Genetic disposition and response of blood lipids to diet

Studies on gene-diet interaction in humans

Rianne M. Weggemans

Promotor:
Prof. Dr M.B. Katan
Persoonlijk hoogleraar bij de Afdeling Humane Voeding & Epidemiologie
Wageningen Universiteit /
Wetenschappelijk directeur
Wageningen Centre for Food Sciences

Co-promotor:
Dr P.L. Zock
Projectleider Voeding en Gezondheid
Wageningen Centre for Food Sciences

Promotiecommissie:
Prof. Dr M.H. Hofker
Universiteit van Maastricht
Prof. Dr Ir R.P. Mensink
Universiteit van Maastricht
Prof. Dr E.G. Schouten
Katholieke Universiteit van Leuven, België / Wageningen Universiteit
Prof. Dr W.M. de Vos
Wageningen Universiteit

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Abstract

Genetic disposition and response of blood lipids to diet.
Studies on gene-diet interaction in humans.
Ph.D. thesis by Rianne M. Weggemans, Division of Human Nutrition and Epidemiology, Wageningen University, The Netherlands. January 17, 2001.

Even though a cholesterol-lowering diet is effective for most people, it is not for all. Identification of genetic determinants of the serum lipid response to diet may be of help in the identification of subjects who will not benefit from a cholesterol-lowering diet. It may also clarify the role of certain proteins in cholesterol metabolism. The *objective* of our research was to determine whether genetic polymorphisms affect the response of serum lipids to diet in humans.

We first assessed sex differences in the response of serum lipids to changes in the diet. Men had larger responses of total and low-density lipoprotein cholesterol to saturated fat and cafestol than women. There were no sex differences in the responses to *trans* fat and dietary cholesterol. We also used these data to study the effect of 11 genetic polymorphisms on responses of serum lipids to the various dietary treatments. Apoprotein E, A4 347 and 360, and cholesteryl-ester transfer protein TaqIb polymorphisms affected the lipid response to diet slightly.

We further studied the effect of the apoprotein A4 360-1/2 polymorphism on response of serum lipids to dietary cholesterol in a controlled trial specially designed for this purpose. The apoprotein A4 360-1/2 polymorphism did not affect the response of serum lipids to a change in the intake of cholesterol in a group of healthy Dutch subjects who consumed a background diet high in saturated fat.

Although it is not directly related to genetic polymorphisms and lipid response, we finally reviewed the effect of dietary cholesterol on the ratio of total to high-density lipoprotein cholesterol, which is a more specific predictor of coronary heart disease than either lipid value alone. Dietary cholesterol raised the ratio of total to high-density lipoprotein cholesterol.

In conclusion, the effect of genetic polymorphisms on serum lipid response to diet is small. It is therefore not possible to identify individuals who will not benefit from a cholesterol-lowering diet on the basis of a specific genetic polymorphism.

List of abbreviations

APO	Apoprotein
CI	Confidence interval
CE	Cholesteryl esters
CETP	Cholesteryl ester transfer protein
FABP2	Intestinal fatty acids binding protein
HDL	High-density lipoprotein
IDL	Intermediate-density lipoprotein
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LRP	LDL-receptor related protein
MTP	Microsomal triglyceride transfer protein
SD	Standard deviation
SE	Standard error
TG	Triglycerides
VLDL	Very low-density lipoprotein

Contents

1

General introduction

Background

World wide, cardiovascular diseases are a substantial source of chronic disability and health costs (1). In the Netherlands, coronary heart disease is the most prevalent cardiovascular disease. During the past 20 years, death as a result of cardiovascular disease has decreased. This decrease is caused by a reduced incidence of cardiovascular disease and by improved care of patients with cardiovascular disease. However, one of the side effects is that the number of patients with cardiovascular disease has increased. In addition, the improved prognosis of coronary heart disease has increased the probability of another cardiovascular event (2). Prevention of cardiovascular disease should be targeted to the general population to postpone time of onset of disease. In addition, people with cardiovascular disease should be targeted for secondary prevention to improve quality of life.

The pathological condition that underlies coronary heart disease is atherosclerosis, which involves lesions in the arterial vessel wall. These lesions contain large amounts of lipids, a large proportion of which is cholesterol that comes from the blood. In blood, most of the cholesterol is transported in low-density lipoprotein (LDL) and a small proportion is transported in high-density lipoprotein (HDL). High concentrations of serum LDL-cholesterol are a risk factor of coronary heart disease. However, on the contrary, high concentrations of HDL-cholesterol protect against coronary heart disease (3).

Subjects who suffer from overt cardiovascular disease or who are at high risk of cardiovascular disease should be targeted for lifestyle intervention and, where appropriate, drug therapies. One of the recommended changes in lifestyle is a lipid-lowering diet. In this diet, total energy intake should comprise less than 30 % fat, total fat intake should comprise less than one third as saturated fat, and intake of dietary cholesterol should be less than 300 mg per day. The prevention goals for blood lipids are concentrations of total cholesterol less than 5.0 mmol/L and of LDL-cholesterol less than 3.0 mmol/L (4).

However, even though a lipid-lowering diet is effective for most people, it is not for all. The effect of a lipid-lowering diet is to some extent reproducible in a subject, which indicates that it may be in part an innate characteristic of a subject (5,6). Identification of genetic factors that are related to the lipid response to diet may be of help in the identification of subjects who will not benefit from a cholesterol-lowering diet. It may also clarify the role of certain proteins in cholesterol metabolism.

This introduction gives a concise overview of cholesterol metabolism and the effect of diet on serum lipids. It then discusses the role of genetic polymorphisms in determining the response of serum lipids to diet and presents a number of candidate genes that are studied in this thesis. The general objective and outline of the thesis are given at the end of this chapter.

Cholesterol metabolism

The steroid cholesterol is an essential component of the cell membrane and is a precursor in the synthesis of bile acids and steroid hormones (7). In addition to cholesterol obtained from diet, there is also de nova synthesis of cholesterol from acetate in the liver and in peripheral tissues.

As reviewed in (8), cholesterol metabolism consists of two pathways, the exogenous pathway and the endogenous pathway (Figure 1.1). The exogenous pathway concerns the transport of dietary cholesterol and triglycerides from the intestine to the liver. Dietary cholesterol and triglycerides are processed in the intestine and packaged into chylomicrons. Intestinal fatty acid binding protein (FABP2) is essential for the uptake, metabolism and/or transport of long-chain fatty acids. Microsomal triglyceride transfer protein (MTP) is essential for the assembly and secretion of chylomicrons from intestinal cells into lymph. These chylomicrons subsequently enter the circulation. The capillary vessel wall of peripheral tissues contains lipoprotein lipase (LPL). Apolipoprotein (apo) C-II, which is part of the chylomicron, activates LPL. Activated LPL hydrolyzes the triglycerides in the core of chylomicrons into free fatty acids, which subsequently enter fat and muscle cells. In this way, chylomicrons are converted into chylomicron remnants, which then pick up cholesteryl-esters from HDL (this is part of endogenous pathway). These cholesterol-enriched chylomicron remnants are efficiently cleared by LDL-receptor and LDL-receptor related protein (LRP) (9), which involves apoB48 and apoE on the surface of remnants. Cholesterol is used in a number of ways in the liver. It may be esterified and stored in liver cells or used for synthesis of cell membranes. It may also be converted into bile acids and subsequently excreted from the body.

The endogenous pathway consists of two interrelated processes. One co-ordinates movement of cholesterol and triglycerides from the liver to peripheral tissues and the other concerns their transport from peripheral tissues back to the liver. This is called the reverse cholesterol transport. As part of the former process, liver cells secrete cholesterol and triglycerides into blood in the form of very low-density lipoproteins (VLDL). MTP is essential for the assembly and secretion of VLDL. Triglycerides in VLDL are hydrolyzed by LPL, similarly to those in chylomicrons. This results in the formation of smaller intermediate density lipoproteins (IDL), which can be cleared from plasma by the liver through LDL-receptors and LRP or is converted to LDL by LPL or hepatic lipase. LDL is then either directly removed by peripheral cells or liver cells through the interaction of apoB-100 with LDL-receptors.

As part of the other process of the endogenous pathway, the reverse cholesterol transport, nascent high-density lipoprotein (HDL), which is synthesized in liver and intestinal cells, changes into HDL_3 by taking up free cholesterol from extrahepatic cells. This

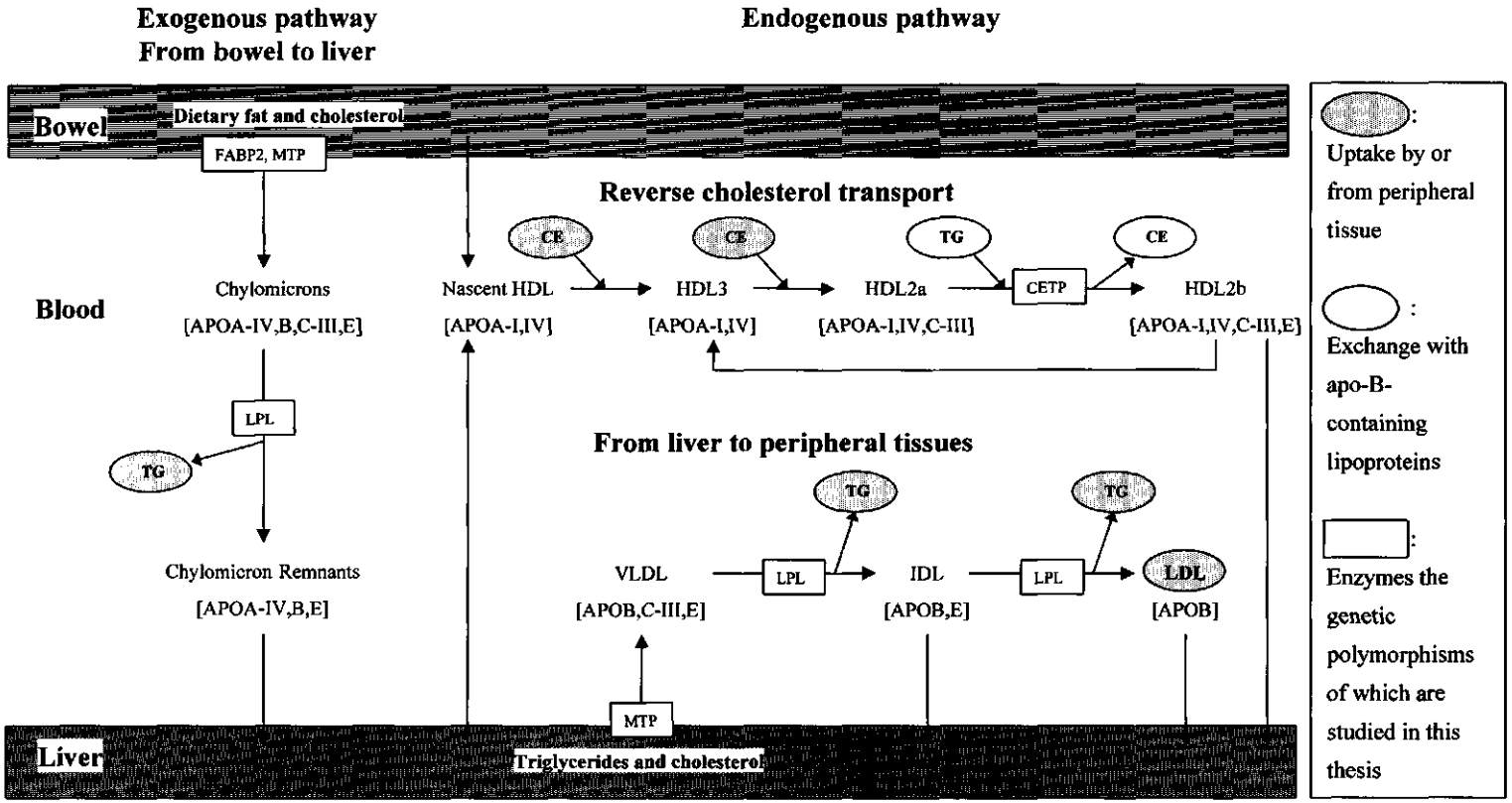

Figure 1.1 Outline of cholesterol metabolism.

Apoproteins that form lipoproteins and the genetic polymorphisms of which are studied in this thesis are shown between brackets; APO, apoprotein; CE, cholesteryl esters; CETP, cholesteryl ester transfer protein; FABP2, intestinal fatty acids binding protein; HDL high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; TG, triglycerides; VLDL very low-density lipoprotein.

Stellingen

1. Een cholesterolverlagend dieet is effectiever bij mannen dan bij vrouwen. *Dit proefschrift.*

2. De conclusie van McCombs et al (*N Eng J Med 1994; 331:706-10*) dat het apoproteïne A4 360-2 allel de cholesterolrespons op voedingscholesterol vermindert, is waarschijnlijk gebaseerd op een toevalsbevinding. *Dit proefschrift.*

3. Voor de primaire preventie van hart- en vaatziekten is het niet van belang of je regelmatig een lange duurloop doet of korte sprints, zolang het energieverbruik maar gelijk is. *Sesso et al. Circulation 2000; 102:981-6.*

4. De behandeling van essentiële hypertensie kan effectiever worden door medicatie af te stemmen op het 24-uurs ritme van de bloeddruk. *Smolanski & Portaluppi. Am Heart J 1999; 137:S14-24.*

5. Slechts 5 tot 10 % van alle vrouwen in de overgang heeft baat bij hormoonsuppletie. *Barrett-Connor. De Anatomische Les, 2000.*

6. Voor de preventie van hart- en vaatziekten zijn de meeste mensen meer gebaat bij een verandering in leefstijl (lichaamsbeweging, niet roken, goede voeding) dan bij het gebruik van vitamine - en mineralensupplementen.

7. Voor de behandeling van RSI (repetitive strain injuries, muisarm) is de verandering in fysieke werkhouding net zo belangrijk als de verandering in mentale werkhouding.

8. Het succes van vernieuwingen is afhankelijk van de mate waarin ze aansluiten bij de bestaande situatie.

Stellingen bij het proefschrift
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Rianne M. Weggemans. Wageningen, 17 januari 2001

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cholesterol is esterified by lecithin:cholesterol acyltransferase (LCAT), which uses apoA-I and apoA-IV as co-factors. The cholesteryl esters migrate to the core of the HDL_3. Further uptake of cholesterol and action of LCAT results in larger-sized and cholesteryl-rich HDL_{2a}. As HDL_{2a} becomes more enriched with cholesteryl esters, apoproteins C-II and C-III are picked up from other lipoproteins. Subsequently, cholesteryl esters are transferred to chylomicrons, VLDL, IDL, and LDL in exchange for triglycerides by the action of cholesteryl ester transfer protein (CETP). This process results in triglycerides-rich HDL_{2b} and enables the hepatic uptake of cholesteryl esters from VLDL, IDL, and LDL. HDL_{2b} can be removed from circulation by the HDL-receptor, also known as scavenger receptor B1. HDL_{2b} can also be converted into the form of HDL_3 by hepatic lipase, which hydrolyzes the triglycerides in HDL_{2b} (8,10).

Effect of diet on lipid concentrations

Diet affects lipid concentrations in the blood. At a population level, replacement of carbohydrates by saturated fat and the addition of dietary cholesterol increase concentrations of LDL- and HDL-cholesterol, whereas replacement of carbohydrates by *cis*-unsaturated fat decreases LDL-cholesterol and increases HDL-cholesterol concentrations (11-14). *Trans* fat increases LDL-cholesterol concentrations in the same way as saturated fat, whereas it decreases HDL-cholesterol concentrations as compared to saturated fat (15). In a similar way, addition of cafestol to diet increases LDL-cholesterol and slightly decreases HDL-cholesterol concentrations (16).

The precise mechanisms determining the response of serum lipids to diet are not known. The response of LDL-cholesterol to saturated fat and dietary cholesterol may be mediated in part by the number of hepatic LDL-receptors that affect the clearance of LDL-cholesterol from the blood. Down-regulation of the cholesterol synthesis in liver, increased CETP-activity, and enhanced synthesis of bile acids may also play a role in the mechanism that determines this lipid response (17,18). The most plausible mechanism by which cafestol affects LDL-cholesterol concentrations is through the sterol regulatory element binding protein (SREBP) pathway (19).

The response of serum lipids to diet varies considerably among subjects. A large part of this heterogeneity in responses is not reproducible and is due to random fluctuations within subjects. Another part of the heterogeneity in response is reproducible and may partly be an innate characteristic of a subject (5,6).

The mechanism underlying the differences in response between subjects is still obscure. It is probably heterogeneous, because various stages in cholesterol metabolism, such

Table 1.1 Overview of genes, function of protein, studied polymorphisms, and the changes in the gene and in the protein.

Gene	Function of resulting protein	Polymorphism	Change in gene	Change in protein
APOA1	Major protein of HDL; co-factor of LCAT.	APOA1 –75G/A	G → A at -75 bp from transcription start site, within promoter region	No
		APOA1 83C/T or 84G/A	C → T at 83 bp and/or G → A at 84 bp from transcription start site within intron 1	No
APOA4	Protein of chylomicrons and HDL; fat absorption; metabolism of HDL and triglyceride-rich particles.	APOA4 Thr347Ser	A → T at codon 347 within exon 3	Thr → Ser
		APOA4 Gln360His	G → T at codon 360 within exon 3	Gln → His
APOB	Protein of chylomicrons, VLDL, IDL, LDL; ligand for LDL-receptor.	APOB EcoRI	G → A at codon 4154 within exon 29	Glu → Lys
APOC3	Protein of VLDL and HDL; metabolism triglycerides.	APOC3 SstI	G → C at 3238 bp within the 3' non-coding region	No
APOE	Protein of chylomicrons, VLDL, IDL, HDL; ligand for LDL-receptor and LRP.	APOE 2/3/4	E2: C → T at codon 158 within exon	Arg → Cys
			E4: T → C at codon 112 within exon	Cys → Arg
CETP	Reverse cholesterol metabolism.	CETP TaqIB	Silent base change at 227 bp within inton 1	No
FABP2	Uptake, intracellular metabolism and/or transport of long-chain fatty acids.	FABP2 Ala54Thr	A → T at codon 54 within exon 2	Ala → Thr
LPL	Hydrolysis of triglcyerides.	LPL Ser447Stop	C → G at codon 447 within exon 9	SerGly → Stop
MTP	Assembly and secretion of chylomicrons and VLDL.	MTP –493G/T	G → T at -493 bp from transcription start site, within promoter region	No

APO, apoprotein; CETP, cholesteryl ester transfer protein; FABP2, intestinal fatty acids binding protein; HDL high-density lipoprotein; IDL, intermediate-density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein; LPL, lipoprotein lipase; LRP, LDL-receptor related protein; MTP, microsomal triglyceride transfer protein; VLDL very low-density lipoprotein.

as absorption of cholesterol, inhibition of cholesterol synthesis, excretion of steroids, receptor-mediated clearance of LDL, LDL production, and accumulation of cholesterol in the body, may all contribute to response (20).

Possible role of genetic variation

Genes are parts of DNA that encode an enzyme or structural protein. Within a gene, coding sequences are interrupted by intervening sequences that are no part of the final gene product. Small flanking regions of DNA at both ends of the gene are important in the initiation and control of transcription, and mutations in these regions can affect the functioning of the gene. Many different forms of a gene may exist as a result of individual mutations. These are called alleles. Genes are called polymorphic when at least two alleles occur at a frequency of more than 1 % in a population (21).

Variation in DNA or genetic polymorphisms may affect the response of serum lipids to diet by influencing the production, composition and/or function of proteins in the cholesterol metabolism. There are many proteins (and thus genes) that play a role in various pathways of cholesterol metabolism. This thesis describes the effect of 11 polymorphisms of 9 genes on the lipid response to diet (Figure 1.1, Table 1.1).

As reviewed in detail by others (22-24), evidence that polymorphisms affect lipid responses is growing in the case of the APOE polymorphism, which has been most extensively studied, and for polymorphisms in the APOA1, APOA4, APOB, CETP, and LPL genes. However, results of studies of the effects of genetic polymorphisms on response are often inconsistent. There are several explanations for these inconsistencies. One possible explanation is that most of the studies lacked statistical power to detect existing effects of polymorphisms on lipid response due to small numbers of subjects. Another explanation is that polymorphisms only affect lipid responses to specific dietary changes, such as a change in dietary cholesterol, a change in dietary fat, or a combination of a change in dietary cholesterol and fat. Yet another explanation is that polymorphisms have sex-specific effects, they may affect the response only in men or only in women.

Objective and outline of the thesis

The *objective* of this research was to determine whether genetic polymorphisms affect the response of serum lipids to diet in humans.

In *Chapter 2* we assess sex differences in the response of serum lipids to changes in the diet. For this purpose, we pooled data on the serum lipid response to diet from 26 former

dietary trials involving 248 men and 243 women. These data were also used to study the effect of genetic polymorphisms on response of serum lipids to diet. *Chapter 3* considers the effect of the APOE2/3/4 polymorphism on serum lipid response and reviews the effect of this polymorphism as determined in other studies. *Chapter 4* describes the effects of 10 other candidate polymorphisms on serum lipid response. In *Chapter 5*, we describe the results of a controlled dietary trial of the effect of APOA4 360-1/2 polymorphism on response of serum lipids to dietary cholesterol. Although it is not directly related to genetic polymorphisms and lipid responses, the data of this trial were also used in a meta-analysis of the effect of dietary cholesterol on the ratio of total cholesterol to HDL-cholesterol (*Chapter 6*). Finally, the main outcomes of the studies of the effects of genetic polymorphisms on serum lipid response are discussed in *Chapter 7*. This chapter also focuses on methodological issues of studies on gene-diet interaction and issues in comparing such studies. In addition, it discusses the feasibility of genetic tests to detect diet-sensitivity and possible directions for future research with regard to gene-diet interactions in lipid metabolism.

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2

Differences between men and women in the response of serum cholesterol to dietary changes

Rianne M. Weggemans, Peter L. Zock, Rob Urgert, Martijn B. Katan

Abstract- Mild hypercholesterolemia is initially treated by diet. However, most studies of diet and cholesterol response were done in men, and it is unknown whether women react to diet to the same extent as men. We therefore studied sex differences in the response of serum cholesterol and lipoproteins to diet.

We measured responses of serum cholesterol to a decrease in dietary saturated fat in seven trials involving 126 men and 147 women, to a decrease in dietary *trans* fat in two trials (48 men and 57 women), and to a decrease in dietary cholesterol in eight trials (74 men and 70 women). We also measured responses to the coffee diterpene cafestol, which occurs in unfiltered coffee, in nine trials (72 men and 61 women). All subjects were lean and healthy.

The response of total cholesterol (± standard deviation) to a decrease in the intake of saturated fat was larger in men (-0.62 ± 0.39 mmol/L) than in women (-0.48 ± 0.39 mmol/L; 95% confidence interval (CI), 0.04 to 0.23 mmol/L). The response of total cholesterol to a decrease in the intake of cafestol was also larger in men (-1.01 ± 0.49 mmol/L) than in women (-0.80 ± 0.49 mmol/L; 95% CI, 0.04 to 0.39 mmol/L). Responses to *trans* fat and to dietary cholesterol did not differ between men and women.

In conclusion, men have larger responses of total cholesterol and LDL-cholesterol to saturated fat and cafestol than women.

Eur J Clin Invest 1999; 29:827-834

Introduction

Mild hypercholesterolemia, which is defined as a total cholesterol level over 5.2 mmol/L, is initially treated by a diet low in saturated fat and cholesterol, regardless of the sex of the patient (1-4). However, this dietary approach is based on evidence from trials most of which only comprised men (5-8). It is therefore not known whether dietary treatments to lower cholesterol should differ between men and women.

Some studies showed that the sex of a subject affects responses of total, low-density lipoprotein (LDL-), and high-density lipoprotein (HDL-) cholesterol to diets low in fat and cholesterol or with a high content of polyunsaturated fat (9-13), but others failed to confirm this (14-16).

We therefore compared diet-induced responses of serum total cholesterol and LDL- and HDL-cholesterol in 248 women and 243 men who participated in 26 controlled trials performed at our department between 1976 and 1996.

Materials and methods

Subjects, diets and experimental design

We pooled data of 26 controlled trials on diet and response, which had all been performed at our department between 1976 and 1996. In seven trials saturated fatty acids were exchanged for *cis*-unsaturated fatty acids or carbohydrates (referred to below as *saturated fat trials*) (17-23), in two trials *trans* fatty acids were exchanged for *cis*-unsaturated fatty acids (*trans fat trials*) (21-22), in eight trials the amount of dietary cholesterol was modified (*dietary cholesterol trials*) (24-28), and in nine trials the coffee diterpenes cafestol and kahweol were given (*cafestol trials*) (29-34) (Figure 2.1, Table 2.1). These diterpenes are responsible for the cholesterol-raising effect of unfiltered coffee. The amounts which were consumed by the subjects of these trials were within the normal range of consumption (35). Results of individual trials have been published elsewhere (17-34). Seven trials had a cross-over design (18, 21-23, 33), 14 a parallel design with a control group (17, 19, 20, 24, 26, 27, 28, 31, 32, 34), and five a before-and-after or linear design without a control group (25, 29-32).

Subjects received all their foods in 12 trials with saturated fat, *trans* fat, or dietary cholesterol (17-24, 26-28). In four trials of dietary cholesterol subjects received eggs as a supplement during the treatment period and guidelines for a diet low in cholesterol during the control period (24-27). In one other trial of dietary cholesterol, subjects received all foods

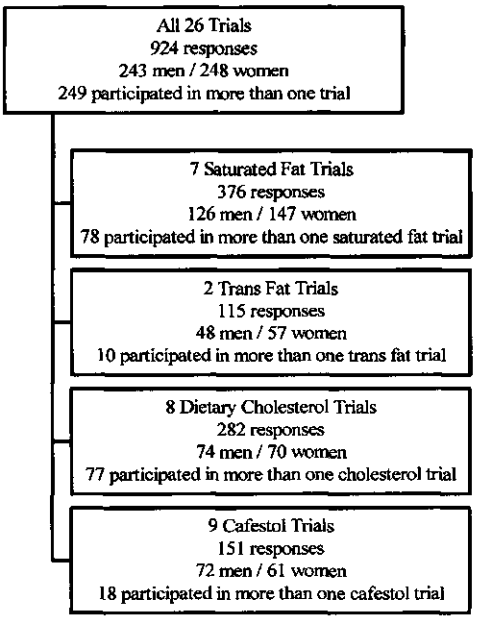

Figure 2.1 Overview of the trials indicating the number of responses measured, the number of participating men and women, and the nature of the dietary intervention. Saturated fat trial, exchange of saturated fatty acids for *cis*-unsaturated fatty acids or carbohydrates; *trans* fat trial, exchange of monounsaturated *trans*-fatty acids for *cis*-unsaturated fatty acids; dietary cholesterol trial, addition of dietary cholesterol to the diet; and cafestol trial, supplementation with the coffee diterpenes cafestol and kahweol.

during the treatment period and received dietary guidelines during the control period (28). In the nine cafestol trials subjects received coffee, coffee grounds, coffee oil, or cafestol and kahweol as a supplement and consumed their habitual diet throughout the trial (29-34). Participants were asked to maintain their usual patterns of activity, their smoking habits, and their use of oral contraceptives during the trials. They recorded in diaries any signs of illness, medications used, or other factors that might have affected the outcome of the study. The number of participants per trial ranged from 3 to 94 (median of the number of participants was 23), and the duration of the treatment ranged from 1.5 to 14 weeks (median duration was 3 weeks).

In the cross-over trials, subjects were randomized to the sequence of the various treatments (18, 21-23, 33) and in the parallel trials, subjects were randomized to one

Table 2.1 Design of the trials, amount and type of dietary intervention, number of subjects, and some baseline characteristics (means) of the subjects.

Reference	Design	Nature of intervention diet	Average duration of treatment (weeks)	Number of men/women	Dietary intervention*	Subject characteristics		
						Baseline cholesterol (mmol/L)†	Body mass index (kg/m^2)	Age (years)
Saturated fat trials					Change in intake of saturated fat (energy percent)			
17.	Parallel	Controlled	4	8/6	8	4.27	20.8	-
18.	Cross-over	Controlled	3	24/23	12	5.04	23.4	44
19.	Parallel	Controlled	3	12/12	10	5.15	22.4	27
20.	Parallel	Controlled	3	14/15	6	4.77	21.7	24
				13/16	6	4.86	21.6	25
21.	Cross-over	Controlled	3	25/34	9	4.75	22.0	25
22.	Cross-over	Controlled	3	26/30	9	4.84	21.5	25
23.	Cross-over	Controlled	3	23/36	10	4.82	22.4	29
Cholesterol trials					Change in intake of cholesterol (mg per day)			
24	Parallel	Free-living	3	25/19	537	-	24.4	52
		Free-living	4	17/17	586	-	25.3	53
24.		Controlled	4	3/3	526	5.80	25.0	54
25.	Before-after	Free-living	1.5	3/3	1596	4.48	23.5	36
26, 27, 28.	Parallel	Controlled	2	46/48	504	5.12	22.3	33
		Controlled	2	22/19	567	5.16	22.4	32
		Free-living	4	21/11	860	5.08	-	32
		Free-living and controlled‡	3	14/9	760	4.93	-	36
				9/4	742	5.58	-	36

Table 2.1 continued

Reference	Design	Nature of intervention diet	Average duration of treatment (weeks)	Number of men/women	Dietary intervention*	Subject characteristics: Baseline cholesterol (mmol/L)†	Subject characteristics: Body mass index (kg/m^2)	Subject characteristics: Age (years)
Trans fat trials					Change in intake of trans fat (energy percent)			
21.	Cross-over	Controlled	3	25/34	11	4.75	22.0	25
22.	Cross-over	Controlled	3	26/30	10	4.84	21.5	25
Cafestol trials					Change in intake of cafestol (mg per day)			
29.	Before-after	Free-living	6	5/5	64	4.70	22.0	28
30.	Before-after	Free-living	3	2/3	72	4.58	22.1	24
			3	3/3	40	5.15	22.9	22
31.	Parallel	Free-living	4	8/8	85	4.47	21.8	22
			4	8/7	81	4.43	22.0	22
			4	8/8	22	4.39	21.7	24
31.	Parallel	Free-living	4	6/9	57	4.52	21.7	22
31.	Before-after	Free-living	6	3/0	73	5.10	24.4	49
32.	Before-after	Free-living	4	9/6	42	4.45	22.0	26
32.	Parallel	Free-living	3	3/4	39	4.82	21.6	24
33.	Cross-over	Free-living	4	10/0	61	4.59	21.3	23
34.	Parallel	Free-living	4	12/11	38	4.58	23.1	30

* Decreases in the amount of saturated or *trans* fat, cholesterol, or cafestol.

† To convert values for baseline cholesterol levels from mmol per liter to miligram per deciliter, divide mmol per liter by 0.02586.

‡ The diet in the low-cholesterol period was the habitual diet and the intervention diet in the high cholesterol period controlled.

of the various treatments (17, 19, 20, 24, 26-28, 31, 32, 34). In five of the nine cafestol trials participants and investigators were blinded to the nature of the treatment (30-33). Lab personnel were never aware of the subject's treatment. Cholesterol levels were determined in at least two serum samples per treatment which were obtained on separate days. All sera from one subject were analyzed within the same run. The coefficient of variation within one run for control samples ranged from 0.7 to 2.9 %. In all trials the accuracy was checked by the analysis of three serum pools of known value provided by the Centers for Disease Control (Atlanta, GA). The mean bias with regard to the target values of the Centers for Disease Control pools ranged from -2 % to 1.1 % for total cholesterol, from -3.2 % to 3.3 % for HDL-cholesterol, and from -1.5 % to 10 % for triglycerides (17-34).

The subjects were healthy as indicated by a medical questionnaire, and by the absence of anemia, glucosuria, and proteinuria. The protocols, which were approved by the appropriate Ethical Committee, were explained to the subjects and informed consent was obtained from all subjects.

In order to protect the privacy of subjects and assure blinding we assigned new identification numbers to all subjects. To check for errors introduced during the coding and during the amalgamation of the data from the 26 trials, we determined the presence of DNA sequences unique to the X and Y chromosome (36). Out of 512 subjects for whom data were available one woman had been erroneously coded as a man; the sex of the other 511 subjects agreed with that in the pooled data files.

For the present analyses we pooled serum cholesterol responses to saturated fat of 126 men and 147 women, responses to *trans* fatty acids of 48 men and 57 women, responses to dietary cholesterol of 74 men and 70 women, and responses to cafestol of 72 men and 61 women (Table 2.2). We excluded data from subjects who received a control diet or a placebo treatment throughout a trial. We did not analyze responses of LDL-cholesterol to dietary cholesterol, because these were available for only 47 of the 144 subjects. Age was recorded and body mass index, weight change, and serum cholesterol levels were measured as described (17-34). Baseline cholesterol levels were measured before the start of the study, when subjects consumed their habitual diets.

Statistical analysis

The way we calculated individual responses of cholesterol depended on the design of each trial, which was either a cross-over, a parallel, or a before-and-after design. In the trials with a cross-over or parallel design we defined individual responses of cholesterol as the level of serum cholesterol at the end of the treatment that lowered cholesterol minus the level at the end of treatment that increased cholesterol. In the trials with a before-and-after

Table 2.2 Baseline characteristics (mean ± standard deviation) of men and women in the various trials.

	Men	Women
Saturated fat trials		
N	126	147
Total cholesterol level (mmol/L)	4.88 ± 0.93	5.12 ± 0.89
Body mass index (kg/m^2)	22.5 ± 2.7	22.4 ± 2.8
Age (years)	28 ± 12	28 ± 12
Trans fat trials		
N	48	57
Total cholesterol level (mmol/L)	4.88 ± 0.77	4.91 ± 0.72
Body mass index (kg/m^2)	21.8 ± 1.9	21.7 ± 2.4
Age (years)	24 ± 8	25 ± 8
Dietary cholesterol trials		
N	74	70
Total cholesterol level (mmol/L)	5.05 ± 0.81	5.42 ± 1.43
Body mass index (kg/m^2)	23.5 ± 3.2	23.2 ± 3.3
Age (years)	37 ± 16	39 ± 17
Cafestol trials		
N	72	61
Total cholesterol level (mmol/L)	4.54 ± 0.66	4.58 ± 0.60
Body mass index (kg/m^2)	22.1 ± 2.0	22.0 ± 2.5
Age (years)	26 ± 8	24 ± 5

To convert values for total cholesterol level from mmol per liter to mg per deciliter, divide mmol per liter by 0.02586.

design in which the treatment lowered cholesterol, we defined individual responses as the level of cholesterol at the end of the treatment minus the level of cholesterol before the treatment. In the trials with a before-and-after design in which the treatment increased cholesterol, we defined individual responses as the level of cholesterol before the treatment minus the level at the end of the treatment. In one dietary cholesterol trial (25) and seven cafestol trials (29, 31,32, 33, 34), we used the level of baseline cholesterol to calculate the response.

We used different methods to estimate the differences between men and women in their responses of cholesterol. To adjust for potentially confounding factors, we used the average individual response as independent variable in a two-factor and multiple regression model. We calculated the average response of each subject over trials with a similar dietary treatment, as well as their average body mass index and age, because 28 per cent of the subjects participated in two or more trials with a similar dietary treatment; 51 per cent of all subjects participated in more than one trial.

Two-factor regression model

The two-factor regression model included as independent factors the sex of the subject and additional factors indicating in which trial the individual's cholesterol response had been measured. If a trial consisted of more than one treatment, we created factors indicating each treatment within a trial. Inclusion of these trial factors allowed us to correct for differences between trials in e.g. characteristics of the background diet, whether the diet was controlled or uncontrolled, or time of the year during which the trial had been performed. If subjects participated in more than one trial with a similar dietary treatment the factors of these trials were set to a value reciprocal to the number of times a subject had participated. For instance, if a subject participated in two trials, the factor for each trial was set to ½. In this way, we could adjust the individual's mean response for the effect of various trials. In the two-factor regression model, the mean cholesterol response of men and women was calculated by a least squares method after correction for the trial in which the response was measured.

Multiple regression models

In a multiple regression model, differences in responses between sexes were estimated after adjustment for trial and for the subjects' age, body mass index, and change in weight during the trial. We lacked information about the age of 21 subjects, body mass index of 65 subjects, and change in weight during the trial of 48 subjects. For these subjects we set age to 25 years, body mass index to 25 kg/m^2, and change in weight to 0.0 kg. For all subjects we created dummy variables that indicated whether the information about a characteristic was available or not. In this way data on the response of cholesterol of all subjects could be included in the multiple regression model.

We did not adjust for baseline cholesterol level in our primary analyses because baseline cholesterol level was part of the response in one dietary cholesterol trial and seven cafestol trials. We excluded these eight trials to study the effect of baseline cholesterol on response of cholesterol. In a regression analysis we estimated the difference in response between men and women after adjustment for trial, age, body mass index, change in weight during the trial, and baseline cholesterol level.

We also analyzed the data using various other models. For a crude analysis, the mean responses of the men and women were calculated for each treatment within a trial. For each type of treatment - saturated fat, *trans* fat, cholesterol, and cafestol - these means were averaged weighting for the reciprocal of their standard error. In this way differences between trials in the precision of the observed mean responses were taken into account. A mixed-model regression analysis was applied to the trials with dietary cholesterol and we analyzed all trials without taking into account that some subjects had participated more than once, thus treating all responses of the same subjects as independent observations. We also used predicted responses as independent factors in a regression model. These predicted responses were calculated by coefficients from published meta-analyses on changes in serum cholesterol and lipoproteins to changes in the amount of dietary fatty acids and cholesterol (5, 6, 35, 37, 38). In yet other models, we expressed the cholesterol response as percentage change from the serum cholesterol level at the end of the control or baseline period and also as Z-scores relative to the average response of the group on the same treatment. All these models yielded similar differences in responses between men and women, which confirmed the robustness of the models presented here (39).

To check that differences in the design of trials did not bias our results, we also performed the analyses without data from trials with a before-and-after design, one involving dietary cholesterol and four involving cafestol, and without data from three saturated fat trials with a parallel design. Again results were similar to the estimated differences when all trials were included. All analyses were performed with SAS statistical software (40).

Results

The baseline cholesterol levels (mean ± standard deviation) in the saturated fat trials were significantly smaller in men (4.88 ± 0.93 mmol/L) than in women (5.12 ± 0.89; P=0.03) (Table 2.2).

Two-factor regression model

Nevertheless, the adjusted responses of serum total cholesterol and LDL-cholesterol to *saturated fat* were larger in men than in women; the difference was 0.14 ± 0.05 mmol/L (mean ± standard error) for total cholesterol (95% confidence interval (CI), 0.04 to 0.23 mmol/ L) and 0.08 ± 0.04 mmol/L for LDL-cholesterol (95% CI, 0 to 0.17 mmol/L) (Figure 2.2, Table 2.3).

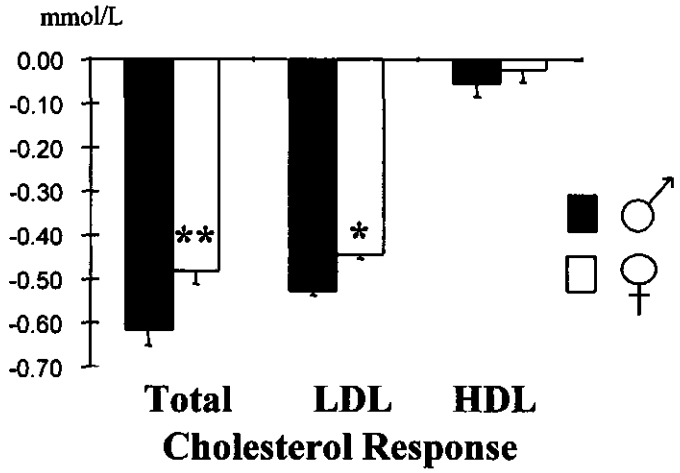

Figure 2.2 Adjusted responses of serum total, low-density lipoprotein (LDL-), and high-density lipoprotein (HDL-) cholesterol in men (N=126) and women (N=147) when nine energy percent dietary saturated fat was replaced by mono- or polyunsaturated fat, or carbohydrates. Responses were adjusted differences between trials in a two-factor model. Error bars indicate one standard error. * $P < 0.05$; ** $P < 0.01$ for differences between men and women.

The adjusted response of total cholesterol to *trans fat* did not differ significantly between men and women, although both the decrease in LDL-cholesterol and the increase in HDL-cholesterol tended to be smaller in men than in women. The difference in response between men and women was -0.02 ± 0.06 mmol/L for LDL-cholesterol (95% CI, -0.13 to 0.10 mmol/L), and 0.03 ± 0.02 mmol/L for HDL-cholesterol (95% CI, -0.02 to 0.07 mmol/L).

The adjusted response to *dietary cholesterol* was not significantly different for men and women. The difference in response between men and women was 0.01 ± 0.05 mmol/L for total cholesterol (95% CI, -0.08 to 0.11 mmol/L) and -0.01 ± 0.02 mmol/L for HDL-cholesterol (95% CI, -0.05 to 0.02 mmol/L).

The adjusted response of total cholesterol to *cafestol* was 0.22 ± 0.09 mmol/L larger in men than in women (95% CI, 0.04 to 0.39 mmol/L) and responses of LDL-cholesterol to cafestol were 0.22 ± 0.08 mmol/L larger in men than in women (95% CI, 0.06 to 0.37 mmol/L) (Figure 2.3).

Table 2.3 Mean responses (± standard deviation) of serum total, low-density lipoprotein (LDL-) and high-density lipoprotein (HDL-) cholesterol to a decrease in the intake of saturated or *trans* fat, dietary cholesterol, or the coffee diterpene cafestol in men and women.

	Response of total cholesterol		Response of LDL-cholesterol		Response of HDL-cholesterol	
	Men	Women	Men	Women	Men	Women
			mmol/L			
Saturated fat	-0.62± 0.39	-0.48 ± 0.39*	-0.53 ± 0.34	-0.44 ± 0.33†	-0.05 ± 0.14	-0.02 ± 0.13
Trans fat	-0.22 ± 0.35	-0.20 ± 0.35	-0.29 ± 0.31	-0.31 ± 0.31	0.12 ± 0.11	0.14 ± 0.11
Cholesterol	-0.39 ± 0.28	-0.38 ± 0.28	‡	‡	-0.06 ± 0.11	-0.07 ± 0.10
Cafestol	-1.01 ± 0.49	-0.80 ± 0.49†	-0.78 ± 0.43	-0.57 ± 0.44*	0.05 ± 0.15	0.02 ± 0.16

To convert values for response of total cholesterol and LDL- and HDL-cholesterol from mmol per liter to mg per deciliter, divide mmol per liter by 0.02586.

All responses were adjusted for differences between trials in a two-factor model.

* $P < 0.05$ for difference in response between men and women.

† $P < 0.01$ for difference in response between men and women.

‡ Responses of LDL-cholesterol to dietary cholesterol were not analyzed because of a too small number of observations.

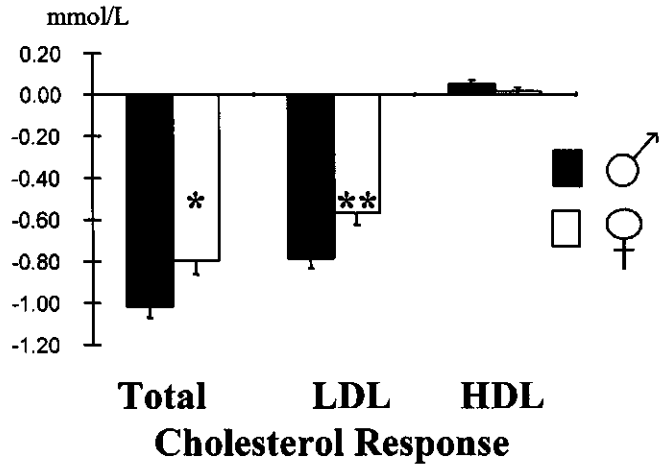

Figure 2.3 Adjusted responses of serum total, low-density lipoprotein (LDL-), and high-density lipoprotein (HDL-) cholesterol in men (N=72) and women (N=61) to a decrease in the intake of the coffee diterpene cafestol. All responses were adjusted for differences between trials in a two-factor model. Error bars indicate one standard error. * $P < 0.05$; ** $P < 0.01$ for differences between men and women.

Multiple regression models

In a multiple regression model we adjusted for trial, age, body mass index, and change in weight during the trial. This yielded differences between the sexes in response of cholesterol similar to those found with the two-factor model. One of the largest differences in results between the two models was the difference between men and women in response of total cholesterol to cafestol, which was 0.19 mmol/L in the multiple regression model as opposed to 0.22 mmol/L in the two-factor model.

We excluded the eight trials in which baseline cholesterol was part of the response to estimate the difference in response between men and women after adjustment for trial, age, body mass index, change in weight during the trial, and baseline cholesterol level. This model yielded differences in response between men and women similar to those found with the two-factor model. The largest difference in results between the two models was the difference between men and women in the response of LDL-cholesterol to saturated fat, which was 0.12 mmol/L in the multiple regression model as opposed to 0.08 mmol/L in the two-factor model. Thus, different models of statistical analysis yielded similar results.

Discussion

We found that healthy men have larger responses of total cholesterol and LDL-cholesterol to dietary saturated fat and to the coffee diterpene cafestol than healthy women. Adjustment for age, body mass index, change in weight during the trial, and baseline cholesterol levels did not affect the difference in response of cholesterol between men and women. However, it may not be suitable to adjust sex differences in cholesterol response for baseline cholesterol level. Baseline cholesterol level may not be a confounder of the relationship between sex differences and cholesterol response as the cholesterol response may affect the cholesterol level instead of being affected by the level (39). Other factors that might affect the cholesterol response to diet are smoking, menstrual cycle, and use of oral contraceptives (41,42,43). The exclusion of all smokers from our analyses (19 % of all subjects) did not affect the results. Thus, smoking does not appear to be responsible for the sex difference in cholesterol response. Because most of the women were pre-menopausal, both the menstrual cycle and use of oral contraceptives might have affected the response of cholesterol. However, the women entered the trials at different points of their menstrual cycle. In this way, cyclic effects on response of cholesterol were averaged out and could not have affected the mean response observed in women as an aggregate. Also, sex differences in response of cholesterol were not affected when we excluded the 33 % of women who used oral contraceptives.

Reports about sex differences in cholesterol response to diet have been contradictory. The *saturated fat* trials which we pooled comprised a much larger number of subjects (126 men and 147 women) than previous studies (22 to 82 men and 22 to 57 women) (9-15). Also, our estimation of individual responses was improved because 78 subjects participated in several trials with saturated fat. This may explain why we were able to show a clear-cut effect of sex on cholesterol responses to saturated fat where others were not.

The difference in response to *trans fat* between men and women was not significant and the responses of LDL- and HDL-cholesterol tended to be smaller in men than in women rather than larger as they were for saturated fat. This agrees with earlier observations that metabolic pathways for *trans* fat are different from those for saturated fat (44, 45). However, the results for *trans* fat must be interpreted with caution, because the mean cholesterol response in the *trans* fat trials was smaller and the number of participants was smaller than in the saturated fat trials.

Some studies reported that subjects who have a large response to dietary fat also have a large response to *dietary cholesterol* (18, 46). However, we found no difference between men and women in cholesterol response to dietary cholesterol. Although both the response to dietary cholesterol and the number of participants in the dietary cholesterol trials were smaller

than in the saturated fat trials, the estimation of the response was improved by 77 subjects who participated in at least two trials with dietary cholesterol. Our results may suggest that saturated fat and dietary cholesterol affect cholesterol metabolism through different mechanisms. This is in line with observations that different genes seem to be involved in determining the responses to dietary fat and cholesterol, the apoprotein E genotype seems to affect the response to dietary fat but not to dietary cholesterol (47) and the apoprotein A4 360 genotype seems to affect the response to dietary cholesterol (48,49).

Responses of total cholesterol and LDL-cholesterol to *cafestol* were larger in men than in women, which is similar to the effects of saturated fat. Our observations agree with epidemiological observations that the effect on cholesterol levels of drinking more than nine cups of boiled coffee per day tends to be larger in men than in women (50).

One possible explanation for the observed differences between men and women in response of cholesterol to the fat composition of the diet is that men had a larger energy intake and thus consumed more saturated fat when expressed in absolute amounts (grams) than women. To investigate this, we adjusted our analysis for the level of energy intake during the last four weeks of each trial in a subset of the saturated fat trials for which data on energy intake were available (19-23). The response of LDL-cholesterol to saturated fat was 0.08 mmol/L larger in men than in women both without and after adjustment for energy intake. Thus the larger response in men was not due to their larger overall food intake. In the cafestol trials men and women received the same absolute amount of cafestol, irrespective of their energy intake. However, if the amount of cafestol was adjusted for the energy intake, differences in cholesterol response between men and women would become even larger.

It could be argued that women complied less with the diets than men, and as a result showed a smaller change in serum cholesterol. However, all subjects were highly motivated and conscious of the aim of the trial. In the controlled dietary trials, subjects received 90 percent of all foods, and women as well as men consumed the hot meals under supervision. Also, adherence to the diets according to anonymous questionnaires was similarly high for women and men (19-23). Thus, we do not have any reason to believe that women were less compliant than men. Nevertheless changes in fatty acid composition of cholesterol esters following changes in the intake of saturated and *trans* fat were smaller in women than in men (unpublished observations). This might at first sight suggest that women were less compliant than men. However, it is also possible that differences between men and women in changes in fatty acid composition of cholesterol esters were caused by the same metabolic processes that caused differences in the responses of serum cholesterol.

Animal studies support the notion that sex hormones affect the response of serum cholesterol to diet. Thus diets high in saturated fat and dietary cholesterol raised serum

cholesterol levels in male, but not in female hamsters. When the females were sterilized, their serum cholesterol became as responsive to diet as that of the male hamsters (51).

The present findings are based on lean and healthy subjects with normal cholesterol levels. It is unknown whether they also apply to elderly, more obese, or hypercholesterolemic subjects.

Our results imply that men will benefit more from a reduction in the intake of saturated fat than women. Nevertheless, the responses of women to reductions in the intake of saturated fat are still considerable. Therefore, dietary treatment should still be recommended for both men and women with hypercholesterolemia.

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3

Apoprotein E genotype and the response of serum cholesterol to dietary fat, cholesterol, and cafestol

Rianne M. Weggemans, Peter L. Zock, Jose M. Ordovas, Juan Pedro-Botet, Martijn B. Katan

Abstract- Previous studies on the effect of the apoprotein (APO) E polymorphism on the response of serum lipids to diet showed inconsistent results.

We therefore studied the effect of the APOE polymorphism on responses of serum cholesterol and lipoproteins to various dietary treatments. To this end, we combined data on responses of serum cholesterol and lipoproteins to saturated fat, to *trans* fat, to dietary cholesterol, and to the coffee diterpene cafestol with newly obtained data on the APOE polymorphism in 395 mostly normolipidemic subjects.

The responses of low-density lipoprotein (LDL-) cholesterol to saturated fat were 0.08 mmol/L larger in subjects with the APOE3/4 or E4/4 genotype than in those with the APOE3/3 genotype (95% confidence interval –0.01 to 0.18 mmol/L). In contrast, responses of LDL-cholesterol to cafestol were 0.11 mmol/L smaller in subjects with the APOE3/4 or E4/4 genotype than in those with the APOE3/3 genotype (95% confidence interval –0.29 to 0.07 mmol/L). Responses to dietary cholesterol and *trans* fat did not differ between subjects with the various APOE genotypes.

In conclusion, the APOE genotype may affect the response of serum cholesterol to dietary saturated fat and cafestol in opposite directions. However, the effects are small. Therefore, knowledge of the APOE genotype by itself may be of little use in the identification of subjects who respond to diet.

Atherosclerosis, in press

Introduction

The response of serum cholesterol to changes in the diet varies considerably between subjects. Within subjects, this response to diet is to some extent reproducible (1) and may in part be an innate characteristic. Identification of genetic factors that affect the response may help to select an effective therapeutic approach for individual patients with an atherogenic lipid profile.

Variation in one candidate genetic factor, the apoprotein E (APOE) gene, is known as the APOE polymorphism (2). The common allele of the APOE gene is the ε3-allele, which encodes for cysteine at amino acid residue 112 and for arginine at residue 158. The ε4-allele encodes for arginine at both residues and the ε2-allele encodes for cysteine at both residues. The various ApoE isoforms differ in binding affinity for the LDL-receptor and the LDL-receptor related protein, for high-density lipoprotein (HDL-) cholesterol, and for triglyceride-rich particles (3-6). In industrialized societies, carriers of the ε4-allele have the highest levels of serum total cholesterol and low-density lipoprotein (LDL-) cholesterol, carriers of the ε3-allele have intermediate, and carriers of the ε2-allele have the lowest levels (3). In addition, subjects with the various APOE genotypes differ in the absorption efficiency of cholesterol from the intestine, in the synthesis rates of cholesterol and bile acids, and in the production of LDL apoprotein B (7-9). This suggests that also the response of serum cholesterol to diet may be affected by the APOE polymorphism.

Some studies show that subjects with an ε4-allele are more responsive to changes in the amount of dietary cholesterol (10-13), the fat composition (13-16), or the amount of dietary cholesterol and fat (8,17-22) than those without an ε4-allele. However, other studies did not find a difference in responsiveness between subjects with an ε4-allele and those without (23-36).

There are several explanations for these contradictory results. One is that most of the studies lacked sufficient power to detect an effect of the APOE polymorphism on the response of cholesterol due to a small number of subjects. Some other explanations are that the APOE polymorphism affects the response of cholesterol in men only and not in women, or only in populations, in which baseline levels of serum total cholesterol and LDL-cholesterol differ between the various APOE genotypes. Yet, another explanation is that the APOE polymorphism may only affect the responses of serum cholesterol to specific changes in dietary cholesterol, dietary fat, or both.

We therefore studied the effect of the APOE polymorphism on the response of serum cholesterol to the exchange of saturated fat for *cis*-unsaturated fat or carbohydrates, to the exchange of *trans* fat for *cis*-unsaturated fat, to supplementation with dietary cholesterol, and to supplementation with the coffee diterpene cafestol in 201 men and 194 women.

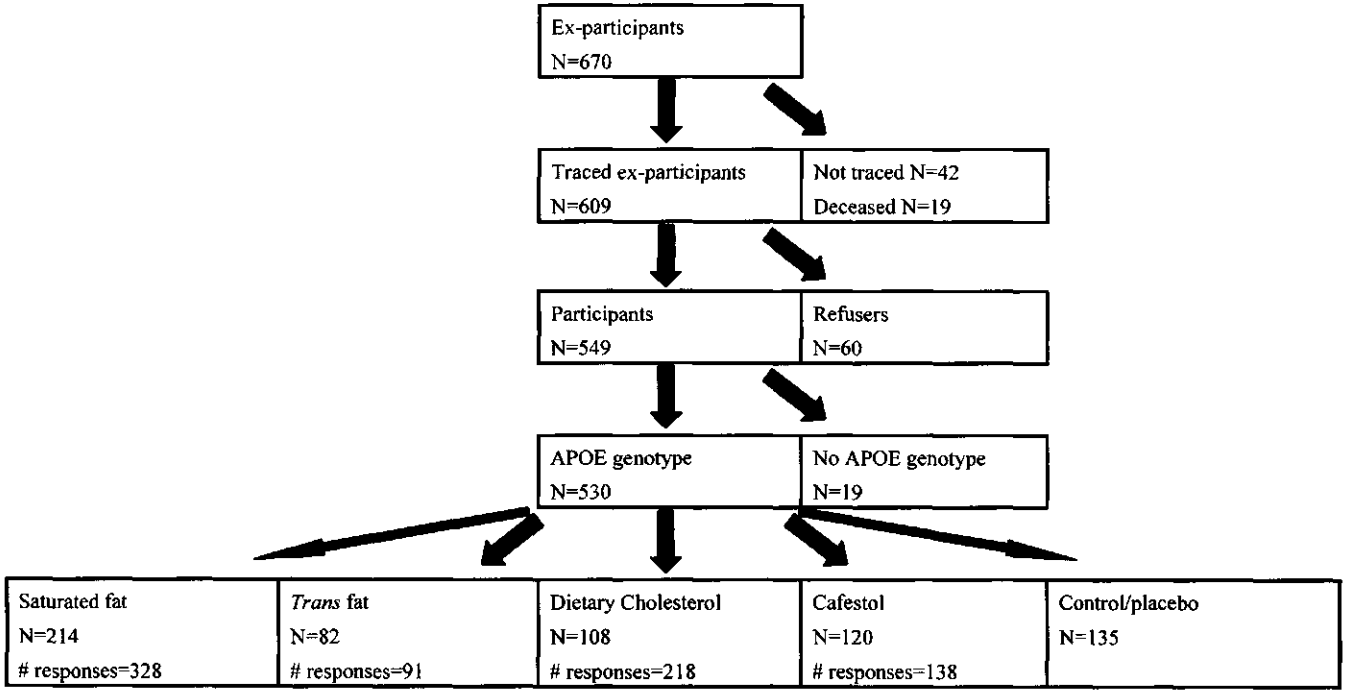

Figure 3.1 Overview of the trials indicating the selection, the number of responses measured, the number of participants, and the nature of the treatment. Saturated fat trials, exchange of saturated fat for *cis*-unsaturated fat or carbohydrates; *trans* fat trials, exchange of *trans* fat for *cis*-unsaturated fat; dietary cholesterol trials, addition of dietary cholesterol to the diet; cafestol trials, supplementation with the coffee diterpenes cafestol and kahweol.
113 subjects participated in two trials that vary in treatment (for instance a saturated fat and a cafestol trial); 8 subjects participated in three trials which vary in treatment.

Materials and methods

Subjects

Our department has performed 26 controlled trials on diet and blood lipids with a total of 670 subjects between 1976 and 1996. Details about the design and methods of these trials are described elsewhere (37). The data of these trials have been carefully maintained. Therefore, we were able to pool the data and to calculate individual responses as well as mean responses of treatment groups. These mean responses agreed with those published at the time trials were done, showing that our data retrieval and cleanup had been successful. At the time of the trials, no DNA samples were collected. In order to obtain DNA samples, we traced the former participants in 1996 and 1997 and managed to find 609 of them. The protocol of the present study, which was approved of by the Ethical Committee of Wageningen Agricultural University, was explained to them. We obtained informed consent and collected DNA of 549 subjects. We sampled blood from 486 subjects and collected mouth swabs from the other 63 In the present study, we used data on 395 subjects, 113 of whom had participated in two trials with a different treatment (e.g. saturated fat and cafestol) and 8 of whom had participated in three trials with a different treatment. Thus, our data consisted of 775 responses to the various treatments in a total of 201 men and 194 women (Figure 3.1). At the time of the trials the subjects were healthy as indicated by a medical questionnaire, and by the absence of anemia, glucosuria, and proteinuria.

Characteristics of trials

All trials were designed to study responses of serum cholesterol to changes in the diet and had been approved by the appropriate medical ethical committees. We pooled data on response of serum cholesterol to saturated fat of seven trials, to *trans* fat of two trials, to dietary cholesterol of eight trials, and to the coffee diterpenes cafestol and kahweol of nine trials. These diterpenes are the substances that are responsible for the cholesterol-raising effect of unfiltered coffee, such as Scandinavian-style boiled coffee, Turkish coffee, and cafetiere coffee (38). Seven trials had a crossover design, 14 a parallel design with a control group, and five a before-and-after or linear design without a control group. The number of participants per trial ranged from 3 to 94 (median was 23) and the duration of the treatment ranged from 1.5 to 14 weeks (median was 3 weeks).

Diets and supplements

All food was supplied in the seven trials with saturated fat, in the two trials with *trans* fat, and in three of the trials with dietary cholesterol. In the saturated fat and *trans* fat trials, the saturated or *trans* fat was exchanged for an equal energy amount of mono- or

polyunsaturated fat, or, in one saturated fat trial, for carbohydrates. In the four trials of dietary cholesterol without complete food supply, subjects received eggs as a supplement during the treatment period and guidelines for a diet low in cholesterol during the control period. In one other trial of dietary cholesterol, subjects received all foods during the treatment period and received dietary guidelines during the control period. In the nine cafestol trials subjects received coffee, coffee grounds, coffee oil, or cafestol and kahweol as a supplement and consumed their habitual diet throughout the trial. The observed responses in the various trials agreed with those expected on the basis of the change in diet. This indicates that compliance was excellent in the trials.

Available data

Information on APOE genotype and responses of total cholesterol and HDL- and LDL-cholesterol to saturated fat was available for 214 subjects, to *trans* fat for 82 subjects, to dietary cholesterol for 108 subjects, and to cafestol for 120 subjects. We did not analyze responses of LDL-cholesterol to dietary cholesterol because data on LDL-cholesterol and APOE genotype were available for only 40 of the 108 subjects.

For the present study we also used data on sex, age, body mass index, and change in weight during the trial, which we considered to be potential confounders of the relationship between response of cholesterol and APOE genotype (17,28,39-45). In addition, we used data on serum cholesterol levels, which were measured when subjects consumed their habitual diets (Table 3.1).

Laboratory analyses

Lab personnel were never aware of the subject's treatment. Serum cholesterol levels were determined in at least two serum samples per treatment, which were obtained on separate days. All sera from one subject were analyzed within the same run. The coefficient of variation within one run for control samples ranged from 0.7 to 2.9 %. In all trials accuracy was checked by the analysis of three serum pools of known value provided by the U.S. Centers for Disease Control (Atlanta, AG). The mean bias with regard to the target values of the Centers for Disease Control pools ranged from -2 % to 1.1 % for total cholesterol and from -3.2 % to 3.3 % for HDL-cholesterol. LDL-cholesterol was calculated by means of Friedewald's equation (46).

Table 3.1 Characteristics of the subjects with various apoprotein (APO)E genotypes in the trials with saturated and *trans* fat, dietary cholesterol, and cafestol.

	APOE2/2 and 2/3	APOE3/3	APOE3/4 and 4/4*
Saturated fat			
Men/Women	15/16	60/70	24/25
Age (years)	29 ± 12	28 ± 12	27 ± 11
Cholesterol level on habitual diet (mmol/l)	4.54 ± 0.68	5.09 ± 0.96	5.13 ± 0.94
Trans fat			
Men/Women	5/8	18/28	12/11
Age (years)	24 ± 7	26 ± 10	22 ± 3
Cholesterol level on habitual diet (mmol/l)	4.51 ± 0.58	4.99 ± 0.69	5.05 ± 0.81
Dietary cholesterol			
Men/Women	12/6	29/33	15/8
Age (years)	32 ± 11	35 ± 14	34 ± 12
Cholesterol level on habitual diet (mmol/l)	4.59 ± 0.94	5.30 ± 0.87	5.54 ± 1.36
Cafestol			
Men/Women	7/11	39/31	15/14
Age (years)	28 ± 12	24 ± 6	25 ± 6
Cholesterol level on habitual diet (mmol/l)	4.40 ± 0.60	4.36 ± 1.06	4.39 ± 1.07

*Subjects with the APOE2/4 genotype, four with a response to saturated fat, five with a response to dietary cholesterol, and three with a response to cafestol, were excluded.

We isolated DNA from fresh blood and from mouth swabs by "salting-out" procedures (47-49). We used the method described by Hixson and Vernier (50) for assessment of the APOE genotype in 500 samples. Forty-nine other samples could not be amplified by the method of Hixson and Vernier (50). We succeeded in genotyping 30 of these 49 samples by another method, a mutagenically separated PCR as described by Rust et al (51). Two independent investigators interpreted all gels and, in case the interpretation differed, the APOE genotype was reanalyzed. We analyzed 35 DNA samples in duplicate to check the accuracy of both procedures for the analysis of the APOE genotype. The investigators who interpreted the gels did not know which samples were the duplicates. The APOE genotypes of all duplicate samples agreed.

Statistical methods

In all trials we defined individual responses of cholesterol as the level of serum cholesterol at the end of the treatment that increased cholesterol minus the level either at the end of the treatment that lowered cholesterol, the placebo treatment, or the diet without the cafestol or cholesterol supplement. In crossover trials with three treatments, for instance a saturated, mono-unsaturated, and poly-unsaturated fat diet, we calculated one response to the substitution of mono-unsaturated for saturated fat and one response to the substitution of poly-unsaturated for saturated fat. Because the cholesterol level on the saturated fat diet is used in the calculation of the two responses, these are dependent. This does not affect the validity of the estimate, but it may slightly affect the standard error and thus the *P*-value.

In the crude analysis, we studied the effect of APOE genotype on response to each of the four treatments, irrespective of whether a subject participated in more than one trial with a similar treatment. We calculated the sum of the APOE-subscript and its correlation (Pearson product-moment correlation coefficient) with the response of cholesterol.

In the adjusted analyses, we adjusted the response for subject and trial. We adjusted for subject because 41 % of the subjects in the saturated fat trials and 56% of the subjects in the dietary cholesterol trials participated in more than one trial with a similar treatment or in a crossover trial with three treatments (37) (Figure 3.1). We adjusted for trial because there were background differences between the trials in background diet, duration of treatment, and time of the year the trial was performed. If a trial consisted of more than one treatment, we created factors indicating each treatment within a trial. In additional analyses we also adjusted for the subject characteristics sex, age, body mass index, and change in weight, because these were potential confounders of the relationship between the APOE genotype and serum cholesterol response (17,28,39-44). Subjects with the APOE2/4 (N=13) genotype were excluded from the analyses. We tested the *adjusted* differences in response of cholesterol between subjects with the APOE2/2 or E2/3, E3/3, and E3/4 or E4/4 genotype by analysis of variance. In case of significant differences, group means were compared by Fisher's Least Significant Difference test for multiple comparisons.

We also analyzed the data using various other regression models. We calculated the mean response of each individual over trials with a similar treatment and estimated differences in this mean response between subjects with the various APOE genotypes. In yet other models, we expressed the cholesterol response as the percentage change from the serum cholesterol level at the end of the control or baseline period. All these models yielded similar differences in response between the APOE genotype groups, which confirmed the robustness of the models presented here (52).

Results

In our subjects the frequency of the ε2 allele was 0.09, of the ε3 allele 0.79, and of the ε4 allele 0.13. The frequency distribution was similar to those observed in Dutch and other Caucasian populations (3,53). Baseline characteristics were similar for subjects with the APOE2/2 or E2/3, E3/3, and E3/4 or E4/4 genotype, except for levels of total cholesterol while subjects consumed their habitual diet, which was lowest in subjects with the APOE2/2 or E2/3 and highest in those with APOE3/4 or E4/4 genotype (Table 3.1). The level of total cholesterol on the habitual diet was 4.54 ± 0.07 mmol/L (mean ± standard error) in subjects with the APOE2/2 or E2/3 genotype, 5.03 ± 0.04 mmol/L in subjects with the APOE3/3 genotype, and 5.08 ± 0.07 mmol/L in those with APOE3/4 or E4/4 genotype. Mean body mass index was 22 kg/m^2 and mean age was 25 years. The body weight of the subjects did not change significantly during the trials.

The responses of LDL-cholesterol to *saturated fat* tended to be smallest in subjects with the APOE2/3 genotype and largest in those with the APOE3/4 or E4/4 genotype (Figure 3.2). The correlation between the sum of APOE-subscripts and the response of LDL-cholesterol was 0.06 (P=0.29). After adjustment for subject and trial the response of LDL-cholesterol to saturated fat was 0.08 mmol/L larger in subjects with the APOE3/4 or 4/4 genotype than in those with the APOE3/3 genotype (95% confidence interval (CI) –0.01 to 0.18 mmol/L) and 0.10 larger than in those with the APOE2/3 genotype (95% CI –0.04 to 0.24).

In contrast, responses of LDL-cholesterol to *cafestol* tended to be largest in subjects with the E3/3 genotype and smallest in those with the APOE2/3 and those with the APOE3/4 genotype (Figure 3.3). The trial-adjusted response of LDL-cholesterol to cafestol was 0.11 mmol/l smaller in subjects with the APOE2/3 genotype (95% CI –0.35 to 0.13 mmol/L) and in subjects with the APOE3/4 or 4/4 genotype (95% CI –0.29 to 0.07 mmol/L) than in those with the APOE3/3 genotype. Responses of HDL-cholesterol to cafestol were –0.07 mmol/L smaller in subjects with the APOE3/4 or 4/4 genotype than in those with the APOE3/3 genotype (95% CI –0.14 to 0 mmol/L) and the responses of HDL-cholesterol in subjects with the APOE2/3 genotype were –0.05 mmol/L smaller than in those with the APOE3/3 genotype (95% CI –0.14 to 0.04 mmol/L). Responses of HDL-cholesterol to saturated fat did not clearly differ between subjects with the various APOE genotypes, as was the case for responses of LDL- and HDL-cholesterol to *trans fat* and to *dietary cholesterol* (Table 3.2).

The differences in response of serum cholesterol between subjects with the various APOE genotypes remained similar after adjustment for each of the following factors sex, age, body mass index, and change in weight.

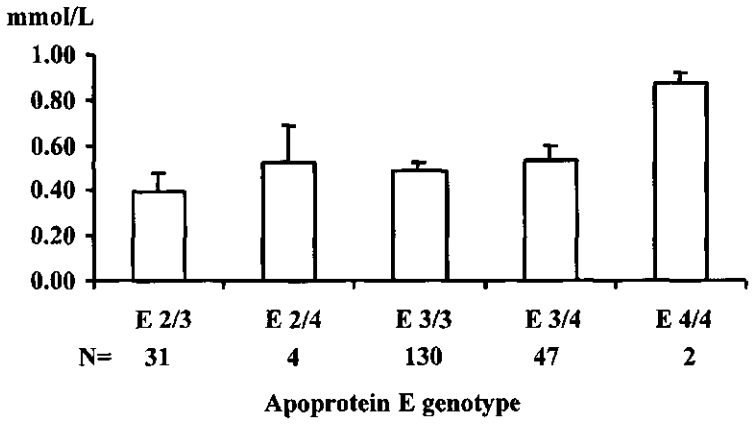

Figure 3.2 The mean response of low-density lipoprotein (LDL-)cholesterol to saturated fat in subjects with various apoprotein (APO)E-genotypes. Error bars indicate one standard error. Values of N indicate the number of subjects per genotype group.

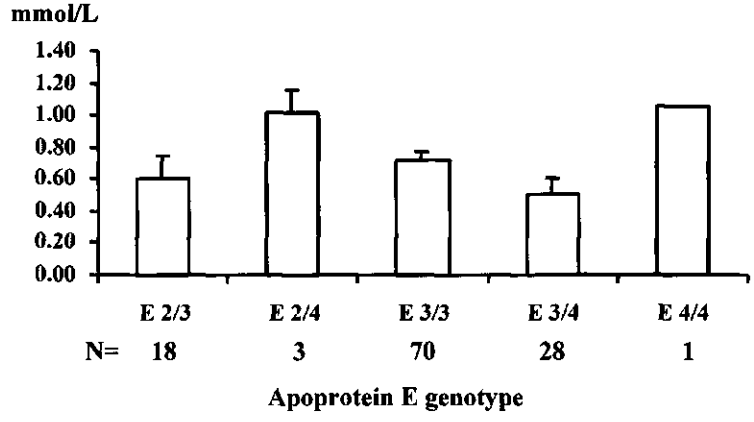

Figure 3.3 The mean response of low-density lipoprotein (LDL-)cholesterol to cafestol in subjects with various apoprotein (APO)E-genotypes. Error bars indicate one standard error. Values of N indicate the number of subjects per genotype group.

Table 3.2 Adjusted responses (mean ± standard error) of total cholesterol, high-density lipoprotein (HDL-) and low-density lipoprotein (LDL-) cholesterol to saturated fat, *trans* fat, cholesterol, and cafestol in subjects with the apoprotein (APO)E2/2 or 2/3, 3/3, and 3/4 or 4/4 genotypes. The mean responses of men and women are between brackets.

	Total cholesterol		HDL-cholesterol		LDL-cholesterol	
	All	(Men; Women)	All	(Men; Women)	All	(Men; Women)
Saturated fat[*,†]			*mmol/L*			
APOE2/2 and 2/3	0.49 ± 0.07	(0.53; 0.44)	0.05 ± 0.02	(0.04; 0.05)	0.49 ± 0.06	(0.54; 0.45)
APOE3/3	0.51 ± 0.04	(0.54; 0.48)	0.04 ± 0.01	(0.04; 0.03)	0.51 ± 0.03	(0.51; 0.52)
APOE3/4 and 4/4	0.61 ± 0.05	(0.62; 0.58)	0.04 ± 0.02	(0.04; 0.06)	0.59 ± 0.04	(0.62; 0.55)
Trans fat[‡]						
APOE2/2 and 2/3	0.33 ± 0.10	(0.31; 0.34)	-0.11 ± 0.03	(-0.13ab; -0.10)	0.40 ± 0.08	(0.40; 0.39)
APOE3/3	0.17 ± 0.05	(0.14; 0.19)	-0.15 ± 0.02	(-0.14a; -0.16)	0.28 ± 0.05	(0.25; 0.29)
APOE3/4 and 4/4	0.22 ± 0.07	(0.28; 0.16)	-0.11 ± 0.02	(-0.06b; -0.15)	0.29 ± 0.07	(0.30; 0.28)
Dietary cholesterol[*,§]						
APOE2/2 and 2/3	0.37 ± 0.08	(0.36; 0.30)	0.13 ± 0.02	(0.13a; 0.08)		
APOE3/3	0.44 ± 0.06	(0.43; 0.48)	0.11 ± 0.01	(0.11ab; 0.12)		
APOE3/4 and 4/4	0.36 ± 0.08	(0.34; 0.38)	0.08 ± 0.02	(0.07b; 0.10)		
Cafestol[‡]						
APOE2/2 and 2/3	0.89 ± 0.14	(1.00; 0.66)	-0.08 ± 0.04ab	(-0.14; -0.03ab)	0.61 ± 0.12	(0.78; 0.37)
APOE3/3	0.99 ± 0.07	(1.06; 0.84)	-0.03 ± 0.02a	(-0.06; 0.05a)	0.72 ± 0.06	(0.79; 0.62)
APOE3/4 and 4/4	0.85 ± 0.09	(1.01; 0.56)	-0.10 ± 0.03b	(-0.09; -0.11b)	0.61 ± 0.08	(0.78; 0.36)

Significant differences ($P<0.05$, least-significant difference test) between responses of subjects with the APOE2/2 or 2/3, 3/3, and 3/4 or 4/4 genotypes are indicated by different fonts.

[*] Adjusted responses to saturated fat and dietary cholesterol are least-squares estimations of the response after adjustment for subject and trial.

[†] Responses of LDL-cholesterol to saturated fat were missing for three subjects with the APOE2/3, three subjects with the 3/3, and four subjects with the 4/4 genotype.

[‡] Adjusted responses to *trans* fat and cafestol are least-squares estimations of the response after adjustment for trial.

[§] Responses of HDL-cholesterol to dietary cholesterol were missing for one subject with the APOE3/3 and four subjects with the 3/4 genotype. Responses of LDL-cholesterol to dietary cholesterol were not analysed because the number of observations was too low.

Some authors, however, suggest that the effect of the APOE polymorphism on response differs between men and women. We therefore also analyzed men and women separately. The effect of the APOE polymorphism on the response of LDL-cholesterol to saturated fat was similar in men and women. Responses of HDL-cholesterol to *trans* fat were -0.07 mmol/L smaller in men with the APOE3/4 or 4/4 genotype than in men with the APOE3/3 genotype (95% confidence interval -0.01 to -0.14 mmol/L). Responses of HDL to dietary cholesterol were -0.07 mmol/L smaller in men with the APOE3/4 or 4/4 genotype than in men with the APOE2/3 genotype (95% confidence interval -0.01 to -0.13 mmol/L). Responses of HDL-cholesterol to cafestol were 0.16 mmol/L larger in women with the APOE3/3 genotype than in women with the APOE3/4 or 4/4 genotype (95% confidence interval 0.04 to 0.28 mmol/L) (Table 3.2).

Discussion

The present study showed that normolipidemic subjects with the APOE3/4 or E4/4 genotype tended to have a larger response of LDL-cholesterol to saturated fat than those with the APOE3/3 genotype. In contrast, they had similar responses to *trans* fat and dietary cholesterol and they tended to have a smaller response to cafestol.

We pooled data of 26 trials in order to obtain a large number of subjects to study the relation between APOE genotype and response of cholesterol to specific dietary changes. We used rigorously standardized laboratory procedures and multiple measurements per subjects. The precision of the estimation of serum cholesterol response to saturated fat and dietary cholesterol was further improved in the subjects who participated in more than one trial with a similar treatment. The precision of the responses reported here is therefore higher than in many other trials. The total number of subjects in our study vastly exceeded that in other studies of the relation between APOE polymorphism and cholesterol response, and even the number of subjects per treatment, i.e. saturated fat or dietary cholesterol, was higher than in any other previous study.

Factors such as a subject's sex and body mass index may affect the association between APOE genotype and response (17,28,39-43). However, results remained similar after adjustment for the subject's sex, age, body mass index, or change in weight. When we analyzed men and women separately, the effect of the APOE polymorphism on the response of HDL-cholesterol to various dietary treatments differed between men and women. However, the differences between men and women may be due to chance, because the examination of several subgroups will increase the risk of chance associations.

Table 3.3 The mean baseline level* (± standard error) of serum total cholesterol by apoprotein (APO)E genotype.

	APOE2/2 or 2/3	APOE3/3	APOE3/4 or 4/4
		mmol/L	
Saturated fat	4.06 ± 0.15a	4.63 ± 0.07b	4.78 ± 0.11b
Trans fat	4.22 ± 0.21a	4.84 ± 0.15b	4.92 ± 0.18b
Dietary cholesterol	4.57 ± 0.19a	5.33 ± 0.11b	5.43 ± 0.18b
Cafestol	4.43 ± 0.16a	4.62 ± 0.09a	4.96 ± 0.14b

Values with different fonts differ significantly (P<0.05).

* The baseline level was measured during the treatment that lowered serum cholesterol, the placebo treatment or the diet without the cafestol or cholesterol supplement.

Some authors suggested that effects of the APOE polymorphism on response may be more pronounced when levels of baseline cholesterol differ between subjects with the various APOE genotypes. In the present study, levels of total cholesterol measured while subjects consumed their habitual diet (Table 3.1) and baseline levels of total cholesterol (Table 3.3) were lower in subjects with the APOE2/2 or E2/3 genotype and somewhat higher in subjects with the APOE3/4 or E4/4 genotype than in those with the APOE3/3 genotype. These differences between subjects with various APOE genotypes in levels of cholesterol mirrored the differences in responses of cholesterol to saturated fat, but not to *trans* fat, dietary cholesterol, or cafestol. It is possible that the differences in levels mainly reflect differences in response to the amount of saturated fat, because saturated fat has a larger effect on the level of serum cholesterol than the amount of dietary cholesterol, *trans* fat, or cafestol in the habitual or baseline diet.

In other studies in which the amount of *saturated fat* was changed, subjects with the ε4-allele had either larger (13-16), similar or lower (24,25,34,54) responses of serum cholesterol than those with the APOE3/3, E3/2, or E2/2 genotype. Only two of these studies had at least 10 subjects with an ε2-allele (16,24). In one of these two studies, subjects with the ε2-allele had a smaller response to changes in dietary fat than those with the APOE3/3 genotype (16), whereas responses were similar or larger in the other (24) (Figure 3.4). Only four studies had at least 10 subjects with an ε4-allele (13,16,24,34). Subjects with an ε4-allele had a larger response than those with the APOE3/3 genotype in one study with normolipidemic men (16) and in one out of two studies with hyperlipidemic men and women (13), but they had a similar response in one study with normolipidemic men and women (24). It may be that the effect of the APOE polymorphism is easier to detect in men than in women, because men are more responsive to saturated fat than women (37). Results of the study of Sarkkinen et al (13) suggest that the response is larger in subjects with the APOE4/4 genotype

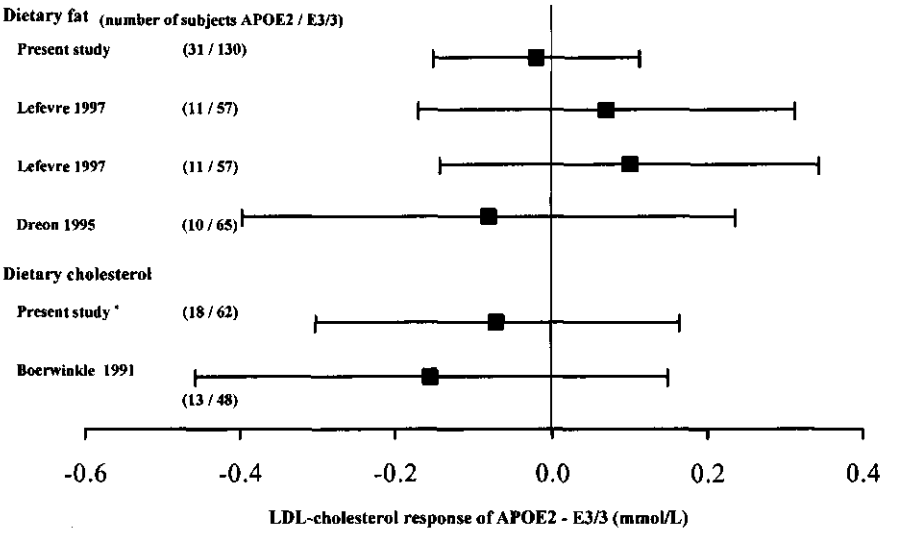

Figure 3.4 The difference in response of low-density lipoprotein (LDL-)cholesterol to an increase in dietary fat and dietary cholesterol between subjects with the apoprotein (APO)E2/2 or 2/3 genotype and those with the APOE3/3 genotype and 95% confidence intervals of the difference in response.

[a] We used data on the response of total cholesterol to dietary cholesterol instead of LDL-cholesterol, because of the small number of subjects with an LDL-cholesterol response.

than in those with the APOE3/4 genotype (Figure 3.5). Thus, the data from the present study and those from previous studies indicate that the APOE2/3 genotype may not affect the response of LDL-cholesterol to a change in saturated fat, the APOE3/4 genotype slightly enhances the response, whereas the APOE4/4 genotype strongly enhances the response.

In the present study, the response to a decrease in the amount of *dietary cholesterol* was not related to APOE genotype. One problem in studying dietary cholesterol is that its effect on serum cholesterol level is smaller than that of saturated fat (55). Therefore, one may need more subjects to show a possible effect of the APOE genotype on response of serum cholesterol to dietary cholesterol. Other studies either found subjects with the ε4 allele to be more responsive to dietary cholesterol than subjects without the ε4 allele (10,12,13,56), or that there were no differences (23,25,29,57). Only one study had at least 10 subjects with the ε2-allele and showed that subjects with the APOE2/3 genotype had a somewhat smaller response to dietary cholesterol than those with the APOE3/3 genotype (29)(Figure 3.4). Only three of these studies had at least 10 subjects with an ε4-allele (13,23,29). None of the three studies

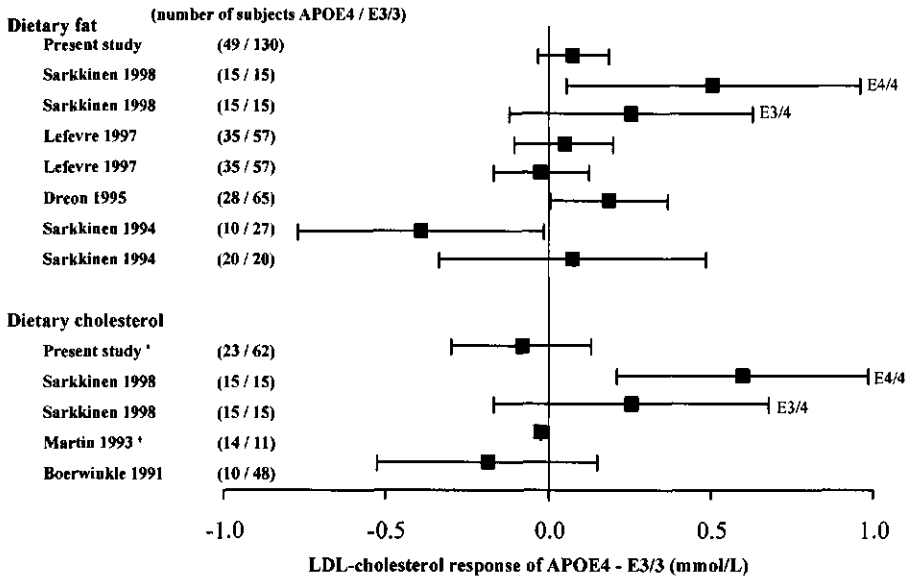

Figure 3.5 The difference in response of low-density lipoprotein (LDL-)cholesterol to an increase in dietary fat and dietary cholesterol between subjects with the apoprotein (APO)E3/4 or 4/4 genotype and those with the APOE3/3 genotype and 95% confidence intervals of the difference in response.

* We used data on the response of total cholesterol to dietary cholesterol instead of LDL-cholesterol, because of the small number of subjects with an LDL-cholesterol response.

† Martin et al did not report sufficient data to calculate a 95% confidence interval.

found a significant difference in response between normo- and hyperlipidemic men and women with the APOE3/3 and 3/4 genotype. The study of Sarkkinen et al (13) also included subjects with the APOE4/4 genotype. These subjects had a significantly larger response than those with the APOE3/3 or 3/4 genotype (Figure 3.5). Because all studies that found an effect of the APOE polymorphism on response to dietary cholesterol were Finnish, the high prevalence of the ε4-allele in Finland might be an explanation for the seemingly inconsistent results. Therefore, the small number of subjects with the APOE4/4 genotype (N=2) in the present study might explain the absence of any effect in the present study. Thus, despite the fact that we did not find any significant difference in response between the various APOE genotypes, the ε4-allele may still enhance the response of LDL-cholesterol to a change in dietary cholesterol but only in subjects with the APOE4/4 genotype.

We did not find a clear effect of the APOE genotype on the response of serum cholesterol to *trans* fat. However, the relatively small effect of *trans* fat on serum total cholesterol and LDL-cholesterol might have obscured such an effect.

The response of LDL-cholesterol to *cafestol* tended to be lower in subjects with the ε4 allele than in subjects with the APOE3/3 genotype. The response of LDL-cholesterol to a change in the intake of oat or wheat bran or to lipid-lowering drugs pravastatin and lovastatin was also smaller in subjects with the ε4 allele than in those without the ε4 allele (58-60). These findings confirm that cafestol raises LDL via pathways different from those for dietary cholesterol or fat (61).

In the present study, we investigated the effect of the APOE polymorphism on the response of serum cholesterol by itself and not in combination with other genetic polymorphisms. It is possible that knowledge of the APOE polymorphism in combination with knowledge of other polymorphisms may be of use in the identification of subjects who respond to diet. At this regard, one study showed that the APOC3 SstI polymorphism affected the expression of hyperlipidemia in subjects with the APOE2/2 genotype (62).

In conclusion, the APOE effects were small. In view of these results, knowledge of the APOE genotype by itself in individual patients with high cholesterol levels may be of little use in the selection of an effective therapeutic approach.

Acknowledgments

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4

Associations between 10 genetic polymorphisms and the serum lipid response to dietary fat, cholesterol, and cafestol in humans

Rianne M. Weggemans, Peter L. Zock, Jose M. Ordovas,
Jennifer Ramos-Galluzzi, Martijn B. Katan

Abstract- The response of serum cholesterol to diet may in part be an innate characteristic. However, previous studies on the effects of polymorphisms in candidate genes on response to dietary treatments were not always consistent and often involved a small number of subjects.

We now studied the effect of 10 genetic polymorphisms on responses of serum cholesterol and lipoproteins to diet. To this end, we used data on responses of serum cholesterol to dietary saturated and *trans* fat, cholesterol, and the coffee diterpene cafestol as measured in 26 previous dietary trials. We combined these responses with newly obtained data on 10 genetic polymorphisms from 405 mostly normolipidemic former participants in these trials.

The response of serum low density lipoprotein (LDL-) cholesterol to diet was somewhat smaller in subjects with the apoprotein (APO)A4 347-1/1 genotype than in those with the APOA4 347-2 allele and in subjects with the APOA4 360-2/2 genotype than in those with the APOA4 360-1 allele. Subjects with the cholesteryl ester transfer protein (CETP) TaqIb-1 allele had smaller responses of high-density lipoprotein (HDL-)cholesterol to diet than those with the CETP TaqIb-2/2 genotype. The effects of the other seven candidate polymorphisms were either inconsistent with results in previous studies or need to be replicated in other studies.

In conclusion, the APOA4 347 and 360 and CETP TaqIb polymorphisms may affect the response of serum cholesterol to diet. The effects, however, are small. Therefore, knowledge of these genotypes by themselves is of little use in the identification of subjects who do not benefit from dietary treatment.

Submitted

Introduction

The response of serum cholesterol to dietary changes is to some extent reproducible within a subject and varies considerably between subjects (1). Theoretically the response may be affected by polymorphisms in genes which encode proteins that play a role in the cholesterol metabolism. Identification of these genetic polymorphisms may be of help in the identification of hypercholesterolemic subjects who will or will not benefit from dietary treatment. It may also clarify the role of certain proteins in the cholesterol metabolism.

Evidence is growing that variation at several loci affects lipid responses. The APOE polymorphism, which has been most extensively studied, may explain some of the variation in response. In addition, polymorphisms in the APOA1, APOA4, APOB, CETP and LPL genes may affect the response (as reviewed in (2-4)). However, in most instances the evidence for these relations is limited to few studies that vary in kind and duration of dietary treatment and in subject characteristics. Furthermore, the strength of these studies is often limited by the small number of subjects.

We now studied in 405 subjects the relation between 10 genetic polymorphisms and the response of serum cholesterol and lipoproteins to dietary factors known to affect plasma lipoprotein levels.

Methods

Subjects

Our department has performed 26 controlled trials on diet and blood lipids with a total of 670 subjects between 1976 and 1996 (1,5-21). The data of these trials have been carefully archived. Therefore, we were able to pool the data and to calculate individual responses as well as mean responses of treatment groups. These mean responses agreed with those published at the time trials were done, showing that our data retrieval and cleanup had been successful. At the time of the trials, no DNA samples were collected. In order to obtain DNA samples, we traced the former participants in 1996 and 1997 and managed to find 609 of them. Nineteen former participants were seriously ill or had died. We could not trace another 42 subjects. The protocol of the present study, which was approved of by the Ethical Committee of Wageningen Agricultural University, was explained to the other 609 subjects. Sixty of the 609 subjects refused to participate. Of those 60, 16 did not want to participate because of the genetic aspect of the study, the other 44 because of various other reasons. We obtained informed consent and collected DNA of the remaining 549 subjects. We sampled blood from 486 subjects and collected mouth swabs from the other 63, because these 63 subjects did not live in the Netherlands anymore or could not give blood for other reasons. We

excluded 144 subjects, who had only received a control diet or a placebo treatment. Of the remaining 405 subjects, 117 had participated in two trials with a different treatment (e.g. saturated fat and cafestol), and 8 had participated in three trials with a different treatment. In addition, 40 % of the subjects in the saturated fat trials participated in more than one saturated fat trial or in a cross-over trial with three treatments, from which two responses were calculated. This also held for 10 % of the subjects in the *trans* fat trials, 55 % of those in the dietary cholesterol trials, and 15 % of those in the cafestol trials. Thus, our data consisted of 903 responses to the various treatments from a total of 206 men and 199 women. The mean age of the subjects was 29 ± 12 years (mean ± standard deviation), mean cholesterol level while subjects consumed their habitual diet was 4.95 ± 0.88 mmol/L, mean body mass index 22 ± 3 kg/m^2, and 19 % of the subjects were smokers. At the time of the trials the subjects were healthy as indicated by a medical questionnaire, and by the absence of anemia, glucosuria, and proteinuria.

Characteristics of trials

All trials were designed to study responses of serum cholesterol to changes in the diet and had been approved by the appropriate medical ethical committees. We pooled data on response of serum cholesterol to saturated fat of seven trials (5-11), to *trans* fat of two trials (9,10), to dietary cholesterol of eight trials (1,12-15), and to the coffee diterpenes cafestol and kahweol of nine trials (16-21). Seven trials had a cross-over design (6,9-11,20), 14 a parallel design with a control group (1,5,7,8,12,14,15,18,19,21), and five a before-and-after or linear design without a control group (13,16,17,19). The number of participants per trial ranged from 3 to 94 (median was 23) and the duration of the treatment ranged from 1.5 to 14 weeks (median was 3 weeks). All trials are described in more detail elsewhere (22).

Diets and supplements

All food was supplied in the seven trials with saturated fat (5-11), in the two trials with *trans* fat (9,10), and in three of the eight trials with dietary cholesterol (1,12,14,15). In the saturated fat and *trans* fat trials, the saturated or *trans* fat was exchanged for an equal amount of energy as mono- or polyunsaturated fat, or, in one saturated fat trial, for carbohydrates. In the four trials on dietary cholesterol without complete food supply, subjects received eggs as a supplement during the treatment period and guidelines for a diet low in cholesterol during the control period (1,12-14). In one other trial on dietary cholesterol, subjects received all foods during the treatment period and received dietary guidelines during the control period (15). In the nine cafestol trials subjects received coffee, coffee grounds, coffee oil, or cafestol and kahweol as a supplement and consumed their habitual diet throughout the trial (16-21).

Genetic polymorphisms and cholesterol response

Available data

Information on genotype and responses of total cholesterol and HDL- and LDL-cholesterol to saturated fat was available for 221 subjects, to *trans* fat for 86 subjects, to dietary cholesterol for 110 subjects, and to cafestol for 121 subjects.

For the present analysis we also used data on sex, age, body mass index, and change in weight during the trial, which might affect the relation between response of cholesterol and genetic polymorphisms (23-32). In addition, we used data on serum cholesterol levels, which were measured when subjects consumed their habitual diets.

Laboratory analyses

Laboratory personnel were never aware of the subject's treatment. Serum cholesterol levels were determined in at least two serum samples per treatment, which were obtained on separate days. All sera from one subject were analyzed within the same run. The coefficient of variation within one run for control samples ranged from 0.7 to 2.9 %. In all trials accuracy was checked by the analysis of three serum pools of known value provided by the U.S. Centers for Disease Control (Atlanta, GA). The mean bias with regard to the target values of the Centers for Disease Control pools ranged from –2.0 % to 1.1 % for total cholesterol and from -3.2 % to 3.3 % for HDL cholesterol (1,5-21). LDL cholesterol was calculated using the Friedewald's equation (33).

We isolated DNA from fresh blood and from mouth swabs by "salting-out" procedures (34-36). We used various methods for the assessment of the genotypes (Table 4.1) (37-48). Results of analyses on the effect of the APOE polymorphism on response have been published previously (49). Two investigators independently interpreted all gels and, in case the interpretation differed, the genotype was reanalyzed. We analyzed 35 DNA samples in duplicate to check accuracy of analytical procedures. The investigators who interpreted the gels did not know which samples were duplicates. The genotypes of all duplicate samples agreed. We labeled the most frequent allele of each polymorphism with "1" and the least frequent allele with "2".

Statistical methods

In all trials we defined individual responses of cholesterol as the level of serum cholesterol at the end of the treatment that increased cholesterol minus the level either at the end of the treatment that lowered cholesterol, the placebo treatment, or the diet without the cafestol or cholesterol supplement. In cross-over trials with three treatments, for instance a saturated, mono-unsaturated, and poly-unsaturated fat diet, we calculated one response to the substitution of mono-unsaturated for saturated fat and one response to the substitution of

Table 4.1 Overview of polymorphisms at candidate genes, the change in the gene and in the protein, the most frequent allele and its frequency, and reference for method used.

Polymorphism	Change in gene	Change in protein	Most frequent allele	Allele frequency	Reference for method used (PCR & restriction)
APOA1 –75G/A	G → A at –75 bp from transcription start site, within promoter region	No	G	0.83	(37,38)
APOA1 83C/T or 84G/A	C → T at 83 bp and/or G → A at 84 bp from transcription start site within intron 1	No	C	0.91	(37,38)
APOA4 Thr347Ser	A → T at codon 347 within exon 3	Thr → Ser	A	0.79	(39,40)
APOA4 Gln360His	G → T at codon 360 within exon 3	Gln → His	G	0.90	(40)
APOB EcoRI	G → A at codon 4154 within exon 29	Glu → Lys	G	0.80	(41)
APOC3 SstI	G → C at 3238 bp within the 3' non-coding region	No	G	0.94	(42)
APOE 2/3/4	E2: C → T at codon 158 within exon	Arg → Cys	E2	0.09	(43,44)
	E4: T → C at codon 112 within exon	Cys → Arg	E4	0.13	
CETP TaqIB	Silent base change at 227 bp within inton 1	No	T+	0.54	(45)
FABP2 Ala54Thr	A → T at codon 54 within exon 2	Ala → Thr	A	0.77	(46)
LPL Ser447Stop	C → G at codon 447 within exon 9	SerGly → Stop	C	0.94	(47)
MTP –493G/T	G → T at –493 bp from transcription start site, within promoter region	No	G	0.71	(48)

APO, apoprotein; CETP, cholesteryl ester transfer protein; FABP2, intestinal fatty acids binding protein; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; A, Adenine; C, Cytosine; T, Thymine; G, Guanine; + denotes restriction site present; - denotes restriction site absent.

poly-unsaturated for saturated fat. Because the cholesterol level on the saturated fat diet in this instance was used in the calculation of the two responses, these are dependent. This does not affect the validity of the estimate, but it may slightly affect the standard error and thus the P-value.

In the crude analysis, we studied the effect of each genotype on response to each of the four treatments, irrespective of whether a subject participated in more than one trial with a similar treatment.

In the adjusted analyses, we adjusted the relation between genetic polymorphisms and responses to saturated fat and dietary cholesterol for potential confounders subject, trial, and APOE genotype and we adjusted the response to *trans* fat and cafestol for trial and APOE genotype. We adjusted for subject because 40 % of the subjects in the saturated fat trials and 55 % of the subjects in the dietary cholesterol trials participated in more than one trial with a similar treatment or in a cross-over trial with three treatments (7,8,11). We adjusted for trial because there were background differences between the trials in background diet, duration of treatment and time of the year the trial was performed. If a trial consisted of more than one treatment, we created factors indicating each treatment within a trial. We also adjusted for APOE genotype, because the APOE genotype may affect the response to diet (50). In additional analyses we further adjusted for the subject characteristics age, body mass index, and change in weight, because these are potential confounders of the relation between the various genotypes and serum cholesterol response (27-32). We also analyzed interactions between the various genotypes and sex in relation to response, because some previous studies reported sex-specific effects of some of the genotypes on response (24-27). In case of significant interaction we performed the analyses for men and women separately. We tested differences in the response of serum cholesterol between subjects with various genotypes by analysis of variance. In case of significant differences, group means were compared by Fisher's Least Significant Difference test for multiple comparisons (51).

Results

Overall, serum LDL-cholesterol increased upon replacement of unsaturated fat for saturated fat by 0.46 ± 0.39 mmol/L (mean ± standard deviation), upon replacement of *cis*-unsaturated fat for *trans* fat by 0.30 ± 0.32 mmol/L, upon addition of dietary cholesterol by 0.31 ± 0.55 mmol/L, and upon suppletion of cafestol by 0.68 ± 0.50 mmol/L. The level of serum HDL-cholesterol increased in response to saturated fat by 0.04 ± 0.16 mmol/L and to dietary cholesterol by 0.08 ± 0.12 mmol/L, it decreased in response to *trans* fat by –0.13 ± 0.11 mmol/L and to cafestol by –0.04 ± 0.16 mmol/L.

For all polymorphisms, the genotype distributions were in accordance with Hardy-Weinberg equilibrium and the rare allele frequencies were similar to those reported in other European Caucasian populations (Table 4.1).

Saturated fat

The response of total cholesterol to *saturated fat* was significantly affected by the APOA4 360 polymorphism, being the lowest in the three subjects with the 2/2 genotype (crude P and P after adjustment for subject, trial and APOE-genotype = 0.05). The small number of subjects with the APOA4 360-2/2 genotype, however, limit these results (Table 4.2). In addition, the response of LDL-cholesterol was significantly influenced by the APOB EcoRI polymorphism, being lower in those with the APOB EcoRI-1/2 genotype than in those with the 1/1 genotype (crude P=0.03; adjusted P=0.05) and by the MTP –493 polymorphism, being larger in those with the 2/2 genotype than those with the 1 allele (crude P=0.08; adjusted P=0.04) (Table 4.3).

The response of HDL-cholesterol to *saturated fat* was associated with three polymorphisms, the APOC3 SstI (crude P=0.11; adjusted P=0.04), CETP TaqIb (crude and adjusted P=0.04) and LPL447 (crude P=0.08; adjusted P=0.03). Subjects with APOC3 SstI-1/1, CETP TaqIb-2/2 and LPL 447-1/1 genotype were more responsive than those with the respective other genotypes (Table 4.4).

Trans fat

The effect of the APOB polymorphism on the response of LDL-cholesterol to *trans fat* was similar to that on its response to *saturated fat,* with those with the APOB EcoRI-1/1 genotype tending to be more responsive than those with the 2 allele (crude P=0.12; adjusted P=0.05) (Table 4.3).

Dietary cholesterol

Determinants of the response of total cholesterol and LDL-cholesterol to *dietary cholesterol* were the CETP TaqIb and the FABP2 54 polymorphisms. Those with the CETP TaqIb-1/1 and 1/2 genotypes had smaller responses than those with the 2/2 genotype (total cholesterol response crude P=0.02; adjusted P=0.01; LDL-cholesterol response *1/1 vs 2/2* crude P=0.03; adjusted P=0.03; *1/2 vs 2/2*, crude P=0.06; adjusted P=0.01), whereas the response of total cholesterol was smaller in subjects with the FABP2 54-1/1 genotype than in those with the 1/2 genotype (crude P=0.05; adjusted P=0.01) but somewhat larger than in those with the 2/2 genotype (Tables 4.2 and 4.3).

Table 4.2 Unadjusted response of serum total cholesterol (mean ± standard error) to saturated fat, *trans* fat, dietary cholesterol, and the coffee diterpene cafestol in subjects with various genotypes.

		Saturated fat		*Trans* fat		Dietary cholesterol		Cafestol
Genotype	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE
APO-A1 –75G/A								
11	208	0.55 ± 0.03	63	0.18 ± 0.05	154	0.40 ± 0.04	89	0.89 ± 0.06
12	69	0.54 ± 0.06	22	0.27 ± 0.08	58	0.46 ± 0.07	43	0.89 ± 0.09
22	12	0.38 ± 0.12	2	0.19 ± 0.18	2	0.50 ± 0.26	2	1.39 ± 0.31
APO-A1 83C/T or 84G/A								
11	267	0.54 ± 0.03	81	0.21 ± 0.04	200	0.42 ± 0.03	124	0.93 ± 0.05*a*
12	22	0.60 ± 0.12	6	0.11 ± 0.21	14	0.44 ± 0.13	10	0.46 ± 0.11*b*
APO-A4 Thr347Ser								
11	176	0.53 ± 0.04	47	0.16 ± 0.06	126	0.41 ± 0.04	76	0.84 ± 0.07
12	84	0.59 ± 0.05	31	0.30 ± 0.06	69	0.44 ± 0.05	34	0.96 ± 0.10
22	13	0.48 ± 0.13	5	0.26 ± 0.26	4	0.67 ± 0.18	2	1.28 ± 0.62
APO-A4 Gln360 His								
11	231	0.55 ± 0.03A	69	0.23 ± 0.05	176	0.44 ± 0.04	112	0.92 ± 0.06
12	50	0.52 ± 0.06A,B	16	0.13 ± 0.09	28	0.36 ± 0.09	19	0.76 ± 0.09
22	3	0.12 ± 0.02B	1	-0.22	1	0.58	1	-0.06
APO-B EcoRI								
11	199	0.56 ± 0.03	59	0.24 ± 0.04	146	0.43 ± 0.04	89	0.89 ± 0.06
12	104	0.47 ± 0.05	32	0.15 ± 0.07	65	0.41 ± 0.06	44	0.89 ± 0.09
22	8	0.55 ± 0.11	2	0.12 ± 0.36	9	0.27 ± 0.09	2	0.85 ± 0.16
APO-C3 SstI								
11	271	0.54 ± 0.03	78	0.21 ± 0.04	191	0.42 ± 0.03	120	0.88 ± 0.06
12	35	0.50 ± 0.07	14	0.17 ± 0.14	29	0.38 ± 0.08	15	0.93 ± 0.12

Table 4.2 continued		Saturated fat		*Trans* fat		Dietary cholesterol		Cafestol
Genotype	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE
CETP TaqIb								
11	70	0.58 ± 0.06	23	0.26 ± 0.09	65	0.36 ± 0.06A	34	0.88 ± 0.09
12	135	0.53 ± 0.04	43	0.26 ± 0.05	113	0.40 ± 0.04A,B	63	0.90 ± 0.08
22	70	0.57 ± 0.06	18	0.06 ± 0.09	29	0.59 ± 0.08B	17	0.90 ± 0.16
FABP2 Ala54Thr								
11	157	0.50 ± 0.04	53	0.22 ± 0.05	109	0.32 ± 0.04A	59	0.81 ± 0.08
12	103	0.54 ± 0.05	26	0.26 ± 0.07	64	0.53 ± 0.06B	41	0.99 ± 0.10
22	12	0.35 ± 0.18	5	-0.03 ± 0.14	13	0.26 ± 0.11A,B	5	0.86 ± 0.18
LPL Ser447Stop								
11	251	0.55 ± 0.03	74	0.20 ± 0.04	188	0.43 ± 0.03	111	0.92 ± 0.06
12	45	0.42 ± 0.07	15	0.23 ± 0.10	19	0.23 ± 0.10	18	0.70 ± 0.12
22	1	0.54			1	0.40		
MTP –493 G/T								
11	151	0.55 ± 0.04	41	0.19 ± 0.07	118	0.45 ± 0.04	56	0.96 ± 0.06
12	141	0.50 ± 0.04	46	0.23 ± 0.05	89	0.39 ± 0.05	67	0.86 ± 0.08
22	9	0.72 ± 0.18	4	0.16 ± 0.16	8	0.32 ± 0.11	7	0.86 ±0.27

#, number of responses; APO, apoprotein; CETP, cholesteryl ester transfer protein; FABP2, intestinal fatty acids binding protein; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein.

Values with different fonts differ significantly ($0.01 \leq P \leq 0.05$), *small fonts* indicate significant differences between genotypes in unadjusted values; *italic fonts* indicate significant differences after adjustment for subject, study and APOE genotype in the saturated fat and dietary cholesterol trials and for study and APOE genotype in the *trans* fat and cafestol trials; *capitals* indicate significant differences in unadjusted and adjusted response.

Table 4.3 Unadjusted response of serum LDL-cholesterol (mean ± standard error) to saturated fat, *trans* fat, dietary cholesterol, and the coffee diterpene cafestol in subjects with various genotypes.

	Saturated fat		*Trans* fat		Dietary cholesterol		Cafestol	
Genotype	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE
APO-A1 –75G/A								
11	208	0.48 ± 0.03	63	0.27 ± 0.04	36	0.35 ± 0.08	89	0.68 ± 0.05
12	69	0.48 ± 0.05	22	0.37 ± 0.08	18	0.42 ± 0.14	43	0.63 ± 0.08
22	12	0.27 ± 0.08	2	0.34 ± 0.18			2	0.84 ± 0.42
APO-A1 83C/T or 84G/A								
11	258	0.47 ± 0.02	81	0.30 ± 0.04	52	0.36 ± 0.07	124	0.69 ± 0.04A
12	22	0.49 ± 0.10	6	0.23 ± 0.22	2	0.74 ± 0.50	10	0.34 ± 0.11B
APO-A4 Thr347Ser								
11	171	0.47 ± 0.03	47	0.26 ± 0.05	32	0.34 ± 0.10	76	0.60 ± 0.06
12	81	0.51 ± 0.04	31	0.38 ± 0.06	17	0.41 ± 0.11	34	0.72 ± 0.08
22	12	0.43 ± 0.11	5	0.32 ± 0.23	2	0.74 ± 0.30	2	1.03 ± 0.60
APO-A4 Gln360 His								
11	226	0.48 ± 0.03	69	0.30 ± 0.04	43	0.39 ± 0.08	112	0.67 ± 0.05
12	46	0.45 ± 0.05	16	0.31 ± 0.07	8	0.34 ± 0.17	19	0.61 ± 0.09
22	3	0.15 ± 0.04	1	0.05			1	0.04
APO-B EcoRI								
11	194	0.50 ± 0.03A	59	0.34 ± 0.04*a*	33	0.41 ± 0.09	89	0.67 ± 0.05
12	100	0.40 ± 0.04B	32	0.23 ± 0.06*b*	19	0.34 ± 0.13	44	0.65 ± 0.08
22	7	0.44 ± 0.08A,B	2	0.20 ± 0.28*a,b*	2	0.11 ± 0.07	2	0.58 ± 0.08
APO-C3 SstI								
11	262	0.46 ± 0.02	78	0.31 ± 0.03	45	0.39 ± 0.08	120	0.66 ± 0.05
12	34	0.49 ± 0.07	14	0.24 ± 0.13	9	0.30 ± 0.12	15	0.67 ± 0.09

Table 4.3 continued		Saturated fat		*Trans* fat		Dietary cholesterol		Cafestol
Genotype	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE
CETP TaqIb								
11	68	0.51 ± 0.06	23	0.35 ± 0.09	18	0.27 ± 0.14A	34	0.65 ± 0.07
12	129	0.47 ± 0.03	43	-0.33 ± 0.04	29	0.35 ± 0.08A	63	0.64 ± 0.07
22	69	0.47 ± 0.05	18	0.19 ± 0.08	7	0.75 ± 0.15B	17	0.71 ± 0.13
FABP2 Ala54Thr								
11	153	0.47 ± 0.03	53	0.31 ± 0.04	28	0.23 ± 0.09	59	0.57 ± 0.06
12	98	0.49 ± 0.04	26	0.35 ± 0.06	16	0.49 ± 0.14	41	0.74 ± 0.08
22	12	0.32 ±0.12	5	0.14 ± 0.16	4	0.23 ± 0.08	5	0.66 ±0.11
LPL Ser447Stop								
11	244	0.47 ± 0.02	74	0.29 ± 0.04	49	0.36 ± 0.07	111	0.69 ± 0.05
12	44	0.41 ± 0.06	15	0.32 ± 0.09	3	0.16 ± 0.35	18	0.50 ± 0.08
22	1	0.55						
MTP -493 G/T								
11	148	0.46 ± 0.03*a*	41	0.30 ± 0.06	29	0.52 ± 0.09	56	0.74 ± 0.06
12	136	046 ± 0.03*a*	46	0.31 ± 0.05	1	0.05	67	0.63 ± 0.07
22	8	0.71 ± 0.14*b*	4	0.27 ± 0.15			7	0.57 ± 0.22

#, number of responses ; APO, apoprotein; CETP, cholesteryl ester transfer protein; FABP2, intestinal fatty acids binding protein; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein.

Values with different fonts differ significantly ($0.01 \leq P \leq 0.05$), *small fonts* indicate significant differences between genotypes in unadjusted values; *italic fonts* indicate significant differences after adjustment for subject, study and APOE genotype in the saturated fat and dietary cholesterol trials and for study and APOE genotype in the *trans* fat and cafestol trials; *capitals* indicate significant differences in unadjusted and adjusted response.

Table 4.4 Unadjusted response of serum HDL-cholesterol (mean ± standard error) to saturated fat, *trans* fat, dietary cholesterol, and the coffee diterpene cafestol in subjects with various genotypes.

	Saturated fat		*Trans* fat		Dietary cholesterol		Cafestol	
Genotype	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE
APO-A1 -75G/A								
11	214	0.06 ± 0.01	63	-0.12 ± 0.01	132	0.06 ± 0.01	89	-0.05 ± 0.02
12	71	0.01 ± 0.02	22	-0.13 ± 0.03	51	0.09 ± 0.02	43	-0.03 ± 0.03
22	12	0.01 ±0.03	2	-0.19 ± 0.03	2	0.10 ± 0.04	2	0.16 ± 0.12
APO-A1 83C/T or 84G/A								
11	267	0.04 ± 0.01	81	-0.12 ± 0.01	175	0.07 ± 0.01	124	-0.03 ± 0.02
12	22	0.06 ± 0.04	6	-0.21 ± 0.04	10	0.07 ± 0.04	10	-0.08 ± 0.05
APO-A4 Thr347Ser								
11	176	0.05 ± 0.01	47	-0.14 ± 0.02	108	0.07 ± 0.01	76	-0.05 ± 0.02
12	84	0.05 ± 0.02	31	-0.11 ± 0.02	60	0.07 ± 0.01	34	-0.02 ± 0.02
22	13	0.03 ± 0.04	5	-0.14 ± 0.03	4	0.13 ± 0.05	2	0.06 ± 0.10
APO-A4 Gln360 His								
11	231	0.04 ± 0.01	69	-0.12 ± 0.01	150	0.08 ± 0.01	112	-0.03 ± 0.02
12	50	0.08 ± 0.02	16	-0.15 ± 0.03	25	0.04 ± 0.02	19	-0.06 ± 0.03
22	3	-0.03 ± 0.05	1	-0.32	1	0	1	-0.21
APO-B EcoRI								
11	199	0.04 ± 0.01	59	-0.14 ± 0.02	122	0.07 ± 0.01	89	-0.04 ± 0.02
12	104	0.03 ± 0.01	32	-0.13 ± 0.02	60	0.07 ± 0.02	44	-0.06 ± 0.02
22	8	0.11 ± 0.05	2	-0.10 ± 0.03	8	0.08 ± 0.04	2	-0.06 ± 0.08
APO-C3 SstI								
11	271	0.04 ± 0.01*a*	78	-0.14 ± 0.01	162	0.08 ± 0.01	120	-0.04 ± 0.02
12	35	-0.01 ± 0.02*b*	14	-0.11 ± 0.04	28	0.04 ± 0.02	15	-0.07 ± 0.02

Table 4.4 continued		Saturated fat		*Trans* fat		Dietary cholesterol		Cafestol
Genotype	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE	#	Mean response ± SE
CETP Taq1b								
11	70	0.04 ± 0.02A,B	23	-0.12 ± 0.02	58	0.06 ± 0.01	34	-0.04 ± 0.03
12	135	0.03 ± 0.01A	43	-0.12 ± 0.02	96	0.07 ± 0.01	63	-0.03 ± 0.02
22	70	0.08 ± 0.02B	18	-0.15 ± 0.03	26	0.09 ± 0.02	17	-0.05 ± 0.04
FABP2 Ala54Thr								
11	157	0.03 ± 0.01	53	-0.13 ± 0.02	95	0.06 ± 0.01	59	-0.04 ± 0.02
12	103	0.06 ± 0.02	26	-0.11 ± 0.02	56	0.08 ± 0.02	41	-0.03 ± 0.03
22	12	-0.01 ± 0.06	5	-0.18 ± 0.04	11	0.07 ± 0.03	5	0.06 ± 0.06
LPL Ser447Stop								
11	251	0.05 ± 0.01*a*	74	-0.13 ± 0.01	165	0.08 ± 0.01	111	-0.03 ± 0.01
12	45	0 ± 0.03*b*	15	-0.13 ± 0.04	14	0.03 ± 0.04	18	-0.04 ± 0.04
22	1	-0.08			1	0.02		
MTP –493 G/T								
11	151	0.04 ± 0.01	41	-0.11 ± 0.02	103	0.06 ± 0.01	56	-0.06 ± 0.02
12	141	0.02 ± 0.01	46	-0.14 ± 0.02	76	0.09 ± 0.01	67	-0.03 ± 0.02
22	11	0.02 ± 0.04	4	-0.17 ± 0.01	1	0.05	7	-0.05 ± 0.05

#, number of responses; APO, apoprotein; CETP, cholesteryl ester transfer protein; FABP2, intestinal fatty acids binding protein; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein.

Values with different fonts differ significantly ($0.01 \leq P \leq 0.05$), *small fonts* indicate significant differences between genotypes in unadjusted values; *italic fonts* indicate significant differences after adjustment for subject, study and APOE genotype in the saturated fat and dietary cholesterol trials and for study and APOE genotype in the *trans* fat and cafestol trials; *capitals* indicate significant differences in unadjusted and adjusted response.

Genetic polymorphisms and cholesterol response

The response of HDL-cholesterol to *dietary cholesterol* was, like its response to *saturated fat*, somewhat larger in subjects with the APOC3 SstI-1/1 genotype than in those with the 2 allele (crude P=0.07; adjusted P=0.06)(Table 4.4).

Cafestol

The only polymorphism that affected the response of total cholesterol and LDL-cholesterol to *cafestol* was the APOA1 83 polymorphism, subjects with the APOA1 83-1/1 genotype had larger responses than those with the APOA1 83-1/2 genotype (crude P=0.01; adjusted P=0.01) (Tables 4.2 and 4.3).

Confounding and effect modification

The differences in response were not materially affected by adjustment for subject, trial, and APOE genotype. In addition, previous studies suggested that the effect of some genetic polymorphisms on the response to diet are sex-specific (24-27). However, most of the effects of the genetic polymorphisms on response were in the same direction in men and women, with the exception of the APOA4 347 and 360 and CETP TaqIb polymorphisms: The effect of the APOA4 347 polymorphism on response was larger in women than in men. In women with the 1/1 genotype the response of serum LDL-cholesterol to *trans fat* was –0.22 mmol/L smaller than that in women with the 1/2 genotype (95% confidence interval (CI), -0.44 to 0 mmol/L), whereas in men, the difference in response to *trans fat* was 0.02 mmol/L (95% CI, -0.21 to 0.25 mmol/L) (P for interaction = 0.07). The difference in response of LDL-cholesterol to *cafestol* was –0.38 mmol/L in women (95 % CI, -0.72 to -0.03 mmol/L) and 0.09 mmol/L in men (95% CI, -0.14 to 0.32) (P for interaction = 0.008).

The effect of the APOA4 360 polymorphism was opposite in men and women (P for interaction = 0.07), men with the APOA4 360-1/1 genotype had a 0.28 mmol/L larger response of total cholesterol to *dietary cholesterol* than men with the 1/2 polymorphism (95% CI, 0.03 to 0.53 mmol/L), whereas women with the 1/1 polymorphism had –0.14 smaller responses than women with the 1/2 polymorphism (95% CI, -0.43 to 0.14 mmol/L).

The effect of the CETP TaqIb polymorphism on the response of total cholesterol and LDL-cholesterol to *trans fat* was limited to women (P for interaction = 0.05), the response of LDL-cholesterol was 0.39 mmol/L larger in women with the 1/1 genotype than in those with the 2/2 genotype (95% CI, 0.06 to 0.72), whereas in men the difference was –0.07 (95% CI, -0.34 to 0.20 mmol/L).

Discussion

The present study shows that genetic polymorphisms may affect responses of serum lipids to various dietary changes in healthy humans. However, there was not one single genotype that largely determined a subject's lipid response to diet.

Quality of the data

We pooled data of 26 trials in order to obtain a large number of subjects to study the relation between genetic polymorphisms and response of cholesterol to specific dietary changes. We used rigorously standardized laboratory procedures and multiple measurements of cholesterol level per subject. The precision of the estimation of serum cholesterol response to saturated fat and dietary cholesterol was further improved in the subjects who participated in more than one trial with a similar treatment. The precision of the responses reported here is therefore higher than in many other trials. The total number of subjects in our study vastly exceeded that in other studies of the relation between genetic polymorphisms and cholesterol response, and even the number of subjects per treatment, i.e. saturated fat or dietary cholesterol, was higher than in any other previous study.

Confounding and effect modification

Subject characteristics such as body mass index, APOE genotype, and sex may affect the association between genetic polymorphisms and response (23,27-32). However, the present results were not materially affected by adjustment for age, body mass index, or change in weight. This might have been due to the narrow range in the distribution of these subject characteristics and does not rule out the possibility that these factors affect the relationship between genetic polymorphisms and response. Adjustment for APOE genotype did not affect the results. However, it may be that the APOE genotype, like possibly sex, does not act as a confounder but rather as an effect modifier of the relation between genetic polymorphisms and lipid response (52). If so, it is not appropriate to adjust for APOE genotype. However, analysis of effect modification by APOE genotype was not attainable, because of the small number of subjects within various sub-groups. When we analyzed men and women separately, the effect of the APOA4 347 and 360 and CETP polymorphisms were either opposite in men and women or limited to one of the two sexes. The differences between men and women, however, might have been due to chance, because the examination of several subgroups will increase the risk of chance associations.

Genetic polymorphisms and cholesterol response

Risk of chance findings

Overall, the risk of chance findings is 5 % in the present study that involved four dietary treatments, ten polymorphisms and three serum lipid values. This means that six out of 120 hypotheses tested might be false positive. To check that present results are not chance findings, we took into account the results of previous studies on response and on possible mechanisms by which the polymorphism affects the response. In addition, promising relations should be checked in dietary trials, which are designed to study the effect of a genetic polymorphism on the response of serum cholesterol to diet.

APOA1 polymorphisms

In the present study, there were no differences in response of serum LDL- and HDL-cholesterol between subjects with the various APOA1 –75 genotypes, which is in line with three previous studies (53-55), but not with all (56,57). The APOA1 –75 polymorphism is situated in the promoter region of the APOA1 gene. Studies on its effect on the cholesterol metabolism are also inconsistent (58-63). Therefore, it may be that the APOA1 –75 polymorphism does not directly affect the cholesterol response, but is in linkage disequilibrium with a functional mutation in the APOA1 or a nearby gene.

One of the polymorphisms that is related to the APOA1 –75 polymorphism is the APOA1 83 polymorphism (64,65). We found that the response of total and LDL-cholesterol to cafestol was smaller in subjects with the APOA1 83 1/2 genotype than in those with the 1/1 genotype. We do not know of any other study that related this polymorphism to the response to diet. The APOA1 83 polymorphism is located in the first intron. Therefore we cannot exclude the possibility that the APOA1 83 polymorphism is a marker of another functional mutation that possibly affects the LDL-cholesterol response.

APOA4 polymorphisms

In the present study, the response of LDL-cholesterol in subjects with the APOA4 347-2 allele was somewhat larger than in those with the 1/1 genotype. This effect was larger in women than in men. The overall effects are in the same direction as those in some other studies (66,67). In contrast, two other studies did not find any effect (68,69). The substitution of serine for threonine at position 347 of the apo-AIV produces changes in the secondary structure of the protein and a slight increase in hydrophilic profile in this position (70). However, the precise mechanism by which the APOA4 347 polymorphism may affect the response to diet is unknown. Thus, the APOA4 347-2 allele may, if anything, slightly enhance the response of LDL-cholesterol to diet.

In the present study, subjects with the APOA4 360-1/2 genotype had only slightly smaller responses of LDL-cholesterol to saturated and *trans* fat and cafestol than those with the 1/1 genotype, whereas subjects with the 2/2 genotype, who were all women, had considerably smaller responses of LDL-cholesterol. However, the number of subjects with the 2/2 genotype was very small. Furthermore, in our previous study the response to increased cholesterol intake of one man with the 2/2 genotype was similar to that of those with the other genotypes (71). The present differences in response between subjects with the 1/1 and 1/2 genotype are in line with those observed in some of the previous studies (71,72), albeit that other studies found a larger effect (26,27,73), whereas again other studies found a small opposite effect (66,68). In the present study, men with the 1/2-genotype were less responsive to dietary cholesterol than men with the 1/1-genotype. This sex-specific effect was also found in several other studies (26,27) and may be an explanation for the inconsistent results between the study of McCombs et al (73) and our previous study (71). The apoA-IV-2 isoform has more α-helical structure, is more stable in solutions and is more hydrofobic than the apoA-IV-1 isoform (70). Nevertheless, the mechanism by which the APOA4 360 polymorphism may affect the response in men is not known (39). In conclusion, the attenuating effect of the APOA4 A360-1/2 genotype on the cholesterol response to dietary cholesterol may be limited to men.

APOB EcoRI polymorphism

The response of LDL-cholesterol to diet was somewhat smaller in those with the APOB EcoRI-1/2 genotype than in those with the 1/1 and 2/2 genotype. In contrast with these findings, one extensive meta-analysis (74) showed that there were no differences between subjects with the APOB EcoRI-1/2 and 1/1 genotype, whereas subjects with the 2/2 genotype tended to have larger responses than those with the 1/1 genotype. One explanation for the opposite findings on the effect of the 2/2 genotype may be the small number of subjects with the APOB EcoRI-2/2 genotype in all studies. The APOB EcoRI polymorphism in exon 29 changes the amino acid sequence, but its functional role is unclear (74-76). We conclude for the present that the APOB EcoRI polymorphism may not affect the lipid response to diet.

APOC3 SstI polymorphism

In the present study, the response of HDL-cholesterol to saturated fat and dietary cholesterol was smaller in those with the APOC3 SstI-1/2 genotype than in those with the 1/1 genotype. In contrast, one previous study found no effect of the APOC3 polymorphism on response of serum lipids (54). In addition, another showed that there were no differences between the genotypes in response of HDL-cholesterol whereas the LDL-cholesterol response

of subjects with the 1/2 genotype was smaller than that of subjects with the 1/1 genotype (77). The APOC3 SstI polymorphism is situated in the 3' non-coding region of the APOC3 gene and may be functional, but may also be neutral or act as a genetic marker for another functional polymorphism (78). Thus studies so far are inconsistent and provide no convincing evidence that the APOC3 SstI polymorphism affects the response.

CETP TaqIb polymorphism

The present study showed that the response of LDL-cholesterol is smaller in those with the CETP TaqIb-1/2 than in those with the 1/1 genotype. In one other study, the response of LDL-cholesterol to fat was also somewhat smaller in those with the 1/2 than in those with the 1/1 genotype (66). In the present study, subjects with the 2/2 genotype had a somewhat larger response of HDL-cholesterol than those with the 1/1 and 1/2 genotype. In contrast, one previous study found that the response of HDL-cholesterol in patients with Type I diabetes was larger in those with the 1/1 genotype than in those with the 1/2 genotype upon consumption of a lipid-lowering diet (79), whereas another did not find any difference (66). If we assume that a larger lipid response leads to a higher lipid level, then the present effect of the CETP TaqIb polymorphism on HDL-cholesterol response will be in line with its effect on HDL-cholesterol levels in previous studies (25,45,80-83). These studies, however, are inconsistent with regard to whether the effect of the polymorphism on the cholesterol metabolism is sex-specific (25,80,82,84). Because the CETP TaqIB mutation is situated in intron 1, it is unlikely that this mutation is functional. Thus, the CETP genotype may be a marker for a mutation that affects the responses of serum HDL-cholesterol and LDL-cholesterol to dietary changes. One mutation that is in linkage disequilibrium with the CETP TaqIb polymorphism is a functional mutation in the promoter region of the CETP gene, CETP/-629 (85).

FABP2 54 polymorphism

The present study showed that subjects with the FABP2 54-1/2 genotype were more responsive and those with the 2/2 genotype less responsive than those with the 1/1 genotype. In contrast with the present findings, one study on the response of serum lipids to dietary fiber found that subjects with the 1/2 genotype were less responsive than those with the 1/1 genotype (86). However, another study found no effect of the FABP2 54 polymorphism on the levels of serum lipids (87). The FABP2 54 polymorphism gives rise to a structural change in the protein and may be functional, because the binding affinity for long-chain fatty acids in vitro is larger for the 2-isoform than for the 1-isoform (46). Nevertheless, the mechanism by

which this difference may affect the response is still unclear. Thus, the present findings should first be confirmed in another study.

LPL 447 polymorphism

In the present study, subjects with the LPL 447-2 allele had smaller responses of HDL-cholesterol to saturated fat than those with the 1/1 genotype. In contrast, one other study did not find any effect on the HDL-cholesterol response to dietary fat, whereas the response of LDL-cholesterol was significantly larger in subjects with the 1/2 genotype than in those with the 1/1 genotype (66). Furthermore, other studies showed that subjects with the LPL 447-2 allele had higher HDL-levels (88,89) than those with the 1/1 genotype. The polymorphism gives rise to a structural change in the protein and may be functional as the production of the 2 isoform is greater than that of the 1 isoform, leading to higher levels of LPL activity (90). However, the present effect of the LPL 447 polymorphism on response of HDL-cholesterol may have been a chance finding.

MTP –493 polymorphism

We found that subjects with the MTP –493-2/2 genotype had a larger LDL-response to saturated fat than those with the 1 allele. However, the number of subjects with the 2/2 genotype is small. We do not know of any other studies on the effect of this polymorphism on the response. Results of studies on the effect of the MTP polymorphism on the level of LDL-cholesterol are inconsistent (48,91). Although the MTP –493 polymorphism, which is situated in the promoter region of the MTP gene, may be of functional importance because it regulates the transcriptional activity by influencing allele-specific binding of nuclear proteins (48), evidence of any effect of the MTP polymorphism on response is weak.

Gene-gene interaction

In the present study, we investigated the effect of 10 polymorphisms by themselves. Overall, the individual polymorphisms explained up to 8 % of the variation in response of LDL-cholesterol and up to 4 % of the variation in response of HDL-cholesterol. We did not assess interactions between genetic polymorphisms, except for the interaction with sex. It is likely that knowledge of gene-gene interaction is of additional use in the identification of subjects who do not respond to diet.

Extrapolation

The subjects in the present study were mostly young and had normal or moderately elevated cholesterol levels. Therefore, we do not know whether the gene-diet interactions are similar in an older, hyperlipidemic population.

In conclusion, the APOA4 347 and 360 and CETP TaqIb polymorphism may affect the response of serum cholesterol to diet in healthy humans. However, the effects were small. Therefore, information on each of these genotypes alone is not sufficient to predict an individual's response to dietary treatment.

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5

The apoproteinA4 360-1/2 polymorphism and response of serum lipids to dietary cholesterol in humans

Rianne M. Weggemans, Peter L. Zock, Saskia Meyboom, Harald Funke, Martijn B. Katan

Abstract- The response of serum lipids to dietary changes is to some extent an innate characteristic. One candidate genetic factor that may affect the response of serum lipids to a change in cholesterol intake is variation in the apoproteinA4 gene, known as the APOA4 360-1/2 or apoA-IVGln360His polymorphism. However, previous studies showed inconsistent results.

We therefore fed 10 men and 23 women with the APOA4 360-1/1 genotype and 4 men and 13 women with the APOA4 360-1/2 or 2/2 genotype (carriers of the APOA4 360-2 allele) two diets high in saturated fat, one containing cholesterol at 12.4 mg/MJ, 136.4 mg/day, and one containing cholesterol at 86.2 mg/MJ, 948.2 mg/day. Each diet was supplied for 29 days in cross-over design.

The mean response of serum low-density lipoprotein cholesterol was 0.44 mmol/L (17 mg/dL) in both subjects with the APOA4 360-1/1 genotype and in subjects with the APOA4 360-2 allele (95 % confidence interval (CI) of difference in response, -0.20 to 0.19 mmol/L (-8 to 7 mg/dL)). The mean response of high-density lipoprotein cholesterol was also similar, 0.10 mmol/L (4 mg/dL), in the two APOA4 360 genotype groups (95 % CI of difference in response, -0.07 to 0.08 mmol/L (-3 to 3 mg/dL)).

Thus, the APOA4 360-1/2 polymorphism did not affect the response of serum lipids to a change in the intake of cholesterol in this group of healthy Dutch subjects who consumed a background diet high in saturated fat. Knowledge of the APOA4 360-1/2 polymorphism is probably not a generally applicable tool for the identification of subjects who respond to a change in cholesterol intake.

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Introduction

The response of serum lipids to dietary cholesterol varies between subjects. In some subjects, the response of serum lipids to an increased cholesterol intake is considerable, whereas in others the response is small. The response to dietary cholesterol is to some extent reproducible within a subject and may be in part an innate characteristic of a subject (1). There are a large number of candidate genetic factors that may affect the response (2). Identification of these genetic factors may contribute to the development of new tests to predict whether a subject with high serum lipid levels will benefit from a diet low in cholesterol. This may contribute to a more efficient treatment of subjects with high serum lipid levels. In addition, knowledge of genetic factors that determine the response of serum lipids to diet will help to gain insight into the mechanisms by which diet affects serum lipid levels.

One of the candidate genetic factors which may affect the response of serum lipids is the apoprotein (APO)A4 gene, which encodes the apoA-IV protein. ApoA-IV is synthesized in the intestine (3). While the precise function of apoA-IV is still unknown, some studies suggest that it plays a role in the absorption of dietary fat (4) and in the metabolism of high density lipoprotein (HDL-) cholesterol and triglyceride-rich particles. In vitro studies showed that apoA-IV activates lecithine:cholesterol acyltransferase (5) and regulates the activity of cholesteryl ester transfer protein (6) and lipoprotein lipase (7). One polymorphism in the APOA4 360 gene, the APOA4 360-1/2 polymorphism, is caused by a G to T substitution in exon 3 of the gene, which causes the glutamine-to-histidine substitution at position 360 in the apoA-IV protein (8). The apoA-IV-2 isoform has more α-helical structure, is more stable in solutions and is more hydrophobic than the apoA-IV-1 isoform. These distinctive features are associated with a higher affinity for phospholipid surfaces and increased catalytic efficiency of the lecithine:cholesterol acyltransferase activation in vitro (9,10). In addition, carriers of the apoA-IV-2 isoform have lower activity of plasma cholesteryl ester transport protein, higher apoA-IV concentrations (11), and slower apoA-IV catabolic rate in vivo (12).

Studies on the effect of the APOA4 360-1/2 polymorphism on the response of serum lipids to diet are not consistent. Some studies showed that the APOA4 360-2 allele attenuates the response of low density lipoprotein (LDL-) cholesterol to dietary cholesterol (13) or dietary cholesterol plus fat (14), whereas other studies did not show a difference between subjects with the APOA4 360-1/1 genotype and those with the APOA4 360-2 allele in terms of response of LDL-cholesterol to dietary cholesterol plus fat (15,16) or to dietary fat (17). These results may indicate that the APOA4 360-1/2 polymorphism affects the response of serum LDL-cholesterol to dietary cholesterol, but not to dietary fat.

We therefore tested the effect of the APOA4 360-1/2 polymorphism on the response of serum LDL-cholesterol to dietary cholesterol in a controlled experiment.

Methods

Subjects

The Ethics Committee of the Division of Human Nutrition and Epidemiology (Wageningen University, Wageningen, The Netherlands) approved of the study protocol. We recruited 200 subjects through advertisements in local newspapers and university and public buildings. We explained the aims and protocol of the study to the subjects. All subjects gave their written informed consent. We screened the subjects, mostly students living in or near the city of Wageningen, for the APOA4 360-1/2 polymorphism and identified 24 carriers of the APOA4 360-2 allele. We selected these 24 carriers and 47 subjects with the APOA4 360-1/1 genotype for a medical screening. The medical screening consisted of a medical questionnaire, hemocytometry, and the assessment of triglycerides and total cholesterol in serum and of protein and glucose in urine after a 12-hours fast. We excluded one subject with serum triglyceride levels over 3.0 mmol/L, two subjects with disease of the gastro-instestinal tract, one subject with glucosuria and two subjects with proteinuria. All other subjects were apparently healthy, as indicated by the medical questionnaire. None of them had anemia, glucosuria, or proteinuria and none were taking medications known to affect blood lipids. During the period between the medical screening and the beginning of the dietary trial, 13 subjects withdrew. Nineteen carriers of the APOA4 360-2 allele and 33 subjects with the APOA4 360-1/1 genotype started the dietary trial. Two carriers of the APOA4 360-2 allele dropped out during the dietary trial, one for personal reasons and one because of appendicitis. Seventeen carriers of the APOA4 360-2 allele, 16 Caucasians and one Hispanic, and 33 subjects homozygous for the APOA4 360-1 allele, 32 Caucasians and one Hispanic, completed the dietary trial (Table 5.1).

The two genotype groups had similar baseline characteristics, except that the APOE4-allele and APOA4 347-T allele were more common in subjects with the APOA4 360-1/1 genotype than in those with the APOA4 360-2 allele (Table 5.2). All subjects who completed the dietary trial received a financial reward.

Table 5.1 Selection of subjects with the apoprotein (APO) A4 360-1/1 genotype and carriers of the APOA4 360-2 allele.

	APOA4 360-1/1		APOA4 360-1/2 or 2/2	
Subjects recruited	176		24	
Excluded after genetic screening		129		0
Excluded after medical screening*		4		2
Withdrawal before the trial		10		3
Subjects entering the trial	33		19	
Drop out during the trial		0		2
Subjects finishing the trial	33		17	

* The medical screening consisted of a medical questionnaire, hemocytometry, and the assessment of triglycerides and total cholesterol in serum and of protein and glucose in urine after a 12-hours fast.

Design

The dietary trial was designed to detect a significant difference ($P<0.05$) in response of LDL-cholesterol between subjects with the APOA4 360-1/1 genotype and subjects with the APOA4 360-2 allele with a power of 80 % if the real population effect exceeded 0.27 mmol/L (10 mg/dL). This power calculation was based on a within-subject standard deviation of 0.27 mmol/L (10 mg/dL). In other studies at our laboratory, within-subject standard deviation was 0.35 mmol/L (13 mg/dL) (18). We expected that the four blood collections per period, instead of two, would decrease the within-subject standard deviation by about 0.08 mmol/L (3 mg/dL) (19).

The dietary trial consisted of two periods of 29 days, during which each subject consumed the diet low in cholesterol and the diet high in cholesterol in cross-over design (20). We included a 6-day wash-out period between the two periods (Figure 5.1).

One group of 26 subjects (18 APOA4 360-1/1, 8 APOA4 3601/2 or 2/2; 7 men,19 women) first received a diet low in cholesterol and then a diet high in cholesterol, the other group of 24 subjects (15 APOA4 360-1/1, 9 APOA4 3601/2 or 2/2; 7 men, 17 women) received the diets in reverse order. All subjects participated simultaneously. None the subjects and staff, except for one investigator (RMW), were aware of the APOA4 360 and 347 and APOE genotypes.

Table 5.2 Baseline characteristics of the subjects with the APOA4 360-1/1 genotype and carriers of the APOA4 360-2 allele.

	APOA4 360-1/1	APOA4 360-1/2 or 2/2*
Men/Women (N)	10 / 23	4 / 13
Age (years)	24 ± 9	24 ± 13
Body mass index (kg/m^2)	23 ± 2	22 ± 3
Total cholesterol (mmol/L) †	4.8 ± 0.9	4.6 ± 0.8
Triglycerides (mmol/L) ‡	1.1 ± 0.4	1.1 ± 0.4
Smokers (N)	2	3
Users of oral contraceptives (N of women)	9	6
APOE genotype (N)		
E2/2	1	0
E3/2	3	4
E3/3	22	12
E4/2	1	1
E4/3	6	0
APOA4 347 genotype (N)		
A/A	18	13
A/T	12	4
T/T	3	0

* The subject with the APOA4 360-2/2 genotype was a man with the APOA4 347-A/T genotype and the APOE2/4 genotype . † To convert serum lipid values from mmol/L to mg/dL multiply mmol/L by 38.67. ‡ To convert serum triglyceride values from mmol/L to mg/dL multiply mmol/L by 88.54.

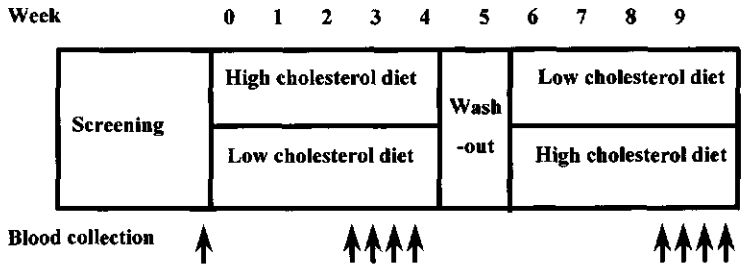

Figure 5.1 Design of the controlled dietary trial.

Diets

Before the trial, the habitual energy intake of the subjects was estimated by a food-frequency questionnaire (21,22). The study diets were formulated at 18 levels of energy intake, ranging from 7 to 24 MJ/day, so that each subject received a diet that met his or her energy needs. Body weights were recorded twice per week and, if necessary, energy intake was adjusted to maintain a stable weight.

The diets consisted of conventional foods and 29 different menus were provided over the course of each period. The nutrient composition of the low and high cholesterol diet was similar, except for dietary cholesterol (Table 5.3).

Table 5.3 Composition of the low-cholesterol diet and of the high-cholesterol diet.

	Low cholesterol diet	High cholesterol diet
Energy (MJ per day)	11	11
Protein (% of energy)	14.9	15.4
Fat (% of energy)	37.8	39.6
Saturated fat	16.7	17.5
Monounsaturated fat	11.1	11.7
Polyunsaturated fat	7.7	8.2
Carbohydrates (% of energy)	46.2	43.8
Alcohol (% of energy)	1.3	1.3
Cholesterol (mg/MJ)	12.4	86.2
Cholesterol (mg/day)	136.4	948.2
Fiber (g/MJ)	3.0	3.1
Fiber (g/day)	33.0	34.1

Dietary cholesterol was added in the form of eggs and egg yolk powder. The egg yolk powder was used for baking bread and preparing salad dressings and deserts. Egg white powder and groundnut oil were used in the diet low in cholesterol to adjust for the added fat and protein from eggs and egg yolk powder in the high-cholesterol diet. Because the response of serum lipids to dietary cholesterol may be enhanced by a background diet high in saturated fat (23-25), both diets were high in saturated fat.

All food items were weighed or counted out for each subject. On weekdays at noon, hot meals were served and consumed in the dining room for metabolic studies of the Division. All other food was supplied daily as a package. Food for the weekend and guidelines for its preparation were provided on Fridays. Approximately 90 % of the energy intake was from supplied foods, the remaining 10 % was from foods chosen by the subjects from a list of 'free-choice' food items without cholesterol or fat.

Subjects were urged not to change their selection of the 'free-choice' food items throughout the study and not to change their smoking habits or physical activities. The participants kept diaries in which they recorded their daily selection of 'free-choice' food items, any sign of illness, medication used, phase of the menstrual cycle, and any deviations from their diets and the protocol. According to these diaries, adherence to the diets and protocol was excellent.

Duplicate portions of each study diet were collected every day for an imaginary participant with a daily energy intake of 11 MJ, stored at -20 ^{0}C, and pooled and analyzed after the study. The energy and nutrient content of each subject's selection of the 'free-choice' food items were calculated and combined with the analyzed values of the duplicate portions.

Blood collection and biochemical analyses

All participants were assigned a random number that was used for labeling blood and serum tubes. In this way, the laboratory technicians did not know the subject's diet sequence or genotype. Blood samples were taken after a 12-hours fast on days 22, 24, 27 and 29 of each dietary period. We took a number of measures to reduce within-subject variation in serum cholesterol. Subjects remained standing while waiting for the blood collection. During the two dietary periods, venipunctures were performed by the same technicians, in the same location, at the same time of the same days of the week and with each subject always in the same position, which was either sitting or lying. Serum was obtained by low speed centrifugation between 0.5 to 1 hour after venipuncture, stored at -80 ^{0}C, and analyzed enzymatically for total cholesterol, high-density lipoprotein (HDL-) cholesterol, and triglycerides (26). All samples from one subject were analyzed within the same run. The coefficient of variation within runs was 0.5 % for total cholesterol, 1.2 % for HDL-cholesterol, and 0.7 % for triglycerides. The mean bias with regard to target values of serum pools (Cholesterol Reference Method Laboratory Network) was -0.07 mmol/L (-3 mg/dL) for total cholesterol and -0.02 mmol/L (-1 mg/dL) for HDL-cholesterol. LDL-cholesterol was calculated (27).

Genotyping

DNA was isolated from fresh blood by a 'salting-out' procedure (28). The DNA was amplified for the assessment of the APOA4 360-1/2, APOA4 347-A/T and APOE2/3/4 polymorphisms by mutagenically separated polymerase chain reactions (MS-PCR) (29). In each MS-PCR, the normal and mutant alleles were amplified in the same reaction tube, using allele-specific primers that differ in length. The MS-PCR-products were made visible by UV light on an agarose gel. We used 17 duplicate samples as a quality control measure for the

assessment of the genotypes. The investigators who assessed the genotypes did not know which samples were the duplicates. The genotypes of all 17 duplicate samples agreed.

Statistical analyses

The four values of serum lipids obtained for each subject at the end of each dietary period were averaged and then used for the calculation of the individual differences in serum lipid levels between the diets. Differences in response of serum lipids to dietary cholesterol between the subjects with the APOA4 360-1/1 genotype and subjects with the APOA4 360-2 allele were analyzed by a two-tailed Student's t-test. We used the General Linear Models (GLM) procedure of the SAS program to check the effect of potential confounders, such as sex, body mass index, age, APOE2/3/4 polymorphism and APOA4 347-A/T polymorphism on differences in response between subjects with the various APOA4 360 genotypes (30).

Results

Overall, the switch from a diet low in cholesterol to a diet high in cholesterol increased levels of serum total cholesterol by 0.55 ± 0.32 mmol/L (21 ± 12 mg/dL) (mean ± standard deviation) or 12 %, levels of LDL-cholesterol by 0.44 ± 0.32 mmol/L (15 ± 12 mg/dL) or 17 %, and levels of HDL-cholesterol by 0.10 ± 0.13 mmol/L (4 ± 5 mg/dL) or 6 %.

The mean difference in response between subjects with the APOA4 360-1/1 genotype and subjects with the APOA4 360-2 allele was 0.01 mmol/L (0 mg/dL) for total cholesterol and HDL-cholesterol and 0 mmol/L (0 mg/dL) for LDL-cholesterol (Table 5.4).

Adjustment for either sex, body mass index, age, baseline cholesterol level, change in body weight during the trial, APOE2/3/4 polymorphism, or APOA4 347-A/T polymorphism did not materially affect the difference in response of serum lipids between the APOA4 360 genotype groups. The largest effect of adjustment was that for the APOE2/3/4 polymorphism; the adjusted response of LDL-cholesterol was 0.02 ± 0.09 mmol/L (1 ± 3 mg/ dL) (estimated mean ± standard error) larger in subjects with the APOA4 360-1/1 genotype than in subjects with the APOA4 360-2 allele.

Table 5.4 Mean levels of serum total, low-density (LDL-) and high-density (HDL-) cholesterol (± standard deviation) during the low cholesterol and high cholesterol diets, difference in response, and its 95 % confidence interval (CI) in subjects with the apoprotein(APO)A4-1/1 genotype (N=33) and carriers of the APOA4 360-2 allele (N=17).

	APOA4 360 genotype	Low cholesterol diet	High cholesterol diet	Difference in response (95% CI)
			*mmol/L**	
Total cholesterol				
	1/1	4.53 ± 0.84	5.08 ± 0.97	0.01 (-0.19 to 0.20)
	1/2 or 2/2	4.46 ± 0.57	5.00 ± 0.64	
LDL-cholesterol				
	1/1	2.62 ± 0.72	3.06 ± 0.85	0.00 (-0.20 to 0.19)
	1/2 or 2/2	2.54 ± 0.47	2.98 ± 0.63	
HDL-cholesterol				
	1/1	1.52 ± 0.32	1.63 ± 0.33	0.01 (-0.07 to 0.08)
	1/2 or 2/2	1.52 ± 0.20	1.62 ± 0.19	

* To convert serum lipid values from mmol/L to mg/dL multiply values in mmol/L by 38.67.

Discussion

We found that the APOA4 360-1/2 polymorphism did not affect the response of serum lipids to an increased intake of cholesterol against a background diet high in saturated fat in Dutch subjects with normal cholesterol levels. The average response of serum total cholesterol to dietary cholesterol, 0.55 mmol/L (21 mg/dL), was in line with responses estimated from prediction equations (25,31,32). There were no significant differences between subjects with the APOA4 360-1/1 genotype and subjects with the APOA4 360-2 allele in the potentially confounding factors sex, body mass index, age, and baseline level of total cholesterol. In addition, the intake of total fat, fatty acids, and cholesterol during the trial was the same in the two groups, as was the average change in body weight. In the present study, however, the APOA4 347-T allele and APOE4-allele, which may enhance the response of serum lipids to diet (33-35), were more prevalent in subjects with the APOA4 360-1/1 genotype than in subjects with the APOA4 360-2 allele. However, this did not lead to a larger response in subjects with the APOA4 360-1/1 genotype than in those with the APOA4 360-2 allele.

We did not assess other genetic polymorphisms than the APOA4 360-1/2 and 347-A/T and APOE-polymorphisms. Therefore, we cannot exclude the possibility that one of these other genetic polymorphisms biases our results. However, because most of these other candidate polymorphisms are not closely linked to the APOA4 360-1/2 polymorphism (2), the

various genotypes of these polymorphisms are likely to be randomly distributed over the subjects with the various APOA4 360 genotypes and may thus not bias the results of the present study. In addition, the APOA4 347-A/T polymorphism, which is linked to the APOA4 360-1/2 polymorphism (36), did not bias the present results.

In contrast with the present study, other studies found that the APOA4 360-2 allele attenuated the response of serum LDL-cholesterol significantly (13,14) or not significantly (15,17), whereas one other study found that the APOA4 360-2 allele enhanced the response (16) (Figure 5.2) (data for the calculation of 95 % confidence intervals: personal communication with dr R.B. Weinberg and dr J.M. Ordovas, 1999).

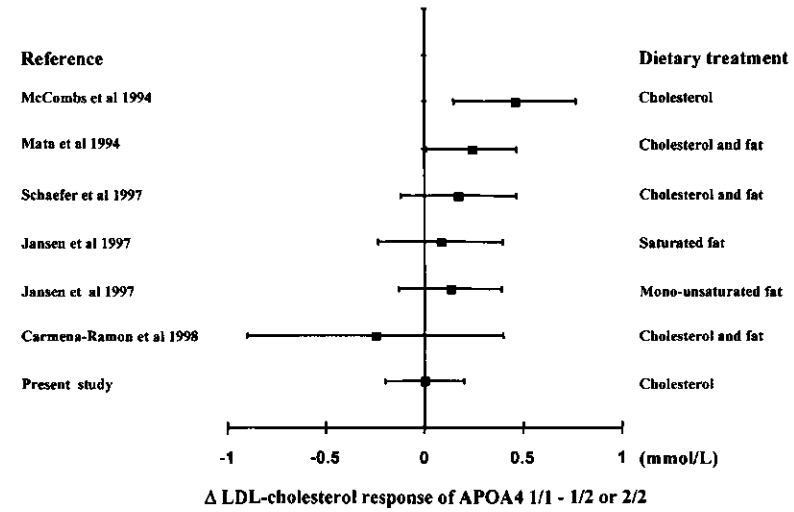

Figure 5.2 Differences in response of serum low density lipoprotein (LDL-) cholesterol to dietary cholesterol and/or fat between subjects with the apoprotein (APO) A4-1/1 genotype or ApoA-IV-1/1 phenotype and subjects with the APOA4 360-2 allele or ApoA-IV-2 isoform and 95 % confidence intervals of the difference in LDL-cholesterol response in six studies.

In the study of Mata et al (14) men with the apoA-IV-2 isoform had smaller responses of LDL-cholesterol to a decrease in the intake of cholesterol plus saturated fat than men with the apoA-IV-1/1 phenotype. In another study (15), which used in part some of the data of Mata et al (14), the differences between men with the various apoA-IV phenotypes were somewhat smaller. One explanation for the different findings in the study of Mata et al and the present study is that in the study of Mata et al not only the intake of cholesterol, but also the intake of fat was changed (14). This may indicate that the APOA4 360-1/2 polymorphism affects the response of LDL-cholesterol to a change in the intake of fat. However, people who

overrespond to dietary cholesterol also tend to overrespond to dietary fat (37). In addition, in a study of Jansen et al (17) responses of LDL-cholesterol were slightly smaller in men with the APOA4 360-2 allele than in men with the APOA4 360-1/1 genotype when carbohydrates were replaced by saturated fat or mono-unsaturated fat. Nonetheless, it remains possible that the APOA4 360-1/2 polymorphism affects the response of LDL-cholesterol to a change in the intake of both cholesterol and fat.

Another explanation for the different findings are differences in subjects' characteristics. The subjects in the study of Mata et al (14) were middle-aged and moderately hyperlipemic, whereas the subjects in the present study were young and had normal cholesterol levels at baseline. However, responsiveness to dietary cholesterol does not differ between older and younger people (38), it is if anything more marked in people with higher cholesterol levels (39). In addition, in one study with subjects with familial hypercholesterolemia the response of serum LDL-cholesterol to an increased intake of cholesterol plus fat was not attenuated but somewhat enhanced by the APOA4 360-2 allele (16). It might be that the effect of mutations in the LDL-receptor on the response of cholesterol overshadowed the effects of the APOA4 360-1/2 polymorphism in these subjects.

McCombs et al (13) showed that 11 young and normolipemic subjects with the apoA-IV-2 isoform had a smaller response of LDL-cholesterol than 12 subjects with the apoA-IV1/1 phenotype to an increased cholesterol intake. These results differed significantly from those in the present study (95% CI for difference in response 0.03 to 0.46 mmol/L (1 to 18 mg/dL)).

A possible explanation for the difference in results between the studies of McCombs et al (13) and Mata et al (14) and the present study is that the APOA4 360-1/2 polymorphism affects the response of LDL-cholesterol in men only and not in women. In the study of McCombs et al (13), 74% of the subjects were men, whereas in the study of Mata et al (14) the APOA4 360-1/2 polymorphism affected the response in men, but not in women. In the present study, 28 % of the subjects were men and only four of them had the APO4-1/2 or 2/2 genotype. Because of this small number we did not have sufficient power to analyze the effects of the APOA4 360-1/2 polymorphism in men only. The response of LDL-cholesterol was –0.04 ± 0.11 mmol/L (2 ± 4 mg/dL) (mean ± standard error) (P = 0.73) smaller in women with the APOA4 360-2 allele than in those with the APOA4 360-1/1 genotype, whereas it was 0.17 ± 0.19 mmol/L (7 ± 7 mg/dL) (P = 0.39) larger in men with the APOA4 360-2 allele than in those with the APOA4 360-1/1 genotype.

Another explanation for the difference in results is that we used a background diet high in saturated fat to enhance the response of serum cholesterol to dietary cholesterol. It is possible that the effect of the high saturated fat diet overwhelmed the effect of the APOA4 360-1/2 polymorphism on cholesterol metabolism and response. However, we do not think

this is likely, because levels of total cholesterol on the low cholesterol, high saturated fat diet were still fairly low (mean 5.06 mmol/L (196 mg/dL)) and increased by 11% on addition of cholesterol to the high saturated fat diet.

In the present controlled dietary trial the lipid response to dietary cholesterol was not affected by the APOA4 360-1/2 polymorphism in 37 women and 13 men with normal cholesterol levels, who were on a background diet high in saturated fat. This suggests that the APOA4 360-1/2 polymorphism may not be a generally applicable tool for the identification of subjects who respond to dietary cholesterol.

Acknowledgments

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6

Dietary cholesterol from eggs increases the ratio of total cholesterol to high-density lipoprotein cholesterol in humans. A meta-analysis.

Rianne M. Weggemans, Peter L. Zock, Martijn B. Katan

Abstract- Several epidemiological studies did not find an effect of egg consumption on risk of coronary heart disease. It is possible that the adverse effect of dietary cholesterol from eggs on total and low-density lipoprotein cholesterol is offset by its favorable effect on high-density lipoprotein cholesterol.

We reviewed the effect of dietary cholesterol on the ratio of total cholesterol to high-density lipoprotein cholesterol, which is a more specific predictor of coronary heart disease than either lipid value alone. Studies were identified by MEDLINE and Biological Abstracts searches (1974- June 1999) and by reviewing reference lists. We added data from a study, which we recently published ourselves. Studies were included if they had a cross-over or parallel design with a control group, the experimental diets only differed in the amount of dietary cholesterol or eggs and were each fed for at least 14 days, and concentrations of high-density lipoprotein cholesterol were reported. Of the 222 studies identified, 17 studies met these criteria. Extraction of data on design of the study, subject characteristics, composition and duration of the diets, and concentrations of serum lipids was done by the same investigator.

The addition of 100 mg dietary cholesterol daily increased the ratio of total to high-density lipoprotein cholesterol by 0.020 units (95% confidence interval (CI), 0.010 to 0.030), and the concentrations of total cholesterol by 0.056 mmol/L (2.2 mg/dL) (95% CI, 0.046 to 0.065 mmol/L (1.8 to 2.5 mg/dL)) and of high-density lipoprotein cholesterol by 0.008 mmol/L (0.3 mg/dL)(95% CI, 0.005 to 0.010 mmol/L (0.2 to 0.4 mg/dL)).

In conclusion, dietary cholesterol raises the ratio of total cholesterol to high-density lipoprotein cholesterol and therefore adversely affects the cholesterol profile. The advice to limit the intake of cholesterol by reducing the consumption of eggs and other cholesterol-rich foods may therefore still be valid.

Conditionally accepted

Introduction

One of the dietary recommendations in the prevention of coronary heart disease is to limit the intake of eggs (1). The rationale behind this recommendation is that eggs are a major source of dietary cholesterol (2). Dietary cholesterol increases serum low-density lipoprotein (LDL-) cholesterol (3-5), an established risk factor for coronary heart disease (6). However, several epidemiological studies did not find a relation between egg consumption and the risk of coronary heart disease (7,8). The absence of a relationship may imply that the recommendation to lower egg consumption is only of little use in the prevention of coronary heart disease. One egg contains approximately 200 mg of cholesterol. Although it is obvious that dietary cholesterol increases concentrations of total cholesterol (3-5), several studies showed that dietary cholesterol increases not only concentrations of LDL-cholesterol but also concentrations of high-density lipoprotein (HDL-) cholesterol (3,4). As HDL-cholesterol may be protective against coronary heart disease, the adverse effects of dietary cholesterol on total cholesterol and LDL-cholesterol might be attenuated by the favorable effects on HDL-cholesterol.

The ratio of total cholesterol to HDL-cholesterol integrates the opposing effects on coronary heart disease risk of LDL- and HDL-cholesterol. As a result, it is a better predictor of the risk of coronary heart disease than the individual lipoprotein concentrations (6,9,10). Therefore, it may be more appropriate to study the effect of dietary cholesterol on the ratio of total cholesterol to HDL-cholesterol than on the individual lipoprotein concentrations.

We now selected well-controlled studies to review the effect of dietary cholesterol from eggs on the ratio of total cholesterol to HDL-cholesterol in humans. We added data from a hitherto unpublished study of our own.

Subjects and methods

Selection of studies

We screened MEDLINE (1974-June 1999) and Biological Abstracts (1989-June 1999) for experimental studies on the effects of dietary cholesterol and eggs on total cholesterol and lipoproteins. We did not screen MEDLINE before 1974 as measurements of HDL-cholesterol, which is part of our main outcome's measure, were not available at the time. For the literature searches, the key words egg, eggs, and dietary cholesterol were each intersected with the words serum (plasma) lipoprotein, serum (plasma) cholesterol, HDL, and LDL. We found 1190 citations in MEDLINE and 883 in Biological Abstracts (Figure 6.1). In addition, we checked the reference lists of several meta-analyses (3-5,11,12) and selected studies. A scan of

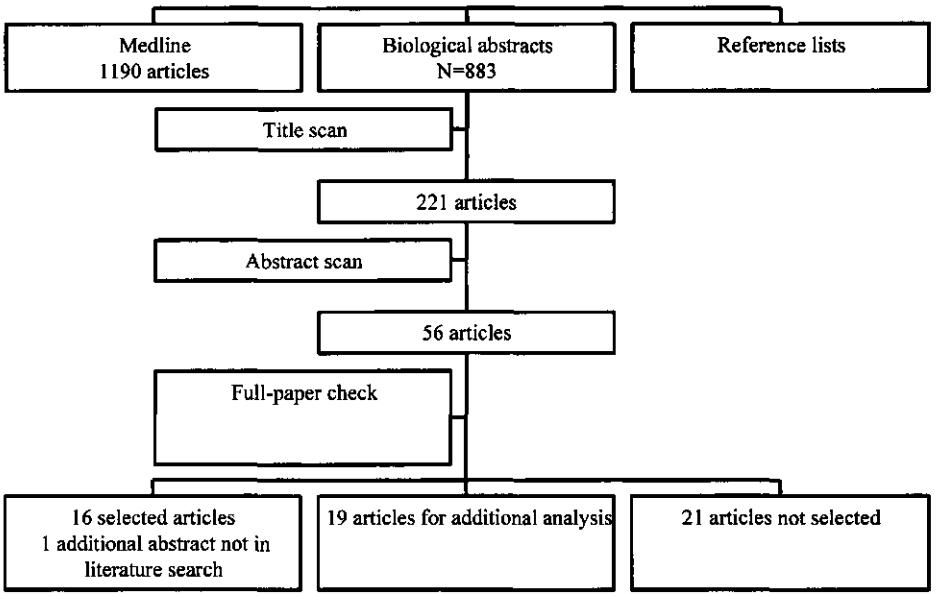

Figure 6.1 The selection of the articles for the meta-analysis and for the additional analysis.

the titles led to the selection of 221 citations. The abstracts of these citations were examined for compliance with the following inclusion criteria. The studies had to be published in English. Within a study, the composition of the experimental diets should differ only in the amount of cholesterol or in the amount of eggs. The subjects should be weight stable throughout the study. The design had to eliminate the effect of nonspecific drifts of the outcome variable with time. This is accomplished by either feeding different groups of volunteers different diets side by side (parallel design) or feeding each volunteer several diets in random order (cross-over or Latin-square design). Studies with before-and-after designs or linear designs without a control group were excluded. The feeding periods had to last at least 14 days, in order to attain equilibrium in concentrations of total cholesterol and lipoproteins. Further, studies had to report fasting concentrations of total cholesterol and lipoproteins. Of the 221 articles passing the title scan, 56 passed the abstract scan. Because most of the 56 abstracts did not provide sufficient information on the selection criteria, we checked the full-text of these articles. Sixteen of the 56 articles (28 %) met the inclusion criteria (13-28). Most other studies were not selected because they did not provide information on concentrations of HDL-cholesterol or had a linear design without a control group. In addition to the data of

Table 6.1 Characteristics of studies and diets and the effects of dietary cholesterol on serum cholesterol and lipoproteins.

						Diet characteristics			Changes in serum cholesterol (mmol/L)				
First author	Year	Men/Women	Design*	Controlled/ Free-living	# per arm	Change in cholesterol (mg/day)	Energy (MJ)	P/S-ratio	Total	HDL	LDL	Total/HDL	HDL/LDL
Chenoweth (13)	1981	32 vs 0	X	Controlled	16†	554	12.5	0.52	0.54	0.09	0.37	0.20	-0.01
					16†	542	12.9	1.22	0.35	0.02	0.22	0.15	-0.02
Buzzard (14)	1982	20 vs 0	//	Free-living	10	563	-	-	0.27	-0.05	-	0.36	-
					10(c)‡	*0*	-	-	*0.15*	*-0.02*	-	*0.08*	-
Applebaum-Bowden (15)	1984	6 vs 3	X	Controlled		897	9.6	0.82	0.29	0.00	0.29	0.18	-0.05
Sacks (16)	1984	4 vs 13	X	Free-living		321	7.7	0.57	0.19	-0.08	0.29	0.25	-0.09
Flynn (26)	1986	54 vs 16	X	Free-living		682	8.6	-	0.41	0.04	-	0.19	-
Bowman (17)	1988	14 vs 0	//	Controlled	7	294	11.8	0.42	0.02	-0.03	0.08	0.07	-0.03
					7(c)‡	*0*	*11.8*	*0.40*	*-0.13*	*0.00*	*-0.05*	*-0.09*	*0.01*
Johnson (18)	1990	10 vs 0	X	Controlled		400	-	1.5	0.26	0.03	0.24	0.10	-0.04
Vorster (19)	1992	70 vs 0	//	Free-living	19	167	13.5	0.7	0.06	0.03	0.15	-0.04	-0.01
					25	408	14.6	0.7	0.24	-0.01	0.16	0.22	-0.03
					26(c)‡	*30*	*14.0*	*0.8*	*-0.16*	*-0.02*	*-0.15*	*-0.07*	*0.02*
Duane (20)	1993	12 vs 0	X	Controlled		825	-	-	0.31	0.02	0.39	0.20	-0.02
Martin (21)	1993	30 vs 0	X	Controlled		783	13.7	0.39	0.61	0.09	0.51	0.23	-0.06

Table 6.1 continued						Diet characteristics			Changes in serum cholesterol (mmol/L)				
First author	Year	Men/Women	Design*	Controlled/ Free-living	# per arm	Change in cholesterol (mg/day)	Energy (MJ)	P/S-ratio	Total	HDL	LDL	Total/HDL	HDL/LDL
Ginsberg (22)	1994	24 vs 0	X	Controlled		215	9.4	0.78	0.14	-0.01	0.19	0.16	-0.04
						430	9.4	0.78	0.16	-0.01	0.17	0.17	-0.04
						860	9.4	0.78	0.29	0.03	0.30	0.15	-0.04
Ginsberg (23)	1995	0 vs 13	X	Controlled		169	7.6	0.87	0.16	0.04	0.10	0.03	-0.01
						559	7.6	0.86	0.42	0.09	0.31	0.10	-0.05
Knopp (28)	1997	55 vs 24	//	Free-living	44	467	8.0	0.71	0.15	0.10	0.07	-0.24	0.02
					35(c)‡	*-30*	*8.1*	*0.82*	*0.07*	*0.02*	*-0.01*	*-0.02*	*0.01*
		31 vs 21	//	Free-living	31	467	8.0	0.71	0.31	0.08	0.32	-0.13	0
					21(c)‡	*-30*	*8.1*	*0.82*	*0.21*	*0.02*	*0.12*	*0.08*	*0*
Blanco-Molina (24)	1998	15 vs 0	X	Controlled		457	11.2	0.54	0.31	0.01	0.19	0.22	-0.04
Romano (27)	1998	11 vs 0	X	Free-living		800	-	-	0.46	0.09	0.31	0.06	0.00
		10 vs 0	X	Free-living		800	-	-	0.29	0.03	0.46	0.15	-0.04
Sehayek (25)	1998	10 vs 8	X	Controlled		335	11.7	0.35	0.30	-0.03	0.30	0.28	-0.07
Weggemans (29)	2000	14 vs 36	X	Controlled		803	11.0	0.40	0.55	0.11	0.43	0.15	-0.05

P/S-ratio, ratio of poly-unsaturated to saturated fat; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

To convert serum lipid values from mmol per liter to mg per deciliter, divide mmol per liter by 0.02586.

* X, cross-over design or Latin square design; //, parallel design ; † the 32 subjects were distributed over two cross-over studies, thus 16 per study; ‡ (c) and *italics*, control group.

the 16 studies, we used data of our own study on the response to egg yolk cholesterol as a function of the apoprotein A-4 1/2 polymorphism, which was recently published (29) (Table 6.1).

The 17 studies yielded 24 dietary comparisons and 5 control treatments. The studies included 422 men and 134 women. Ten trials were carried out in men only, six included both men and women, and one study included women only. None of the studies reported the race of the subjects. The age of the volunteers ranged from 18 to 75 years, mean body mass index ranged from 20.8 to 28 kg/m^2, and mean baseline cholesterol concentration ranged from 4.06 to 5.92 mmol/L (157 to 229 mg/dL). Not all studies reported mean body mass index (13,15-17,20,26) or baseline cholesterol concentration (20,21,25,28). Eleven were metabolic ward studies, in which all food was provided and five employed free-living subjects who were provided with eggs, high cholesterol products, or egg-free substitutes. The change in cholesterol intake ranged from 137 to 897 mg per day. Values for the concentrations of total cholesterol, LDL- and HDL-cholesterol in plasma were multiplied by 1.029 to convert them to serum values (30).

Statistical analysis

We subtracted the mean concentration of serum cholesterol at the end of the low-cholesterol diet from that at the end of the high-cholesterol diet to calculate the change in serum cholesterol. Only six studies reported the means of individual ratios of total cholesterol to HDL-cholesterol (15,18,20,24,29,31) and only four studies reported the means of the individual ratios of HDL- to LDL-cholesterol (15,20,27,29). Therefore, we used mean concentrations in total, LDL- and HDL-cholesterol concentrations at the end of each diet to estimate the mean ratios of total cholesterol to HDL-cholesterol and HDL- to LDL-cholesterol. According to the Taylor-approximation, this procedure to calculate the ratios caused an underestimation of the true ratio. The size of the underestimation is dependent on the total variation in the numerator and denominator and the correlation between the numerator (x) and denominator (y), $E(x/y) \approx Ex/Ey * [1+CVy * \{CVy - corr(x,y) * CVx\}]$ (32). From an independent and large set of data (33), we calculated the coefficients of variation (CV) of total cholesterol, 0.21, HDL-cholesterol, 0.22, and LDL-cholesterol, 0.25, and the correlation coefficients of HDL-cholesterol with total cholesterol, 0.194, and with LDL-cholesterol, 0.195. Therefore, the ratio of mean total cholesterol to mean HDL-cholesterol as used by us was approximately 4 % lower than the mean of the individual ratios, similarly the ratio of mean HDL- to mean LDL-cholesterol was approximately 7 % lower. We assumed that the underestimation varied at random by treatment and study. This implies that the changes in ratios in the present study are marginally smaller than those obtained when the

mean change in individual ratios would be used. We did not adjust the ratios and their changes for this minute underestimation.

For studies with a cross-over or latin-square design, the observed changes could be fully attributed to the change in dietary cholesterol or egg consumption, because the study design eliminates drift of variables over time. For studies with a parallel design, we adjusted for drift of variables over time by subtracting the changes in total cholesterol and lipoproteins in the control group from those in the treatment group. For instance in the study of Buzzard et al (14), total cholesterol concentrations increased by 0.27 mmol/L (10.4 mg/dL) in the treatment group and by 0.15 mmol/L (5.8 mg/dL) in the control group. We subtracted the 0.15 mmol/L (5.8 mg/dL) from the 0.27 mmol/L (10.4 mg/dL) to obtain the actual increase in the treatment group, 0.12 mmol/L (4.6 mg/dL).

Regression analysis

We used linear regression models (General Linear Models procedure) (34) to study the effect of dietary cholesterol on total cholesterol and lipoproteins. We did not use any non-linear regression models, because the number of studies in our data set was limited. Moreover, the present analysis comprised only three studies (15,20,21) with a cholesterol intake just over 1000 mg per day, whereas the relation between cholesterol intake and cholesterol concentrations appears linear up to a cholesterol intake of 1000 mg per day (5). We applied several linear models. In one, the change in total cholesterol and lipoproteins (mmol/L) was expressed as a function of the absolute change in dietary cholesterol in mg per day. Regression lines were forced through the origin, because a zero change in cholesterol intake will by definition produce no change in lipoprotein cholesterol attributable to dietary cholesterol. Thus, we applied the following model

Change in serum cholesterol = β x *(Change in dietary cholesterol)*

The change in serum cholesterol is expressed in mmol/L for concentrations and in dimensionless units for ratios. The change in dietary cholesterol is expressed in units of 100 mg per day.

We also expressed dietary cholesterol in milligram per megajoule (1 megajoule equals 238 kilocalories). For these analyses, we excluded four studies, which did not provide data on energy intake (14,18,20,27). There were no large differences in the average energy intake between the various studies and the results did not materially alter when we expressed the dietary cholesterol in milligram per megajoule instead of milligram per day. Therefore, we only report the effects of a change in dietary cholesterol in milligram per day.

Although studies were selected on the basis of the design and duration of the treatments, there were still considerable differences between the studies. The number of subjects per study ranged from 9 to 131. To take this into account, it is usual in meta-analyses

to weight each study by the reciprocal of the squared standard error. However, the standard errors of the changes in cholesterol and lipoprotein concentrations were not reported in some of the studies. We therefore weighted each study by the number of subjects, which is inversely proportional to the squared standard error. Further, the ratio of poly-unsaturated to saturated fat of the background diet varied between studies. A high ratio of poly-unsaturated to saturated fat, which is an indicator of a background diet relatively low in saturated fat, may attenuate the change in total cholesterol upon an increase in dietary cholesterol (5,35,36). In additional analyses we checked whether the ratio of poly-unsaturated to saturated fat affected the relation of dietary cholesterol with total cholesterol and lipoproteins. Analysis of the residuals was performed to check the appropriateness of each model.

To detect publication bias, we explored heterogeneity in funnel plots visually. To this end, we plotted the response of serum lipids to 100 mg dietary cholesterol against the sample size by study. In the absence of bias, the plots will resemble a symmetrical inverted funnel, as results of small studies will scatter at the left side of the plot with the spread narrowing among larger studies on the right side of the plot (37).

Results

All 17 studies reported values for total cholesterol and HDL-cholesterol, but two studies did not report values for LDL-cholesterol (14,19) (Table 6.1). Most studies presented comparisons of two diets, but four studies presented comparisons of three or four diets (13,19,22,23). In two studies, various groups of subjects were studied side by side. In one study diabetics were compared with healthy subjects (27), whereas in another study hyperlipemic subjects were compared with combined-hyperlipemic subjects (28).

The ratio of total cholesterol to HDL-cholesterol and the concentrations of total and LDL-cholesterol increased relative to control groups or treatments upon an increase in dietary cholesterol in all but one of the studies, whereas the concentrations of HDL-cholesterol increased in 19 of the 24 dietary comparisons. The ratio of HDL- to LDL-cholesterol decreased in all but one of the studies.

If we assume that one egg contains 200 mg of cholesterol (2), consumption of one additional egg daily will increase the ratio of total cholesterol to HDL-cholesterol by 0.041 ± 0.011 units (mean ± standard error of the estimate), the concentrations of total cholesterol by 0.111 ± 0.010 mmol/L (4.3 ± 0.4 mg/dL), LDL-cholesterol by 0.100 ± 0.008 mmol/L (3.9 ± 0.3 mg/dL), and HDL-cholesterol by 0.016 ± 0.003 mmol/L (0.6 ± 0.1 mg/dL) (Figure 6.2). One additional egg daily will decrease the ratio of HDL- to LDL-cholesterol by 0.011 ± 0.002 units (Table 6.2).

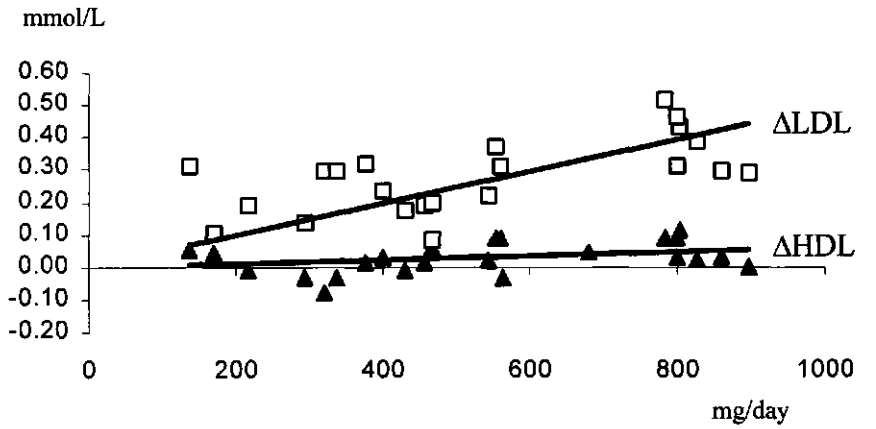

Figure 6.2 Changes in serum LDL-cholesterol (□) and HDL-cholesterol (▲) upon cholesterol feeding in 17 studies providing 24 dietary comparisons.

Table 6.2 The predicted changes (± standard error of the estimate) in serum total cholesterol and lipoproteins induced by a 100 mg increase in dietary cholesterol and the 95 % confidence interval of the predicted change.

Predicted change in serum cholesterol	100 mg/day increase in dietary cholesterol	95% confidence interval
Total cholesterol (mmol/L)	0.056 ± 0.005	0.046 to 0.065
HDL-cholesterol (mmol/L)	0.008 ± 0.001	0.005 to 0.010
LDL-cholesterol (mmol/L)	0.050 ± 0.004	0.042 to 0.058
Total/HDL-cholesterol	0.020 ± 0.005	0.010 to 0.030
HDL-/LDL-cholesterol	-0.006 ± 0.001	-0.008 to –0.004

HDL, high-density lipoprotein; *LDL*, low-density lipoprotein.

To convert serum lipid values from mmol per liter to mg per deciliter, divide mmol per liter by 0.02586.

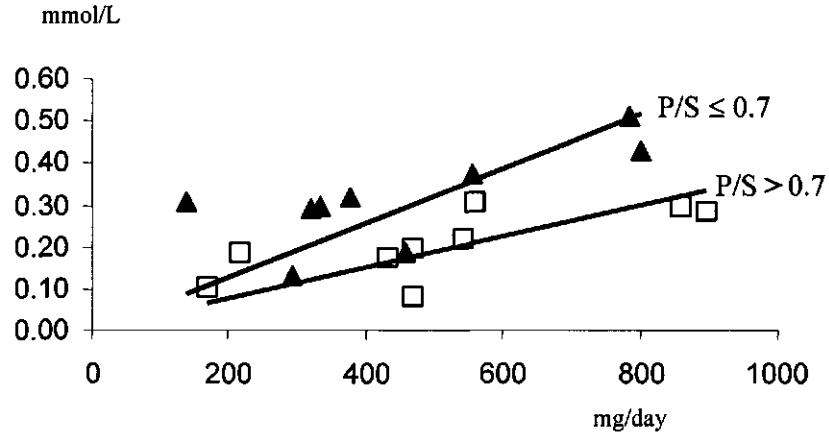

Figure 6.3 The effect of a change in cholesterol intake on serum LDL-cholesterol in studies with a ratio of poly-unsaturated to saturated fat less than or equal to 0.7 (▲) and more than 0.7 (□).

We divided the studies into those with a ratio of poly-unsaturated to saturated fat less than or equal to the median, 0.7, indicative of a background diet relatively high in saturated fat and those more than 0.7, indicative of a background diet relatively low in saturated fat. The response of LDL-cholesterol to a change in dietary cholesterol was somewhat weaker in the studies with a background diet low in saturated fat than in those with a background diet high in saturated fat (Figure 6.3). We estimated that each additional 100 mg of dietary cholesterol will increase serum LDL-cholesterol by 0.036 ± 0.004 in the studies low in saturated fat and by 0.061 ± 0.006 in the studies high in saturated fat (P = 0.03). The fatty acid composition of the background diet did not affect the response to dietary cholesterol of HDL-cholesterol or of the ratio of total cholesterol to HDL-cholesterol or HDL- to LDL-cholesterol.

We did not detect publication bias as indicated by the absence of heterogeneity in funnel plots (results not shown).

We checked whether our results also applied to other studies. For this purpose, we selected 19 articles that did report concentrations of HDL-cholesterol but had failed to meet other inclusion criteria, such as the design (Figure 6.1). These 19 studies provided 33 dietary comparisons (36,38-55). In 20 out of these 33 dietary comparisons, the ratio of total cholesterol to HDL-cholesterol increased, whereas in the other 13 the ratio decreased when cholesterol intake increased. Regression analysis showed that a 100 mg per day increase in dietary cholesterol increased the ratio of total cholesterol to HDL-cholesterol by 0.014 ± 0.003 units in these studies, whereas the increase was 0.020 units in the studies that fulfilled our selection criteria (Figure 6.4).

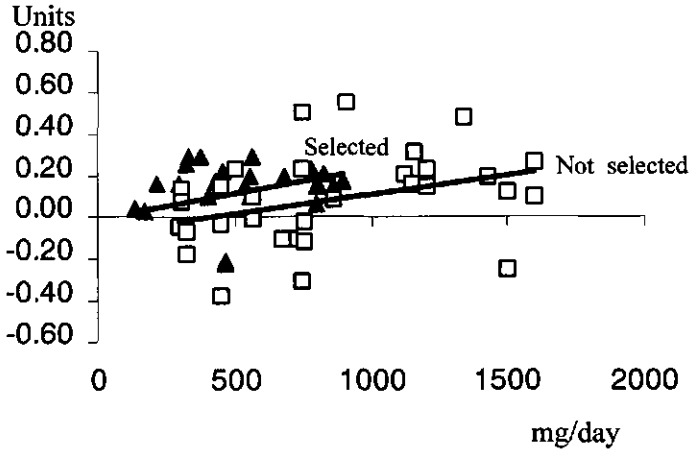

Figure 6.4 The effect of an increase in dietary cholesterol on the ratio of total cholesterol to HDL-cholesterol in 17 studies that fulfilled the selection criteria (▲) and 19 studies that did not fulfill our selection criteria (□).

Discussion

Our meta-analyses of 17 trials showed that dietary cholesterol increased the ratio of total cholesterol to HDL-cholesterol. The effect was highly significant ($P < 0.0009$) and the 95% confidence interval was narrow. This suggests that the favorable rise in HDL-cholesterol upon increased cholesterol intake fails to compensate for the adverse rise in total cholesterol and LDL-cholesterol and that therefore increased intake of dietary cholesterol may raise the risk of coronary heart disease. Our meta-analysis covered men and women from North America (13-18,20-23,26,28), Europe (24,25,27,29), and South Africa (19) with a wide range of ages. The consistency of the findings between studies suggests that our conclusions are valid for much of the white populations of affluent countries. However, the absence of data on the race of the subjects does not allow confident extrapolation to other populations.

In the present study, we used a regression model without an intercept, because a zero change in cholesterol intake will by definition produce no change in serum cholesterol attributable to dietary cholesterol. However, in studies that change the intake of eggs, not only the intake of dietary cholesterol, but also the intake of other egg components such as fat and lecithin is changed. These factors may also affect concentrations of serum cholesterol and for such studies, it may therefore not be valid to force the regression line through the origin. To check this, we performed an analysis excluding studies that changed the intake of eggs (14,16,19,26) or that did not report whether the change in the intake of fat was adjusted for in the control diet (20,24). This did not materially alter the results and we therefore included these studies in our analysis.

Dietary cholesterol and blood lipids

Stratification of the studies for study design (cross-over or latin-square versus parallel) or for setting (metabolic ward versus free-living) or adjustment of the change in dietary cholesterol for energy intake did not materially alter the results. A high ratio of poly-unsaturated to saturated fat, indicating a background diet relatively low in saturated fat, attenuated the change in LDL-cholesterol induced by an increase in dietary cholesterol. Some other studies also found that a background diet low in saturated fat attenuated the effect of dietary cholesterol on serum total cholesterol and LDL-cholesterol (35,36,56), whereas others did not (13,31,42,50,57-59). In some of the latter studies the change in dietary cholesterol might have been too small to show an effect of the fat-composition of the background diet on the change in serum cholesterol. The ratio of poly-unsaturated to saturated fat, however, does not take into account the absolute amount of fat in a diet. Thus, a diet with 5 energy percent poly-unsaturated fat and 10 energy percent saturated fat has the same ratio as a diet with 10 energy percent poly-unsaturated fat and 20 energy percent saturated fat. Differences between studies in the absolute amount of fat may therefore be also be an explanation for some of the inconsistent results.

We did not identify publication bias in our meta-analysis by use of funnel plots. In the studies that failed to fulfill our selection criteria the effect of dietary cholesterol on the ratio of total cholesterol to HDL-cholesterol was somewhat smaller than in those included in our meta-analysis. This might be due to lack of dietary control resulting in a larger error in the amount of dietary cholesterol that was changed. This attenuates the estimated effect of dietary cholesterol on serum cholesterol towards the null (60). However, it may also be due to the lack of adjustment for the change in fat intake that is induced by the change in egg consumption. Only three (40,46,49) of these 19 studies adjusted for the change in fat intake, whereas 11 of the 17 studies included in our meta-analysis did. Nevertheless, the effect of dietary cholesterol on the ratio of total cholesterol to HDL-cholesterol in the studies that failed to fulfill our selection criteria was in the same direction as the effect in our meta-analysis. This indicates that the present results are not due to a biased selection of the studies.

Effects in hyperlipemic subjects

Cholesterol-lowering diets are usually prescribed to hyperlipemic subjects, with concentrations of total cholesterol over 5.0 mmol/L (193 mg/dL) (61). However, the mean baseline cholesterol concentrations of subjects in the studies that fulfilled our selection criteria were below 5.0 mmol/L (193 mg/dL), except for two studies (13,28). The moderately hyperlipemic subjects in the study of Chenoweth et al (13) showed an 0.20 units increase in the ratio of total cholesterol to HDL-cholesterol upon an increase in dietary cholesterol of 554 mg per day, whereas the hyperlipemic subjects in the study of Knopp et al (28) showed an

0.22 units decrease and the combined hyperlipemic subjects an 0.21 units decrease upon an increase in dietary cholesterol of 437 mg per day. The additional analysis with studies that failed to fulfill our selection criteria included five studies with mostly moderately hyperlipemic subjects (39,50,51,53,54). Due to the limited number of studies, we could not analyze these studies separately. Nevertheless, the results of these studies did not clearly differ from those in subjects with normal cholesterol concentrations. Therefore, the results of the present meta-analysis appear also applicable to hyperlipemic subjects.

Effects on total cholesterol and on LDL-cholesterol

The estimated change in total cholesterol of 0.056 mmol/L (2.2 mg/dL) for each 100 mg per day increase in dietary cholesterol agrees well with changes estimated from other meta-analyses (3-5,12). Figure 6.2 suggests that a simple linear model may predict group mean changes in LDL-cholesterol concentrations rather well over the normal range of dietary cholesterol intakes. Because diet-induced changes in total cholesterol and lipoproteins vary considerably between individuals (40,62,63), our results cannot reliably predict changes in total cholesterol and lipoproteins in individual subjects or patients.

Dietary cholesterol and risk of coronary heart disease

We showed that consumption one additional egg daily, will increase the ratio of total cholesterol to HDL-cholesterol by 0.040 units, which would imply an increase in the risk of myocardial infarction by 2.1 % (9). The calculated increase in risk may be small in an individual patient, but in view of the widespread consumption of diets high in cholesterol it may still be substantial at the population level.

Of course, these calculations do not take into account the effects of other nutrients in eggs that may be beneficial in preventing coronary heart disease, such as vitamin E, folate and other B vitamins, and unsaturated fatty acids (2). Hu et al (8) calculated that in the USA eggs contribute to the intake of many nutrients, such as retinol (4 %), alpha-tocopherol (3 %), folate (4 %), other B vitamins (3 % or less), mono-unsaturated fat (3 %), and linoleic acid (2 %). However, eggs contributed to 32 % of total dietary cholesterol. Thus, in view of the relatively small contribution of eggs to the intake of nutrients that may be beneficial in preventing coronary heart disease, the recommendation to limit consumption of eggs may still be valid for the prevention of coronary heart disease. Other major sources of dietary cholesterol are dairy fats and meat, but these are already considered less favorable for heart disease risk because of their saturated fat content.

In conclusion, the consumption of cholesterol increases the ratio of total cholesterol to HDL-cholesterol, which would predict increased risk of coronary heart disease. Therefore, the

advice to limit consumption of eggs and other foods rich in dietary cholesterol may still be of importance for the prevention of coronary heart disease.

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7

General discussion

Introduction

The main *objective* of our research was to determine whether genetic polymorphisms affect the response of serum lipids to diet in humans. We found that the effect of genetic polymorphisms on lipid response to diet is small.

The first part of this chapter summarizes the main findings of the studies. The second part concerns methodological aspects of studies of the effect of genetic polymorphisms on serum lipid response, such as statistical power, multiple testing, effect modification and confounding, and extrapolation to other populations. The discussion further focuses on issues in comparing these studies, such as the use of different diets and different study populations and the possibility of chance findings, using apoprotein (APO)A4 360-1/2 polymorphism as an example. And finally, the feasibility of genetic tests to detect diet sensitivity is discussed. Recommendations for further research, conclusion, and implications are presented at the end of this chapter.

Main findings

The pooled analysis of 26 dietary trials showed differences in serum lipid response to diet between men and women, and between subjects with various APOE, APOA4, and cholesteryl ester transfer protein (CETP) genotypes. Men had larger responses of serum lipids to saturated fat and the coffee diterpene cafestol than women. There were no sex differences in response to *trans* fat or dietary cholesterol (*Chapter 2*). Subjects with the APOE3/4 or 4/4 genotype tended to have a larger response of low-density lipoprotein (LDL-) cholesterol to saturated fat than those with the APOE3/3 genotype. On the contrary, they had similar responses to *trans* fat and dietary cholesterol and they tended to have a smaller response to cafestol (*Chapter 3*). Furthermore, subjects with the APOA4 347-1/1 genotype had smaller responses of LDL-cholesterol to diet than those with the APOA4 347-1/2 or 2/2 genotype and subjects with the APOA4 360-2/2 genotype had smaller responses than those with the APOA4 360-1 allele. Subjects with the CETP TaqIb-1 allele had smaller responses of HDL-cholesterol to diet than those with the CETP TaqIb-2/2 genotype. The effects of seven other candidate polymorphisms were either inconsistent with results in previous studies or need to be replicated in other studies (*Chapter 4*). Thus, none of the studied polymorphisms had a major effect on the response of serum lipids to diet.

The controlled dietary trial showed that, unlike in some of the previous studies (1,2), APOA4 360-1/2 polymorphism did not affect the lipid response to dietary cholesterol in healthy women and men (*Chapter 5*).

The meta-analysis that involved 17 studies showed that dietary cholesterol increases the ratio of total cholesterol to HDL-cholesterol, which may be a better marker of coronary heart disease risk than individual lipid concentrations (3,4)(*Chapter 6*).

Methodological issues in studies of genetic polymorphisms and lipid response

There are several methodological aspects that are important when studying effects of genetic polymorphisms on serum lipid response. Below, issues on statistical power, multiple testing, confounding and effect modification, and the extrapolation into other populations are discussed.

Number of subjects

The number of subjects with the rare allele is often a limiting factor in studies of gene-diet interaction. The smallest group determines the statistical power to detect significant effects of a genetic polymorphism on the lipid response to diet (5).

Especially when the frequency of the rare allele is low, it is hard to find sufficient subjects with the rare allele. One way to find sufficient subjects with the rare allele is to pool data of various dietary trials. Another way is to screen large numbers of subjects with reference to their genotype before the start of a study and select all available subjects with the rare genotype. It is also possible to pool subjects heterozygous for the rare allele with those homozygous for the rare allele. However, this may not always be appropriate because the allele effect may differ between heterozygous and homozygous subjects.

Multiple testing

Using the pooled data, we tested differences in response of total cholesterol, HDL-, and LDL-cholesterol to saturated fat, *trans* fat, dietary cholesterol, and cafestol between men and women and between genotype groups of 11 polymorphisms. Thus, we performed 144 statistical tests. The probability of a spurious finding (α) was 0.05. This means that at least 7 associations may have been chance findings. If we assume that any relation in the data is attributable to chance, the probability of at least one statistically significant spurious finding will be near 100 %. However, we did not adjust α for multiple testing to reduce the probability of chance findings, because the pooled analysis was exploratory rather than hypothesis testing, and adjustment for multiple testing would reduce the power to detect existing associations (5).

We knew beforehand that we could not rule out the possibility that some of the findings in the pooled analysis might have been due to chance. These findings should

therefore be compared to those in previous studies and/or replicated in new dietary trials that are especially designed to test the relation between serum lipid response and a candidate gene. In addition, the mechanism by which a polymorphism may affect the response, should be clarified.

Effect modification and confounding

Effect modification and confounding, though different concepts, both involve the effect of one extraneous variable on the association between two or more other variables, for instance between genetic polymorphism and lipid response. Effect modification will occur if the association between genotype and response differs between subjects in various categories of the extraneous variable. Confounding will occur if the association is similar between subjects in the various categories and the extraneous variable is not evenly distributed between subjects with various genotypes (6). Potential effect modifiers and confounders of the relationship between genetic polymorphisms and lipid response are body mass index, sex, age, and smoking (7-13). Whether an extraneous variable acts as a confounder or as an effect modifier can be determined by comparing the association between genotype and response in various categories of the extraneous variable. However, this requires a group of subjects that is even larger than the one used when studying the main effect of a genetic polymorphism on lipid response. Sub-group analyses were not feasible in this study, due to the fact that the large numbers of subjects that are needed were not even attained in the pooled analyses. Effect modification of the relationship between a genetic polymorphism and response should be described. By including confounders as co-variables in a regression model, the independent effect of a genotype on response can be estimated.

Several authors suggest that baseline cholesterol concentration may confound the association between genotype and serum lipid response (14,15). One way to adjust for the effect of baseline cholesterol concentration is to analyze relative responses, e.g. the percentage change from baseline, rather than absolute responses. However, by definition, the variance of the relative response is larger than that of the absolute response. This reduces the power to detect significant effects of a polymorphism on response. Another way to adjust for baseline cholesterol concentration is to use it as a co-variable in a regression model. However, if one assumes that differences in response to diet *cause* differences in concentrations of baseline cholesterol, it is, by definition, inappropriate to adjust for baseline cholesterol concentration (6).

Extrapolation into different populations

Subjects in the pooled analysis (*Chapters 2 to 4*) and the controlled dietary trial (*Chapter 5*) were mostly young, lean, and had normal cholesterol concentrations. It is not sure whether the results also apply to older subjects, obese subjects, or subjects with moderate hypercholesterolemia. However, responsiveness to diet does not differ between older and younger people (16). If anything, it is less marked in people with a higher body mass index (17) and more marked in those with higher cholesterol concentrations (18). Thus, differences in lipid response between subjects with various genotypes may be smaller in obese subjects than in lean subjects and larger in those with high cholesterol concentrations than in those with low cholesterol concentrations. However, several studies found that subject characteristics such as age and body mass index, act as effect modifiers on the relationship between genotype and serum lipid concentration (10,12). If this is true, the results of the studies in this thesis cannot be extrapolated into older subjects with higher body mass index and cholesterol concentrations.

Issues in comparing studies of the effect of a genetic polymorphism on lipid response

There are several issues to consider when comparing various studies of the effect of a genetic polymorphism on lipid response, such as differences in type of dietary treatments between studies, differences between men and women, and the possibility of chance findings. These issues are also briefly discussed in *Chapters 3* to *5*. Since the publication of our paper on the APOA4 360-1/2 polymorphism from the dietary trial (*Chapter 5*), several other studies have been published. In addition, data regarding our pooled analysis of APOA4 360-1/2 polymorphism and lipid response (*Chapter 4*) have become available. Using all data now available, we will examine some of the issues in comparing studies on genetic disposition and serum lipid response to diet.

The APOA4 360-1/2 polymorphism and lipid response to diet

Two independent studies published in 1994 showed that subjects with the APOA4 360-2 allele had smaller lipid response to diet than those with the APOA4 360-1/1 genotype (1,2). However, in several later studies, the difference in lipid response between subjects with the various APOA4 360 genotypes was less (19-21), not present (22), or in the opposite direction (21,23-25) (Figure 7.1) (95% confidence intervals: personal communications with dr R.B. Weinberg and dr J.M. Ordovas, 1999, and Ms L. Heilbronn, 2000).

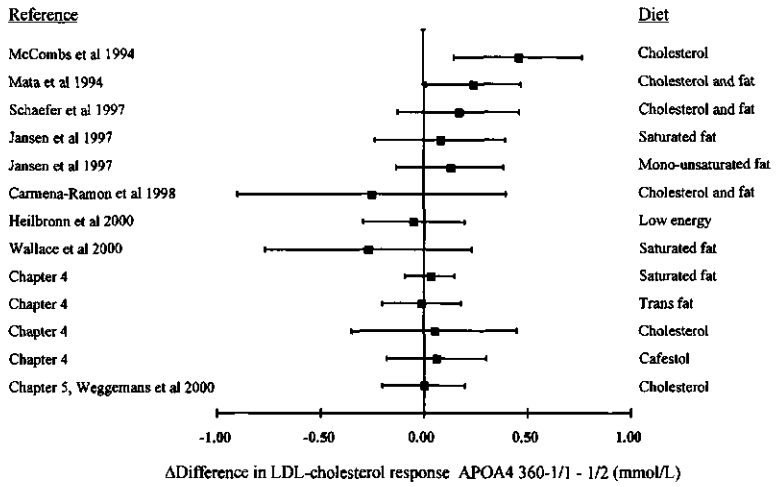

Figure 7.1 Mean difference (with 95% confidence interval) in response of LDL-cholesterol to diet between subjects with the apoprotein (APO) A4 360-1/1 and 1/2 genotype in various studies.

Dietary treatment

One explanation for these inconsistent findings is that the APOA4 360-1/2 polymorphism only affects the response of LDL-cholesterol to specific changes in diet. There are considerable differences in dietary treatments between studies. The studies of McCombs et al (1) and Mata et al (2) showed that the APOA4 360-2 allele attenuates the response of LDL-cholesterol to diet. In the study of McCombs et al (1), subjects received additional eggs in the high cholesterol period, which might have unintentionally changed fat intake as well. In the study of Mata et al (2) the intake of dietary cholesterol and fat was changed, similar to two (19,23) of the later studies. Schaefer et al (19) combined some of the data from Mata et al (2) with data from other controlled dietary trials and found somewhat less effects than Mata et al (2), but in the same direction. On the contrary, Carmena-Ramon et al (23) found that the APOA4 360-2 allele, if anything, increases the response of LDL-cholesterol to diet. However, the subjects in this study had familial hypercholesterolemia and it is possible that the effects of mutations in the LDL-receptor overshadowed the effects of the APOA4 360-1/2 polymorphism on lipid response.

Later studies, in which only the intake of cholesterol was changed showed no differences between subjects with various APOA4 360 genotypes (21,22). Furthermore, in later studies that changed the intake of fat, cafestol, or energy, there were no consistent differences between subjects (21,24,25).

Thus, effects of the APOA4 360-1/2 polymorphism on the lipid response to dietary cholesterol alone, or to fat alone, are small and probably absent. However, it remains possible that the APOA4 360-1/2 polymorphism affects the response of serum LDL-cholesterol to a change in both saturated fat and dietary cholesterol.

Sex differences

Another explanation for discrepancies between studies is that the APOA4 360-1/2 polymorphism affects the response of LDL-cholesterol in men but not in women. In the study of McCombs et al (1), there were 17 men, and only 6 women. In the studies of Mata et al (2) and Schaefer et al (19), the effect of the APOA4 360-1/2 polymorphism was limited to men. However, in the study of Schaefer et al (19), there were only 5 women with the APOA4 360-1/2 genotype. The limited number of men and women in most of the other studies does not allow for any conclusions to be drawn as to possible sex differences in the effect of APOA4 360-1/2 polymorphism on lipid response (9,20,21,23,24). Few studies (21,25) comprised more than 5 men and 5 women with the APOA4 360-2 allele. Opposite to previous findings (1,2,19), men with the APOA4 360-1/2 genotype were slightly more responsive to a low energy diet than those with the 1/1 genotype, whereas women with APOA4 360-1/2 genotype were slightly less responsive (25). This was also the case with the response of LDL-cholesterol to *trans* fat in the pooled analysis (21). There were no differences in the effect of the APOA4 360-1/2 polymorphism on the response to cafestol between men and women. Similar to the studies of McCombs et al (1) and Mata et al (2), the APOA4 360-2 allele attenuated responses of LDL-cholesterol to saturated fat by -0.10 mmol/L in men, whereas the 2 allele increased it by 0.03 mmol/L in women (21). However, all these findings were the result of subgroup analyses that are limited by a small number of subjects with the least-frequent allele.

Hence, it is possible that the effect of the APOA4 360-1/2 polymorphism on the response to saturated fat and dietary cholesterol is present in men, but not in women. It is also possible that the effect of the APOA4 360-1/2 polymorphism may be easier to detect in men than in women, because men are more responsive to saturated fat than women (9).

Chance findings

The effect of the APOA4 360-1/2 on response of LDL-cholesterol becomes less as time goes by (Figure 7.1). This may be an indication that the first studies yielded chance findings that could not be replicated in later studies. Such a trend also occurred in other studies that were designed to replicate the results of previous studies, as was the case with the supposed cholesterol-lowering effect of Lactobacillus Acidophilus (26). This issue stresses

the need for multiple studies to assess an effect. An effective way to combine data from prior studies with new evidence is the use of Bayesian methods, such as the Bayes Factor (27,28).

Conclusion

APOA4 360-1/2 polymorphism probably does not affect the lipid response to diet, at least not in every population. It possibly affects the response of LDL-cholesterol to a change in saturated fat and dietary cholesterol, and such an effect may be limited to men. However, there is no known mechanism that could explain the association between APOA4 360-1/2 polymorphism and lipid response, and thereby support its possible existence. Therefore, the effect of APOA4 360-1/2 polymorphism on lipid response observed in previous studies may well have been due to chance.

Use in clinical practice: prediction of serum lipid response to diet by a genetic test

One of the rationales for the studies in this thesis was that identification of genetic polymorphisms affecting lipid response to diet might help to identify patients with high cholesterol concentrations who do not benefit from dietary treatment. A genetic test to predict an individual's response to diet would thus allow for a targeted treatment of high cholesterol concentrations.

Before developing such a test, several criteria must be met in establishing medically useful links between genetic polymorphisms and serum lipid response (29). The first criterion, that the polymorphism causes a relevant functional and/or structural change in the protein, does not hold for all polymorphisms studied in this thesis (Table 1.1). The second criterion, that the number of subjects with the rare allele is sufficient, is met in most, but not all cases. The third criterion, that there should be clear-cut differences in lipid response between subjects with various genotypes, is not met either, as there were no major gene effects. The last criterion, that there must be a plausible underlying mechanism, does not hold either, because the mechanism by which each of the studied polymorphisms may affect the response is still unclear.

All in all, a genetic test on the basis of a single genetic polymorphism to predict an individual's response to diet is not feasible in the general population. Patients with familial hypercholesterolemia are an exception, due to the fact that testing for the known genetic defect in the LDL-receptor meets all the above criteria.

The question remains whether it will eventually be possible to accurately predict an individual's response to diet on the basis of genetic testing. The response to diet depends on a combination of genetic and environmental factors. The correlation of lipid response to diet

with genetic polymorphisms is weak and different combinations of genetic and environmental factors may lead to a similar response. Nevertheless, we cannot rule out the possibility that in the future there will be a test that predicts an individual's response to diet on the basis of a combination of genetic and environmental factors. This test may be useful in clinical practice, because screening subgroups that are prone to high cholesterol concentrations will help to select the most suitable preventive measures or therapy. However, there is no basis for population-wide screening. It is highly unlikely that the costs will counterbalance the benefits. Besides, one should be aware that genetic testing is a sensitive issue that will encounter many ethical barriers. Therefore it should be used with caution (30).

Recommendations for future research

We suggest several directions for future research.

In general, studies of genetic polymorphisms and lipid response to diet may provide new data with regard to the role of proteins involved in cholesterol metabolism and may thus contribute to additional insight in the cholesterol metabolism.

Our studies have been performed in healthy and lean subjects. Dietary studies using different populations, such as diabetic subjects, would also be of interest, because these conditions may modify the association between genetic polymorphisms and lipid response to diet.

The APOA4 360-1/2 polymorphism may affect the response of LDL-cholesterol to a change in saturated fat and dietary cholesterol, and such an effect may be limited to men. A new controlled dietary trial with sufficient numbers of men and women per genotype group, should be performed to test these hypotheses.

Several candidate genes were not studied in this thesis, such as the genes encoding scavenger receptor B1, 7-α-hydroxylase, ATP-binding cassette 1, and peroxisome proliferator activated receptor-α. Polymorphisms of these genes should be studied further in relation to lipid metabolism and lipid response to diet (31,32,33,34).

Cholesterol metabolism involves a large number of proteins, and thus, genes. There are several methods available in animal studies to identify which genes play a role in the response to a specific dietary component. Serial analysis of gene expression and micro-array analyses are methods for obtaining a complete inventory of expressed genes in a particular organ or cell type. Human genes, that are homologous to the genes identified with these tools in animals, are candidate-genes for studies on gene-diet interaction. The first step is to identify single-nucleotide polymorphisms of a candidate gene. The next step is to define haplotypes of the candidate gene, which are series of alleles found at linked loci on a single

genotype, possibly by using parental DNA of each participant. The last step consists of testing genetic polymorphisms and lipid response to diet in randomized trials under carefully controlled dietary conditions.

The mechanism by which dietary components increase serum lipids is still not understood. Evaluation of specific molecular processes underlying dietary responsiveness remains a major challenge.

The long-term goals of the studies described above should be to determine the diet-gene interaction affecting atherosclerosis in humans.

Main conclusion and implication

The effect of genetic polymorphisms on serum lipid response to diet is small. It is therefore not possible to identify individuals who will not benefit from a cholesterol-lowering diet on the basis of a specific genetic polymorphism.

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Summary

World wide, cardiovascular diseases are a substantial source of chronic disability and health costs. Subjects at high risk of cardiovascular disease or who suffer from overt cardiovascular disease should be targeted for lifestyle intervention and, where appropriate, drug therapies. One of the changes in lifestyle is a lipid-lowering diet, which is low in dietary cholesterol and saturated fat. However, even though a lipid-lowering diet is effective for most people, it is not for all. Identification of genetic factors that are related to the dietary-induced change in cholesterol concentrations, the lipid response, may be of help in the identification of subjects who will not benefit from a cholesterol-lowering diet. It may also clarify the role of certain proteins in cholesterol metabolism.

The *objective* of our research was to determine whether genetic polymorphisms affect the response of serum lipids to diet in humans.

We first assessed sex differences in the response of serum lipids to changes in the diet (*Chapter 2*). For this purpose, we pooled data on the serum lipid response to diet from 26 former dietary trials. We used lipid responses to dietary saturated fat in seven trials involving 126 men and 147 women, to dietary *trans* fat in two trials (48 men and 57 women), and to dietary cholesterol in eight trials (74 men and 70 women). We also measured responses to the coffee diterpene cafestol, which occurs in unfiltered coffee, in nine trials (72 men and 61 women). All subjects were lean and healthy. The response of total cholesterol to saturated fat was 0.14 mmol/L (mean) larger in men than in women (95% confidence interval (CI), 0.04 to 0.23 mmol/L). The response of total cholesterol to cafestol was 0.22 mmol/L larger in men than in women (95% CI, 0.04 to 0.39 mmol/L). Responses to *trans* fat and to dietary cholesterol did not differ significantly between men and women. In conclusion, men have larger responses of total cholesterol to saturated fat and cafestol than women.

We also used these data to study the effect of apoprotein (APO) E (*Chapter 3*) and 10 other genetic polymorphisms (*Chapter 4*) on responses of serum lipids to various dietary treatments. For this purpose, we combined data on lipid responses to saturated fat, to *trans* fat, to dietary cholesterol, and to cafestol with newly obtained data on 11 genetic polymorphisms in 405 mostly normolipidemic subjects. The responses of low-density lipoprotein (LDL-) cholesterol to saturated fat were 0.08 mmol/L larger in subjects with the APOE3/4 or E4/4 genotype than in those with the APOE3/3 genotype (95% CI, –0.01 to 0.18 mmol/L). In contrast, responses of LDL-cholesterol to cafestol were 0.11 mmol/L smaller in subjects with APOE3/4 or E4/4 genotype than in those with APOE3/3 genotype (95% CI, –0.29 to 0.07 mmol/L). Responses to dietary cholesterol and *trans* fat did not differ significantly between

subjects with various APOE genotypes. The response of serum LDL-cholesterol to diet was somewhat smaller in subjects with the APOA4 347-1/1 genotype than in those with APOA4 347-2 allele and it was smaller in subjects with APOA4 360-2/2 genotype than in those with APOA4 360-1 allele. Subjects with cholesteryl ester transfer protein (CETP) TaqIb-1 allele had smaller responses of high-density lipoprotein (HDL-) cholesterol to diet than those with CETP TaqIb-2/2 genotype. The effects of the other seven candidate polymorphisms were either inconsistent with results in previous studies or need to be replicated in other studies. In conclusion, polymorphisms in APOE, APOA4, and CETP genes may affect the lipid response to diet.

We further studied the effect of the APOA4 360-1/2 polymorphism on response of serum lipids to dietary cholesterol in a controlled dietary trial specially designed for this purpose (*Chapter 5*). To this end, 10 men and 23 women with the APOA4 360-1/1 genotype and 4 men and 13 women with the APOA4 360-1/2 or 2/2 genotype (carriers of the APOA4 360-2 allele) were fed two diets high in saturated fat, one containing cholesterol at 136 mg/day, and one containing cholesterol at 948 mg/day. Each diet was supplied for 29 days in a crossover design. The mean response of serum LDL-cholesterol was 0.44 mmol/L in both subjects with the APOA4 360-1/1 genotype and in those with the APOA4 360-2 allele (95 % CI of difference in response, -0.20 to 0.19 mmol/L). The mean response of HDL-cholesterol was also similar, 0.10 mmol/L, in the two APOA4 360 genotype groups (95 % CI of difference in response -0.07 to 0.08 mmol/L). In conclusion, the APOA4 360-1/2 polymorphism did not affect the response of serum lipids to a change in cholesterol intake in this group of healthy Dutch subjects who consumed a background diet high in saturated fat.

Although it is not directly related to the relation between genetic factors and serum lipid response, we also used the data of this trial to review the effect of dietary cholesterol on the ratio of total cholesterol to HDL-cholesterol, which is a more specific predictor of coronary heart disease than either lipid value alone (*Chapter 6*). The other studies were identified by MEDLINE and Biological Abstracts searches (1974 - June 1999) and by reviewing reference lists. Studies were included if they had a crossover or parallel design with a control group, if the experimental diets only differed in the amount of dietary cholesterol or eggs and were each fed for at least 14 days, and if concentrations of HDL-cholesterol were reported. Of the 222 studies identified, 17 studies met all of these criteria. Extraction of data on design of the study, subject characteristics, composition and duration of the diets, and concentrations of serum lipids was done by the same investigator. Addition of 100 mg dietary cholesterol to daily intake increased the ratio of total cholesterol to HDL-cholesterol by 0.020

units (95% CI, 0.010 to 0.030), the concentration of total cholesterol by 0.056 mmol/L (95% CI, 0.046 to 0.065 mmol/L), and the concentration of HDL-cholesterol by 0.008 mmol/L (95% CI, 0.005 to 0.010 mmol/L). In conclusion, dietary cholesterol raises the ratio of total cholesterol to HDL-cholesterol, which would predict increased risk of coronary heart disease. Therefore, the advice to limit consumption of eggs and other foods rich in cholesterol may still be of importance for the prevention of coronary heart disease.

In *conclusion*, the effect of genetic polymorphisms on serum lipid response to diet is small. It is therefore not possible to identify individuals who will not benefit from a cholesterol-lowering diet on the basis of a specific genetic polymorphism.

Samenvatting

Wereldwijd zijn hart- en vaatziekten de meest voorkomende chronische ziekte. Mensen die een hoog risico op hart- en vaatziekten hebben of die aan hart- en vaatziekten lijden, krijgen het advies hun leefstijl te veranderen. Verder ontvangen zij, indien nodig, medicatie. Eén van de veranderingen in leefstijl is een cholesterolverlagend dieet, dat weinig voedingscholesterol en verzadigd vet bevat. Een cholesterolverlagend dieet is effectief voor de meeste mensen maar niet voor iedereen. Wanneer erfelijke factoren die gerelateerd zijn aan de effectiviteit van een dieet bekend zijn, zal dit het identificeren van mensen die niet gebaat zijn bij een cholesterolverlagend dieet vergemakkelijken. Het kan verder bijdragen aan kennis over de rol van verschillende eiwitten in het cholesterolmetabolisme.

Het *doel* van ons onderzoek was om te bepalen of genetische polymorfismen veranderingen in het serum cholesterolgehalte, de cholesterolrespons, die het gevolg zijn van veranderingen in de voeding beïnvloeden.

Als eerste onderzochten we of mannen en vrouwen verschillend reageren op veranderingen in de voeding (*Hoofdstuk 2*). Hiervoor combineerden we gegevens over de cholesterolrespons op voeding uit 26 vroegere dieetstudies. We beschikten over gegevens over de cholesterolrespons op verzadigd vet uit zeven studies met 126 mannen en 147 vrouwen, op *trans* vet uit twee studies (48 mannen en 57 vrouwen) en op voedingscholesterol uit acht studies (74 mannen en 70 vrouwen). Verder beschikten we over gegevens over de respons op het koffiediterpeen cafestol, dat voorkomt in ongefilterde koffie, uit negen studies (72 mannen en 61 vrouwen).

De respons van totaalcholesterol op verzadigd vet was 0,14 mmol/L (gemiddelde) groter in mannen dan in vrouwen (95% betrouwbaarheidsinterval (bti), 0,04 tot 0,23 mmol/L). De respons van totaalcholesterol op cafestol was 0,22 mmol/L groter in mannen dan in vrouwen (95% bti, 0,04 tot 0,39 mmol/L). De responsen op *trans* vet en voedingscholesterol verschilden niet tussen mannen en vrouwen. Hieruit concluderen we dat mannen een grotere respons van totaalcholesterol op verzadigd vet en cafestol hebben dan vrouwen.

We gebruikten deze gegevens ook om het effect te bestuderen van het apoproteïne (APO) E (*Hoofdstuk 3*) en tien andere genetische polymorfismen (*Hoofdstuk 4*) op de cholesterolrespons op verschillende dieetbehandelingen. Hiervoor combineerden we de gegevens over de cholesterolrespons op verzadigd vet, *trans* vet, voedingscholesterol en de koffiediterpeen cafestol met nieuw verkregen gegevens over 11 genetische polymorfismen in 405 personen van wie het merendeel normale cholesterolwaardes had.

De respons van lage-dichtheid liproteïne (LDL-) cholesterol op verzadigd vet was 0,08 mmol/L groter in personen met het APOE3/4 of 4/4 genotype dan in degenen met het APOE3/3 genotype (95% bti, -0,01 tot 0,18 mmol/L). Daarentegen was de respons van LDL-cholesterol op cafestol 0,11 mmol/L kleiner in personen met het APOE3/4 of 4/4 genotype dan in degenen met het APOE3/3 genotype (95% bti, -0,29 tot 0,07 mmol/L). De responsen op voedingscholesterol en *trans* vet verschilden niet tussen personen met de verschillende APOE genotypen. De respons van LDL-cholesterol was enigszins kleiner in personen met het APOA4 347-1/1 genotype dan in degenen met het APOA4 347-2 allel en het was kleiner in personen met het APOA4 360-2/2 genotype dan in degenen met het APOA4 360-1 allel. Personen met het cholesterylestertransfer-eiwit (CETP) TaqIb-1 allel hadden kleinere responsen van hoge-dichtheid lipoproteïne (HDL-) cholesterol op voeding dan degenen met het CETP TaqIb-2/2 genotype. De effecten van de andere zeven kandidaat polymorfismen kwamen niet overeen met die uit vorige studies of moeten eerst nog worden bevestigd in nieuwe studies. Hieruit concluderen we dat het APOE, het APOA4 347, het APOA4 360 en het CETP TaqIb polymorfisme de cholesterolrespons op voeding mogelijk beïnvloeden.

We bestudeerden het effect van het APOA4 360-1/2 polymorfisme op de cholesterolrespons op voedingscholesterol verder in een gecontroleerde dieetstudie die hiervoor speciaal was opgezet (*Hoofdstuk 5*). Hiervoor aten 10 mannen en 23 vrouwen met het APOA4 360-1/1 en 4 mannen en 13 vrouwen met het 1/2 of 2/2 genotype (dragers van het 2 allel) twee diëten hoog in verzadig vet, één met 136 mg/dag aan cholesterol en één met 948 mg/dag aan cholesterol. Elk dieet werd verstrekt gedurende 29 dagen in cross-over vorm. De gemiddelde respons van LDL-cholesterol was 0,44 mmol/L in zowel personen met het APOA4 360-1/1 genotype als personen met het APOA4 360-2 allel (95% bti van het verschil in respons, -0,20 tot 0,19 mmol/L). De gemiddelde respons van HDL-cholesterol was eveneens gelijk, 0,10 mmol/L, in de twee APOA4 360 genotypengroepen (95% bti van het verschil in respons, -0,07 tot 0,08 mmol/L). Hieruit concluderen we dat het APOA4 polymorfisme de cholesterolrespons niet beïnvloedt in gezonde Nederlanders die een achtergrondvoeding aten met veel verzadigd vet.

Hoewel het niet direct gerelateerd is aan de relatie tussen erfelijke factoren en de cholesterolrespons, gebruikten we de gegevens van deze studie verder voor een meta-analyse naar het effect van voedingscholesterol op de ratio totaal- ten opzichte van HDL-cholesterol (*Hoofdstuk 6*). Deze ratio is een specifiekere maat voor het risico op hart- en vaatziekte dan de afzonderlijke lipideniveaus. We verzamelden de andere studies met behulp van MEDLINE en Biological Abstracts (1974 - juni 1999) en met het bekijken van referentielijsten. De

insluitingcriteria waren dat de studies een cross-over of parallelle opzet met een controlegroep hadden, dat de diëten in de studies alleen verschilden in de hoeveelheid voedingscholesterol of eieren en dat ze minstens 14 dagen verstrekt werden, en dat de HDL-cholesterol concentraties werden vermeld. Van de 222 geïdentificeerde studies voldeden er 17 aan deze criteria. Het verzamelen van de gegevens over studieopzet, persoonskenmerken, samenstelling en duur van de voedingen en de serum cholesterol concentratie is uitgevoerd door één onderzoeker.

Een dagelijkse consumptie van 100 mg cholesterol per dag extra verhoogde de ratio van totaal- ten opzichte van HDL-cholesterol met 0,020 eenheden (95% bti, 0,010 tot 0,030), de totaalcholesterol concentratie met 0,056 mmol/L (95% bti, 0,046 tot 0,065 mmol/L) en de HDL-cholesterol concentratie met 0,008 mmol/L (95% bti, 0,005 tot 0,010 mmol/L). We concluderen dat voedingscholesterol de ratio van totaal- ten opzichte van HDL-cholesterol verhoogt, wat samenhangt met een verhoogd risico op coronaire hartziekte. Dus, het advies om matig te zijn met het eten van eieren is zeker van belang voor de preventie van coronaire hartziekte.

De *conclusie* van dit proefschrift is, dat de invloed van genetische polymorfismen op de cholesterolrespons klein is. Het is dan ook niet mogelijk om op basis van informatie over een specifiek genetisch polymorfisme mensen te identificeren die niet gebaat zijn bij een cholesterolverlagend dieet.

Dankwoord

Dit proefschrift is tot stand gekomen met de directe en indirecte hulp van een groot aantal mensen dat ik hiervoor wil bedanken.

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Tijdens het eerste anderhalf jaar van dit project moesten er 670 mensen worden opgespoord, die in de periode 1976-1996 ooit aan een voedingsproef op de afdeling Humane Voeding en Epidemiologie hadden meegedaan. Van deze groep mensen was namelijk al de cholesterolrespons op voeding bekend en we wilden nu hun DNA verzamelen voor onderzoek naar de effecten van erfelijke factoren op deze cholesterolrespons. Miranda Mul, je hebt hierbij geweldig geholpen. Het was een kwestie van 'doorbellen' en doorzetten, maar samen hebben we toch maar mooi 94 % van deze oud-deelnemers getraceerd.

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In het derde jaar volgde een gecontroleerde voedingsproef, de EXPRES-proef. In deze proef zijn 200 mensen op het apoproteïne A4 360-1/2 polymorfisme gescreend en zijn er uiteindelijk 50 mensen geweest die acht weken lang hebben meegedaan. Saskia Meyboom, je hebt een groot aandeel geleverd aan de proef door voedingen te ontwerpen die niet alleen hoog (of laag) in cholesterol waren, maar ook nog een keer hoog (of laag) in verzadigd vet. Els Siebelink, jouw steun bij de praktische uitvoering en het contact met de deelnemers was

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About the author

Rianne Margriet Weggemans was born on December 3, 1971, in Laag-Zuthem, the Netherlands. In 1990, she passed secondary school, gymnasium-β, at 'Gymnasium Celeanum' in Zwolle. In the same year she started the study 'Human Nutrition' at the former Wageningen Agricultural University. As part of that study she conducted an epidemiologic research project on the relation between heart rate and heart rate variability and risk of cardiovascular disease at the former Department of Epidemiology and Public Health (Aug-Dec 1993) and an experimental research project on the validity and reproducibility of bio-electrical impedance measurements at the former Department of Human Nutrition at Wageningen Agricultural University (May – Oct 1994). She spent her practical time studying the relation between nutrition and atopic eczema at Booth Hall Children's Hospital at the University of Manchester in Manchester, United Kingdom (Feb-April 1994). In November 1994, she received the MSc degree.

In 1995, she worked as a research assistant for the former Department of Human Nutrition at Wageningen Agricultural University on the 'SENECA study on nutrition and the elderly in Europe'. Within the framework of this project, she studied changes in vitamin status, mental health, and the determinants of vitamin B12 and folic acid status in elderly Europeans at Hofmann-La Roche, AG, in Basel, Switzerland.

In February 1996, she started as a PhD-fellow on the project described in this thesis. She joined the education programme of the Graduate School VLAG (advanced courses in Food Technology, Agrobiotechnology, Nutrition and Health Sciences). In June 1996 she attended the Annual New England Epidemiology Summer Program at Tufts University, Boston, USA. She was a member of the executive board of the Division of Human Nutrition and Epidemiology and of the PhD-excursion committee that organised a two-week study tour to Scandinavia in 1997.

Since October 2000, she has worked as a scientist cardiovascular health at Unilever Health Institute of Unilever Research in Vlaardingen, The Netherlands.

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