristic acid is about 1.5 times as cholesterol-raising as palmitic acid, which is much less than the factor of 4 to 6 suggested by meta-analytical studies [4,13]. The high values found in meta-analyses [4,13] could be a statistical artifact, because in experiments that use natural fats the intake of myristic acid is strongly correlated with that of either lauric or palmitic acid. Such "collinearity" [34] may produce unreliable outcomes of multiple regression analyses. Other investigators reported that palm oil, rich in palmitic acid, causes remarkably lower cholesterol levels than coconut oil, which is rich in lauric and myristic acid [14-16,33]. Ng et al. [16] therefore suggested that myristic acid is the major contributor to the cholesterol-raising effect of saturated fatty acids and that palmitic acid may be neutral, just like stearic and oleic acid. This, however, is not confirmed by the present study. Hayes and Khosla [35,36] proposed that palmitic acid might have a conditional effect on serum cholesterol, with palmitic acid being cholesterol-raising in hypercholesterolemic subjects (>5.8 mmol/L or 225 mg/dL), but not in normocholesterolemic subjects. Our findings do not support this: the increase of total cholesterol on the palmitic acid diet relative to the oleic acid diet of subjects in the lowest tertile of initial cholesterol level (initial level, 3.78 mmol/L; increase 0.37 mmol/L) was similar to that of subjects in the highest tertile (initial level, 5.97 mmol/L; increase, 0.42 mmol/L). Myristic acid did raise cholesterol more than palmitic acid, but about one half of the effect was due to HDL cholesterol. Both myristic and palmitic acids

markedly raised LDL cholesterol and apoB levels compared with oleic acid. This accords with other well-controlled studies in which palmitic acid was shown to raise LDL cholesterol relative to that produced by oleic acid [5,7,12].

It must be noted that we investigated a synthetic myristic acid-rich fat in which myristic acid is randomly attached to glycerol molecules. In contrast, the triglycerides in natural coconut oil or dairy fat have a specific fatty acid distribution that might modulate the effect of myristic acid on cholesterol metabolism [37]. However, in our experience two dietary fats with equal total fatty acid composition but contrasting positional distribution of saturated fatty acids resulted in highly similar serum lipid and lipoprotein levels in volunteers (Chapter 6). It is therefore unlikely that myristic acid in synthetic fats would have a totally different effect on serum lipoproteins than in coconut oil or in dairy fat.

Changes in HDL cholesterol

Myristic acid increased HDL cholesterol by about 9% as compared with palmitic acid and oleic acid. The apoA-I to HDL cholesterol ratio was lower on the myristic acid diet than on both other diets which indicates an increase in the less dense HDL particles and suggests that the change in HDL cholesterol occurred mainly in HDL_2. A specific HDL cholesterol-raising effect of myristic acid has not been reported before, but myristic acid-rich coconut oil diets, apart from increasing total and LDL cholesterol, generally produce higher HDL cholesterol levels than other saturated fat diets [14-16,33,38]. A large cross-cultural study [39] showed that men in Finland, who consume large amounts of myristic acid from dairy fat [40], not only have high total cholesterol but also very high HDL cholesterol concentrations. Although these epidemiological data provide no direct evidence, they are in line with the present observation that myristic acid raises HDL cholesterol. In spite of their high HDL levels, the Finnish men still had very high rates of coronary heart disease. Thus, the beneficial effect (if any [41]) of raising HDL cholesterol through diet is evidently more than undone by the unfavorable effect on LDL cholesterol, a lipoprotein that is well-known to be atherogenic.

Gender-specific effects

It is often believed that women are less suitable subjects for studying dietary effects on serum lipids because of confounding effects of the menstrual cycle [42,43] or the use of oral contraceptives [44]. With a proper study design, however, this is not the case. In our study different women entered the trial at different points of their cycle; moreover, the diets were fed in random sequence. In this way effects of menstrual cycle canceled each other and averaged out and thus could not have systematically biased the comparisons of the diets. Unlike the means, one would expect the SDs of the responses to be enlarged because cyclic effects add a random positive or negative term to each lipid data point of an individual woman. However, we found that the SDs of the mean responses of the 36 women to the diets did not differ significantly from those of the 23 men or were even smaller (on average, 0.42 mmol/L vs. 0.41 mmol/L for total cholesterol; 0.16 vs. 0.14 mmol/L for HDL cholesterol; and 0.23 vs. 0.28 mmol/L for triglycerides). Moreover, the SDs of the mean serum lipid changes of the women using oral contraceptives (0.45 mmol/L for total cholesterol, 0.16 mmol/L for HDL cholesterol, and 0.22 mmol/L for triglycerides) were similar to those of the men. The present observation that women show no substantially larger within-subject variation in their serum lipid levels than men is in accord with our previous studies and again shows that female volunteers are as suitable subjects as men for studies on diet and lipoproteins (Chapter 2).

The mean responses of total and LDL cholesterol to the two saturated fat diets tended to be nonsignificantly higher for men than for women, whereas the HDL levels of men and women responded similarly. We have earlier reported gender-specific effects of dietary fats on HDL cholesterol levels [23,45]. It is possible that we missed small gender-specific effects in the present study. However, in other trials both sexes were also found equally responsive to changes in dietary fat saturation [15,16,18,20,46]. At the very least, lipoprotein levels of both sexes respond in the same direction and with the same order of magnitude. It therefore appears that diets low in saturated fat can be recommended for both male and female patients. Furthermore, mean effects of diet on lipoproteins in the 11 women using and the 25 women not using oral contraceptives were identical. Oral contraceptives may influence absolute serum lipid levels [44], but apparently they do not affect responsiveness of lipoproteins to diet. This is in line with our previous experience (Chapter 2).

Dosage

The differences in intake of myristic and palmitic acid between the diets amounted to about 10% of total daily energy. For palmitic acid this is a realistic amount; it is similar to the difference between an affluent high-saturated fat diet and the American Heart Association Step I diet [47]. The difference in myristic acid, however, was much higher than is achievable with natural fat sources. Thus, when extrapolating our findings for myristic acid to lower levels of intake one must assume that the amount of myristic acid in the diet and its effects on serum lipids and lipoproteins are related in a linear way. Such a linear relation has in fact been observed for saturated fatty acids in general [3,4,13].

Comparison with other dietary fatty acids

Fig. 2 puts our findings into the context of other studies which directly compared effects of individual fatty acids in humans. It must be noted that Fig. 2 merely summarizes data currently available from a few well-controlled trials; it is not meant to quantify the exact differences in cholesterolemic effect between the various fatty acids. Nevertheless, the estimates suggest that myristic and palmitic acids are the most important cholesterol-raising saturated fatty acids. Palmitic acid appears to be more potent than lauric acid [12] but less potent than myristic acid. *Trans* fatty acids [17,18](Chapter 2) raise total cholesterol less than lauric, myristic and palmitic acids, but they simultaneously raise LDL cholesterol and lower HDL cholesterol, which also results in an unfavorable lipoprotein risk profile. Stearic acid [7](Chapter 2) produces lower cholesterol levels than the other saturates and nearly equals oleic acid, i.e. it is approximately neutral in its effects on serum total and lipoprotein cholesterol.

The effects of different fatty acids on LDL levels in humans summarized in Fig. 2 correspond well with the effects of dietary fatty acids on LDL metabolism in the model developed by Spady and coworkers [8] In this model LDL cholesterol levels are affected through changes in the activity of the hepatic LDL receptor. The liver enzyme acyl coenzyme A:cholesterol acyl transferase, which converts free cholesterol into cholesteryl esters, has a much lower activity towards saturated than towards unsaturated fatty acids. As a consequence, feeding saturated fatty acids to hamsters decreases hepatic cholesteryl ester content [48,49]. It is

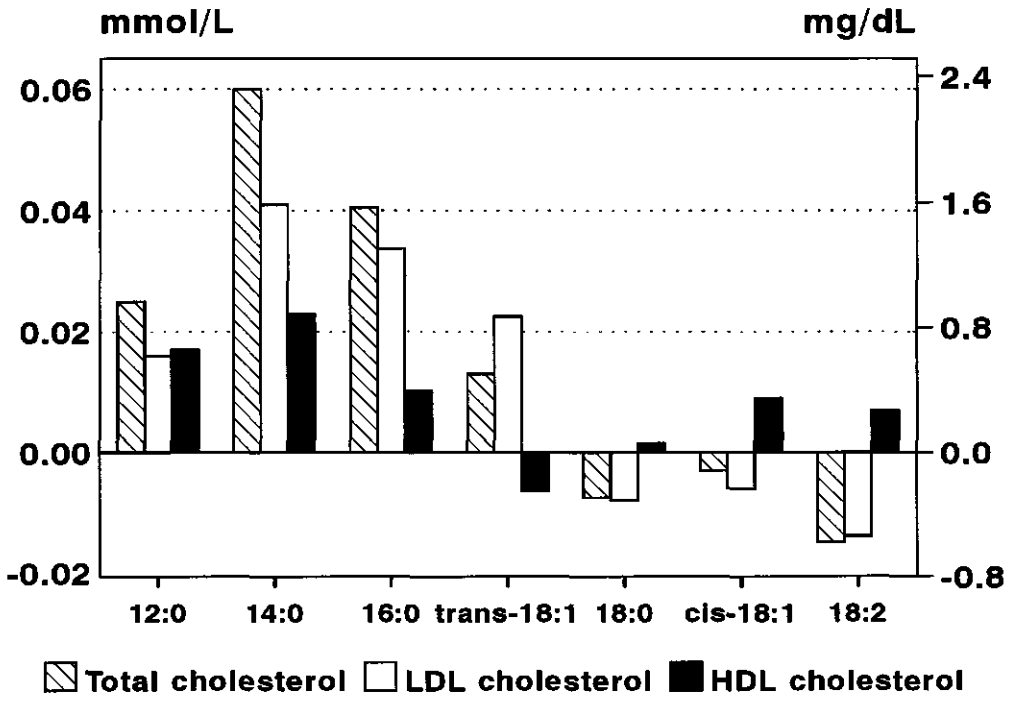


Fig. 2. Overview of the effects of individual dietary fatty acids on serum total, HDL, and LDL cholesterol in recent trials, expressed as the changes that occur when 1% of dietary energy as carbohydrates is replaced by a specific fatty acid. The values for lauric acid (12:0) were obtained in a direct comparison with oleic acid [12]; values for myristic acid (14:0) are those of the current study (Table 4). The estimates for palmitic acid (16:0) are averages of results from the present plus three other strictly controlled trials in which palmitic acid was isoenergetically exchanged for oleic acid [5,7,12]. The values for monounsaturated *trans* fatty acids (*trans*-18:1) are averages from reference [18] and Chapter 2. Values for stearic acid (18:0) are also derived from two direct comparisons with oleic acid [7] and linoleic acid (Chapter 2). Reported plasma levels were multiplied by 1.03 to convert them to serum values, and the amount of fatty acids in total dietary fat was set at 0.96 g/g. Data for oleic acid (*cis*-18:1) and linoleic acid (18:2) are based on regression coefficients derived from a meta-analysis of 27 trials [13]. These regression coefficients were also used to recalculate the effects of all fatty acids relative to carbohydrates.

assumed that at the same time the amount of free cholesterol increases in certain hepatocyte compartments, including the putative pool that controls the expression of the LDL receptor, presumably via the LDL receptor mRNA level in the cell. This leads to down-regulation of the receptor activity and the accumulation of LDL in plasma and increased formation of LDL from its precursor VLDL [50,51]. In this way, saturated fatty acids raise LDL cholesterol levels relative to unsaturated oleic and linoleic acid [48,49]. Oleic acid (*cis*-C18:1) loses its ability to increase receptor activity when it is converted to its *trans* isomer, elaidic acid (*trans*-C18:1n-9), which might explain why *trans* fatty acids result in higher LDL cholesterol levels than oleic acid [8]. The exact mechanisms involved in regulating plasma lipoprotein cholesterol levels in humans, however, require additional investigation.

Limitations of the data presented in Fig. 2 need to be stressed. First, estimates are based on a limited number of studies and for lauric and myristic acids on only one trial each. The precise differences in cholesterolemic effects among the various saturated fatty acids still need to be established. Second, we assumed a linear dose-response relationship between the amount of fatty acids in the diet and their effects on serum total and lipoprotein cholesterol. Previous studies do support this assumption [3,4,13]. Third, the values for lauric, myristic, and stearic acids are based on studies in which the fatty acids were supplied as synthetic or interesterified fats. These triacylglycerols contain one third of the saturated fatty acids at each of the three positions of the glycerol molecule. In contrast, the estimates for palmitic acid in Fig. 2 are derived from studies which used natural palm oil, in which palmitic acid is predominantly esterified at the C1 or C3 position of glycerol [37,52]. However, in our experience the intramolecular position of palmitic acid in dietary triglycerides had no effect on fasting lipid and lipoprotein concentrations in volunteers (Chapter 6).

In conclusion, both myristic and palmitic acids are dietary constituents that powerfully raise serum total and LDL cholesterol levels. Myristic acid as well as palmitic acid should be reduced in diets for the treatment of hypercholesterolemia.

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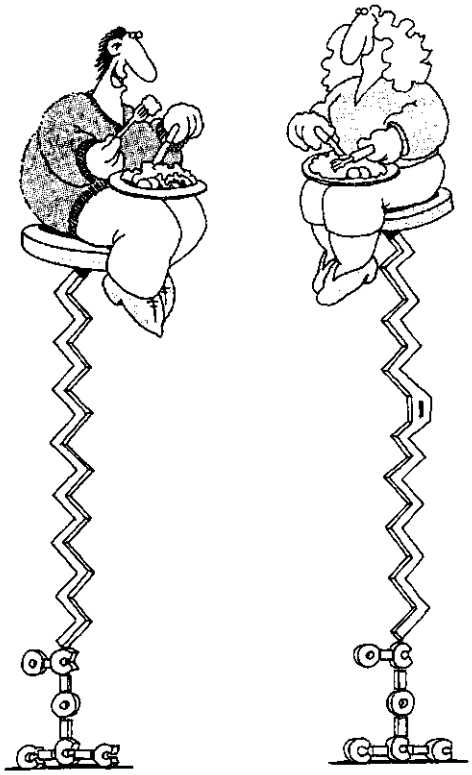
CHAPTER 6

Positional distribution of fatty acids in dietary triglycerides: effects on fasting blood lipoprotein levels in humans



Zock PL, de Vries JHM, de Fouw NJ, Katan MB.
The American Journal of Clinical Nutrition 1995; **61**: in press.

ABSTRACT

Aim. We examined the effect of the positional distribution of fatty acids within dietary triglycerides on serum lipoproteins.

Methods. Sixty subjects consumed two diets of equal fatty acid compositions for three weeks each. In the palm oil diet 82% of palmitic acid was attached to the outer two carbon atoms of glycerol, and 18% to the middle carbon. In the diet rich in enzymatically modified palm oil these figure were 35% and 65%, respectively.

Results. On the modified fat, average lipoprotein levels showed nonsignificant ($P > 0.13$) rises of 0.06 mmol/L for total, 0.03 mmol/L for HDL, and 0.04 mmol/L for LDL cholesterol compared with palm oil. The small increases in total and LDL cholesterol reached statistical significance in the men ($n = 23$) but not in the women ($n = 37$). The HDL to LDL cholesterol ratio and serum triglyceride levels were unchanged.

Conclusion. Thus, a large difference in dietary fatty acid configuration had little effect on lipoprotein levels in humans.

INTRODUCTION

Dietary fatty acids may differ from each other in four aspects: number of double bonds, chain length, configuration of the double bonds, and position of the fatty acid on the glycerol molecule (**Fig. 1**). The number of double bonds modulates the effects of fatty acids on cholesterol levels; more specifically, saturated fatty acids (with zero double bonds) raise serum cholesterol [1,2]. The cholesterol-raising effect of saturated fatty acids depends on their chain length [2-5] (Chapter 5).

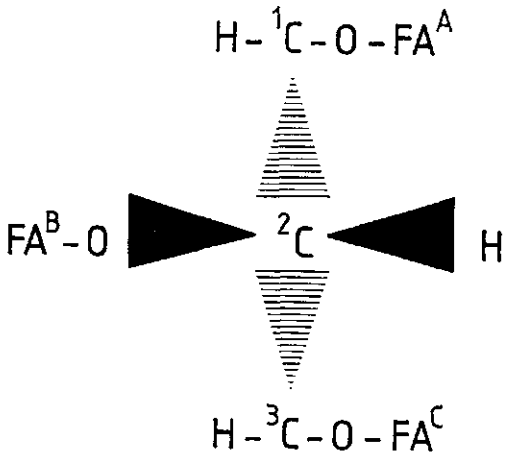


Fig. 1. Structure of a triglyceride molecule showing the stereospecific numbering (*sn*) of the carbon atoms of glycerol. If the fatty acid of the middle carbon (sn-2-FA^B) is drawn to the left (above the plane of the paper), then the top carbon is numbered 1 and the bottom carbon 3 (behind the plane of the page). Different fatty acids on the 1 and 3 positions make carbon atom 2 asymmetric, and then 2 optical isomers are possible; 1-FA^A,2-FA^B,3-FA^C-glycerol and 1-FA^C,2-FA^B,3-FA^A-glycerol.

The geometry of the double bonds of unsaturated fatty acids, in particular the *trans* versus the *cis*-configuration, also influences lipoprotein cholesterol levels [6-8](Chapter 5). However, little is known about the influence of the position of fatty acids within dietary triglycerides on lipoprotein levels and cholesterol

metabolism. More than 20 years ago, Hegsted and coworkers [9] found that stearic acid (C18:0) in synthetic fats, in which this saturate is randomly distributed over each of the 3 positions of glycerol, raised cholesterol levels [9]. In contrast, the cholesterol-raising effect of stearic acid is less when fed as cocoa fat [1,3], in which it is predominantly esterified to the 1 and 3 position. In laboratory animals randomization of fats with specific fatty acid distributions, such as peanut oil, can alter their atherogenicity and cholesterolemic effect [10-12]. This has raised interest in the influences of the fatty acid configuration of triglycerides on the biologic effects of dietary fat [12-15], but at present few data on this topic are available.

By itself it is not implausible that the positional distribution of dietary fatty acids could affect lipoprotein levels. Triglycerides are absorbed in the intestine after hydrolysis to *sn*-2-monoglycerides and fatty acids [16], and are then resynthesized into triacylglycerols and secreted in chylomicrons. The chylomicron triglycerides largely retain the original fatty acid in the 2 position [16,17]. Fatty acids attached to the *sn*-2 position might then be preferentially transported to the liver instead of to the extra-hepatic organs because lipoprotein lipase, like pancreatic lipase, primarily attacks the 1 and 3 positions of triglycerides [18]. Since the hepatocyte is the major site of action of fatty acids on LDL metabolism, saturated fatty acids in the *sn*-2 position of dietary triglycerides might elevate LDL levels more than the same fatty acid in the *sn*-1 or 3 position [14].

Common dietary fats show large differences in the positional distribution of their constituent fatty acids [13]. In palm oil and other vegetable fats, palmitic acid (C16:0) is predominantly esterified at the 1 and 3 positions, while pork fat contains palmitic acid mainly on the 2 position [19]. The fatty acid configuration of dietary triglycerides can also be altered to produce confectionary and other fats with better texture or certain desired physical properties. Palmitic acid is the most abundant saturate in the diet, and it is thus important to know whether its position in the triglyceride molecule modulates its effects on total and LDL cholesterol levels. In newborn piglets a synthesized fat with palmitic acid mainly in the 2 position raised fasting total and HDL cholesterol levels as compared with palm oil [20]. However, direct comparisons of the effects of palmitic acid in different triglyceride positions in humans are lacking. We therefore compared the effects of two dietary fats with equal amounts of individual fatty acids but different positional distribution on serum lipid and lipoprotein levels in healthy male and female volunteers.

METHODS

Subjects

In response to announcements via local newspapers and posters in University and community buildings, 47 women and 28 men applied for enrollment in the study. They were invited to participate in a medical and dietary screening. Three women withdrew during the screening phase. Two men and one woman were excluded because their cholesterol levels exceeded 7.1 mmol/L (275 mg/dL) or their blood pressure was over 140/90 mmHg, one woman because she had a low hemoglobin level, and three women and one man because their food records revealed irregular dietary habits or an alcohol intake over 10% of daily energy. Two men withdrew after the screening procedure because their spouses had been excluded for one of the reasons mentioned above. We accepted all 23 remaining men. Of the 39 eligible women, two were excluded by lot because only 60 subjects could be admitted to the trial. All 23 men and 37 women completed the study. None suffered from anemia, glycosuria, or proteinuria, and they were apparently healthy, as indicated by a medical questionnaire. Pre-experimental fasting total cholesterol ranged from 2.80 to 6.98 mmol/L (mean, 4.62 mmol/L), HDL cholesterol from 0.76 to 2.42 mmol/L (mean, 1.52 mmol/L), and triglycerides from 0.41 to 2.24 mmol/L (mean, 0.92 mmol/L). The men were aged from 19 to 67 years (mean, 29 years), they weighed between 63 and 108 kg (mean, 79 kg), and their body mass indexes ranged from 18.5 to 30.9 kg/m^2 (mean, 22.9 kg/m^2). The women were between 20 and 67 years old (mean, 32 years), they weighed between 54 and 84 kg (mean, 66 kg); their body mass indexes ranged from 18.1 to 29.7 kg/m^2 (mean, 22.4 kg/m^2). The habitual diet of the subjects as measured by self-recorded food intake on 2 working and 1 weekend day supplied on average 10.7 MJ/day (2560 kcal), of which 37% was fat, 13% was protein, 48% was carbohydrates, and 2% was alcohol. They consumed on average 280 mg cholesterol and 35 grams of dietary fiber per day. Nine women used oral contraceptives and 5 were post-menopausal. Nine women and 4 men smoked. A majority of the subjects were university students, and most of the others had college degrees. They lived in or around Wageningen, a small university town in the center of the Netherlands. The protocol and goals of the study were fully explained to the subjects, who gave their written consent. Before the study, they were thoroughly instructed about the daily routines during the trial and the necessity of

full compliance, and they were strongly advised not to take part if not highly motivated or if they anticipated any problems in adhering to the protocol. No payment was given except for the study diets, which were free. The study had been approved by the Ethics Committee of the Department of Human Nutrition.

Design

The objective of the study was to investigate the effects of the positional distribution of fatty acids in the dietary triglyceride molecules on serum lipid and lipoprotein levels. The trial was designed to detect a significant ($P<0.05$) effect of modified fat versus palm oil on total cholesterol with a power of 80% if the real population effect exceeded 0.13 mmol/L. This power calculation was based on the within subject variation (SD) in cholesterol response to diet of 0.35 mmol/L observed in previous studies [6](Chapters 2 and 5).

All subjects participated simultaneously from February 15 to March 29, 1993. The trial consisted of two consecutive 3-week periods, during which each participant followed both diets, in different order (cross-over). One diet was high in palm oil, with palmitic acid predominantly esterified at the *sn*-1 and *sn*-3 position, and the other diet was high in an enzymatically modified palm oil (Betapol, Loders-Croklaan, Wormerveer, The Netherlands) which contains palmitic acid mainly in the *sn*-2 position. Before the study began, subjects were categorized according to sex, and women also according to the use of oral contraceptives. Subjects within each subcategory were then randomly allocated to one of the two diet sequences, except members of a couple living together, who were assigned to the same sequence group. Both sequence groups had a nearly equal number of each category, and they were well-balanced for age, baseline total and HDL cholesterol level, and body mass index. Use of this cross-over design eliminated possible bias due to the order in which the diets are consumed or to drift of variables over time [21]. As described earlier (Chapters 2 and 5), this experimental design also eliminates bias due to lipid variation during the hormonal cycle. This enables the measurement of the influence of diet on serum lipids in women, without confounding effects of menstrual cycle or use of oral contraceptives (Chapters 2 and 5). Foodstuffs and packages were color-coded so as to keep subjects unaware of the nature of their diets.

Diets

The diets consisted of conventional solid foods, and menus were changed daily during each 3-week dietary period. The amount and type of foodstuffs were the same for the two diets. The differences in the positional distribution of the fatty acids were achieved by the use of special margarines and oils with highly distinct fatty acid configurations (**Table 1**). A fat rich in *sn*-1,3-palmitate was obtained by solvent fractionation of natural, unprocessed palm oil. Subsequently 87.5 parts of this fraction were blended with 11 parts of high-oleic acid sunflower oil and 1.5 parts of high-linoleic acid sunflower oil. This yielded an oil blend with palmitic acid predominantly in the 1 and 3 position. An oil with palmitic acid mainly in the 2 position was derived by *sn*-1,3-enzymatic interesterification of a palm oil fraction with sunflower fatty acids using immobilized *Rhizomucor miehei* lipase (Novo Industries, Copenhagen, Denmark), followed by solvent fractionation. The palm oil-based fat had a slightly higher melting point than the enzymatically modified fat, but both were liquid at 37 °C.

Table 1. Total, sn-2, and sn-1,3 fatty acid composition of the special oils and margarines.

Fatty Acid	Palm Oil Rich			Modified Fat Rich		
	Total	*Sn*-2[a]	*Sn*-1,3[a]	Total	*Sn*-2[a]	*Sn*-1,3[a]
	g/100 g of fatty acids					
Saturated	39.9	13.7	53.0	38.2	78.1	18.2
Lauric acid (C12:0)	0.4	0.3	0.4	0.6	0.7	0.5
Myristic acid (C14:0)	1.0	0.5	1.3	1.2	1.9	0.9
Palmitic acid (C16:0)	28.8	6.4	40.0	28.9	66.9	9.9
Stearic acid (C18:0)	9.1	6.5	10.4	7.1	8.4	6.5
Monounsaturated	47.8	63.5	39.9	49.5	18.5	65.0
Oleic acid (*cis*-C18:1)	47.2	63.4	39.1	48.0	18.1	62.9
Polyunsaturated	12.0	22.4	6.8	10.5	3.3	14.1
Linoleic acid (C18:2)	11.8	22.1	6.6	10.1	3.2	13.5
Unknown	0.3	0.4	0.3	1.8	0.1	2.7
Total	100	100	100	100	100	100

The margarine provided 64% and oil 6% of all dietary triglycerides; together they provided 28% of total energy intake.

[a] Out of every 100 g of total fatty acids 33.3 g is in the *sn*-2 and 66.7 g is the *sn*-1 or *sn*-3 position.

Margarines were made from a blend of 93 parts of these special oils with 7 parts of fully hydrogenated sunflower oil high in stearic acid. These margarines had similar textures and were easy to spread. They contained equal total amounts of individual fatty acids, but differed substantially in the positional distribution of the fatty acids over the triglyceride molecules. Table 1 shows that the palm oil-rich fats contained mainly oleic and linoleic acid in the 2 position of the triglycerides, while the 2 position in the modified fat product was predominantly occupied by palmitic acid. These margarines were used as spreads with the bread meals, in sauces and gravies, and for baking cookies and special bread containing on average 8% fat. The oils from which the margarines had been made were also used to prepare salad dressings. The margarines and oils provided 70% of all triglycerides in the study diets.

The diets were formulated at 30 levels of energy intake, ranging from 5.5 to 20 MJ/day, so that each subject received a diet that met his or her energy needs. The energy requirements of each participant were estimated from 3-day food records kept by the subjects before the trial. As such food records underestimate actual energy requirements [22], we provided the subjects with an initial amount of energy 10% higher than his or her reported intake. Body weights were recorded twice weekly, and the energy level was adjusted when necessary, so as to maintain a stable weight. Over the 42 days of the trial, average body weight fell by 0.3 ± 1.1 kg (range, −2.6 to 1.6 kg). Mean body weight at the end of the dietary treatments was 0.1 ± 0.8 kg (range, −1.5 to 1.9 kg) higher on the modified fat diet than on the palm oil diet.

All foodstuffs were weighed out for each individual subject. On weekdays at noon, hot meals were served and eaten at the department. All other foods were supplied daily as a package. Foods for the weekends and guidelines for its preparation were provided on Fridays. In addition to the food supplied, the subjects chose each day a limited number of food items free from fat and cholesterol, which provided 8 − 9% of total daily energy. Subjects were urged not to change their selection of free-choice items between the dietary periods. They were also instructed to maintain their habitual pattern of physical activity and not to change their smoking habits, consumption of coffee, or use of oral contraceptives. The participants kept diaries in which they recorded their selection and amount of free-choice items, any sign of illness, all medications used, and any deviation from their diets. At the end of the trial, subjects completed an anonymous questionnaire relating to the blinding of the diets and problems and noncompliance during the study.

Food composition

Duplicate portions of both study diets were collected on each of the 42 days for an imaginary participant with a daily energy intake of 11 MJ/day, stored at −20 °C, and pooled and analyzed after the study for protein [23], total fat [24], total fatty acid composition [25], dietary fiber [26], and cholesterol [27]. Available carbohydrate was calculated by difference. *Sn*-2 fatty acid composition was determined enzymatically [28]. After partial hydrolysis of triglycerides by *sn*-1,3 specific porcine pancreatic lipase (Type II, EC 3.1.1.3, Sigma, St. Louis, MO), *sn*-2-monoglycerides were isolated on aminopropyl columns (Bond Elut, Varian, Harbor City, CA) [29] and hydrolyzed, and the fatty acids were methylated and then separated by gas liquid chromatography. This yielded the fatty acid composition of one third of all dietary fatty acids, namely those esterified to the *sn*-2 position (Table 1 and **Table 2**, columns 2 and 5). The composition of the *sn*-1,3 fatty acids, which made up the other two thirds of dietary fatty acids (Tables 1 and 2, columns 3 and 6), was calculated from the total and the *sn*-2 fatty acid compositions. The following example illustrates this calculation:

$$sn\text{-}1,3 \text{ oleic acid} = [3 \times (\text{total oleic acid}) - (sn\text{-}2 \text{ oleic acid})] / 2.$$

We also calculated percentages "horizontally". For instance, the amount of palmitic acid present on the 2 position as a percentage of all dietary palmitic acid was calculated from the data in Tables 1 and 2 as:

$$[sn\text{-}2 \text{ palmitic acid} / (3 \times \text{total palmitic acid})] \times 100\%,$$

and the percentage present on the 1 or 3 position as:

$$[100\% - \text{percentage on the 2 position}].$$

The energy and nutrient content of the free choice-items, which contained negligible amounts of fat, was calculated [30] and combined with the analyzed values of the foods supplied (**Table 3**).

Table 2. Total, sn-2, and sn-1,3 fatty acid composition all triglycerides in the two diets.

Fatty Acid	Palm Oil Diet			Modified Fat Diet		
	Total	*Sn*-2[a]	*Sn*-1,3[a]	Total	*Sn*-2[a]	*Sn*-1,3[a]
	g/100 g of fatty acids					
Saturated	42.1	26.5	49.9	40.6	67.8	26.9
Lauric acid (C12:0)	1.0	1.1	0.9	1.0	1.4	0.8
Myristic acid (C14:0)	2.5	3.5	2.0	2.7	4.3	1.9
Palmitic acid (C16:0)	27.7	14.6	34.2	27.4	53.5	14.3
Stearic acid (C18:0)	9.2	6.2	10.8	8.1	6.9	8.6
Monounsaturated	42.8	52.0	38.1	44.4	22.3	55.5
Oleic acid (*cis*-C18:1)	41.3	51.0	36.4	42.1	20.9	52.7
Polyunsaturated	13.2	20.6	9.6	12.3	8.4	14.2
Linoleic acid (C18:2)	12.2	19.6	8.5	11.2	7.2	13.1
Unknown	1.9	0.9	2.4	2.7	1.5	3.3
Total	100	100	100	100	100	100

Special oils and margarines supplied 70% of total fat. The remaining 30% came from conventional foodstuffs, largely dairy products and meat.

[a] *Out of every 100 g of total fatty acids 33.3 g is in the sn-2 and 66.7 g is the sn-1 or sn-3 position.*

Blood sampling and analysis

Subjects were assigned a random number that was used for labeling blood and serum tubes. In this way, technicians were blinded as to the subjects' diets. Blood was sampled after a 12-hour fast on days 1, 17, and 21 (period 1), and days 38 and 42 (period 2). All venipunctures of a particular subject were performed by the same technician, in the same location, and at the same time of the same days of the week for the two dietary periods. Serum was obtained by low-speed centrifugation within one hour of venipuncture, stored at −80 °C, and analyzed enzymatically for total and HDL cholesterol and triglycerides [31-33]. All samples from a particular subject were analyzed within one run. The coefficient of variation within runs was 1.4% for total, 0.7% for HDL, and 1.3% for triglycerides. Mean bias with regard to the target values of serum pools provided by the Centers for Disease Control (Atlanta, GA) was −0.04 mmol/L for total cholesterol, 0.01 mmol/L for HDL cholesterol, and 0.12 mmol/L for triglycerides. LDL cholesterol was calculated using the Friedewald equation [34].

Statistical analysis

The two lipid and lipoprotein values obtained for each subject at the end of each dietary period were averaged for statistical analyses. The data were analyzed with the General Linear Models (GLM) procedure of the Statistical Analysis System [35], using a 2-way ANOVA with subject and diet as class variables. This way of testing is equivalent to the paired *t*-test [21]. Carry-over effects of previous diet were checked by introducing a diet-by-period interaction term in the model. Differences in responses between men and women or between women using or not using oral contraceptives were compared with unpaired *t*-tests [35].

RESULTS

Diets and dietary adherence

Table 2 shows that the two diets contained equal amounts of each specific fatty acid (columns 1 and 4), but markedly differed in their positional fatty acid distribution. In the modified-fat diet, 65% of the palmitic acid was on the 2 position, and the remaining 35% on the 1 and 3 position. In contrast, 82% of the palmitic acid in the palm-oil diet was on the 1 and 3 position and only 18% was found on the 2 position. Sixty-eight percent of the *sn*-2 fatty acids in the modified fat diet was saturated, mainly palmitic acid, whereas 73% of the *sn*-2 fatty acids in the palm oil diet were unsaturated, the majority being oleic acid (Table 2, columns 2 and 5). Table 3 shows that the intake of energy and all other relevant food components was similar for both diets.

Inspection of the diaries and the anonymous questionnaires that were handed in at the end of the trial revealed only minute deviations from the study protocol. The diaries showed that consumption of foods outside the dietary regime provided at most 0.2 g of fat per day. The magnitude and the frequency of deviations reported in the anonymous questionnaires were in line with those reported in the diaries. This indicated that compliance was high, and that deviations probably did not materially affect the results. A two-choice question about the color-coding of the diets yielded 30 correct and 25 false answers ($P=0.25$). Thus, blinding was successful.

Table 3. Mean daily intake of energy and nutrients of the 60 subjects on the two diets.

Energy/Nutrient	Palm Oil Diet	Modified Fat Diet
Energy		
MJ/d	12.4 ± 3.1	12.3 ± 3.0
kcal/d	2958 ± 740	2941 ± 725
Protein (% of energy)	13.0	13.4
Total fat (% of energy)	40.8	40.1
Saturated fatty acids	16.5	15.6
Lauric acid (C12:0)	0.4	0.4
Myristic acid (C14:0)	1.0	1.0
Palmitic acid (C12:0)	10.8	10.5
Stearic acid (C18:0)	3.6	3.1
Monounsaturated fatty acids	16.7	17.1
Oleic acid (*cis*-C18:1)	16.2	16.2
Polyunsaturated fatty acids	5.3	4.8
Linoleic acid (*cis,cis*-C18:2)	4.8	4.3
Carbohydrates (% of energy)	44.9	45.4
Alcohol (% of energy)	1.1	1.0
Cholesterol		
mg/d	368	367
mg/MJ	29.8	29.8
Dietary fiber		
g/d	44.6	43.0
g/MJ	3.6	3.5

Values are based on chemical analyses of complete duplicate diets plus the calculated contribution of free-choice items. Each value represents the mean of two independent duplicates collected and pooled in the two different periods during which each diet was consumed by one half of the subjects. Variations between periods were negligible.

Serum lipids and lipoproteins

The diets had little effect on serum lipoprotein cholesterol levels (**Fig. 2**). Thirty-six participants showed higher total cholesterol values on the modified fat diet than on the palm oil diet, one showed no change, and 23 participants had lower values on modified fat (**Fig. 3**). The changes in total cholesterol were not related to initial cholesterol level (Pearson correlation coefficient (r) $=0.13$, $P=0.34$), age ($r=0.07$, $P=0.61$), or body mass index ($r=-0.11$, $P=0.40$).

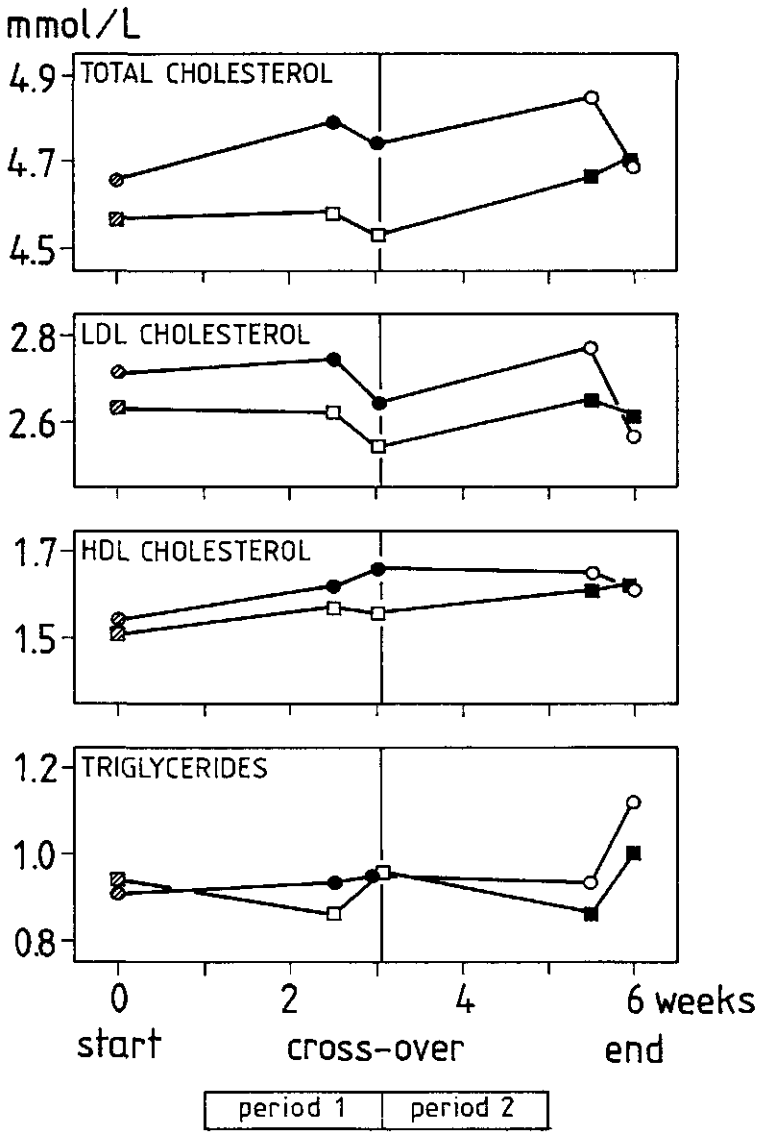


Fig. 2. Mean concentration of serum total, LDL, and HDL cholesterol and of serum triglycerides throughout the experiment. During the first 3 weeks, thirty subjects consumed a diet rich in sn-2-saturated triglycerides (modified fat, ●) and in the subsequent period a diet rich in sn-2-unsaturated triglycerides (palm oil, ○). Thirty other subjects consumed these diets in reverse order (palm oil, □; modified fat, ■). The two diets had the same total fatty acid composition (see Table 3).

The average rise in total cholesterol of 0.06 ± 0.32 mmol/L or 2.3 ± 12.4 mg/dL on modified fat relative to palm oil was not significantly different from zero ($P=0.15$). The mean changes in HDL (0.03 mmol/L or 1.2 mg/dL, $P=0.13$), non-HDL (VLDL+LDL) cholesterol (0.03 mmol/L or 1.2 mg/dL, $P=0.39$), and LDL cholesterol (0.04 mmol/L or 1.5 mg/dL, $P=0.23$) were also not statistically significant (**Table 4**). In addition, The HDL to LDL cholesterol ratio ($P=0.89$) and the triglyceride levels did not differ ($P=0.35$) between the diets. The absence of significant diet-by-period interactions regarding the lipid and lipoprotein levels indicated that there were no important carry-over effects from previous diet. Confidence intervals for changes in lipoprotein levels (Table 4) were narrow, and left little room for major effects.

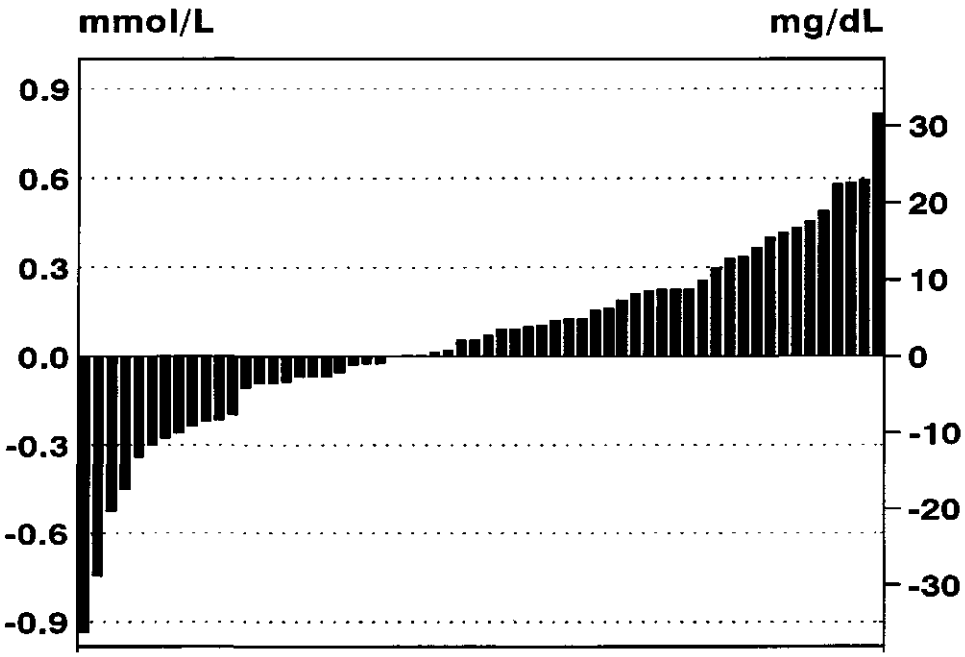


Fig 3. Individual differences in serum total cholesterol level between the end of the modified fat diet and the end of the palm oil diet, sorted by magnitude. The average change was 0.06 mmol/L ± 0.32 mmol/L ($P=0.15$). The 60 subjects consumed both diets for 3 weeks each.

Table 4. Serum lipid and lipoprotein cholesterol levels in 60 subjects on diets high in palm oil or in an enzymatically modified palm oil analogue in which palmitic acid is attached mainly to the middle (*sn*-2) instead of the outer two carbon atoms (*sn*-1,3) of glycerol.

	Palm Oil Diet	Modified Fat Diet	Change	(95% CI)
Total cholesterol		*mmol per liter*		
All	4.66 ± 0.90	4.72 ± 0.94	0.06	(−0.02 to 0.14)
Women	4.89 ± 0.84	4.92 ± 0.88	0.03	(−0.09 to 0.16)
Men	4.31 ± 0.89	4.41 ± 0.96	0.10[a]	(0.02 to 0.18)
HDL cholesterol				
All	1.60 ± 0.33	1.63 ± 0.37	0.03	(−0.01 to 0.07)
Women	1.77 ± 0.24	1.79 ± 0.31	0.03	(−0.04 to 0.09)
Men	1.33 ± 0.26	1.37 ± 0.29	0.04	(−0.00 to 0.08)
Non-HDL cholesterol				
All	3.07 ± 0.85	3.09 ± 0.86	0.03	(−0.04 to 0.09)
Women	3.12 ± 0.78	3.13 ± 0.79	0.01	(−0.08 to 0.10)
Men	2.98 ± 0.94	3.04 ± 0.97	0.06	(−0.02 to 0.13)
LDL cholesterol				
All	2.62 ± 0.78	2.66 ± 0.80	0.04	(−0.03 to 0.10)
Women	2.69 ± 0.76	2.71 ± 0.76	0.01	(−0.08 to 0.11)
Men	2.51 ± 0.81	2.59 ± 0.86	0.08[a]	(0.00 to 0.15)
HDL to LDL ratio				
All	0.67 ± 0.29	0.67 ± 0.30	0.00	(−0.02 to 0.03)
Women	0.70 ± 0.20	0.70 ± 0.19	0.00	(−0.04 to 0.04)
Men	0.62 ± 0.39	0.63 ± 0.41	0.00	(−0.02 to 0.03)
Triglycerides				
All	0.97 ± 0.42	0.94 ± 0.40	−0.03	(−0.08 to 0.03)
Women	0.92 ± 0.33	0.91 ± 0.38	−0.02	(−0.08 to 0.05)
Men	1.04 ± 0.54	0.99 ± 0.44	−0.04	(−0.16 to 0.07)

Values are mean ± SD. The twenty-three men and thirty-seven women consumed both diets for 3 weeks each, in different order. To convert values for total, HDL, non-HDL (VLDL + LDL), and LDL cholesterol to milligrams per deciliter, multiply by 38.67. To convert values for triglycerides to milligrams per deciliter, multiply by 88.54.

[a] Significantly different from zero, $P<0.05$.

The small increases in total (0.10 mmol/L) and LDL cholesterol (0.08 mmol/L) on the modified-fat diet reached statistical significance in the men (Table 4). However, the men's non-HDL (or VLDL + LDL) cholesterol was not significantly different ($P=0.39$) between the two diets. None of the responses in lipid and

lipoprotein levels was significantly different between men and women ($P > 0.30$). Also, no significant differences were detected between responses of women using or not using oral contraceptives.

Before the sera were analyzed, we had identified 5 women who experienced events that probably influenced their lipoprotein levels: one received estrogen therapy during part of the trial, one had a sinus infection and was treated with antibiotics, one reported frequent deviations from the study diets, one suffered a knee injury and stopped daily vigorous exercise, and one lost more than 3 kg of body weight during the trial. Statistical analysis without the data of these 5 women ($n = 55$ men and women) did not yield substantially different results. The mean effect of modified fat relative to palm oil for the remaining 32 women was somewhat, but not significantly, larger for total cholesterol (0.08 mmol/L; 95% CI, −0.04 to 0.21 mmol/L) and for HDL cholesterol (0.06 mmol/L; 95% CI, 0.01 to 0.12 mmol/L) than for the 37 women in the initial analysis (Table 4). Again, the responses in women and men were not significantly different. The mean effects of the modified-fat diet versus the palm oil diet in the 55 subjects who experienced no health problems or material changes in body weight were 0.09 mmol/L or 3.5 mg/dL (95% CI, 0.01 to 0.17 mmol/L) for total cholesterol, 0.05 mmol/L or 1.9 mg/dL (95% CI, 0.02 to 0.09 mmol/L) for HDL, and 0.05 mmol/L or 1.9 mg/dL (95% CI, −0.02 to 0.12 mmol/L) for LDL cholesterol. As in the analysis with all 60 subjects, the HDL to LDL ratio was identical on both diets.

DISCUSSION

Fasting serum lipids and lipoproteins

We found that two dietary fats with an extreme contrast in the type of fatty acid on the 2 position, specifically palmitic versus oleic acid, resulted in minimal differences in serum total or lipoprotein cholesterol levels. The only statistically significant effects were observed in the men, who showed small rises in LDL and total cholesterol on the diet with palmitic acid on the *sn*-2 position. In view of the more modest differences in positional distribution that can be achieved in everyday diets, the influence on serum cholesterol levels in free-living people should be much smaller than the 0.1 mmol/L for men observed here. The design and the size of the trial provided a high statistical power to detect real population differences. It is

therefore unlikely that we failed to pick up any major effect through chance fluctuations. Also, most of the effect of dietary lipids on serum lipoproteins is established within one or two weeks [3,36-38]; therefore, 3 weeks should have been amply sufficient to observe an important effect if there had been one. We used solvent fractionation of palm oil to increase the proportion of triglycerides with unsaturated fatty acids in the *sn*-2 position, which is typical for vegetable oils. Solvent fractionation is a physical process that does not produce changes in molecular structures. Our results therefore suggest that fat with palmitic acid mainly on the *sn*-1,3 position does not confer any major advantage in terms of effects on serum lipoproteins over other fats with the same fatty acid composition but a different positional distribution of fatty acids.

Innis and coworkers [20] fed formulas containing palm oil or enzymatically modified fat to newborn piglets. At 18 days of age the modified fat formula high in *sn*-2-palmitate triglycerides resulted in higher plasma total and HDL cholesterol levels than the palm-oil formula high in *sn*-2-oleate glycerides [20]. These changes were in the same direction, but much larger than observed in the present study. However, the effects of dietary fat on lipid metabolism of newborn piglets are difficult to compare with those of adult humans, not only because of species differences, but also because in newborn animals and humans the absorption of palmitic acid in the 2 position might be more efficient than that of palmitic acid in the 1 and 3 positions [39-41].

Other potential effects of positional fatty acid distribution

Although dietary fatty acid configuration had no substantial effect on fasting lipoprotein levels, it might still influence postprandial lipoprotein concentrations. Indeed, Mortimer and Redgrave [42,43] injected chylomicrons or lipid emulsions into rats and found that hydrolysis of triglycerides by lipoprotein lipase and liver uptake of remnant particles were slower when the fatty acid in the 2 position was saturated than when it was unsaturated. If these findings can be extrapolated to humans, then eating *sn*-2-saturated triglycerides, such as lard, might result in elevated plasma remnant concentrations. Myher and coworkers [44] found that in human volunteers fed a lard-rich breakfast the chylomicron triglycerides contained considerably more palmitic acid in the 2 position than those in subjects who consumed a control breakfast. This shows that in humans the *sn*-2 position of

palmitic acid in lard is at least partly retained after hydrolysis by pancreatic lipase in the gut. Myher et al. [44], however, did not report effects of lard on chylomicron concentrations. Recently, Zampelas and coworkers [45] examined the effects of positional distribution of dietary palmitic acid on postprandial plasma lipids. Sixteen healthy men consumed the same dietary fats as studied in our trial, and plasma total and chylomicron triglyceride levels were monitored over 6 hours after the liquid test meals. No differences between the palm oil and the modified-fat meal were detected. The authors concluded that the positional distribution of fatty acids in dietary triglycerides is not an important determinant of postprandial lipemia [45].

Kritchevsky et al. [10-12] found that randomization of fats with a specific fatty acid distribution such as peanut oil reduces their atherogenicity in laboratory animals. Peanut oil contains 4 – 7% of saturated C20:0, C22:0 en C24:0, almost all of it in the *sn*-3 position [46]. Although these very long chain saturates can slow down chylomicron metabolism in rats [43], this cannot explain why randomized peanut oil, in which these fatty acids are present in the 2 position as well as in the 1 and 3 position, should be less atherogenic than native peanut oil. The atherogenesis in animals fed native peanut oil does not seem to be mediated by elevated plasma cholesterol levels [10,11]. It has been suggested that the presence of C20-24 saturates in the 3 position of peanut oil might render linoleic acid in the 2 position relatively unavailable, which could promote the atherogenicity of the oil [46].

Conclusion

The fatty acid configuration of food fats can be altered to produce confectionary and other fats with better texture or certain desired physical properties. Our study provides no evidence that changing the position of palmitic acid in this "structuring" process has important health consequences. However, future studies should also investigate the positional effects of other fatty acids.

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CHAPTER 7

Partial conservation of the *sn*-2 position of dietary triglycerides in fasting plasma in humans

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ABSTRACT

Aim. We investigated the effect of the position of fatty acids within dietary triglycerides on the composition of plasma triglycerides and cholesteryl esters.

Methods. Sixty volunteers consumed two diets of equal fatty acid compositions for three weeks each. In the palm oil diet 82% of palmitic acid (C16:0) was attached to the *sn*-1 and *sn*-3, and 18% to the *sn*-2 position of glycerol. In the diet rich in a palm oil analogue, Betapol, these figures were 35% and 65%, respectively.

Results. Consumption of Betapol increased palmitic plus palmitoleic acid (*cis*-C16:1) in the plasma cholesteryl esters by about 1 g/100 g fatty acids ($P<0.0001$) at the expense of oleic and linoleic acid. The proportion of palmitic acid in the 2-position of fasting plasma triglycerides was 10.2 g/100 g on palm oil and 12.3 ($P<0.0001$) on Betapol; that of oleic acid (*cis*-C18:1) was 46.9 versus 43.6 ($P<0.0001$). Overall oleic acid content of triglycerides was 1.4 g/100 g fatty acids lower on Betapol than on palm oil ($P=0.002$), while palmitoleic and polyunsaturated fatty acids showed small increases.

Conclusion. The fatty acid configuration of dietary fat is partially reflected in fasting plasma triglycerides. Dietary palmitic acid is incorporated more efficiently into plasma cholesteryl esters when attached to the middle then when attached to the outer positions of dietary triglycerides. The positional distribution of fatty acids in dietary fat has effects on lipid metabolism that persist beyond fat absorption and chylomicron clearance.

INTRODUCTION

Every triglyceride (triacylglycerol) molecule contains three distinct and non-equivalent attachment sites for fatty acids. These are numbered *sn*-1, *sn*-2, and *sn*-3, where *sn* stands for stereospecific numbering. Natural fats and oils in the human diet differ in the positional distribution of their constituent fatty acids (**Table 1**). The influence of the stereospecific distribution of fatty acids within dietary triglycerides on lipid metabolism is largely unknown, especially for the in vivo situation in humans.

Table 1. Approximate total and *sn*-2 fatty acid composition (g/100 g) and the percentage of each type of fatty acid at the 2 position (%*sn*2)[a] in triglycerides of major dietary fats.

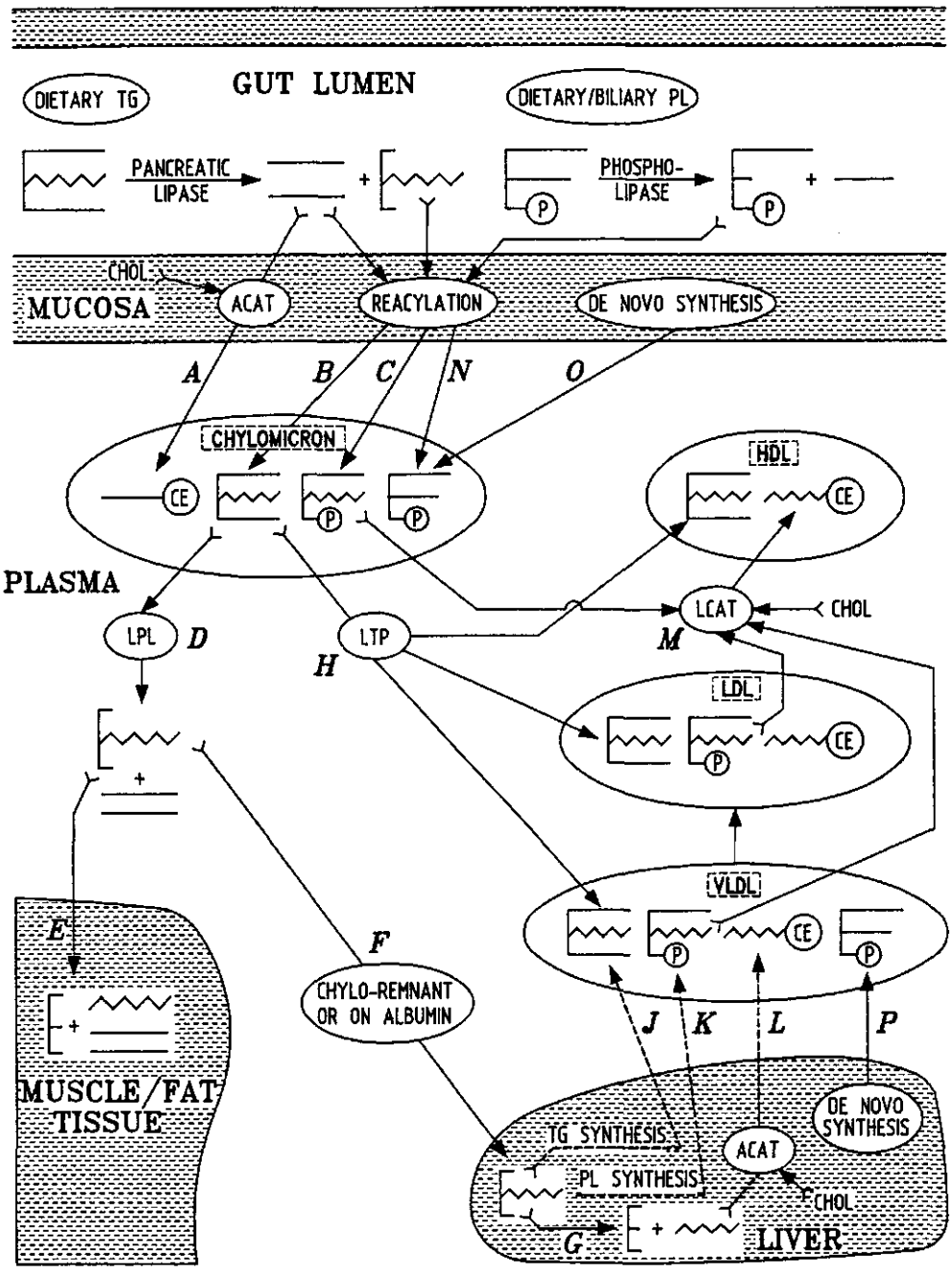
Fat	Saturates			Monounsaturates			Polyunsaturates		
	Total	*Sn*-2	%sn2	Total	*Sn*-2	%sn2	Total	*Sn*-2	%sn2
Beef (tallow)	54	29	18	44	64	49	3	7	88
Dairy fat[b]	68	73	36	28	26	31	3	1	11
Pork (lard)	36	71	65	50	22	16	14	7	17
Palm kernel oil	49	13	9	40	70	59	11	17	52
Soybean oil	13	0	0	27	42	53	60	58	33
Cocoa butter	60	10	5	36	88	81	4	2	15

Values are derived from references [1-3].

[a] Calculated as (*sn*-2 fatty acids / 3 x total fatty acids). When fatty acid distribution would be random this figure would equal 33%.

[b] Short chain fatty acids (C4-C10) and stearic acid (C18:0) in milk are mainly at the *sn*-1 and *sn*-3 positions, and the C12-C16 saturates predominantly at the *sn*-2 position [1].

The major pathways of lipid metabolism as currently envisaged (**Fig. 1**) do not allow for effects of dietary triglyceride configuration on plasma lipid composition beyond the postprandial phase. After partial hydrolysis by pancreatic lipase in the intestine, dietary triglycerides are absorbed as 2-monoglycerides and free fatty acids [4,5]. Reacylation of the monoglycerides in the mucosal cells results in chylomicron triglycerides which largely retain the original fatty acid in the middle or *sn*-2 position [6] (route *B* in fig. 1). When these triglycerides are hydrolyzed in the capillary bed, free fatty acids, glycerol, and 2-monoglycerides are formed


GUT LUMEN
DIETARY TG
DIETARY/BILIARY PL
PANCREATIC LIPASE
PHOSPHO-LIPASE
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MUCOSA
ACAT
REACYLATION
DE NOVO SYNTHESIS
A
B
C
N
O
CHYLOMICRON
CE
HDL
PLASMA
LPL
D
LTP
H
LCAT
CHOL
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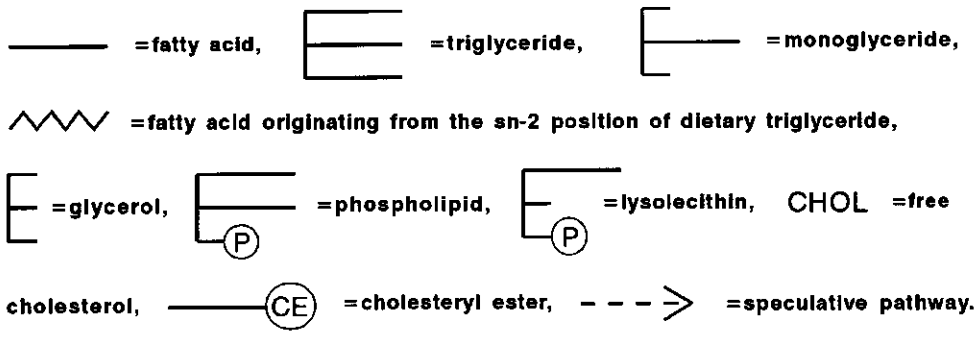
LDL
VLDL
E
F
CHYLO-REMNANT OR ON ALBUMIN
J
K
L
P
MUSCLE/FAT TISSUE
TG SYNTHESIS
PL SYNTHESIS
ACAT
DE NOVO SYNTHESIS
CHOL
G
LIVER

Fig.1 (left page). Potential metabolic routes of fatty acids attached to the *sn*-2 position of dietary triglycerides. The phosphatidic acid pathway of triglyceride synthesis in the mucosa and the liver, and the exchange of cholesteryl esters and triglycerides between HDL, LDL, and VLDL are not shown. The majority of the fasting plasma lipids do *not* contain (*sn*-2) fatty acids which originate from the 2 position of dietary triglycerides; these lipids have been left out for reasons of clarity. *A:* formation of chylomicron cholesteryl esters by intestinal ACAT [7], *B:* reacylation of absorbed 2-mono-acylglycerols to chylomicron triglycerides [6], *C:* reacylation of 2-mono-acylglycerols to chylomicron phosphatidylcholine [8], *D:* peripheral hydrolysis of fatty acids on the *sn*-1 and *sn*-3 position of chylomicron triglycerides by LPL [9], *E:* tissue uptake of lipolytic products generated by LPL [10], *F:* transport to the liver of 2-mono-acylglycerols formed by LPL [11], *G:* hydrolysis of 2-mono-acylglycerols reaching the liver [11,12], *H:* transfer of chylomicron triglycerides to other lipoproteins by LTP [13], *J:* hepatic triglyceride synthesis from 2-mono-acylglycerols transported to the liver (speculative), *K:* hepatic phospholipid synthesis from 2-mono-acylglycerols transported to the liver (speculative), *L:* formation of plasma cholesteryl esters by hepatic ACAT [7], *M:* formation of plasma cholesteryl esters by transfer of the *sn*-2 fatty acid from phosphatidylcholine to free cholesterol by LCAT [14], *N:* plasma phospholipid synthesis by intestinal reacylation of absorbed lysolecithin [15,16], *O: de novo* phospholipid synthesis in the intestine [17], *P: de novo* phospholipid synthesis in the liver [18]. ACAT, acyl:CoA acyltransferase; LCAT, lecithin:cholesterol acyltransferase; LPL, lipoprotein lipase; LTP, lipid transfer protein.



because lipoprotein lipase, like pancreatic lipase, primarily attacks the 1 and 3 position [9] (route *D*). The fate of the monoglycerides is not exactly known, but it is believed that they are removed quickly from the circulation after further

hydrolysis by lipoprotein lipase [10] or monoglyceride hydrolase [19] (route *E*). Alternatively, the monoglycerides may be transported to the liver attached to albumin or in the core of the chylomicron remnants [11] (route *F*). Upon uptake in the liver they are probably further degraded [11,12] (route *G*). Thus, according to these pathways, any memory of the original *sn*-2 position of the dietary triglycerides should be erased by the time chylomicron clearance has been completed.

Plasma cholesteryl esters are mainly formed by the lecithin:cholesterol acyltransferase (LCAT) reaction on HDL, which transfers the *sn*-2 acyl chain from phosphatidylcholine to cholesterol [14] (route *M* in fig. 1). Phosphatidylcholine in plasma is synthesized *de novo* in the liver [18] (route *P*) and the intestine (route O), or by mucosal reacylation of lysolecithin absorbed from the intestinal lumen [15,16] (route *N*). Cholesteryl esters are also formed from cholesterol and fatty acids by the Acyl:CoA acyltransferase (ACAT) reaction in hepatic (route *L*) and intestinal mucosa cells (route *A*) and excreted into plasma in VLDL and chylomicron particles [7]. None of these routes predicts an effect of dietary triglyceride configuration on the fatty acid composition of cholesteryl esters.

Therefore, neither endogenous VLDL triglycerides in fasting blood nor plasma cholesteryl esters are expected to reflect changes in the position of fatty acids in dietary triglycerides as long as the overall mix of dietary fatty acids remains constant. However, current knowledge about the pathways by which dietary fat influences lipid metabolism is largely based on animal and in vitro experiments (Fig 1). We now compared the effect of the positional distribution of dietary fatty acids on the fatty acid composition of fasting plasma lipids in a large group of human subjects.

METHODS

Design and statistical analyses

The aim of the study was to investigate the effects of the positional distribution of fatty acids in the dietary triglyceride molecules on fasting blood lipids and lipid metabolism in humans. The effects on serum total and lipoprotein cholesterol levels have been reported elsewhere (Chapter 6).

The trial consisted of two consecutive periods, during which each participant consumed both diets, in random order (cross-over). The diets were high in either palm oil, with palmitic acid predominantly in the *sn*-1 and *sn*-3 positions, or in Betapol, which contains palmitic acid mainly in the *sn*-2 position. The two sequences contained a nearly equal number of male and female subjects, and of women using and not using oral contraceptives. In this way, bias due to treatment order, drift of variables over time, or effects of hormonal cycle was eliminated [20]. The subjects were masked as to the nature of their diets.

Effects of diet were analyzed statistically by comparison of each subject's data on the two diets using paired t-tests [21].

Subjects

All 23 men and 37 women who were admitted completed the trial successfully (Chapter 6). None suffered from anemia, glycosuria, or proteinuria, and they were apparently healthy, as indicated by a medical questionnaire. At the beginning of the study fasting serum total cholesterol ranged from 2.80 to 6.98 mmol/L (mean, 4.62 mmol/L), HDL cholesterol from 0.76 to 2.42 mmol/L (mean, 1.52 mmol/L), and triglycerides from 0.41 to 2.24 mmol/L (mean, 0.92 mmol/L). The subjects were between 19 and 67 years old (mean, 31 years), and weighed between 54.4 and 108.4 kg (mean, 71.3 kg); their body mass index (mean ± SD) was 22.6 ± 2.8 kg/m^2. Approval for the study had been obtained from the Ethics Committee of the department. The protocol and aims of the study were thoroughly explained to the subjects, who gave their written consent. No financial reward was given, except for the study diets, which were free.

Diets

The diets were composed of conventional solid foodstuffs, and menus were changed daily during each 3-week dietary period. On week days at noon hot meals were served and eaten at the department, and other foods were supplied daily as a package. All foods were weighed out individually, according to each person's energy requirement. Body weights were essentially constant during the 6 weeks of the study (Chapter 6).

Both diets supplied on average 12.4 MJ (2950 kcal) per day, of which 41% as fat, 45% as carbohydrates, 13% as protein, and 1% as alcohol. Cholesterol content was 30 mg per MJ (368 mg/day). The differences in the positional distribution of fatty acids were achieved by the use of special margarines and oils, developed by the Unilever Research Laboratory, Vlaardingen, the Netherlands. **Table 2** shows that the fat in the two diets contained equal amounts of each specific fatty acid (columns 1 and 4), but differed widely in the positional fatty acid distribution. In the Betapol diet, 65% of the palmitic acid was attached to the 2 position, and the remaining 35% to the 1 and 3 position. In contrast, 82% of the palmitic acid in the palm-oil diet was on the 1 and 3 position, while only 18% was found on the 2 position. Sixty-eight percent of the *sn*-2 fatty acids in the Betapol diet was saturated, mainly palmitic acid, whereas 73% of the *sn*-2 fatty acids in the palm-oil diet were unsaturated, the majority being oleic acid (Table 2, columns 2 and 5).

Table 2. Total, sn-2, and sn-1,3[a] fatty acid composition of the triglycerides in the experimental diets.

Fatty acid	Palm oil diet			Betapol diet		
	Total	*Sn*-2	*Sn*-1,3	Total	*Sn*-2	*Sn*-1,3
	g/100 g of dietary fatty acids					
Saturated	42.1	26.5	49.9	40.6	67.8	26.9
Lauric acid (C12:0)	1.0	1.1	0.9	1.0	1.4	0.8
Myristic acid (C14:0)	2.5	3.5	2.0	2.7	4.3	1.9
Palmitic acid (C16:0)	27.7	14.6	34.2	27.4	53.5	14.3
Stearic acid (C18:0)	9.2	6.2	10.8	8.1	6.9	8.6
Monounsaturated	42.8	52.0	38.1	44.4	22.3	55.5
Oleic acid (*cis*-C18:1)	41.3	51.0	36.4	42.1	20.9	52.7
Polyunsaturated	13.2	20.6	9.6	12.3	8.4	14.3
Linoleic acid (C18:2)	12.2	19.6	8.5	11.2	7.2	13.1
Unknown	1.9	0.9	2.4	2.7	1.5	3.3
Total	100	100	100	100	100	100

The special oils supplied 70% of the total fat in the study diets, of which 64% as margarine. The remaining 30% came from conventional foodstuffs, largely dairy products and meat.

[a] Calculated from the total and *sn*-2 fatty acid composition.

Blood sampling and analysis

Blood was sampled after a 12 h fast on days 17 and 21 of each dietary period. Serum was obtained by low speed centrifugation within one hour of venipuncture and used for enzymatic analysis of total and HDL cholesterol and of total triglyceride levels [22-24]. On day 21 of each period blood was also drawn into tubes with EDTA as anticoagulant. These tubes were immediately centrifuged at low speed and 4 °C to separate plasma from the red blood cells. The cells were washed twice with ice-cold isotonic saline, transferred into glass tubes, and hemolyzed at −80 °C. Samples were stored at −80 °C until analysis.

For the isolation of cholesteryl esters and triglycerides, 3 mL of isopropanol and 1 mL of distilled water were added to 0.65 mL plasma, the sample was mixed for 30 s, 3 mL of *n*-octane were added and this mixture was shaken for 15 min. After centrifugation for 3 min. at 1580 x *g* the (upper) octane layer was removed and evaporated to dryness in a stream of nitrogen. The lipids were redissolved in 2 mL 2.5 vol% diethyl ether in hexane, applied to a silica column (Varian, Analytichem Bond Elut) and eluted twice with 2 mL of the same solvent to obtain the cholesteryl ester fraction. Subsequently, the triglycerides were eluted with 2 mL of 15 vol% diethyl ether in hexane. Thin-layer chromatography [25] of the fractions showed that separation was essentially complete.

1.5 mL of the eluate that contained the triglycerides was dried down under N_2. If the amount of triglycerides was less then 0.3 mg, then more plasma lipids was extracted until sufficient material was obtained. The triglycerides were dissolved in 0.05 ml of hexane and incubated with 2.53 mL of buffered porcine pancreatic lipase emulsion (EC 3.1.1.3, Sigma), plus 0.125 mL sodium cholate solution and 0.05 mL calcium chloride solution to hydrolyse the *sn*-1 and *sn*-3 fatty acids. The reaction was stopped after 3 min. by adding 1 mL of hydrochloric acid, 6 mol/L. The lipids were extracted with diethyl ether, dried, dissolved in chloroform and applied to an aminopropyl column (Varian, Analytichem Bond Elut). Diglycerides plus triglycerides were eluted with 4 mL of 15 vol% ethyl acetate in hexane and the 2-monoglycerides with 4 mL of chloroform/2-propanol, 2/1 (vol/vol). The 2-monoglyceride fraction and an aliquot of the original triglyceride fraction were saponified and methylated for gas chromatographic analysis [26].

Fatty acid compositions of monoglyceride fractions obtained at different lipase concentrations were highly similar, but absolute amounts recoverd differed. We reduced the concentration of lipase from 10 mg per mL [27] to 2.5 mg/mL in order

Table 3. Total and *sn*-2 fatty acid composition (mean ± SD) of the fasting plasma triglycerides in the 60 subjects at the end of the two dietary periods.

Fatty acid	Total fatty acid composition			*Sn*-2 fatty acid composition		
	Palm oil diet	Betapol diet	Difference	Palm oil diet	Betapol diet	Difference
	g/100 g of total triglyceride fatty acids			*g/100 g of sn-2 triglyceride fatty acids*		
C14:0	1.4 ± 0.4	1.4 ± 0.5	−0.0 ± 0.5	1.1 ± 0.5	1.0 ± 0.5	−0.0 ± 0.4
C16:0	24.1 ± 2.0	23.9 ± 3.1	−0.2 ± 2.6	10.2 ± 2.2	12.3 ± 3.6	2.1 ± 3.0[b]
C16:1	3.7 ± 1.2	4.0 ± 1.1	0.3 ± 1.0[a]	4.5 ± 1.8	4.8 ± 1.7	0.3 ± 1.6
C18:1	41.8 ± 2.9	40.4 ± 3.2	−1.4 ± 3.4[b]	46.9 ± 3.4	43.6 ± 4.5	−3.3 ± 4.8[b]
C18:2n-6	19.9 ± 3.6	20.8 ± 5.2	0.9 ± 5.1	30.3 ± 4.8	30.7 ± 6.6	0.4 ± 6.2
C18:3n-3	1.5 ± 1.0	1.9 ± 1.2	0.4 ± 1.4[a]	1.4 ± 0.9	1.7 ± 1.0	0.3 ± 1.1[a]
C20:4n-6	1.2 ± 0.3	1.3 ± 0.3	0.1 ± 0.2[b]	1.7 ± 0.4	1.7 ± 0.4	0.0 ± 0.4

[a] Significantly different from zero, $P<0.05$.
[b] Significantly different from zero, $P<0.01$.

to optimize the proportion of total fatty acids recovered in the monoglyceride fraction. The *sn*-2 fatty acid compositions of Betapol and palm oil as determined by the official method [27] were similar to the compositions as determined by our method; differences were less than 1.4 g/100 g for every *sn*-2 fatty acid. A serum pool with a triglyceride concentration of 1 mmol/L and a solution of 0.3 mg Betapol in 0.65 mL hexane were used as internal controls and for calculation of the reproducibility. The analytical coefficient of variation within runs of the main fatty acids (C16:0, C18:1, C18:2, and C20:4) was on average 1.3% for total fatty acids and 4.8% for *sn*-2 fatty acids in triglycerides. All samples of a particular subject were analyzed within one run.

Erythrocyte lipids were extracted from hemolyzed cells into isopropanol/hexane 3/4 (vol/vol); the hexane contained 50 mg 2,6-di-tert-butyl-p-cresol (BHT; BDH Biochemicals, Poole, UK) per L as an anti-oxidant. The lipids from erythrocytes were transmethylated for 18h at 60 °C and the cholesteryl esters from plasma for 1h at 90 °C, both with 40 mL H_2SO_4/L methanol [26]. The coefficients of variation within runs for the major fatty acids was 0.7% for cholesteryl esters and 1.9% for erythrocyte phospholipids.

RESULTS

Fasting serum triglyceride levels were similar ($P=0.35$) on the palm oil diet (0.97 ± 0.42 mmol/L) and the Betapol diet (0.94 ± 0.40 mmol/L). Lipoprotein cholesterol concentrations were also highly similar (Chapter 6). **Table 3** shows the total and *sn*-2 fatty acids compositions of the plasma triglycerides. On the Betapol diet, in which oleic acid was attached to the outer positions of the dietary triglycerides, the proportion oleic acid of total fatty acids in triglycerides was 1.4 g/100 g fatty acids lower ($P=0.002$; 95% confidence interval, −2.3 to −0.5 g/100 g) than on the palm oil diet. There were small increases in the proportions of palmitoleic and polyunsaturated fatty acids (Table 3, column 3). Changes were largely located in the *sn*-2 position: column 6 of Table 3 shows that the Betapol diet raised the proportion of palmitic acid on the *sn*-2 position of plasma triglycerides by 2.1 g/100 g *sn*-2 fatty acid ($P<0.0001$; 95% confidence interval, 1.4 to 2.9 g/100 g) and lowered oleic acid by 3.3 g/100 g *sn*-2 fatty acids ($P<0.0001$; 95% confidence interval, −4.5 to −2.0 g/100 g). **Fig. 2** depicts the

individual differences in *sn*-2 fatty acids between the two diets. Out of all 60 subjects, 45 had a higher proportion of palmitic acid and 46 had a lower proportion of oleic acid on the *sn*-2 position of plasma triglycerides on the Betapol diet relative to the palm oil diet.

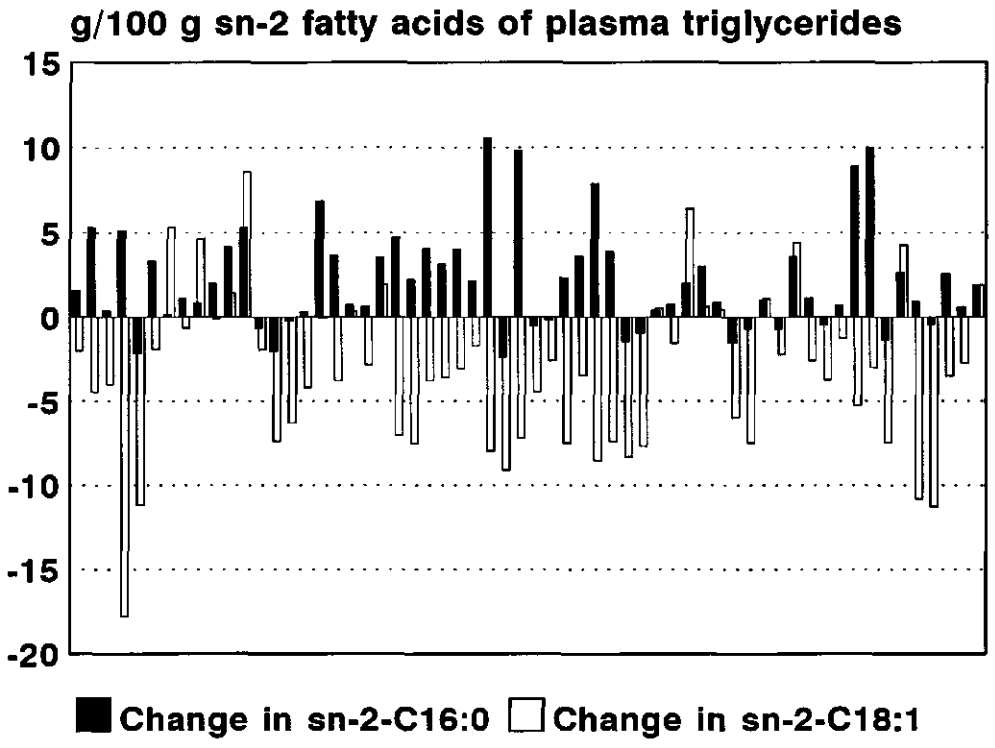


Fig. 2. Individual differences in the proportions of palmitic (C16:0) and oleic acid (C18:1) on the *sn*-2 position of fasting plasma triglycerides between the Betapol and the palm oil diet.

Table 4 shows the fatty acid compositions of the plasma cholesteryl esters. Betapol increased the proportion of palmitic acid by 0.7 ($P<0.0001$) and palmitoleic acid by 0.3 ($P<0.0001$) g/100 g cholesterol ester fatty acids, and decreased oleic by 0.4 ($P=0.014$), linoleic by 0.6 ($P=0.029$), and arachidonic acid by 0.2 ($=0.004$) g/100 g cholesteryl ester fatty acids.

Table 4. Fatty acid composition (mean ± SD) of the plasma cholesteryl esters in the 60 subjects at the end of the two dietary periods.

	Palm oil diet	Betapol diet	Difference	(95% CI)
		g/100 g of cholesteryl esters fatty acids		
C14:0	0.43 ± 0.10	0.45 ± 0.11	0.02 ± 0.08[a]	(0.00 to 0.04)
C16:0	10.09 ± 0.64	10.76 ± 0.64	0.67 ± 0.57[b]	(0.53 to 0.81)
C16:1	2.34 ± 0.96	2.63 ± 0.88	0.29 ± 0.48[b]	(0.16 to 0.41)
C18:0	0.78 ± 0.15	0.72 ± 0.15	−0.06 ± 0.12[b]	(-0.09 to -0.03)
C18:1n-9	18.61 ± 1.52	18.18 ± 1.46	−0.43 ± 1.20[b]	(-0.72 to -0.14)
C18:1n-7	0.99 ± 0.13	1.05 ± 0.17	0.06 ± 0.15[a]	(0.02 to 0.10)
cis-C18:1	19.60 ± 1.53	19.23 ± 1.51	−0.37 ± 1.20[a]	(-0.66 to -0.08)
C18:2n-6	55.16 ± 3.68	54.52 ± 3.44	−0.64 ± 2.19[a]	(-1.20 to -0.07)
C18:3n-3	0.45 ± 0.10	0.49 ± 0.10	0.04 ± 0.09[b]	(0.01 to 0.06)
C20:4n-6	6.01 ± 1.08	5.83 ± 1.01	−0.18 ± 0.50[b]	(-0.30 ± -0.06)

[a] Significantly different from zero, $P<0.05$.
[b] Significantly different from zero, $P<0.01$.

Fig. 3 shows that 55 out of 60 subjects had a higher proportion of palmitic and 43 had a lower proportion of oleic acid in their cholesteryl esters on the Betapol diet than on the palm oil diet.

Fatty acid compositions of the erythrocyte membranes are given in **Table 5**. On Betapol the proportion of palmitic acid increased by 0.2 g/100 g fatty acids ($P=0.038$), while the proportions of polyunsaturated fatty acids were somewhat, although not significantly, lower than on the palm oil diet.

Five women experienced events during the trial that could have influenced their lipid metabolism, e.g. estrogen therapy during part of the trial, treatment with antibiotics, and weight loss (Chapter 6). However, statistical analyses without the data of these 5 women produced essentially similar results.

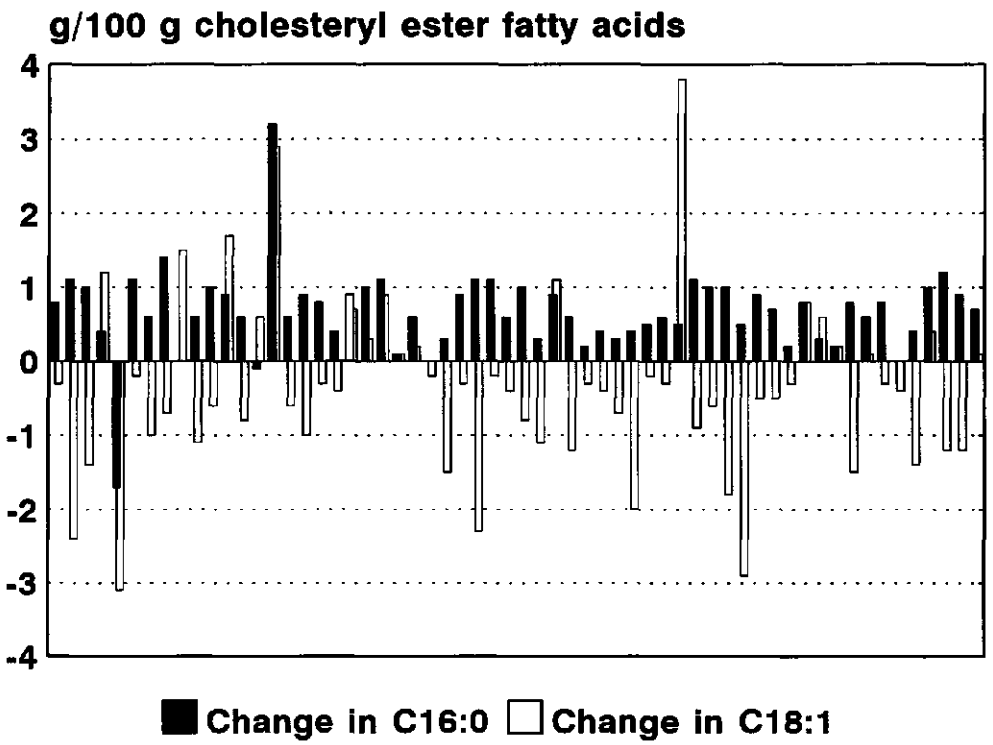


Fig. 3. Individual differences in the proportions of palmitic (C16:0) and oleic acid (C18:1) in fasting cholesteryl esters between the Betapol and the Palm oil diet.

DISCUSSION

Plasma triglyceride configuration

Our findings show that the positional distribution of dietary fatty acids significantly influences the fatty acid configuration and composition of fasting plasma lipids. Although the differences in plasma triglyceride configuration between the diets were much smaller than the differences in the dietary triglycerides, the changes in *sn*-2 fatty acids in plasma triglycerides clearly reflected changes in the *sn*-2 fatty acids in dietary fat. Myher et al. [28] found an increase in palmitic acid at the *sn*-2 position of plasma triglycerides 4 hours after subjects consumed a large

Table 5. Fatty acid composition (mean ± SD) of the erythrocyte membranes in the 60 subjects at the end of the two dietary periods.

	Palm oil diet	Betapol diet	Difference	(95% CI)
	g/100 g of erythrocyte membrane fatty acids			
C14:0	0.35 ± 0.08	0.36 ± 0.07	0.01 ± 0.09	(-0.01 to 0.03)
C16:0	20.83 ± 0.60	21.00 ± 0.62	0.17 ± 0.64 [a]	(0.01 to 0.34)
C16:1	0.36 ± 0.11	0.39 ± 0.10	0.03 ± 0.11 [a]	(0.00 to 0.06)
C18:0	14.10 ± 0.62	13.99 ± 0.72	−0.11 ± 0.66	(-0.06 to -0.28)
C18:1n-9	11.59 ± 0.73	11.59 ± 0.62	−0.00 ± 0.46	(-0.09 to 0.09)
C18:1n-7	1.25 ± 0.18	1.31 ± 0.16	0.06 ± 0.11 [b]	(0.04 to 0.08)
cis-C18:1	12.85 ± 0.73	12.91 ± 0.62	0.06 ± 0.45	(-0.04 to -0.16)
C18:2n-6	9.89 ± 1.10	9.92 ± 1.14	0.04 ± 0.53	(-0.10 to 0.17)
C18:3n-3	0.29 ± 0.43	0.34 ± 0.42	0.05 ± 0.41	(-0.06 to 0.15)
C20:3n-6	1.42 ± 0.37	1.43 ± 0.40	0.01 ± 0.28	(-0.06 to 0.08)
C20:4n-6	12.82 ± 0.85	12.74 ± 0.92	−0.08 ± 0.50	(-0.20 to -0.05)
C22:0	1.81 ± 0.43	1.86 ± 0.30	0.05 ± 0.49	(-0.07 to 0.17)
C22:4n-6	3.02 ± 0.46	3.01 ± 0.45	−0.01 ± 0.15	(-0.04 to 0.03)
C22:5n-3	2.04 ± 0.38	2.04 ± 0.36	−0.00 ± 0.25	(-0.06 to 0.05)
C24:0	4.83 ± 0.40	4.76 ± 0.45	−0.07 ± 0.23 [a]	(-0.13 to -0.00)
C22:6n-3	3.84 ± 0.80	3.86 ± 0.81	0.01 ± 0.41	(-0.09 to -0.11)
C24:1	4.37 ± 0.48	4.32 ± 0.39	−0.04 ± 0.28	(-0.11 to 0.02)

[a] Significantly different from zero, $P<0.05$.
[b] Significantly different from zero, $P<0.01$.

single dose of lard, which, like Betapol, contains palmitic acid mainly at the *sn*-2 position. This effect was somewhat larger than observed here but still modest. A change of this kind was to be expected because Myher measured postprandial triglycerides. Our observation that fasting plasma triglycerides partly reflect the dietary fatty acid configuration is new and suggests an effect beyond chylomicron clearance.

The positional distribution of dietary fatty acids was also reflected in the total fatty acid composition of plasma lipids; when a fatty acid was mainly on the *sn*-2 position of the dietary fat, its proportion in both total triglycerides and cholesteryl esters was increased. Changes in the pattern of the fatty acids in erythrocyte membranes – mainly phospholipids – were in the same direction as seen in the triglycerides and cholesteryl esters. Although these effects were smaller than those

seen when the overall pattern of dietary fatty acids is changed [29](Chapter 5), they were highly consistent.

Innis and co-workers [30] found that in newborn piglets Betapol increased palmitic and decreased linoleic acid in the plasma triglycerides, while the proportion of oleic acid was unaltered. We found that Betapol did not affect total palmitic acid in the plasma triglycerides, and decreased the oleic acid content. Innis et al. [30] reported an increase in palmitic acid and decreases in unsaturated fatty acids in cholesteryl esters on the Betapol diet. This agrees with our findings, although the differences seen in piglets were larger. However, the effects of dietary fat on lipid metabolism of newborn piglets are difficult to compare with those of adult humans, not only because of species differences, but also because in neonates the absorption of palmitic acid in the *sn*-2 position is much more efficient than that of palmitic acid in the *sn*-1 and *sn*-3 position [31,32].

It is unlikely that the differences in the configuration of triglycerides in plasma were due to residual chylomicrons. Blood samples were taken 11.5 to 14.0 hours after the last meal. Compliance of the subjects with instructions for fasting was high (Chapter 6), and the fasting state was confirmed by low triglyceride levels. The observed differences in *sn*-2 fatty acids must thus be due to triglycerides in lipoproteins other than chylomicrons. A plausible explanation is that chylomicron triglycerides absorbed after a meal escape lipolysis through transfer to VLDL, LDL, or HDL by lipid transfer proteins [13] (route *H* in fig. 1). VLDL particles are converted to LDL [33] and LDL triglycerides cannot be hydrolyzed by lipoprotein lipase because LDL lacks apolipoprotein CII. Furthermore, catabolism of VLDL, LDL and HDL is much slower than that of chylomicron (remnant) particles [33]. Observations that substantial amounts of retinyl ester are transferred from chylomicrons to VLDL [34,35] supports this explanation.

An alternative explanation is that newly synthesized hepatic triglycerides reflect the positional distribution of dietary triglycerides (route *J* in fig. 1). Theoretically, 2-monoglycerides resulting from peripheral lipoprotein lipase action on exogenous triglycerides (route *D*) could be transported to the liver by chylomicron remnants or albumin [11] (route *F*) and then be used for synthesis of VLDL triglycerides in the microsomes (route *J*). However, this presumes that 2-monoglycerides are not hydrolyzed by hepatic lipases (route *G*) and that the 2-monoacylglycerol pathway of triglyceride synthesis (route *J*) is active in the adult human liver. Significant activity of microsomal monoacylglycerol acyltransferase, the enzyme which defines this pathway, has been observed in neonatal but not in

adult rat liver [36]. It is possible that the high proportion of energy obtained from dietary fat by humans induces this pathway, but this mechanism remains speculative [37], and lipid transfer from chylomicrons to other lipoproteins seems the most plausible explanation for our findings.

Fatty acid composition of cholesteryl esters

The majority of cholesteryl esters in plasma are generated by LCAT, which transfers a fatty acid from phosphatidylcholine to free cholesterol (route *M* in fig. 1). LCAT has a preference for the unsaturated *sn*-2 fatty acids of phosphatidylcholine. Most phospholipids circulating in plasma are synthesized in the liver (route *P*), but intestinal mucosal cells also produce phosphatidylcholine, either by reacylation of absorbed lysolecithin [16] (route *N*), or by *de novo* synthesis [38] (route *O*). An early study [17] with isolated microsomes from rat intestinal cells suggested that mucosal phosphatidylcholine is synthesized only via the phosphatidic acid pathway (route *O*), but it was recently shown that rat and hamster mucosal cells can also utilize 2-monoacylglycerols for phospholipid production in vitro [8] (route *C*). We are not aware of any human data on this subject. If route *C* operates *in vivo* in man, then dietary *sn*-2 fatty acids might end up in the *sn*-2 position of chylomicron phosphatidylcholine and be subsequently transferred to cholesteryl esters by LCAT in plasma (route *M*). This explanation is supported by the fatty acid composition of erythrocyte phospholipids, which showed a small but significant enrichment of palmitic acid on the Betapol diet.

Our data do not support formation of chylomicron phospholipids through reacylation of the 2-position of absorbed lysolecithin (*sn*-1-acylglyceryl-phosphorylcholine) in mucosal cells in man in vivo (route *N* in fig. 1). During digestion and absorption of fat, dietary *sn*-2 fatty acids remain esterified to glycerol – at least partly – and are thus less available for reacylation of the 2 position of lysolecithin than are *sn*-1 or *sn*-3 fatty acids, and by inference they would also be less available for cholesteryl ester formation by LCAT (route *M*). However, we observed enrichment of dietary *sn*-2 fatty acids in cholesteryl esters; therefore selective reacylation of lysolecithin in the human gut does not explain our findings. The same reasoning suggests that intestinal ACAT is not a major route towards formation of plasma cholesteryl esters (route *A*): *sn*-1 and *sn*-3 fatty acids from dietary triglycerides are more available for mucosal ACAT than the *sn*-2 fatty acids,

but we observed an enrichment rather than depletion of *sn*-2 fatty acids in plasma cholesteryl esters. Therefore, synthesis of chylomicron phosphatidylcholine from absorbed 2-monoacylglycerols (route *C*) and subsequent transfer of the *sn*-2 fatty acid to cholesterol esters by LCAT in plasma (route *M*) is the most likely pathway for the observed effects of the dietary fatty acid configuration on the composition of plasma cholesteryl esters.

Alternatively, some of the *sn*-2-mono-palmitoyl-glycerols produced by lipoprotein lipase action on chylomicrons after a Betapol-rich meal might be transported to the liver by albumin or in chylomicron remnants (route *F* in fig. 1) [11]. The ensueing higher availability of palmitic acid for cholesterol esterification by hepatic cells could enhance production and secretion of cholesteryl palmitate in VLDL particles (route *L*). Increased delivery of palmitic acid to the liver could also explain the minimal rises in serum LDL cholesterol seen on Betapol [30] (Chapter 6), because the low affinity of ACAT for palmitic acid may cause a rise in a pool of unesterified (chole)sterol, resulting in down-regulation of the hepatic LDL receptor and a rise in plasma LDL concentrations [39].

A third possible mechanism is that exogenous 2-monoacylglycerols reaching the liver might be converted into phosphatidylcholine (route *K* in Fig. 1). Secretion of these phospholipids in VLDL particles and transfer of the *sn*-2 fatty acids to cholesteryl esters by LCAT (route *M*) might explain the increase in plasma cholesteryl palmitate that we observed on the Betapol diet. However, this mechanism is less likely because the 2-monoacylglycerol pathway of glycerolipid synthesis is probably not active in the adult liver [36,37].

In conclusion, we found that the positional distribution of fatty acids in dietary fat had a small but distinct and significant influence on the fatty acid configuration and composition of fasting plasma triglycerides and cholesteryl esters in humans. The most plausible explanation for retention of the *sn*-2 position in dietary triglycerides in fasting plasma lipids is that chylomicron triglycerides with the original fatty acid on the *sn*-2 position are transferred to other lipoproteins and in this way escape postprandial lipolysis by lipoprotein lipase. The enrichment of cholesteryl esters in dietary *sn*-2 fatty acids points to formation of phospholipids in the intestine from absorbed 2-monoacylglycerols and subsequent transfer of the *sn*-2 fatty acids to cholesteryl esters by LCAT in plasma. However, other explanations cannot be excluded.

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CHAPTER 8

General discussion

The current thesis embodies three controlled dietary trials in which we studied the influence of fatty acids on serum lipoprotein cholesterol levels and some other risk factors for CHD. The studies were aimed at investigating effects of the *cis/trans* configuration of the double bonds, the chain length, and the triglyceride configuration of fatty acids. The first section of this chapter summarizes the main findings and the results are put into perspective with those of other studies. Next, some methodological aspects of the trials are discussed. The main conclusions are given at the end of this chapter.

MAIN FINDINGS AND PERSPECTIVE WITH OTHER STUDIES

Monounsaturated *trans* fatty acids

LDL and HDL cholesterol

The first study in this thesis confirms that monounsaturated *trans* fatty acids from hydrogenated vegetable oils simultaneously raise LDL and lower HDL cholesterol as compared with *cis* unsaturated fatty acids. Several dietary trials on *trans* fatty acids have been reported since our study was published [1-3]. **Fig. 1** shows the effects of monounsaturated *trans* fatty acids relative to their *cis* isomer oleic acid across five trials. Differences in other fatty acids between the *trans* enriched diets and the reference diets were adjusted for using the meta-analysis of Mensink [4]. Thus, the changes we observed for on the *Trans*-diet versus the Linoleate diet (Chapter 2) are now expressed relative to oleic acid, taking into account the modest differences between oleic and linoleic acid. When the data from references [1-3,5] and our study are combined in a linear model, every additional percent of dietary energy as *trans* fatty acids results in an increase in LDL cholesterol of 0.040 mmol/L or 1.55 mg/dL ($R^2 = 0.86$, $P = 0.028$) and a decrease in HDL cholesterol of 0.013 mmol/L or 0.50 mg/dL ($R^2 = 0.88$, $P = 0.019$). These combined data indicate that the effects of monounsaturated *trans* fatty acids on LDL cholesterol is about similar to that of saturated fatty acids [4]. Although more studies would be needed to define the true shape of the dose-response curves, the available data fit well in linear relations. The regression coefficients are in good agreement with the estimates of 0.03 mmol/L for LDL and −0.015 mmol/L for HDL derived from the study of Mensink [5] and ours (Chapter 2). Fig. 1 shows

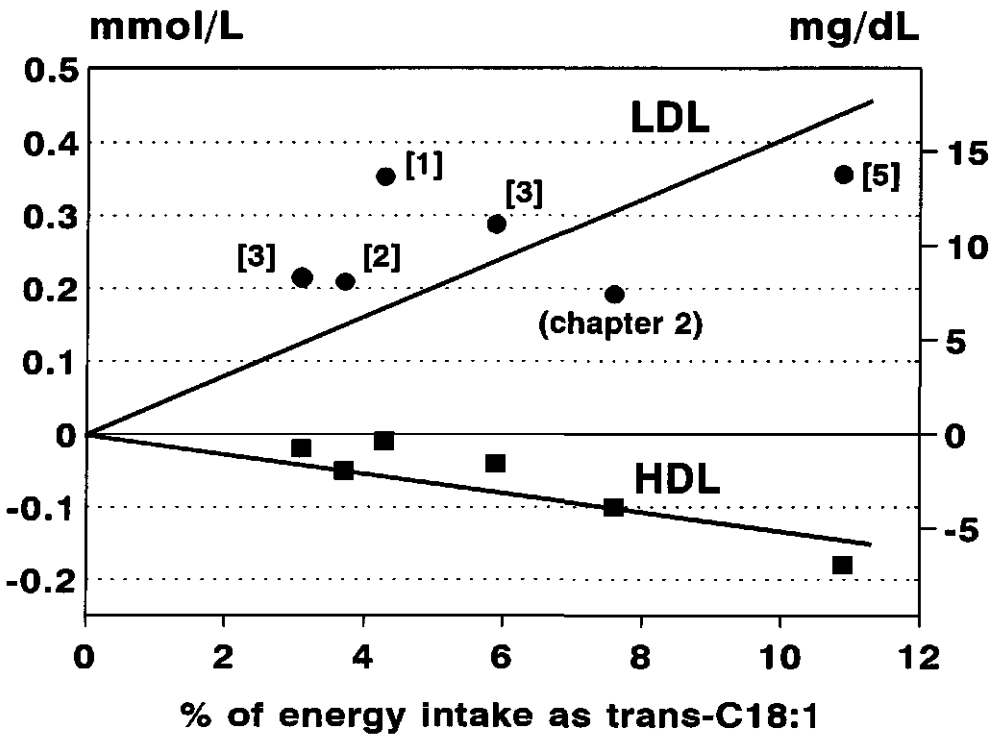

Fig. 1. Effects of monounsaturated *trans* fatty acids (*trans*-C18:1) on lipoprotein cholesterol levels relative to oleic acid (*cis*-C18:1). Data are derived from 6 dietary comparisons between *trans* monounsaturates and *cis* unsaturated fatty acids [1-3,5] (Chapter 2); differences between diets in fatty acids other than *trans* and *cis* monounsaturates were adjusted for using regression coefficients from a meta-analysis of 27 controlled trials [4]. The regression lines were forced through the origin because a zero change in intake will produce a zero change in lipoprotein levels. Regression coefficients per percent contribution of *trans* to daily energy are 0.040 mmol/L per en% for LDL ($R^2 = 0.86$, $P = 0.0028$) and −0.013 mmol/L per en% ($R^2 = 0.88$, $P = 0.0019$) for HDL cholesterol.

that the increase in LDL seen in our study was somewhat smaller than predicted by the regression line. However, the predicted value fell within the 95% confidence interval of the change in LDL as observed by us, and the difference thus may well be due to chance.

It has been proposed that HDL cholesterol responses to *trans* fatty acids may be dependent on the dietary dose, with a threshold below which *trans* monounsaturates have no effect [1,3]. However, Fig. 1 suggests that the effect on HDL increases with the amount of *trans* fatty acids consumed. It appears that to date there is no evidence for a threshold below which *trans* fatty acids do not affect HDL cholesterol.

Hydrogenation alternatives

Grundy [6] suggested that stearic acid, a fatty acid known to be 'neutral' in its effects on serum lipids [7-12], might be a favorable alternative to *trans* fatty acids for the hardening of edible fats. We followed this lead by comparing stearic acid and *trans* fatty acids with each other, and with linoleic acid, the fatty acid from which stearic and *trans* fatty acids are formed during hydrogenation. The Stearate-diet in our study slightly elevated the HDL to LDL cholesterol and the apolipoprotein A-I to B ratios as compared with the *Trans*-diet (Chapter 2), but the differences in HDL and LDL cholesterol levels were small and not statistically significant. Thus, our data do not support the idea that substituting stearic acid for *trans* fatty acids yields much health benefits. It is possible, however, that our study somewhat underestimated the effect of *trans* monounsaturates on LDL cholesterol (Fig. 1), and that the true differences between stearic acid and *trans* fatty acids are larger than observed by us.

Linoleic acid, like stearic acid, has been reported to equal oleic acid in its effects on serum lipids [13-18]. Therefore, we hypothesized that the effects of stearic acid would be similar to those of linoleic acid. This was not confirmed by the results; stearic acid moderately raised total and LDL cholesterol as compared with linoleic acid. The average increase in men plus women was 0.15 mmol/L for total cholesterol. This is smaller than the 0.25 mmol/L estimated by the equation of Keys [7], but slightly larger than the rise of 0.11 mmol/L predicted for stearic versus linoleic acid according to the meta-analysis of Mensink [4]. Unfortunately, we did not study oleic acid in our trial, and it is thus impossible to tell whether the difference between the Stearate- and the Linoleate-diet was caused by stearic acid being slightly cholesterol-raising or linoleic acid being cholesterol-lowering relative to oleic acid. In any event, stearic acid increases total and LDL cholesterol less than lauric, myristic, and palmitic acid [7-12]. Furthermore, we observed a small HDL cholesterol-lowering effect of stearic acid. Others also reported somewhat lower HDL cholesterol levels on stearic acid diets than on diets rich in unsaturated

fatty acids [9,19]. The differences in these latter studies were not statistically significant, but together with our data they suggest that stearic acid may have some HDL cholesterol-depressing effect.

In summary, this study does not support the idea that complete hydrogenation of linoleic acid to stearic acid is much more beneficial for the serum lipid profile than partial hydrogenation to *trans* fatty acids. However, the potential of stearic acid as a replacer of *trans* fatty acids in hard fats needs more investigation.

Blood pressure

Monounsaturated *trans* fatty acids did not affect systolic or diastolic blood pressure (Chapter 3). This is consistent with findings from the study by Mensink and Katan [20]. Lichtenstein and co-workers [2] also reported no effect of *trans* fatty acids on systolic and diastolic blood pressure. Thus, it seems that *trans* fatty acids from hydrogenated vegetable oil do not influence blood pressure in normotensive subjects.

Lipoprotein(a)

Two dietary trials from our department show that consumption of *trans* fatty acids may increase the serum Lp(a) concentration relative to saturated or *cis* unsaturated fatty acids (Chapter 4). This finding confirms the study of Nestel and co-workers [1], in which a *trans* fatty acid-rich diet led to elevations in Lp(a) levels compared with diets rich in palmitic or oleic acid. Lichtenstein et al. [2] did not observe a rise in Lp(a) after consumption of *trans* fatty acids. However, this lack of effect might be explained by the lower dose of *trans* fatty acids, the relatively small number of subjects, and the high variability of Lp(a) levels, resulting in a limited power. Hornstra and co-workers [21] showed that replacement of the habitual fat in a Dutch diet by palm oil lowered Lp(a) levels in healthy men. These findings are in line with the notion that *trans* fatty acids increase Lp(a), as the replacement of habitual fat by palm oil decreased the intake of *trans* fatty acids by more than 50% [4].

Additional comments

Consumption of monounsaturated *trans* fatty acids results in an unfavorable lipoprotein risk profile for CHD by concurrently increasing LDL and decreasing HDL cholesterol levels relative to *cis* unsaturated fatty acids. Moreover, *trans* fatty acids might enhance CHD risk by raising the serum Lp(a) concentration. It should be

mentioned that, in addition to partially hydrogenated vegetable oils, partially hydrogenated fish oils are an important source of *trans* fatty acids in Western Europe. Such hardened fish oils contain mainly polyunsaturated *trans* isomers with a chain length of 20 or 22 rather than 18 carbon atoms. The metabolic effects of hardened fish oils in humans have not been studied in depth, and could possibly be different from those of fats rich in monounsaturated *trans* isomers of oleic acid (*trans*-C18:1).

The mechanisms by which *trans* fatty acids raise LDL and lower HDL cholesterol are as yet unclear. A report by Abbey and Nestel [22] suggests that the activity of cholesterol ester transfer protein (CETP), an enzyme which transfers cholesterol from HDL to lower density lipoproteins, is involved. This explanation is supported by measurements of the activity levels of CETP in sera from our study. The CETP activities after the stearic acid diet and the linoleic acid diet were identical, despite the higher (VLDL + LDL) cholesterol on stearic acid. The *trans* fatty acid diet was accompanied by a mean increase of 18% in CETP activity. The rise was seen in 52 out of 55 individuals, one subject showed no change, and two showed a decrease (van Tol A, Zock PL, van Gent T, Scheek LM, Katan MB. Submitted for publication).

Epidemiologic studies sustain the concept that *trans* fatty acid intake contributes to CHD risk. In a cross-sectional study of 748 men consumption of *trans* fatty acids was positively related to LDL and inversely related to HDL cholesterol [23]. In several case-control studies, patients were found to have higher intakes of *trans* fatty acids than controls [24-26]. The most suggestive evidence derives from a prospective study of 85,000 women, in which consumption of *trans* fatty acids from partially hydrogenated vegetable oils was significantly associated with CHD risk.

Although not fully conclusive, the metabolic and epidemiologic data together provide considerable evidence that a high intake of *trans* fatty acids has adverse health effects.

Specific saturated fatty acids

Myristic versus palmitic acid

The trial on specific saturated fatty acids clearly showed that myristic as well as palmitic acid are dietary constituents that raise serum total and LDL cholesterol levels (chapter 5). Myristic acid was about 1.5 times as cholesterol-raising as palmitic acid, due to increases in both LDL and HDL cholesterol on the myristic versus the palmitic acid diet. Few studies have investigated the relative effects of lauric, myristic, and palmitic acid by direct comparison. In three trials lauric acid was exclusively exchanged for palmitic acid [27-29]. These studies, however, did not produce a conclusive answer; lauric acid resulted in either lower [27], the same [28], or higher [29] cholesterol levels than palmitic acid. Our study is the only trial next to that by McGandy and co-workers [30] in which myristic acid was directly compared with palmitic acid. McGandy et al. concluded that both myristic and palmitic acid raise total cholesterol. They did not find a consistent difference between these two saturates [30], possibly because the number of subject was small. Other human studies on the relative cholesterol-raising potencies of myristic and palmitic acid either applied a meta-analytical approach [4,8], or studied the effects of coconut oil, rich in both lauric and myristic acid, versus those of palm oil, which is rich in palmitic acid [31-33]. Findings from the meta-analyses suggest that myristic acid might be 4 to 6 times more cholesterol-raising than palmitic acid [4,8]. This large difference, however, may be partly explained by the type of studies included in the regression analyses. Butterfat and coconut oil, the main sources of myristic acid, were the primary saturated fats studied, the intake of lauric acid (coconut oil) and palmitic acid (butterfat) were thus closely linked with that of myristic acid. The high correlations of the various fatty acids could compromise the ability of regression analysis to accurately assess the relative potencies [34].

Palmitic acid from palm oil

The trials in which palm oil was compared with coconut oil found that palmitic acid has a much smaller cholesterol-raising effect than a mixture of lauric and myristic acid [31-33], and the authors therefore suggested that palmitic acid might be neutral (i.e. equivalent to oleic acid). The reasons for the discrepancy with our finding on palmitic acid are not entirely clear, but one explanation may be that the dietary control in the other trials was less strict than in our study. Hayes and

Khosla [35-37] propose that palmitic acid raises serum cholesterol in hypercholesterolemic subject (total cholesterol, >5.8 mmol/L or 225 mg/dL), especially in those with a high cholesterol intake, but not in normocholesterolemic subjects. This is not confirmed by our study. The subjects in our trial were normocholesterolemic and had an average cholesterol intake; yet their cholesterol levels were clearly elevated on the palmitic versus the oleic acid diet. Furthermore, the magnitude of the individual responses to palmitic acid were not related to the subjects' initial cholesterol levels (Chapter 5). Our findings are in accordance with a number of other studies in which palmitic acid from palm oil was shown to increase total and LDL cholesterol relative to oleic acid [1,9,13,27,38]. **Table 1** summarizes the results of three strictly controlled trials and our study. The cholesterol-raising effect of palmitic acid ranges from 0.034 to 0.047 mmol/L per percent of energy of palmitic acid substituted for oleic acid. The effect does not seem to be dependent on the average cholesterol level of the subjects or the amount of cholesterol in the diets. However, the average rise of 0.04 mmol/L per percent of energy as palmitic acid is lower than the values of 0.055 to 0.065 mmol/L for C12-C16 saturates estimated in the meta-analyses of Keys [7], Hegsted [8], and Mensink [4]. This difference can be partly explained by myristic acid, and perhaps also lauric acid [29], being more cholesterol-raising than palmitic acid. It is also possible that the consumption of lauric and myristic acid modifies the effect of palmitic acid, and that the combined effect of C12-C16 saturates is larger than the sum of the indiviual effects of each fatty acid. Moreover, the amount of dietary

Table 1. Cholesterol intake, average serum total cholesterol level, and the proportional change in total cholesterol on strictly controlled diets in which palmitic acid from palm oil was iso-energetically substituted for oleic acid.

Study	Cholesterol intake	Average cholesterol level during the trial (mmol/L)	Change in cholesterol per energy % of palmitic acid (mmol/L)
Mattson [13]	very low[a]	5.28	0.046
Bonanome [9]	<100 mg/day	4.79	0.034
Denke [27]	very low[a]	4.85	0.047
Zock (Chapter 5)	350 mg/day	4.89	0.043

[a] Actual cholesterol intake was not reported, but vegetable oils (containing no cholesterol) were the sole fat sources in the experimental diets.

cholesterol in some of the studies included in the early meta-analyses [7,8] was much higher than in the studies in Table 1. A very high cholesterol intake might enhance the rise in serum cholesterol induced by saturated fatty acids [35]. It has also been claimed – but not proven – that minor constituents in palm oil could have effects on cholesterol metabolism in humans [39].

Whatever the possible modulating effects, a diet rich in palmitic acid as present in palm oil obviously has a cholesterol-raising potential. It is possible, however, that the background composition of the diet modulates the magnitude of the effect of palmitic acid.

In conclusion, myristic acid produces a higher cholesterol level than palmitic acid. However, the difference is caused by rises in both LDL and HDL cholesterol, resulting in similar unfavorable HDL to LDL cholesterol ratios on either saturate. Our study indicates that palmitic should remain classified as a cholesterol-raising fatty acids. Most epidemiologic studies have not reported the consumption of specific saturated fatty acids, and it is thus very difficult to estimate their individual impact on CHD risk. More specific intake data would be needed to further investigate this point. The available studies indicate the lauric, myristic, and palmitic acid each raise total and LDL cholesterol levels. Therefore, it seems prudent to consider a high intake of these three fatty acids as an important CHD risk factor.

Chain length or number of double bonds?

The common cholesterol-raising fatty acids in the human diet are saturated and differ in chain length from 12 to 16 carbon atoms, whereas the predominant 'neutral' or cholesterol-lowering fatty acids have one or two double bonds and are 18 carbons in length. A pertinent question is whether the distinctive effects of these two classes of fatty acids are primarily due to their differences in chain length or to the presence or absence of double bonds. This question is difficult to answer because the precise mechanisms by which the molecular structure of fatty acids influence cholesterol metabolism are still unknown [40]. Our and other studies on 12, 14, and 16 carbon fatty acids indicate that the chain length modulates their cholesterol-raising effect. Saturated stearic acid (18 carbon atoms) raises serum cholesterol much less than the shorter C12-C16 saturates, which also suggests that length is important. Yet, the presence of one or more double bonds

also seems to have some metabolic effect, as the 18 carbon stearic, oleic, and linoleic acids differ slightly in their effects on lipoprotein cholesterol levels [4,7,8](Chapter 2). Recently, Nestel and co-workers [38] directly addressed the issue of chain length vs. double bonds. They compared the effects of palmitoleic acid, a 16 carbon fatty acid with one double bond (*cis*-C16:1n-7), with those of palmitic (C16:0) and of oleic acid (*cis*-C18:1n-9). Nestel et al. hypothesized that palmitoleic acid would be as effective as oleic acid in lowering cholesterol, because they expected palmitoleic acid to be elongated to oleic acid in the body. However, it was found that palmitoleic acid produced the same total and lipoprotein cholesterol levels as palmitic acid, and that both 16 carbon fatty acids raised total and LDL cholesterol relative to oleic acid [38].

Thus, it appears that chain length is more important than the double bond in determining the effects of a fatty acid on cholesterol metabolism. However, the modest relative effects of 18 carbon acids with different numbers of double bonds remain to be explained.

Positional distribution of fatty acids

Fasting serum lipid and lipoprotein cholesterol levels

Our study on triglyceride configuration suggests that the position of fatty acids, specifically palmitic and oleic acid, on the glycerol backbone does not influence its effect on fasting serum lipid and lipoprotein levels (Chapter 6). In the field of lipid research, possible effects of differences in triglyceride configuration have received much less attention than the overall fatty acid composition of dietary fats. No other study has directly examined the influence of the positional distribution of fatty acids on fasting serum lipids in humans, and thus it is difficult compare our findings with those of others. Nevertheless, Zampelas and co-workers [41] recently examined the effects on postprandial lipids of the same two dietary fats as in our study. They found no differences in lipids and lipoproteins over the 6 h following their liquid test meals [41]. A tentative conclusion from this and our study is that the differences between dietary fats in the positional distribution of their component fatty acids have no important consequences for CHD risk. However, our findings need to be confirmed by future studies and, in addition, positional effects of fatty acids other than palmitic acid should be investigated.

Fatty acid composition and configuration of plasma lipids

The data on the fatty acid composition and configuration of blood lipids showed that the position of a fatty acid is partly retained in fasting plasma triglycerides and cholesteryl esters. Although small, these effects were distinct and statistically significant.

It is unlikely that the partial conservation of the *sn*-2 position of dietary triglycerides in fasting plasma lipids has any substantial health effect. Yet, our study shows that the positional distribution of dietary fatty acids has effects that persist beyond fat absorption and clearance. This finding could not be predicted by the current knowledge about the pathways of fat metabolism. Several speculations about the possible mechanisms can be made. The most plausible explanation for retention of the dietary *sn*-2 position in fasting plasma triglycerides seems the following: the chylomicron triglycerides, which enter the blood stream after a meal, contain fatty acids on the original dietary *sn*-2 position. These triglycerides can be transferred to higher density lipoproteins and in this way escape postprandial lipolysis, resulting in fasting lipoproteins that still contain triglycerides with fatty acids in the initial *sn*-2 position. The enrichment of cholesteryl esters in fatty acids from the *sn*-2 position of dietary fat could be explained by formation of phospholipids in the intestinal wall from absorbed 2-monoglycerides and subsequent transfer of the *sn*-2 fatty acids to cholesteryl esters by lecithin:cholesterol acyl transferase in plasma.

Effects of dietary fat on other risk factors for CHD

Dietary recommendations are not based exclusively on the effects of diet on serum lipoprotein and blood pressure levels. Fatty acids may also exert other effects that modulate CHD risk. One aspect is the proneness of LDL particles to oxidative modification [42]. According to this plausible, although not yet proven theory, LDL particles damage the arterial wall once they have become modified by certain oxidative agents [43]. Several studies have shown that fatty acids [44-46] and other food components [47-49] influence the *in vitro* susceptibility of LDL to oxidative modification. However, the possible role of oxidative damage to LDL does by no means discard the importance of the level of circulating LDL cholesterol for CHD risk. Another risk factor for CHD that appears to be influenced by dietary fat is the blood coagulation tendency and fibrinolysis. Several epidemiologic studies

suggest that certain parameters of hemostasis are independent risk factors [50-52], and both epidemiologic [53-55] and experimental data [56-59] indicate that these parameters might be modified by diet.

The possible influence of dietary fat on risk factors other than serum lipoproteins or blood pressure should be taken into account when evaluating the effect of diet on the risk for CHD.

METHODOLOGY OF THE STUDIES

The effects of dietary fatty acids on risk factors for CHD were studied by means of controlled trials in which we supplied specific diets to healthy volunteers. The purpose of this section is to briefly discuss some aspects of the methods used, and to indicate the potentials and limitations of extrapolating our findings to public health practice.

Duration

The subjects in our studies consumed each experimental diet for three weeks. The choice of this period is based on experience from trials with longer duration, which indicates that lipid and lipoprotein levels stabilize within two weeks after a dietary change [7,60-62]. Some studies have suggested that the effects of dietary fat on certain serum lipid levels, specifically triglyceride and HDL cholesterol levels, might be transient [63-65]. However, others have shown that changes in triglyceride and HDL cholesterol after modifying dietary fat are sustained for periods of several months [61,66,67] up till one year [68]. These data are consistent with epidemiologic observations of free-living subjects who have been eating their customary diets for a very long period [69,70]. Moreover, it has never been reported that diet-induced changes in total and LDL cholesterol levels might be temporary. Thus, the 3-week periods in our trials seem long enough to study the relation between fatty acid consumption and serum lipids and lipoproteins in the general population.

We can not rule out the possibility that the dietary periods were too short to induce a change in blood pressure, although some studies show that blood pressure levels stabilize within 3 weeks after a dietary change [71,72]. Also, we

are not sure whether the observed changes in Lp(a) (Chapter 4) were transient or, alternatively, underestimated and not yet at their maximum after three weeks. Another consequence of the short-term character of our studies is that we can not prove whether the dietary changes will indeed modify CHD risk. To this end long-term intervention studies are needed. Nevertheless, the axiom that lowering the LDL cholesterol level –an endpoint in our studies– reduces CHD risk seems legitimate (see Chapter 1).

Design

The three studies described in this thesis applied a cross-over design to compare the effects of various fatty acids. The feature that distinguishes the cross-over trial from other trials is that measurements on different treatments –in our case diets– are obtained from each subject. The main advantage is that the diets are compared 'within' subjects, and any variation due to differences between the subjects is removed from the comparison. Since the statistical tests of differences between diets are based on within-subject variation only, a cross-over design provides a much higher power to detect true differences than, for example, a parallel design. Another advantage is that differences in measurements between periods, for example due to systematic drift of variables over time, are eliminated by supplying the diets in different order [73]. If the order of the diets is completely balanced, as was the case in our studies, then period effects can not bias the comparisons of the diets [74]. A potential disadvantage of a cross-over design is the possibility that the effect of a diet given in one period might still be present at the end of the following period. This 'carry-over' effect could attenuate the estimates of the differences between diets. However, as argued on the previous page, it is reasonable to assume that lipid and lipoprotein levels reach a steady state within two weeks after a dietary change. Thus it is unlikely that carry-over effects biased our findings on total, LDL, and HDL cholesterol. Possible carry-over effects on the measurements of Lp(a) and blood pressure levels can not be totally excluded. Nevertheless, the absence of significant diet-by-period interactions in either of the studies indicates that there were no large carry-over effects [74]. At the most, small carry-over effects, if any, could have biased the estimated differences in blood pressure and Lp(a) levels to the null hypothesis, i.e. an underestimation of the true population effect.

Diets

The study diets consisted of conventional foodstuffs, such as bread, meat, vegetables, fruits, dairy products, cookies, etcetera. The differences in fatty acid composition between the diets were achieved by using specially designed and manufactured margarines. The tailored compositions of these margarines enabled us to compose diets in which the fatty acids under study were iso-energetically exchanged, while all other nutrients and food components were kept constant. Thus, the changes observed between the diets were due to the differences in fatty acid composition only, without confounding effects of other food components.

The supply of a variety of foodstuffs resulted in experimental diets which resembled every-day food intake in the normal population. The overall nutrient composition came close to that of an average Dutch diet [75]. Some investigators applied liquid formula diets to study the influence of fatty acids on serum lipoproteins, e.g. [9,13,27]. The advantage of using conventional solid food diets rather than liquid formula diets is that effects of fatty acids are studied in the context of a more realistic food intake, and that any possible artefact due to the use of liquid formula diets is eliminated [76,77]. It must be noted, however, that the absolute amount of fat in our diets was quite high (37% to 40% of energy intake). We do not know for certain whether the magnitude of the observed dietary effects would be different at lower levels of fat intake.

Body weight influences serum lipid and lipoprotein levels [78,79]. Therefore, it was important to keep our subjects at stable weights during the trials. The study diets were formulated at different levels of energy intake, so that each subject received the amount of food that met his or her energy needs. The subjects' weights were closely monitored and the level of energy intake was adjusted when necessary. We succeeded in keeping body weights constant, thus weight changes could not have affected the results.

Subjects

Characteristics of the volunteers

Our subjects were on average young, lean, healthy men and women with normal serum lipid and blood pressure levels. It is reasonable to assume that our findings will apply to similar people in the general population. However, one must

be cautious in extrapolating our results to other population groups, such as people with high lipoprotein levels, overweight, hypertension, or the elderly. For example, it has been proposed that a persons cholesterol response to a change in saturated fat intake depends on his or her initial cholesterol level [35]. We could not support this view. Our subjects' starting cholesterol levels, which ranged from 3 to 6.5 mmol/L, were not related to cholesterol responses in either of the trials. However, our results do not tell whether diet responsiveness of people with moderate to severe hyperlipidemia is different from that of normolipidemic subjects. Possibly, cholesterol levels of hyperlipidemic subjects are more sensitive to dietary change [80-82]. A high body weight has also been associated with a diminished response of lipid levels to dietary changes [65,82-84]. Furthermore, some studies suggest that older subjects are more diet responsive than younger ones [82,85].

Gender differences

Women are often considered to be less suitable subjects for studying dietary effects on serum lipoproteins because of supposed confounding effects of menstrual cycle [86,87] or the use of oral contraceptives [88]. As argued in Chapter 2 and 5, the design of our studies excluded possible bias due to cyclic effects. Thus, the differences in serum lipoproteins observed between the dietary treatment were not confounded by hormonal variations.

It has been questioned whether women and men have similar responses to changes in dietary fat [89,90]. Specifically, some studies indicate that the HDL cholesterol levels of women are less sensitive to dietary modification than those of men [14,62]. However, other studies suggest the opposite [82,85]. In our three trials we did not find any substantial gender-specific effect of diet on HDL. We therefore think that HDL cholesterol levels of men and women respond similarly to changes in dietary fatty acid composition. If anything, the responses of total and LDL cholesterol on saturated fat tended to be somewhat larger for the men than for the women, although this difference was statistically significant only for the changes on stearic versus linoleic acid (Chapter 2). In a number of other trials both sexes were equally responsive to saturated fat [3,5,32,91,92].

The precise gender differences in diet responsiveness remain unclear. In any case, women as well as men show a considerable decrease in the total and LDL cholesterol level when saturated fat intake is diminished (Chapter 5). At present, there seems to be no need for different dietary treatments of men and women with hypercholesterolemia.

Compliance

When dietary effects are investigated in human volunteers, it is of obvious importance that the subjects adhere to the study diets. On weekdays during the trials, hot meals were served at the department and eaten under our supervision. Other foods as well as foods for the weekends were supplied as a package and consumed at home. The following actions were taken to maximize dietary compliance in this relatively 'free-living' situation. First of all, the recruitment was aimed at selecting volunteers who were well aware of the restraints of participating in a diet study and who were motivated to adhere to the protocol. No payment was offered to ensure that subjects did not participate for personal financial gain. One-hundred-seventy-five of the 178 participants completed the trials successfully. This illustrates that we succeeded in recruiting highly motivated subjects. Also, the daily visits to the department ensured frequent contact between the volunteers and the dietary staff. Consultancy hours offered ample opportunity to privately discuss difficulties with the protocol and to receive personal advice if needed. Subjects were encouraged to talk about problems related to the diets, and the importance of reporting any deviation was carefully explained. We told the participants to contact us whenever they faced problems or were not sure about the protocol. The investigators could always be reached, including the evenings and the weekends. Probably the most effective way to improve dietary compliance is to provide the subjects with high quality foods. Our dietary staff made large efforts to present a high variety in the menus and to prepare palatable foods. A large number of volunteers indicated that they appreciated the foods and diversity of the diet.

In order to obtain an estimate of the actual degree of compliance, subjects were not only asked to record deviations from the protocol in their diaries, but also in anonymous questionnaires which were handed out at the end of the studies (Chapter 5 and 6). The anonymously reported deviations were in accordance with those recorded in the diaries. Consumption of foods outside the dietary protocol was very low. For example, it provided at most 0.2 grams of fat per day in the trial on triglyceride configuration (Chapter 6). In the studies on *trans* fatty acids (Chapter 2) and specific saturates (Chapter 5) we also measured the fatty acid compositions of serum cholesteryl esters as indicators of compliance. Individual changes in cholesteryl ester fatty acids consistently followed the differences in fatty acid composition between the diets. These quantative data confirmed that dietary adherence was very high.

CONCLUSIONS

Monunsaturated *trans* fatty acids

Trans fatty acids as present in hydrogenated vegetable oils produce an unfavorable lipoprotein risk profile for CHD by raising LDL and lowering HDL cholesterol. Use of stearic acid to produce semi-solid and solid fats does not seem to offer much health benefit over *trans* fatty acids, but more study on this point is needed. Monounsaturated *trans* fatty acids have no substantial effects on blood pressure, but they may increase CHD risk by raising serum Lp(a). It should be kept in mind that the consumption of *trans* fatty acids is low compared with that of saturates, and that the reduction of saturated fat and cholesterol intake remains a primary goal in the dietary prevention of CHD.

Specific saturated fatty acids

Myristic acid is about 1.5 times more cholesterol-raising than palmitic acid. This difference is due to LDL as well as HDL cholesterol, and as a consequence myristic and palmitic acid result in similar unfavorable HDL to LDL cholesterol ratios. Both saturated fatty acids strongly raise total and LDL cholesterol. Since palmitic acid is the most abundant saturate in our diet, it can be regarded as the principle cholesterol-raising fatty acid.

Positional distribution of fatty acids

Changing the position of palmitic acid on the glycerol molecule does not materially affect fasting serum lipid and lipoprotein levels. However, differences in the positional distribution do have small but consistent effects on the fatty acid configuration and composition of fasting plasma triglycerides and cholesteryl esters. Nevertheless, at present there is no solid evidence that the 'structuring' of dietary triglycerides has important health consequences.

General conclusion and implication

The studies in this thesis show that the *cis* or *trans* configuration of double bonds modulates the effect of dietary fatty acids on serum lipoproteins. Chain length modifies the cholesterol-raising properties of saturated fatty acids, but the differences are not as large as has been suggested. The positional distribution of fatty acids over the triglyceride molecule is probably not an important determinant of serum lipid levels.

We conclude that consumption of monounsaturated *trans* fatty acids and the saturates myristic and palmitic acid has adverse effects on the serum lipoprotein risk profile for CHD. Dietary therapy and public health measures to lower total and LDL cholesterol levels should aim at reducing the intake of both *trans* and saturated fatty acids. In practical terms, people at increased risk for CHD should replace the hard fats in their diets by carbohydrates or unsaturated oils.

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Summary

Elevated levels of total or LDL cholesterol, lipoprotein(a) and blood pressure, and low levels of HDL cholesterol are known to increase the risk for coronary heart disease (Chapter 1). Differences in the chain length and the number and geometry of the double bonds in dietary fatty acids may affect these risk factors. Thus, coronary heart disease risk might be altered by modifying the amount and type of fat in the human diet.

The aim of the studies described in this thesis was to investigate the effects of dietary fatty acids on risk factors for coronary heart disease. In each of three trials we fed conventional solid food diets with tailored fatty acid composition to about 60 healthy male and female volunteers. The different diets within one trial were supplied to every subject for 3 weeks each, in random order (cross-over).

In the first study we investigated whether stearic acid could be a favorable alternative to *trans* fatty acids for the production of semi-solid and solid fats. We compared the effects of 8% of energy intake as monounsaturated *trans* fatty acids with those of linoleic acid, the fatty acid from which *trans* fatty acids are formed upon partial hydrogenation, and with those of stearic acid, a product of complete hydrogenation of linoleic acid. As compared with linoleic acid, *trans* fatty acids raised LDL by 0.24 mmol/L and lowered HDL cholesterol by 0.10 mmol/L. Stearic acid raised LDL by 0.17 mmol/L and lowered HDL by 0.06 mmol/L relative to linoleic acid. Differences between the *trans* fatty acid and the stearic acid diet were not statistically significant. Thus, hydrogenation of linoleic acid to either *trans* fatty acids or stearic acid produced fatty acids that may increase LDL and decrease HDL cholesterol relative to linoleic acid itself (Chapter 2). These findings do not support the use of stearic acid as a preferable alternative to *trans* fatty acids for the production of hard fats. Systolic and diastolic blood pressure were not affected by *trans* fatty acids or stearic acid (Chapter 3). This and an earlier study from our department show that *trans* fatty acids may produce modest increases in serum lipoprotein(a) levels relative to other dietary fatty acids (Chapter 4).

The second study addressed the relative cholesterol-raising properties of two specific saturates. Three diets differed from each other in myristic, palmitic, or oleic acid by 10% of energy intake. As compared with oleic acid, myristic acid increased LDL by 0.50 mmol/L and HDL cholesterol by 0.15 mmol/L. Palmitic acid

raised LDL cholesterol by 0.38 mmol/L, but not HDL. The differences between myristic and palmitic acid were statistically significant. Nevertheless, both saturates caused high total and LDL cholesterol levels and raised the LDL to HDL cholesterol ratio (Chapter 5). Thus, myristic and palmitic acid have disadvantageous effects on serum lipids.

In the third study we examined the effect of the positional distribution of fatty acids within dietary triglycerides. Two diets had identical total fatty acid composition. One diet, rich in palm oil, contained 82% of its palmitic acid on the two outer carbon atoms of glycerol, and 18% on the middle carbon. In the other diet, rich in modified palm oil (Betapol), these figures were 35% and 65%, respectively. Total, LDL, and HDL cholesterol levels were the same on Betapol and on palm oil. Thus, a large contrast in the fatty acid configuration of dietary fat did not influence lipid and lipoprotein levels (Chapter 6). However, the positional distribution of the fatty acids did have consistent but small effects on the fatty acid configuration and composition of fasting plasma triglycerides and cholesteryl esters (Chapter 7). Taken together, the positional distribution of fatty acids in dietary fat do not seem to have important health effects.

Trans fatty acids as present in hydrogenated vegetable oils produce an unfavorable lipoprotein profile by raising LDL cholesterol and lipoprotein(a) levels and by lowering HDL cholesterol levels. Myristic and palmitic acid differ in their effects on serum lipids, but they both increase total and LDL cholesterol. The position of a fatty acid on the glycerol molecule does not materially affect serum lipid and lipoprotein levels.

In conclusion, consumption of monounsaturated *trans* fatty acids and of saturated fatty acids has adverse effects on the serum lipoprotein risk profile for coronary heart disease. The positional distribution of fatty acids over the triglyceride molecule is probably not an important determinant of coronary heart disease risk. Dietary therapy and public health measures to lower total and LDL cholesterol levels should aim at reducing the consumption of both *trans* and saturated fatty acids. In practice, this implies that people at risk for coronary heart disease should avoid high intakes of hard fats and incorporate more carbohydrates or unsaturated oils in their diets.

Samenvatting

Een hoog gehalte aan totaal- of LDL-cholesterol en lipoproteïne(a), een verhoogde bloeddruk, en een laag HDL-cholesterolgehalte verhogen het risico voor coronaire hartziekte (Hoofdstuk 1). Verschillen in de ketenlengte en het aantal en de plaats van dubbele bindingen van voedingsvetzuren kunnen deze risicofactoren beïnvloeden. De kans op coronaire hartziekte kan dus veranderen wanneer de hoeveelheid en de soort vet in de voeding wordt gewijzigd.

Het doel van het onderzoek in dit proefschrift was de uitwerking van vetzuren in de voeding op risicofactoren voor coronaire hartziekte te bestuderen. In drie proeven kregen telkens 60 mensen alledaagse voedingen met nauwkeurige vetzuursamenstelling te eten. Elk van de verschillende voedingen binnen een proef werd gedurende 3 weken verstrekt aan iedere deelnemer, in verschillende volgorde.

In de eerste proef onderzochten we of stearinezuur een gunstig alternatief voor *trans*-vetzuren kan bieden bij de produktie van geharde vetten. We vergeleken de effecten van enkelvoudig onverzadigde *trans*-vetzuren met die van linolzuur, het vetzuur waaruit *trans*-vetzuren tijdens gedeeltelijke hydrogenering worden gevormd, en met die van stearinezuur, dat bij volledige hydrogenering van linolzuur ontstaat. Het verschil in de hoeveelheid van deze vetzuren tussen de drie voedingen bedroeg 8 % van de dagelijkse energiebehoefte (energie%). Vergeleken met linolzuur verhoogden *trans*-vetzuren het LDL-cholesterol met 0.24 mmol/L en verlaagden het HDL-cholesterol met 0.10 mmol/L. Stearinezuur verhoogde LDL met 0.17 mmol/L en verlaagde HDL met 0.06 mmol/L. De verschillen tussen de *trans*-vetzuur- en de stearinezuurvoeding waren niet statistisch significant. Het hydrogeneren van linolzuur tot zowel *trans*-vetzuren als tot stearinezuur levert dus vetzuren op die het LDL verhogen en het HDL verlagen vergeleken met linolzuur zelf. Onze resultaten kunnen het gebruik van stearinezuur als gunstige keuzemogelijkheid bij het maken van harde vetten niet ondersteunen (Hoofdstuk 2). De systolische en diastolische bloeddruk werden niet beïnvloed door *trans*-vetzuren of stearinezuur (Hoofdstuk 3). Deze proef laat samen met andere proeven van onze vakgroep zien dat *trans*-vetzuren wel het lipoproteïne(a)-gehalte kunnen verhogen (Hoofdstuk 4).

De tweede proef was gericht op het verschil in cholesterolverhogende werking van twee afzonderlijke verzadigde vetzuren. Drie voedingen verschilden 10 energie% van elkaar in myristine-, palmitine- of oliezuur. Ten opzichte van oliezuur

verhoogde myristinezuur het LDL met 0.50 mmol/L en het HDL-cholesterol met 0.15 mmol/L. Palmitinezuur verhoogde het LDL met 0.38 mmol/L, maar niet het HDL. De verschillen tussen myristine- en palmitinezuur waren statistisch significant. Beide verzadigde vetzuren veroorzaakten echter een hoog totaal- en LDL-cholesterol en verhoogden de LDL / HDL verhouding. Zowel myristinezuur als palmitinezuur hebben dus een ongunstige uitwerking op de bloedlipiden (Hoofdstuk 5).

In de derde proef onderzochten we de invloed van de positie van vetzuren in het triglyceridemolecuul. Twee voedingen hadden precies dezelfde totale vetzuursamenstelling. Eén voeding was rijk aan palmolie, en bevatte 82% van het palmitinezuur op de twee buitenste koolstofatomen van het glycerol, en 18% op het middelste koolstofatoom. In de andere voeding, rijk aan gewijzigde palmolie, was dit 35% en 65%, respectievelijk. De totaal-, LDL- en HDL- cholesterolgehalten waren op beide voedingen gelijk. Een groot verschil in de vetzuurconfiguratie van voedingsvet had dus geen invloed op de lipidengehalten (Hoofdstuk 6). De positie van het vetzuur had echter wel duidelijke, zij het kleine, effecten op de vetzuurconfiguratie en -samenstelling van nuchtere triglyceriden en cholesterolesters in plasma (Hoofdstuk 7). Over het geheel gezien hebben verschillen in positionele verdeling van voedingsvetzuren geen grote invloed op de serumlipiden.

Trans-vetzuren in gehydrogeneerde plantaardige oliën veroorzaken een ongunstig lipoproteïnebeeld doordat ze het LDL-cholesterol en lipoproteïne(a)-gehalte verhogen en het HDL-cholesterolgehalte verlagen. Myristinezuur en palmitinezuur hebben verschillende effecten, maar beide verhogen het totaal- en LDL-cholesterol. De positie van een vetzuur op het glycerolmolecuul is van weinig betekenis voor de lipoproteïnegehalten.

De conclusie is dat de consumptie van enkelvoudig onverzadigde *trans*-vetzuren en van verzadigde vetzuren een ongunstige uitwerking heeft op het risicobeeld van de lipoproteïnen voor coronaire hartziekte. De positionele verdeling van vetzuren over het triglyceridenmolecuul heeft waarschijnlijk geen belangrijke invloed op het risico. Dieetbehandeling en algemene maatregelen om het totaal- en LDL-cholesterolgehalte te verlagen zouden gericht moeten zijn op verminderde consumptie van zowel *trans*- als verzadigde vetzuren. In de praktijk betekent dit dat mensen met een hoge kans op coronaire hartziekte het gebruik van harde vetten dienen te vermijden en meer koolhydraten of onverzadigde oliën in hun voeding moeten opnemen.

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Curriculum vitae

Petrus Laurens Zock was born on September 21, 1960, in Ridderkerk, the Netherlands. In 1978, he passed secondary school at the 'Katholieke Scholengemeenschap' in Etten-Leur. In the same year, he started his studies at the Wageningen Agricultural University. As part of the training in Human Nutrition, he spent 9 months of practical research at the Division of Gastroenterology, Department of Internal Medicine, St. Radboud Hospital, University of Nijmegen. He received the Parke-Davis Award 1988 for the report of this research entitled "Dietary fiber and colonic cancer". In september 1987, he obtained the MSc-degree in Human Nutrition (cum laude), with Human Nutrition and Physiology as extended major, and Methods and Techniques of Social Scientific Research as minor topic. From August 1988 to March 1990, he worked at the Department of Human Nutrition, Wageningen Agricultural University, so as to replace his military duty by civil services. In this period he conducted animal and human experiments on the relation between coffee consumption and blood lipids. In April 1990, he started as a PhD-fellow in the Netherlands Postgraduate Programme in Human Nutrition. The studies of his PhD-project, entitled "Influence of structure and chain length of dietary fatty acids on serum lipoproteins in humans", were conducted at the Department of Human Nutrition in Wageningen. From July 1991 to December 1994, he was a member of the Standing Research Committee of the Wageningen Agricultural University. In July 1993, he attended the Annual New England Epidemiology Summer Program, a 3-week course at Tufts University, Boston, USA. He participated in the first European Nutrition Leadership Programme, March 1994, Echternach, Luxembourg. From April to December 1994, he was appointed as an associate investigator at the Department of Human Nutrition in Wageningen. He is currently completing an educational programme, of which this thesis is a part, to become registered as 'Epidemioloog B' by the Netherlands Epidemiology Society.

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