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Trans fatty acids, HDL-cholesterol, and cardiovascular disease risk: Effects of dietary changes on vascular reactivity

stelling 1
Vervanging van verzadigd vet in de voeding door transvet vermindert de vaatwandfunctie.
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stelling 2
Het risico op hart- en vaatziekten kan even goed worden verlaagd door het vervangen van verzadigd vet door olie rijk aan enkelvoudig onverzadigde vetzuren als door zetmeelbronnen, groente en fruit.
o.a. dit proefschrift

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stelling 4
Een experiment waarin geen verschil in effect wordt gevonden tussen behandeling en placebo heeft alleen waarde als de dieet- of therapietrouw gemeten is.

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BMJ 2001;322:530

stelling 6
Het placebo-effect is overschat.
N Engl J Med 2001;344:1549-1601

stelling 7
Eerst een hond of kat en dan een kind is een verstandige keuze.
o.a. Clin Exp Allergy 1999;29:611-7

Trans fatty acids, HDL-cholesterol, and cardiovascular disease risk

Effects of dietary changes on vascular reactivity

Nicole M. de Roos

Promotoren

Prof. dr. M.B. Katan, Persoonlijk Hoogleraar bij de Afdeling Humane Voeding en Epidemiologie, Wageningen Universiteit

Prof. dr. E.G. Schouten, Deeltijdhoogleraar Epidemiologie van de Voeding, Katholieke Universiteit Leuven, België

Promotiecommissie

Prof. Dr. A.H. Lichtenstein
Tufts University, Boston, United States

Prof. dr. M. Müller,
Wageningen Universiteit

Prof. dr. J.C. Seidell,
Vrije Universiteit Amsterdam

Prof. dr. C.D.A. Stehouwer,
Vrije Universiteit Amsterdam

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Proefschrift

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Abstract

Trans fatty acids, HDL-cholesterol and cardiovascular disease risk: Effects of dietary changes on vascular reactivity

PhD thesis by Nicole M de Roos, Division of Human Nutrition and Epidemiology, Wageningen University, The Netherlands

Intake of *trans* fatty acids increases the risk of coronary heart disease, even more so than saturated fatty acids. We wanted to investigate whether this higher risk was caused by the decrease in serum HDL-cholesterol by *trans* fatty acids. To do this, we studied the effect of diet-induced changes in HDL-cholesterol on vascular reactivity, a surrogate endpoint for coronary heart disease. Vascular reactivity was measured as flow-mediated vasodilation: the percentage increase in arterial diameter after a provoked increase in blood flow. The extent of flow-mediated vasodilation appears to be predictive of future coronary heart disease. The studies were performed in healthy men and women.

Replacement of 9.2% of energy (en%) from saturated fatty acids by *trans* fatty acids lowered serum HDL-cholesterol after 4 weeks by 0.39 (95%CI 0.28, 0.50) mmol/L and impaired flow-mediated vasodilation from 6.2% to 4.4%, a decrease of 1.8%-points (0.4, 3.2). The activity of serum paraoxonase, an HDL-bound esterase which might protect against atherosclerosis, decreased by 6% (2%, 10%). We then verified whether a different HDL-lowering diet also impaired flow-mediated vasodilation. In this study, we replaced ≈20en% monounsaturated fatty acids with carbohydrates: HDL-cholesterol decreased by 0.21 (0.17, 0.26) mmol/L and flow-mediated vasodilation increased from 4.1% to 4.8%, an increase of 0.7% (-0.6, 1.9). This result did not support our hypothesis that decreases in HDL-cholesterol increase risk of cardiovascular disease; however, the decrease might have been too small to cause an effect. We therefore investigated in an oral fat-loading test whether *trans* fatty acids could impair flow-mediated vasodilation while HDL-cholesterol was constant. This was not the case; flow-mediated vasodilation after an oral fat load of 1g/kg bodyweight was 3.1% versus 2.6% before, and *trans* fatty acids and saturated fatty acids had similar effects. Serum paraoxonase activity parallelled the change in flow-mediated vasodilation, and was slightly increased after an oral fat load with either *trans* or saturated fatty acids.

We conclude that replacement of saturated fatty acids by *trans* fatty acids impairs vascular function within 4 weeks. This may explain why *trans* fatty acids relate more strongly to risk of cardiovascular disease than saturated fatty acids. Whether the effects on vascular function are caused by changes in HDL-cholesterol remains to be resolved.

Contents

1

General Introduction

Rationale for the studies in this thesis

The average diet of a Dutch adult contains 100g of fat[1]. A small part, 2-5% of total fat, is formed by *trans* fatty acids; unsaturated fatty acids with one or two double bonds in the *trans* configuration. The main source of *trans* fatty acids - or *trans* fats - are partially hydrogenated vegetable oils that are formed when liquid oils are hardened to produce shortenings, frying fats, and other solid fats. A smaller amount of *trans* fat is derived from meat or dairy products of ruminant animals, such as cows, sheep, and goats.

A high intake of *trans* fat increases the risk of coronary heart disease[2], and some investigators believe that their effect is stronger than that of saturated fatty acids. This difference in risk cannot be explained by differences in effect on LDL-cholesterol, because both types of fatty acids increase LDL-cholesterol to a similar extent. However, *trans* fatty acids decrease serum HDL-cholesterol when they replace saturated fatty acids, and a low HDL-cholesterol is associated with increased risk of cardiovascular disease. We postulated that if *trans* fats have indeed a stronger effect than saturated fatty acids on risk of cardiovascular disease, this would be caused by differences in effect on HDL-cholesterol. The underlying question of the studies in this thesis therefore was:

Does the decrease in serum HDL-cholesterol by *trans* fatty acids explain their stronger effect on cardiovascular disease as compared to saturated fatty acids?

We have not answered this question directly. Instead, we studied effects of *trans* fatty acids and of changes in HDL-cholesterol on a surrogate endpoint of cardiovascular disease: flow-mediated vasodilation. Thus, our research question was whether a decrease in HDL-cholesterol or a high intake of *trans* fatty acids impaired flow-mediated vasodilation.

Flow-mediated vasodilation is the widening of arteries in response to increased blood flow[3]. It is a measure of integrity and function of the vascular endothelium[4]. We used this marker because it appears to be predictive of future cardiovascular events; it correlates with known risk factors for cardiovascular disease; it quickly responds to treatment; and it can be used in a non-medical setting. All studies were performed in healthy volunteers.

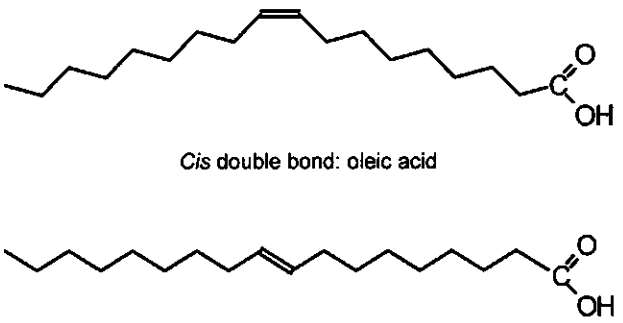
In the following paragraphs the relation between *trans* fatty acids, HDL-cholesterol, and coronary heart disease will be discussed.

Sources of *trans* fatty acids

The majority of *trans* fats in western-type diets are the partially hydrogenated vegetable and marine oils[5]. Their contribution to *trans* fat intake in the Netherlands is ≈80%[5,6]. Main sources of partially hydrogenated oils are fats (frying and cooking fats, solid margarines and spreads), baked goods (biscuits, cakes, buns), chips, french fries, and savoury snacks[5].

The most common *trans* fatty acids in partially hydrogenated vegetable oils are the *trans* isomers of the monounsaturated octadecenoic acid (C18:1)[5]. They include elaidic acid, which is the *trans* isomer of oleic acid (*cis*-C18:1n-9). In contrast, partially hydrogenated marine oils contain mainly *trans* fatty acids with 20-22 carbon atoms.

Figure 1.1:
Chemical structure of *cis*-C18:1n-9 (oleic acid) and *trans*-C18:1n-9 (elaidic acid)



The remainder of *trans* fat is largely derived from ruminant animal sources; they contribute ≈20% of total *trans* fat intake in the Netherlands[5,6]. Main sources are meat and meat products, cheese, and milk. Milk fats mainly contain vaccenic acid (C18:1n-7) and its positional isomers[7]. An additional small amount of *trans* fat is found in liquid vegetable oils; they are formed during the process of deodorisation of the oils and contain mainly *trans*-polyunsaturated fatty acids such as *trans*-alpha-linolenic acid (C18:3n-3)[8]. Intake of *trans*-alpha-linolenic acid is estimated to be 20-600 mg/d in European countries[9].

The intake of partially hydrogenated vegetable oils has decreased from 4.3% of energy (en%) in 1985 to 1.5 en% in 1995 in the Netherlands. In contrast, the intake of *trans* fats from ruminant animal sources has been stable at about 0.7en%[6]. Reports on the adverse effects of *trans* fats led food manufacturers to remove them from margarines for the consumer market[10]. Margarines for the catering industry, shortenings for bakers, and frying fats for fast food restaurants, however, still contain considerable amounts of *trans* fats. The reason is that hydrogenated oils add firmness and texture to products and can therefore not always be replaced by oils in their natural fluid state. Another advantage of hydrogenation is that it makes oils less susceptible to oxidation (spoilage) and therefore more stable upon deep fat frying. Replacement of partially hydrogenated vegetable oils by solid tropical fats is a technical possibility, but these fats are rich in saturated fatty acids and therefore undesirable from a public health view.

Trans fatty acids and coronary heart disease risk

Our knowledge on health effects of *trans* fats is based on observational and experimental diet studies. The strength of most observational studies is their endpoint: coronary heart disease, often in the form of fatal or non-fatal myocardial infarction or stroke. Their weaknesses include errors in estimating *trans* fatty acid intake and the limited possibilities to correct for confounding variables, such as saturated fat intake and a sedentary lifestyle.

These weaknesses can be eliminated in experimental diet studies: the intake of dietary components can be controlled, and randomising volunteers to treatment or control group eliminates bias due to e.g. lifestyle factors. However, a weakness of diet studies is their limited length and number of volunteers which makes it impractical to study effects on 'hard' coronary heart disease endpoints. Instead, effects on risk markers such as serum cholesterol have been studied.

observational studies

prospective observational follow-up studies

Four large prospective observational follow-up studies have estimated the effects of intake of *trans* fats on risk of coronary heart disease (**Table 1.1**). In the only study with women, the Nurses' Health Study[11], over 80,000 nurses were followed-up for 14 years; 939 coronary events were recorded in this period. Their mean intake of *trans* fats at baseline, estimated with food frequency questionnaires, was 2.2% of energy. The relative risk of coronary heart disease, in a multivariate model, was 1.62 (95% CI, 1.23 to 2.13) for each increase of 2% of energy from *trans* fats. A smaller effect was seen in the Health Professionals follow-up study[12]. During a 6-year follow-up of nearly 44,000 men, 734 coronary events - including 229 coronary deaths - were recorded. Median intake of *trans* fats ranged from 1.5 g/d in the lowest quintile to 4.3 g/d in the highest quintile of intake; the relative risk of coronary heart disease was 1.13 (95% CI, 0.81 to 1.58) for each additional 2% of energy from *trans* fats. A comparable effect was seen in the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study that was performed in Finland[13]. In this study, nearly 22,000 smoking men were followed-up for 6.1 years; 2034 coronary events - including 635 coronary deaths - were recorded. Median intakes of *trans* fats were slightly higher in this Finnish population than in the US Health Professionals, and ranged from 1.3 g/d in the lowest quintile to 6.2 g/d in the highest. The relative risk for each increase in *trans* fatty acid intake by 2% of energy was 1.14 (95% CI, 0.96 to 1.35). The Dutch Zutphen Elderly Study confirmed the positive association between *trans* fatty acid intake and risk of coronary heart disease. In this study 667 men with a mean *trans* fatty acid intake of 4.3% of energy at baseline were studied prospectively [6]. After 10 years of follow-up 98 coronary events had been recorded; the relative risk of coronary heart disease for each increase in *trans* fatty acid intake by 2% of energy was 1.28 (95% CI, 1.01 to 1.61). Oomen et al pooled the data of these four prospective studies and estimated that each 2% increase in energy intake from *trans* fats increased risk of cardiovascular disease by 25%[6].

Table 1.1 Relative risks of coronary heart disease for each additional 2% of energy from *trans* fatty acids in 4 prospective cohorts

study	number of subjects	follow-up (y)	number of events	relative risk (95% CI)
Nurses' Health Study	80,082 women	14	939	1.62 (1.23 to 2.13)
Health Professionals	43,757 men	6	734	1.13 (0.81 to 1.58)
Alpha-Tocopherol Beta-Carotene Cancer Prevention Study	21,930 men	6.1	2034	1.14 (0.96 to 1.35)
Zutphen Elderly Study	667 men	10	98	1.28 (1.01 to 1.61)

Data on effects of the different sources of *trans* fats are limited. It is known, however, that dietary fatty acids with small differences in structure may have large differences in effect, and therefore we cannot assume that all *trans* fats will act identically[8]. In the Dutch Zutphen Elderly Study; the effects of *trans* fats from animal origin appeared to be similar to those of partially hydrogenated fats[6]. In the Nurses' Health Study, however, *trans* fats from ruminant animal fats appeared to be inversely related to risk, while *trans* fats from vegetable fats increased risk of coronary heart disease[14]. In this study, margarine, beef, pork, or lamb as a main dish, and cookies and white bread contributed most to *trans* fatty acid intake. Of these products, margarine, cookies and white bread had positive associations with risk while the meat sources had not, indicating that only the *trans* fats from partially hydrogenated vegetable oils increased risk of coronary heart disease. In the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study the risk associated with *trans* fat intake was largely attributable to elaidic acid intake from partially hydrogenated fat, and not to *trans* fats from animal sources.

Case-control studies

Another approach to investigating whether *trans* fats are related to coronary heart disease is to compare *trans* fat intake across patients and healthy controls in a case-control study. Ascherio et al compared 239 men and women who were admitted to hospital for a first myocardial infarction with 282 population controls. Intake of *trans* fats was 4.36 g/d or 1.6% of energy in men and 3.61 g/d or 1.7% of energy in women; hydrogenated fats contributed 74% of the total *trans* fat intake. Control subjects had a lower intake of *trans* fats than patients: 3.78 g/d versus 4.68 g/d (P for difference <0.001). The relative risk of myocardial infarction after adjustment for known risk factors, other fats, and cholesterol intake, was 2.03 (95% CI, 0.98 to 4.22) for the highest versus the lowest quintile of *trans* fat intake[15]. Plasma concentrations of LDL- and HDL-cholesterol had been measured in 458 patients and controls, and the investigators evaluated whether the association between *trans* fat intake and myocardial infarction was mediated by these

risk factors. The relative risk of myocardial infarction among these subjects, comparing the highest versus the lowest quintile of *trans* fat intake, was 2.23 (95% CI, 1.19 to 4.18) before adjustment for plasma LDL- and HDL-cholesterol, and 2.12 (95% CI, 1.09 to 4.11) after adjustment. Thus, a higher *trans* fat intake appeared to increase risk of myocardial infarction independent of its effects on lipoproteins.

No such clear relation between *trans* fat intake and myocardial infarction was seen in the European multicentre EURAMIC study[16]. In this study, *trans* fatty acid content of adipose tissue of 671 men with a first myocardial infarction was compared with that of 717 age-matched controls without a history of myocardial infarction. The C18 *trans* fatty acid content of adipose tissue was used as a biomarker of *trans* fat intake, and was shown to be 1.60% in patients and 1.59% in controls, with a difference of 0.01% (95% CI, –0.02 to 0.04). Thus, there was no overall difference between cases and controls. Of the ten centres that collaborated in this study, two were from Spain, and the men from these two centres had very low C18 *trans* fatty acid contents in their adipose tissue; ranging from 0.40 to 0.47%. After exclusion of these two centres, the second highest and the highest quintile of *trans* fats were associated with higher risks of myocardial infarction, but the relation was not significant. The investigators suggested that the contribution of *trans* fats to risk of myocardial infarction might be restricted to countries with high *trans* fat intakes. A comparable study in the UK, with 66 men who had died from sudden cardiac arrest and 286 healthy controls, showed no relation between adipose tissue C18:1 or C18:2 *trans* fats and cardiac death[17]. This study was apparently too small to detect any difference between cases and controls; the confidence intervals were wide, and diabetes, an established risk factor for coronary heart disease, was not associated with events.

Thus, the relation between *trans* fat intake and risk of myocardial infarction or cardiac death is inconsistent in these case-control studies. However, this may have been due to lack of power in some of the studies.

Cross-sectional studies

Only one cross-sectional study of *trans* fat intake and coronary heart disease in western subjects was found[18]. The study was conducted in 10,359 Scottish men and women, who were divided in three groups: those who reported prevalent coronary heart disease (369 men and 235 women), those with symptoms of coronary heart disease that had not been diagnosed previously (659 men and 795 women), and those who were free of symptoms and who did not use anti-hypertensive, cholesterol-lowering or other drugs related to coronary heart disease (3720 men and 3749 women). Intake of *trans* fats had no clear effect on the risk of coronary heart disease, neither in the patients with prevalent coronary heart disease nor in the symptomatic but undiagnosed subjects. The intake of *trans* fats was high and ranged from 3.8 g/d in the lowest quintile to 11.9 g/d in the highest quintile for men, and from 3.1 to 11.0 g/d for women. About 60% of all *trans* fats in the diet was derived from partially hydrogenated fats. The lack of relation between *trans* fats intake and coronary heart disease might be due to the design of this study; patients with coronary heart disease might have changed their dietary habits, and are likely to use margarines rich in polyunsaturated fats instead of *trans* fats. This may have obscured an existing relation.

experimental diet studies

Serum lipoproteins

Experimental diet studies with *trans* fats have focussed on their effect on serum lipoproteins, especially since Mensink and Katan showed that these fats differ from saturated fats in their effects on serum HDL-cholesterol[19]. When data of controlled diet studies are combined, it can be estimated that replacement of 1% of energy from carbohydrates by *trans* fats increases serum LDL-cholesterol by 0.034 mmol/L and decreases serum HDL-cholesterol by 0.004 mmol/L[20,21]. Such a change in cholesterol concentrations can be translated in change in risk: according to Willett, an increase in total cholesterol of 0.5 mmol/L increases risk of coronary heart disease by about 25%[22]. Thus, each additional percent from *trans* fats will increase risk of coronary heart disease by 1-2%. This estimate is much less than the increase in risk of 25% for each 2% of energy from *trans* fats that was estimated from epidemiological studies. Thus, other effects appear to contribute to the risk associated with high intakes of *trans* fats.

Other effects of trans fatty acids

In addition to their effects on serum LDL- and HDL-cholesterol, *trans* fats have consistently been shown to increase serum lipoprotein(a)[23-26]. This particle, which is composed of an LDL particle attached to apolipoprotein(a), has been associated with coronary heart disease in epidemiological studies[27]. Its concentration in human serum is largely genetically determined[28] but can be modified by diet. The increase in serum lipoprotein(a) is about 0.5 mg/dL per 2en% increase in *trans* fat intake[2]. This appears to be an adverse effect, but exactly how this translates into risk of coronary heart disease is unclear because of the complex relation between lipoprotein(a) with cardiovascular disease[28].

Other effects of *trans* fats that have been studied in human studies are blood pressure, which was unaffected[29], and incorporation of certain *trans* fats in blood platelets[30], which might be increased in patients with more severe coronary artery disease. This latter effect, however, needs confirmation, and the composition of platelets as a marker of disease needs to be validated.

In conclusion, changes in serum lipoprotein concentrations only partly explain why a high intake of *trans* fats increases risk of coronary heart disease. This suggests that additional mechanisms are involved in the pathway between *trans* fat intake and coronary heart disease.

Serum HDL-cholesterol and coronary heart disease risk

Unlike saturated fatty acids, *trans* fats decrease serum HDL-cholesterol when they replace other fats in the diet. We hypothesised that this might explain why *trans* fats relate more strongly to risk of cardiovascular disease than saturated fatty acids, because there is increasing evidence that HDL-cholesterol is causally related to coronary heart disease[31].

Cross-sectional studies and prospective follow-up studies

Several cross-sectional studies have shown that coronary heart disease is more common in men and women with low serum HDL-cholesterol concentrations than in those with high serum HDL-cholesterol[32-34]. Moreover, a low concentration is predictive of future cardiovascular and coronary events: Two papers summarised the results of six cohort studies (FHS, LRCF, CPPT, MRFIT, BRHS, and BIRNH)[1] and found risk reductions for coronary heart disease between 1.3-4.2% for each 1 mg/dL (0.026 mmol/L) higher baseline serum HDL-cholesterol[35,36]. The effects on cardiovascular disease were somewhat smaller, but consistent across studies. The protective effect of HDL-cholesterol is not restricted to subjects in Europe and the United States but was also seen in a group of Japanese men[37].

Drug trials

It is widely recognised that lowering serum LDL-cholesterol reduces the risk of cardiovascular disease with 1-2% for each percent lowering[38]. Several drug studies suggest a similar effect of increases in serum HDL-cholesterol. However, most drug trials aimed at lowering serum LDL-cholesterol; changes in HDL-cholesterol were no priority. The only exception is the Veterans Affairs High-Density Lipoprotein Intervention Trial (VA-HIT), which was specifically designed to study effects of increasing HDL-cholesterol with gemfibrozil. In this trial, 2531 men with a history of coronary heart disease were randomly allocated to treatment or placebo. After a median follow-up of 5.1 years, the number of primary coronary events (nonfatal myocardial infarction or death from coronary heart disease) was 275 in the placebo group and 219 in the drug group[39]. Serum HDL-cholesterol was 6% higher and serum triglycerides 31% lower in the treatment group than in the placebo group after 1 year; serum LDL-cholesterol remained stable. The investigators calculated that a 5 mg/dL (0.13 mmol/L) increase in HDL-cholesterol reduced the number of coronary events by 11%; changes in HDL-cholesterol therefore contributed to the beneficial effect of gemfibrozil[40].

Further evidence for a protective effect of increases in serum HDL-cholesterol is derived from drug trials that were designed for studying effects of lowering LDL-cholesterol or triacylglycerols on coronary heart disease risk. These studies, typically with 1000-4000 subjects, report risk reductions up to 6% per mg/dL or 23% per 0.1 mmol/L[41-43].

Studies with intermediate endpoints of coronary heart disease also show a better outcome when HDL-cholesterol concentrations increase during the follow-up period[44] or when baseline HDL-cholesterol concentrations are higher[45].

In summary, HDL-cholesterol shows an inverse relation with risk of coronary heart disease. Drugs that increase serum HDL-cholesterol reduce risk of coronary heart disease.

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[1] Framingham Heart Study, Lipid Research Clinics Follow-up study, Coronary Primary Prevention Trial, Multiple Risk Factor Intervention Trial, British Regional Heart Study, and Belgian Interuniversity Research on Nutrition and Health.

Why was flow-mediated vasodilation chosen as an outcome of the studies?

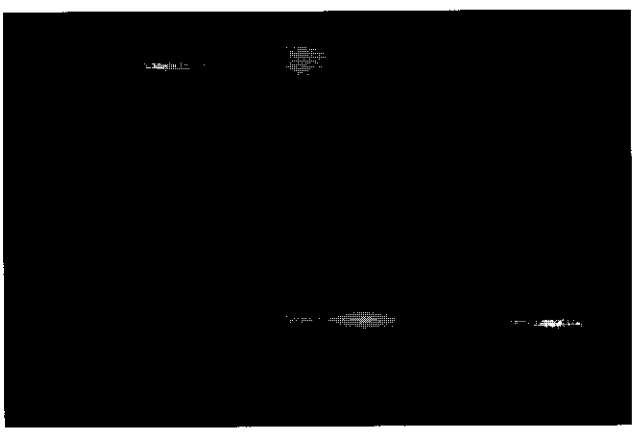
Our aim was to investigate whether *trans* fats increase risk of coronary heart disease more than saturated fats because *trans* fats decrease HDL-cholesterol. Ideally we would have studied this in a long-term trial with hard endpoints, such as myocardial infarction, with the volunteers on controlled diets rich in either saturated fat or *trans* fat. However, even if such a study would be feasible - despite the huge numbers of volunteers and the long follow-up period - it would be unethical because we know that diets high in saturated fat increase risk of cardiovascular disease. Thus, we looked for a risk marker that would add new information to our knowledge of the effect of *trans* fats but would respond within weeks. Flow-mediated vasodilation appears to be such a marker.

Flow-mediated vasodilation is the increase in arterial diameter that is induced by a local increase in arterial blood flow. Such an increase in blood flow (hyperemia) is produced by inflating a blood pressure cuff around the arm to occlude arterial flow for 5 min. Then, release of the cuff leads to hyperemia that increases the shear stress on the endothelial cells. This stimulates the endothelial cells to releasing nitric oxide, a potent vaodilator. The vasodilation is measured non-invasively with high-resolution ultrasound, a technique first described by Celermajer and co-workers[46]. The measurement depends on reproducible measurements of the lumen of the brachial artery (**Figure 1.2**).

In our studies with healthy volunteers, 5 minutes of ischemia induced by a pressure cuff around the lower arm, inflated to a pressure of 240 mmHg, induced a flow-mediated vasodilation of 5% of the baseline diameter.

Flow-mediated vasodilation depends on the release of nitric oxide by the endothelial cells[4]. If nitric oxide release is blocked by chemical agents, for example by N-monomethyl-L-arginine, flow-mediated vasodilation is abolished[47].

Figure 1.2:
Ultrasound image of the brachial artery.



Flow-mediated vasodilation and risk of cardiovascular disease

Flow-mediated vasodilation correlates with all known risk factors of cardiovascular disease[48]. Thus, a high serum LDL-cholesterol, homocysteine, smoking, diabetes, hypertension, and old age are all associated with impaired flow-mediated vasodilation. Changes in these risk factors to more desirable values often improves flow-mediated vasodilation[49,50]. Patients with coronary heart disease show less flow-mediated vasodilation than healthy controls[51]. More important, however, is the comparison of flow-mediated vasodilation with validated markers of coronary heart disease.

Such comparisons showed that flow-mediated vasodilation in the brachial artery correlates with the number of diseased coronary vessels in patients with chest pain [51,52]. It also correlates with flow-mediated vasodilation in the coronary arteries[53] and with intima media thickness of the common carotid artery[54]. Although these correlations suggest that flow-mediated vasodilation might be predictive of future coronary events, follow-up studies are needed to prove this. One study with 73 patients with chest pain showed that cardiovascular events occurred more during a 5-year follow-up in 46 patients with an initial flow-mediated vasodilation of <10% than in 27 other patients with a vasodilation >10%[55]. However, this result was biased by the fact that patients with low initial flow-mediated vasodilation had more severe stenosis at baseline - and therefore a higher risk - than patients with high flow-mediated vasodilation. In another study, flow-mediated vasodilation of the coronary arteries was shown to be predictive of future coronary events[56]. Up to now, these are the only reports in the literature on the predictive value of flow-mediated vasodilation.

In conclusion, it is likely but not proven that changes in flow-mediated vasodilation reflect changes in cardiovascular disease risk. The results of the studies described in this thesis should therefore be balanced against the existing knowledge on the relation between *trans* fats, HDL-cholesterol and coronary heart disease.

Outline of the studies in this thesis

Before we started the diet studies we assessed the within-subject variability of flow-mediated vasodilation in a group of healthy young volunteers (**Chapter 2**). This variability was crucial for our estimate of sample size in the next studies. We then performed three diet studies: two dietary controlled studies and one test meal study. In the two dietary controlled studies all food was prepared for the volunteers during two periods of 3-4 weeks. Effects on flow-mediated vasodilation were measured at the end of each period (**Chapters 3-6**). In the test meal study we studied changes in flow-mediated vasodilation in response to oral fat loads on 4 occasions (**Chapter 7**). The results of all studies will be combined in the final chapter (**Chapter 8, General Discussion**).

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2

Within-subject variability in flow-mediated vasodilation of the brachial artery in healthy men and women

NM de Roos, ML Bots, EG Schouten, MB Katan

ABSTRACT

Background Flow-mediated vasodilation (FMD) of the brachial artery is used as a marker of cardiovascular disease risk. It is defined as the percentage dilation from baseline diameter in response to a provoked increase in blood flow. The within-subject variability, crucial in the design of trials with FMD as an endpoint, appears to vary widely between studies.

Aim To assess the within-subject variability of FMD in healthy subjects and to estimate the number of subjects needed to detect various treatment effects in intervention trials and observational studies.

Design FMD was assessed with B-mode high-resolution ultrasound. Thirteen subjects were measured on 6 occasions, after they had fasted overnight.

Results The mean (±SD) FMD was 5.60±2.15% of the baseline diameter. The within-subject standard deviation was 2.8%-points, which resulted in a coefficient of variation (CV) of 2.8/5.6×100%=50.3%. The CVs for the baseline and maximum diameter were much smaller: 4.8% (SD 0.193 mm, mean 4.060 mm) for the baseline and 5.2% (SD 0.222 mm, mean 4.285 mm) for the maximum. The number of subjects needed to detect a treatment difference of 2%-points in FMD with a probability of 0.05 and a power of 0.90 would be 42 in a cross-over design and 83 per group in a parallel design for comparison of group changes.

Discussion The within-subject variability of FMD is large, about 50% of the mean response. This includes biological and technical variation. Repeated measurements and repeated readings of recorded measurements are recommended to reduce variability.

Introduction

Non-invasive assessment of flow-mediated vasodilation (FMD) is used to evaluate endothelial function and is used as a surrogate endpoint for cardiovascular disease in observational and experimental studies. It is based on the ability of vascular endothelial cells to respond to changes in shear stress. The response consists of a release of nitric oxide, a potent vasodilator that relaxes the smooth muscle cells in the vascular wall[1], resulting in an increase in lumen diameter. FMD can be non-invasively assessed from high-resolution ultrasound images of the brachial artery at baseline and at maximum dilation[2]. In healthy people FMD is 5-15% of the baseline diameter, but in patients with cardiovascular disease FMD is impaired or absent[3,4]. An impaired FMD appears to be predictive of future events: patients with chest pain and impaired FMD were shown to be more likely to experience cardiovascular events in a 5-year period than patients with preserved FMD[5].

The diameter of the brachial artery can be measured reproducibly with high-resolution (7.5-13 MHz) ultrasound: in the literature we found coefficients of variation (within-subject standard deviation divided by mean baseline diameter) of 1.5-6%. At a mean baseline diameter of 4.0 mm this corresponds with a within-subject standard deviation of 0.06-0.24 mm. However, the reported within-subject variability of the FMD is less consistent: coefficients of variation ranging from 1.2% to 13.6% (**Table 2.1**) have been reported. Instead of coefficients of variation, some investigators report alternative measures of reproducibility, that are not well defined. Moreover, most reproducibility studies were based on only two repeated measurements in less than 10 subjects. Therefore we designed a study that would adequately estimate within-subject variability in FMD. We used this information to estimate sample sizes for experimental studies that compare treatment differences on flow-mediated vasodilation.

Subjects and Methods

The Medical Ethics Committee of Wageningen University approved the study protocol. We explained aim and design of the study to the volunteers who all signed informed consent forms.

Number of subjects and measurements

We wanted to estimate the within-subject variance with a certain precision, defined by the width of the 95% confidence interval (CI). We first determined the desired width of the 95% CI, which has a Chi-square distribution that becomes narrower with more degrees of freedom (df)[6,7]. The distribution is skewed: up to 40 df the confidence interval narrows markedly when the number of df increases but over 40 the interval narrows much slower. We chose $0.72\sigma^2$-$1.48\sigma^2$ as the desired width of the 95% CI, corresponding with 60 df. The number of df for a within-subject variance is the number of subjects times {the number of repeated measurement minus 1}. We decided to perform 6 repeated measurements and therefore needed 12 subjects ($12\times\{6-1\}=60$ df).

Table 2.1 Measures of reproducibility of flow-mediated vasodilation of the brachial artery as reported in various studies

number of subjects	number of repeated measurements per subject	reproducibility	reference
3	≥4 times	average CV of 13.6%	11
40 m/f	4	between-scan variance 3.45; overall CV 1.8%	2
8 m	5 within 1 month	CV for repeated measures of FMD% (±SE) 9.77±0.82%	12
15	2 times	intraobserver variability CV 1.2% to 4.2%	13
7 m	2 within 1 day	mean difference 0.88, SD of difference 0.82 FMD%	14
5	2 times, 2 weeks apart	mean difference 1 FMD%, SD of differences 2 FMD%, with a CV of 1.4%	15
10 f	2 on different days	mean difference 1.4 FMD%, SE 0.3 FMD% (SD 0.94 FMD%)	16
3 m	2 on different days	mean difference 3.34 FMD%, SD of difference 2.68 FMD%	14
30 m/f	2 times	repeatability coefficient* 6.93; CV 10.3%	17
10 m/f	2 times on different days	reproducibility 5.5%	18
?	2 times on separate days	variability 1.05±0.35%	19
48	2 times separated by 7 days	variability 1.9±0.3%	20

* $2\times\sqrt{(\Sigma D_i^2/n)}$ with *D=difference between paired scans*

We recruited 15 healthy volunteers from university students and staff. Health was assessed by means of a medical questionnaire; all 15 volunteers were enrolled in the study. Two volunteers had to withdraw from the study after the first visit because we could not obtain good quality measurements of their brachial artery. The 13 remaining volunteers (3 men, 10 women) aged 18-43 (mean age 24) years successfully completed the study.

Design

Each volunteer was measured on 6 occasions, with a maximum of 16 d (mean 5.4 d) in between. They all completed the study within 6 weeks. No food and caloric drinks were allowed after 8pm and no water after 10pm the night before the measurements. The measurements were carried out between 7.15am and 10.30am and appointment times were kept constant for each subject throughout the study. They recorded illness and use of medication in a diary.

FMD measurement

We obtained images of the brachial artery with a 7.5 MHz linear-array transducer of an Ultramark-5 ultrasound system (ATL Woerden, the Netherlands). All images were recorded on super VHS videotape for off-line analysis. One sonographer performed all the measurements and one reader analysed all the videotapes.

The method we used was similar to the one described by Celermajer et al[8]. The measurements were done at the brachial artery of the right arm, at the site of the antecubital crease, with an inflatable cuff around the forearm. Arm and ultrasound transducer were held in position with a specially designed fixture (TAF® method developed by Meijer et al. Vascular Imaging Center, Julius Center for Patient Oriented Research UMC Utrecht Heidelberglaan 100, 3584 CX Utrecht). We chose a segment of the artery of at least 5 mm in length with clear lumen and distinctive vessel walls. We first recorded 3 baseline images of the brachial artery. We then inflated the cuff around the lower arm to a pressure of 240 mm Hg. After 4 minutes we deflated the cuff and started image recording. In the next 5 minutes, images of the brachial artery were frozen every 15 seconds. Others showed that maximal dilation is reached within 5 minutes[2,9,10].

Off-line analysis and quality of ultrasound images

The reader traced the trailing edge of the adventitia-media interface at the near wall and the leading edge of the media-adventitia interface at the far wall of the brachial artery over a length of at least 3 mm. The distance between these interfaces reflects the lumen diameter. All ultrasound images were given a quality score (1=excellent, 2=moderate, or 3=insufficient) by the reader. These quality scores were based on the presence of clear vessel wall boundaries and whether the arm and thus the brachial artery was held in the same position. Only quality scores 1 and 2 were used for data analysis.

Statistical analysis

Baseline and maximum diameters were measured in millimeters. We report means, standard deviations (SD), variances (Var=SD^2), and coefficients of variation (CV=SD/mean×100%). We used the SAS (SAS System for Windows Release 6.12, SAS Institute Inc., Cary, North Carolina, USA) procedure 'Proc Anova' with 'subject' as main effect to calculate the within-subject variance (MSE=Mean Square Error in SAS output).

FMD is computed as the increase in diameter divided by the baseline diameter:

$$\text{FMD} = \{(\text{maximum}-\text{baseline})/\text{baseline}\} \times 100\%.$$

The formula can also be written as the ratio between maximum and baseline diameter minus 1:

$$\text{FMD} = \{(\text{maximum}/\text{baseline})-1\} \times 100\%.$$

The latter formula shows that the FMD is a rescaled ratio between the two diameters. Because rescaling by subtraction does not change the variance, only the mean, we also calculated the coefficient of variance for the ratio between maximum and baseline diameter. We did this because this might explain the small coefficients of variation reported by others (**Table 2.1**). For reasons of clarity we use 'FMD%' as a unit of FMD-measurements and '%' for other percentages.

We estimated the number of subjects needed to detect treatment differences in a cross-over study and a parallel study, and group means in an observational study {ref Snedecor}. For a cross-over study, we used the estimate $N \approx \text{factor} \times SD^2/D^2$ with N=total number of subjects, SD=standard deviation of the difference, estimated as $\sqrt{2}$×within-subject SD, and D=treatment difference. The factor is derived from $(Z_\alpha+Z_\beta)^2$ with probability α=5% and (1-β) power=80% (factor=7.9) or 90% (factor=10.6). For a parallel study that compares treatment responses between groups, we used $N \approx 2 \times 8 \times SD^2/D^2$ with N=number of subjects per group, SD=standard deviation of the response within a group, estimated as $\sqrt{2}$×within-subject SD, and D=differences between responses.

Results

The number of measurements of sufficient quality ranged from 2 to 6 per subject (**Table 2.2**), with a total of 62 out of 78 scans. The remaining 16 scans with low quality were excluded from the analysis. The coefficient of variation of the baseline diameter was 4.8% and that of the maximum diameter 5.2%, in contrast to the much higher coefficients of variation of the absolute increase in diameter (CV=51.1%) and the flow-mediated vasodilation (CV=50.3%) (**Table 2.3**). The coefficient of variation of the ratio between maximum and baseline diameter was 2.7% (**Table 2.3**).

Table 2.2 Means with standard deviations (SD) of brachial artery measurements of 13 healthy volunteers, measured 2-6 times each after an overnight fast

		brachial artery diameter (mm)				Flow-mediated vasodilation	
		at rest (baseline)		maximum		(% of baseline)	
subject	N[#]	average	SD	average	SD	average	SD
1	2	3.526	0.144	3.760	0.117	6.67	1.03
2	4	4.309	0.362	4.527	0.359	5.09	1.53
3	4	3.761	0.187	4.108	0.285	9.19	3.87
4	6	4.189	0.242	4.437	0.220	5.98	1.96
5	5	4.034	0.186	4.325	0.180	7.26	3.15
6	2	4.851	0.090	4.908	0.105	1.16	0.30
7	3	4.619	0.188	4.966	0.339	7.46	3.97
8	6	3.855	0.182	4.118	0.198	6.82	2.53
9	6	3.704	0.128	3.810	0.141	2.90	2.67
10	6	4.519	0.120	4.719	0.169	4.43	2.00
11	6	3.871	0.118	4.030	0.086	4.16	2.52
12	6	4.293	0.186	4.490	0.242	4.62	4.58
13	6	3.682	0.218	3.944	0.280	7.07	2.09

[#] N = number of used measurements (high and moderate quality)

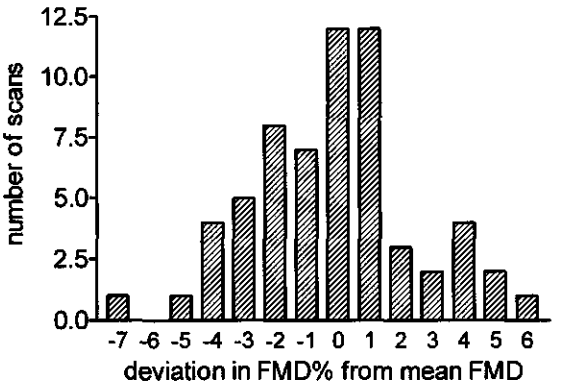
Table 2.3 Overview of brachial artery dimensions, within-subject variabilities, and coefficients of variation (CV) of 13 healthy volunteers measured on 6 occasions, each occasion after an overnight fast

	mean	SD (root MSE[†])	CV[‡]
baseline	4.060 mm	0.193 mm	4.8%
maximum	4.285 mm	0.222 mm	5.2%
absolute change[¶]	0.225 mm	0.115 mm	51.1%
FMD[$]	5.6 FMD%	2.8 FMD%	50.3%
ratio[#]	1.056	0.028	2.7%

[†] MSE = mean square error
[‡] CV = root MSE / mean ×100%
[¶] maximum - baseline
[$] {(maximum-baseline)/baseline}×100% or {(maximum / baseline)-1} ×100%
[#] maximum / baseline

For each subject we subtracted his or her average FMD from the corresponding 2-6 individual FMD-measurements and we plotted all 62 differences in a histogram (**Figure 2.1**). The figure shows that 87% (54/62) of repeated measurements were within 4 FMD% of a subject's mean FMD (range in differences –6.8 to 6.1 FMD%).

Figure 2.1:
Deviations of each of the 2-6 repeated measurements per subject from their corresponding mean



Because we had 62 measurements of 13 subjects, the number of degrees of freedom allocated to the within-subject variance was {62-1}-{13-1}=49. The corresponding lower limit of the 95% CI for the within-subject variance was $0.70\sigma^2$ and the upper limit $1.55\sigma^2$[27]. The corresponding 95% CI for the within-subject SD (square root of variance) was therefore 2.4 to 3.5 FMD%.

We calculated sample sizes needed to detect differences in cross-over studies and parallel studies (**Table 2.4**). The number of subjects needed to detect a difference of 2 FMD% with a probability of 0.05 and a power of 0.90 would be 42 in a cross-over trial and 83 per group when group changes (e.g. responses to treatment) are compared.

Table 2.4 number of subjects needed in a cross-over study or a parallel study to compare treatment effects ranging from 0.5-3.0 FMD%

minimum difference	number of pairs in cross-over study[†]		number of subjects per group when comparing responses[‡]	
	power 0.80	power 0.90	power 0.80	power 0.90
0.5	495	665	991	1330
1.0	124	166	248	332
1.5	55	74	110	148
2.0	31	42	62	83
2.5	20	27	40	53
3.0	14	18	28	37

[†] calculated using the SD of differences between pairs, estimated as √2×within-subject SD = √2×2.8 FMD%=3.96 FMD%
[‡] calculated using the SD of responses within groups, estimated as √2×within-subject SD = √2×2.8 FMD%=3.96 FMD%

Discussion

Within-subject variability of flow-mediated vasodilation is large: at a mean FMD of 5.60 FMD% of the baseline diameter we found a within-subject SD of 2.82 FMD%. The corresponding coefficient of variation, the within-subject SD divided by the mean, was 50%, which is larger than those described in the literature (**Table 2.1**). However, the coefficients of variation that we found for the baseline and maximum diameter were about 5%, comparable to those found by others[2,21]. Therefore we think that previously reported coefficients of variation of FMD may have been calculated differently. A possibility is that the coefficient of variation of the ratio between maximum and baseline diameter has been reported. This ratio is in fact a rescaled FMD, because the FMD can be calculated as the ratio minus 1. In our dataset, the ratio between maximum and baseline diameter was 1.056 with a within-subject SD of 0.028 and a corresponding CV of 2.7%, which is close to the reported CVs (**Table 2.1**).

The fact that the coefficient of variation of FMD is larger than that of the baseline or maximum diameters can be explained mathematically. By definition, the coefficient of variation is the standard deviation divided by the mean. The standard deviation of the absolute change in diameter is about the same as that of the baseline and maximum diameter (**Table 2.3**), and will therefore not cause the difference in coefficient of variation. However, the mean of the absolute change in diameter is about twenty times smaller than the baseline and maximum diameters. A smaller mean in the denominator increases the CV, and the CV could even approximate infinity if the mean FMD would be close to zero, for example in patients with coronary heart disease. Thus, a large coefficient of variation for FMD measurements is not surprising.

The mean FMD in our study was small compared to that found in some other studies, and this affects the size of the coefficient of variation. Larger values for FMD have been reported by investigators that placed the cuff around the upper arm instead of the lower arm, a difference that can be as large as 10 FMD%[22,23]. Thus, differences in methodology may account for some of the difference in coefficients of variation.

Our estimate of within-subject variability includes biological and analytical variation. We could not separate the two because all images were made by one sonographer and read by one reader. Therefore it is possible that biological within-subject variability, or day-to-day variability, is smaller than the 2.8 FMD% we estimated. We therefore estimated the variation in repeated readings of flow-mediated vasodilation, using data of 13 subjects of a later study (**Chapter 3**). Each subject's measurement was read twice by one observer; and for each subject the mean and SD for these two readings were calculated. The mean ± SD of these 13 averaged readings was 3.73±1.98 FMD%. The within-subject SD, or between-readings SD, was 1.27 FMD%, which corresponded with a CV of 1.27/3.73×100%=34%. For comparison, the CV of measurements performed on different days was 50%, suggesting that reading variation might be more important than biological variation. However, this also means that the variation can be reduced both by multiple measurements and by multiple readings of the same measurement.

The sample size calculations showed that large numbers of subjects are needed to detect small treatment differences, e.g less than 2FMD%. The number of subjects could be reduced if the SD that is used in the sample size equation is reduced. This may be accomplished by repeated measurements, because the variance of k repeated measures is $1/k$ times the original variance[6]. Another possibility is pre-study selection of subjects on the basis of image quality, because inclusion of images with low quality increases the within-subject variability (data not shown). However, this might introduce bias in the outcome of the study, because we do not know whether subjects with higher image quality respond differently to treatment than other subjects.

In conclusion, studies with flow-mediated vasodilation as an outcome variable may give us insight on the effects of drugs or diet components on cardiovascular disease risk. The variability within-subjects is crucial for the calculation of sample sizes, and is about 50% of the mean FMD. Previous reports of coefficients of variation for FMD in the literature are much smaller but may have been erroneous. To improve reproducibility of FMD-measurements we recommend duplicate measurements and duplicate reading of the tapes.

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3

Replacement of dietary saturated fatty acids by *trans* fatty acids lowers serum HDL cholesterol and impairs endothelial function in healthy men and women

N.M. de Roos, M.L. Bots, M.B. Katan

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ABSTRACT

Objectives We tested whether *trans* fatty acids and saturated fatty acids had different effects on flow-mediated vasodilation (FMD), a risk marker of coronary heart disease (CHD).

Background Consumption of *trans* fatty acids is related to increased risk of CHD, probably through effects on lipoproteins. *Trans* fatty acids differ from most saturated fatty acids because they decrease serum high-density lipoprotein (HDL) cholesterol, and this may increase risk of CHD.

Methods We fed 29 volunteers two controlled diets in a 2×4 weeks randomized cross-over design. The 'Trans-diet' contained 9.2 energy percent of *trans* fatty acids; these were replaced by saturated fatty acids in the 'Sat-diet'.

Results Mean serum HDL cholesterol after the Trans-diet was 0.39 mmol/L (14.8 mg/dL) or 21% lower than after the Sat-diet (95% CI, 0.28 to 0.50 mmol/L). Serum LDL and triglycerides concentrations were stable. Mean±SD FMD was 4.4±2.3% after the Trans-diet and 6.2±3.0% after the Sat-diet (difference -1.8%, 95% CI, -3.2 to -0.4).

Conclusions Replacement of dietary saturated fatty acids by *trans* fatty acids impaired FMD of the brachial artery, which suggests increased risk of CHD. Further studies are needed to test whether the decrease in serum HDL cholesterol caused the impairment of FMD.

Introduction

When liquid oils are partially hydrogenated to form solid margarines and shortenings, *trans* isomers of fatty acids are formed. In countries such as the USA[1,2] and the Netherlands[3], *trans* fatty acids constitute 4-7% of dietary fat intake. A high intake of *trans* fatty acids is associated with an increased risk of coronary heart disease (CHD)[4-6]. One probable cause is the effect of *trans* fatty acids on serum lipoproteins. Like saturated fatty acids, *trans* fatty acids increase the concentration of serum low-density lipoprotein (LDL) cholesterol[7,8]. Moreover, and unlike saturated fatty acids, *trans* fatty acids decrease serum high-density lipoprotein (HDL) cholesterol[7-11]. This might be harmful because there is increasing evidence that HDL cholesterol is inversely related to CHD[12,13].

We investigated whether the intake of *trans* fat would indeed increase the risk of CHD more than the intake of saturated fat by comparing the effects of these fats on endothelial function, a surrogate cardiovascular endpoint[14-16]. We assessed endothelial function as flow-mediated vasodilation (FMD) of the brachial artery, because this is a non-invasive measurement which correlates well with known risk factors[17-22] and other markers of CHD[23-25]. Moreover, two longitudinal studies show an association between FMD in the past with future CHD events[26,27]. The diets were given for a minimum of 3 weeks; a time period long enough to establish changes in serum lipids[28] and FMD[21]. We hypothesized that FMD would be lower after the diet rich in *trans* fat than after the diet rich in saturated fat because of the expected difference in serum HDL cholesterol.

Methods

Subjects

The Medical Ethical Committee of Wageningen University approved the study aim and design. Each volunteer signed an informed consent form. We recruited 39 non-smoking men and women and assessed their health using a questionnaire; we eliminated 1 person because of use of medication, 2 because of missing information, and 1 because of poor veins for venipuncture. All subjects had normal concentrations of serum cholesterol and triglycerides and normal amounts of protein and glucose in their urine. We excluded 2 subjects because we could not obtain a clear ultrasound image of their brachial artery. One other subject withdrew before the start of the study; in the end, 32 subjects were enrolled. They all completed the study.

Study design

We provided 2 controlled diets for 4 weeks each in a randomized cross-over design. The diets consisted of conventional food items supplemented with special margarines, and were given in a 28-day menu cycle. On Mondays through Fridays subjects came to our dining room and ate a hot meal under our supervision. All other foods (bread; margarine; meat and/or cheese; honey, jam, or sprinkles; fruit; milk and/or yogurt) were packed for consumption at home, as was food for the weekends.

Habitual energy intake of the subjects was estimated from a food frequency questionnaire. We designed menus for 14 levels of energy intake, ranging from 7-20 MJ/d, and allocated the subjects to an intake level close to their habitual energy intake. We provided 90 percent of energy (en%); all this food was weighed out for each subject. We measured body weight twice a week; if body weight changed >1 kg subjects were switched to a different energy intake level. The remaining 10 en% had to be chosen from a list of low-fat food items and recorded in a diary. Subjects received the diets for 21-32 days (mean 27.5 days).

Diets

The experimental diets differed in margarine only (**Table 3.1**). The margarine in the diet rich in *trans* fatty acids was a blend of 70 parts partially hydrogenated soy oil, containing 44% *trans*-C18:1 (Gouda's Glorie, Van Dijk Foods, Lopik, the Netherlands); 14 parts vegetable oil containing 63% linoleic acid and 23% oleic acid (Becel, Unilever, Vlaardingen, the Netherlands) and 16 parts water. The margarine in the diet rich in saturated fat was a blend of 60 parts palm kernel fat (Loders Croklaan, Wormerveer, the Netherlands) and 40 parts commercially available margarine made from a blend of vegetable oils and solid vegetable fats (Blue Band, Van den Bergh BV, Rotterdam, the Netherlands). Both margarines were produced at NIZO Food Research (Ede, the Netherlands). The margarines were used as shortenings in bread and cookies, in sauce and gravy, and as a spread. They supplied 62% of fat in the diets, the remaining 38% was mainly derived from meat, cheese and other dairy products, eggs, and salad dressings.

Table 3.1 Fatty acid composition of the margarines used in the diet rich in *trans* fatty acids and the diet rich in saturated fatty acids

Fatty Acid	g/100 g Fatty Acid	
	Trans margarine	Sat margarine
Saturated	30.5	63.1
Lauric acid (C12:0)	not detected	24.5
Myristic acid (C14:0)	0.1	10.2
Palmitic acid (C16:0)	10.5	17.0
Stearic acid (C18:0)	18.5	7.4
cis-Monounsaturated	18.6	20.9
Oleic acid (*cis*-C18:1n-9)	8.0	19.9
trans-Monounsaturated	41.4	0.6
trans-C18:1	40.9[1]	0.3
Polyunsaturated	8.7	15.0
Linoleic acid (*cis,cis*-C18:2)	8.2	14.6
Others	1.3	0.6

[1] mainly n-10 (22%), n-9 (20%), and n-11(17%) isomers

The composition of the diets was calculated using food composition tables[3,29,30] and checked by collecting duplicates of all meals (**Table 3.2**). The analyzed values were similar to the calculated composition.

Table 3.2 Analyzed composition of the two experimental diets

Fatty Acid	Trans-diet	Sat-diet
Carbohydrate (energy%)	48.6	45.6
Protein (energy%)	14.0	13.5
Total fat (energy%)	37.4	41.0
Saturated	12.9	22.9
Lauric acid (C12:0)	0.3	6.8
Myristic acid (C14:0)	0.8	3.8
Palmitic acid (C16:0)	5.7	7.8
Stearic acid (C18:0)	5.3	3.1
Monounsaturated, total	18.2	8.8
cis-C18:1	8.4	7.9
trans-C18:1	9.2	0.3
total *trans*	9.4	0.4
Polyunsaturated	4.7	6.9
Linoleic acid (*cis,cis*-C18:2)	4.1	5.9
Linolenic acid (*cis,cis,cis*-C18:3)	0.3	0.7
Cholesterol (mg/MJ)	27.0	26.8
Cholesterol (mg/day)	248.4	253.5
Fibre (g/MJ)	3.2	3.1
Fibre (g/day)	29.4	29.3
Energy (MJ/day)	9.20	9.46
Energy (kcal/day)	2199	2261

Blood lipids

We took blood samples after an overnight fast on two separate days after day 19 of each diet. All 4 blood samples of each subject were analyzed in duplicate within 1 run. Total cholesterol and triglycerides (Cholesterol Flex™ and Triglycerides Flex™ reagent cartridge, Dade Behring, Newark USA) and HDL cholesterol (*liquid* N-geneous™ HDL-C assay, Instruchemie BV, Hilversum, the Netherlands) were measured, and LDL cholesterol was calculated with the Friedewald formula. The coefficient of variation of 64 duplicate measurements was 0.4% for total cholesterol, 1.5% for triglycerides, and 1.1% for HDL cholesterol.

Brachial artery measurements

All subjects had an overnight fast of at least 12 h before the measurements. We measured FMD of the brachial artery as described by Celermajer *et al.*[22,31]. We used the diameter of the artery at rest and at maximum vasodilation to calculate the percentage increase or FMD. All measurements were done at end diastole by the use of the R-wave of the electrocardiogram. The ultrasound images were made by one technician with a 7.5 MHz linear array transducer of an Ultramark™ 9 HDI duplex scanner. All images were stored on super-VHS videotapes for off-line analysis.

Subjects were made to lie down in a temperature-controlled room (range 20-23°C) with the right arm in two arm support cushions. An inflatable cuff was placed around the lower arm. The transducer was held in position at the site of the antecubital crease with a specially developed transducer arm fixature (TAF® method developed by Meijer et al. Vascular Imaging Center, The Julius Center for Patient Oriented Research UMC Utrecht Heidelberglaan 100, 3584 CX Utrecht).

We first obtained an optimal two-dimensional B-mode ultrasound image of the brachial artery at rest and recorded three images to measure the diameter. We then inflated the cuff to 250 mm Hg and kept this pressure constant for 5 minutes to induce ischemia in the forearm and hand. After 5 minutes the cuff was deflated. The image of the brachial artery was optimized and changes in the diameter of the artery were recorded during the next 5 minutes. Every 15 seconds a frozen image was stored on videotape. At the end of the second feeding period we also measured endothelium-independent vasodilation after a sublingual dose of 400 μg of nitroglycerin.

One reader who was blinded to the treatment read all images at the Vascular Imaging Center of the University Medical Center in Utrecht. The reader rated the quality of the images from class 1 (perfect) to class 4 (unfit for use). All 32 subjects were measured twice on both diets, so we had 4 measurements per subject. Of these 128 measurements, 24 were rated as perfect, 71 as fair, 26 as marginal, and 2 as unfit. Five measurements were missing. We only used measurements rated perfect or fair, which left us with 29 subjects for whom we had observations on both diets. At a mean FMD of 5.3% of the resting diameter, the SD within-subjects was 2.6%-points so the corresponding CV was 49%. The largest difference in a duplicate FMD-measurement was 18.2%-points (FMD 2.6 and 20.8% of the resting diameter); the smallest difference 0.16%-points (measurements: 7.2 and 7.4% of the resting diameter). The CV of the resting and maximum diameter was 8%.

Statistics

We averaged the duplicate measurements in each dietary period and tested for order effects by ANOVA, with diet and order as main effects in the model[32]. Because the order of the two diets did not significantly contribute to the model, we then calculated for each subject the difference between treatments. We tested whether these differences were significantly different from zero by the Student *t* test for paired samples. We give two-sided 95% confidence intervals for the differences.

Results

We analyzed data of 29 subjects, 10 men and 19 women. Their mean (±SD) age was 30±16 years, their mean weight 69±9 kg, and their mean body mass index 22.5±2.4 kg/m^2. Pre-study serum cholesterol concentrations were 5.1±1.1 mmol/L and serum triglycerides 1.2±0.7 mmol/L.

Body weight

During the 4-week feeding periods, body weight remained basically stable, with mean decreases of 0.4 kg during the Trans-diet and 0.6 kg during the Sat-diet (P=0.43 for difference in change between diets).

Blood lipids

Serum HDL cholesterol decreased from 1.87±0.46 mmol/L (73.1±17.8 mg/dL) on the diet rich in saturated fats to 1.49±0.33 mmol/L (56.5±12.8 mg/dL) on the diet rich in *trans* fats (**Table 3.3**). The decrease was 0.39 mmol/L (95% CI, -0.50 to -0.28) or 21%. Serum LDL cholesterol and triglycerides remained stable. The order of the two diets hardly affected the change in HDL cholesterol: the mean change was 0.35±0.25 mmol/L in subjects who went from the Trans-diet to the Sat-diet and 0.43±0.32 mmol/L in the subjects who received the diets in the reverse order.

Brachial artery measurements

The diameter of the brachial artery at rest was 4.02±0.70 mm on the Sat-diet and 4.08±0.73 mm on the Trans-diet. The maximum diameter was 4.33±0.80 mm on the Sat-diet and 4.19±0.73 mm on the Trans-diet. FMD was 6.2±3.0% on the Sat-diet and 4.4±2.3% on the Trans-diet (P=0.015). Thus, FMD was 1.8 percent (95% CI –3.2 to -0.4) or 29% lower on the Trans-diet than on the Sat-diet (**Figure 3.1**). The order of the two diets hardly affected the results: 15 subjects went from an FMD of 4.8% after the Trans-diet to 6.4% after the Sat-diet whereas 14 other subjects went from 5.9% after the Sat-diet to 4.2% after the Trans-diet.

All subjects showed vasodilation after nitroglycerin (range 4.4 to 20.8%). Diet had no effect on nitroglycerin-mediated vasodilation, which was 14.3±3.4% on the Trans-diet and 13.4±5.3% on the Sat-diet (unpaired t test, P=0.64).

A decrease in HDL cholesterol went together with a decrease in FMD in 18 of 29 subjects. The correlation between changes in HDL cholesterol and FMD was positive but not significant (r=0.12, 95% CI –0.26 to 0.46, P=0.55).

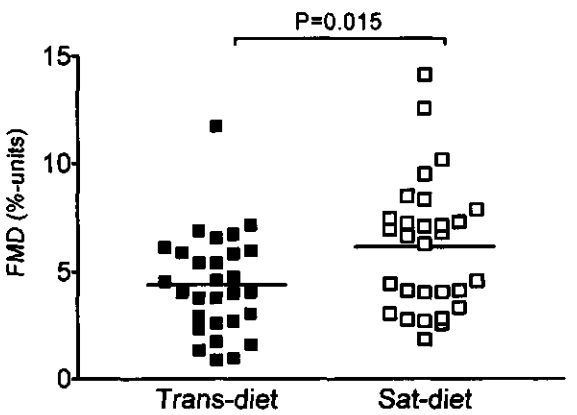
Table 3.3 Concentration of serum lipids after 4 weeks consumption of the two diets

	Trans-diet	Sat-diet	Difference (95% CI)
	concentrations in mmol/L		
total cholesterol	4.97 ± 0.94	5.34 ± 0.95	–0.37 (–0.24 to -0.50)
high-density lipoproteins	1.48 ± 0.33	1.87 ± 0.45	–0.39 (–0.28 to -0.50)
low-density lipoproteins	3.04 ± 0.80	3.05 ± 0.81	–0.01 (–0.14 to 0.11)
triglycerides	0.98 ± 0.41	0.90 ± 0.36	0.08 (–0.04 to 0.20)

Values are means ± SD. The 29 subjects consumed both diets for 4 weeks in random order. To convert values for total, HDL, and LDL cholesterol to milligrams per deciliter, multiply by 38.67. To convert triglycerides to milligrams per deciliter, multiply by 88.54

Figure 3.1:

Flow-mediated vasodilation of the 29 subjects after the diet rich in *trans* fatty acids (■) and after the diet rich in saturated fatty acids (□). The subjects consumed both diets for 21-32 days in randomized order.



Discussion

Consumption of *trans* fatty acids resulted in lower HDL cholesterol and a smaller flow-mediated vasodilation than consumption of saturated fatty acids. This might explain the increased risk of cardiovascular disease at high intakes of *trans* fatty acids. However, the question remains whether the impaired vasodilation was attributable to the decrease in HDL cholesterol.

HDL cholesterol, other dietary factors, and endothelial function

There is some evidence that changes in HDL cholesterol concentration could change endothelial function. First, higher serum HDL cholesterol is associated with better endothelial function[24,33,34]. This might be due to the proposed antioxidant capacity of HDL[35] which might prevent oxidation of LDL and therefore prevent adverse effects of oxidatively modified LDL on endothelial function. We know of no other interventions aimed at HDL, but other antioxidants, such as vitamin C[36,37] were shown to improve FMD. Second, there is ample evidence that reductions in other known risk factors, such as LDL cholesterol[21,25,38] or homocysteine[39], improve FMD, suggesting that changes in HDL cholesterol could have similar effects. The fact that we did not find a significant correlation between changes in HDL cholesterol and FMD does not rule out a causal relation, because the data were too scarce to correct for possible confounding variables such as gender and age. On the other hand, a significant correlation would be no proof of a causal relation.

Other factors in the diets might account for the effect on FMD. As shown in **Table 3.2** there was a small difference in linoleic acid between the two diets, and studies with rats show that *trans* fatty acids have stronger effects at low intakes of linoleic acid[40]. Although this might apply to man, those rat studies were done at very high intakes of *trans* fatty acids (20en%), and the adverse effects could be counteracted with a linoleic acid intake as low as 2en%. Thus, the 4.1en% provided by linoleic acid in our 9.2en% Trans-diet was not low as compared to the rat studies. Also, we think that the difference in linoleic acid between the Sat en Trans-diet was too small to fully explain the effects seen on FMD. Another factor is vitamin E; the different fat mixtures likely differed by 10-20 mg/100g. However, studies that showed an effect of vitamin E on FMD[41] used much higher doses, and even at these high doses most studies did not show an effect[42-44]. Lastly, FMD is impaired in diabetes[45] and if *trans* fatty acids and saturated fatty acids have different effects on insulin metabolism this could have biased the results. However, it is unlikely that fasting serum insulin was different between the two diets[46].

We do not know of studies that compared long-term effects of different fats on FMD. Postprandial effects of saturated and *cis*-monounsaturated fats seem to be similar; they all appear to impair FMD compared to pre-prandial values or compared to low-fat control meals[36,47,48]. However, some of these studies[36,47] are flawed because the low-fat meals had a higher vitamin C content than the fat-enriched meals which might have improved FMD[49]. We know of no short-term effects of *trans* fatty acids on FMD.

Study limitations

We used a cross-over design to eliminate variation due to differences between subjects. The order of the two diets was balanced and randomized per subject to eliminate bias due to a systematic drift of the outcome variables over time[32]. Although we did not include a wash-out period, we did not find a significant order effect on any of the blood lipoproteins or for FMD.

We were only interested in differences between the two test diets; not in changes from the habitual diet, and therefore no baseline data were collected. We can only speculate on changes in blood lipoproteins and FMD from baseline. Both experimental diets differed in fat content from habitual diets: The amount of *trans* fatty acids in the Trans-diet was about 23 g/d, which is 5-fold higher than the estimated 4.8 g/d for men and 3.8 g/d for women in the Netherlands[3]. The amount of saturated fat in the Sat-diet was 58 g/d which is also higher than the habitual intake of 42 g/d for men and 32 g/d for women in the Netherlands. Because of the low habitual intake of *trans* fatty acids, replacing them all by saturated fatty acids would probably hardly improve endothelial function. Conversely, our findings imply that replacing all saturated by *trans* fatty acids could impair FMD and should therefore be discouraged.

The inclusion of women in the study may have increased the variation in FMD response, because changes in serum oestradiol concentrations affect FMD[50]. However, we minimized this variation with 4-week study periods, the length of a menstrual cycle. The women appeared to respond stronger to the diets, with a 2.3%-units (95% CI, 0.4 to 4.2) smaller FMD on the Trans-diet than on the Sat-diet, than the men in who the difference was 0.8%-units (95% CI, –1.3 to 3.0). However, the number of men was small (n=10) and therefore our study was not powered to test for gender differences. Further studies with larger numbers of men and women are needed to test for differences in response.

Repeatability of the FMD-measurement

We found a mean FMD of 5.3%. This is somewhat lower than values for healthy volunteers reported by others[16,36,50], but differences in methodology, for example the position of the inflatable cuff[51], could account for this. The variability in FMD was high, we found a coefficient of variation of 49%. This is comparable with the variability found in some studies[52,53], but higher than values reported by others[23,31,50,54,55]. However, in most papers it is unclear how the values for variability have been calculated.

In conclusion, we showed that replacement of saturated fatty acids by *trans* fatty acids in the diet lowered serum HDL cholesterol and impaired FMD. This suggests that *trans* fatty acids increase risk of CHD more than intake of saturated fats with similar effects on LDL cholesterol. Further studies are needed to verify whether decreases in HDL cholesterol indeed impair endothelial function and thereby explain the increased risk of CHD at high intakes of *trans* fats.

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4

Flow-mediated vasodilation is not impaired when HDL cholesterol is lowered by substituting carbohydrates for monounsaturated fat

N.M. de Roos, M.L. Bots, E. Siebelink, E.G. Schouten, M.B. Katan
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ABSTRACT

Background Low-fat diets, in which carbohydrates replace some of the fat, decrease serum cholesterol. This decrease is due to decreases in LDL cholesterol but in part to possibly harmful decreases in HDL cholesterol. High-oil diets, in which oils rich in monounsaturated fat replace some of the saturated fat, decrease serum cholesterol mainly through LDL cholesterol.

Methods We used these two diets to investigate whether a change in HDL cholesterol would change flow-mediated vasodilation, a marker of endothelial function. We fed 32 healthy volunteers two controlled diets in a 2×3½ weeks randomised cross-over design to eliminate variation in changes due to differences between subjects. The low-fat diet contained 58.2 percent of energy (en%) as carbohydrates and 25.0 en% as fat (7.5 en% as oil); the oil-rich diet contained 36.5 en% as carbohydrates and 42.4 en% as fat (18.4 en% as oil).

Results Average (±SD) serum HDL cholesterol after the low-fat diet was 0.21±0.12 mmol/L (8.1 mg/dL) lower than after the oil-rich diet. Serum triacylglycerols were 0.22±0.28 mmol/L (19.5 mg/dL) higher after the low-fat diet than after the oil-rich diet. Serum LDL and homocysteine concentrations remained stable. Flow-mediated vasodilation was 4.8±2.9% after the low-fat diet and 4.1±2.7% after the oil-rich diet (difference 0.7%; 95% CI, –0.6 to 1.9%).

Conclusion Although the low-fat diet produced a lower HDL cholesterol than the high-oil diet, flow-mediated vasodilation, an early marker of cardiovascular disease, was not impaired.

Introduction

Diets low in saturated fats and high in carbohydrates are often advocated to reduce the risk of cardiovascular disease (CVD) because they lower serum total and LDL cholesterol[1-3]. However, there has been a debate about whether to lower the intake of saturated fats by decreasing total fat intake or by replacing them with *cis*-unsaturated fats. Supporters of low-fat diets argue that replacement of fat by carbohydrates will not only decrease risk of CVD through lowering of serum cholesterol but will also help people lose weight[4] and thus prevent obesity[5,6]. However, others argue that low-fat diets might not be the wisest recommendation because these diets lower HDL cholesterol[7], which may increase the risk of coronary heart disease[8-17]. HDL cholesterol is not lowered when saturated and *trans* fatty acids are replaced by unsaturated vegetable oils, and therefore diets rich in vegetable oils might be a good alternative to low-fat diets[7].

To investigate whether the difference in HDL cholesterol after a low-fat diet and a high-oil diet would affect risk of cardiovascular disease, we used flow-mediated vasodilation of the brachial artery as an outcome variable. Flow-mediated vasodilation is a measure of endothelial function, which is believed to be an early stage of cardiovascular disease[18,19]. Flow-mediated vasodilation of the brachial artery is mediated by nitric oxide released by the endothelial cells[20] and can be measured non-invasively. We chose flow-mediated vasodilation (FMD) because it appears to be predictive of cardiovascular events[21].

In a previous study (Chapter 3) we showed that intake of *trans* fatty acids reduced serum HDL cholesterol and impaired flow-mediated vasodilation in healthy men and women. Although the results of that study seemed to be compatible with a causal relationship between HDL cholesterol and FMD, a verification of the results was needed. Thus, we investigated whether the difference in HDL cholesterol after a low-fat diet and a oil-rich diet affected flow-mediated vasodilation. We applied Bayesian methods to integrate the existing evidence for a protective effect of HDL cholesterol with the present data.

Methods

The study was approved by the Medical Ethics Committee of Wageningen University. Each volunteer signed an informed consent form.

Subjects

We recruited 39 non-smoking men and women by advertising in the university paper and by personally inviting subjects who had taken part in previous studies. We selected subjects on the basis of a medical questionnaire, serum cholesterol (<8 mmol/L) and triacylglycerol (<2 mmol/L) , urinary protein (<0.3 g/L) and glucose (<5.5 mmol/L), and a good quality ultrasound image of the brachial artery. We enrolled 35 subjects. One subject withdrew from the study after 1 week because he was unable to comply with the study protocol. The study was completed by 34 subjects; 13 men and 21 women with mean age 27 (range 19-59) years. Their

mean (±SD) baseline body weight was 68±9 kg, body mass index 22±2.3 kg/m², fasting total cholesterol 4.6±0.8 mmol/L and triacylglycerols 1.2±0.5 mmol/L.

Study design

Our aim was to test whether a difference in HDL cholesterol induced by two different diets would result in a difference in FMD. To minimize the variation in the differences we chose a cross-over design. The order in which the two diets were given was randomly allocated.

We provided 2 controlled diets for 3½ weeks each without a wash-out period. The diets were given in a 28-day menu cycle. On Mondays to Fridays subjects came to our dining room and ate a hot meal under our supervision. All other foods (bread; margarine; meat and/or cheese; honey, jam, or sprinkles; fruit; milk and/or yogurt) were provided in a package for consumption at home, as was food for the weekends.

On 2 days during the last week of each diet period, after subjects had consumed the diets for at least 22 days, we measured flow-mediated vasodilation and serum lipids of the subjects. The measurements were performed 1-2 days apart. Because not all subjects could be measured on the same day, they received the diets for 24-27 days (mean 25 days).

Diets

The two diets consisted of conventional food items. The composition of the two diets was calculated to change the concentration of HDL cholesterol without changing LDL cholesterol. Therefore, it was impossible to match the two diets for saturated and poly-unsaturated fatty acid intake. We used a low-fat margarine, low-fat dairy products, and extra carbohydrate in the low-fat diet, and olive oil, margarine, and full-fat dairy products in the oil-rich diet (**Table 4.1**). The composition of the experimental diets was calculated using food composition tables[22,23]. We checked the composition of the diets by collecting duplicates of all meals. The analyzed values were similar to the calculated composition (**Table 4.2**).

Habitual energy intake of the subjects was estimated from a food frequency questionnaire. We designed menus for 14 levels of energy intake, ranging from 7 to 20 MJ per day. The subjects were allocated to an energy intake level close to their habitual energy intake. We provided 90 percent of energy and all food was weighed out for each subject. The remaining 10 percent of energy had to be chosen from a list of low-fat food items. Subjects recorded their choice from this low-fat food list in a diary. We measured body weight twice a week; if body weight changed more than 1 kg subjects were switched to a different energy intake level.

Table 4.1 Food items (g/day) provided in a 11-MJ menu of the low-fat- and oil-rich diet

	food item	low-fat diet	oil-rich diet
food items that differed in amount and composition	bread*	233.0	200.0
	cookies*	30.0	45.0
	sauce and gravy*	70.0	70.0
	salad dressing†	15.0	15.0
	dessert‡	250.0	125.0
	table spread	26.0	35.0
foods that differed in amount only	starch (potatoes, rice, pasta, bulgur)	270.0	180.0
	vegetables	230.0	180.0
	fruit	248.0	124.0
	salad	38.0	38.0
	meat	82.0	68.0
	milk, 1.5% fat	250.0	200.0
	eggs	17.5	28.0
	cheese, 31% fat	16.0	32.0
	meat (filling)	36.0	36.0
	sweet fillings (honey, jam, sprinkles, etc)	39.0	26.0
	crisps	9.0	9.0

* Made with margarine (Blueband, Unilever, Vlaardingen, the Netherlands) in the low-fat diet and with olive oil (Carbonell, Cordoba, Spain) in the oil-rich diet.

† Made with low-fat salad dressing (5 g fat/100 g) in the low-fat diet and with olive oil in the oil-rich diet.

‡ Low-fat desserts in the low-fat diet and full-fat desserts in the oil-rich diet.

§ Low-fat margarine (35 g fat/100 g) in the low-fat diet and full-fat margarine (80 g fat/100 g) in the oil-rich diet.

Table 4.2 Diet composition in the low-fat and in the unsaturated oil-rich period. In both periods 90% of energy was provided and duplicate meals were analyzed. The remaining 10% of energy was chosen from a list of low-fat food items and the composition of these was calculated

Component	low-fat diet	oil-rich diet
Energy (MJ/day)	11.4	11.3
Energy (kcal/day)	2702	2693
Carbohydrate (energy%)	59.7	37.8
Protein (energy%)	13.4	16.4
Total fat (energy%)	25.7	44.4
Saturated	10.3	15.5
Lauric acid (C12:0)	0.8	1.3
Myristic acid (C14:0)	0.9	1.5
Palmitic acid (C16:0)	5.7	7.9
Stearic acid (C18:0)	2.2	3.7
Monounsaturated, total	7.8	19.3
Polyunsaturated	6.9	8.8
Cholesterol (mg/MJ)	25.9	34.1
Cholesterol (mg/day)	294	386
Fibre (g/MJ)	2.6	2.4
Fibre (g/day)	29.5	27.1
Alcohol (en%)	1.3	1.2

Blood lipids

We took fasting blood samples on two separate days after day 22 of each diet. All 4 blood samples of each subject were analyzed in duplicate within 1 run. Total cholesterol and triacylglycerols (Cholesterol Flex™ and Triglycerides Flex™ reagent cartridge, Dade Behring, Newark USA) and HDL cholesterol (*liquid* N-geneous™ HDL-C assay, Instruchemie BV, Hilversum, the Netherlands) were measured, and LDL-cholesterol was calculated with the Friedewald-formula. The coefficient of variation of 64 duplicate measurements was 0.4% for total cholesterol, 1.5% for triacylglycerols, and 1.1% for HDL cholesterol.

Brachial artery measurements

All brachial artery measurements were done in subjects after an overnight fast. We assessed endothelial function as flow-mediated vasodilation (FMD) of the brachial artery as described elsewhere [24,25]. We measured the diameter of the artery at rest and at maximum vasodilation, and calculated the FMD as the percentage increase. All measurements were done at end-diastole by the use of the R-wave of the ECG.

The ultrasound images were made with a 7.5 MHz linear array transducer of an Ultramark™ 9 HDI duplex scanner. All images were stored on super-VHS videotapes for off-line analysis. All measurements were done by one technician in a temperature-controlled room (range 20°C to 24°C). Subjects were lying down with the right arm in two arm support cushions. An inflatable cuff was placed around the forearm. The measurements were done at the site of the antecubital crease. The position of the transducer was held constant during the measurements with a specially developed transducer arm fixature (TAF® method developed by Meijer et al, Vascular Imaging Center, The Julius Center for Patient Oriented Research, UMC Utrecht, the Netherlands).

We first obtained an optimal two-dimensional B-mode ultrasound image of the brachial artery at rest. The search was for a good trailing edge of the adventitia interface of the near wall and a leading edge of the media-adventitia interface of the far wall of the artery. Three optimal images were frozen at the R-wave of the ECG, at end-diastole, and stored on videotape. These images were used to calculate the resting diameter of the artery. We then inflated the cuff to 250 mmHg and kept this pressure constant for 5 minutes to induce ischemia in the forearm and hand. After 5 minutes the cuff was deflated. The image of the brachial artery was again optimised and changes in the diameter of the artery were recorded during the next 5 minutes. Every 15 seconds a frozen image was stored on videotape. At the end of the second feeding period we also measured endothelium-independent vasodilatation after a sublingual dose of 400 μg of nitroglycerin.

All images were read at the Vascular Imaging Center of the University Medical Center in Utrecht by one reader who was blinded to the treatment. The reader rated the quality of the images as class 1 (perfect), class 2 (fair), class 3 (marginal) to class 4 (unfit for use). All 34 subjects were measured twice on both diets, so we had 4 measurements per subject. Of these 136 measurements, 16 were rated as marginal and 3 as unfit. We only used measurements rated perfect or fair, which left us with 32 subjects for whom we had observations on both diets. At a mean FMD of 4.5%-units, the within-subjects SD was 2.9%-units so the corresponding coefficient of variation was 65%. The biggest difference between duplicate FMD-measurements was 9%-units (measurements: -8.05 and 0.95%-units); the smallest difference 0.01%-units (measurements: 2.83 and 2.82 %-units). The coefficient of variation of the diameter of the brachial artery at rest was 6.9%.

Serum homocysteine

Total homocysteine concentrations in serum were measured with high-performance liquid chromatography and fluorimetric detection[26,27]. The coefficient of variation was 3.2% within and 8% between runs.

Statistics

We averaged the duplicate measurements in each dietary period and then calculated for each subject the difference between treatments. We tested whether these differences were significantly different from zero by the Student *t* test for paired samples. We give two-sided 95% confidence intervals for the differences. All statistical analyses were performed with the SAS System for Windows (SAS Institute Inc., Cary, NC, USA), release 6.12.

We used Bayesian statistics to combine existing evidence for a protective effect of HDL cholesterol with the present data. The existing evidence was used to postulate a prior probability (i.e. *before* the present study) for a direct effect of HDL cholesterol on flow-mediated vasodilation. We postulated a prior probability of 75%. The effect-size was estimated from data of our previous study (**Chapter 3**): we hypothesised that flow-mediated vasodilation would be 1 %-unit lower on the low-fat diet than on the high-oil diet. The rationale behind this hypothesis was that in the previous study a decrease in HDL cholesterol of 0.36 mmol/L went together with a decrease in FMD of 1.8 %-units. In the present study we expected to see a decrease in HDL cholesterol of 0.20 mmol/L on the low-fat diet and therefore a decrease in FMD of 1 %-unit (1/0.2=1.8/0.36). We used the Bayes factor, which was derived from the p-value of the Student *t* test, to evaluate whether the data from the present study changed the prior probability [28].

Results

All results refer to 11 men and 21 women for whom data were complete (data for 2 men were incomplete). They had a mean age of 26.8±12.8 (SD) years, a mean pre-study weight of 68.5±8.6 kg, and a mean body mass index of 22.1±2.2 kg/m^2.

Body weight and food intake

Body weight was fairly constant during the study and hardly differed between the two diet periods: the average body weight was 68.7±8.7 kg after the oil-rich diet and 68.6±8.7 kg after the low-fat diet. On average subjects consumed 10.2 MJ/d of the experimental diets that were provided by us. They consumed an additional 1.1 MJ/d of free choice low-fat food items.

Blood lipids

Serum HDL cholesterol was 0.21 mmol/L (8.1 mg/dL) lower after the low-fat diet than after the oil-rich diet (95% CI, -0.26 to –0.17 mmol/L). Serum total cholesterol was 0.14 mmol/L lower after the low-fat diet than after the oil-rich diet (95% CI, –0.27 to –0.01 mmol/L). In contrast, serum triacylglycerols were 0.22 mmol/L higher after the low-fat diet than after the oil-rich diet (95% CI, 0.12 to 0.32 mmol/L). Serum LDL-cholesterol remained stable (**Table 4.3**).

The order of the two diets hardly affected the change in HDL cholesterol: the mean change was -0.23±0.14 mmol/L in subjects who changed from the low-fat to the oil-rich diet and 0.20±0.13 mmol/L in subjects who received the diets in the reverse order.

Table 4.3 Concentration of serum lipids (mmol/L) after consumption of the two diets

	oil-rich diet		low-fat diet		Difference (95% CI)
	mean	SD	mean	SD	
total cholesterol	4.48	0.87	4.34	0.84	–0.14 (–0.27 to –0.01)
HDL cholesterol	1.66	0.39	1.44	0.35	–0.21 (–0.26 to –0.17)
LDL cholesterol	2.45	0.65	2.42	0.67	–0.03 (–0.12 to 0.07)
triacylglycerols	0.81	0.41	1.03	0.52	0.22 (0.12 to 0.32)

Values are means±SD. The 32 subjects consumed both diets for 3½ weeks in random order. To convert values for total, HDL, and LDL cholesterol to milligrams per deciliter, multiply by 38.67. To convert triacylglycerols to milligrams per deciliter, multiply by 88.54

Table 4.4 Brachial artery measurements after both diets. None of the differences between the diets was statistically significant ($P<0.05$)

	oil-rich diet		low-fat diet	
	mean	SD	mean	SD
resting diameter (mm)	3.91	0.68	3.95	0.55
maximum diameter (mm)	4.07	0.69	4.13	0.55
absolute vasodilation (mm)	0.16	0.10	0.18	0.10
flow-mediated vasodilation (%)*	4.13	2.72	4.80	2.94
endothelium-independent dilation (%)	11.9	7.3	10.0	5.1
systolic blood pressure (mm Hg)	121.9	11.2	120.4	11.5
diastolic blood pressure (mm Hg)	72.2	9.1	71.0	7.4

* calculated for each subject as absolute vasodilation divided by resting diameter ×100%.

Table 4.5 Change in prior probabilities, ranging from weak to strong, to posterior probabilities using data of the present study. Prior probabilities were first converted to prior odds. The prior odds were then multiplied by the Bayes factor to obtain a posterior odds. Finally, the posterior odds were converted to posterior probabilities

prior probability	prior odds	posterior odds	posterior probability
0.75 (strong)	0.75/(1–0.75) = 3	3× Bayes factor* = 0.12	0.12/(1+0.12) = 0.11
0.50 (equivocal)	0.50/(1–0.50) = 1	1× Bayes factor = 0.042	0.042/(1+0.042) = 0.04
0.25 (weak)	0.25/(1–0.25) = 0.33	0.33× Bayes factor = 0.014	0.014/(1+0.014) = 0.014

* Bayes factor = e to the power $z^2/2$, where z is the z-score of the p-value for obtaining a result as large as +0.67 %-units under the hypothesis that the result would be -1.0 %-units. P-value = 0.0119, z-score = 2.52

Brachial artery measurements

The resting and maximum diameter of the brachial artery were hardly affected by the type of diet (**Table 4.4**). Flow-mediated vasodilatation was slightly better after the low-fat diet than after the oil-rich diet: 4.8±2.9% versus 4.13±2.7% (P=0.29), which was a difference of -0.67 %-units (95% CI, –1.94 to 0.61). Subjects who changed from the oil-rich diet to the low-fat diet showed a bigger change in FMD (1.26 %-units) than subjects who received the diets in reversed order (0.08 %-units).

All subjects showed vasodilation after nitroglycerin (range 1.1 to 26.4%), indicating that their smooth muscle cells were able to respond to nitric oxide. The type of diet had hardly any effect on nitroglycerin-mediated vasodilation, which was 10.0±5.1% after the low-fat diet and 11.9±7.3% after the oil-rich diet.

Serum homocysteine measurements

Serum homocysteine concentrations were not affected by the difference in the two diets: concentrations after the low-fat diet were 10.0±2.5 μmol/L and after the oil-rich diet 10.1±2.7 μmol/L (difference 0.2 μmol/L, 95% CI, -0.3 to 0.6 μmol/L).

Bayesian interpretation

Before the study we postulated that flow-mediated vasodilation would be 1 %-unit lower after the low-fat diet than after the oil-rich diet. We gave this hypothesis a prior probability of 75%, which corresponds with a prior odds of 0.75/(1-0.75)=3. From our data we calculated that the probability P of finding an effect of -0.67 %-units under this hypothesis was 0.29. A P-value of 0.29 corresponds with a z-score of 2.52 and a minimum Bayes-factor of 0.04. This Bayes-factor was used to correct the prior odds into a posterior odds by multiplication. Thus, the posterior odds for the hypothesis was 0.04×3=0.12, which corresponded with a posterior probability of the hypothesis of 0.12/(1+0.12)=0.11 or 11% (**Table 4.5**). Consequently, smaller prior probabilities of 50% or 25% corresponded with even smaller posterior probabilities.

Discussion

We found that a change in HDL cholesterol induced by two different diets, one low in fat and one high in oil, did not change flow-mediated vasodilation, one of the markers of endothelial function. This suggests that the reduction in HDL cholesterol by a low-fat, high-carbohydrate diet does not have an adverse effect on vascular functioning in individuals of the type studied here.

Does a reduction in HDL cholesterol impair endothelial function? From prior to posterior probability

We expected to find a smaller flow-mediated vasodilatation after the low-fat diet than after the high-oil diet. We based this on data of our previous study and on data of studies of others. In our previous study, a decrease in serum HDL cholesterol of 0.36 mmol/L went together with a decrease in FMD of 1.8%-units. We designed the diets in the present study in such a way that a difference in HDL cholesterol of 0.20 mmol/L could be expected, and thus a difference in FMD of 0.20/0.36 ×1.8%-units or 1%-units. This expectation is based on a positive, linear relation between HDL cholesterol and endothelial function. Indeed, many[18,29-34] but not all[35] cross-sectional studies showed a positive relation between serum HDL cholesterol and endothelial function. Another reason why we expected to see a decrease in endothelial function after a decrease in HDL cholesterol is that other studies showed changes in endothelial function when risk factors for CVD were changed. For example, lowering of elevated homocysteine by folic acid improved endothelial function after 6 weeks[36]. Also lowering of LDL-cholesterol by statins[37-41] or diet and cholestyramine[42] was shown to improve endothelial function. Based on these previous studies, we hypothesised that a predicted decrease in serum HDL cholesterol of 0.2 mmol/L would lower FMD by at least 1 %-unit. We gave this hypothesis a prior probability of 75%, but evidently different prior probabilities may be postulated (**Table 4.5**). Based on our data, the hypothesis that a diet low in fat would decrease flow-mediated vasodilatation by the postulated amount became less likely; the posterior probability was only 11%. Moreover, a recent study in Australian men and women showed that a low-fat diet decreased serum HDL cholesterol but did not affect arterial elasticity when compared to a diet high in monounsaturated fats[43].

We did not measure HDL composition or particle size. However, it is possible that different diet-induced decreases in HDL cholesterol have different effects on HDL composition or particle size. Indeed, studies in which fat was replaced by carbohydrates show a change in the composition of HDL particles, with a larger decrease in the antiatherogenic HDL2 subfraction than in the HDL3 subfraction[44,45]. In contrast, replacement of saturated fat by trans fat decreased serum HDL cholesterol without changing the composition of the HDL particles[46] and with only a slight decrease in apolipoprotein A-I [47,48]. However, these differences point at a more atherogenic change in HDL induced by a low-fat diet than by a diet rich in *trans* fatty acids, and this is not reflected in the changes in flow-mediated vasodilation.

Other factors in the diets that might have affected endothelial function

The goal of the two study diets was to achieve a difference in HDL cholesterol while keeping the diets as equal as possible. Although that goal was reached, there were a number of differences between the diets that might have counteracted an effect of HDL cholesterol. First, there was a difference in fatty acid composition between the two diets because we wanted to keep serum LDL-cholesterol constant. If we had replaced 20 en% of carbohydrates with 20 en% of monounsaturated fatty acids, serum LDL-cholesterol would have decreased by 0.12 mmol/L[49]. Thus, the high-oil diet was higher in saturated fat (5% of energy) and polyunsaturated fat (2% of energy). The higher intake of saturated fat might have impaired endothelial function, but this is only suggested by short-term studies that compared high-fat with low-fat meals[50,51]. On the other hand, the higher intake of polyunsaturated fats might have improved endothelial function because these fats were shown to improve arterial compliance, although at higher intakes[52]. The mechanism by which fats might affect endothelial function is unclear, because not all studies show an impairment of endothelial function after a high-fat meal[53]. It is possible that high concentrations of triacylglycerols in serum cause the impairment because intravenous dosing of triacylglycerols results in impaired endothelial function[54]. However, others suggest that especially used fats, rich in degradation products of heated fat, may impair endothelial function[53]. Although in our study the concentration of fasting triacylglycerols in serum was higher after the low-fat diet than after the high-oil diet, it is unlikely that this had an effect on endothelial function[55] .

The two diets not only differed in fat and carbohydrate content: the intake of fruits and vegetables was also higher on the low-fat diet than on the high-oil diet. We could have kept the intake of fruits and vegetables equal on the two diets, but then the amount of starchy foods, such as potatoes, rich and pasta, would have been too bulky to be appetizing. Thus, the intake of some vitamins was different between the diets. We estimate that the daily intake of folate from fruits and vegetables was 25-50 μg higher from the low-fat diet than from the high-oil diet[56]. Consequently[56,57], serum homocysteine concentrations were slightly (0.2 μmol/L) lower after the low-fat diet than after the high-oil diet. This decrease was probably too small to have improved flow-mediated vasodilation[36,58]. Another difference between the two diets was vitamin C: the low-fat diet contained about 30 mg per day more vitamin C than the high-oil diet. This difference is unlikely to have had an effect on endothelial function because studies that showed an effect of vitamin C used amounts of 500-1000 mg/d[59-62]. In contrast to vitamin C and folic acid, which were higher on the low-fat diet, vitamin E intake was higher on the high-oil diet, mainly because we used olive oil. However, vitamin E does not appear to have strong effects on endothelial function[63] and the difference between diets was only 10 mg/day, probably too small to have had any effect.

In conclusion, we showed that flow-mediated vasodilation, one of the markers of endothelial function, was not affected when HDL cholesterol was lowered by substituting carbohydrates for monounsaturated oil. Thus, our data provide no evidence for an adverse effect of low-fat diets on vascular functioning.

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5

Consumption of a solid fat rich in lauric acid results in a more favorable serum lipid profile in healthy volunteers than consumption of a solid fat rich in trans fatty acids

N.M. de Roos, E.G. Schouten, M.B. Katan
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ABSTRACT

Background Solid fats are used in food manufacturing to provide texture and firmness to foods. Such fats are rich in either saturated or *trans* fatty acids, which both increase risk of coronary heart disease. Epidemiological and experimental studies suggest that *trans* fatty acids increase risk more than saturates because they lower serum HDL cholesterol. However, there appear to be differences between saturates in their effect on HDL-cholesterol.

Aim We investigated whether consumption of a solid fat rich in lauric acid (C12:0) would result in a more favorable blood lipid profile than consumption of a solid fat rich in *trans* fatty acids.

Methods We fed 32 healthy volunteers 2 controlled diets in a 2×4 weeks randomized cross-over design. The diets consisted of a background diet supplemented with margarines. In the Trans-diet, 9.2% of energy was provided by *trans* fatty acids and 12.9% by saturated fatty acids. In the Sat-diet, energy intake from *trans* fatty acids was 0% and from saturated fatty acids 22.9 %. Lauric acid comprised one third of all saturates in the Sat-diet.

Results Serum HDL-cholesterol was 0.36 mmol/L lower at the end of the Trans-diet than at the end of the Sat-diet (95% CI, -0.46 to -0.26) while serum LDL-cholesterol and triglycerides remained stable. Serum total cholesterol was 0.31 mmol/L (95% CI, -0.48 to -0.14) lower at the end of the Trans-diet than at the end of the Sat-diet. Consumption of a solid fat rich in lauric acid gives a more favorable serum lipoprotein pattern than consumption of partially hydrogenated soybean oil rich in *trans* fatty acids.

Conclusion Solid fats rich in lauric acids, such as tropical fats, appear to be preferable to *trans* fats in food manufacturing where hard fats are indispensible.

Introduction

Margarines and vegetable shortenings are important sources of *trans* fatty acids in industrialized countries[1]. The intake of vegetable shortenings exceeds that of margarines and is likely to increase even further because of their use in ready-to-eat foods and for deep-fat frying. This is an undesirable development, because intake of *trans* fatty acids is related to increased risk of cardiovascular disease[2-4]. Experimental studies support this relation: many metabolic studies showed that *trans* fatty acids have an unfavorable effect on blood lipids because, like saturated fatty acids, they increase serum LDL-cholesterol[5-8]. Moreover, replacement of saturated by *trans* fatty acids was shown to decrease serum HDL-cholesterol in many studies[9-12], which suggests an additional increase in risk of cardiovascular disease[13-21]. In a response to the negative health effects of *trans* fatty acids, many European manufacturers have decreased the *trans* fatty acid content of most stick margarines to amounts of less than 2% of energy[22,23]. The *trans* fatty acid content can be decreased by reducing the degree of hydrogenation, which results in softer margarines. Alternatively, solid margarines can be produced from tropical fats instead of from partially hydrogenated vegetable oils: the amount of saturated fatty acids will then be higher but the amount of *trans* fatty acids will be negligible[22].

Studies suggest that replacement of 10% of energy from *trans* fat by saturated fat will increase serum HDL-cholesterol by 0.15 mmol/L[7,24]. However, this prediction is based on studies in which palmitic acid is the major saturated fatty acid, and some, although not all[10], studies suggest that lauric acid might increase serum HDL cholesterol more than palmitic acid[7,25]. Palm kernel fat and coconut fat are rich sources of lauric acid (USDA Nutrient Database for Standard Reference, Release 13). We investigated whether replacement of a margarine rich in *trans* fatty acids by a margarine rich in lauric acid would produce a stronger increase in serum HDL-cholesterol than the 0.15 mmol/L per 10 en% that was estimated before.

Methods

The protocol of the study was approved by the Medical Ethical Committee of Wageningen University. We gave each volunteer a written and oral presentation of the purpose and execution of the studies. Each volunteer signed an informed consent form.

Subjects

We enrolled 11 men and 21 women with a mean age of 30 years (range, 18 to 69 years) in the study. All of the volunteers were non-smokers and all were healthy as assessed by means of a medical questionnaire. Their initial serum cholesterol was 5.0 mmol/L (range, 3.0 to 7.1 mmol/L); their body mass index 22.8±2.5 kg/m^2. The volunteers had no history of any chronic illness and were not taking any medication known to affect blood lipid metabolism. They all completed the study.

Study design

We provided 2 controlled diets for 4 weeks each in a randomized cross-over design. One diet was rich in *trans* fatty acids (Trans) and one was diet rich in saturated fatty acids (Sat). There was no wash-out period between the two diets. The two diets were equal except for supplemental margarines, and were given in a 28-day menu cycle. The background diet consisted of conventional food items.

The margarine that was used in the diet rich in *trans* fatty acids was a blend of 70 parts of partially hydrogenated soy oil, containing 44% *trans*-C18:1 (Gouda's Glorie, Van Dijk Foods, Lopik, the Netherlands); 14 parts of a vegetable oil containing 63% linoleic acid and 23% oleic acid (Becel, Unilever, Vlaardingen, the Netherlands) and 16 parts of water (**Table 5.1**). The margarine that was used in the diet rich in saturated fat was a blend of 60 parts of palm kernel fat (Loders Croklaan, Wormerveer, the Netherlands) and 40 parts of commercially available margarine made from a blend of unhydrogenated rapeseed oil, soybean oil, sunflower oil, palm kernel fat, coconut oil, and palm oil (Blue Band, Van den Bergh BV, Rotterdam, the Netherlands) (**Table 5.1**).

Table 5.1 fatty acid composition (g/100 g fatty acid) of the margarines used in the diet rich in *trans* fatty acids and in the diet rich in saturated fatty acids

Fatty Acid	Trans margarine	Sat margarine
Saturated	30.5	63.1
Lauric acid (C12:0)	not detected	24.5
Myristic acid (C14:0)	0.1	10.2
Palmitic acid (C16:0)	10.5	17.0
Stearic acid (C18:0)	18.5	7.4
Monounsaturated	59.5	21.3
cis-Monounsaturated	18.6	20.9
trans-Monounsaturated	40.9[1]	0.3
Polyunsaturated	8.7	15.0
Linoleic acid (*cis*,*cis*-C18:2)	8.2	14.6
Unknown	1.3	0.6

[1] All *trans*-monounsaturated fatty acids were *trans*-C18:1

Both supplemental margarines were produced at NIZO Food Research (Ede, the Netherlands). The margarines were used as a spread, as shortening in bread and cookies, and as fat in sauce and gravy. They supplied 77% of total fat in the Trans-diet and 68% of total fat in the Sat-diet. The composition of the experimental diets was calculated using food composition tables[26-28]. To check the composition of the diets we collected duplicates of all meals (**Table 5.2**). The analyzed values were similar to the calculated composition.

We designed menus for 14 levels of energy intake, ranging from 7 to 20 MJ per day. The subjects were allocated to an energy intake level close to their habitual energy intake, which was estimated from a food frequency questionnaire. We provided 90 percent of energy; all food was weighed out for each subject. The remaining 10 percent of energy had to be chosen from a list of low-fat food items. Subjects recorded their choice from this low-fat food list in a diary. Monday through Friday of each week subjects ate a hot meal under our supervision. All other foods (bread; margarine; meat and/or cheese; honey, jam, or sprinkles; fruit; milk and/or yogurt) were packaged for consumption at home, as was food for the weekends. They received the diets for 21-32 days (mean 27.5 days).

We weighed the subjects twice a week and increased or decreased their energy intakes as needed to maintain stable body weights.

Table 5.2 Analyzed composition of the two experimental diets

Fatty Acid	Trans-diet	Sat-diet
Carbohydrate (energy%)	48.6	45.6
Protein (energy%)	14.0	13.5
Total fat (energy%)	37.4	41.0
Saturated	12.9	22.9
Lauric acid (C12:0)	0.3	6.8
Myristic acid (C14:0)	0.8	3.8
Palmitic acid (C16:0)	5.7	7.8
Stearic acid (C18:0)	5.3	3.1
Monounsaturated, total	18.2	8.8
cis-C18:1	8.4	7.9
trans-C18:1	9.2	0.3
total *trans*	9.4	0.4
Polyunsaturated	4.7	6.9
Linoleic acid (*cis,cis*-C18:2)	4.1	5.9
Linolenic acid (*cis,cis,cis*-C18:3)	0.3	0.7
Cholesterol (mg/MJ)	27.0	26.8
Cholesterol (mg/day)	248.4	253.5
Fibre (g/MJ)	3.2	3.1
Fibre (g/day)	29.4	29.3
Energy (MJ/day)	9.20	9.46
Energy (kcal/day)	2199	2261

Biochemical analysis

We took blood samples on two separate days after day 19 of each diet. All 4 blood samples of each subject were analyzed in duplicate within 1 run. Total cholesterol and triglycerides (Cholesterol Flex™ and Triglycerides Flex™ reagent cartridge, Dade Behring, Newark USA) and HDL cholesterol (*liquid* N-geneous™ HDL-C assay, Instruchemie BV, Hilversum, the Netherlands) were measured, and LDL-cholesterol was calculated with the Friedewald-formula. The coefficient of variation of 64 duplicate measurements was 0.4% for total cholesterol, 1.5% for triglycerides, and 1.1% for HDL cholesterol.

The fatty acid composition of the margarines and the experimental diets was analyzed by gas-liquid chromatography (GLC) of the fatty acid methyl esters (FAME) (Metcalfe 1966) and, for 18 carbon *trans* fatty acids, by GLC of fatty acid 4,4-dimethyloxazoline (DMOX) derivatives[29].

Statistics

We averaged the duplicate measurements in each dietary period and then calculated for each subject the difference between diets. We tested whether these differences were significantly different from zero by the Student *t* test for paired samples. We give two-sided 95% confidence intervals for the differences.

Results

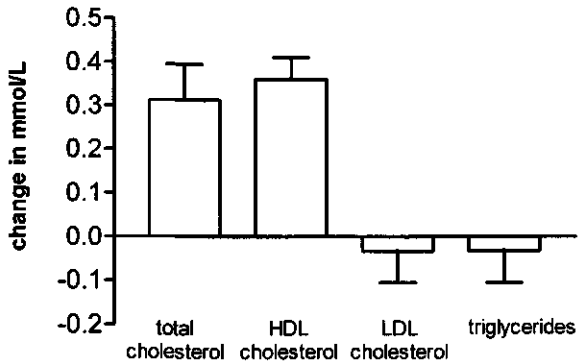
Body weight and compliance to study protocol

Changes in body weight during the study were small and non-significant: -0.4±0.9 kg during the Trans-diet and -0.6±0.9 kg during the Sat-diet (P value for difference between diets >0.5). We checked the diaries for deviations from the study protocol but only minor deviations, unlikely to interfere with the results of the study, had been reported.

Blood lipids

Total cholesterol was 0.31 mmol/L (95% CI, 0.14 to 0.48 mmol/L, *P*=0.0007) or 12.0 mg/dL lower at the end of the Trans-diet period than at the end of the Sat-diet period (**Figure 5.1**). This difference was mainly due to a difference in HDL-cholesterol, which was 1.89±0.46 mmol/L (73.1±17.8 mg/dL) at the end of the Sat-diet and 1.46±0.33 mmol/L (56.5±12.8 mg/dL) at the end of the Trans-diet, a difference of 0.36 mmol/L (95% CI, 0.26 to 0.46, P<0.0001) (**Figure 5.1**). Serum LDL-cholesterol did not differ between the diets and was 3.05±0.81 mmol/L at the end of the Sat-diet and 3.04±0.80 mmol/L at the end of the Trans-diet (*P*=0.64). Serum triglycerides were slightly lower at the end of the Sat-diet, with a concentration of 0.90±0.36 mmol/L, than at the end of the Trans-diet, when the concentration was 0.98±0.41 mmol/L (*P*=0.66) (**Figure 5.1**).

Figure 5.1:
Differences in fasting lipoproteins and lipids in 32 fasting subjects when 9.2 en% of *trans* fatty acids in the diet were replaced by saturated fatty acids. Bars represent means with SEM. To convert values for total, HDL, and LDL cholesterol to mg/dL, multiply by 38.67. To convert triglycerides to mg/dL, multiply by 88.54



The order of the two diets hardly affected the change in HDL cholesterol: the mean change was 0.33 ±0.26 mmol/L in subjects who went from the Trans-diet to the Sat-diet and 0.39±0.31 mmol/L in the subjects who received the diets in the reverse order (P=0.5).

The LDL:HDL ratio was significantly higher after the Trans diet, with a ratio of 2.2, than after the Sat diet, when the ratio was 1.8 (difference –0.41, 95% CI –0.54 to –0.27, P<0.0001).

Discussion

We investigated whether replacement of a margarine rich in *trans* fatty acids by a margarine rich in lauric acid would produce a stronger increase in serum HDL-cholesterol than the 0.15 mmol/L per 10 en% that was estimated before[30]. Indeed, serum HDL-cholesterol was 0.36 mmol/L higher at the end of a 4-week period in which 9.2% of energy was provided by *trans* fatty acids and 12.9% by saturated fatty acids than after a 4-week period in which energy intake from *trans* fatty acids was 0% and from saturated fatty acids 22.9 %. Although the diets did not provide equal amounts of polyunsaturated fatty acids (a difference of 2.2% energy), this difference could only account for about 0.015 mmol/L in HDL cholesterol, using the Mensink and Katan (1992) equation. Thus, lauric acid or C12:0 appears to increase serum HDL-cholesterol more than myristic or C16:0 and palmitic acid or C14:0[7,12,25]. The major *trans* fatty acids in our study were the n-10, n-9, and n-11 isomers of C18:1, as is usual in partially hydrogenated soybean oil. These *trans* fatty acids were also the major *trans* fatty acids that used in many other studies[5,6,11,31].

The difference in serum HDL-cholesterol at the end of the two diets is in line with results of epidemiological studies that suggest that risk of cardiovascular disease is increased more by consumption of *trans* fatty acids than by consumption of saturated fatty acids[1]. For example, in the Health Professionals follow-up study the multivariate relative risk for myocardial infarction was 1.12 (95% CI, 0.97 to 1.28) for each 5% increase in intake of saturated fatty acids and 1.36 (95% CI, 1.03 to 1.81) for each 2% increase in intake of *trans* fatty acids[32]. In the Nurses' Health Study, intake of foods rich in *trans* fatty acids, such as margarines, was also significantly

associated with higher risk of coronary heart disease[2]. In the same study, each increase of 5% of energy intake from saturated fat was associated with a multivariate relative risk of 1.17 (95% CI, 0.97 to 1.41) of coronary heart disease. This was less than the relative risk associated with a 2% increase of energy intake from *trans* fatty acids, which was 1.93 (95% CI, 1.43 to 2.61) [4]. These studies show that the intake of saturated and especially *trans* unsaturated fatty acids should be reduced in order to reduce the risk of coronary heart disease. Moreover, we found that the LDL:HDL ratio was significantly higher after the diet rich in *trans* fatty acids than after the diet rich in saturated fatty acids, indicating a higher risk of coronary heart disease.

The consumption of saturated fat and *trans* fat should not be encouraged. However, in products that need solid fats for their texture or firmness, replacement of *trans* fat by solid, tropical fats rich in lauric acids appears to be prudent.

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6

Replacement of dietary saturated fat with *trans* fat reduces serum paraoxonase activity in healthy men and women

N.M. de Roos, E.G. Schouten, L.M. Scheek, A. van Tol, M.B. Katan
Submitted

ABSTRACT

Background A high intake of saturated fat and of *trans* isomers of unsaturated fat is associated with increased risk of cardiovascular disease.
Methods Recently we found that replacement of saturated fat by *trans* fat in a dietary controlled study with 32 volunteers decreased serum HDL cholesterol and impaired endothelial function, suggesting that *trans* fats have stronger adverse effects than saturated fats. To investigate this further, we measured the activity of paraoxonase (PON1) in serum after both diets, because PON1 protects lipoproteins from oxidative damage and higher PON1 activity appears to be related to lower cardiovascular disease risk.
Results PON1 activity was 195.9 U/L after 4 weeks of a diet with 22.9% of energy (en%) from saturated fat and decreased to 184.5 U/L when 9.3 en% from saturated fat was replaced by *trans* fat (P=0.006).
Conclusion Replacement of dietary saturated fat by *trans* fat not only decreased serum HDL cholesterol and impaired endothelial function, but also decreased the activity of serum paraoxonase.

Introduction

Intake of *trans* and saturated fatty acids is associated with increased risk of cardiovascular disease[1], probably because of their effects on serum lipoproteins. *Trans* fatty acids increase serum low-density lipoprotein (LDL) cholesterol to a similar extent as saturated fatty acids. In addition, they decrease serum high-density lipoprotein (HDL) cholesterol[2-9], resulting in a undesirably high LDL:HDL ratio.

In a recent study[10] we compared the effects of saturated and *trans* fatty acids on serum HDL-cholesterol and endothelial function in a controlled dietary intervention. Endothelial function was assessed as flow-mediated vasodilation of the brachial artery, a non-invasive marker for coronary artery disease risk[11]. We found that serum HDL-cholesterol was 20% lower on the diet rich in *trans* fatty acids and that flow-mediated vasodilation was impaired. Serum concentrations of LDL-cholesterol and triglycerides remained constant. It is probable that the decrease in HDL-cholesterol paralleled a decrease in the concentration of high-density lipoproteins in serum, because *trans* fatty acids have also been shown to decrease the concentration of apolipoprotein A-I, the major protein of HDL, in serum[4,8]. HDL can inhibit the formation of oxidized LDL in vitro[12-15], and LDL isolated from subjects with low HDL concentrations appears to be more susceptible to oxidation than LDL isolated from subjects with high HDL concentrations in serum[16]. In subjects whose LDL is more susceptible to oxidation, endothelial function appears to be impaired[16]. Thus, a decrease in HDL might result in impaired endothelial function through its antioxidant effect on LDL.

The antioxidant capacity of HDL is thought to be largely[17-19] although not entirely[15] effected by paraoxonase-1 (PON1), an esterase which is closely attached to the HDL-particle. PON1 was shown to catalyze hydrolysis of lipid peroxides in oxidized lipoproteins[17]. The activity of serum PON1 is genetically determined[19], but can be influenced by changes in the diet[20-23] and smoking[24]. There are several indications that serum PON1 activity is associated with cardiovascular disease. First, PON1-knockout mice are more susceptible to atherosclerosis than their wild type littermates[25]. This is in accordance with several epidemiological studies showing that cardiovascular disease patients have lower serum PON1 activity or concentrations than healthy controls[19]. In addition, a transgenic mouse model carrying the human PON1 gene showed higher PON1-concentrations and less atherosclerosis than the wild type after feeding both types of mice an atherogenic diet for 16 weeks[26]. There is also some evidence that PON1 polymorphisms are associated with cardiovascular disease[19]. For example, a prospective study among participants in the Kuopio ischemic heart disease risk factor study showed that men who were MM homozygous for the Met54Leu polymorphism had a more than threefold risk of a first myocardial infarction than men who were LL homozygous[27].

We hypothesized that a diet rich in *trans* fatty acids that lowers serum HDL-cholesterol would also decrease PON1 activity in serum. We tested this in the same study for which we previously reported serum lipoproteins and flow-mediated vasodilation (**Chapter 3**).

Subjects and Methods

The study was approved by the Medical Ethics Committee of Wageningen University. Each volunteer signed an informed consent form after oral and written explanation of the study.

Design

We performed a dietary controlled study with a 2×4 weeks cross-over design. Subjects were 11 men and 21 women aged 18 to 69 years who were free of cardiovascular or kidney disease and who were not taking any drugs known to alter lipid metabolism. At baseline their mean fasting serum cholesterol was 5.1±1.1 mmol/L and triacylglycerols 1.3±0.6 mmol/L. Serum lipids, flow-mediated vasodilation, and serum paraoxonase activity were measured at the end of the 4-week diet periods. Other details of the study may be found elsewhere (**Chapter 3**).

Diets

The two controlled diets consisted of regular foods supplemented with special margarine. We used the margarine to obtain a difference in *trans* and saturated fatty acids of about 10% of energy. We collected duplicate diets for analysis of macronutrients. The diet rich in *trans* fat contained 48.6 en% of carbohydrates, 14.0 en% of protein, and 37.4 en% of fat, with 9.3 en% from *trans* fatty acids and 12.9 en% from saturated fatty acids. The diet rich in saturated fat contained 45.6 en% of carbohydrates, 13.5 en% of protein, and 41.0 en% of fat, with 0.3 en% from *trans* fatty acids and 22.9 en% from saturated fatty acids. Body weight was kept stable throughout the study.

Analyses

Blood was collected after an overnight fast in evacuated collection tubes (Venoject II, Terumo, Leuven, Belgium) from an antecubital vein and allowed to clot for 30 minutes at room temperature. Serum was obtained by centrifugation at 1187×*g* for 10 minutes at 4°C. To eliminate inter-assay variation, serum was stored at –75°C and all analyses were done at the end of the study. PON1 activity was determined using paraoxon as a substrate at pH 8.0 [20]. PON1 activity was expressed in U/L, which is equal to 1 μmol of hydrolyzed paraoxon/L/min.

Statistics

We used the average of 2 repeated measurements of serum HDL-cholesterol and 1 measurement of PON1 activity in serum for statistical analysis. Differences between the diets were tested for normality using the Kolmogorov-Smirnov test [28]. We give means with 95% confidence intervals of the change between the two diets. In addition we used the one-sample *t* test to test whether changes were significantly ($P<0.05$) different from zero and we calculated the Pearson correlation coefficient between changes in HDL-cholesterol and changes in serum paraoxonase activity. We used GraphPad Prism (version 3.0 for Windows) for statistical analyses and graphs (GraphPad Software, Inc., San Diego, U.S.A.).

Results

Mean PON1 activity was 184.5 U/L on the diet rich in *trans* fatty acids and 195.9 U/L on the diet rich in saturated fatty acids (P=0.006). The difference between the two diets was 11.5 U/L (95% CI: 3.6 to 19.3) or 6%. The effect was consistent: in 26 of the 32 subjects PON1 activity was lower on the *trans* fat diet than on the saturated fat diet.

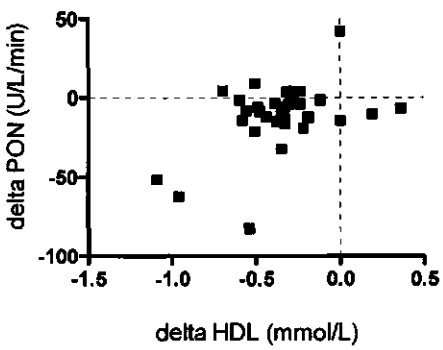
Serum HDL-cholesterol was 1.49 mmol/L (57.6 mg/dL) on the diet rich in *trans* and 1.85 mmol/L (71.5 mg/dL) on the diet rich in saturated fatty acids, while other serum lipids remained constant (**Table 6.1**). As shown in **Figure 6.1**, changes in HDL-cholesterol correlated with changes in PON1 activity (r=0.47, 95% CI: 0.15 to 0.71). We did not find a significant correlation between changes in PON1 activity and changes in the percentage flow-mediated vasodilation (r=–0.18, 95% CI: –0.5 to 0.2).

Table 6.1 Serum lipoproteins and lipids (mmol/L[1]) of the 32 volunteers at the end of the 4-week controlled diets. We show means±SD for each diet and the mean with 95% CI for the difference between the two diets

	Diet rich in saturated fat	Diet rich in *trans* fat	Difference (95% CI)
Total cholesterol	5.32 ± 0.92	5.01 ± 0.92	0.31 (0.14 to 0.48)
HDL-cholesterol	1.85 ± 0.46	1.49 ± 0.33	0.36 (0.26 to 0.46)
LDL-cholesterol	3.04 ± 0.78	3.07 ± 0.79	–0.03 (–0.18 to 0.12)
Triacylglycerols	0.94 ± 0.51	0.97 ± 0.41	–0.03 (–0.18 to 0.11)

[1] To convert mmol/L to mg/dL multiply by 38.67 for total, HDL and LDL cholesterol and multiply by 88.57 for triacylglycerols

Figure 6.1.
Correlation between changes in HDL-cholesterol and changes in paraoxonase activity in serum. Positive changes indicate higher values on the diet rich in saturated fatty acids.



Discussion

A diet rich in *trans* fatty acids resulted in a significantly lower PON1 activity towards paraoxon than consumption of a diet rich in saturated fatty acids. This lower enzyme activity might explain the impaired endothelial function we observed previously in the same population after consumption of the diet rich in *trans* fatty acids.

The size of the effect (6%) was similar to that of alcohol consumption compared to water consumption [20] and about half the difference reported between smokers and non-smokers [24]. In these studies the 'high-risk' categories (*trans* fat, water, smoking) had lower serum paraoxonase activities.

It is still unclear whether the activity of PON1 towards paraoxon is a good marker of the physiological function of the enzyme [29]. Evidence in favor of this was given by a case-control study that showed that PON1 activity towards paraoxon was significantly lower in 106 male cardiovascular disease patients than in 106 age-matched controls [30]. Also, a study of patients with familial hypercholesterolemia showed that increases in PON1 activity after simvastatin therapy resulted in lower lipid peroxide concentrations in serum [31]. Thus, an increase in the activity of serum PON1 towards paraoxon appears to reflect a physiological reduction in lipid peroxides and could therefore act as a marker of lipid peroxidation.

We were not able to show a relation between changes in paraoxonase activity and changes in flow-mediated vasodilation in our subjects. However, our study was not powered to find such a relation, and the confidence interval for the correlation coefficient was therefore wide. Others have found that serum paraoxonase activity was correlated with endothelial function in 27 patients with angiographically confirmed atherosclerosis: the percentage constriction following a serotonin provocation test was smaller in patients with higher paraoxonase activity towards paraoxon [32].

In conclusion, we showed that consumption of *trans* fatty acids, which is related to increased risk of coronary heart disease, decreased serum PON1 activity. A decreased activity of this enzyme might result in increased concentration of lipid peroxides in serum, which could impair vascular functioning. This effect could be part of the mechanism by which *trans* fatty acids exert adverse effects on coronary heart disease risk.

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7

Trans monounsaturated fatty acids and saturated fatty acids have similar effects on postprandial flow-mediated vasodilation

N.M. de Roos, E. Siebelink, M.L. Bots, A. van Tol, E.G. Schouten, M.B. Katan

ABSTRACT

Background Consumption of *trans* fatty acids is associated with increased risk of coronary heart disease (CHD). We previously showed that replacement of saturated fatty acids by *trans* fatty acids impaired flow-mediated vasodilation (FMD), a marker of CHD risk. In that study, FMD was measured in fasting subjects. We wanted to investigate whether the same impairment would be seen after a single meal.

Objective To study the postprandial effects of meals rich in saturated fatty acids or *trans* fatty acids on flow-mediated vasodilation.

Methods We fed 25 healthy men two different testmeals with 0.9-1.0 g fat/kg bodyweight: one rich in saturated fatty acids (Sat), mainly from palm kernel fat, and one rich in *trans* fatty acids (Trans) from partially hydrogenated soy bean oil.

Results FMD increased from a fasting value of 2.3±2.0% of the baseline diameter to 3.0±1.7% after the Sat-testmeal (95% CI for change –0.33, 1.70) and from 2.7±2.3% to 3.1±2.0% after the Trans-testmeal (95% CI for change –0.57, 1.29). Differences between testmeals were not significant. Serum triacylglycerols increased by 0.46±0.36 mmol/L after the Sat-testmeal and by 0.68±0.59 mmol/L after the Trans-testmeal; a difference of 0.23 (0.07, 0.39) mmol/L. Serum HDL-cholesterol was hardly affected by the testmeals. The activity of serum paraoxonase, an esterase bound to HDL, increased slightly after the two test meals but the difference between meals was not significant.

Conclusion FMD was not impaired and not different after test meals with saturated or *trans* fatty acids. Thus, differences in long-term effects of these fats are not caused by differences in acute effects on the vascular wall.

Introduction

Consumption of a diet rich in *trans* fatty acids, found in fried fast foods, bakery goods, potato chips, french fries, and solid margarines, is associated with increased risk of coronary heart disease (CHD)[1]. Pooled data from prospective follow-up studies suggest that each 2% increase in energy intake from *trans* fatty acids is associated with a 25% increase in risk[2]. This increase in risk is higher than that for a 2% increase in energy intake from saturated fats[1], although their effect on LDL-cholesterol is similar. The difference in risk could be caused by effects on HDL-cholesterol, which is decreased when saturated fats are replaced by *trans* fats[3,4]. We used metabolic studies with flow-mediated vasodilation as primary endpoint to study the causal role of HDL-cholesterol in coronary heart disease. Flow-mediated vasodilation is the percentage increase in arterial diameter after provoked increases in blood flow; impaired dilation appears to be an early stage of atherosclerosis[5]. Moreover, an impaired flow-mediated vasodilation has been related to increased risk of recurrence of cardiovascular events in 73 patients with chest pain[6].

In the first study done at our lab we showed that replacement of ≈9% of energy from saturated fat by *trans* fat decreased serum HDL-cholesterol by 0.39 mmol/L and impaired flow-mediated vasodilation (**Chapter 3**). This result was in line with the observation that *trans* fatty acids are more strongly related to cardiovascular disease than saturated fatty acids, and suggested that this was caused by the decrease in HDL-cholesterol. We verified the effect of a decrease in HDL-cholesterol in a subsequent study with a low-fat versus a high-oil diet, but this time flow-mediated vasodilation was not impaired by the decrease in HDL-cholesterol (**Chapter 4**). This result appeared to weaken the evidence for a causal role of HDL-cholesterol but we may have missed an effect, because the decrease in HDL-cholesterol was half of that observed in our first study.

We performed the present study to investigate whether differences in effect of *trans* and saturated fatty acids might be independent of HDL-cholesterol. We postulated that the increased risk with consumption of *trans* fatty acids might be due to acute effects of circulating *trans* fatty acids in serum. Indeed, it has been shown by others that consumption of fat-enriched meals impairs flow-mediated vasodilation (**Table 7.1**), but we found no data on effects of *trans* fatty acids. Therefore we compared the effects on flow-mediated vasodilation of a testmeal with *trans* fatty acids with those of a testmeal with saturated fatty acids. We also measured the activity of the HDL-associated enzyme paraoxonase, an esterase related to cardiovascular disease risk. We had found earlier that replacement of saturated fatty acids by *trans* fatty acids reduced the activity of this enzyme (**Chapter 6**). A high serum paraoxonase activity prevents oxidation of lipids in LDL in vitro[7]; if this occurs in vivo paraoxonase might improve vascular function[8]. We hypothesized that the activity of serum paraoxonase would parallel changes in flow-mediated vasodilation.

Subjects and methods

The study was approved by the Medical Ethics Committee of Wageningen University. All subjects signed an informed consent form.

Table 7.1 *Postprandial effects (3-4 h after a meal) of oral fat loads on flow-mediated* vasodilation in men (m) and women (w).

subjects	test meals	change from fasting	ref
16m	2 isocaloric meals: low-fat (5 g fat) vs high-oleic acid (50 g fat)	decrease	13
5m, 5w	2 isocaloric meals: low-fat (0 g fat) vs. high-fat (50 g fat, 14 g saturated). Low-fat ±90 mg more vitamin C	decrease	14
13	2 isocaloric high-fat fast-food meals rich in saturated and *trans* fatty acids	decrease	20
10m	1 high-fat meal (65g/m^2 body surface area) saturated fat	decrease	17
7w, 5m	1 fat tolerance test (1480 kcal, 80 g saturated fat)	decrease	31
7m, 13w	4 isocaloric meals: low-fat (0 g fat) and high-fat (50 g fat, 14 g saturated) with or without vitamins C and E	decrease, but not with added vitamins	19
10m	3 meals (milkshakes): 1 control (low-fat) and 2 with 46 g extra fat (saturated), either unused or used for deep-fat frying	no decrease after unused fat; decrease after used fat	26
12	2 isocaloric meals (1030 kcal, 61 g fat): saturated fatty acids versus monounsaturated fatty acids	no effect	30
10m	5 isocaloric meals (900 kcal, 50 g fat): olive oil (+/– vitamins C, E or vegetables), canola oil, salmon	decrease after olive oil only	23
25m, 25w	3 isocaloric meals high in fat (mainly saturated fat)	small increase	29

Subjects

We recruited 40 non-smoking men aged ≥35 y by advertising in local papers and by inviting subjects who had taken part in previous studies. We excluded 9 men because of diabetes, thrombosis, or use of cholesterol-lowering drugs. All 31 remaining subjects had normal urinary glucose and protein (<0.3 g/L) concentrations. Five men were excluded because of high serum cholesterol (>8 mmol/L) and/or fasting triacylglycerols (>3 mmol/L), because we wanted to exclude men with an impaired lipid metabolism. Of the remaining 26 subjects 25 completed the study. Pre-study mean (±SD) serum cholesterol of these 25 men was 5.8±0.8 mmol/L, triacylglycerols 1.2±0.7 mmol/L, bodyweight 83.5±10.0 kg and body mass index 25.4±2.6 kg/m^2. Their habitual diet, assessed by a validated questionnaire[9], contained 11 MJ/d (2620 kcal/d) with 40.4% of energy (%en) from fat (saturated fat 14.9 en%). Habitual daily intake of cholesterol was 274 mg/d.

Study design

The two test meals were served in random order on separate days within one week; this was repeated in reverse order the next week to obtain duplicate measurements. Thus, we had a total of four test days, separated by at least 1 d.

A test day was preceded by a controlled evening meal and an overnight fast. The evening meal was prepared by us and packaged for consumption at home. Subjects consumed on average 3047kJ with this evening meal, with 20.5en% from fat (9.5en% saturated fat), 22.9en% from protein, and 56.5en% from carbohydrates. They were asked to refrain from sports activities on the day preceeding the test day and in the morning before the measurements.

In the morning, after an overnight fast, subjects came to our lab between 7:00 and 9:30am; the appointment time was kept constant for each subject throughout the study. Some subjects came by bike, others by car; so we had everyone rest for at least 10 minutes before the start of the measurements. After subjects had rested, we measured flow-mediated vasodilation and took blood samples. Then, subjects were given the test meal which had to be consumed within 15 minutes. Postprandial measurements were done 3 h after the end of the test meal. Subjects were not allowed food or drink between test meal and postprandial measurements, and were asked to refrain from strenuous physical activities.

Test meals

The two test meals differed only in source of fat. The fat used in the saturated fat meal was derived mainly from palm kernel fat, and was rich in lauric acid (C12:0) and myristic acid (C14:0); the fat used in the *trans* fat meal was mainly partially hydrogenated soy bean oil rich in *trans* monoenes with 18 carbon atoms (**Table 7.2**).

Table 7.2 Fatty acid composition (g/100g fatty acids) of the fat rich in saturated fatty acids and the fat rich in *trans* fatty acids

	fat rich in saturated fat	fat rich in *trans* fat
	fatty acid composition[†], %	
C8:0 caprylic acid	1.9	0.0
C10:0 capric acid	2.2	0.0
C12:0 lauric acid	39.6	0.0
C14:0 myristic acid	16.3	0.1
C16:0 palmitic acid	13.3	9.5
C18:0 stearic acid	4.2	16.8
C18:1 *cis*	12.0	25.5
C18:1 *trans*[‡]	0.4	33.8
C18:2 *cis,cis* linoleic acid	8.6	11.9

[†] only those with >1% of mass in one of the test fats

[‡] mainly (n–10), (n–9), and (n–11)

Table 7.3 Macronutrient composition and vitamin content of average portions of the two test meals

	milkshake+bread (21 subjects)	porridge (4 subjects)
Energy (kJ)[·]	4947 (1178 kcal)	4103 (977 kcal)
Protein (g)	16	14
Fat (g)[†]	79	82
Carbohydrate (g)	102	49
Mono- and disaccharides (g)	59	23
Polysaccharides (g)	43	26
Fibre (g)	7	3

[·] the average portion of porridge was smaller because subjects who consumed the porridge were lighter

[†] 1% dairy fat and 99% test fats (rich in either saturated or *trans* fat)

Flow-mediated vasodilation of the brachial artery

We measured flow-mediated vasodilation of the brachial artery, a non-invasive technique using ultrasound, as set up by Celermajer et al [10]. The ultrasound images were made with a linear array transducer (range 8-14 MHz) of an Esaote AU5 scanner (Pie Medical Benelux B.V., Maastricht, the Netherlands). All images were stored on super-VHS videotapes for off-line analysis. We inflated a cuff around the forearm to a pressure of 240 mmHg to induce ischemia for 5 min. Changes in brachial artery diameter at the site of the antecubital crease were recorded for 5 minutes after cuff release. The maximum diameter that was reached within 5 minutes was used to calculate the percentage flow-mediated vasodilation from the baseline diameter. Other details can be found elsewhere (Chapter 2).

The images were analyzed with special software developed by the Wallenberg Institute of Cardiovascular Research and the Chalmers University of Technology (Gothenburg, Sweden)[11] at the Vascular Imaging Center of the University Medical Center in Utrecht. Of the 200 measurements (25 subjects measured 8 times each) 31 (16% of total) had insufficient quality due to movement of the arm or, because we had recorded a vein instead of an artery in 1 case. To calculate within-subject variability we used the 4 pre-meal measurements of each subject's resting diameter, maximum diameter, and FMD. At a mean resting diameter of 4.763 mm, the SD within-subjects was 0.311 mm and the corresponding CV 6.5%. The maximum diameter was 4.883 mm, SD within-subjects 0.330 mm and the corresponding CV 6.8%. Pre-meal FMD was 2.58% of the baseline diameter; the SD within-subjects was 2.18%-points and the corresponding CV was 84% of the mean FMD. The within-subject variability of the FMD was larger than the 50-60% in our previous studies but this was due to a smaller FMD, not to a larger SD.

Measurement of serum triacylglycerols and lipoproteins

Blood was collected in evacuated collection tubes (Venoject II, Terumo, Leuven, Belgium) from an antecubital vein and allowed to clot for 30-45 minutes at room temperature. Serum was obtained by centrifugation at 1187×g for 10 minutes at 4 °C. To eliminate inter-assay variation, serum was stored at –75 °C and all analyses were done in one assay at the end of the study.

Total cholesterol and triacylglycerols (Cholesterol Flex™ and Triglycerides Flex™ reagent cartridge, Dade Behring, Newark USA) and HDL cholesterol (liquid N-geneous™ HDL-C assay, Instruchemie BV, Hilversum, the Netherlands) were measured in one run, and LDL-cholesterol in fasting samples was calculated with the Friedewald formula. The analytical within-run coefficient of variation for all analyses was <1.5%.

Measurement of serum paraoxonase activity

Serum paraoxonase activity was measured in the serum samples of the first study week, so for each subject we had 1 pre-meal (fasting) and 1 postprandial sample per type of fat. PON1 activity was determined using paraoxon as a substrate at pH 8.0[12]. Serum paraoxonase activity was expressed in U/L, which is equal to 1 μmol of hydrolyzed paraoxon/L/min.

Statistics

We calculated for each person the mean of the 2 brachial artery measurements per type of fat. We used SAS for statistical tests and calculations (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism for graphing the data.

Results

Serum triacylglycerol concentrations were significantly increased from baseline at 3.5h after consumption of the test meals (**Table 7.4**), with a 0.23 mmol/L stronger increase after *trans* fat than after saturated fat (95% confidence interval (CI) for difference 0.07 to 0.39). The concentration of serum HDL-cholesterol increased slightly after consumption of the meal rich in saturated fat but not after the meal rich in *trans* fat; with a slight but statistically significant difference of –0.03 mmol/L between meals (95% CI, –0.06 to 0.008). The increase in serum total cholesterol was not significantly different between the two meals.

The results for the brachial artery measurements are based on 21 subjects of whom we had paired fasting and postprandial measurements for both meals. Consumption of the test meals had little effect on the baseline diameter of the brachial artery, i.e. the diameter before occlusion; differences between fasting and postprandial diameters and between the two types of fat were small (**Table 7.4**). The maximum diameter that was reached after cuff release was larger after the meals than before, and the effect was similar for both types of fat. Thus, the absolute increase in mm was larger postprandially for both types of fat, as was the percentage flow-mediated vasodilation. The type of fat did not affect any of the changes in brachial artery dimensions.

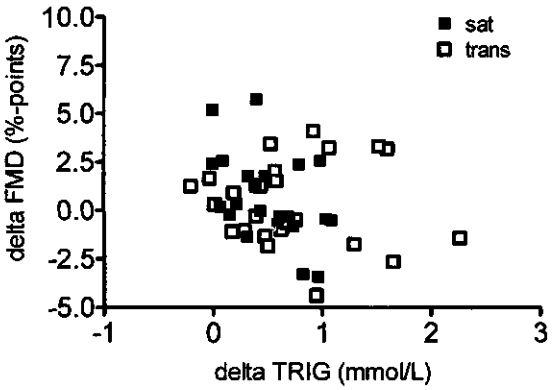
Changes in serum triacylglycerols were significantly correlated with changes in flow-mediated vasodilation after the Sat-meal (Pearson r –0.45, 95% CI, –0.73 to –0.03) but not after the Trans-meal (Pearson r –0.06, 95% CI, –0.45 to 0.35) (**Figure 7.1**).

Serum paraoxonase activity showed a large variability between subjects, with a range of 50.2-340.5 U/L. The variability within-subjects was small: at a mean fasting serum paraoxonase activity of 130.1 U/L the SD within-subjects was 5.9 U/L, corresponding with a CV of 4.5% (25 subjects, 2 fasting samples per subject). Serum paraoxonase activity was slightly higher 3.5h after the meals than fasting, with similar effects for both types of fat (**Table 7.4**).

Table 7.4 Serum lipoproteins and lipids for all 25 subjects, and brachial artery dimensions for 21 subjects, before (fasting) and 3 h after consumption of the testmeals (postprandial)

	testmeal rich in saturated fat			testmeal rich in trans fatty acids			
	fasting	post-prandial	difference (95% CI)	fasting	post-prandial	difference (95% CI)	95% CI for difference trans–sat
total cholesterol (mmol/L)	5.54	5.67	0.13 (0.08, 0.17)	5.60	5.68	0.08 (–0.02, 0.18)	–0.03 (–0.15, 0.08)
HDL-cholesterol (mmol/L)	1.33	1.35	0.02 (0.01, 0.04)	1.34	1.33	–0.01 (–0.04, 0.01)	–0.03 (–0.06, –0.01)
triacylglycerols (mmol/L)	1.23	1.69	0.46 (0.31, 0.61)	1.14	1.82	0.68 (0.44, 0.92)	0.23 (0.07, 0.39)
baseline diameter (mm)	4.783	4.776	–0.006 (–0.144, 0.130)	4.774	4.825	0.051 (–0.085, 0.186)	0.032 (–0.143, 0.208)
maximum diameter (mm)	4.890	4.916	0.027 (–0.102, 0.155)	4.902	4.972	0.072 (–0.078, 0.219)	0.025 (–0.173, 0.222)
increase (mm)	0.107	0.140	0.033 (–0.011, 0.077)	0.127	0.147	0.019 (–0.025, 0.064)	–0.008 (–0.076, 0.060)
FMD (% of baseline)	2.32	3.00	0.69 (–0.33, 1.70)	2.70	3.06	0.36 (–0.57, 1.29)	–0.0217 (–1.615, 1.181)
paraoxonase activity (U/L)	131.1	133.2	2.1 (–0.8, 5.0)	129.2	132.5	3.3 (0.3, 6.4)	1.2 (–3.1, 5.6)

Figure 7.1:
Correlation between postprandial changes in triacylglycerols and flow-mediated vasodilation (FMD) in 21 subjects after two testmeals rich in *trans* or saturated fatty acids.



Discussion

We found no difference in effect on postprandial flow-mediated vasodilation in 21 healthy men between high doses of *trans* fatty acids and saturated fatty acids. Thus, our previous observation that replacement of saturated fatty acids by *trans* fatty acids impaired flow-mediated vasodilation after 4 weeks could apparently not be explained by differences in acute effects of the fatty acids.

Postprandial effects of fats on flow-mediated vasodilation

We found a slight improvement from fasting values of flow-mediated vasodilation after the test meals. Although previous studies yielded conflicting results, most of them showed marked reductions in flow-mediated vasodilation after an oral fat load[13-15]. Studies that showed an impairment of flow-mediated vasodilation by an oral fat load used similar doses of fat (0.9-1.0 g/kg bodyweight) and study designs (measurements fasting and 3-4h postprandially) to ours (**Table 7.1**). We mixed the test fats with a milkshake, as was done by others[14,16,17]. The test meals were consumed under our supervision, and therefore non-compliance could not explain the unexpected outcome of the study. A difference with most other studies, however, is that our subjects were allowed to take either a 125 mL cup of coffee or tea (one choice throughout the whole study) with the test meals. If the corresponding dose of caffeine (60 mg for coffee and 40 mg for tea[18] had strong vasodilatory effects, up to 3 hours after consumption, this might have counteracted the effect of the test fats. However, we found no evidence for an effect of caffeine in the literature[19,20]. Thus, the fact that we did not find an impairment in flow-mediated vasodilation after a meal rich in either saturated or *trans* fat cannot be explained by differences in dose or study design.

Flow-mediated vasodilation was not impaired by the test fats in our study, but was already quite low in our subjects: the fasting values were 2.6% of the resting diameter. This value is about half of that observed in our previous studies, but those studies were done in younger subjects, and flow-mediated vasodilation appears to decline with age[21]. Ong et al found a similar

value for lean, young, healthy men[13], but most investigators reported higher values. Part of the higher values can be explained by the position of the cuff; because higher values for flow-mediated vasodilation are found when the cuff is placed around the upper arm than around the lower arm, the position we used[22].

Correlation between changes in serum triacylglycerols and flow-mediated vasodilation

In agreement with results of others[17,23] we found that changes in serum triacylglycerols showed a negative correlation with changes in flow-mediated vasodilation, although statistically significant for saturated fatty acids only. This result appears to contradict our finding that flow-mediated vasodilation was slightly higher 3h after the meals, when serum triacylglycerols were higher, than before the meals. Indeed, from **Figure 7.1** it can be estimated that a zero change in serum triacylglycerols corresponded with an increase in flow-mediated vasodilation. This would suggest that flow-mediated vasodilation improves during the morning, because the first measurement was before 9:30am and the second was 3h later. Such a diurnal variation has been reported by others, with a change in flow-mediated vasodilation from 4.0% at 8:00am to 5.3% at 12:00noon in healthy young men[24], although a different diurnal variation was found in women[25]. Maybe the increase in serum triacylglycerols in our study was too small to counteract such a diurnal variation in flow-mediated vasodilation; the increase 3.5h after the meals was moderate, with 0.46 mmol/L after the Sat-meal and 0.68 mmol/L after the Trans-meal. However, many studies that did show an effect on flow-mediated vasodilation reported similar increases in serum triacylglycerols within the same time frame[17,19,20,26].

Long-term versus postprandial effects of *trans* fats and saturated fats

We performed the present study to investigate whether *trans* fatty acids had acute effects on flow-mediated vasodilation besides the long-term adverse effects we had seen in our previous study (Chapter 3). Postprandial effects, however, were not different for saturated fatty acids and *trans* fatty acids. This is in agreement with the similar effects of *trans* fatty acids and saturated fatty acids on postprandial concentrations of serum triacylglycerols[27,28], chylomicron triacylglycerols, serum HDL-cholesterol, the activity of cholesteryl ester transfer protein[27], and Factor VII coagulant activity and activated Factor VII[28]. Moreover, we showed that postprandial changes in serum paraoxonase activity were not different between the two fats despite a 6% difference in fasting values after 4 weeks consumption of meals enriched with either of the two fats. Our hypothesis was that changes in paraoxonase activity would parallel changes in flow-mediated vasodilation. This proved to be correct, but the changes in paraoxonase activity were probably too small to have caused the changes in flow-mediated vasodilation. Thus, postprandial differences between *trans* fatty acids and saturated fatty acids appear to be small or absent.

In conclusion, we found no impairment of flow-mediated vasodilation after test meals rich in either saturated or *trans* fatty acids. It is more likely that the impairment of flow-mediated vasodilation after 4 weeks consumption of a diet rich in *trans* fatty acids was mediated by a decrease in HDL-cholesterol.

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8

General discussion

Main conclusion from the studies in this thesis

We investigated the difference in effect of *trans* fatty acids and saturated fatty acids on a marker of coronary heart disease risk. Our hypothesis was that the difference in HDL-cholesterol might be responsible for differences in risk of coronary heart disease; *trans* fatty acids reduce serum HDL-cholesterol, and low concentrations of HDL-cholesterol are related to high risk of coronary heart disease. We tested our hypothesis in diet studies using healthy volunteers. Endpoint of the studies was flow-mediated vasodilation, a marker of coronary heart disease risk.

Our main conclusion from the studies in this thesis is:

Replacement of saturated fatty acids in the diet by *trans* fatty acids reduces serum HDL-cholesterol and impairs flow-mediated vasodilation, which suggests an increased risk of cardiovascular disease. The evidence for a causal relation between changes in HDL-cholesterol and flow-mediated vasodilation, however, is inconsistent.

This conclusion implies that we are uncertain whether the decrease in HDL-cholesterol is the mechanism behind the stronger relation with cardiovascular disease of *trans* fatty acids than of saturated fatty acids.

We demonstrated, however, that replacement of saturated fatty acids by *trans* fatty acids impaired flow-mediated vasodilation. This supports the results of observational studies that intake of *trans* fatty acids relates more strongly to risk of cardiovascular disease than intake of saturated fatty acids. Moreover, this shows that adverse effects of *trans* fatty acids are not limited to serum lipoproteins, but extend to vascular reactivity. Our finding therefore supports the recommendation to limit the intake of *trans* fatty acids.

Study limitations

The effects of decreases in HDL-cholesterol on flow-mediated vasodilation were inconsistent. This is disturbing, because there are several indications that decreases in HDL-cholesterol do increase risk of coronary heart disease. Our results should therefore be examined against the background of strong evidence for a causal role of HDL-cholesterol in coronary heart disease: we might have missed an effect if flow-mediated vasodilation is not a valid risk marker.

The results of the studies described in this thesis heavily depend on the validity of the use of flow-mediated vasodilation as an outcome in diet studies. In the following paragraphs the reproducibility and several technical aspects of the use of flow-mediated vasodilation will be discussed, because differences in technique may account for some of the discordant outcomes between studies. Some other techniques for the assessment of vascular reactivity will be discussed as well. After the discussion on technical aspects, a discussion on the possible mechanisms by which diet can affect vascular function will be given. Finally, suggestions for future studies will be made.

Reproducibility of flow-mediated vasodilation

When we started this project, little was known about the variation in repeated measurements of flow-mediated vasodilation. Knowledge of this variation was crucial for choosing appropriate sample sizes for the diet studies, and therefore we started with the reproducibility study as described in Chapter 2. As turned out, the variation in repeated measurements of flow-mediated vasodilation was considerable. A large part of this variation could be explained by variation in the off-line analysis of the recorded images; the coefficient of variance for 13 measurements that were analysed twice was 30%. This analytical variation could be reduced by analysing all measurements two or more times and using the average of the analyses , as is done by some research groups[1-3].

Biological variation and variations in measurement performance can be reduced by repeated measurements per volunteer for each treatment. The measurement site could be marked, but if measurements are several days apart this is not practical. We used the antecubital crease as a marker for our measurements, because this crease is easy to find and the artery is easy to locate at this site. Biological variation and variations in measurement performance can be further reduced by standardising the protocol as much as possible, as will be discussed below.

Technical variations in the assessment of flow-mediated vasodilation

In contrast to - for example - serum cholesterol concentrations, there is no consensus on 'normal' values for flow-mediated vasodilation. Values that are considered to be an indication of coronary heart disease in one study may be considered normal in another. Some of the variation can be explained by differences in how flow-mediated vasodilation is measured. Although all non-invasive methods use high-resolution ultrasound to measure changes in the brachial artery diameter upon increased blood flow, several variations exist.

Position of the pneumatic cuff

The cuff that is used for artery occlusion can be placed around the forearm (wrist) or upper arm. When placed around the forearm, it induces ischemia distal to the antecubital fossa; the site of measurement. When placed around the upper arm, it induces ischemia in the forearm (proximal to measurement site) plus the site of measurement, and a larger area of ischemia is likely to produce a larger increase in blood flow. However, some investigators argue that ischemia of the brachial artery itself, by upper arm occlusion, might lead to abnormal endothelial function and nitric oxide release[4]. For that reason we chose forearm occlusion in our studies.

Several investigators compared the effect of the two cuff positions on flow-mediated vasodilation. Vogel et al found that placement of the cuff around the upper arm resulted in a larger blood flow and a larger flow-mediated vasodilation than placement of the cuff around the forearm (13.4±5.3% vs. 5.6±3.4% in 10 healthy subjects and 7.9±3.5% vs. 3.9±2.2% in 10 subjects with one risk factor for coronary heart disease)[5]. This was later confirmed by investigators who found a flow-mediated vasodilation of 9.0±4.8% after upper arm and 5.9±2.4% after lower arm occlusion in 16 subjects[6].

They also found a larger increase in blood flow after release of the upper arm cuff (from 2.4±0.2 to 33.1±3.1 mL/min per 100 mL) than after release of the lower arm cuff (from 2.5±0.5 to 22.8±2.2 mL/min per 100 mL). Although a difference in flow-mediated vasodilation had not been found in earlier - smaller - studies with 6 subjects (upper arm cuff 4.8%±2.7% vs forearm cuff 5.4±4.8%)[4] and 7 subjects (upper arm cuff 6.6±3.6% vs forearm cuff 8.3±4.9%)[7], it appears that cuff position may explain some of the large variation in flow-mediated vasodilation between studies.

Cuff pressure and occlusion time

An occlusion time of 4.5-5 min and a cuff pressure of at least 50 mmHg suprasystolic are commonly used to measure flow-mediated vasodilation; we used 5 min of 230-250 mmHg. Several investigators have found that longer occlusion times hardly increase vasodilation; they do however increase discomfort to the volunteers[4,8,9]. An occlusion time of 1 or 3 minutes, however, is too short for maximal vasodilation in healthy volunteers[4,7].

Cuff pressure varies between studies, but is minimally 50 mmHg suprasystolic (≈170 mmHg) and maximally 300 mmHg. We found no data on effects of graded cuff pressures on flow-mediated vasodilation, but studies in which 300 mmHg was used do not seem to produce larger values of flow-mediated vasodilation than studies that used lower cuff pressures.

Choice of 'maximum' diameter

Immediately after cuff release the brachial artery starts to dilate, to reach a maximum within 5 min[4,6,7]. To calculate the percentage dilation, some investigators use the largest diameter that is reached; others use the diameter after 60 or 90 seconds. We used the largest diameter that was reached within 5 minutes after cuff-release, but in most of our volunteers this maximum was reached within 2 minutes. Others have shown that in healthy volunteers the maximum diameter is reached after approximately 1 min of cuff release[4,6,8,10]. In other words, the diameter 60 or 90 s after cuff release will be very close to the maximum diameter, and flow-mediated vasodilation will be similar. This difference in study protocols will therefore not result in large differences in the percentage flow-mediated vasodilation.

Changes in resting (pre-occlusion) diameter in repeated measurements

The percentage flow-mediated vasodilation is calculated as the percentage increase in diameter from the resting diameter. Differences between resting diameter before a meal and after a meal complicate the interpretation of changes in flow-mediated vasodilation. For example, in a study that compared pre- with post-prandial flow-mediated vasodilation, the resting diameter changed from 3.9±0.6 mm in the fasting subjects to 4.3±0.6 mm 2h postprandially. The corresponding maximum diameters were 4.2±0.6 and 4.6±0.6 mm, resulting in %flow-mediated vasodilation of 8% and 5.9%[11]. Thus, flow-mediated vasodilation was lower after the meals than before, but this may have been due to the smaller resting diameters before the meals. The same was found by other investigators[12].

Wall-tracking versus B-mode ultrasound

In our studies all recorded longitudinal images of the brachial artery were stored on super-VHS videotapes for off-line analysis. Off-line analysis was done largely by hand: an observer marked the anterior and posterior walls over a length of at least 3 mm and a software program computed the average distance between the markings. This method is time-consuming and prone to substantial variation, as we showed in Chapter 2. However, it allows the observer to rate image quality and to detect possible movements of the arm. Another method is the wall-tracking system; a wall movement detector system that detects and images radiofrequency signals from the vessel walls. From these signals, the distance at end-diastole is calculated for 4-5 cardiac cycles. The distance is calculated for one cross-section only (M-mode), as opposed to the longitudinal section of at least 3 mm in B-mode. The reproducibility of the wall-tracking system is hardly described in the literature; a coefficient of variation of 13.9% was reported for an unknown number of volunteers and repeated measurements[13].

Transducer frequency

Ultrasound images are made with high-frequency emitting probes. Deeper structures are imaged with lower frequency probes than structures near the surface[14]. For example, 2 MHz probes are used for imaging the heart, and 13 MHz probes are used for imaging the carotid or brachial artery. For imaging of the brachial artery probes ranging from 7.5-14 MHz are commonly used. The probe frequency affects the axial resolution; the ability to resolve objects that lie one above the other. Although the axial resolution of images will be better when a higher frequency probe is used (resolution or wavelength equals velocity divided by frequency), the resolution also depends on other factors, such as the resolution of the monitor and the scan depth. In theory, a 14 MHz probe will yield a resolution of 0.11 mm (using an ultrasound velocity of 1540 m/s in soft tissue).

Measurement arm

When flow-mediated vasodilation is measured repeatedly, it is always measured at the same side of a subject or patient; either at the left or the right arm. Earlier studies have shown that blood flow response after ischemia is higher in the dominant arm than in the non-dominant arm[15,16]. It therefore makes sense to choose either the dominant or the non-dominant arm in studies that compare flow-mediated vasodilation between groups. In our studies flow-mediated vasodilation was compared within subjects, and it is unlikely that changes in flow-mediated vasodilation are different between arms. Therefore we did all measurements at the right arm, which was more practical than switching from right to left arm if a subject was left-handed. Moreover, left-handedness in adults has been shown to be less than 10% in English and German population samples[17,18], which is probably not much different from the frequency in dutch adults. Thus, the majority of our measurements was done at the dominant (right) arm.

Fasting or fed subjects: circadian rhythm and meal effects

There are some reports on a possible circadian rhythm in flow-mediated vasodilation, but whether this is due to being in a fasting or fed state or to other factors is unknown. For example, flow-mediated vasodilation in healthy young men was 4.0% at 8:00am, 5.3% at 12:00noon, 9.7% at 5:00pm, and 6.9% at 9:00pm[19]. A different diurnal variation of flow-mediated vasodilation was found in 16 young women: 3.9% at 8:00h, 3.1% at 2:00pm, 4.4% at 8:00pm, and a peak of 5.1% at 2:00am[20]. These women consumed a nitrate/nitrite-poor diet during the study periods, and refrained from drinking caffeine-containing beverages. The times, amounts, and compositions of the meals, however, were not controlled, and these might have increased the variation in flow-mediated vasodilation during the day in both studies. Thus, it is unclear to what extent this circadian rhythm interferes with study protocols.

Other measures of vascular structure and reactivity

Flow-mediated vasodilation is not the only method for measuring vascular reactivity that is used as an intermediary outcome in clinical and diet studies. Other measures - including measures of vascular structure - are intima media thickness, venous plethysmography, and several measures of systemic or coronary arterial compliance. The similarities and differences with flow-mediated vasodilation will be discussed in the following paragraphs.

Intima media thickness (IMT) is a measure of vascular wall thickness, and is commonly measured in the carotid artery, where it can be measured with external ultrasound[21]. IMT of the carotid artery increases with age, by 3-10μm/y[10,22]. IMT is positively associated with coronary events[23] and stroke[24]. Several studies have shown that flow-mediated vasodilation is smaller when intima media thickness is larger[25,26]. This may be due to increased stiffness of the carotid arteries, because the response to a sublingual dose of nitroglycerin was also less with increased intima media thickness[26]. We did not choose IMT as and endpoint for our studies because of the large number of subjects needed for an adequate sample size and the long duration of follow-up.

Forearm plethysmography is another technique for measuring flow-mediated vasodilation, based on the same principle that an increase in blood flow triggers nitric oxide release. Plethysmography does not use ultrasound but uses special sensors that detect swelling of the forearm[27]. Because the principle of the two techniques is the same, flow-mediated vasodilation as assessed by forearm plethysmography is likely to respond similarly to food or drug treatment as flow-mediated vasodilation assessed by ultrasound. Statin treatment, for example, was shown to improve flow-mediated vasodilation measured by venous occlusion plethysmography[28] and by high-resolution ultrasound[29].

Several non-invasive techniques have been developed to measure arterial stiffness, which is the reciprocal of arterial distensibility or arterial compliance. These techniques all depend on meaurements of changes in blood flow, volume, or vessel area induced by differences in blood pressure from end systole to end diastole. Like flow-mediated vasodilation, arterial compliance decreases with age[10,30]. Arterial compliance can be improved, as was shown after supplementation with flaxseed oil[31] and soy isoflavones[32]. Changes in arterial compliance/distensibility do not need to imply changes in flow-mediated vasodilation: soy

isoflavone supplementation improved systemic arterial compliance in 9 women, but did not improve acetylcholine-mediated vasodilation[32]. Moreover, no correlation was found between arterial compliance and flow-mediated vasodilation in 50 healthy men and women (r=0.10, P>0.05)[10]. We did not choose any of the compliance measurements as an endpoint, because flow-mediated vasodilation appeared to be better correlated with known risk factors for coronary heart disease.

In conclusion, flow-mediated vasodilation as assessed by ultrasound is likely to yield the same results as when assessed by venous occlusion plethysmography. Changes in flow-mediated vasodilation not necessarily need to imply changes in intima media thickness or arterial compliance. The measurement of flow-mediated vasodilation is prone to a high variation; standardisation of the technique is crucial.

Diet, lipids and flow-mediated vasodilation

Dietary modulation of endothelial function is regarded as a means to preventing cardiovascular disease[33]. Flow-mediated vasodilation is one of several assessments of endothelial function; it depends on nitric oxide release by the endothelial cells[34,35]. It is thought that effects on flow-mediated vasodilation are mediated through changes in the bioavailability of nitric oxide. As a consequence, many studies have looked at the role of antioxidant vitamins or oxidised lipoproteins[33,36,37].

It is unlikely that antioxidant vitamins played a role in our studies, because any difference in vitamin content between the diets used in our studies would have been too small to elicit an effect. Oxidisability of lipoproteins might have played a role, but we did not include markers of oxidative stress in our studies[38], so any discussion on this topic would be mere speculation. The discussion will therefore be limited to what is known about HDL-cholesterol, paraoxonase, and acute effects of fatty meals on vascular function.

Serum HDL-cholesterol and vascular function

HDL-cholesterol is positively related to flow-mediated vasodilation in some cross-sectional studies[39-43]. However, effects of changes in HDL-cholesterol on vascular reactivity have hardly been studied; they are limited to a drug trial. In this trial, 100 patients with low initial HDL-cholesterol (<1 mmol/L) but normal LDL-cholesterol (<4 mmol/L) were randomised to treatment with gemfibrozil and niacin or cholestyramin. The aim was to increase HDL-cholesterol by at least 25% and to lower LDL-cholesterol to <2.8 mmol/L. Although serum lipoprotein concentrations significantly improved in the treatment group - HDL-cholesterol increased by 0.25 mmol/L and LDL-cholesterol decreased by 0.8 mmol/L - flow-mediated vasodilation did not change[44]. Statin therapy, on the other hand, improved vascular reactivity, but hardly increased serum HDL-cholesterol in the same studies[28,45]. Thus, effects of changes in HDL-cholesterol on vascular function, as assessed by flow-mediated vasodilation, are inconsistent.

Paraoxonase and vascular function

Paraoxonase is a component of HDL. It has been shown to inhibit copper-catalysed oxidation of LDL in vitro[46]. If such an inhibition also occurs in vivo, this might have an effect on vascular function, because oxidised LDL is thought to reduce nitric oxide production and bioavailability[47]. Indeed, there are suggestions that LDL isolated from serum of subjects with low paraoxonase activity is more susceptible to oxidation than LDL from subjects with high serum paraoxonase activity[48,49]. This could lead to differences in vascular reactivity, because oxidised LDL has many in vitro effects that might affect vascular reactivity in vivo[37]. However, paraoxonase is not the only enzyme involved in the formation of oxidised LDL[50,51]. This may be one of the reasons that relations between paraoxonase activity and coronary heart disease are complex and inconsistent.

We found only minimal differences in serum paraoxonase activity induced by the diets in our studies. Whether these differences had an effect on flow-mediated vasodilation remains unknown; no other data on paraoxonase activity and vascular function were found in the literature.

Acute effects of high-fat meals

Several studies have found that dietary lipids decrease flow-mediated vasodilation, as is reviewed in the introduction of Chapter 7. The mechanism for this effect is not yet understood[52], and finding the mechanism is complicated by studies that reported no change in flow-mediated vasodilation after oral fat loads, such as our own study. In one such study, only fat that had been used for deep-fat frying caused an impairment of flow-mediated vasodilation[53]. This suggests that not the triacylglycerols, but rather their breakdown products are responsible for the impairment of flow-mediated vasodilation[52]. In contrast to that result, infusion of triacylglycerols decreased flow-mediated vasodilation within 1 h in 7 healthy volunteers. However, no control infusion was included to check whether infusion of fluids had an effect on flow-mediated vasodilation[54]. In summary, this research area has produced inconsistent results. Furthermore, it is unclear whether a disturbed flow-mediated vasodilation after an oral fat load is predictive of future cardiovascular disease.

Suggestions for future studies

Predictive value of flow-mediated vasodilation measurements

Flow-mediated vasodilation is widely used as an endpoint in clinical and diet studies. However, hard data on the value of flow-mediated vasodilation as a predictor of future coronary heart disease is lacking. At present there is no consensus on a 'normal' value for flow-mediated vasodilation; thus, it is unclear which treatment effects are physiologically relevant. Large cohort studies that relate flow-mediated vasodilation at present with future coronary heart disease could provide these data.

Effects of fats on vascular reactivity

Acute effects of high doses of fat on vascular reactivity have been inconsistent. Future studies should use stricter test meals, differing only in fat content or fatty acid composition. Dose response studies might help understand the importance of dietary fat on vascular function[52].

There is no data on the predictive value of an impaired postprandial vascular reactivity. Thus, we do not know whether it predicts the risk of future coronary heart disease. To resolve this, cohort studies that are designed to relate fasting flow-mediated vasodilation with disease could include measurements of flow-mediated vasodilation after standardised meals.

Trans fatty acids

Most of what we know about *trans* fatty acids is based on research with elaidic acid; there is little data on other isomers. Experimental diet studies with specific *trans* fatty acids may ascertain whether the deleterious effects hold for all *trans* fatty acids or only a subset. This could also resolve whether *trans* fatty acids of animal origin, e.g. vaccenic acid, have the same effects as elaidic acid.

Experimental studies with *trans* fatty acids have used doses far outside the range of ordinary intake. Epidemiological studies suggest that different populations have remarkable differences in *trans* fatty acids intake, and that adverse effects might be restricted to those populations with a high intake. Dose-response studies could provide data in which amounts of *trans* fatty acids can be correlated with changes in serum lipoproteins and thus in risk.

The mechanism by which *trans* fatty acids increase serum LDL-cholesterol and decrease HDL-cholesterol are not fully understood. Mechanistic studies, as have been done for e.g. cafestol and kawheol, the cholesterol-raising lipids in coffee, would provide insight in this mechanism.

Could *trans* fatty acids be replaced? Many tropical fats, rich in saturated fats, are solid at room temperature. They can add firmness and texture to foods just like *trans* fats. Intake of saturated fats as a whole is related to cardiovascular disease, but whether this holds for the separate fatty acids, e.g. for palmitic and lauric acids, remains to be resolved. Different fatty acids have different effects on serum lipoproteins and thrombosis tendency; it is likely that they have different effects on disease risk. Further comparison of individual saturated fatty acids in experimental diet studies might help find out which fats could be a prudent replacement of *trans* fats.

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Summary

Aim of the studies in this thesis

Consumption of *trans* fatty acids increases risk of cardiovascular disease; presumably by 25% for each additional 2% of energy from these fatty acids. This increase in risk is stronger than that of saturated fatty acids, despite similar effects on LDL-cholesterol. We investigated whether the decrease in HDL-cholesterol could explain the additional risk of cardiovascular disease with consumption of *trans* fatty acids. We used flow-mediated vasodilation, a marker of cardiovascular disease risk, as principal outcome of our studies. Flow-mediated vasodilation is the percentage increase in the diameter of an artery after a provoked local increase in blood flow. An impaired flow-mediated vasodilation appears to predict future coronary events. In all studies in this thesis flow-mediated vasodilation was measured non-invasively with high-resolution ultrasound in the brachial artery.

Reproducibility and sample size (Chapter 2)

In a pilot study with 13 volunteers measured 6 times each we found a mean flow-mediated vasodilation of 5.6% of the baseline diameter. The within-subject standard deviation was 2.8 percentage points, so the corresponding coefficient of variation was 50%. From these data we estimated that we would need 32 volunteers in future cross-over designs to detect a treatment effect of 2 percentage points with 5% probability and a power of 80%.

The TransSat-study (Chapters 3, 5, and 6)

The TransSat-study was a dietary controlled study with 32 men and women in a cross-over design with two diets. The aim of this study was to decrease serum HDL-cholesterol with *trans* fatty acids and to measure the effect on flow-mediated vasodilation. The Trans-diet provided 9.2% of energy from *trans* fatty acids, these were replaced by saturated fatty acids in the Sat-diet. After 4 weeks, serum HDL-cholesterol was 1.87 mmol/L after the Sat-diet and 1.48 mmol/L after the Trans-diet, a difference (95% CI) of 0.39 (0.28 to 0.50) mmol/L. The low HDL-cholesterol was parallelled by an impaired flow-mediated vasodilation, which was 4.4% after the Trans-diet; significantly lower than the 6.2% after the Sat-diet with a difference of 1.8%-points (0.4, 3.2). This result suggested that decreases in HDL-cholesterol impair flow-mediated vasodilation, and thereby increase risk of cardiovascular disease. However, this finding had to be confirmed.

The CarbOil-study (Chapter 4)

We sought confirmation for the impairment of flow-mediated vasodilation after a decrease in HDL-cholesterol in a subsequent cross-over study in which we lowered serum HDL-cholesterol

by replacement of monounsaturated fat by carbohydrates. Although serum HDL-cholesterol was lowered by 0.21 (0.17, 0.26) mmol/L, flow-mediated vasodilation was not impaired. Instead, flow-mediated vasodilation was 4.1% after the diet rich in monounsaturated fat and 4.8% after the diet rich in carbohydrates, a difference of 0.7%-points (-0.6, 1.9). We checked whether the carbohydrate-rich diet, with its 124g larger fruit and 80g larger vegetable content, had reduced serum homocysteine, which may have improved flow-mediated vasodilation, but this was not the case. Thus, either the decrease in serum HDL-cholesterol had been too small to elicit an effect, or HDL-cholesterol was not causally related to flow-mediated vasodilation. In that case, the effect of *trans* fatty acids on flow-mediated vasodilation may have been mediated by other factors, such as serum paraoxonase activity.

Serum paraoxonase activity (Chapter 6)

We decided to measure serum paraoxonase activity in serum samples of the TransSat- study. Paraoxonase is an HDL-bound enzyme that can hydrolyse oxidised lipids in LDL and HDL particles; and oxidised lipids may impair endothelial function as suggested by in vitro and animal studies. Moreover, serum paraoxonase activity is inversely related to risk of coronary heart disease in cross-sectional studies, and to vasoreactivity in patients with coronary heart disease. We found that serum paraoxonase activity was 196μmol/L/min after the Sat-diet and 6% (2%, 10%) lower, or 185μmol/L/min, after the Trans-diet. This difference is about half that between smokers and non-smokers, who also differ markedly in flow-mediated vasodilation. Thus, consumption of *trans* fatty acids for 4 weeks not only reduced serum HDL-cholesterol but also reduced the activity of serum paraoxonase. Whether this contributed to the impairment of flow-mediated vasodilation, however, remains to be determined. The last question we wished to answer was whether replacement of saturated fat by *trans* fat could impair flow-mediated vasodilation independent of changes in HDL-cholesterol. We did this by comparing postprandial effects of the two fats on flow-mediated vasodilation.

The Before&After-study (Chapter 7)

Based on reports by others we hypothesised that meals rich in saturated or *trans* fat would impair postprandial flow-mediated vasodilation, and based on the TransSat study we expected to see a larger effect with *trans* fat. Twenty-five men were given oral fat loads (0.9-1.0 g fat/kg bodyweight) with saturated or *trans* fatty acids, and flow-mediated vasodilation was measured before and 3 h after the fat load. In contrast to reports by others we found a vasodilation of 3.1% after the meals, which was higher than the 2.6% before the meals. Moreover, we found no differences in change in flow-mediated vasodilation between the two types of fat.

Serum paraoxonase activity parallelled the change in flow-mediated vasodilation, and was about 2% higher after the meals than before. In accordance with reports by others we found that postprandial changes in serum triacylglycerols were inversely related with changes in flow-mediated vasodilation.

Conclusion

In conclusion, we found that replacement of dietary saturated fatty acids by *trans* fatty acids for several weeks impaired flow-mediated vasodilation, a marker of cardiovascular disease risk. This effect may have been caused by the concomitant decrease in serum HDL-cholesterol, but this was not confirmed in our second diet study in which we decreased HDL-cholesterol by a low-fat diet. The effects of *trans* fatty acids on flow-mediated vasodilation appeared to be long-term only: single meals rich in *trans* fatty acids or saturated fatty acids did not act differently on postprandial flow-mediated vasodilation. Serum paraoxonase activity parallelled changes in flow-mediated vasodilation in both studies with *trans* fatty acids, but whether this is causal remains to be determined. Thus, evidence for a causal role of HDL-cholesterol in coronary heart disease remains convincing but tentative.

Samenvatting

Deze Nederlandse samenvatting is bedoeld voor de geïnteresseerde leek. Een meer beknopte samenvatting voor wetenschappers wordt gegeven in de Summary.

Wat was het doel van het onderzoek?

Dit proefschrift draait om de vraag waarom consumptie van *trans*-onverzadigd vet gepaard gaat met een hogere kans op het krijgen van hart- en vaatziekten dan consumptie van verzadigd vet. *Trans*-onverzadigd vet, vanaf hier aangeduid met transvet, vormt 2-5 procent van het vet in de Nederlandse voeding. Fast foods, snacks, koekjes en crackers zijn belangrijke bronnen van transvet in een westerse voeding. Consumptie van transvet verhoogt het gehalte aan LDL-cholesterol in het bloed, net als consumptie van verzadigd vet, en dit is een ongunstig effect. Bovendien verlaagt consumptie van transvet het gehalte aan HDL-cholesterol, een effect dat niet optreedt bij consumptie van verzadigd vet. We hebben onderzocht of deze verlaging van het HDL-cholesterolgehalte bijdraagt aan de verhoging van de kans op hart- en vaatziekten door consumptie van transvet.

Wat was de hypothese?

Onze hypothese was dat transvetzuren de kans op het ontstaan van hart- en vaatziekten meer verhogen dan verzadigd vet omdat ze het gehalte aan HDL-cholesterol in het bloed verlagen. Uit grote bevolkingsstudies is bekend dat een laag gehalte aan HDL-cholesterol in het bloed meer voorkomt bij personen met hart- en vaatziekten dan bij gezonde personen. Dit is tegengesteld aan de relatie tussen het LDL-cholesterolgehalte en hart- en vaatziekten, want hiervoor geldt juist dat een hoog gehalte vaker voorkomt bij personen met hart- en vaatziekten. Het is bekend dat een verlaging van het LDL-cholesterolgehalte in het bloed met medicijnen of een dieet de kans op hart- en vaatziekten verlaagt. Het is echter nog onbekend of een verhoging van het gehalte aan HDL-cholesterol hetzelfde effect heeft.

Hoe werd de kans op hart- en vaatziekten onderzocht?

Om nu te onderzoeken of *trans*vetzuren de kans op het ontstaan van hart- en vaatziekten verhogen door verlaging van het HDL-cholesterolgehalte hebben we een aantal voedingsexperimenten gedaan. De voedingsexperimenten duurden maximaal 8 weken. Daardoor was het onmogelijk om te onderzoeken of de proefpersonen meer of minder hart- en vaatziekten kregen; dit is immers een proces dat jaren duurt. In plaats daarvan hebben we een surrogaat eindpunt gekozen in de vorm van vaatwandfunctie. Vaatwandfunctie kan worden gedefinieerd als de mate van verwijding van een bloedvat als gevolg van een verhoogde bloeddoorstroming door dat vat.

Om de vaatwandfunctie te bepalen hebben we bij proefpersonen de diameter van de armslagader gemeten voor- en nadat de bloeddoorstroming experimenteel werd verhoogd. Het meten van de diameter gebeurt met ultra-geluidsgolven ('echogolven'); er wordt een echo gemaakt van de armslagader ter hoogte van de elleboogplooi. Vervolgens wordt de bloeddoorstroming experimenteel verhoogd: een bloeddrukband wordt om de onderarm aangebracht, opgeblazen tot de armslagader wordt afgesloten, en vervolgens, na 5 minuten, weer leeggelaten. Als reactie op de toegenomen doorstroming van de armslagader neemt de diameter toe, en bereikt een maximum binnen 5 minuten. Uit de maximale diameter en de aanvangsdiameter wordt het percentage verwijding berekend; dit is de FMD die in dit proefschrift centraal staat. FMD staat voor *flow-mediated vasodilation*, ofwel vaatverwijding door toegenomen doorstroming.

Het is bekend dat patienten met hart- en vaatziekten een slechtere vaatwandfunctie en dus een lagere FMD hebben dan gezonde mensen. Bovendien lijkt het erop dat een slechte vaatwandfunctie voorspellend is voor het optreden van hart- en vaatziekten. Ook is bekend dat vrijwel alle bekende risicofactoren voor hart- en vaatziekten samengaan met vaatwandfunctie. Een hoog serum cholesterolgehalte, roken, en suikerziekte gaan bijvoorbeeld allemaal samen met een verlaagde FMD. Tevens is bekend dat veranderingen in die risicofactoren naar meer gunstige waarden, bijvoorbeeld een verlaging van het cholesterolgehalte, een verbetering van de vaatwandfunctie tot gevolg hebben. Dit was voor ons voldoende reden om te onderzoeken hoe transvet de vaatwandfunctie beinvloedt. Een effect op de FMD zou immers aanwijzingen geven over het effect op de kans op het ontstaan van hart- en vaatziekten.

Welke onderzoeken zijn uitgevoerd?

We zijn begonnen met een schatting te maken van de dag-tot-dag variatie in vaatwandfunctie (Hoofdstuk 2). Het is nodig om die variatie te weten, want hoe groter de variatie, dus hoe meer schommeling in vaatwandfunctie, hoe meer proefpersonen er nodig zijn om veranderingen in vaatwandfunctie aan te tonen. Om de variatie te schatten hebben we bij 13 proefpersonen de vaatwandfunctie 6 maal gemeten. Uit die metingen konden we afleiden dat de gemiddelde FMD 0.225 mm was, ofwel 5.6% van de aanvangsdiameter, en dat de dag-tot-dag variatie binnen een proefpersoon 2.8% (0.115 mm) was, dus de helft van de gemiddelde FMD. Hieruit konden we berekenen dat we 32 proefpersonen nodig hadden als we een verschil van 2 FMD% als werkelijk verschil en niet als toeval wilden kwalificeren.

De TransSat-proef

In de eerste voedingsproef vergeleken we het effect op vaatwandfunctie van een voeding rijk aan transvetzuren met een voeding rijk aan verzadigde vetzuren (Hoofdstuk 3). Om die vergelijking zo zuiver mogelijk te houden werd de gehele dagvoeding aan de proefpersonen verstrekt; zowel warm eten als broodmaaltijden en tussendoortjes. De proefpersonen kregen eerst 4 weken de ene voeding te eten, daarna 4 weken de andere voeding. Het gehalte aan HDL-cholesterol in het serum was aanzienlijk verschillend aan het eind van de twee voedingen: 1.87 mmol/L na de voeding rijk aan verzadigde vetzuren en 1.48 mmol/L na de voeding rijk aan transvetzuren

(Hoofdstuk 5). Het gehalte aan LDL-cholesterol bleef constant. Onze verwachting was dat een verlaging van HDL-cholesterol door transvetzuren de vaatwandfunctie zou verminderen, en dat klopte: de FMD was 6.2% na de voeding rijk aan verzadigde vetzuren en 4.4% na de voeding rijk aan transvetzuren. Hoewel nu zichtbaar was gemaakt dat transvetzuren inderdaad een sterker effect op de kans op hart- en vaatziekten hadden dan verzadigde vetzuren, hadden we nog geen bewijs dat dat verschil in risico het gevolg was van het verschil in HDL-cholesterol. In de CarbOlie-proef probeerden we dat bewijs te leveren.

De CarbOlie-proef

We onderzochten of een tweede verandering in HDL-cholesterol, bij een nieuwe groep van proefpersonen en met andere voedingen, eveneens voor een verschil in vaatwandfunctie zou zorgen (Hoofdstuk 4). De twee voedingen die nu werden vergeleken waren een voeding rijk aan enkelvoudig verzadigde vetzuren (voornamelijk uit olijfolie) en een voeding die arm was aan vetzuren maar rijk aan koolhydraten. Net als in de eerste voedingsproef werd al de gehele dagvoeding weer verstrekt aan de proefpersonen. Zoals verwacht daalde het HDL-gehalte wanneer de voeding rijk aan koolhydraten werd gegeten; een daling van 1.66 naar 1.44 mmol/L. De vaatwandfunctie nam echter niet af, maar nam zelfs iets toe, van 4.1% naar 4.8%, hoewel dat waarschijnlijk toeval was. We konden dus niet bevestigen dat een daling van het HDL-cholesterol een vermindering van de vaatwandfunctie tot gevolg had.

Serum paraoxonase

Vervolgens hebben we geprobeerd het effect van transvetzuren op de vaatwandfunctie te verklaren uit activiteitsmetingen van het enzym paraoxonase, een enzym dat betrokken lijkt te zijn bij de antioxidantwerking van HDL (Hoofdstuk 6). Een lage activiteit van dit enzym wordt gezien bij patienten met hart- en vaatziekten en bij rokers. De voeding rijk aan transvetzuren bleek niet alleen de concentratie aan HDL-cholesterol in het bloed te verlagen, maar ook de activiteit van het paraoxonase te verminderen, en dit lijkt een ongunstig effect. Het zou kunnen betekenen dat HDL een lagere antioxidantwerking had tijdens consumptie van transvetzuren. Mogelijk had dit een ongunstig effect op vaatwandfunctie, maar dit is nog onvoldoende uitgezocht. Het is ook mogelijk dat de activiteit van serum paraoxonase een maat was voor de hoeveelheid HDL in serum, en geen causale rol speelde.

De Voor&Na-studie

Ter afsluiting leek het ons verstandig te onderzoeken of een eenmalige hoge dosis van transvetzuren de vaatwandfunctie kon verminderen, onafhankelijk van veranderingen in HDL-cholesterol (Hoofdstuk 7). Voor deze proef moesten de proefpersonen op 4 meetdagen nuchter blijven en kregen ze van ons een milkshake waaraan 60-90 gram vet was toegevoegd. Tweemaal kregen ze een milkshake rijk aan verzadigde vetzuren en tweemaal een milkshake rijk aan transvetzuren, en we bepaalden hun vaatwandfunctie voordat en 3 uur nadat ze de milkshake hadden gedronken. Het bleek dat hun vaatwandfunctie na de milkshake iets beter was dan ervoor, een effect wat tegengesteld was aan wat andere onderzoekers hadden gevonden. Bovendien was er geen verschil in effect tussen transvetzuren en verzadigde vetzuren. Hoewel

vervanging van verzadigd vet in de voeding door transvet in de TransSat-studie leidde tot een vermindering van vaatwandfunctie, was dit dus niet het geval na een enkele maaltijd met deze vetten.

Welke consequenties hebben de resultaten van de onderzoeken?

We hebben niet kunnen aantonen dat transvetzuren de kans op hart- en vaatziekten meer verhogen dan verzadigde vetzuren omdat ze het HDL-cholesterolgehalte verlagen. We hebben echter wel kunnen aantonen dat een hoge consumptie van transvetzuren gedurende enkele weken nadelige effecten heeft op de vaatwandfunctie. Een verminderde vaatwandfunctie leidt op lange termijn mogelijk tot hart- en vaatziekten, hoewel dit nog niet onomstotelijk bewezen is. Onze resultaten ondersteunen het advies om zo min mogelijk producten rijk aan transvetten te consumeren. Dit betekent vooral een beperkte consumptie van snacks, koekjes en fast foods, de belangrijkste leveranciers van transvetzuren in de westerse voeding.

Dankwoord

Het dankwoord. Als laatste geschreven en als eerste gelezen. Bedoeld om iedereen te bedanken die heeft bijgedragen aan het onderzoek, maar ook om een persoonlijke punt te zetten achter deze leerzame en unieke periode.

Die periode begon met de aanmoediging van professor Hautvast om AIO te worden. Zijn vertrouwen in mij leidde tot een opdracht van de toenmalige Voedingsraad. Onder begeleiding van Willem Bosman deed ik literatuuronderzoek; hij leerde mij vraagtekens te zetten bij ogenschijnlijk logische conclusies. Eén van de literatuuronderzoeken betrof probiotica, en toen onze afdeling werd gevraagd de cholesterolverlagende eigenschappen hiervan te onderzoeken kreeg ik mijn eerste onderzoekscontract. Sinds die tijd is Martijn Katan mijn begeleider geweest, en mijn ontzag voor hem veranderde al snel in respect en waardering. Respect voor zijn enorme kennis, heldere denken, en vermogen om de vinger op de zwakke plek te leggen. Waardering voor zijn nauwgezette nakijkwerk van manuscripten, zijn hulp bij dreigend onheil, en zijn relativerende uitspraken.

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Bij de overgang van probiotica op voedingsvetten kreeg ik niet alleen een extra jaar maar ook een extra begeleider; Evert Schouten. Naast zijn interesse in de theoretische achtergrond toonde hij ook belangstelling voor de praktische uitvoering van het onderzoek, en dat heb ik erg gewaardeerd. Met zijn rustige en sympathieke natuur had ik er een bijzonder prettige begeleider bij.

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Onderzoek bedenken is één maar uitvoeren is een heel ander verhaal. Achter elk getal in de resultaten schuilen proefpersonen die voor de wetenschap anoniem zijn maar die voor mij een gezicht hebben. Ik ben ze stuk voor stuk dankbaar voor hun medewerking en doorzettingsvermogen.

Vaatwandfunctie was nog niet eerder gemeten op de afdeling dus moest er iemand worden gevonden die zich de metingen eigen kon maken. Jan Harryvan heeft dat uitstekend gedaan. Met zijn enorme geduld, nauwgezetheid en technisch inzicht verkreeg hij in korte tijd het certificaat om vaatwandfunctiemetingen te doen. Ik ben hem dankbaar dat hij - in drie studies op een rij - elke ochtend weer om kwart over zeven klaarstond om de eerste slaperige proefpersoon te meten. Vera Fierkens en Trudy Jansen deden dat in de laatste studie, en ook hen wil ik bedanken voor hun stiptheid, inzet, en bereidheid tot het leren van nieuwe technieken. Rudy Meijer en Michiel Bots van het Julius Centrum voor Patiëntgebonden Onderzoek in Utrecht ben ik zeer erkentelijk voor hun hulp bij het opzetten en verwerken van de ultrasound-metingen, en Stevan Stuit van PieMedical in Maastricht voor zijn inspanningen om apparatuur te leveren toen de laatste proef werd verlengd.

De gecontroleerde voedingen uit de 'TransSat'proef werden samengesteld door onderzoeksdiëtiste Saskia Meyboom. Ze kon daarvoor deels teruggrijpen op beproefde receptuur, maar had toch nog heel wat rekenwerk aan de proef. En niet zonder resultaat: de door Saskia berekende voedingen kwamen precies overeen met de chemische analyses door Truus Kosmeyer van het lab. Bij de twee laatste voedingsproeven lag de verantwoordelijkheid voor het rekenwerk en 'de keuken' bij Els Siebelink: zij rustte niet voor de ideale samenstelling uit de printer rolde. Haar praktische blik op onderzoeksvoorstellen en haar strakke hand in de keuken namen mij heel wat zorgen uit handen. Ik ben dan ook blij dat ze me naast Liesbeth Zandstra als paranimf zal bijstaan. De overige diëtistes - Marieke, Herma, Kirsten, Dieneke en Irma - bleken onmisbare en gezellige hulpen in keuken en eetzaal.

Studenten bleken ook geïnteresseerd in vaatwandfunctie en zo kregen we zeven Wageningse studenten en zeven diëtetiekstudenten te begeleiden. Door het enthousiasme, de kritische vragen en de betrokkenheid van Anke, Halime, Irene, Irma, Judith, Karen, Louise, Marieke, Nadège, Rosemarijn, Sabine, 2 Saskia's en Simone kreeg het AIO-leven een menselijk gezicht met alle ups en downs die daarbij horen. Ypie Blauw, bedankt voor je steun bij de downs!

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Het ga jullie goed!

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Curriculum vitae

Nicole Michelle de Roos werd op 10 juni 1971 geboren in Rozenburg, Zuid-Holland. In 1989 slaagde ze voor het eindexamen VWO aan de Rijksscholengemeenschap Petrus Hondius in Terneuzen. In datzelfde jaar begon ze aan de studie Voeding van de Mens aan de Landbouwuniversiteit Wageningen. In 1995 rondde zij deze studie af met een doctoraalonderzoek Fysiologie en twee doctoraalonderzoeken en een stage Voedingsleer. De stage Voedingsleer werd uitgevoerd aan de Human Nutrition Unit van het Rowett Research Institute in Aberdeen, UK.

Na haar afstuderen in 1995 werkte ze een jaar als medewerker literatuuronderzoek voor de toenmalige Voedingsraad. Van 1996-1997 was ze aangesteld als toegevoegd onderzoeker op een project waarin de cholesterolverlagende werking van een probiotische yoghurt werd onderzocht. In april 1997 volgde een aanstelling als assistent in opleiding (AIO) op het project getiteld 'Gezondheidseffecten van probiotica'. Dit project resulteerde in een overzichtsartikel maar werd niet voltooid wegens de geringe verwachtingen voor wetenschappelijk resultaat bij gezonde proefpersonen. In april 1998 werd besloten een nieuw AIO-project te starten getiteld 'Voedingsvetten en endotheelfunctie' - de resultaten zijn in dit proefschrift beschreven.

Graduate School

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