The effects of constituents and the food matrix of dairy products postprandial metabolism in overweight subjects

THE EFFECTS OF CONSTITUENTS AND THE FOOD MATRIX OF DAIRY PRODUCTS ON POSTPRANDIAL METABOLISM IN OVERWEIGHT SUBJECTS

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

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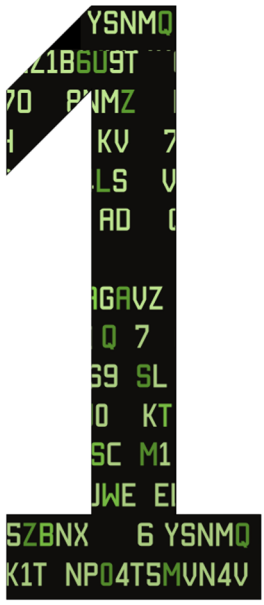
CONTENTS



GENERAL INTRODUCTION

INTRODUCTION

The incidence of the metabolic syndrome, a cluster of metabolic risk markers including elevated plasma glucose levels, an atherogenic lipid profile (i.e. increased triacylglycerols (TAG) and decreased high-density lipoprotein (HDL) cholesterol concentrations), abdominal obesity, low-grade systemic inflammation, and elevated blood pressure (1) has increased dramatically during the last decades (2). It is frequently diagnosed based on criteria formulated by the National Cholesterol Education Program's Adult Treatment Panel III (NCEP-ATP III; **Table 1.1**). If three or more of these conditions are present, people are diagnosed as suffering from the metabolic syndrome, which is a major risk factor for developing cardiovascular disease (CVD) and type 2 diabetes mellitus (3, 4).

Table 1.1: NCEP-ATP III criteria for the diagnosis of metabolic syndrome (5)

Criteria	Cut-off values
Waist circumference, *cm*	> 102 (men)
	> 88 (women)
Blood pressure, *mm Hg*	≥ 130 / ≥85
Triacylglycerols, $mmol \cdot L^{-1}$	≥ 1.7
HDL cholesterol, $mmol \cdot L^{-1}$	< 1.03 (men)
	< 1.30 (women)
Fasting glucose, $mmol \cdot L^{-1}$	≥ 6.1

Cardiovascular disease

Although the term CVD classifies all diseases of the heart and blood vessels, e.g. heart failure, stroke and myocardial infarction, it is generally used to describe atherosclerosis. Atherosclerosis is the continuous process of hardening of the arteries, which already starts at an early age, and is the leading cause of stroke, infarction and peripheral vascular disease (6). The trigger for this process is endothelial damage, which is often induced by - but not restricted to - high circulating concentrations of low-density lipoprotein (LDL) cholesterol, proinflammatory cytokines, glucose, and non-esterified fatty acids. The inflicted damage increases the permeability of the endothelium and stimulates endothelial cells to induce chemotaxis by the release of chemokines, e.g. monocyte chemotactic protein (MCP)-1 (**Figure 1.1**). In addition, the increased permeability may lead to extravasation of atherogenic LDL particles and TAG-rich lipoproteins (TRL), which may undergo oxidation and stimulate endothelial cells to express cellular adhesion molecules (CAM). As a result of both chemotaxis and the expression of CAM, monocytes are drawn to the damage site, resulting in diapedesis and

extravasation of monocytes. Once migrated into the intima layer, monocytes differentiate into macrophages, producing an array of proinflammatory cytokines, including interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)-α. Additionally, these macrophages are capable of scavenging large amounts of oxidized LDL and TRL inside the intima layer through scavenger receptors, hereby transforming into lipid-laden foam cells and drastically increasing the production of proinflammatory cytokines. The endothelial cells and smooth muscle cells surrounding the affected area are stimulated to produce proinflammatory cytokines and growth factors, stimulating the proliferation of smooth muscle cells and the formation of a fibrous cap. At this stage the formation of the mature atherosclerotic plaque is completed, which may partially or completely block arterial blood flow. Eventually, this atherosclerotic plaque may rupture and result in a thrombus, which may obstruct a cerebral artery (cerebrovascular infarction) or coronary artery (myocardial infarction).

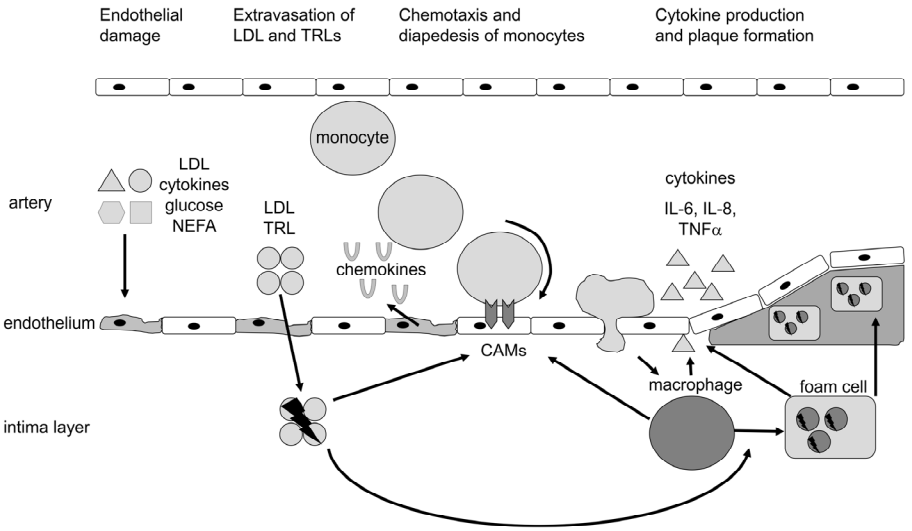


Figure 1.1: Schematic overview of the atherosclerotic process. LDL: low density lipoprotein, TRL: triacylglycerol-rich lipoprotein, CAM: cellular adhesion molecule, IL-6/8: interleukin-6/8, TNFα: tumor necrosis factor α.

DAIRY CONSUMPTION AND METABOLIC SYNDROME AND CVD

Several epidemiological studies (7-10) have found that a high intake of dairy products, such as milk, cheese, cream, butter and yogurt is associated with a reduced risk of developing the metabolic syndrome. Some studies (11, 12), however, have found a protective health effect only for low-fat dairy products. In addition, these protective effects were less pronounced compared to those of other studies (7-10). Moreover, consumption of dairy products was negatively related with the development of cardiovascular disease (CVD) and type 2 diabetes mellitus in case-control studies and prospective cohort studies (13, 14).

Dairy components and disease risk

The neutral to beneficial effects of the consumption of full-fat dairy products in relation to disease risk are surprising, because dairy products contain high amounts of cholesterol and saturated fatty acids, which are well known for their adverse effects on the serum lipoprotein profile. Saturated fatty acids increase LDL cholesterol concentrations by down-regulation of LDL-receptor mediated catabolism (15, 16), which may ultimately increase the risk of cardiovascular disease (17, 18). On the other hand, dairy products also contain high amounts of calcium and protein. Dietary calcium is well-known for its beneficial blood pressure modulation, showing on average a decrease of 0.5 mm Hg or 0.4 mm Hg in diastolic blood pressure in women and men respectively from one quartile of calcium intake to the next (19). In that study (19), calcium intake ranged between 688 and 1826 mg/day, with an average consumption of 1109 mg/day for men and 980 mg/day for women. Moreover, higher intakes of calcium were associated with a lower prevalence of the metabolic syndrome in middle-aged and older US women (20). In addition, calcium is known for its intestinal fat binding capacity, inducing the formation of insoluble calcium soaps thereby reducing intestinal fat absorption and increasing fecal fat excretion (21, 22). By this, calcium may positively affect lipoprotein metabolism.

Dairy products are also an important source of dietary protein. It has been shown that replacing dietary carbohydrates by proteins favorably reduced the total:HDL cholesterol ratio (23, 24). One of the most important characteristics of dietary protein is probably its potential of modifying body composition in relation to weight loss. Because of its satiating properties, dietary protein decreases energy intake and as a result fat mass, whilst sparing fat free mass thereby inducing a shift in body composition.

From the overview presented above it is clear that dairy products are complex food products and that their health effects may not be assigned to single dairy constituents alone. The findings that also high-fat dairy products may

decrease the risk for the metabolic syndrome suggest that the composition of dairy products may play a role.

POSTPRANDIAL METABOLISM OF DIETARY FAT

After food intake, dietary fat, which is mainly composed of TAG, is partially digested into free fatty acids and monoacylglycerol by gastric lipase in the stomach and later in the duodenum and jejunum by pancreatic lipase. Fatty acids can be divided into short-chain (less than 6 carbon atoms (C)), medium-chain (6-12 C), long-chain (13-20 C), and very long-chain fatty acids (more than 20 C). Short-chain and medium-chain fatty acids are absorbed passively in the intestine and released directly from the intestinal capillaries into the portal circulation. The release of these fatty acids into the portal circulation does not result in a lipemic response. In contrast, long-chain and very long-chain fatty acids are at least partially absorbed actively by specific fatty acid transport proteins, including Fatty Acid Transport Protein-4, Fatty Acid Translocase/CD36 and plasma membrane-bound Fatty Acid Binding Protein (25).

After absorption, the long-chain and very long-chain fatty acids are re-esterified into TAG and incorporated into apolipoprotein B-48-rich chylomicrons in the intestine. These chylomicrons travel through the lymphatic vessels and enter the circulation in the vena subclavia. Whilst the intestinally formed chylomicrons travel through the circulation towards the liver, the TAG content is hydrolyzed by lipoprotein lipase (LPL). The released fatty acids are delivered either as an energy source to fat-utilizing tissues, e.g. muscle tissue, or to fat-storing tissues, for example adipose tissue, where these fatty acids are re-esterified to TAG. Hydrolysis, in combination with exchange of TAG between chylomicrons and the free fatty acid pool, leads to the formation of chylomicron remnants (26). The chylomicron remnants, which predominantly contain intestinally absorbed cholesterol after TAG clearance by LPL, are removed from the circulation mainly by the LDL receptor and LDL receptor-related protein (LRP) (27-29).

Due to the increased flux of free fatty acids towards the liver and the rise of cholesterol levels inside the liver cells as a consequence of TRL clearance, very low-density lipoproteins (VLDL) synthesis increases. Inside the endoplasmatic reticulum, cholesteryl esters and TAG are incorporated into a growing particle. This particle also contains apolipoprotein B-100 and is exocytosed into the circulation as VLDL.

Importance of postprandial metabolism and food matrix

By far most of the nutritional intervention studies are carried out in the fasted state. However, the evaluation of postprandial responses is of great importance, since people spend most of the day in a postprandial state. Considering that in Western countries on average at least three meals per day are consumed and that the duration of postprandial lipemia is on average 6-8 hours depending on the fat content of the meal, people spend most of the day in the postprandial state. Many studies have shown a positive correlation between the level and duration of postprandial lipemia and cardiovascular disease (30-32). Postprandial inflammation is tightly related to postprandial lipemia, since TRL augment cytokine production ex vivo by endothelial cells and in vitro by macrophages (33, 34). Atherosclerosis may therefore also be a postprandial process. It is thought that the most important particles involved in the interactions between postprandial lipemia, inflammation and endothelial activation are chylomicron remnants (35) and VLDL particles (33).

Besides the proposed effects on the long-term, as discussed in the paragraph above, dairy constituents may also alter the direct responses after meal consumption. In this, dairy protein, which consists mainly of casein, may decrease postprandial TAG concentrations (36) and as a result inflammatory responses. Moreover, in addition to its hypothesized effects on fat absorption, calcium may also decrease the formation of reactive oxygen species by altering mitochondrial uncoupling status through the suppression of calcitriol (37).

Since dairy products are complex food products, also the food matrix, which consists of the structure of the dairy product as a whole, the molecular arrangement and also the interrelationship between its constituents may play a substantial role in the postprandial responses. For instance, for carbohydrate-rich foods it is known that the physical structure of the food product determines postprandial glucose and insulin responses (38, 39), which may arguably also be the case for lipemic responses to fat-rich foods. For example, when different dairy products, e.g. milk, butter and cheese, are compared, differences in the food matrix may play a crucial role on the postprandial lipemic response, because differences in the physical state of the dairy fat might affect absorption kinetics and bioavailability. Therefore, the postprandial response to a specific dairy product may not be the net effect of all its constituents, but may be modified by the matrix in which it is consumed.

OUTLINE OF THE THESIS

This thesis describes the results of several human intervention studies addressing the role of dairy products, their components and their food matrix on postprandial metabolic risk markers for CVD, including postprandial lipemia, and markers for inflammation and endothelial activation. In **chapter 2,** an overview of the literature on the effects of macronutrients on the different stages of digestion, absorption and metabolism, and postprandial risk markers is presented. The observational study in **chapter 3** was designed to measure the protein distribution profiles of the prominent cholesterol and fatty acid transport proteins along the human gastrointestinal tract, to gain insight in the main loci of transporter-mediated uptake of cholesterol and fatty acids. The human intervention study in **chapter 4** was designed to compare postprandial responses in TAG and markers for inflammation and endothelial activation between saturated and (n-6) polyunsaturated fatty acids in overweight men. In **chapter 5** the effects of milk and its main constituents - total milk protein and calcium – on postprandial inflammatory and endothelial responses in overweight men are described, while **chapter 6** presents the results of a human intervention study on the effects of the food matrix of milk on postprandial risk markers. Finally, the main findings and conclusions based on the studies described in this thesis are discussed in **chapter 7**.

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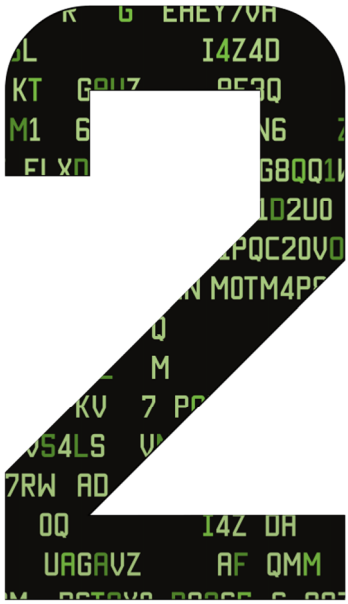

2 THE EFFECTS OF MACRONUTRIENTS ON POSTPRANDIAL LIPEMIC, INFLAMMATORY AND ENDOTHELIAL RESPONSES

CJ MASSON AND RP MENSINK

SUBMITTED

ABSTRACT

Postprandial lipemia is positively associated with cardiovascular disease risk. Moreover, the importance of postprandial inflammatory and endothelial processes is becoming more and more evident. Obviously, dietary fatty acids are a prerequisite for a postprandial lipemic response, and since postprandial lipemia and the inflammatory and endothelial responses are related, fatty acids may also affect these processes. Fat-specific determinants of lipemia include the amount of fat and the fatty acid composition, e.g. chain length, degree of saturation, triacylglycerol structure and solid fat content. Additionally, both dietary protein and carbohydrates may affect postprandial metabolism at various steps of digestion, absorption, and metabolic processing. Considerable effects may be found on fatty acid handling, mainly through insulinotropic effects, hereby decreasing the hepatic output of very low density lipoprotein (VLDL) particles. Regarding the inflammatory and endothelial responses, there are clear indications that meal consumption can result in activated endothelium and inflammatory signaling. However, data on macronutrient-specific differences is limited. This review presents an overview of the literature on the influence of the different macronutrients on postprandial lipemic, inflammatory and endothelial responses in human subjects.

INTRODUCTION

Many intervention studies have examined the effects of diets on fasting concentrations of metabolic risk markers. However, it may take 6-8 hours to process a meal and many people in Western societies consume at least three meals a day. Thus, we spend most time of the day in the postprandial state. In fact, obese subjects and people suffering from diabetes mellitus and the metabolic syndrome (MetS) often display increased postprandial triacylglycerol (TAG) concentrations(1-4) which are positively associated with cardiovascular disease (CVD). Therefore, many recent studies have focused on postprandial metabolism, concentrating on lipemic, glycemic and inflammatory responses and on endothelial function. Postprandial metabolism is influenced by many factors including the macronutrient composition of the diet, which may affect the absorption and clearance rate of TAG-rich lipoproteins (TRLs). For fat, chain length, degree of saturation, the TAG structure, and the solid fat content might be important (5-9). Also, the composition of the habitual diet may influence the postprandial response by increasing fasting TAG levels, which are positively associated with postprandial TAG responses (10, 11).
Information on the role of macronutrients on postprandial inflammatory and endothelial responses is scarce. We therefore decided to review human studies on the influence of macronutrients on postprandial lipemia and inflammatory and endothelial processes, which might be involved in the etiology of the MetS and CVD.

METABOLISM OF MACRONUTRIENTS IN RELATION TO TRI-ACYLGLYCEROL-RICH LIPOPROTEIN FORMATION

Digestion and absorption of macronutrients

After food intake, dietary fat, which is mainly composed of TAG, is partially digested into free fatty acids and monoacylglycerol by gastric lipase in the stomach and later in the duodenum and jejunum by pancreatic lipase. The concentrations of the digestive enzyme gastric lipase may depend on the amount of fat in the diet. This was suggested by Armand et al. (12), who measured levels of gastric lipase in the gastric fluid of healthy subjects after an overnight fast, following 2-week-periods of either a high-fat diet (50% of energy (En%) from fat) or a low-fat diet (25 En% fat). Levels of gastric lipase were increased after the high-fat diet. This indicates that enzyme synthesis and secretion adapt to habitual fat consumption.

Digestion and absorption of dietary fatty acids varies according to type and chain length of the fatty acids involved. Fatty acids can be divided into short-chain (less than 6 carbon atoms (C)), medium-chain (6-12 C), long-chain (13-20 C), and very long-chain fatty acids (more than 20 C). Short-chain and medium-chain fatty acids are absorbed passively in the intestine and released directly from the intestinal capillaries into the portal circulation. The release of these fatty acids into the portal circulation does not result in a lipemic response. In contrast, long-chain and very long-chain fatty acids are at least partially absorbed actively by specific fatty acid transport proteins, including Fatty Acid Transport Protein-4, Fatty Acid Translocase/CD36 and plasma membrane-bound Fatty Acid Binding Protein (13) .
After absorption, the long-chain and very long-chain fatty acids are re-esterified into TAG and incorporated into apolipoprotein B-48-rich chylomicrons in the intestine. These chylomicrons travel through the lymphatic vessels and enter the circulation in the vena subclavia.
Dietary oligosaccharides and polysaccharides are hydrolyzed into monosaccharides by pancreatic and intestinal glycosidases. In the intestine, several transporters are responsible for carbohydrate uptake (reviewed in (14)). The intestinal sodium/glucose cotransporter (SGLT-1), which has the highest affinity for glucose and galactose, is responsible for the majority of the uptake of monosaccharides from the intestine into the enterocyte, a process that depends on extracellular sodium. On the other hand, fructose absorption occurs by glucose transporter-5 (GLUT-5) facilitated diffusion. After absorption, the monosaccharides are released into the circulation by basolateral membrane-located glucose transporter-2 (GLUT-2) or GLUT-5.
For protein absorption, proteases, peptidases and specific peptide and amino acid transporters are involved (15). Proteins are degraded into amino acids and taken up by the enterocytes. From here, the amino acids are released into the circulation. However, the postprandial amino acid composition in plasma does not necessarily resemble the composition of the protein ingested, due to differences in the absorption rate and rate of release of the different amino acids into the circulation (16). Additionally, the amino acids from ingested protein are mixed with endogenously synthesized proteins and amino acids, and amino acids derived from proteins of shed intestinal mucosa cells (17).

Formation and clearance of triglyceride-rich lipoproteins

Whilst the intestinally formed chylomicrons travel through the circulation towards the liver, the TAG content is hydrolyzed by lipoprotein lipase (LPL), which is the main enzyme for chylomicron clearance and therefore partially determines the extent of postprandial lipemia. The released fatty acids are

delivered either as an energy source to fat-utilizing tissues, e.g. muscle tissue, or to fat-storing tissues, for example adipose tissue, where these fatty acids are re-esterified to TAG. LPL activity is thought to depend mainly on plasma TAG levels (18). Hydrolysis, in combination with exchange of TAG between chylomicrons and the free fatty acid pool, leads to the formation of chylomicron remnants (19). The chylomicron remnants, which predominantly contain intestinally absorbed cholesterol after TAG clearance by LPL, are removed from the circulation mainly by the LDL receptor and LDL receptor-related protein (LRP) (20-22).

Due to the increased flux of free fatty acids towards the liver and the rise of cholesterol levels inside the liver cells as a consequence of TRL clearance, very low-density lipoproteins (VLDL) synthesis increases. Inside the endoplasmatic reticulum, cholesteryl esters and TAG are incorporated into a growing particle. This particle also contains apolipoprotein B-100 and is exocytosed into the circulation as VLDL. Insulin plays an important role in this process, since it acutely suppresses the production of hepatic apolipoprotein B-100-containing lipoproteins (23-25) and intestinally-derived lipoproteins (26), partly by its suppressive effects on free fatty acid concentrations, but also when free fatty acids are not affected (27, 28). This may occur by rerouting the free fatty acids towards adipose tissue and by increasing its clearance into tissues (29). Therefore, due to insulin-stimulating effects, also dietary carbohydrates and proteins may affect these processes.

There is evidence that TAG in TRLs directly after the consumption of a meal originates from the previous meal and that chylomicron TAG may therefore peak twice after a meal (30, 31). Evans *et al*. (31) have shown that after a standardized high-fat breakfast (54 En% fat; 4.3 MJ), the early TAG response (45-60 min) after a consecutive lunch, which was consumed 5 hours after the breakfast, was comparable after a low-fat (1 En% fat; 1.4 MJ) and a high-fat (60 En% fat; 3.5 MJ) lunch, while no response was observed after water ingestion or saline infusion. The second peak after 120 min, however, was much more pronounced after the high-fat than after the low-fat lunch. These findings indicate that the postprandial TAG response is primed by the previous meal and therefore comparisons between test meals may be confounded by the previous meal. Whether this also applies to the meal consumed on the previous day remains unclear. The exact mechanism by which the fat from the previous meal appears in chylomicrons of the subsequent meal, is not fully understood. It has been suggested that the subsequent meal triggers an increase in intestinal lymph flow, which may accelerate the entry of pre-formed chylomicrons into the circulation (31).

FATTY-ACID RELATED FACTORS INFLUENCING FATTY ACID META-BOLISM

Amount of intake

Various studies have shown that postprandial TAG concentrations are linearly related to the amount of fat in the meal. Relatively low intakes of fat in the meal of 5-15 g did not significantly increase postprandial TAG concentrations (32), whereas intakes varying between 30-80 g stimulated postprandial lipemia dose-dependently (32-35). Cohen *et al*. (35) measured serum TAG concentrations after consumption of 40 g, 80 g or 120 g fat from cream and found that the increase in postprandial lipemia from 40 g to 80 g was larger than that from 80 to 120 g. However, since TAG concentrations had not reached baseline concentrations after 8 hours, the effects calculated as the area under the curve (AUC) might have been underestimated, especially at the higher intakes (35). Compared with the intake of 40 g fat, the maximum serum TAG concentration was twice as high after the intake of 80 g, but was only 2.4 times higher after the consumption of 120 g fat. For this observation, no obvious explanation could be given by the authors, since fat absorption, even at high doses (36), is nearly complete. It was however speculated that high-fat loads might increase the clearance rate of TAG from the circulation (35).

Fatty acid type

Since medium and short-chain fatty acids are secreted into the portal vein and are not incorporated into chylomicrons, these fatty acids have limited effects on postprandial lipemia. Indeed, in healthy subjects (BMI 20.1-27.8 kg/m^2) postprandial lipemia (*A*UC) was lower after a medium-chain TAG (MCT)-rich meal (90 g fat, of which 42% MCT) as compared to long-chain TAG (LCT)-rich meals (90 g fat) containing predominantly oleate, elaidate or palmitate (37). Recently, Poppitt *et al*. (38), however, did not report differences in postprandial lipemia for 3 hours after the intake of short-chain triacylglycerols (SCT), MCT or LCT-rich breakfasts in lean men. However, the postprandial period may have been too short, while the difference in the intake of SCT or MCT vs. that of LCT was only 10 g.

Also, the degree of saturation of long-chain fatty acids may modulate postprandial lipemia, but studies examining differences in postprandial TAG concentrations between saturated fatty acids, monounsaturated fatty acids and n-6 PUFA are conflicting (39). On the other hand, marine n-3 PUFA may reduce postprandial TAG. Beneficial effects of realistic amounts of n-3 long-chain polyunsaturated fatty acids (LCPUFA) in the diet on fasting TAG concentrations have been found in numerous studies, especially in subjects

with hypertriacylglycerolemia (reviewed in (40)). Effects of n-3 LCPUFA on postprandial TAG concentrations are less clear. A single evening meal rich in fish oil (n-3 long-chain PUFA) resulted in lower postprandial TAG iAUCs as compared to a saturated fat-rich meal, but not to an n-6 PUFA meal (41). Multiple studies, however, found no reductions in postprandial lipemia, as evaluated by changes in iAUCs, peak changes, peak concentrations (not corrected for baseline) and time to peak, when fish oil (doses varying from 2.3-16.0 g EPA+DHA) was added to a test meal with varying lipid compositions (42-44), while supplementation of the background diet with n-3 LCPUFA from fish oil was shown to decrease postprandial TAG concentrations in healthy subjects (44-47). Moreover, after long-term consumption of diets with varying n-3 or n-6 PUFA contents, decreased fasting TAG concentrations (48), and a decreased postprandial TAG response after a standardized test meal containing 50 g of fat (49) were found after the n-3 LCPUFA diets as compared to the n-6 PUFA diets. On average doses of 3-4 g n-3 LCPUFA/day reduce fasting serum TAG concentrations by approximately 25% (40). Also, changing the n-6/n-3 ratio of fatty acids in the meal during 6 months resulted in decreased fasting TAG and postprandial TAG iAUC in men and post-menopausal women, although postprandial LPL activity was unchanged (49). This finding seems contradictory to the findings by Park *et al.(50)* that n-3 LCPUFA accelerate postprandial clearance of chylomicrons, which was suggested to be due to increased LPL activity, as an increased preheparin LPL activity was found after n-3 LCPUFA supplementation.

Several other studies have analyzed whether LPL activities did depend on fatty acid type. In one study, lower increases in LPL activity were seen after the consumption of diets high in stearic acid or palmitic acid than after the intake of meals with linoleic acid or trans oleic acid (5). Changes in LPL activity corresponded with changes in TAG (5). LPL activity may be determined by chylomicron size, which in rats is larger after a meal containing mainly LCPUFA than after a meal containing mainly lauric acid or mainly MCT (51). Based on studies in rats, Martins *et al.* (52) have shown that increased particle size increases the clearance rate, but that the number of particles might be more important than particle size. This was explained through the competition for clearance, since plasma clearance of chylomicrons and remnants is a saturable process (52).

Triacylglycerol structure and solid fat content

Studies in animals and infants have suggested that TAG structure is important in the process of fat uptake, chylomicron synthesis, and clearance. Since pancreatic lipase preferably hydrolyzes fatty acids at the sn-1 and sn-3

positions of the TAG structure (53), fatty acids in the sn-2 position are retained in the resulting 2-monoacylglycerol, which is a substrate for the resynthesis of TAG within the enterocyte. The non-esterified fatty acids from the sn-1 and sn-3 positions may form insoluble calcium/magnesium soaps, which may lower the absorption of these fatty acids. In adults, however, TAG structure does not seem to determine the extent of absorption (reviewed in (54)).
Whether TAG structure influences postprandial hyperlipidemia is not known, TAG structure is highly related to the melting curve of a fat and Berry *et al.*(8, 54) have proposed that a high solid fat content at body temperature lowers the postprandial TAG response. The solid fat particles are suggested to form micelles less readily, thereby delaying the absorption process and consequently lowering postprandial lipemia (8, 9).

MACRONUTRIENT-RELATED FACTORS INFLUENCING FATTY ACID METABOLISM

Acute interaction between dietary carbohydrates and fatty acid metabolism

Carbohydrate-rich meals may lower the absorption of fatty acids in the intestine by changing pancreatic enzyme synthetic rates. In rats, a meal rich in glucose and starch increased the pancreatic synthesis of amylase, but decreased lipase and protease mRNA expression and synthesis (55) (reviewed in (56)). These animal data probably reflect a physiological adaptive response, since a relative increase in carbohydrates coincides with a relative decrease in other macronutrients, resulting in decreased synthesis of their respective catabolic enzymes.
Several studies showed that the addition of carbohydrates to a fat load, resulted in a decreased lipemic response. Decreases in the iAUC for total serum TAG were found after glucose addition to a fat load as compared to a fat load alone, when the amount of glucose was increased from 50 g to 100 g (57) or from 50 g to 75 g or 100 g (58). Westphal et al.(58) extended these findings by looking at different lipoproteins. They found comparable concentrations of TAG in chylomicrons after a fat load alone or in combination with 75 g glucose, though the chylomicron response was delayed due to delayed gastric emptying after the fat load in combination with glucose. Moreover, these authors showed a suppressed postprandial VLDL-TAG increase after the combination of fat with glucose, which was hypothesized to be due to an insulin-mediated decrease in free fatty acid flux towards the liver and an increased flux towards the adipose tissue.

The type of carbohydrate may also affect postprandial lipemia. In an early study by Cohen *et al*. (59), the addition of 50 g of fructose or 100 g of sucrose, providing 50 g of fructose and 50 g of glucose, to a high-fat load increased both the AUC for TAG and the maximum increase, whereas postprandial lipemia was unchanged after the addition of 50 g glucose. From these findings it was concluded that fructose or fructose-containing carbohydrates increase postprandial lipemia, whereas insulinogenic carbohydrates do not. Increases in postprandial lipemia after fructose addition to a fat load have been confirmed in several other studies (60, 61).

Acute interaction between dietary protein and fatty acid metabolism

It has been suggested that dietary protein may modulate postprandial TAG by affecting hepatic VLDL synthesis through insulinotropic effects, resulting in the rerouting of free fatty acids from the liver towards adipose tissue. However, especially plant proteins, including soy protein (62, 63), lupin protein (64, 65), chickpea protein and lentil protein (66) decreased serum TAG concentrations as compared to casein protein in rats when included in the habitual diet, while plasma insulin concentrations were comparable or higher after the casein diets as compared to the plant proteins. As LPL activity was either unchanged after 22 days of 200 g/kg lupin protein as compared to casein (65), or increased in the liver after 20 days of 50 g/kg lupin protein (64) or decreased in adipose tissue after 28 days of 200 g/kg chickpea or lentil protein as compared to casein (66), the lower serum TAG concentrations were probably due to the consistently decreased VLDL synthesis after the plant proteins. This decrease may have resulted in, or be caused by, a decreased expression of sterol regulatory element-binding protein (SREBP), which is a key transcription factor for several genes involved in FA synthesis and esterification (67). This reduction may have been caused by the decreased insulin concentrations after the plant proteins, since insulin selectively stimulates SREBP mRNA (68). For humans, information on the effects of dietary protein on postprandial TAG concentrations is limited. Westphal and coworkers (69) have found that the addition of casein to a high-fat meal or to a high-fat meal with oligosaccharides significantly reduced postprandial plasma free fatty acid concentrations, possibly through the insulinotropic effects of amino acids (70, 71). Postprandial lipemia, however, was only significantly decreased in the chylomicron TAG fraction in the presence of oligosaccharides, which arguably may have contributed to the decreased lipemic response through inhibition of gastric lipase, as discussed earlier. However, a difference between the meals in gastric emptying and delayed fat absorption due to precipitation by casein may also have contributed to the delay and reduction in TAG response, mainly from the reduced and delayed

chylomicron TAG response. Due to the decreased flow of free fatty acids to the liver, a decrease in hepatic VLDL synthesis would be expected, resulting in decreased concentrations of VLDL TAG. However, this was not confirmed in the study by Westphal *et al*. (69), which may indicate that this is an effect in the longer term.

The role of longer-term intake of different macronutrients on fatty acid metabolism

The effects on postprandial fatty acid metabolism after longer-term consumption of a high-fat diet have also been studied. Sharman et al. (72) switched 12 of in total 20 healthy normolipemic male subjects from their background diet (17 En% protein, 47 En% carbohydrate and 32 En% fat) to a ketogenic, high-fat diet (30 En% protein, 8 En% carbohydrate and 61 En% fat), whereas the remaining 8 subjects remained on their background diet (control group). Whether the background diet was unchanged during the 6-week intervention period in the control group was checked by measurement of body weight, which was not changed in the control group but slightly decreased in the intervention group (-2.2 ± 1.7 kg). After 3 and 6 weeks of intervention, fasting samples of serum lipids were determined. Compared with the control group, the ketogenic diet significantly decreased fasting TAG concentrations after 3 weeks of intervention, but fasted TAG levels did not further decrease after 3 weeks. After both dietary periods, subjects were postprandially challenged by the same high-fat meal (11 En% carbohydrate, 2 En% protein, and 87 En% fat). Postprandial TAG concentrations were significantly lowered from 3-8 hours after the postprandial challenge in the group on the ketogenic diet as compared to the control group. Based upon these findings it was suggested that a short-term, ketogenic, high-fat diet not only beneficially influences fasting concentrations of TAG, but also postprandial concentrations. It should be noted however that protein intake was also higher on the ketogenic diet. Another study with 10 healthy male subjects (73) showed significant reductions in fasting and postprandial TAG after an 8-week low-carbohydrate, high-fat diet (64 En% fat, 7 En% carbohydrates, and 28 En% protein) in combination with supplementation with n-3 LCPUFA (3291 ± 314 mg) as compared to the habitual diet, which was assessed by 7-day food records (29-39 En% from fat; 493 ± 293 mg n-3 PUFA). Unfortunately, it remains unclear what caused the decreases in lipid concentrations, since the high-fat diet was also hypocaloric (8.8 MJ *vs*. 11.1 MJ). Also, these diets were higher in protein content and higher in MUFA content, which may have influenced the results.
Longer-term intake of carbohydrates increases fasting and postprandial TAG (reviewed in (74)). A role for the habitual high amount of carbohydrates in

hyperlipidemia was suggested to be due to an increased *de novo* lipogenesis (75-77), resulting in higher circulating concentrations of TAG. However, when both normolipemic (fasting TAG <1.13 mmol/L) and slightly hyperlipemic (fasting TAG levels 1.13-2.26 mmol/L) subjects switched to a 5-week isocaloric low-fat, high-carbohydrate diet after a 1-week normal diet (35 En% fat), VLDL-TAG clearance rate was reduced (78). This may suggest a role for this diet on a decreased removal of VLDL-TAG.
Unfortunately, to the best of our knowledge, no studies have been performed regarding the effects of longer-term intake of high-protein diets on postprandial TAG concentrations.

MACRONUTRIENTS AND INFLAMMATORY AND ENDOTHELIAL RESPONSES

The relation between postprandial lipemia, and postprandial inflammation

Postprandial inflammation is tightly related to postprandial lipemia, since TAG-rich lipoproteins augment cytokine production by endothelial cells and macrophages (79, 80). The most important particles involved in the interactions between postprandial lipemia, inflammation and endothelial activation are chylomicron remnants (81) and VLDL particles (79). *In vitro* studies have further suggested that interactions between chylomicron remnants and monocytes play an important role in atherogenesis (82, 83).
Several studies in healthy subjects and type 2 diabetic patients have shown that postprandial lipemia results in increased concentrations of circulating leukocytes, adhesion molecules, proinflammatory cytokines and other inflammatory markers (84-86). Moreover, several prospective cohort studies have shown that markers of inflammation (MOI), including C-Reactive Protein (C-RP) and interleukin-6 (IL-6), predict CVD in healthy individuals and in subjects with MetS (87-89). Additionally, increased concentrations of the Cellular Adhesion Molecules (CAMs) such as soluble Intracellular Adhesion Molecule (sICAM-1) as markers for endothelial activation and early atherosclerosis have been demonstrated to precede CHD several years before its clinical manifestations (90). Markers of inflammation, including C-RP, IL-6, Tumor Necrosis Factor α (TNFα) and TNF receptors, are increased in obesity (91-95) and in subjects with insulin resistance (96, 97).

The influence of dietary fat on inflammatory and endothelial markers

Postprandial changes in MOI have been repeatedly found, but results are inconsistent. C-RP is a well-known member of the acute-phase proteins, which is produced as a result of an inflammatory stimulus (98, 99). C-RP is suggested to reflect the baseline activity of circulating cytokines (100, 101) and is produced in the liver in response to increased cytokine concentrations, mainly IL-6 (102, 103). C-RP levels, however, did not change in healthy subjects after consumption of a high-fat meal (62.5 g fat) and in subjects with MetS (1 g fat/kg body weight) (104, 105). In contrast, IL-6 did vary after the consumption of a high-fat meal in obese men (60 g fat/m^2) (96), trained and untrained subjects (80 g of fat in combination with 80 g of carbohydrates)(106), and also in healthy subjects and patients with *type 2* diabetes (84). In the latter study (84), the postprandial variations in the cytokines IL-6 and TNFα, and adhesion molecules ICAM-1 and VCAM-1, were compared between healthy subjects and type 2 diabetic patients after consumption of either a high-fat meal providing 59.2 En% from fat, 12.3 En% from protein and 28.5 En% from carbohydrates with or without vitamins (vitamins C and E), or a high-carbohydrate meal providing 20.6 En% from fat, 6.5 En% from protein and 72.9 En% from carbohydrates. It was found that plasma concentrations of both cytokines and adhesion molecules increased when healthy subjects consumed a high-fat meal, but not when a high-carbohydrate meal was consumed, suggesting that the consumption of a fat-enriched meal is directly associated with endothelial activation. In type 2 diabetic patients, consumption of both meals significantly increased cytokines and adhesion molecule concentrations. When vitamins were consumed directly after the meal, the increments in cytokines and adhesion molecules were no longer found in both subject groups, suggesting that the inflammatory response is either dependent on the oxidation of TRL or that TRL-induced oxidation processes are inhibited by the antioxidant actions of vitamins. Similarly, a high-fat meal increased postprandial MOI as compared to a high-carbohydrate meal in another study in healthy subjects (107). In this study postprandial serum lipid concentrations were compared during 4 hours and endothelial function during 3 hours after intake of 75 g of glucose, a high-fat meal (35 En% fat, with no other macronutrients or energy content specified) or a standard meal (478 kcal, containing 16.4 En% protein, 32.7 En% fat and 50.4 En% carbohydrate. Only the consumption of the high-fat meal increased TAG as compared to the carbohydrate and standard meals. The high-fat meal also significantly lowered forearm blood flow and flow debt repayment, suggesting a deleterious effect of acute fat consumption on endothelial function.

In vitro, it was shown that the n-6 PUFA increase endothelial activation.(108) *In vivo*, however, these acute n-6 PUFA specific inflammatory effects have not

been found. Tulk *et al*. (105) studied the postprandial effects after fat loads with varying n-6/n-3 ratios by blending palm stearin and safflower oil and adding fish oil to the mixture to achieve high or low n-6/n-3 ratios of 20:1 or 2:1 respectively. Irrespective of this ratio, IL-6 concentrations increased postprandially after the fat loads as compared to a water control.
Consumption of both butter and walnuts increased the activity of postprandial NFκB (109), which is a redox-sensitive transcription factor (110). Olive oil on the other hand, which contains antioxidative polyphenols, did not elicit postprandial NFκB activation. However, since walnuts also contain polyphenols, it cannot be concluded whether the absence of postprandial NFκB activation after olive oil was due to the difference in the type of fatty acid consumed (MUFA) or to the higher antioxidative capacity (109).
In an intervention study, healthy occasionally-smoking subjects used either no meal, 3 cigarettes, 3 toasts with 50 g of butter, or the same meal with 3 cigarettes. After the high-fat meals, increased plasma LPS concentrations were found, as compared to when no meal was consumed or only cigarettes were smoked (111). The increased plasma LPS concentrations after the high-fat meals were accompanied by a decrease in LPS neutralization capacity, whereas this was not affected by the other two conditions. These findings may indicate a more efficient transport of bacterial LPS from the intestinal lumen into the blood stream with fat. Since LPS has a high affinity for chylomicrons, LPS is possibly incorporated into chylomicrons by the Golgi complex in the enterocytes before exocytosis (112, 113). The comparison of two fatty acids, i.e. oleic acid and butyric acid supported this hypothesis (114). Short-chain butyric acid, which is absorbed into the hepatic portal vein and consequently does not lead to chylomicron formation, did not induce an increase in cell-associated LPS release. Long-chain oleic acid on the other hand, which is incorporated into chylomicrons, resulted in significantly higher cell-associated LPS release. Moreover, addition of an inhibitor of chylomicron formation blocked the oleic acid-induced effects on LPS levels. Noteworthy, however, since the methodology on plasma LPS measurements is not widely accepted, these findings are not firmly established.

Dietary carbohydrates and postprandial MOI and endothelial function

In a study with lean, young adults, postprandial NFκB activity and concentrations of the oxidative stress marker nitrotyrosine were increased two hours after the consumption of bread with a high glycemic index (GI) when compared with low GI pasta (115). As no correlation was found between NFκB-activity and postprandial insulin levels, these effects were attributed to the increases in plasma glucose concentrations. However, also no correlation between changes in venous glucose concentrations and NFκB activity was

found. However, since the first venous blood was drawn after 1 h, the timing of blood sampling may be the explanation for the lack of a significant correlation. Currently, there is no strong evidence that the glycemic index or load *per se* plays a role in postprandial inflammatory processes, since no alterations in inflammatory markers have been shown after a high GI meal as compared to a low GI meal (116, 117). However, these latter findings are hard to interpret, since there were also large differences in fiber content.
It is also believed that dietary fructose, which is a low-GI carbohydrate, may have pro-inflammatory characteristics as it was previously implicated to contribute to the increased incidence of obesity and MetS (118, 119). Unfortunately, no *in vivo* data is present on the acute effects of fructose on postprandial inflammatory markers in human subjects.

Dietary protein and postprandial MOI and endothelial function

The effects of dietary protein on inflammatory and endothelial responses have mainly been attributed to the ratio of lysine:arginine. In this respect, several studies have shown that the process of atherosclerosis is accompanied by a reduction in nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS) in the arterial wall (120-122). Since L-arginine is a precursor of epithelium-derived NO, several recent human studies (123-126) have examined inflammatory and endothelial processes after L-arginine supplementation, showing beneficial effects on flow-mediated dilation (FMD), but not on inflammatory markers. To the best of our knowledge, only three studies (125, 127, 128) have evaluated the role of dietary protein on postprandial inflammatory and endothelial responses. Two studies found improvements in FMD after acute L-arginine consumption (125, 127). In the third study, the addition of 50 g of either sodium caseinate or soy protein to an acute fat load (1g fat/kg bodyweight) prevented in healthy subjects the decreases in flow-mediated dilation of 58% caused by the fat load alone (128). The authors suggested that both the increased supply of L-arginine, but also the proteins' insulinotrophy played a role in these improvements, since both proteins increased insulin concentrations and the ratio of L-arginine versus asymmetric dimethylarginine (ADMA), a molecule that inhibits eNOS activity and consequently NO production in the vessel wall.
Insulin may have both direct and indirect effects, including vasodilatation, and decreased FFA concentrations, resulting in decreased VLDL production and, as a result, decreased lipemia. These positive effects on FMD were not found when glucose was the stimulator of insulin secretion (129), since glucose increased the production of ROS (e.g. peroxidation of NO by superoxide to form peroxynitrite) (130), which may counteract the beneficial effects of insulin.

CONCLUSION

This review presents an overview on the influence of macronutrients on postprandial lipemic, inflammatory and endothelial responses. These processes are closely related at different levels. For lipemia, evidence is present for a relation with increased CVD risk, while the importance of postprandial inflammatory and endothelial processes is becoming more and more evident. Of course, fatty acids are a prerequisite for postprandial lipemia, but dietary protein and carbohydrates have significant modulatory effects. Fat-specific determinants of lipemia include the amount of fat in the diet and its fatty acid composition. In this respect, chain length, degree of saturation, TAG structure and solid fat content result in various effects on the different steps of digestion, absorption, and metabolic processing. Dietary carbohydrates and protein may have considerable effects on fatty acid handling, mainly through insulinotropic effects, hereby decreasing hepatic VLDL output. Compared with glucose, fructose-containing carbohydrates may however increase postprandial TAG. Concerning inflammation and endothelial activity, studies have used various markers and techniques. There are clear indications that diet can result in activated endothelium and inflammatory signaling. However, there is not much data available on macronutrient-specific differences. There is some evidence that mainly high-fat diets induce these processes and that there are differences between fatty acids. Differences between fatty acids in single meals are poorly understood. Moreover, there are some suggestions that high GI carbohydrates may be proinflammatory, while dietary proteins with high L-arginine contents may have beneficial effects on endothelial activity. In general, however, macronutrient-specific effects on postprandial inflammatory processes and endothelial activity have hardly been studied.

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FATTY ACID- AND CHOLESTEROL TRANSPORTER PROTEIN EXPRESSION ALONG THE HUMAN INTESTINAL TRACT

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ABSTRACT

Protein distribution profiles along the human intestinal tract of transporters involved in the absorption of cholesterol and long-chain fatty acids (LCFA) have been scarcely evaluated. In post-mortem samples from 11 subjects, intestinal transporter protein distribution profiles were determined by Western Blot. Differences in transporter levels were statistically tested using ANOVA and Tukey's Post Hoc comparisons. Levels in all segments were expressed relative to those in duodenum. Except for ABCG5 and FATP4, levels (mean ± SEM) were the highest in the ileum. For ABCA1, ileal levels (1.80 ± 0.26) differed significantly from those in duodenum (P=0.049) and proximal colon (0.92 ± 0.14; P=0.029). ABCG8 levels in ileum (1.91 ± 0.30) differed from those in duodenum (P=0.041) and distal colon (0.84 ± 0.22; P=0.010) and jejunum (1.64 ± 0.26) tended to be higher than distal colon (0.84 ± 0.22; P=0.087). Ileal NPC1L1 levels (2.56 ± 0.51) differed from duodenum levels (P=0.019) and from distal colon (1.09 ± 0.22; P=0.030). There was also a trend (P=0.098) for higher jejunal (2.23 ± 0.37) than duodenal NPC1L1 levels. The levels of ABCG5 did not correlate with those of ABCG8. FAT/CD36 levels in ileum (2.03 ± 0.42) differed from those in duodenum (P=0.017), and proximal and distal colon (0.89 ± 0.13 and 0.97 ± 0.15 respectively; P=0.011 and P=0.014). FABPpm levels in ileum (1.04 ± 0.13) differed from proximal (0.64 ± 0.07; P=0.026) and distal colon (0.66 ± 0.09; P=0.037). The distribution profiles showed a bell-shape pattern along the GI-tract with the highest levels in ileum for ABCA1, ABCG8, NPC1L1, FATCD36 and FABPm, suggesting a role for ileum in transporter-mediated uptake of cholesterol and LCFAs.

INTRODUCTION

The incidence of the metabolic syndrome (MS) has rapidly increased over the last few decades (1, 2). MS is a clustering of metabolic risk markers, including abdominal obesity, elevated plasma glucose levels, and an atherogenic lipid profile, which altogether contribute to the development of cardiovascular disease (CVD) (3, 4). Patients suffering from the MS often show disturbances in fatty acid (FA) metabolism (5) leading to elevated plasma free fatty acid levels which negatively influence insulin-mediated glucose uptake (6, 7). The disturbances in lipoprotein profiles most likely originate from an elevated hepatic production of large triacylglycerol-rich VLDL1 particles, which in combination with increased cholesterylester transfer protein (CETP) mediated lipid fluxes and decreased lipoprotein lipase (LPL) mediated lipolysis results in hypertriglyceridemia and low serum HDL cholesterol concentration (8-10). However, it becomes more and more evident that lipoprotein metabolism is also regulated by absorption characteristics of cholesterol and FA in the intestine. For example, it was recently shown that the level of cholesterol absorption from the intestine was inversely related to reverse cholesterol transport from peripheral tissue macrophages into the feces (11).

Concerning the MS, there is an ongoing discussion whether these patients are characterized by elevated and/or accelerated intestinal cholesterol absorption or not. In this respect, Miettinen et al. (12) have proposed, that subjects can be characterized as cholesterol absorbers (with a low cholesterol synthesis) or as cholesterol synthesizers (with a low cholesterol absorption). Based on circulating levels of plant sterols which can be used as markers for fractional cholesterol absorption, it has been suggested that subjects with the MS are rather cholesterol synthesizers than absorbers (13). Indeed, obese subjects displayed increased cholesterol synthesis and a decreased fractional cholesterol absorption (14).

Intestinal cholesterol absorption is tightly regulated by a number of transporter proteins and whether an individual is a cholesterol absorber or cholesterol synthesizer might be influenced by variations in the intestinal protein levels or activities of these cholesterol transporters. Four important proteins involved in transporting sterols across the intestinal lining are (1) adenosine-triphosphate (ATP) binding cassette A1 (ABCA1), a 226 kDa basolateral membrane protein which is involved in the synthesis of HDL by basolateral donation of cholesterol to its acceptor apolipoprotein A1; (2) ATP binding cassette G5 (ABCG5) and (3) G8 (ABCG8), also called sterolin-1 and sterolin-2 respectively, both with a molecular weight of ~65 kDa. ABCG5 and ABCG8 are expressed apically at the brush border membrane (15) and collaborate in transporting excess sterols out of the enterocyte back into the lumen (16). The fourth protein is Niemann-Pick C1 like 1 (NPC1L1), a 150 kDa protein which

may cooperate with the scavenger receptor class B type 1 (SR-BI) (17) and regulates the intestinal uptake of sterols (**Figure 3.1**).
In addition, some recent studies suggest that several FA transporters, i.e. membrane fatty acid translocase (FAT/CD36) and cytoplasmic fatty acid-binding protein (I-FABP and L-FABP), are also involved in the uptake of cholesterol, making cholesterol and long-chain fatty acid (LCFA) uptake potentially interrelated (18-20).
Important intestinal FA transporters are (1) FABPpm, a 40 kDa protein located peripherally on the plasma membrane and identical to the mitochondrial enzyme aspartate aminotransferase (21); (2) FAT/CD36, an 88 kDa integral membrane glycoprotein with two predicted transmembrane domains, also known as the Class B scavenger receptor CD36 (22) and (3) fatty acid transport protein subtype 4 (FATP4), a 63kDa integral membrane protein which possibly can drive fatty acid uptake or activate FA by trapping them inside the cell as their CoA thioesters (23-27). Once LCFA are taken up, they can be transported to the mitochondria via cytoplasmic fatty acid-binding proteins ($FABP_c$) for ß-oxidation (**Figure 3.2**) (28, 29). These proteins also facilitate the cellular uptake of FA and protect against the cytotoxic effects of free cellular FA (30).
Data concerning differences in the intestinal levels of these transporter proteins between MS patients and healthy controls is not available. However, new strategies to improve serum lipoprotein profiles could focus on the role of the intestine in dyslipidemia (31, 32). Therefore, it is important to know the intestinal protein distribution profiles of these transporter proteins, which are chosen based upon recent up-to-date reviews, in order to develop possible site-specific modulators of intestinal fatty acid and sterol metabolism. For these reasons, we decided to investigate the protein levels of these transporters in different segments of the intestinal tract to visualize their distribution profiles along the human duodenal-colonal axis.

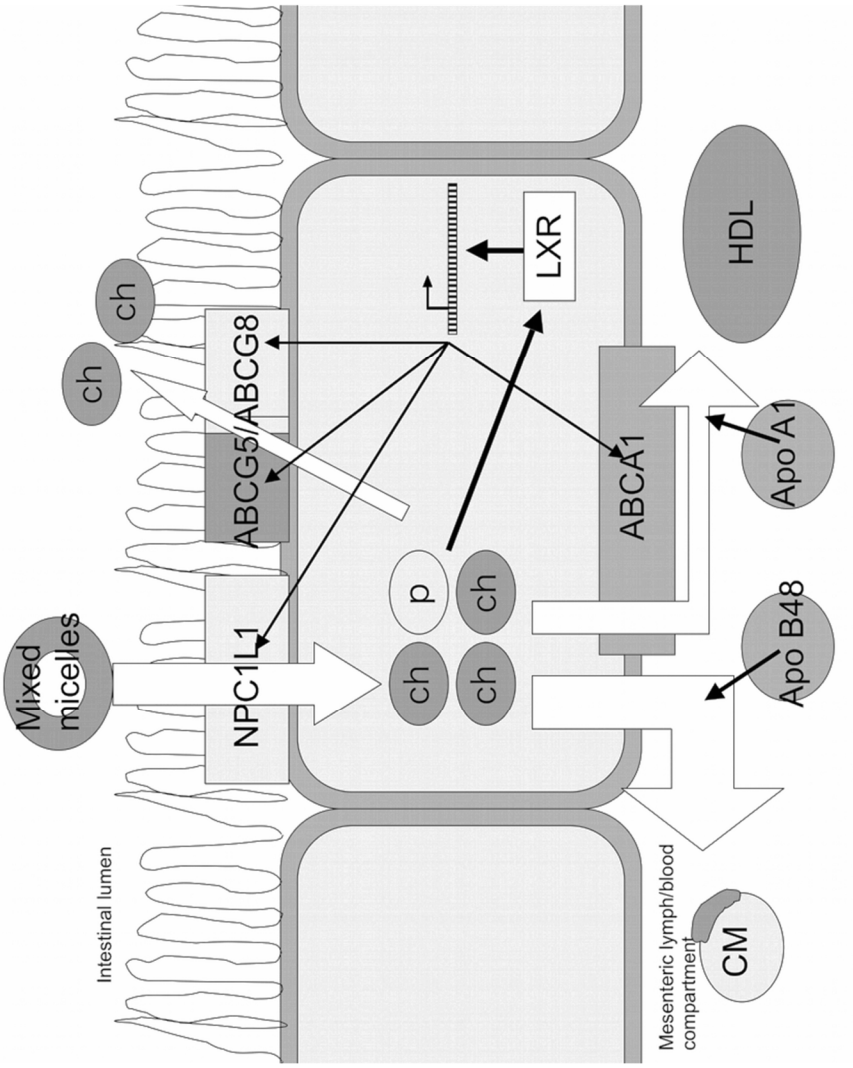

Figure 3.1: Schematic overview of the uptake of dietary cholesterol (chol). Dietary cholesterol and plant sterols/stanols (ps) travel, incorporated in mixed micelles, through the intestinal lumen. The sterols are transported across the brush-border membrane by NPC1L1. Once taken up, sterols are either incorporated in apoB48-rich chylomicrons (CM), which are secreted in the lymph compartment, or used to form apoA1-rich HDL cholesterol, a process that is mediated by ABCA1. Excess amounts of sterols are also excreted into the intestinal lumen by the reverse sterol transporters ABCG5 and G8. The level of transport proteins is under tight control of the liver X receptor (LXR) gene. This gene indirectly measures cellular sterol levels and regulates the transcription of sterol transporters NPC1L1, ABCA1, ABCG5 and G8.

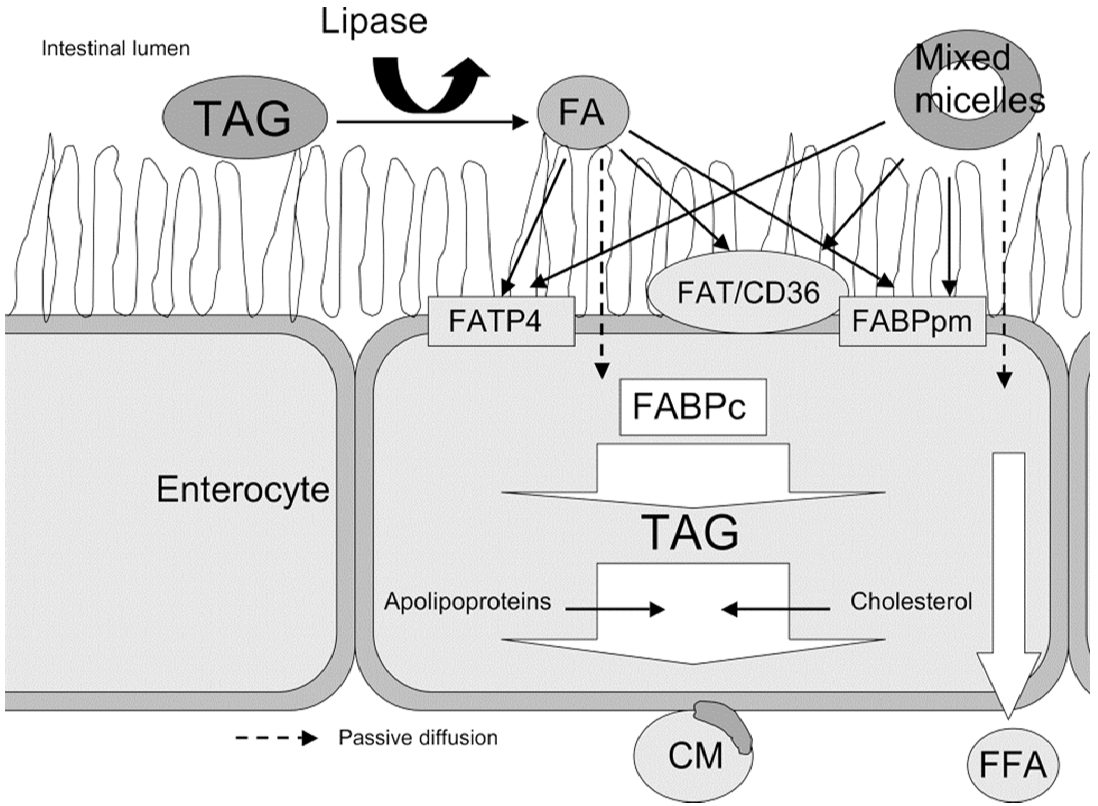

Figure 3.2: Schematic overview of the uptake of LCFA. Dietary fatty acids (FA) pass through the intestinal lumen whilst esterified to triacylglycerols (TAG) or incorporated into mixed micelles. Gastric and hepatic lipases free the fatty acids, which are then receptive to uptake. Fatty acids are either transported across the apical membrane actively by FATP4, FAT/CD36 or FABPpm, or passively diffuse (blocked arrows) through the lipid bilayer. In the enterocyte $FABP_c$ facilitates fatty acid transport through the cytosol. In the cytoplasm, the major part of fatty acids is re-esterified to triacylglycerols and excreted into chylomicrons (CM), whereas a small part is ex-creted as free fatty acids (FFA).

MATERIALS AND METHODS

Human tissue preparation

Human intestinal tissue samples were obtained from autopsies of 7 female and 4 male subjects, aged 37 to 83 years (62.8 ± 5.0 (mean ± SEM); Medical University of Bialystok, Bialystok, Poland and Mental Hospital, Choroszcz, Poland), 18.1 ± 1.5 h after death (**Table 3.1**). The subjects died of non-intestine related diseases, such as cardiac, cerebral, renal or pulmonary events. Samples, which were directly frozen in liquid nitrogen, were taken from the duodenum, jejunum, ileum, proximal colon and distal colon. Subsequent sample preparations were performed at 4°C.

Before analyses, tissues were homogenized (3-16% w/v) in SET-buffer (0.25 M sucrose, 10 mM EDTA, 10 mM Tris, pH 7.4) using an Ultra-Turrax homogenizer (IKA Werke, Breisgau, Germany) and sonificated on ice (4 × 15 s, MSE ultrasonic disintegrator). Total crude homogenate samples were stored at -80° C until analyses. Protein concentrations in the tissue homogenates were quantified with the Pierce micro-BCA assay (Pierce, Rockford, USA).

Table 3.1: Subject information on age at death and biopsy time (mean ± SEM).

	men	women	all	P
n	4	7	11	-
age at death	57.8 ± 9.4	65.7 ± 5.9	62.8 ± 5.0	0.469
time of biopsy	20.0 ± 2.8	17.0 ± 1.7	18.1 ± 1.5	0.360

Western blot analyses of human FABPpm, FATP4, ABCG5/G8, ABCA1 and NPC1L1

For detection of FABPpm, tissue samples containing 30 µg protein were loaded on SDS-PAGE precast gels (4-15% gradient gel, Tris-HCl, 1.0 mm, Biorad, Hercules, USA) applying the Criterion™ system (Biorad, Hercules, USA). For detection of ABCG5, ABCG8 and FATP4, self-cast gels were prepared with a 7.5% gradient. For ABCA1 and NPC1L1 detection, a gel with a gradient of 5% was prepared. Samples were electrophorezed (90 min, 200 V) and blotted on nitrocellulose (0.45 µm) at 4°C. After blotting, membranes were blocked overnight at 4°C with either 5% non-fat dry-milk (NFDM) in TBS-Tween 20 0.1% (TBS-T) for FABPpm, ABCG5, ABCG8 and FATP4, and 3% NFDM in TBS-T for ABCA1 and NPC1L1. After blocking, all blots were washed

5 times with TBS-T and incubated for 1 h with the primary antibodies polyclonal goat anti-FATP4 (prepared, purified and characterized by dr. J. Füllekrug, Department of Gastroenterology, University Hospital Heidelberg, Germany), polyclonal anti-ABCG5 and anti-ABCG8 (Santa Cruz Biotechnology, CA, USA), polyclonal rabbit anti-FABPpm (kind gift from dr. Calles-Escandon, Department of Medicine, University of Vermont, Burlington, Vermont), monoclonal mouse anti-ABCA1 (kind gift from dr. A.K. Groen, Department of Pediatrics / Laboratory Medicine, University Hospital Groningen, Groningen, The Netherlands), polyclonal rabbit anti-NPC1L1 (Cayman Chemical, MI, USA) in either 5% non-fat dry-milk (NFDM) in TBS-Tween 20 0.1% (TBS-T) for FABPpm, ABCG5 and G8 and FATP4, and 3% NFDM in TBS-T for ABCA1 and NPC1L1. Following overnight blocking, secondary antibodies were added and incubated for 60 min. Detection of ABCG5/G8, FABPpm, FATP4, ABCA1 and NPC1L1 was performed by enhanced chemi-luminescence (ECL). Blots were subsequently analyzed by Quantity One software (Biorad, Hercules, USA).

FAT/CD36 ELISA

FAT/CD36 protein level in the different segments was measured via a sandwich ELISA as described previously (33). In short, a polyvinylchloride microtitre plate (Falcon type 3912, Beckton Dickinson, Oxnard, CA) was coated overnight and washed five times. Thereafter, wells were blocked for 30 min with PBS/2% Marvel. Following 5 washing steps with PBT, standard containing 0-1 µg/ml recombinant 6-His FAT in PBS/0.4 %Triton X-100 was added per well. The samples were diluted 1:1 and added to the plate after centrifugation. After incubation for 90 min, wells were washed. Then, phage (2×10^{11} colony forming units (cfu)/well in PBS/2% Marvel) were added and incubated for 90 min. After 5 washes, 1/5000 diluted sheep anti-fd labeled with horseradish peroxidase in PBS/2% Marvel was added. After one-hour incubation and 5 washes, plates were developed with 100 µl TMB/well. The reaction was stopped after 10 min and the absorbance was read at 450 nm using a Titertek Multiscan MkII microplate reader. The CD36 ELISA showed a detection limit of 50 µg/L. Using standards of 2000 and 1000 µg/L, the intra-assay and inter-assay coefficients of variation (CV) were <10% and 15%, respectively.

Standardization

Protein level of each lane was standardized by loading 30 μg of protein for every sample. A duodenal biopsy sample was included in every blot as an internal standard and levels of intestinal protein level of all biopsy samples loaded on the blots were corrected for the variations in the internal standards. Furthermore, duodenal transporter level was set at 1 and levels in the other segments were compared to that in duodenum.
Since intestinal samples contained both enterocytes and myocytes, we also measured H-FABP as a marker of muscle content with a direct non-competitive sandwich-type ELISA using monoclonal antibodies obtained from Hycult biotechnology (HK 403; Uden, the Netherlands) as described previously (34). These data showed significantly higher H-FABP in jejunum (1.73 ± 0.30) as compared to duodenum, ileum (1.09± 0.19), proximal colon (0.97 ± 0.16) and distal colon (0.90 ± 0.17), whereas the levels between the other segments were not different. Because of this, we concluded that the biopsy muscle content was for an unknown reason higher in our jejunum biopsies and this might have resulted in an underestimation of cholesterol transport protein levels. Therefore we corrected the cholesterol transport protein levels for muscle content. Because FA transport proteins are also present in myocytes, differences in distribution patterns of the fatty acid transporters could have been influenced by the higher levels of myocytes in our jejunal biopsies. However, it is not possible to correct FA transporter levels for this possible confounding effect, because of their presence in myocytes and we do not know whether this correction might lead to overestimation or underestimation of the transport protein levels.

Statistical analysis

Data are presented as mean ± SEM. All protein levels were related to the duodenal protein level. All data were normally distributed as tested with Shapiro-Wilk's test for normality. Analysis of variance (ANOVA) was used to compare mean transporter levels between the various segments of the intestine. Alpha inflation due to multiple comparisons was corrected with Tukey's HSD Post Hoc tests and $P<0.05$ was considered to be statistically significant. Independent sample t-tests were used to compare transporter levels between men and women within each segment. Pearson's correlations were determined to evaluate the possible relationship between ageing and intestinal transport protein level within each segment. Moreover, linear regression analysis was performed regarding time of biopsy after death versus expression levels of ABCA1, ABCG5, ABCG8, NPC1L1 and FAT/CD36 to evaluate whether protein degradation was an issue. All tests were performed with SPSS 16.0 (SPSS Inc. Chicago, Illinois).

RESULTS

(Chole)sterol transport proteins

As explained in the method section, the level of each individual protein in duodenum was arbitrarily set at 1. ABCA1 protein level was significantly higher in ileum (1.80 ± 0.26) than in duodenum (P=0.049) and proximal colon (0.92 ± 0.14; P=0.029) (**Figure 3.3A**). For ABCG5, no significant differences between duodenum, jejunum (1.41 ± 0.19), ileum (1.13 ± 0.20), proximal colon (0.98 ± 0.24) and distal colon (0.98 ± 0.19) were found (**Figure 3.3B**). Ileal level of ABCG8 was significantly higher (1.91 ± 0.30) than that in duodenum (P=0.041) and distal colon (0.84 ± 0.22; P=0.010) and also tended to be higher in jejunum (1.64 ± 0.26) than in distal colon (P=0.087) (**Figure 3.3C**). The protein level of the cholesterol transporter Niemann-Pick C1 Like 1 was significantly higher in ileum (2.56 ± 0.51) as compared to that in duodenum (P=0.019) and in distal colon (1.09 ± 0.22; P=0.030), whereas jejunum NPC1L1 level (2.23 ± 0.37) tended to be higher than distal colon (P=0.098) (F**igure 3.3D**). Blots of individual subjects representative for the group are presented in figure 3E. Protein levels of the various sterol transporters did not differ between men and women, and did not correlate with age. There was no significant correlation between ABCG5 and ABCG8.

Concerning the linear regression analysis, no relation was found between time of biopsy after death and expression levels of ABCA1, ABCG8, NPC1L1 and FAT/CD36, whereas even a positive relation was found for ABCG5 (R^2=0.25; P=0.0001). Additionally, this analysis was performed per intestinal segment, but the results were comparable (data not shown).

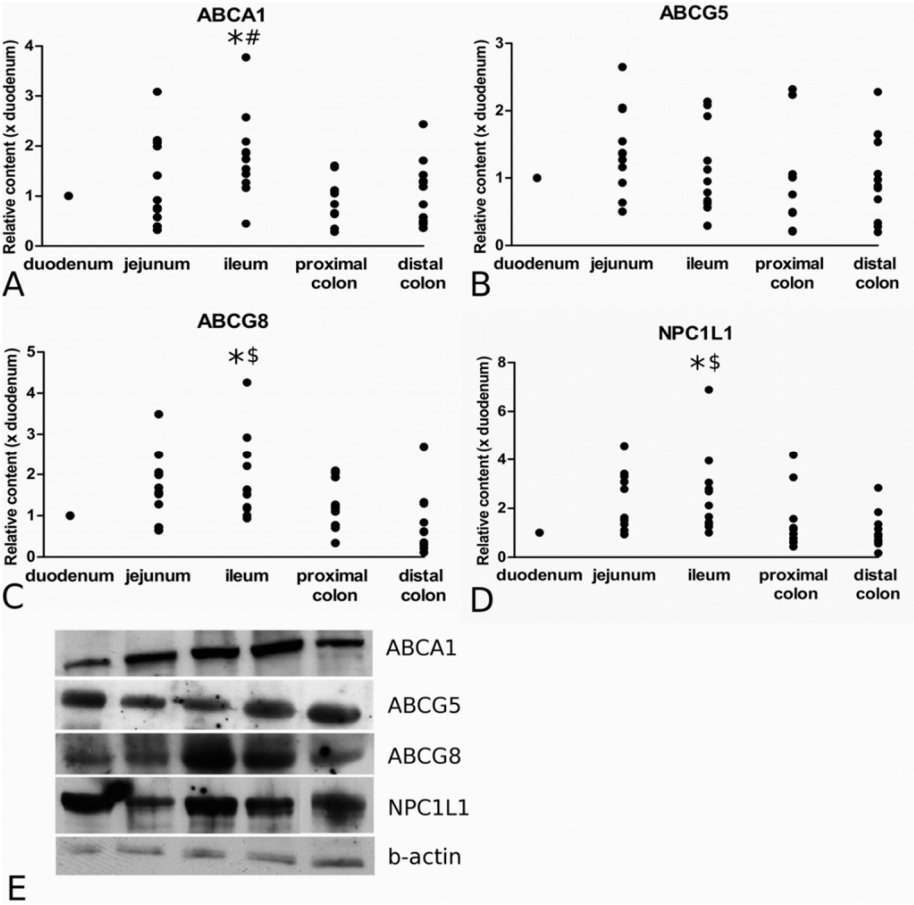

Figure 3.3: Distribution of the cholesterol transport proteins ABCA1 (panel A), ABCG5 (panel B), ABCG8 (panel C) and NPC1L1 (panel D) along the longitudinal axis of the human intestine. Panel E displays Western Blots of single subjects, which are representative for the average distribution pattern of ABCA1, ABCG5 and ABCG8 and NPC1L1. * = P<0.05 compared to duodenum; # = P<0.05 compared to proximal colon; $ = P<0.05 compared to distal colon. D = duodenum, J = jejunum, I = ileum, PC = proximal colon and DC = distal colon.

Long-chain fatty acid transport proteins

FABPpm protein level was significantly higher in ileum (1.04 ± 0.13) than in proximal colon (0.64 ± 0.07; P=0.026) and distal colon (0.66 ± 0.09; P=0.037), and tended to be higher in duodenum than in proximal and distal colon (P=0.059 and P=0.082 respectively) (**Figure 3.4A**). For FATP4, no significant differences were found between any of the segments (**Figure 3.4B**). FAT/CD36 protein level was significantly higher in ileum (2.03 ± 0.42),

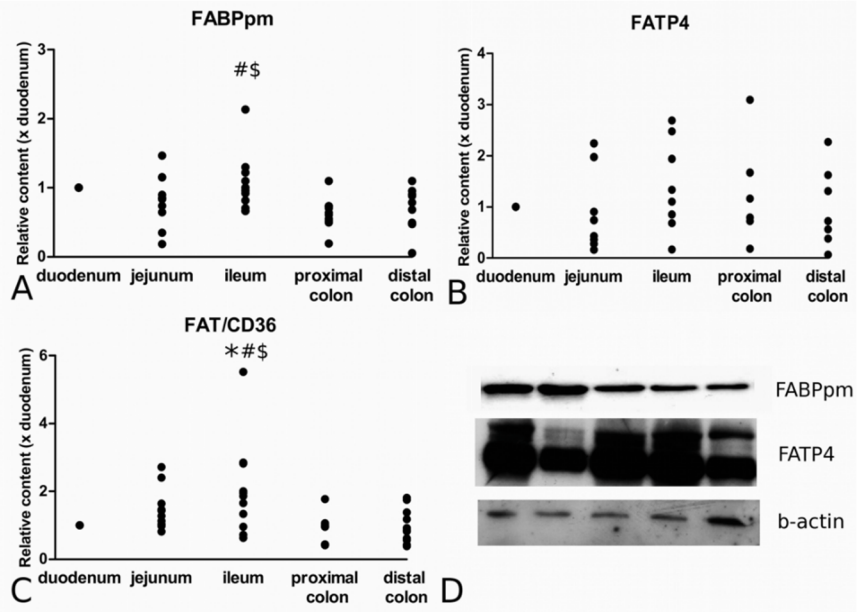

Figure 3.4: Distribution of fatty acid transporters FABPpm (panel A), FATP4 (panel B) and FAT/CD36 (panel C) along the proximal-distal axis of human intestine. Panel D displays Western Blots of the different segments of a single subject, which is representative for the average distribution pattern of FABPpm and FATP4. * = P<0.05 compared to duodenum; # = P<0.05 compared to proximal colon; $ = P<0.05 compared to distal colon. D = duodenum, J = jejunum, I = ileum, PC = proximal colon and DC = distal colon.

when compared to that in duodenum (P=0.017), proximal colon (0.89 ± 0.13; P=0.011) and distal colon (0.97 ± 0.15; P=0.014) (**Figure 3.4C**). Comparable to the sterol transporters, no significant differences were found in intestinal LCFA transporter levels between men and women and no correlations with age could be shown. Blots of individual subjects representative for the group are presented in **Figure 3.4D**.

DISCUSSION

Sterol transporters

Since intestinal cholesterol absorption is tightly regulated by a number of transporter proteins (ABCA1, ABCG5, ABCG8, and NPC1L1) we decided to describe for the first time distribution profiles of these transporter proteins along the duodenal-colonic axis in humans, as a first step to further understand *in vivo* human intestinal cholesterol metabolism. In contrast to humans, some animal data on the importance of different segments for intestinal cholesterol metabolism is available. For example, Sylven et al. (35) have reported that in rats the major part of cholesterol was taken up in the proximal half of the small intestine, when cholesterol was administered in crystallized form. When administered in an oil phase, the gross uptake occurred more distally. In humans, cholesterol is in the duodenum first incorporated into micelles. Thus, if these animal data can be extrapolated to the human situation, it suggests that cholesterol absorption in humans mainly occurs in the distal part of the small intestine. However, from *in vivo* studies with porcine intestine, it was concluded that the predominant site for cholesterol uptake was the jejunum (36-38). Thus, results from animal studies are not uniform and it was therefore a priori unclear where the protein level of cholesterol-transporter proteins like NPC1L1, ABCG5 and ABCG8 would be highest. We now found that the levels of NPC1L1 and ABCG8 were bell-shaped along the proximal-distal axis, with the highest levels in ileum, suggesting that transporter-dependent sterol fluxes in humans are located more distally than in pigs.

For mRNA, Davies et al. (39), showed that NPC1L1 expression was very similar in jejunum, ileum and duodenum, but higher in these segments than in segments of the large intestine. This may suggest that protein levels do not parallel mRNA expression. Another study in rats found the highest NPC1L1 mRNA and protein levels in jejunum (40). Similarly, Sané and coworkers (17) found the highest levels of NPC1L1 in jejunum in human subjects. The differences between this study and our results might be explained by the difference in subject population, since the samples in the study by Sané *et al*. were partially derived from patients with Crohn's Disease, which are often known to display abnormalities in lipid metabolism (41, 42), which presumably might also lead to alterations in protein expression levels and distribution patterns. Finally, it should also be realized that a lot of the data available in the literature is derived from animal studies. However, it was shown for example that there are differences in organ-specific transport protein mRNA levels between mouse, rat and human tissue (40), which may also indicate different distribution patterns between species within the intestine.

Because of the co-localization of ABCG5 and ABCG8 towards the apical membrane (43-45), a similar distribution pattern of these two transporters was expected. However, ABCG5 levels were very similar between the intestinal segments and did not correlate with ABCG8 levels, for which we do not have an explanation.
ABCA1 plays a role in the biogenesis of anti-atherogenic high-density lipoproteins (HDL) in the intestinal lining (46-48). Using enterocyte specific ABCA1 knock-out mice, Brunham et al. (49) showed that in mice approximately 30% of the steady state plasma HDL pool was due to intestinal ABCA1 activity. According to our data, ABCA1 protein levels were the highest in ileum. A similar distribution has been described for ABCA1 mRNA in hamsters (50). This may suggest that the ABCA1-mediated assembly of HDL in the human intestine mainly occurs in ileum.
Except for levels (expressed per mg of total protein) of the active transporters, the actual absorption also depends on the surface areas of the different segments. Estimates for the duodenum are 0.1 m^2, for jejunum 60 m^2, for ileum 60 m^2, and for colon 0.2 m^2 (51, 52). Combining these areas with the segmental transporter levels, it can now be hypothesized that the ileum is the most important site for transporter-dependent sterol fluxes.

Sterol and LCFA transporters

Recent studies have suggested that the LCFA transporter FAT/CD36, like the cholesterol transporter NPC1L1 a member of the CD36 superfamily, plays a crucial role in cholesterol uptake in the proximal intestine of mice (18). In FAT/CD36 deficient mice, *in vitro* cholesterol uptake in the first of three equal intestinal segments was approximately 75% lower as compared to that in wild-type littermates. For the second and third segment no differences were found. The distribution pattern of FAT/CD36 was comparable to the pattern found for NPC1L1, but the highest protein expressions were found more distally than would be expected based upon the hypothesis of their role in proximal cholesterol uptake.
In a different study on the role of FA transporters in cholesterol transport (19), overexpression of cytoplasmic I-FABP in a human intestinal epithelial cell line not only resulted in a decrease in free cholesterol absorption from micelles, but also in a downregulation of NPC1L1, and upregulation of ABCA1, ABCG5 and G8. These findings suggest that overexpression of cytosolic I-FABP favors cholesterol efflux.
Expression of transporter molecules may be changed by the composition of the diet, as in mice the FABP protein level in the distal small intestine was increased after high-fat intakes (53). Similar findings were shown for FAT/CD36 after consumption of LCFAs, but not after consumption of MCFAs

(54). These studies clearly indicate a role for nutrition in regulating mRNA and possibly also protein levels. Unfortunately, we have no data records on dietary habits of the subjects. De Vogel-van den Bosch et al. (20) studied the possible interrelations between FA and cholesterol uptake *in vivo*. A cholesterol-free, high-fat diet suppressed gene expression of the cholesterol transporters NPC1L1, ABCA1, ABCG5 and ABCG8 in the middle segment of the mouse small intestine after 2, 4 and 8 weeks. Thus, these studies suggest that an interrelation exists between FA and cholesterol uptake, potentially mediated by changes in intestinal transporter activity. Although these interrelations are not completely understood, it does suggest that knowledge on distribution profiles of LCFA transporters in combination with those of sterol transporters is needed to optimize potential intervention strategies to lower sterol absorption.

Besides playing a potential role in intestinal cholesterol uptake, FAT/CD36 is also involved in a wide range of physiological processes and disorders (55). FAT/CD36 in particular is important for very long-chain (VLC) FA (more than 18 carbon atoms) metabolism, since intestinal VLCFA uptake was completely abolished in $CD36^{-/-}$ mice (56).

Lobo *et al. (57)* described that in humans, FAT/CD36 protein was restricted to duodenal and jejunal epithelium. On the other hand, Poirier *et al*. (54) also found FAT/CD36 protein in rat ileal epithelium, although in lower amounts, with the highest FAT/CD36 protein expression in jejunum, followed by duodenum. In both of the studies, membrane proteins were separated from the lysates and then analyzed. In contrast, Chen *et al.* (58) used total cell protein, and found a distribution pattern comparable with that of Poirier *et al*. (54), but also found FAT/CD36 protein in the stomach and colon. Another study by Nassir *et al.(18)* found the highest levels of FAT/CD36 in rats in duodenum, with a steep decrease in expression when proceeding more distally.

Since discrepancies were found between species, Wang *et al.* (59) looked at tissues of both rats and humans. These authors also used total lysates and showed ubiquitous CD36 mRNA and protein in all intestinal segments of the rat. In the human samples the mRNA distribution was different from FAT/CD36 protein, since FAT/CD36 protein was highest in ileum, while mRNA was lower in ileum than in duodenum, jejunum and colon. Our data closely resemble the protein data from Wang *et al.*, in which the same technique for tissue preparation was used. The above data indicates that there are differences in the distribution patterns of CD36 between different species and that, at least in humans, protein levels do not resemble mRNA levels, a finding of which there are numerous other examples as reviewed by Glatz *et al.(60)*. Our results suggest a more distally situated (ileum) FAT/CD36-mediated

uptake of LCFA in humans than in rats. Additionally, we found a small amount of FAT/CD36 in colon, but levels were relatively low.

Wang *et al.* (59) even suggested that FABPpm is co-expressed with FAT/CD36. Indeed, there are several indications for an interaction between both transporters at the protein level (61, 62). As levels were the highest in the ileum, our results suggest that the small intestine is more important than the large intestine in FABPpm mediated LCFA uptake. Co-expression of FABPpm and FAT/CD36 is not suggested by our data, since we did not find significant correlations for any of the segments.

Also FATP4 is important in LCFA transport (23, 63). In mice, Stahl et al. (23) showed the highest expression of FATP4 in jejunum and ileum, but there were also detectable amounts present in duodenum. These findings are partly confirmed by our study, since we found FATP4 in all segments. The presence of FATP4 in human colon is not in agreement with the findings of Stahl et al. (23). We suggest that the presence of fatty acid transporters in the distal intestine is a last resort for the absorption of LCFA. In fact, fatty acid uptake is very efficient and approximately 90 - 95% of dietary fatty acids are absorbed. Most of the FA uptake is probably complete in the proximall small intestine (64, 65), but also ileum is capable of absorbing FA (66, 67), which is in line with the locations of the LCFA transport proteins in combination with estimations of segmental surface area. Additionally, based upon the findings mentioned above and the finding that bowel resection of the proximal 75% of the pig small intestine led to adaptive absorption of cholesterol in ileum (36), it may be speculated that the dietary availability of fatty acids and cholesterol is important in understanding these inconsistencies. Relatively, intraluminal availability of (dietary) cholesterol and LCFA is highest in duodenum, where a lot of fatty acids and cholesterol can easily be absorbed by the relevant transport proteins and also passive diffusion of FA is more eligible to occur. More distally, relative cholesterol and fatty acid bioavailability is lower, and therefore more transport proteins are required for the same absolute uptake of cholesterol and fatty acids. Finally, it should be noted that we were not able to correct the fatty acid transport protein levels for the amount of myotubes in the intestinal samples, since variations in fatty acid transporter levels in myocytes along the duodenal-colonic axis cannot be ruled out, which could have influenced the results.

Gender and age differences

Duan et al. (68) showed that estrogens may influence cholesterol uptake through up-regulation of NPC1L1 and possibly by down-regulation of the sterol efflux transporters ABCG5 and ABCG8. It was suggested that estrogen acts as transcription modulator for the target genes through effects on estrogen receptor (ER) α or ER β, two subtypes of the steroid hormone receptor superfamily. Furthermore, a significant positive effect of aging on cholesterol uptake through inhibition of the sterol efflux transporters ABCG5 and ABCG8 has been reported (68). In contrast, fat uptake was suggested to decline with ageing (69-71) and also more recently decreased ileal uptake of palmitic, stearic, oleic and linoleic acid was found with ageing in rats (72). These effects were abolished when mucosal surface area was considered. Other studies however have suggested that age is not related to lipid absorption (73) or to morphological changes of the intestine (74), whilst others have even reported an increased lipid uptake with ageing (75). We did not find a decrease in any of the lipid transport proteins with ageing, nor gender-related differences. However, since the women were probably post-menopausal, no estrogen-related gender difference was expected. Concerning the influence of gender and age, the power of the study may not have been sufficient or the range of age may not have been large enough.

Conclusion

In conclusion, this is the first study that has analyzed the distribution of cholesterol and LCFA transport proteins along the human duodenal-colonal axis. Protein distribution patterns are different from the sites suggested for predominant uptake of cholesterol and LCFA in pigs, mice and rats (18, 35-37), which may arguably indicate that the higher proximal concentrations of cholesterol and LCFA are more easily absorbed and that more distally a higher transporter protein expression is needed. Also, the discrepancy between the distribution patterns of ABCG5 and ABCG8 was not expected. Most of the data available in the literature is derived from animal studies. When considering the human data in this manuscript, it should be realized that human profiles might indeed differ from profiles in animals. Unfortunately, we were not able to determine the subcellular distribution of cholesterol and LCFA transporters or protein activities. Therefore, further research in this field is warranted.

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EXCHANGING SATURATED FATTY ACIDS FOR (N-6) POLYUNSATURATED FATTY ACIDS IN A MIXED MEAL MAY DECREASE POSTPRANDIAL LIPEMIA AND MARKERS OF INFLAMMATION AND ENDOTHELIAL ACTIVITY IN OVERWEIGHT MEN

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ABSTRACT

Postprandial lipemia, low-grade systemic inflammation and endothelial activity are related to metabolic disorders. It is well known that dietary fatty acid composition modulates postprandial lipemia, but information on the other metabolic risk markers is limited. We therefore studied the acute effects of a meal rich in SFA versus those of a meal rich in (n-6) PUFA on postprandial responses in overweight men, who are at an increased risk to develop the metabolic syndrome and its comorbidities. In a crossover design, the effects of 50 g butter (rich in SFA) on lipemia and markers for inflammation and endothelial activity were compared to those of 50 g sunflower oil (rich in (n-6) PUFA) during an 8-h postprandial mixed meal tolerance test in 13 overweight men. Postprandial changes in serum TG were comparable between the meals (*P*=0.38), except for a reduction in the incremental area under the curve (*P*=0.046) in the late postprandial phase after (n-6) PUFA (125 ± 96 mmol·min·L^{-1}) as compared to SFA (148 ± 98 mmol·min·L^{-1}). Compared with the SFA meal, the (n-6) PUFA meal significantly decreased plasma IL-6 (*P*=0.003), TNFα (*P*=0.005), soluble TNF receptors I and II (sTNFr^5; *P*=0.024 and *P*<0.001 respectively) and soluble vascular cell adhesion molecule-1 concentrations (sVCAM-1; *P*=0.030). These results indicate that exchanging SFA from butterfat for (n-6) PUFA in a mixed meal may decrease postprandial lipemia and concentrations of IL-6, TNFα, sTNFr-I and II, and sVCAM-1 in overweight men.

INTRODUCTION

Postprandial lipemia, which is positively related to cardiovascular risk (1), is influenced by the composition of the diet. In this respect, linoleic acid from vegetable oils may be more beneficial than SFA (2). Results, however, are controversial. Especially, conflicting results have been found for butterfat, as both decreased and increased TG responses have been reported compared to those of linoleic acid (3, 4). Results between studies however are difficult to compare due to differences in experimental designs. In the latter studies (3, 4), for example, the test fats were added to an energy-free soup or to tomato sauce, or consumed as a spread. In the soup and tomato sauce, the solid fat content of the meal was probably lowered due to its high temperature, which may aggravate postprandial lipemia (5). Also, genetic background may be important. Recently, it was shown that single nucleotide polymorphisms of the *TCFL2* gene may affect postprandial responses to (n-6) PUFA (6), indicating the complexity of postprandial metabolic responses.

Less is known on effects of dietary composition on markers of low-grade systemic inflammation and endothelial activation, which are both involved in the process of atherosclerosis (7). Several *in vitro* studies have suggested that SFA are pro-inflammatory, whereas PUFA are anti-inflammatory (8, 9). On the other hand, in another *in vitro* study (10), incubation with (n-6) PUFA (linoleic or arachidonic acid) resulted in higher expression of the cellular adhesion molecules (CAM) ICAM-1 and VCAM-1 than incubations with SFA (palmitic acid) and (n-3) PUFA (DHA) did. *In vivo*, it was recently shown that acute ingestion of carbohydrate meals with or without 0.6 g fat/kg body wt resulted in higher late-postprandial IL-6 concentrations in obese than in lean women, but no differences between cream (SFA), olive oil (MUFA+SFA) and canola oil (MUFA+ (n-6) PUFA) meals were found (11). However, other relevant markers of inflammation and endothelial activation were not measured. Another study showed no differences in inflammatory markers in normal weight men, after altering the ratio of saturated to unsaturated fatty acids of a high-fat (3.1 MJ; 70.8 percent of energy (En%) from fat) test meal (12). In addition, the acute effects of different types of fat on markers of inflammation and endothelial activation have not been studied in overweight men, who are at increased risk for developing the metabolic syndrome (13).

We therefore decided to examine in overweight men changes in concentrations of plasma markers related to inflammation and endothelial activation after the consumption of a mixed meal providing 50 g of fat from butter or margarine, which are rich in respectively SFA and (n-6) PUFA (linoleic acid).

MATERIALS AND METHODS

Participants

Volunteers were recruited through announcements in local newspapers or among participants who had participated in earlier studies at the department. Female participants were excluded to avoid any possible variations in postprandial responses due to hormonal effects. Participants were invited for 2 screening visits if they met the following inclusion criteria: aged between 18 and 70 y, BMI between 25 and 30 kg/m^2, stable body weight (weight gain or loss <5% in the previous 3 mo), no intention to change the physical activity pattern during the study, no use of lipid-lowering medication or a prescribed diet, and no participation in another biochemical trial for the past 30 d. Fifteen participants were included (**Table 4.1**). They had mean fasting serum concentrations <8.0 mmol/L for total cholesterol and <1.7 mmol/L for TG; no indications for treatment for hyperlipidemia according to the Dutch Cholesterol Consensus (14); no inflammatory disease; no clinical condition known to affect lipid metabolism; no drug or alcohol abuse; and no history of coronary artery disease, heart failure (class III or IV), cardiomyopathy or kidney, liver, or pancreatic disease or malignancy <5 y ago. All participants gave written informed consent before entering the study. The study protocol was approved by the Ethics Committee of Maastricht University.

Table 4.1: Baseline characteristics of the overweight male participants

n = 13	mean ± SD
age, *y*	51.2 ± 16.7
BMI, $kg \cdot m^{-2}$	29.1 ± 1.28
blood pressure, *mmHg*	
systolic	135 ± 18.1
diastolic	86.4 ± 11.2
glucose, $mmol \cdot L^{-1}$	5.63 ± 0.56
insulin, $pmol \cdot L^{-1}$	117 ± 45.7
TC, $mmol \cdot L^{-1}$	5.73 ± 1.11
HDL-cholesterol, $mmol \cdot L^{-1}$	1.39 ± 0.23
TG, $mmol \cdot L^{-1}$	1.35 ± 0.29

Study design

A randomized, double-blind crossover design was used, in which all participants received two different interventions. On the day before each test, participants were asked not to engage in any strenuous physical exercise or to consume alcohol. Participants were also asked to refrain from high-fat foods, including fried foods and sausages.
After a 12 h overnight fast (from 20 h), participants visited the department by public transport or car. After resting for 20 min in supine position, an intravenous cannula was inserted into a vein of the non-dominant arm. At T = 0 min (T0), a blood sample was collected. Participants were then requested to consume within 10 min, one of the two test meals: a meal rich in butterfat or a meal rich in sunflower oil. Both meals provided 50 g fat. Subsequent blood samples were collected at T= 15 min after meal consumption (T15), and at T = 30 min (T30), T = 45 min (T45), T = 60 min (T60), T = 90 min (T90), T = 120 min (T120) and T = 180 min (T180). After T180, participants received a low-fat lunch. Further blood samples were drawn at T = 240 min (T240), T = 300 min (T300), T = 360 min (T360), T = 420 min (T420) and T = 480 min (T480). After sampling, the cannula was rinsed with 1 mL 1% heparin (LEO Pharma BV, Breda, Netherlands).
During each 8-h test period, participants had to drink 250 mL of water at T0, T180, and T360. A period of at least 7 d separated the test days. Participants were requested not to alter their food intake pattern or physical activity level during the entire study.

Test meals

The participants received 2 muffins providing 50 g butterfat (butter meal) or 40 g margarine plus 10 g safflower oil (margarine meal) and a glass of water (250 mL). The meals (**Table 4.2**) had a comparable energy content (4095 kJ and 4253 kJ, respectively) and macronutrient composition (fat/carbohydrate/protein: 51 En%/43 En%/6 En% for the butter meal vs. 53 En%/41 En%/6 En% for the margarine meal, respectively). The butter meal consisted for a large part of SFA, while in the margarine meal, most of the SFA of the butter meal was replaced by linoleic acid, an (n-6) PUFA. Egg yolk was added to the margarine muffins to standardize the amount of cholesterol between the meals.
One batch of muffins was prepared for the entire study. After baking for 20 min at 180° C and cooling down for 20 min, the muffins were portion packed and frozen at -20° C. The low-fat lunch consisted of 200 mL low-fat yogurt with sweetener and an apple.

Table 4.2: Macronutrient composition of the high-fat mixed test meals and of the low-fat lunch[1]

	butter	margarine	low-fat lunch
energy, *kJ*	4095 (1694)	4253 (1689)	692 (173)
protein, *g*	14.5 (6.0)	16.0 (6.4)	9 (2.25)
CHO, *g*	103 (42.7)	103 (40.8)	50.2 (12.55)
total fat, *g*	56.6 (23.4)	60.5 (24.0)	0.2 (0.05)
SFA, *g*	33.9 (14.0)	12.9 (5.1)	0 (0)
MCFA, *g*	3.7 (1.5)	1.2 (0.5)	0 (0)
TFA, *g*	2.2 (0.9)	0.4 (0.1)	0 (0)
MUFA, *g*	14.5 (6.0)	21.3 (8.5)	0 (0)
PUFA, *g*	2.7 (1.1)	21.8 (8.7)	0 (0)
linoleic acid, *g*	1.7 (0.7)	20.1 (8.0)	0 (0)
cholesterol, *g*	349 (144.3)	349 (138)	0.4 (0.1)

[1] Values between brackets are expressed per 100 g

Blood analyses

EDTA-containing 10 mL vacutainer tubes (Becton Dickinson, Plymouth, UK) and NaF-containing 4 mL vacutainer tubes (Becton Dickinson, Plymouth, UK) were placed on ice directly after blood sampling. Tubes were centrifuged at 1,300xg at 4°C for 15 min. Blood drawn in 5 mL vacutainer serum tubes (Becton Dickinson, Plymouth, UK) was allowed to clot for 30 min at 21°C. Subsequently, the serum tubes were centrifuged at 1,300xg for 15 min at 21°C. Plasma and serum aliquots were directly frozen in liquid nitrogen and stored at -80°C until analysis.

Fasting serum was measured enzymatically by the CHOD-PAP method (Roche Diagnostics, Mannheim, Germany) for serum total cholesterol and for serum HDL cholesterol after precipitation of apoB-containing lipoproteins (Phosphotungstate precipitant; Roche Diagnostics, Mannheim, Germany). Plasma glucose (Roche Diagnostic Systems, Hoffmann-La Roche Ltd., Basel, Switzerland) was measured in NaF plasma at T0, 15, 30, 45, 60, 90, 120, 300 and 480 and serum insulin concentrations were determined 0, 15, 30, 45, 60, 120, 300 and 480 min after meal consumption with a human insulin-specific radioimmunoassay kit (Linco Research Inc., St. Charles, MO, USA).

Serum TG with correction for free glycerol was determined hourly (GPO Trinder; Sigma-Aldrich Corp., St. Louis, MO, USA), and serum apo B-48 concentrations were analyzed 0, 120, 240, 360 and 480 min after meal consumption (Shibayagi Co Ltd, Ishihara, Japan).

Plasma inflammatory markers (IL-6, IL-8 and TNFα) were measured 0, 120, 240, 360 and 480 min after consumption of the test meals with a commercially

available Multi Spot ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA). TNF receptors 1 and 2 were measured at the same time points in plasma with a commercially available Multi Spot ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA). Serum monocyte chemoattractant protein-1 (MCP-1) was measured 0, 60, 120, 240 and 480 min after test meal consumption with a single spot ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA). Cellular adhesion molecules s-ICAM and s-VCAM were measured in plasma at T0, 120, 240, 360 and 480 with a Multi Spot ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA).

Statistical analysis

All results are presented as mean ± SD. Differences in baseline concentrations between test days were tested using a paired t-test. Changes in concentrations were analyzed by ANOVA with diet and time as fixed factors and with diet·time as interaction term. If the term was not statistically significant, it was omitted from the model. If factor time was significant, post hoc tests with Bonferroni correction were carried out to compare concentrations to baseline concentrations. For both meals, the maximum change from baseline (peak change) and the time period between meal consumption and peak change (time to peak) were calculated. Also, the incremental AUC (iAUC; the area above baseline (T0) concentrations) or the decremental AUC (dAUC; the area below baseline concentrations) was calculated using the trapezoidal rule (15). Since serum TG concentrations peaked approximately 4 h after meal consumption, we divided the postprandial period in an early (0-240 min) and a late postprandial phase (240-480 min). Comparable analyses were performed for apoB-48, glucose and insulin concentrations. The differences in peak changes, time to peak, and iAUC or dAUC between the meals were compared using paired samples t-tests. Differences were considered to be statistically significant when $P<0.05$. Statistical analyses were performed using SPSS 16.0 software (SPSS Inc. Chicago, Illinois).

RESULTS

After screening, 15 participants started the study. One subject dropped out due to other commitments and 14 participants completed both postprandial tests. One of these participants was excluded from the statistical analyses, because he was not fasting on one of the two test days, as indicated by a large difference between fasting TG concentrations on these two days (5.08 mmol/L vs. 1.34 mmol/L).

Postprandial lipemia

Fasting TG concentrations were comparable between the test days (*P*=0.60). After meal consumption, postprandial TG concentrations rose until 3-4 h and returned to baseline after 8 h (**Figure 4.1A**). Changes in TG concentrations did not significantly differ between the meals (*P*=0.38 for diet effect). The iAUC of serum TG over the 8h-postprandial period and in the early phase (T0 to T240) were comparable after consumption of the meals, but the iAUC in the late postprandial phase (T240 to T480) was significantly higher (*P*=0.046) after the butter meal (148 ± 98 mmol·min·L^{-1}) than after the margarine meal (125 ± 96 mmol·min·L^{-1}). There was a significant delay in time-to-peak of 55 min after consuming the butter meal (*P*=0.008), whereas the maximum changes from baseline were comparable (**Tables 4.3** and **4.4**). Serum concentrations of apolipoprotein B-48 increased over time (**Figure 4.1B**). The changes in apoB-48 concentrations after the meals did not differ (*P*=0.95) and the iAUC, the iAUC for the early and late postprandial phases (data not shown), maximum changes, or the time to peak did not differ (**Tables 4.3**, **4.4** and **4.5**).

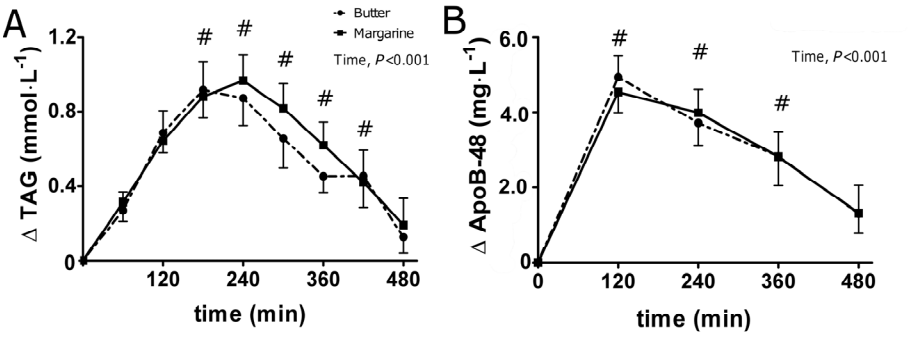


Figure 4.1: Serum concentrations of (A) TAG and (B) apoB-48 before and after a high-fat mixed meal rich in either (n-6) PUFA (margarine) or SFA (butter) in a randomized crossover study with overweight men. Data are presented as mean ± SEM; n=13. #Different from baseline, *P*<0.05

Postprandial glycemia

Glucose (*P*=0.58) and insulin concentrations (*P*=0.24) at baseline did not differ between the test days. Following each meal, there was a rapid increase in both glucose and insulin, which was significant for the factor time (*P*<0.001; **Figure 4.2**). Plasma glucose concentrations were significantly increased during the first 60 min (*P*<0.05 at all time points) and serum insulin during the first 2 h (*P*<0.001 at all time points) after both interventions. The glucose (*P*=0.83) and insulin (*P*=0.65) responses did not differ between the meals. The postprandial responses, also between the early (T0-T120) and late postprandial phase (T120-T480), between the test meals were comparable for all other variables (**Tables 4.3**, **4.4** and **4.5**).

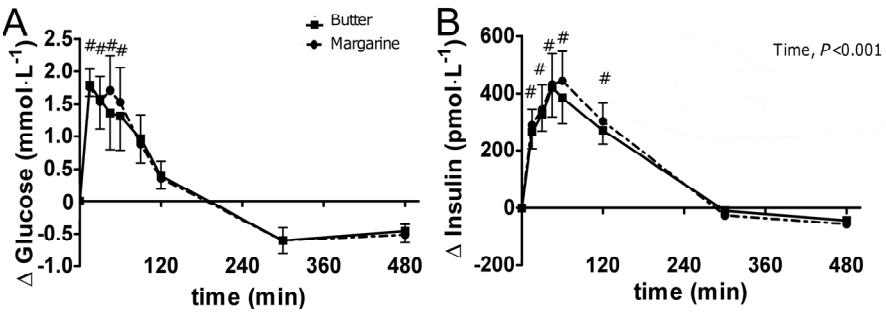


Figure 4.2: Concentrations of (A) plasma glucose and (B) serum insulin before and after a high-fat mixed meal rich in either (n-6) PUFA (margarine) or SFA (butter) in a postprandial, randomized crossover study with overweight men. Data are presented as mean ± SEM; n=13. #Different from baseline, *P*<0.05

Table 4.3: Time between consumption of two single challenge meals rich in either (n-6) PUFA or SFA and maximum change from baseline in glucose, insulin, TG and apoB-48 concentrations in overweight male participants[1]

	(n-6) PUFA	SFA	difference
glucose, *min*	35.8 ± 24.1	27.7 ± 17.2	8.08 ± 21.8
insulin, *min*	43.8 ± 18.8	39.2 ± 18.9	4.62 ± 15.5
TG, *min*	208 ± 90.4*	263 ± 79.5	-55.4 ± 62.3
apoB-48, *min*	157 ± 90.1	185 ± 116	-27.7 ± 87.0

[1] Values are presented as mean ± SD; n=13
* Different from SFA, *P*<0.01

Table 4.4: Maximum changes from baseline in glucose, insulin, TG, apoB-48, IL-6, IL-8, TNFα, TNF receptor I and II, MCP-1, sVCAM-1 and sICAM-1 after consumption of two single challenge meals rich in either (n-6) PUFA or SFA in overweight male participants[1]

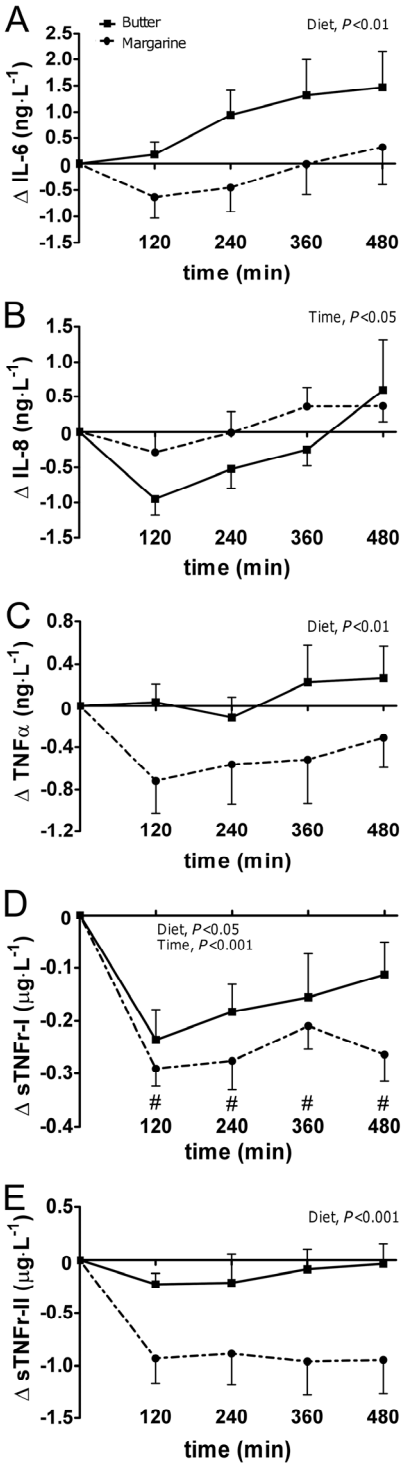
	(n-6) PUFA	SFA	difference
glucose, *mmol·L^{-1}*	2.40 ± 1.56	2.49 ± 1.35	- 0.10 ± 0.88
Insulin, *pmol·L^{-1}*	545 ± 394	523 ± 312	22.0 ± 182
TG, *mmol·L^{-1}*	1.04 ± 0.57	1.13 ± 0.47	- 0.09 ± 0.36
apoB-48, *mg·L^{-1}*	5.34 ± 2.35	4.99 ± 2.20	0.35 ± 1.90
IL-6, *ng·L^{-1}*	0.15 ± 2.93	1.59 ± 2.71	- 1.44 ± 3.68
IL-8, *ng·L^{-1}*	0.04 ± 1.37	0.03 ± 2.92	0.00 ± 2.92
TNFa, *ng·L^{-1}*	-0.85 ± 1.56	0.13 ± 1.45	-0.98 ± 1.96
TNFr-I, *μg·L^{-1}*	-0.39 ± 0.15*	-0.25 ± 0.26	-0.15 ± 0.24
TNFr-II, *μg·L^{-1}*	-1.18 ± 1.15	-0.13 ± 1.05	-1.05 ± 1.80
MCP-1, *μg·L^{-1}*	-0.20 ± 0.08	-0.20 ± 0.08	-0.01 ± 0.07
sICAM-1, *μg·L^{-1}*	-14.6 ± 46.7	6.05 ± 40.28	-20.7 ± 74.4
sVCAM-1, *μg·L^{-1}*	-38.5 ± 84.3	-9.49 ± 86.2	-29.0 ± 142

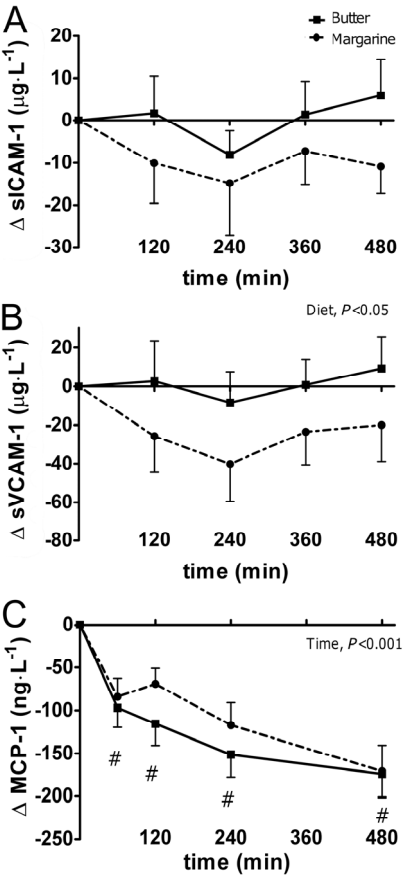
[1] Values are presented as mean ± SD; n=13

Table 4.5: iAUC and dAUC for glucose, insulin, TG, apoB-48, IL-6, IL-8, TNFα, sTNFr-I and II, MCP-1, sICAM-1, and sVCAM-1 after consumption of two single challenge meals rich in either (n-6) PUFA or SFA in overweight male participants[1]

		(n-6) PUFA	SFA	difference
glucose, *mmol·min·L^{-1}*	iAUC	202 ± 184	188 ± 155	14.5 ± 100
insuline, *nmol·min·L^{-1}*	iAUC	67.8 ± 53.2	63.0 ± 38.9	4.74 ± 26.6
TG, *mmol·min·L^{-1}*	iAUC	264 ± 155	287 ± 132	-23.8 ± 53.8
apoB-48, *g·min·L^{-1}*	iAUC	1.47 ± 0.747	1.46 ± 0.802	0.017 ± 0.592
IL-6, *ng·min·L^{-1}*	iAUC	222 ± 324	425 ± 663	-203 ± 613
	dAUC	334 ± 535	44.5 ± 59.0	290 ± 559
IL-8, *ng·min·L^{-1}*	iAUC	175 ± 244	101 ± 200	73.9 ± 320
	dAUC	145 ± 177	271 ± 253	-126 ± 311
TNFa, *ng·min·L^{-1}*	iAUC	56.6 ± 125	135 ± 275	-78.7 ± 272
	dAUC	292 ± 456	102 ± 112	190 ± 411
TNFr-I, *μg·min·L^{-1}*	dAUC	109 ± 58.5	86.6 ± 65.8	22.9 ± 75.3
TNFr-II, *μg·min·L^{-1}*	dAUC	397 ± 417	135 ± 158	262 ± 458
MCP-1, *μg·min·L^{-1}*	dAUC	53.3 ± 29.5	64.6 ± 35.9	-11.3 ± 24.6
sICAM-1, *mg·min·L^{-1}*	dAUC	7.40 ± 10.7	3.95 ± 4.70	3.45 ± 12.6
sVCAM-1, *mg·min·L^{-1}*	dAUC	160 ± 22.0	8.06 ± 8.71	7.91 ± 26.0

[1] Values are presented as mean ± SD; n=13





▲ **Figure 4.4**: Concentrations of (A) plasma sICAM-1, (B) plasma sVCAM-1 and (C) serum MCP-1 before and after a high-fat mixed meal rich in either (n-6) PUFA (margarine) or SFA (butter) in a randomized crossover study with overweight men. Data are presented as mean ± SEM; n=13. *Different from SFA, *P*<0.05; #Different from baseline, *P*<0.001

◄ **Figure 4.3**: Concentrations of (A) IL-6, (B) IL-8, (C) TNFα, (D) sTNFr-I and (E) sTNFr-II before and after a high-fat mixed meal rich in either (n-6) PUFA (margarine) or SFA (butter) in a randomized crossover study with overweight men. Data are presented as mean ± SEM; n=13. *Different from SFA, *P*<0.05; #Different from baseline, *P*<0.01

Postprandial markers of inflammation and endothelial activation

Baseline concentrations of all the markers related to inflammation and endothelial activation were comparable between the test days, except for sTNFr-II, which was significantly lower (*P*=0.049) at the butter test day than at the margarine test day.

Changes in plasma IL-6 concentrations were not significant for time (*P*=0.14), but increased more after butter than after margarine (*P*=0.003; **Figure 4.3A**). The dAUC tended to be higher after margarine than butter (*P*=0.09; data not shown), whereas other variables were comparable (**Tables 4.4** and **4.5**).

Changes in IL-8 concentrations after the meals showed a significant time effect (*P*=0.011; **Figure 4.3B**). After adjustment for multiple comparisons, however, none of the time points was statistically different from baseline. Moreover, there was no significant meal effect (*P*=0.12). Other variables were also comparable between the meals (**Tables 4.4** and **4.5**).

For TNFα, postprandial concentrations were significantly lower after margarine than after butter (*P*=0.005 for diet effect; **Figure 4.3C**), and there was a trend for a higher maximum decrease in plasma TNFα concentrations after the margarine than after the butter meal (*P*=0.10; **Table 4.4**).

sTNFr-I concentrations decreased (*P*<0.001) and sTNFr-II tended to decrease after meal consumption (*P*=0.06 for time effect; **Figure 4.3D**). Plasma sTNFr-I concentrations (*P*=0.024) and sTNFr-II concentrations (*P*<0.001) were lower after margarine consumption than after butter. The maximum decrease in sTNFr-I was significantly higher after margarine than after butter (*P*=0.049). For sTNFr-II, there was a trend for a larger dAUC (*P*=0.06; data not shown), and a higher maximum decrement (*P*=0.06) after margarine consumption (**Tables 4.4** and **4.5**).

Postprandial sICAM-1 and sVCAM-1 concentrations did not change over time (*P*=0.63 and *P*=0.62 respectively; **Figure 4.4A** and **B**). For sVCAM-1 there was a significant diet effect (*P*=0.030) with higher concentrations after butter than after margarine. For sICAM-1, concentrations tended to be lower after consumption of the margarine meal as compared to the butter meal (*P*=0.07). Other variables were comparable (**Tables 4.4** and **4.5**).

Postprandial MCP-1 concentrations decreased after meal consumption over time (*P*<0.001) and were significantly lower than baseline at all time points (*P*<0.001; **Figure 4.4C**), whereas the other variables did not differ between the meals (**Tables 4.4** and **4.5**).

DISCUSSION

Postprandial lipemic response

Serum TG concentrations peaked after approximately 3 h after consumption of the margarine meal, but 4 h after consumption of the butter meal. It should however be realized that postprandial concentrations are the resultant of two main coinciding processes, e.g. absorption and clearance. It has been suggested that absorption is delayed if the melting point of a fat is high, resulting in a higher solid fat content at body temperature (5, 16). The solid fat content of butter fat is close to 0% at body temperature (17). We do not know whether this has affected absorption. For both meals, differences in absorption and clearance were comparable during the early postprandial phase, resulting in similar TG responses. However, it is still possible that (n-6) PUFA (linoleic acid) are absorbed faster and that the clearance rate is higher, as increased postprandial LPL activity was found after consumption of linoleic acid as compared to stearic and palmitic acids (18), which may explain the increased postprandial lipemia in the late postprandial phase after SFA. Additionally, it has been suggested that large chylomicrons are cleared faster from the circulation than smaller chylomicrons when equal fat loads are given (19). In rats safflower oil resulted in larger chylomicrons as compared to coconut oil, rich in SFA (20). In our study however, the equal apoB-48 responses after the meals indicated that the number of intestinally derived lipoproteins was equal, but whether particle size was different is not known. Dworatzek *et al.* (21) have reported similar responses for total serum TG when butter was compared with safflower oil. Chylomicron TG responses however were decreased after the butter meal. The authors speculated that this may have been due to the fact that a part of the TG after the butter meal was packed into smaller, denser lipoproteins, which were lost during isolation. Alternatively, it was mentioned that the lower TG concentrations may have resulted from the absorption of medium-chain fatty acids (MCFA) into the portal circulation, which does not result in the formation of chylomicrons. However, Nielsen *et al.* (22) found no differences in postprandial lipemia when either palm oil or butter was added to a low-fat meal, which suggests that MCFA are not an important determinant for total serum TG responses during the postprandial phase.

Postprandial markers of inflammation and endothelial activation

After the consumption of the butter muffins, IL-6 concentrations increased in our study, whereas IL-6 concentrations decreased after consumption of the margarine muffins. In studies with lean and overweight children (23), men with metabolic syndrome (24), abdominally obese men (25), CHD patients (26) and

healthy participants (27), an increase in IL-6 concentrations was observed after a high-fat meal, whereas a carbohydrate-rich meal did not influence IL-6 concentrations (28). In contrast, no differences in IL-6 responses were found in obese men when the (n-6)/(n-3) fatty acid ratio was decreased from 20:1 to 2:1 (24), or in lean and obese women when SFA was exchanged for MUFA or (n-6) PUFA (11). Reasons for inconsistencies may be found in differences between study populations, i.e. men vs. women. Additionally, we have compared mixed meals with a low (3% of total fatty acids) or a high (33% of total fatty acids) linoleic acid content, whereas in the study by Manning *et al*. (11) differences in linoleic acid content between the cream meal and canola oil meal were less pronounced (4% vs. 19%).

For IL-8 we did not find significant changes from baseline. In contrast, van Oostrom *et al.* (27) found increased concentrations of IL-8 (50% increase) 1 h after ingestion of a glucose bolus, but no statistically significant effects after intake of a fat bolus. The latter effect, however, was much more pronounced (130% increase) and the lack of statistical significance was therefore related to high inter-subject variations. The discrepancy between our study and the findings by van Oostrom *et al*. (27) might be explained by the timing of sampling. In the latter study, IL-8 concentrations were only measured at 0, 1 and 2 h after the glucose bolus and after the fat bolus. We measured IL-8 concentrations at 2-h intervals during 8 h after the intake of a mixed meal. Therefore, it is possible that we have missed an early increment in IL-8 concentrations. Our findings in overweight men, however, are in agreement with the aforementioned study of Manning *et al*. (11), who found no early peak in IL-8 concentrations nor time or meal dependent variations in IL-8 concentrations after 1, 4 and 6 h. These findings suggest that the type of fatty acid consumed does not acutely affect IL-8 concentrations during an 8-h postprandial period.

In our study, we found a potentially favorable reduction in TNFα concentrations after the muffins rich in linoleic acid, whereas TNFα concentrations remained stable after the butter muffins and slightly increased 6-8 h postprandially after both meals. In studies with abdominally obese men (25) and with both men and women covering a broad range of adiposity (29), TNFα concentrations were significantly reduced. Unfortunately, the compositions of the test meals used in these two studies (25, 29) were not reported, which makes a comparison with our study difficult. On the other hand, Nappo *et al*. (28) found a time-dependent increase in TNFα concentrations in both healthy participants and *type 2* diabetic patients after consumption of a test meal rich in SFA and MUFA.

With respect to variations in TNFα concentrations, the responses in sTNFr-I and -II should also be considered, although their exact roles are under debate

(30-34). In our study, the response in plasma sTNFr-II after consumption of both meals followed that of plasma TNFα. sTNFr-I concentrations, however, were significantly reduced after consumption of both types of muffins. To the best of our knowledge, no other intervention studies have compared postprandial responses in sTNFr-I and sTNFr-II between meals with a different fatty acid composition.

Postprandial serum MCP-1 concentrations decreased significantly after both meals, irrespective of the type of fat consumed. In contrast, endothelial MCP-1 mRNA expression was previously shown to increase after incubation with fasting or postprandially derived TG-rich lipoproteins from hypertriglyceridemic participants (35) or chylomicron remnants (36). *In vivo*, high intake of mainly saturated fat increased MCP-1 concentrations in hypertriglyceridemic patients with metabolic syndrome (37). In contrast, in HIV-patients (38), postprandial MCP-1 was not significantly affected after an oral fat load. The reason for these inconsistencies is unknown.

In our study, we did not find time-dependent differences in sICAM-1 and sVCAM-1. However there was a significant meal-dependent reduction in sVCAM-1 concentrations and a trend for lower sICAM-1 concentrations after the margarine muffins. In contrast, most other studies found postprandial increases in sICAM-1 and sVCAM-1 after fat loads mainly providing SFA in healthy participants, type 2 diabetic patients and hyperlipidemic participants (28, 39, 40). In contrast, Rubin *et al.* (41) did not find postprandial variations in sICAM-1 and sVCAM-1 after a mixed meal with mainly SFA and MUFA. The authors, however, noted that this could have been the result of the high retinol content of the test meal, as increases in sICAM-1 and sVCAM-1 were found when retinol was excluded from the test meal. On the other hand, no increases or rather decreased sICAM-1 and sVCAM-1 concentrations were found in premature CHD patients and healthy controls (26) or mildly obese men (42) after a high-fat meal with MUFA or (n-6) PUFA. Thus, large differences exist between studies for which we do not have an explanation.

In summary, our data indicate that exchanging SFA from butterfat for (n-6) PUFA in a mixed meal may decrease postprandial lipemia and affects several postprandial markers of inflammation and endothelial activation in overweight men. Postprandial glucose and insulin concentrations were not affected differently. The impact of these findings on long-term health remains to be elucidated.

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THE EFFECTS OF MILK AND MILK CONSTITUENTS PROVIDED WITH A HIGH-FAT MEAL ON POSTPRANDIAL MARKERS FOR INFLAMMATION IN OVERWEIGHT MEN

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SUBMITTED

ABSTRACT

Some epidemiological studies have suggested that an increased intake of dairy products may decrease cardiovascular risk. It is not very likely that this is due to effects on conventional cardiovascular risk markers. We therefore studied the effects of milk and two major milk constituents (protein and calcium) on postprandial markers for inflammation and endothelial activation, which are more and more acknowledged as emerging cardiovascular risk markers.

In a randomized crossover study, 16 overweight men (BMI >27 kg/m^2, age 18-70 y) were given a high-fat meal (44 g fat) with 500 mL skimmed milk, a milk protein drink with similar protein (20.4 g) and calcium contents (700 mg), a calcium drink (700 mg) or water (control). After 60 min, sICAM-1 concentrations were lower after milk than after calcium (14%; P=0.008) or control (16%; P=0.004), and lower after protein than calcium (11%; P=0.030) or control (13%; P=0.016). A comparable pattern was observed for sVCAM-1. After 60 min, TNFα was lower after control than after calcium (10%; P=0.018), and after 120 min lower after milk and control than after protein (19% and 23%, respectively; P<0.001 for both) and calcium (17% and 21%; P=0.027 and P<0.001). After 240 min TNFα was lower after control than all other meals (14-19%; P<0.01). This was also reflected by a larger decremental AUC (P<0.050). IL-6 and IL-8 were not different between diets.

Our results indicate that adding skimmed milk to a high-fat meal may beneficially affect postprandial sICAM-1 and sVCAM-1 concentrations, but negatively those of TNFα. The protein fraction may be the most important determinant for the effects on sICAM-1 and sVCAM-1.

INTRODUCTION

Several epidemiological studies have suggested that an increased intake of dairy products reduces the risk to develop cardiovascular disease (CVD) (1-3). In some studies (4, 5), however, only positive associations were found for low-fat dairy products, while it should be noted that in other studies no relationships were observed at all (6). These findings however do suggest that dairy products may contain constituents that negate the potential deleterious effects of saturated fatty acids and cholesterol.

Since CVD is thought to be an inflammatory disease leading to both structural and functional changes of the endothelium, fasting serum concentrations of inflammatory and endothelial markers may be important in cardiovascular risk assessment (7-9). A recent cross-sectional survey in more than 3000 subjects found a significant negative dose-dependent relationship for fasting levels of proinflammatory cytokines C-reactive protein (CRP), interleukin-6 (IL-6) and tumor necrosis factor α (TNFα), when low-fat dairy intake increased from less than 8 servings per week to between 11 and 14 servings, or more than 14 servings per week (10). Relations were less significant when full-fat dairy was included in the model. In an intervention study with overweight and obese subjects, Zemel *et al*. (11) recently found that the consumption of a dairy-based smoothie 3 times per day for 28 days significantly reduced fasting serum CRP, IL-6, TNFα and monocyte chemoattractant protein-1 (MCP-1) concentrations and oxidative stress as compared to consumption of an isocaloric soy-based smoothie. The macronutrient content of the diets was comparable and body weight did not change in that study. However, it remains unknown which dairy constituents may have been responsible for the effects observed, as the experimental products differed not only in the source of protein, but also in calcium content. *In vitro* studies with murine adipose tissue, for example, have shown that leucine, which is more abundant in milk protein than in soy protein, may decrease energy storage and increase fat oxidation, but increase oxidative and inflammatory stress (12). However, since no decrease in body weight was found, it is unlikely that the difference in leucine content can explain the difference in inflammatory markers in that study (11). Secondly, the addition of casein to a high-fat meal decreased the postprandial triacylglycerol (TAG) response (13), which may also affect plasma biomarkers of inflammation, since triglyceride-rich lipoproteins (TRL) have been shown to augment cytokine production by endothelial cells and macrophages (14, 15). Calcium on the other hand may decrease the formation of reactive oxygen species by altering mitochondrial uncoupling status by suppression of calcitriol (16). Additionally, calcium may decrease the absorption of fatty acids by the formation of insoluble calcium soaps in the intestine, resulting in a decreased TAG response (17). Also, these studies (10, 11) did not provide information

on postprandial inflammatory and endothelial responses, which may be modulated by TRL and oxidative stress (18, 19). Since reductions of postprandial excursions of pro-inflammatory cytokines and endothelial markers may be beneficial (20), this can provide a mechanism for the proposed relations between dairy consumption and CVD risk. Therefore, we decided to investigate the effects of adding milk or its two major constituents – milk protein and calcium – to a high-fat meal on these postprandial markers in overweight men.

SUBJECTS AND METHODS

Subjects

Subjects were recruited through announcements in local newspapers or among subjects who had participated in earlier studies at the department. Subjects who were interested in participation were informed about the purposes and requirements of the study and all gave their written informed consent before entering the screening procedure. Subjects were invited for 2 screening visits, if they met the following inclusion criteria: male; age 18-70 y; body mass index (BMI) >27 kg/m^2; stable body weight (weight gain or loss <5% in the previous 3 months); no intention to change the physical activity pattern during the study; no indications for treatment for hyperlipidemia; no inflammatory disease; no clinical condition known to affect lipid metabolism; no drug or alcohol abuse; and no history of or active cardiovascular disease or other medical conditions that might interfere with the study outcomes. During the screening visits, body weight, height and blood pressure were measured and a fasting blood sample was drawn to determine serum lipid and lipoprotein concentrations. Sixteen subjects were enrolled and were asked not to change their dietary habits, use of alcohol, and physical activity level during the study period. All subjects completed the study **(Table 5.1)**. The study protocol was approved by the Medical Ethical Committee of Maastricht University Medical Center+ and was registered at www.ClinicalTrials.gov as NCT00917878.

Table 5.1: Baseline characteristics of the overweight male participants

n = 16		mean ± SEM		
age, *y*		49.8	±	3.88
BMI, $kg \cdot m^{-2}$		31.2	±	0.90
weight, *kg*		102	±	2.65
height, *m*		1.81	±	0.01
blood pressure	systolic, *mm Hg*	129	±	3.08
	diastolic, *mm Hg*	86.0	±	2.03
glucose, $mmol \cdot L^{-1}$		5.66	±	0.09
insulin, $mU \cdot L^{-1}$		23.0	±	2.55
total cholesterol, $mmol \cdot L^{-1}$		5.58	±	0.22
HDL cholesterol, $mmol \cdot L^{-1}$		1.23	±	0.07
LDL cholesterol, $mmol \cdot L^{-1}$		3.57	±	0.18
triacylglycerol, $mmol \cdot L^{-1}$		1.71	±	0.16
IL-6, $ng \cdot L^{-1}$		1.94	±	0.30
IL-8, $ng \cdot L^{-1}$		3.48	±	0.20
TNFα, $ng \cdot L^{-1}$		7.05	±	0.55
sICAM-1, $\mu g \cdot L^{-1}$		252	±	13.8
sVCAM-1, $\mu g \cdot L^{-1}$		420	±	27.5

Study design

In a randomized crossover design, subjects received the different interventions on four separate occasions, which were separated by washout periods of at least 3 days. On each test day, subjects participated in a postprandial test. On the day before each test, subjects were asked not to engage in any strenuous physical exercise or to consume alcohol, and to refrain from high-fat foods.

After an overnight fast, subjects visited the department by public transport or car. After resting for 20 minutes in supine position, an intravenous cannula (Venflon®, Becton Dickinson, Plymouth, UK) was inserted into an antecubital vein of the non-dominant arm. At T = 0 min (T0), a blood sample was collected for analysis of fasting concentrations of metabolic risk markers. Subjects were then requested to consume within 10 minutes one of the test meals. Subsequent blood samples were collected at T= 15 min after meal consumption (T15), and at T = 30 min (T30), T = 45 min (T45), T = 60 min (T60), T = 90 min (T90), T = 120 min (T120), T = 180 min (T180), T = 240 min (T240), T = 300 min (T300), and T = 360 min (T360). After sampling, the cannula was rinsed with 1 mL 1% heparin (LEO Pharma BV, Breda, Netherlands) in 0.9% NaCl. During each 6-hour test period, subjects had to drink 250 mL of water directly after the blood collections of T120 and T240.

Test meals

The subjects received, on each occasion, 168 g butter cake and 500 mL of one of four drinks: water, water with calcium, water with calcium and total milk protein, or milk as reported earlier (21) (**Table 5.2**). In order to standardize lactose content, 29 g lactose (DMV International, Veghel, the Netherlands) was added to the water-based drinks. The milk from the milk intervention was skimmed milk (0% fat; Campina, Woerden, the Netherlands). Compared with the milk drink, the calcium drink was formulated to contain an equal amount of calcium (700 mg; Lactoval®; DMV International, Veghel, the Netherlands), and the protein drink an equal amount of milk protein (20.4 g protein; REFIT®; DMV International, Veghel, the Netherlands) and calcium. The drinks were prepared on the morning of the test day.

Table 5.2: Composition of the control, calcium, milk protein and milk meals

	control	calcium	milk protein	milk
Energy, *kcal*	864	864	948	948
Protein, *g*	8	8	29	29
Carbohydrates, *g*	109	109	109	109
Mono/disaccharides, *g*	74	74	74	74
Lactose, *g*	30	30	30	30
Fat, *g*	44	44	44	44
Calcium, *mg*	49	699	703	697
Phosphorus, *mg*	100	353	407	600

Blood analyses

EDTA-containing vacutainer tubes (Becton Dickinson, Plymouth, UK) were placed on ice directly after blood sampling. Tubes were centrifuged at 1300 g at 4°C for 15 minutes. Blood drawn in vacutainer serum tubes (Becton Dickinson, Plymouth, UK) was allowed to clot for at least 30 minutes at 21°C. Subsequently, the serum tubes were centrifuged at 1300 g for 15 minutes at 21°C. Plasma and serum aliquots were directly frozen in liquid nitrogen and stored at -80°C until analysis. Fasting serum samples were analyzed for triacylglycerol concentrations with correction for free glycerol (GPO Trinder; Sigma-Aldrich Corp., St. Louis, MO, USA), total cholesterol (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany) and HDL cholesterol concentrations (phosphotungstate precipitant; Roche Diagnostics, Mannheim, Germany), and insulin concentrations (Linco Research Inc., St. Charles, MO, USA). Fasting plasma samples were analyzed for glucose concentrations (Horiba ABX, Montpellier, France).

Plasma inflammatory markers (IL-6, interleukin-8 (IL-8) and TNFα) were measured 0, 60, 120, 240, and 360 min after consumption of the test meals

with a commercially available Multi Spot ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA).
Plasma soluble intercellular adhesion molecule (sICAM)-1 and soluble vascular cell adhesion molecule (sVCAM)-1 were measured 0, 60, 120, 240, and 360 min after meal consumption using a Multi Spot ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA).

Statistical analysis

All data are presented as mean ± SEM. Baseline concentrations were compared using univariate ANOVA with diet as fixed factor and subject number as random factor. Changes from baseline were compared using linear mixed models with diet and time as within-subject fixed factors, and with diet·time interaction. If the interaction term was not statistically significant, it was omitted from the model. Based upon the information criteria output, the most suitable covariance model was selected. In case of significant diet effects, the diets were compared pairwise using a Bonferroni correction. If time was significant, time points were compared to baseline values. If the diet·time interaction term reached statistical significance, differences between the diets were tested at each individual time point. For all parameters the incremental or decremental area under the curve (iAUC/dAUC) was calculated using the trapezoidal rule (22). Moreover, the maximum positive and negative change from baseline (peak change), and the time period between meal consumption and peak changes (time to peak) were calculated and compared using ANOVA. In addition, correlations between various parameters were analyzed using partial correlations with correction for subject number.
All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and differences were considered to be statistically significant when $P<0.05$.

RESULTS

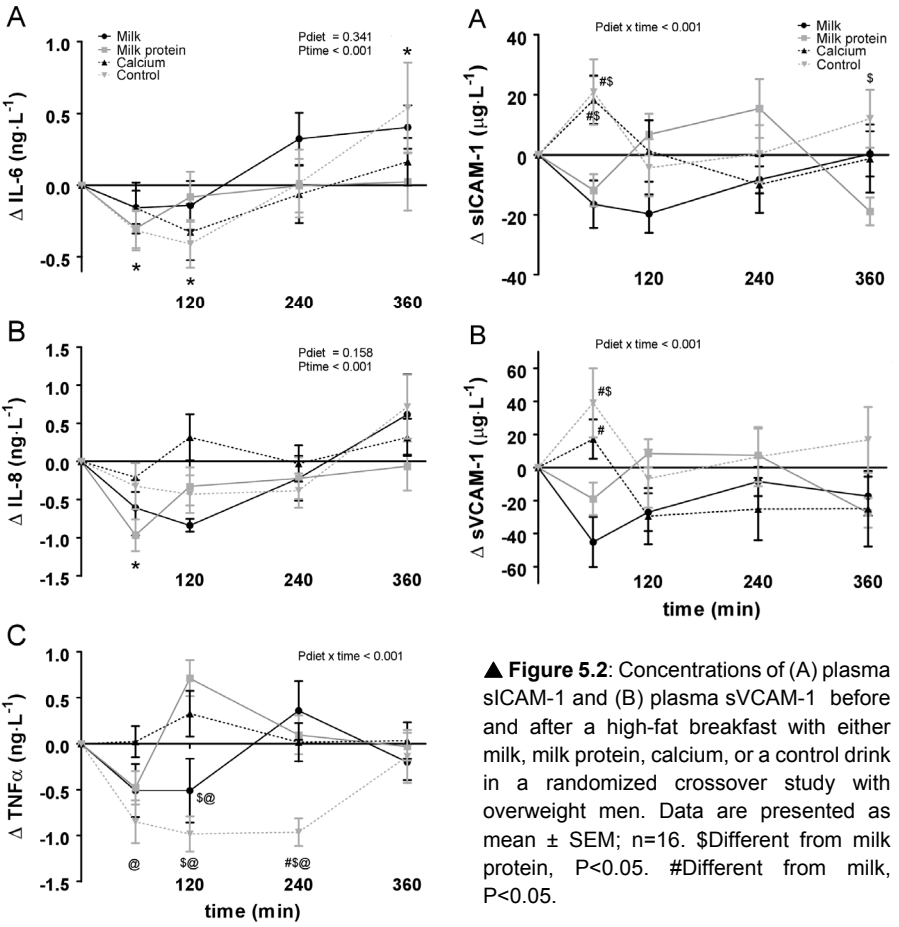
Markers for inflammation

Baseline plasma IL-6 concentrations were comparable between the meals ($P=0.720$). The diet·time interaction term did not reach statistical significance ($P=0.105$). Concentrations of IL-6 changed significantly over time after consumption of the meals ($P<0.001$ for time effect), but time courses were not different between the meals ($P=0.341$ for diet effect) (**Figure 5.1A**). At T60 and T120, IL-6 concentrations were significantly decreased ($P=0.003$ and $P=0.015$, respectively) and at T360 increased ($P=0.026$) as compared to those at baseline (T0). The dAUC, the maximum positive and negative

changes, and the peak times were not significantly different between diets (data not shown).

Baseline plasma concentrations of IL-8 were comparable between the meals (P=0.312), while the diet·time interaction term did not reach statistical significance (P=0.253). Changes in IL-8 differed significantly between the time points (P<0.001), but not between the meals (P=0.158) (**Figure 5.1B**). At T60, concentrations were significantly decreased as compared to baseline (P=0.032). Other parameters did not differ between meals (data not shown). Despite comparable postprandial patterns the peak changes and dAUC of IL-8 did not correlate with those of IL-6 (R=0.168, P=0.189; and R=-0.056, P=0.662, respectively).

Baseline TNFα concentrations were significantly higher before the control meal as compared to milk protein (0.95 ± 0.31 ng·L^{-1}, P=0.021) and calcium (0.77 ± 0.22 ng·L^{-1}, P=0.008). Plasma TNFα time courses (**Figure 5.1C**) differed significantly between diets (P<0.001 for diet·time interaction). At T60, TNFα concentrations were 10% lower (P=0.018) after the control meal than after the calcium meal. As compared to control at T120, TNFα was 23% higher after the milk protein and 21% after the calcium meal (P<0.001 for both). As compared to milk consumption, TNFα concentrations were 19% higher (P<0.001) after the milk protein and 17% higher (P=0.027) after the calcium meal. Compared with control, TNFα concentrations were at T240 significantly higher after the milk meal (19%; P<0.001), milk protein (17%; P=0.002) and calcium meal (14%; P=0.005). Differences between the meals were also found for the dAUC (P<0.001) and in the maximum decrease in TNFα concentrations (P<0.001). The dAUC was significantly larger after the control meal than after the other meals (P<0.050 for all) and the dAUC tended to be larger after milk than after milk protein (P=0.062). Moreover, the maximum decrease in TNFα concentrations was more pronounced after control than after milk protein (P=0.005) or calcium (P<0.001) and after milk than after calcium (P=0.043). Other parameters did not differ between the meals (data not shown).



▲ **Figure 5.2**: Concentrations of (A) plasma sICAM-1 and (B) plasma sVCAM-1 before and after a high-fat breakfast with either milk, milk protein, calcium, or a control drink in a randomized crossover study with overweight men. Data are presented as mean ± SEM; n=16. $Different from milk protein, P<0.05. #Different from milk, P<0.05.

▲ **Figure 5.1**: Plasma concentrations of (A) IL-6, (B) IL-8, and (C) TNFα before and after a high-fat breakfast with either milk, milk protein, calcium, or a control drink in a randomized crossover study with overweight men. Data are presented as mean ± SEM; n=16. *Different from T=0, *P*<0.05. @Different from calcium, P<0.05. $Different from milk protein, P<0.05. #Different from milk. P<0.05.

Markers for endothelial activation

Baseline plasma concentrations of sICAM-1 were comparable between meals (P=0.382). For changes in sICAM-1 (**Figure 5.2A**), a significant diet·time interaction was found (P<0.001). At T60, sICAM-1 concentrations were lower after milk consumption as compared to the calcium (14%; P=0.008) or control meals (16%; P=0.004), and lower after milk protein as compared to the calcium (11%; P=0.030) or control meals (13%; P=0.016). At T120, the sICAM-1 concentration tended to be lower after milk than after milk protein

(10%; P=0.082). Additionally, at T360, sICAM-1 concentrations were 14% lower after milk protein as compared to the control meal (P=0.027). No differences between the meals were found for the other parameters (data not shown).

Baseline plasma concentrations of sVCAM-1 were comparable between meals (P=0.687). Changes in sVCAM-1 concentrations (**Figure 5.2B**) were comparable to changes in plasma sICAM-1 concentrations. A significant diet·time interaction was found (P<0.001). sVCAM-1 decreased 15% more after the milk than after the calcium meal (P=0.011) and 24% more than after the control meal (P<0.001). Additionally, sVCAM-1 concentrations decreased 17% more after milk protein than after the control meal (P=0.021). None of the other parameters differed between meals (data not shown), except for a significantly higher maximum increase in sVCAM-1 after the control meal as compared to the milk meal (P=0.019).

Significant correlations were found between sICAM-1 and sVCAM-1 for positive peaks (R=0.853, P<0.001), negative peaks (R=0.693, P<0.001) and dAUCs (R=0.910, P<0.001).

DISCUSSION

The addition of milk or its constituents to a high-fat meal did not alter the responses in IL-6 or IL-8 concentrations in our study population. Several other studies have investigated differences in postprandial IL-6 and IL-8 concentrations, but mainly examined the effects of various fatty acids as part of a high-fat meal (23-26). These studies found no differences between diets, whereas we found in a previous study differences between saturated fat from butter and (n-6) PUFA from sunflower oil (27). To the best of our knowledge, only two studies have compared postprandial IL-6 responses between dairy products (28) or dairy proteins (29), but no studies have determined postprandial calcium effects. In these studies, Nestel et al. (28) found no differences in 3h-postprandial IL-6 responses when overweight subjects consumed single meals consisting of 50 g butter, 115 mL cream, 110 g cheese, 600 mL yogurt or 400 mL reduced fat milk. The meals were standardized for fat content (except for the reduced fat meal), but varied in carbohydrate and protein content. In accordance, Pal et al. (29) found that postprandial IL-6 concentrations were not differently affected when overweight postmenopausal women consumed a standardized breakfast supplemented with either 45 g sodium casein isolate, 45 g whey protein isolate, or 45 g glucose.

After consumption of the meals, postprandial plasma concentrations of IL-6 decreased during the first 60 min and then increased gradually for the next 5

hours. At the end of the study, concentrations were higher as compared to baseline. Other studies in lean healthy subjects (24, 30), obese men (31), and subjects with metabolic syndrome (25) or coronary heart disease (32) have observed progressively postprandial increases in plasma IL-6 concentrations after a high-fat meal, with peaks mainly after 8 h, whereas we found this progressive increase after 1 h. In our study, time courses of IL-8 concentrations were very similar to those of IL-6. Comparable responses between IL-6 en IL-8 were found in one of our previous studies (27), and also when healthy subjects (24, 26) and obese and lean women (33) consumed a high-fat meal. In the latter study (33), IL-6 concentrations were also decreased after 1 h. Unfortunately, we did not measure IL-6 concentrations after 1 h in our previous study (27). Since both cytokines can be produced by macrophages (34), it is possible that their responses reflect the stimulation of these cells after meal consumption. Based on the results of these studies, however, it seems that the composition of the diet has only small effects on postprandial changes in IL-6 and IL-8.

TNFα decreased after consumption of the high-fat meal in our study. In agreement, TNFα has been shown to decrease after consumption of high-fat meals in various study populations (24, 31, 35), but also increased TNFα concentrations have been reported (18). We found a reduction in TNFα 2 h after the consumption of the control and the milk meals, whereas TNFα was increased after the other two meals. There is no good explanation why TNFα decreased after consuming the high-fat breakfast, but we clearly showed that adding calcium or milk protein affects postprandial TNFα responses. Milk on the other hand did not change the TNFα response.

In the aforementioned study by Pal *et al.* (29) TNFα responses between the diets were not different and concentrations did not change as compared to baseline, indicating that the source of protein is not important for postprandial TNFα responses. Nestel *et al*. (28) also reported no differences in TNFα responses between the diets. The results of these studies (28, 29) are difficult to compare with our study due to differences in study design. Pal *et al*. (29) compared the effects of exchanging glucose for two different protein sources to a standardized breakfast with an equal fat content, while in our study we used a water control. Moreover, Nestel *et al.* (28) compared different dairy products with each other and to a low-fat control meal, whereas we compared it to a breakfast with equal fat content. In the latter study (28), the authors found that increments in insulin did not correlate with changes in inflammatory markers. We also could not find such correlations (data not shown). The large amount of leucine in milk may increase oxidative stress and endothelial activation (12). This may explain the increase in TNFα after the milk protein meal, but in that case it cannot be explained why milk did not increase TNFα.

We have earlier shown in overweight and obese subjects (36) that 8 weeks of daily low-fat dairy consumption (500 ml low-fat milk and 150 g low-fat yogurt) resulted in decreased fasting concentrations of TNFα as compared to carbohydrate-rich control products (600 ml fruit juice and three fruit biscuits). In contrast, in a 6-month dairy intervention study in overweight subjects who were either instructed to increase dairy intake up to 3-5 portions or to maintain habitual intake (37), no effects of dairy consumption on inflammatory markers were found. However, as the authors stated, the subjects already had a relatively high baseline dairy intake and the difference between the dairy intervention and habitual intake may not have been sufficient. The aforementioned beneficial effects (11, 36) were not found in the postprandial responses of IL-6 and TNFα in our study, suggesting that these effects cannot be translated to the postprandial situation or may require longer-term dairy consumption. However, we did find that the consumption of the control and calcium meals caused a small increase in plasma sICAM-1 and sVCAM-1 over time, whereas adding milk or milk protein to the high-fat breakfast reduced concentrations of sICAM-1 and sVCAM-1 up to 2h after meal consumption. Several other studies (18, 38, 39) have found increased concentrations of sICAM-1 and sVCAM-1 after a fat load, whereas others have reported no time-dependent responses (19). Our results indicate that mainly milk protein is responsible for the reductions found in adhesion molecules, since no significant reductions were found when the calcium drink accompanied the breakfast. It must be noted however, that milk protein was ingested together with calcium, so the effects due to milk protein may be dependent on the presence of calcium. However, since milk tended to induce even larger reductions in adhesion molecules, other nutrients may be involved. Arguably, the complex dairy matrix may also have modulated the effects of the individual constituents. We can only speculate on the mechanism by which these adhesion molecules are reduced. As a strong correlation was found between insulin responses and sICAM-1 (19), protein may decrease sICAM-1 through insulin stimulation, since especially the whey fraction is insulinogenic (40). However, we did not find this correlation after consumption of the milk protein or milk meals (data not shown). From animal studies (41) and *in vitro* studies with endothelial cells (42), it is known that TNFα increases the expression of sICAM-1 and sVCAM-1 5 h after injection (41) or 10 h after incubation (42). Possibly, this association is not apparent from our data, because we measured protein concentrations and not RNA expression. Moreover, it may be that the time period was too short to find effects at the protein level.

In conclusion, the results of this study provide insight into the effects of adding milk or one of its two major constituents - protein and calcium - to a high-fat

meal on postprandial markers for inflammation and endothelial activation. Our data show that consuming 500 mL of skimmed milk or a milk protein drink with a high-fat meal may increase concentrations of TNFα in overweight men. On the other hand, postprandial responses of sICAM-1 and sVCAM-1 may be improved by consuming milk, which may be partially, but not solely, attributable to the protein fraction.

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MILK HOMOGENIZATION AND POSTPRANDIAL METABOLISM IN OVERWEIGHT MEN: A RANDOMIZED STUDY

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SUBMITTED

ABSTRACT

The physical structure of foods affects postprandial metabolism, a risk marker for CVD[5]. In animals, homogenization of milk changes postprandial responses, but these effects have never been studied in humans. The objective was to compare postprandial effects of homogenized milk, non-homogenized milk, or skimmed milk plus butter on lipemic, glycemic, inflammatory and endothelial markers. Eighteen overweight men (BMI 25-30 kg/m^2, age 18-70 y) consumed in random order crisp bread with marmalade and 900 mL of homogenized whole milk (H-milk), non-homogenized whole milk (NH-milk), or skimmed milk plus 44 g butter. Macronutrient compositions of the meals were comparable. Compared to butter, postprandial TAG concentrations decreased significantly after NH-milk (P=0.002). Furthermore, glucose concentrations on the NH-milk meal were higher after 30 min (P=0.038), 45 min (P=0.004) and 60 min (P=0.017) and on the H-milk meal after 45 min (P=0.008). Homogenization of milk significantly increased total count (P=0.002) and iAUC (P=0.025) of white blood cells. IL-6 concentrations were significantly higher after the H-milk meal as compared to the butterfat meal at (P=0.026) and T=480 (P=0.001) and at T=240 as compared to NH-milk (P=0.036). TNFα concentrations were higher after NH-milk than after butter (P=0.007). Changes in concentrations of sICAM-1 and sVCAM-1 were comparable between the meals. These findings do not indicate a major role for homogenization of milk on acute postprandial responses. We did however find clear - potentially beneficial and unbeneficial - effects when fat was consumed as butter.

INTRODUCTION

Dietary fat increases the postprandial serum triacylglycerol (TAG) response (1), which may increase cardiovascular risk (2-4). Also, postprandial increases in markers for inflammation and endothelial function are related to CVD risk (5). Whether these effects depend on the fatty-acid composition of the meal is less clear. Butterfat, for example, has been found to increase (1, 6) and to decrease (7-10) postprandial lipemia, as compared to a fat rich in polyunsaturated fatty acids. For carbohydrate-rich foods, the physical structure of the food product also determines postprandial responses (11, 12). This may be true for fat-rich foods as well. Clemente *et al.* (13) studied postprandial responses to a milk meal, a butter meal and a cheese (mozzarella) meal in eight type 2 diabetics. Although the incremental areas (iAUC) under the 6h TAG curve were not different between the meals, a significantly lower 0-3h iAUC and a significantly delayed peak response were found after the butter meal. However, except for the food matrix, the meals also differed in their sources of protein and fats, thereby making the comparison between milk, butter and mozzarella cheese difficult. In contrast, Tholstrup *et al*. (14) found no postprandial differences after consumption of lactose and total milk protein-standardized milk, butter or cheese meals, suggesting that the physical structure of the fat is not important. This study, however, was performed in healthy participants with low serum TAG concentrations. To what extent these results can be extrapolated to overweight participants with increased serum TAG concentrations, who may be more responsive (15, 16), is unknown. In addition, effects on other postprandial risk markers have hardly been studied. To assess the role of the food matrix of dairy on postprandial metabolism into more detail, we therefore decided to compare in overweight men side-by-side the effects of homogenized milk, non-homogenized (unprocessed) milk and butterfat. In unprocessed milk, fat is present in relatively large fat droplets, enclosed by the milk fat globule membrane (MFGM). During homogenization, milk is pressed through a filter under high pressure and under high temperature, thereby reducing milk fat globule size. It is thought that these smaller fat globules are absorbed more easily (17), which may increase postprandial lipemia. Additionally, due to a 4-10 fold increase in fat-water surface area, the MFGM composition undergoes major changes (17). It has been postulated that these changes increase vascular damage by an increased presence of several oxidation-inducing proteins, e.g. bovine xanthine oxidase, in the vasculature (18). There are, however, no human studies addressing the impact of milk homogenization on postprandial metabolism.

PARTICIPANTS AND METHODS

Participants

Ethical approval was obtained from the Medical Ethics Committee of Maastricht University (METC 10-3-089) and the study was registered at www.clinicaltrials.gov as NCT01317524. Before inclusion, informed consent was obtained from all participants. Participants were recruited through announcements in local newspapers or among volunteers who had participated in earlier studies at the department. To exclude any possible variations in postprandial responses due to hormonal effects, female participants were excluded from participation. Fifty-three participants responded to recruitment announcements and 23 participants attended the screening visits. Participants willing to participate were invited for the two screening visits if they met the following inclusion criteria: aged between 18 and 70 y, BMI between 25 and 30 kg/m^2, stable body weight (weight gain or loss <5% in the previous 3 mo), no intention to change their physical activity pattern during the study, no use of lipid-lowering medication or a prescribed diet, and no participation in another biochemical trial for the past 30 d. Three participants did not meet the inclusion criteria: mean fasting serum concentrations <8.0 mmol/L for total cholesterol and of <1.7 mmol/L for TAG; no indications for treatment for hyperlipidemia according to the Dutch Cholesterol Consensus (19); no inflammatory disease; no clinical condition known to affect lipid metabolism; no drug or alcohol abuse; and no history of coronary artery disease, heart failure (class III or IV), cardiomyopathy or kidney, liver, or pancreatic disease or malignancy <5 y ago. Of the remaining 20 volunteers, two participants decided not to participate, as it was not possible to schedule their experimental days. Thus, 18 participants started the study (**Table 6.1**) who all completed the study. For 1 subject retinal images could not be obtained, due to cataract surgery.

Study design

A randomized, single-blind crossover design was used, in which all participants received three different interventions. On the day before each test, participants were asked not to engage in any strenuous physical exercise or to consume alcohol. Participants were also asked to refrain from high-fat foods, including fried foods and sausages. Participants arrived at approximately 7.45 AM and rested in the supine position for 15 min. An intravenous catheter was inserted in an antecubital vein of the non-dominant arm, after which a baseline blood sample was drawn. Participants then

Table 6.1: Baseline characteristics of the overweight male participants

n = 18		mean ± SEM		
age, *y*		57.9	±	1.92
BMI, $kg \cdot m^{-2}$		27.7	±	0.49
weight, *kg*		87.4	±	1.91
height, *m*		1.78	±	0.01
blood pressure	systolic, *mm Hg*	140	±	2.73
	diastolic, *mm Hg*	89	±	1.54
glucose, $mmol \cdot L^{-1}$		5.55	±	0.16
insulin, $mU \cdot L^{-1}$		15.6	±	1.13
HOMAir, $mmol \cdot L^{-1} x\ mU \cdot L^{-1}$		3.87	±	0.32
total cholesterol, $mmol \cdot L^{-1}$		5.83	±	0.21
HDL cholesterol, $mmol \cdot L^{-1}$		1.49	±	0.05
triacylglycerol, $mmol \cdot L^{-1}$		1.28	±	0.08

Table 6.2: Composition of the non-homogenized (NH) milk meal, homogenized (H) milk meal and the butterfat meal[1]

	H- milk	NH- milk	Butterfat
energy, *kJ*	4150	4150	4228
energy, *kcal*	992	992	1011
fat, *g*	41.1	41.1	41.1
carbohydrates, *g*	114.5	114.5	115.9
protein, *g*	41.5	41.5	43.6

[1] All meals provided 37% of energy fat, 46% carbohydrates, and 17% protein

received in random order one of the three experimental meals, which had to be consumed within 15 min. All meals provided two slices of bread with marmalade and 2 pieces of crisp bread. The non-homogenized (NH) milk meal further consisted of 900 mL of whole, pasteurized milk (NIZO food research BV, Ede, the Netherlands) and the homogenized (H) milk meal of 900 mL of whole, homogenized milk (NIZO food research BV, Ede, the Netherlands), made from the same batch of milk as the NH-milk. Both types of milk provided 40 g of milk fat. The butterfat meal consisted of 900 mL of skimmed milk with 44 g of salted butter (to be used on the bread and crisp bread) in order to standardize the fat content between the meals. The meals provided equal amounts of energy (4150 kJ for H-milk and NH-milk and 4228 kJ for butterfat) and equal amounts of energy from fat (37 En%), carbohydrate (46 En%) and protein (17 En%), as calculated with the Dutch food composition table (20) (see **Table 6.2**).

Blood was drawn every 15 min during the first hour after intake of the meal, every 30 min during the second hour, and hourly during the rest of the test. Blood was drawn as much as possible by the same technician, at the same

location, and at the same time of the day between the different participants. The participants consumed an apple after 3 h and a glass of water (200 mL) after 3 h and 6 h.

Blood analyses

EDTA-containing or NaF-containing vacutainer tubes (Becton Dickinson, Plymouth, UK) were placed on ice directly after blood sampling and within 60 minutes centrifuged at 1300 g at 4°C for 15 minutes. Blood drawn in vacutainer serum tubes (Becton Dickinson, Plymouth, UK) was allowed to clot for at least 30 minutes at 21°C and then centrifuged at 1300 g for 15 minutes at 21°C. Plasma and serum aliquots were directly frozen in liquid nitrogen and stored at -80°C until analysis. Serum samples of T0, T60, T120, T180, T240, T300, T360, T420 and T480 were analyzed for TAG concentrations with correction for free glycerol (GPO Trinder; Sigma-Aldrich Corp., St. Louis, MO, USA), and fasting serum samples for total cholesterol (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany) and HDL cholesterol concentrations (phosphotungstate precipitant; Roche Diagnostics, Mannheim, Germany). Insulin concentrations were measured in serum samples of T0, T15, T30, T45, T60, T120, T300 and T480 (Linco Research Inc., St. Charles, MO, USA). NaF plasma samples were analyzed for glucose concentrations (Horiba ABX, Montpellier, France) at T0, T15, T30, T45, T60, T90, T120, T300 and T480 and analyzed for non-esterified fatty acids (NEFA) at T0, T60, T120, T300 and T480 (NEFA kit; WAKO, Neuss, Germany). Concentrations of apolipoprotein B-48 were measured in plasma samples at T0, T60, T120, T240, T360 and T480 (Shibayagi Co Ltd, Ishihara, Japan).
Plasma inflammatory markers (interleukin-6 (IL-6), IL-8 and tumor necrosis factor α (TNFα)), soluble intercellular adhesion molecule (sICAM)-1 and soluble vascular cell adhesion molecule (sVCAM)-1 were measured at T0, T60, T120, T240, T360 and T480 with a commercially available Multi Spot ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA). White blood cell counts (WBC) and differentiations were determined automatically using a Cell-dyn 3500 hematology analyzer (Abbott, Abbott park, IL, USA) at T0, T120 and T480.

Retinal imaging

Retinal vascular images were obtained at T0, T240 and T480 to visualize the response of the microvasculature in the eye. During this test, participants were seated with their head resting on a chinrest, looking directly into the camera (Topcon TRC-NW-300; Topcon Co., Tokyo, Japan). The camera focused on and photographed the retina. Images were digitized and analyzed to calculate the mean artery width (MAW), mean vein width (MVW) and artery:vein ratio (AV-ratio) with the appropriate software (Generalized Dual-Bootstrap Iterative Closest Point (GDB-ICP)), as described in detail elsewhere (21). In short, the software automatically aligns the images based on detected vascular centerlines by iteratively transforming the algorithm. At least 3 arteries and 3 veins were selected, which were at all time points the same set of vessels for a subject.

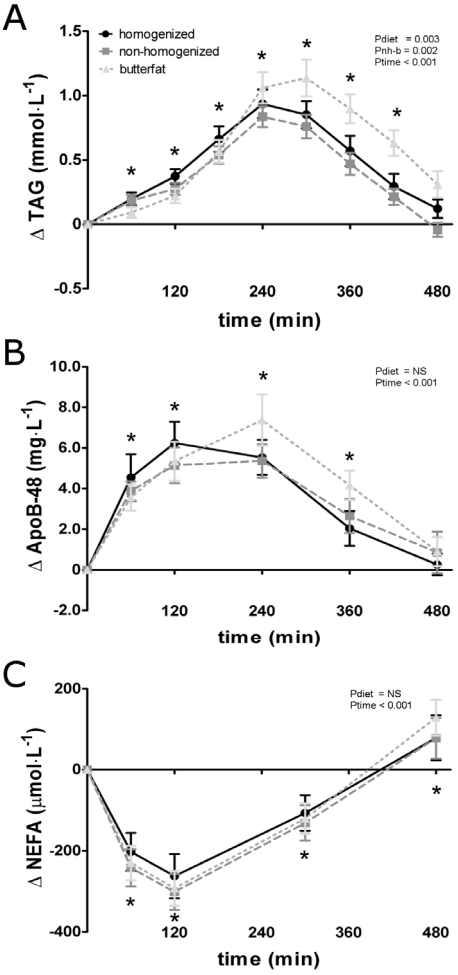
Sample size calculation and randomization

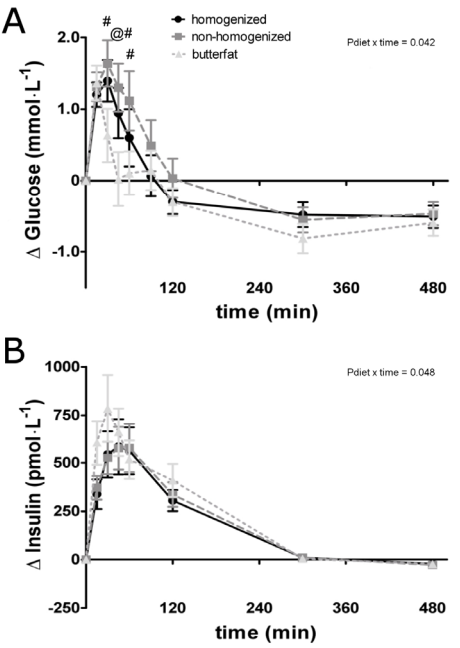
Sample size calculations were based on 18 men completing the study, using the primary outcomes of the incremental area under the curve (iAUC) of postprandial TAG concentrations. This sample size has been selected to give a power of >80% to detect a 0.75 SD unit change at P=0.017 (to account for multiple comparisons between groups) using a crossover design, considering an expected difference of 42 mmol/min/L and an intra-subject variability of 54 mmol/min/L (13). Randomization was performed by an independent person with an online random number generator at http://www.randomization.com using performed balanced permutations.

Statistical analysis

All data are presented as mean ± SEM. Baseline concentrations were compared using univariate ANOVA with diet as fixed factor and subject number as random factor. Changes from baseline were compared using linear mixed models with diet and time as within-subject fixed factors, and with diet·time interaction. If the interaction term was not statistically significant, it was omitted from the model. Based upon the information criteria output, the most suitable covariance model was selected. In case of significant diet effects, the diets were compared pairwise using a Bonferroni correction. If time was significant, time points were compared to baseline values. If the diet·time interaction term reached statistical significance, differences between the diets were tested at each individual time point. For all parameters the incremental or decremental area under the curve (iAUC/dAUC) was calculated using the trapezoidal rule. Moreover, the maximum positive and negative change from baseline (peak change), and the time period between meal consumption and

peak changes (time to peak) were calculated and compared using ANOVA. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and differences were considered to be statistically significant when P<0.05.





▲ **Figure 6.2**: Postprandial changes in plasma glucose concentrations (A) and serum insulin concentrations (B) after consumption of the H-milk meal (●), NH-milk meal (■) and butter-fat (▲) meal in overweight men. # within time point NH-milk significantly different from butterfat. @ within time point H-milk significantly different from butterfat. P<0.05.

◄ **Figure 6.1**: Postprandial changes in serum TAG (A), plasma apoB-48 (B) and plasma NEFA (C) concentrations after consumption of the H-milk meal (●), NH-milk meal (■) and butterfat (▲) meal in overweight men. * significantly different compared to baseline (P<0.05).

Table 6.3: Calculated iAUC or dAUC after H-milk, NH-milk or the butterfat meal

	H-milk			NH-milk			butterfat		
[1]TAG, *mmol·min·L^{-1}*	240	±	28.3^{AB}	198	±	21.3^{A}	289	±	30.7^{B}
[1]apoB-48, *g·min·L^{-1}*	1.88	±	0.283	1.80	±	0.207	2.17	±	0.310
[2]NEFA, *mol·min·L^{-1}*	1.13	±	0.195	1.25	±	0.175	1.17	±	0.154
[1]glucose, *mmol·min·L^{-1}*	96.8	±	23.2	118	±	25.5	70.4	±	22.9
[1]insulin, *nmol·min·L^{-1}*	13.7	±	2.61	14.4	±	2.52	17.0	±	3.31
[1]IL-6, *ng·min·L^{-1}*	297	±	55.5	267	±	65.6	153	±	43.8
[2]IL-8, *ng·min·L^{-1}*	254	±	95.3	198	±	27.2	503	±	175
[2]TNFα, *ng·min·L^{-1}*	169	±	42.9	196	±	35.3	280	±	68.4
[2]sICAM-1, *µg·min·L^{-1}*	8.94	±	1.92	7.39	±	1.44	9.58	±	2.98
[2]sVCAM-1, *µg·min·L^{-1}*	15.3	±	2.89	9.75	±	1.48	13.6	±	3.88
[1]WBC, *10^{9} cells·min·L^{-1}*	231	±	40.4^{A}	119	±	23.5^{B}	135	±	36.0^{AB}
[1]neutrophils, *10^{9} cells·min·L^{-1}*	245	±	32.1	145	±	30.3	192	±	55.4
[2]lymphocytes, *10^{9} cells·min·L^{-1}*	70.9	±	24.4	99.5	±	24.3	99.0	±	32.3
[1]MAW, *µm·min*	506	±	204	471	±	149	469	±	118
[2]MVW, *µm·min*	477	±	132	572	±	149	564	±	134

Values with different superscripts differ significantly; [1] iAUC, [2] dAUC

Table 6.4: Calculated peak changes after H-milk, NH-milk or the butterfat meal

	H-milk			NH-milk			butterfat		
TAG, *mmol·L^{-1}*	1.04	±	0.10^{AB}	0.90	±	0.09^{A}	1.32	±	0.13^{B}
apoB-48, *mg·L^{-1}*	7.74	±	1.06	7.38	±	0.88	8.45	±	1.05
NEFA, µ*mol·L^{-1}*	-271	±	54.1	-308	±	44.3	-293	±	43.2
glucose, *mmol·L^{-1}*	1.95	±	0.26^{AB}	2.37	±	0.29^{A}	1.49	±	0.27^{B}
insulin, *pmol·L^{-1}*	118	±	22.9	122	±	19.4	140	±	28.3
IL-6, *ng·L^{-1}*	1.55	±	0.318	1.21	±	0.236	0.777	±	0.191
IL-8, *ng·L^{-1}*	-0.891	±	0.232	-0.814	±	0.156	-1.29	±	0.492
TNFα, *ng·L^{-1}*	-0.800	±	0.130	-0.754	±	0.145	-1.28	±	0.242
sICAM-1, *µg·L^{-1}*	-44.5	±	8.30	-36.7	±	7.98	-47.7	±	12.0
sVCAM-1, *µg·L^{-1}*	-73.2	±	11.9	-46.1	±	9.71	-69.6	±	14.6
WBC, *10^{9}·L^{-1}*	0.906	±	0.158	0.683	±	0.127	1.16	±	0.586
neutrophils, *10^{9}·L^{-1}*	0.894	±	0.0968	0.544	±	0.100	0.661	±	0.204
lymphocytes, *10^{9}·L^{-1}*	-0.283	±	0.0772	-0.450	±	0.0857	-0.411	±	0.103
MAW, *µm*	1.79	±	0.622	1.95	±	0.562	2.13	±	0.437
MVW, *µm*	-2.10	±	0.477	-2.24	±	0.523	-2.21	±	0.515
AVr, 10^{-2}	1.88	±	0.60	1.88	±	0.587	2.24	±	0.518

Values with different superscripts differ significantly

RESULTS

Postprandial lipids

Fasting baseline serum TAG concentrations were not different between the meals (P=0.891). Also, the diet·time interaction term did not reach statistical significance (P=0.261). Compared to baseline, serum TAG concentrations were significantly increased over time (P<0.001) at all time points (all P<0.001), except after 480 min (**Figure 6.1A**). The diet effect also reached statistical significance (P=0.003) and serum TAG responses were on average 0.18 ± 0.05 mmol/L lower after the NH-milk meal as compared to the butterfat meal (P=0.002). This was also reflected by differences in the iAUC (**Table 6.3**, P=0.022) and peak changes (**Table 6.4**, P=0.01).

Baseline plasma concentrations of apoB-48 were not different between the meals (P=0.886) and the diet·time interaction term did not reach statistical significance (P=0.654). However, apoB-48 increased significantly over time (P<0.01). As compared to baseline, concentrations were significantly increased after 60, 120, 240 and 360 min (all P<0.01; **Figure 6.1B**). No diet effects (P=0.788) or differences in the iAUC (P=0.289) or peak changes (P=0.430) were found, however a trend for a delayed time to peak was found after the H-milk meal (**Table 6.5**, P=0.053).

Baseline concentrations of NEFA were not different between the meals (P=0.288). No significant diet·time interaction was found (P=0.918). Over time, NEFA concentrations changed significantly (P<0.001). Compared to baseline, concentrations were significantly decreased 60, 120 and 300 min after meal consumption and increased after 480 min (P<0.001 for all; **Figure 6.1C**). Diet effects (P=0.694), the dAUC (**Table 6.3**, P=0.656) and other parameters (**Tables 6.4** and **6.5**) were not significantly different between the meals.

Postprandial glycemia

Baseline glucose concentrations were not different between the meals (P=0.647). Time courses for plasma glucose differed significantly between the meals (P=0.044 for diet·time interaction; **Figure 6.2A**). Fifteen min after consumption of the butterfat meal, glucose concentrations decreased and had returned to baseline after 45 min. Compared to the butterfat meal, concentrations on the NH-milk meal were higher after 30 min (0.92 ± 0.37 mmol/L; P=0.038), 45 min (1.19 ± 0.37 mmol/L; P=0.004) and 60 min (1.01 ± 0.36 mmol/L; P=0.017) and on the H-milk meal after 45 min (1.12 ± 0.37 mmol/L; P=0.008). The iAUC tended to be higher after the NH-milk meal as compared to the butterfat meal (**Table 6.3**, P=0.094) and the peak change

was significantly higher after the NH-milk as compared to the butterfat meal (**Table 6.4**, P=0.004).

Baseline insulin concentrations were not different between the meals (P=0.548). A significant diet·time interaction was found (P=0.036; **Figure 6.2B**). Post-hoc analyses however, did not show any significant differences between the diets at the different time points. Insulin concentrations however tended to be higher 15 min after intake of the butterfat meal as compared to the H-milk meal (P=0.062), and 30 min after the butterfat meal as compared to the NH-milk meal (P=0.081). Other parameters were comparable between the meals (**Tables 6.3, 6.4** and **6.5**).

Table 6.5: Calculated peak times after H-milk, NH-milk or the butterfat meal

	H-milk			NH-milk			butterfat		
TAG, *min*	247	±	14.4	268	±	12.7	275	±	17.1
apoB-48, *min*	140	±	20.6^{A}	180	±	23.8^{AB}	210	±	21.3^{B}
NEFA, *min*	110	±	11.1	117	±	3.33	117	±	3.33
glucose, *min*	29.6	±	3.24	31.7	±	5.44	20.3	±	3.14
insulin, *min*	43.3	±	3.41	36.7	±	4.24	35.8	±	5.96
IL-6, *min*	340	±	37.9	373	±	27.2	313	±	41.3
IL-8, *min*	217	±	40.6	187	±	36.3	183	±	35.5
TNFα, *min*	227	±	38.1	193	±	35.8	243	±	35.1
sICAM-1, *min*	207	±	38.9	167	±	37.1	237	±	38.0
sVCAM-1, *min*	193	±	35.8	200	±	40.9	237	±	38.3
WBC, *min*	313	±	43.3	380	±	45.8	303	±	50.5
neutrophils, *min*	280	±	43.4	300	±	49.7	223	±	37.8
lymphocytes, *min*	123	±	26.3	107	±	7.75	103	±	6.52
MAW, *min*	212	±	49.9	184	±	48.4	240	±	46.0
MVW, *min*	282	±	47.1	240	±	46.0	311	±	44.9
AVr, *min*	219	±	41.5	205	±	45.8	219	±	38.8

Values with different superscripts differ significantly

White blood cell counts

Baseline WBC were not different between the meals (P=0.487), but the time courses were (P=0.040 for diet·time interaction; **Figure 6.3A**). Two hours after the H-milk meal, WBC counts were significantly higher as compared to the NH-milk meal (0.58 ± 0.17·10^9 cells/L; P=0.002). The WBC iAUC was significantly higher (129 ± 45.9·10^9 cells·min/L; **Table 6.3**, P=0.025) after the H-milk meal as compared to the NH-milk meal.

Baseline and postprandial neutrophil counts (**Figure 6.3B**) and lymphocyte counts (**Figure 6.3C**) were not different between the meals. The neutrophil count increased after all meals (P<0.001), whereas the lymphocyte count decreased after all meals (P<0.001). Other parameters for the white blood cells were comparable between the meals (**Tables 6.4** and **6.5**).

Inflammatory and endothelial markers

Baseline plasma IL-6 concentrations were not different between the meals (P=0.940). The time courses of IL-6 concentrations were significantly different between the meals (P=0.027 for diet·time interaction; **Figure 6.4A**). IL-6 concentrations were significantly higher after the H-milk meal as compared to the butterfat meal at T=120 (0.64 ± 0.24 ng/L; P=0.026) and T=480 (0.88 ± 0.24 ng/L; P=0.001). Moreover, IL-6 was increased after the NH-milk meal as compared to the butterfat meal at T=240 (0.60 ± 0.24 ng/L; P=0.036). These findings were also reflected by the iAUC, which was significantly higher after the H-milk meal than after the butterfat meal (**Table 6.3**, P<0.001).

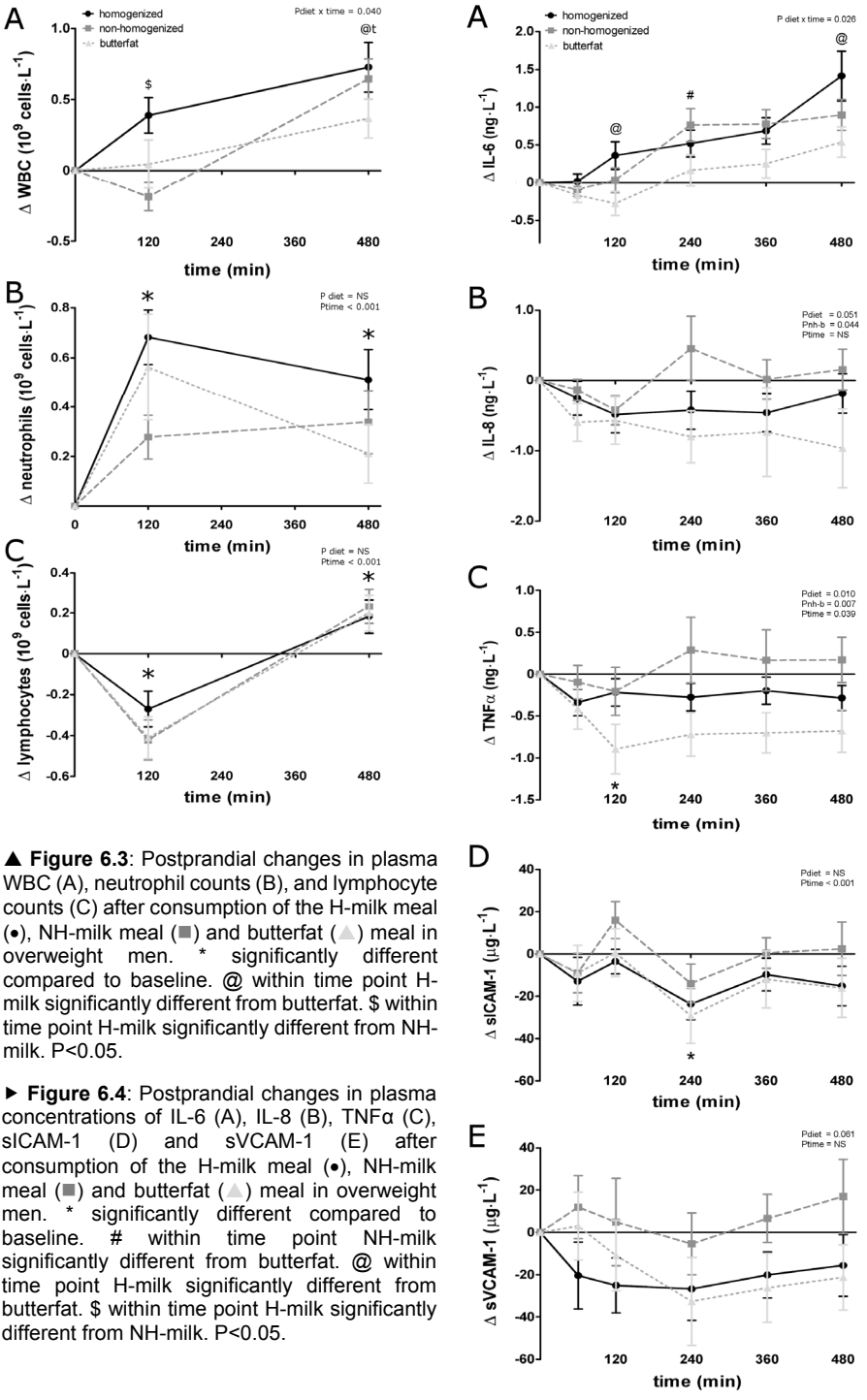
Baseline plasma concentrations of IL-8 were not different between the meals (P=0.293). The diet·time interaction did not reach statistical significance (P=0.290) and the diet effect nearly reached statistical significance (P=0.051; **Figure 6.4B**). IL-8 did not change over time (P=0.195).

Baseline plasma TNFα concentrations were comparable between the meals (P=0.982) and the diet·time interaction term did not reach statistical significance (P=0.254). Concentrations of TNFα were different between the diets (P=0.01; **Figure 6.4C**) and were on average 0.50 ± 0.16 ng/L higher after the NH-milk meal as compared to the butterfat meal (P=0.007). As compared to baseline, TNFα was significantly decreased after 120 min (-0.42 ± 0.15 ng/L; P=0.024).

Baseline plasma concentrations of sICAM-1 were comparable between the meals (P=0.591) and the diet·time interaction term was not significant (P=0.986; **Figure 6.4D**). Concentrations of sICAM-1 changed over time (P<0.001) and were significantly decreased 2 h after meal consumption (-22.1 ± 5.92 µg/L; P=0.001). No diet effects were found (P=0.190).

Baseline plasma sVCAM-1 concentrations were comparable between the meals (P=0.444) and the diet·time interaction term did not reach statistical significance (P=0.872; **Figure 6.4E**). There was a trend for diet effects (P=0.061). Concentrations of sVCAM-1 did not change over time (P=0.281).

All other parameters for IL-6, IL-8, TNFα, sICAM-1 and sVCAM-1 were comparable between the meals (**Tables 6.3, 6.4** and **6.5**).



▲ **Figure 6.3**: Postprandial changes in plasma WBC (A), neutrophil counts (B), and lymphocyte counts (C) after consumption of the H-milk meal (●), NH-milk meal (■) and butterfat (▲) meal in overweight men. * significantly different compared to baseline. @ within time point H-milk significantly different from butterfat. $ within time point H-milk significantly different from NH-milk. P<0.05.

► **Figure 6.4**: Postprandial changes in plasma concentrations of IL-6 (A), IL-8 (B), TNFα (C), sICAM-1 (D) and sVCAM-1 (E) after consumption of the H-milk meal (●), NH-milk meal (■) and butterfat (▲) meal in overweight men. * significantly different compared to baseline. # within time point NH-milk significantly different from butterfat. @ within time point H-milk significantly different from butterfat. $ within time point H-milk significantly different from NH-milk. P<0.05.

Retinal imaging

All baseline variables were comparable between the meals (P>0.05). The mean artery width tended to increase over time (P=0.069; **Figure 6.5A**), but was not different between the meals (P=0.939). As compared to baseline, the mean vein width however, was significantly decreased 4 hours after meal consumption (-1.03 ± 0.402 µm; P=0.024), but no differences between the meals were found (P=0.910; **Figure 6.6B**). These changes were also reflected by the AV-ratio, which was significantly increased after 4 hours as compared to baseline (0.012 ± 0.004; P=0.013). No differences in the AV-ratio were found between the meals (P=0.972; **Figure 6.6C**). All other parameters for retinal imaging were comparable between the meals (**Tables 6.3, 6.4** and **6.5**).

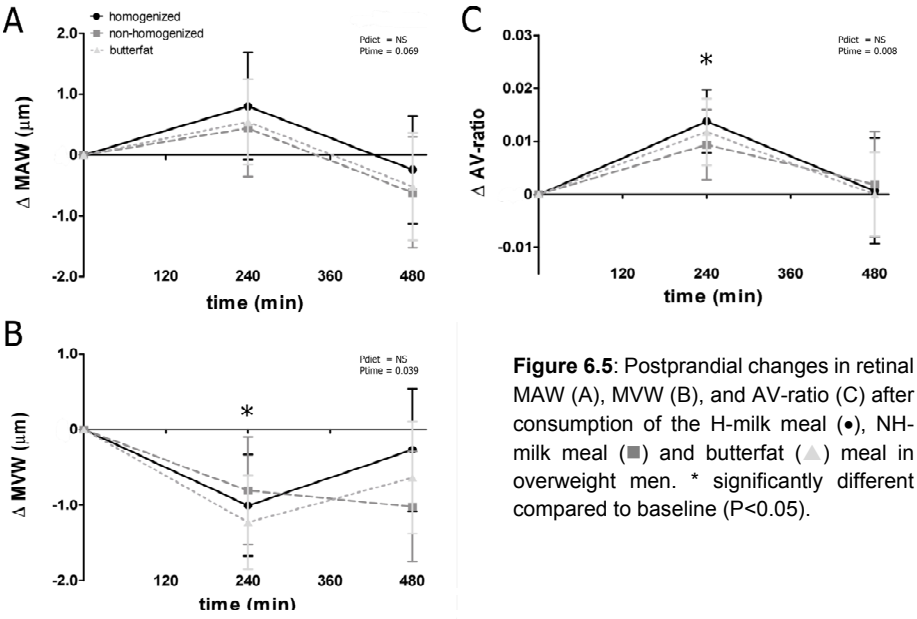


Figure 6.5: Postprandial changes in retinal MAW (A), MVW (B), and AV-ratio (C) after consumption of the H-milk meal (●), NH-milk meal (■) and butterfat (▲) meal in overweight men. * significantly different compared to baseline (P<0.05).

DISCUSSION

Postprandial lipemic response

In this study with overweight men, we found that homogenization of milk did not affect postprandial lipemia. Homogenization decreases milk fat particle size from on average 4 µm to 1 µm (17), thereby greatly increasing its surface area. It could therefore be speculated that intestinal lipolysis rates of TAG would be higher after H-milk as compared to NH-milk (22) and absorption rates faster. However, increased concentrations of NEFA in the intestine may slow down gastric emptying through the stimulation of cholecystokinin (23). On the other hand, due to homogenization, proteins (mainly casein) attach to the MFGM. This translocation may accelerate digestion and absorption, as in a minipig model the aggregation of casein in the stomach was lower for homogenized pasteurized milk than for raw and untreated milk (24), resulting in an increased gastric emptying rate (25). Michalski *et al.* (26), however, force-fed rats dairy formulations with milk fat varying in supramolecular structure. As plasma TAG appearance was lower in rats consuming homogenized fat, the authors hypothesized that gastric emptying was slower after homogenized cream. Thus, based on these conflicting findings, it is difficult to predict effects of homogenization on postprandial lipemia. We found no differences in postprandial TAG concentrations or peak times between homogenized and non-homogenized milk. However, TAG concentrations after butterfat were increased as compared to NH-milk. In contrast, Clemente *et al.* (13) reported in type 2 diabetics increased postprandial TAG concentrations after the consumption of a milk meal as compared to a butter meal (13). In that study, the difference in TAG concentrations between the butter and milk meal was not related to gastric emptying, which was comparable between these meals. However, the source and the type of proteins were different as in the butter meal protein was mainly provided by lean jam and in the milk meal it was provided by milk. Moreover, the jam in the butter meal also provided small part of the fat content, which may have affected the results.

As compared to the H-milk meal, the apoB-48 peak after the butterfat meal was however delayed, which may suggest a delayed absorption of fat from butter into chylomicrons as compared to the two milk meals. Fat in milk is present as small droplets, whereas in butter fat is present as large aggregates, which may have slowed down emulsion and hydrolysis (27).

Postprandial glycemic response

Postprandial glucose concentrations were the highest after NH-milk and H-milk as compared to the butterfat meal. These increased glucose concentrations were likely the result of the lower insulin responses after these two meals as compared to the butterfat meal, since carbohydrate contents and sources were comparable between the meals. Why the insulin response was increased following the butterfat meal is not evident. It is known that protein increases insulin responses, but it is unlikely that the additional 2.1g of protein in the butter fat meal would have increased the maximum insulin response by 30%.

In contrast to our study, Tholstrup *et al.* (14) found in lean healthy men that meals rich in either milk, butter or cheese, with similar amounts of lactose and total milk protein as in our study, comparable peak postprandial insulin concentrations after the butter meal and milk meal. Unfortunately, they did not measure insulin at T=15 min, where we observed the largest difference. It is also possible that the effect is more pronounced in our study population, because our participants were slightly more insulin resistant, as suggested by a higher HOMAir.

Postprandial inflammatory and endothelial response

Compared to NH-milk, we found a higher iAUC and a higher total count for WBC 2 hours after the intake of the H-milk meal. This effect was not longer evident after 8 hours. Effects were mainly due to increases in neutrophils. This would agree with an earlier postprandial study by van Oostrom *et al*. (28) after consumption of a glucose meal, a fat meal or a mixed meal. In that study, water consumption had no effects. In two other studies by the same group (29, 30), WBC increased after a high-fat meal but not after a water control. In these two latter studies neutrophils increased over time, but changes between the high-fat meal and water control did not reach statistical significance. In one study (29), however, several activation markers of mainly neutrophils were increased after the high-fat meal as compared to the water control. Possibly, activation of neutrophils may be more responsive to meal effects than neutrophil counts. Several studies have suggested that activated neutrophils stimulate the activation of endothelial cells (31, 32). Unfortunately we did not measure neutrophil activation markers in our study.

Except for an increased WBC, we did not observe any differences between the H-milk and the NH-milk meal in TNFα, IL-6, IL-8, sICAM-1 and sVCAM-1 and measures of the retinal vasculature, suggesting that the homogenization of milk does not markedly affect inflammatory and vascular responses directly after meal consumption. We did however find consistently lower concentrations of IL-6 and TNFα when fat was presented as butter, suggesting

that the structure of fat in the meal is important. Noteworthy, the butterfat meal showed the highest TAG response. Whether these findings suggest that postprandial TAG may not stimulate the release of plasma inflammatory markers is not known. Several studies have shown that postprandial lipemia correlates with inflammation (28, 33), but a causal effect of postprandial TAG on the release of inflammatory markers has not been established *in vivo*.
Since cytokines, i.e. mainly IL-6 and IL-8, are responsible for the recruitment of WBC (28, 34), we examined whether iAUCs of cytokine concentrations were correlated to those of total WBC, neutrophil and lymphocyte counts. However, no significant relationships were found (data not shown).
Except for a small decrease in retinal vein width and a small increase in the AV-ratio over time, we did not find any differences between the meals in the retinal vasculature. As far as we know, we are the first to study the postprandial retinal vascular responses. Unfortunately, we cannot discriminate whether the measured responses are a result of the fat load consumed or due to circadian variation. More research is therefore warranted to establish the response of the retinal vasculature after a high-fat meal.

Conclusion

The findings of our study do not indicate a major role for homogenization of milk on the acute postprandial responses of lipids, inflammatory markers, endothelial markers and measures of the retinal vasculature. We did however find clear - potentially beneficial and unbeneficial - differences when fat was consumed as butter. To what extent these findings can be extrapolated to the longer-term and translate into health effects warrants further investigation.

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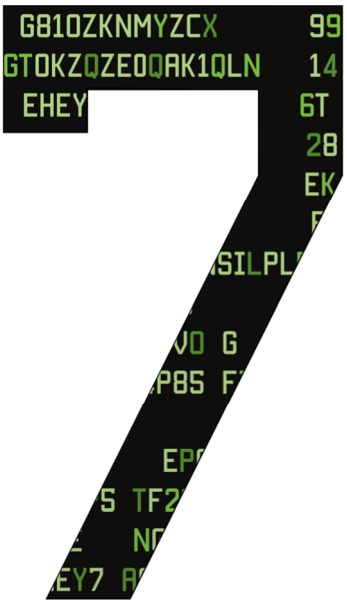

GENERAL DISCUSSION

INTRODUCTION

Cardiovascular disease (CVD) is one of the main causes of death worldwide. Considering that the global incidence of obesity and the metabolic syndrome, major risk factors for developing CVD (1, 2) is rising at an alarming rate (3, 4), researchers from many different disciplines aim to increase our understanding of the causes and progression of these conditions, and on possible intervention strategies to prevent or even cure their clinical manifestations. One important approach is to focus on lifestyle intervention strategies, including nutritional interventions.

Several observational studies have suggested that an increased intake of dairy products may be related to a decreased risk for developing the metabolic syndrome, CVD and type 2 diabetes mellitus (5-11). This relationship may not be restricted to low-fat dairy products alone (6-8). Dairy products may therefore have added value in the development of dietary intervention strategies. However, data from intervention studies is scarce. Further, most intervention studies have been performed in the fasted state and only a few studies have investigated postprandial responses. As most people in the Western world consume on average at least three meals per day and postprandial metabolic processing may take up to 8 hours, most time of the day is spent in a postprandial state. Therefore, the studies described in this thesis were designed to increase our understanding of the effects of dairy products, their components and their food matrix on postprandial metabolic risk markers for CVD.

DAIRY CONSUMPTION AND METABOLIC SYNDROME AND CVD

Several cross-sectional epidemiological studies have related the intake of dairy products to the incidence of the metabolic syndrome or its characteristics and to the risk to develop CVD. A cross-sectional study in 827 Tehranian men and women ranging from 18-74 y showed that subjects within the highest quartile of dairy intake, comprising of milk, yogurt and cheese, had significantly reduced odds ratios (OR) for developing the metabolic syndrome or hypertension or an increased waist circumference, as compared to those within the lowest quartile of dairy intake (5). ORs weakened after adjustment for calcium intake, but remained statistically significant. It should be noted however, that it is hard to differentiate between calcium intake and dairy intake, since dairy products were the main calcium source in the diet. Comparable results were found in a population-based prospective study in 3157 young black and white adults, showing that overweight individuals with ≥35 dairy servings per week had a 79% lower chance of developing the metabolic syndrome as compared to individuals with <10 servings per week

(6). Furthermore, in two studies in middle-aged men, ORs for developing the metabolic syndrome were significantly decreased in the highest quintile (7) or quartile (8) of total dairy intake as compared to the lowest ones. ORs for the metabolic syndrome decreased with increased dairy intake, regardless of the dairy products consumed (6-8). Moreover, decreased risks for developing the metabolic syndrome were found in several recent observational studies (12-15), whereas in 3 studies no association was found for total dairy intake (16-18). In contrast, in some studies ORs for high-fat dairy products were slightly higher than those for low-fat dairy products (6, 8), whereas in one study (18) only full-fat dairy was inversely associated with the risk for developing the metabolic syndrome, whereas low-fat dairy was not.

Regarding CVD, total dairy consumption was inversely associated with mortality in Japanese women (19) and with the incidence of myocardial infarction (20), whereas in a recent meta-analysis only milk, but not total dairy consumption, modestly decreased the relative risk for CVD (21). On the other hand, 3 other observational studies found that total dairy intake was not related to CVD incidence (22) or CVD mortality (23, 24), whereas full-fat dairy was beneficial (24, 25) or harmful (23). Finally, relative risks (9, 10) for type 2 diabetes or acute coronary syndrome (11) decreased only with low-fat dairy products.

Even though several of the earlier studies (5-8, 12-15, 18) have indicated a relation between an increased dairy intake and a decreased incidence of the metabolic syndrome, causal effects can only be derived from intervention studies. Furthermore, regarding the fatty-acid composition of high-fat dairy products, these presumed beneficial effects might be surprising. Therefore, we aimed to increase our understanding of dairy products and some of its characteristics on postprandial metabolism.

ABSORPTION OF CHOLESTEROL AND FATTY ACIDS

Although an increased consumption of high-fat dairy products may be related to a decreased incidence of metabolic syndrome, these products contain relatively large amounts of cholesterol and saturated fatty acids, which are well known for their detrimental effects on the serum lipoprotein profile. As compared to polyunsaturated fatty acids, saturated fatty acids may increase the serum triacylglycerol (TAG) response after a meal (26) and increase LDL cholesterol concentrations by down-regulation of LDL-receptor mediated catabolism (27, 28), which may ultimately increase the risk of cardiovascular disease (29, 30).

Short-chain (<6 carbon atoms (C)) and medium-chain (6-12 C) fatty acids are absorbed passively in the intestine and released directly from the intestinal

capillaries into the portal circulation, which does not result into a lipemic response. In contrast, long-chain (13-20 C; LCFA) and very long-chain (>20 C) fatty acids are at least partially absorbed actively by specific fatty acid transport proteins, including Fatty Acid Transport Protein-4 (FATP-4), Fatty Acid Translocase (FAT)/CD36 and plasma membrane-bound Fatty Acid Binding Protein (FABPpm), and incorporated into chylomicrons, which are released into the vena subclavia via lymphatic vessels, thereby eliciting a lipemic response. Dietary cholesterol, on the other hand, is absorbed passively, actively by Niemann-Pick C1 like 1 (NPC1L1), or excreted back into the intestinal lumen by ATP Binding Cassette G5 (ABCG5) and G8 (ABCG8). Adenosine-triphosphate (ATP) binding cassette A1 (ABCA1) mediates the efflux of cellular cholesterol into lipid-poor apolipoproteins to form nascent HDL. Concerning the metabolic syndrome, there is an ongoing discussion whether these patients are characterized by elevated and/or accelerated intestinal cholesterol absorption or not, since subjects can be characterized as cholesterol absorbers (with a low cholesterol synthesis) or as cholesterol synthesizers (with a low cholesterol absorption) (31). It has been suggested that subjects with the metabolic syndrome are rather cholesterol synthesizers than absorbers (32).

Lipoprotein metabolism is often disturbed in the metabolic syndrome and cholesterol and LCFA transporter levels in the intestine may play a pivotal role. However, since no data was available in humans, we measured the distribution patterns of the most important transport proteins involved in the uptake of cholesterol and LCFA along the human duodenal-colonic axis in human post-mortem intestinal biopsies **(chapter 3)**.

A study in rats (33) suggested that when cholesterol was administered in crystallized form it was absorbed proximally, whereas in an oil phase, which is more similar to micelles in the human situation, uptake occurred more distally. On the other hand, patients undergoing Roux-en-Y gastric bypass surgery showed a significant decrease in cholesterol uptake (34). However, since cholesterol uptake was not completely abolished, it can be concluded that also the more distal segments could absorb cholesterol. Moreover, several in vivo studies in pigs (35-37) have shown that cholesterol uptake predominantly occurred in the jejunum. Therefore, we expected to find the highest levels of transporters in the proximal intestine.

We found for the transport proteins NPC1L1, ABCA1, ABCG8, FAT/CD36 and FABPpm the highest protein levels in the ileum. For ABCG5 and FATP-4, levels were comparable in all segments. Noteworthy, we also found a significant amount of transporters in the colon. Our findings therefore indicate that the ileum plays an important role in transporter-mediated uptake of both cholesterol and LCFA, especially when considering its large surface area as

compared to that of the duodenum and colon. In one study, transporter protein levels were studied in intestinal biopsies, which were derived from healthy subjects and from patients with Crohn's disease. Samples were pooled and the highest protein levels were found in the jejunum (38). Crohn's Disease patients, however, often display decreased cholesterol uptake with a compensatory increase in cholesterol synthesis (39, 40), which may affect the distribution of transporters over the intestinal tract and explain the difference with our study. When membrane proteins were separated from the total lysates, FA transport proteins were found only in the proximal intestine (41, 42). However, if total cell lysates were analyzed as in our study, FA transport proteins were also found in the ileum and colon (43-45). Thus, the transport proteins may not be solely bound to the brush border membrane, but may also be stored within cells and used depending on nutritional needs. In support, meal composition has previously been shown to affect transporter mRNA levels (42, 46).

The relatively high levels of transporter proteins in more distal segments in our study may be explained by the relatively low intraluminal availability of cholesterol and LCFA in these segments. Levels are relatively the highest in the duodenum, and fatty acids and cholesterol can easily be absorbed by the relevant transport proteins. Also, passive diffusion of FA is more likely to occur. More distally, cholesterol and FA bioavailability is lower and more transport proteins may be required to achieve the required uptake of cholesterol and FA.

Conclusions on absorption of fatty acids and cholesterol

Based on the distribution patterns and segmental surface areas, the most important site for transporter-mediated uptake of cholesterol and fatty acids in humans is probably the ileum. However, the uptake of these nutrients may not be restricted to this segment alone. As a matter of fact, transporter proteins were found in all segments, even in colon. It should be considered however, that our protein data only covers the active uptake and not the passive absorption, which may be higher in the proximal intestine due to higher bioavailability. Also, protein activity and functionality were not studied.

POSTPRANDIAL LIPEMIA

After absorption, the long-chain and very long-chain fatty acids are re-esterified into TAG and incorporated into apolipoprotein B-48-rich chylomicrons in the intestine. While these chylomicrons travel towards the liver, the TAG content is hydrolyzed by lipoprotein lipase (LPL) and the released fatty acids are delivered either as energy source to fat-utilizing

tissues, or to fat-storing tissues, where these fatty acids are re-esterified into TAG. Hydrolysis, in combination with exchange of TAG between chylomicrons and the free fatty acid pool, leads to the formation of chylomicron remnants, which are removed from the circulation mainly by the chylomicron-remnant receptor and LDL receptor-related protein (LRP) (47-49). Several studies have shown a positive correlation between the level and duration of postprandial lipemia and CVD. In addition, patients with the metabolic syndrome often show an increased lipemic response (50-53).
There are numerous factors that determine the level of postprandial lipemia. Obviously, dietary fatty acids are a prerequisite for a postprandial lipemic response and fat-related determinants of lipemia include the amount of fat and the fatty-acid composition, e.g. chain length, degree of saturation, TAG structure, and solid fat content.

Effects of fatty acid type on postprandial lipemia

Because of the proposed relation of dairy consumption with CVD and the metabolic syndrome, it is important to know how lipemic responses of dairy fat compare to those of other fats and oils. This is unclear, as both increased and decreased postprandial lipemic responses have been found after butterfat as compared to for example oils rich in linoleic acid (54, 55), which is one of the most common substitutes for saturated fats in the diet. Since in the latter studies test fats were consumed as a spread or were added to tomato sauce (54) or soup (55), the differences in postprandial lipemic responses were probably not merely due to the difference in type of fatty acid consumed, but were also affected by the solid fat content due to the difference in temperature. Therefore, we compared in overweight men the postprandial lipemic responses after the consumption of isoenergetic muffins containing butter or margarine (**chapter 4**). Although the solid fat content still varied between the products as butter has a higher melting point than margarine, the fats were provided in a typical food matrix.
We found a comparable iAUC for serum TAG between the meals. However, peak time was significantly delayed and the iAUC in the late postprandial phase was significantly higher after butterfat. It should be noted however that the lipemic response is a resultant of absorption and clearance and it was therefore not possible to identify which of these two processes was affected. The comparable apoB-48 response suggested that the number of chylomicrons released was comparable between the fats, but chylomicron size may have differed between meals, as in rats larger chylomicrons were found after safflower oil as compared to coconut oil (56). As large chylomicrons are cleared faster from the circulation than small chylomicrons and increased LPL-activity was found after linoleic acid as compared to

palmitic and stearic acids (57), the clearance rate after (n-6) PUFA intake may have been increased. It remains unclear whether also the absorption was different between the meals.

Effects of the food matrix on postprandial lipemia

As the physical structure of carbohydrate-rich foods affects postprandial responses (58, 59), this may also be true for fat-rich foods. Several studies have been performed comparing postprandial lipemic responses to different fats (54, 55) or to different dairy products (60), without adequately accounting for differences in the food matrix. Regarding possible effects of the food matrix of dairy products, there has been a lot of discussion whether homogenization of milk is unhealthy or not. Homogenized milk is characterized by smaller fat globules in the emulsion and alterations in the structure of the milk fat globule membrane (MFGM) (61). Whether consumption of homogenized milk alters postprandial responses as compared to non-homogenized milk has never been studied in humans. Therefore, we compared in overweight men side-by-side the effects of homogenized milk, non-homogenized (unprocessed) milk and butterfat (**chapter 6**). We found no differences in postprandial TAG concentrations or peak times between homogenized and non-homogenized milk, despite the difference in fat structure due to homogenization. Based on changes in TAG, apolipoprotein B-48 and non-esterified fatty acid concentrations, we found no evidence for faster digestion, absorption or intestinal lipolysis rates due to the increased surface area of the fat droplets and alterations to the MFGM, as suggested by a minipig model (62). Unfortunately, since we did not measure gastric emptying, a decrease in this parameter after homogenized milk intake as suggested from a rat study (63) could not be confirmed.

After butterfat, TAG concentrations were increased as compared to NH-milk, which was accompanied by a trend for delayed apolipoprotein B-48 peak concentrations. This suggests a delayed absorption and incorporation of fat from butter into chylomicrons as compared to the two milk meals, which may be explained by the fat structure. In milk, fat is present as small droplets, whereas in butter it is present as large aggregates, which may have slowed down emulsion and hydrolysis (64). In contrast, in type 2 diabetics increased postprandial TAG concentrations after the consumption of a milk meal as compared to a butter meal (60) were found. In that study, the difference in TAG concentrations between the meals was not related to gastric emptying. In this study (60) however, a high-protein jam was added to the butter meal in order to match the protein contents between the meals. Unfortunately, the types of proteins in the jam were not specified. Arguably, these proteins may have resulted in a higher insulinemic response as different proteins or amino

acid mixtures have been shown to affect insulin responses differently (65). This increased insulinemic response may have resulted in the rerouting of free fatty acids from the liver towards adipose tissue, which may have down-regulated hepatic VLDL synthesis.

Conclusions on postprandial lipemia

Despite many studies, effects of dairy fat on postprandial lipemia are inconclusive. As compared to margarine, we found that butter did not affect the iAUC of lipemia nor the peak concentrations, but the peak time was delayed. However, since the TAG response is the resultant of both absorption and clearance, differences in one process may level off differences in the other, but this could not be determined from our study. Furthermore, it is clear that the food matrix affects postprandial lipemia as we found increased postprandial TAG concentrations and also a delayed response as indicated by apoB-48 concentrations after butter as compared to NH-milk. This indicates that the food matrix may be an important confounder when comparing trials studying the effects of fats on postprandial metabolism.

POSTPRANDIAL INFLAMMATION AND ENDOTHELIAL ACTIVATION

The metabolic syndrome is characterized by a low-grade fasting inflammatory state (66) and atherosclerosis is also categorized as an inflammatory disease. As the processes involved in the development of atherosclerosis also occur postprandially, nutrition may play a crucial role in modulating the responses. However, little is known on the effects of dietary composition and the food matrix on markers of low-grade systemic inflammation and endothelial activation, which are both involved in the process of atherosclerosis (67).

Effects of fatty acid type on postprandial inflammatory markers and endothelial activation

Exchanging saturated fatty acids for (n-6) PUFA in overweight men resulted in decreased interleukin (IL)-6, tumor necrosis factor (TNF)α, soluble TNF receptor 2 and decreased concentrations of sVCAM-1. On the other hand, no differences in IL-6 were found in lean and obese women when SFA was exchanged for MUFA or (n-6) PUFA (68). Possibly, these inconsistencies may be explained by gender differences and the dissimilarity in linoleic acid content of the meals between our study (3% vs. 33% of total fatty acids) and their study (4% vs. 19%) (68). A comparable reduction in TNFα concentrations was found in abdominally obese men (69), and in both men and women covering

a broad range of adiposity (70) after consuming a high-fat meal. The lack of a clear description of the study meals, however (69, 70), makes it difficult to relate these results to our findings. On the other hand, when healthy participants and type 2 diabetic patients consumed a test meal rich in SFA and MUFA (71), a time-dependent increase in TNFα concentrations was found. An increase in TNFα after SFA could not be confirmed in our study. Of course, study populations differed and unfortunately in that study no meal with (n-6) PUFA was included.

Although sICAM-1 and sVCAM-1 concentrations did not change over time, we found a meal-dependent reduction in sVCAM-1 concentrations and a trend for lower sICAM-1 concentrations after margarine. In contrast, several other studies with healthy participants, type 2 diabetic patients and hyperlipidemic participants found postprandial increases after fat loads mainly providing SFA (71-73), whereas others found no responses after a mixed meal with mainly SFA and MUFA (74) or even found decreased concentrations in premature CHD patients and healthy controls (75) or mildly obese men (76) after a high-fat meal with MUFA or (n-6) PUFA. Clearly, large differences exist in postprandial sICAM-1 and sVCAM-1 responses, which indicates very large between-subject variations.

Thus, our findings indicate that exchanging SFA from butterfat for (n-6) PUFA in a mixed meal affects several postprandial markers of inflammation and endothelial activation in overweight men, but the impact of these findings for other population groups and on long-term health remains to be elucidated.

Effects of dairy constituents on postprandial inflammatory markers and endothelial activation

Because of the possible relation between dairy consumption and CVD and the metabolic syndrome, it may be hypothesized that dairy products contain constituents that negate the potential deleterious effects of SFA and cholesterol. In a recent cross-sectional survey, it was found that fasting concentrations of C-reactive protein (CRP), IL-6 and TNFα were reduced, when low-fat dairy intake increased from less than 8 servings per week to between 11 and 14 servings, or more than 14 servings per week (77). For full-fat dairy, the differences in IL-6 and TNFα were less significant, but still present. Moreover, in an intervention study with overweight and obese subjects (78), the consumption of a dairy-based smoothie 3 times per day for 28 days significantly reduced fasting serum CRP, IL-6, TNFα and monocyte chemoattractant protein-1 (MCP-1) concentrations and oxidative stress as compared to consumption of an isocaloric soy-based smoothie with a comparable macronutrient composition. Which constituents were responsible

for the effects observed remained unclear, since the experimental products differed not only in the source of protein, but also in calcium content.
The aim of the last study was to identify the constituents from dairy that may modulate postprandial inflammatory and endothelial responses (**chapter 5**). For this, overweight men consumed at four different occasions butter cake with either water, water with calcium, water with calcium and total milk protein, or milk. We found that both the milk meal and the protein meal reduced sICAM-1 and sVCAM-1 concentrations as compared to the other meals, whereas TNFα concentrations were decreased after the control meal as compared to the other meals. Despite an increase over time, IL-6 and IL-8 concentrations were not different between the meals.
No differences in 3h-postprandial IL-6 and TNFα responses were found when overweight subjects consumed single meals consisting of 50 g butter, 115 mL cream, 110 g cheese, 600 mL yogurt or 400 mL reduced fat milk (79). In accordance, Pal et al. (80) found that postprandial IL-6 and TNFα were not differently affected when overweight postmenopausal women consumed a standardized breakfast supplemented with either 45 g sodium casein isolate, 45 g whey protein isolate, or 45 g glucose. The results of these studies (79, 80) are difficult to compare with our study due to differences in study design. Pal *et al*. (80) compared the effects of exchanging glucose for two different protein sources to a standardized breakfast with an equal fat content, while in our study we used a water control. Moreover, Nestel *et al.* (79) compared different dairy products with each other and to a low-fat control meal, whereas we compared it to a breakfast with equal fat content.
We found that the consumption of the control and calcium meals caused a small increase in plasma sICAM-1 and sVCAM-1 over time, whereas adding milk or milk protein to the high-fat breakfast reduced concentrations of sICAM-1 and sVCAM-1 up to 2h after meal consumption, which may indicate that mainly milk protein is responsible for the reductions found in adhesion molecules. Since a strong correlation between insulin responses and sICAM-1 has been shown (74), protein may decrease sICAM-1 through insulin stimulation, but we did not find such a correlation.
It must be noted however, that milk protein was ingested together with calcium, so the effects of milk protein may be dependent on the presence of calcium. However, since milk tended to induce even larger reductions in adhesion molecules, other nutrients may be involved as well. Arguably, the complex dairy matrix may also have modulated the effects of the individual constituents. Our data indicate that consuming 500 mL of skimmed milk or a milk protein drink with a high-fat meal may increase concentrations of TNFα in overweight men. On the other hand, postprandial responses of sICAM-1

and sVCAM-1 may be improved by consuming milk, which may be partially, but not solely, attributable to the protein fraction.

Effects of the food matrix on postprandial inflammatory markers and endothelial activation

In order to test for possible effects of the food matrix on postprandial inflammatory and endothelial responses, we compared meals containing homogenized milk, non-homogenized (unprocessed) milk and butterfat (**chapter 6**). These meals only differed in fat structure. As already stated, fat in butter is present as large aggregates, whereas fat in unprocessed milk is present as relatively large droplets. Due to homogenization and pasteurization fat droplets decrease in size, which results in major changes in the composition of the milk fat globule membrane (MFGM), which has been postulated to increase vascular damage by an increased endothelial presence of several oxidation-inducing proteins, e.g. bovine xanthine oxidase (81). Effects of homogenization on postprandial inflammatory markers and endothelial activation have never been tested before.

We found that homogenization of milk increased the iAUC and total count of white blood cells, which was mainly due to increases in neutrophils. Changes in neutrophils however, were not statistically different between the meals. As activation markers of mainly neutrophils were increased after a high-fat meal as compared to a water control in one study (82), it may be that the activation of neutrophils may be more responsive to meal effects than neutrophil counts. Moreover, several studies have suggested that activated neutrophils stimulate the activation of endothelial cells (83, 84). Unfortunately, we did not measure neutrophil activation markers.

On the other hand, markers of inflammation and endothelial activation, and measures of the retinal vasculature were not affected by homogenization. We did however find consistently lower concentrations of IL-6 and TNFα, when fat was presented as butter, suggesting that the structure of fat in the meal is important. Changes in these markers did not correlate with postprandial TAG concentrations. Whether this suggests that postprandial TAG may not stimulate the release of plasma inflammatory markers is not known. Although correlations between lipemia and inflammation have been found (71, 85), causality has not been established *in vivo*.

Altogether, these findings do not indicate a major role for homogenization of milk on the acute postprandial responses of inflammatory markers and endothelial activation.

Conclusions on postprandial inflammation and endothelial activation

Our findings clearly indicate a role for food composition in modulating postprandial inflammatory and endothelial responses, as exchanging SFA from butterfat for (n-6) PUFA decreased several postprandial markers of inflammation and endothelial activation in overweight men. We also found that the major dairy constituents differently affected postprandial inflammatory and endothelial responses, with possibly beneficial effects of dairy protein on adhesion molecules. An important role for the food matrix was found, as butter affected these responses differently from milk. Homogenization however, did not elicit major changes in postprandial inflammatory and endothelial responses.

Despite the presence of several studies addressing differences in inflammatory and endothelial markers between different fatty acids and various dairy products, the large differences in study populations and test meal compositions makes it difficult to compare the results and warrants the implementation of a standardized study protocol for postprandial tests.

GENERAL CONCLUSIONS

Although an inverse association between dairy intake and the metabolic syndrome and CVD has been shown in several observational studies, causal relations cannot be derived from these studies. In our intervention studies we aimed to increase our understanding and establish possible effects of dairy fat, protein, calcium and also of the food matrix of dairy products on postprandial responses.

We found that the transporter-mediated absorption of fatty acids and cholesterol in humans may predominantly take place in the ileum, but may also take place in the other segments. It should be considered however, that also passive uptake of LCFA occurs, which may be higher in the proximal intestine due to its higher bioavailability.

Regarding dairy products, we showed that exchanging SFA from butterfat for (n-6) PUFA improved postprandial lipemia and inflammatory and endothelial responses. We also showed that especially dairy protein may improve postprandial adhesion molecules and that the food matrix is an important determinant of postprandial lipemia, inflammation and endothelial activation.

FUTURE DIRECTIONS

In our studies we focused on the acute effects. Future studies should therefore be undertaken, in which postprandial responses are compared when dairy fat

is consumed in different food matrices over a longer period of time, for instance a few weeks. Also, since our results indicated that milk differently affected postprandial responses when compared to a mixture of calcium and milk protein, other dairy constituents may be important as well. Future studies should try to define if this assumption is true and try to determine these constituents. As low-fat dairy products may be part of a diet to prevent the metabolic syndrome, it is also important to understand underlying mechanisms. As we performed our tests in a population at risk for the metabolic syndrome and CVD, it would also be interesting to study effects of dairy in subjects who already have developed the metabolic syndrome or CVD, in order to see if dairy products may slow down the progression or even regress the clinical manifestations of these conditions.

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SUMMARY

The global incidence of the metabolic syndrome, a major risk factor for developing cardiovascular disease (CVD), is rising at an alarming rate. This warrants a detailed understanding of the causes and progression of this metabolic aberration and on possible nutritional intervention strategies to prevent or even cure their clinical manifestations. Within this context, dairy products may fulfill a special role.

Several observational studies have suggested that an increased intake of dairy products may be related to a decreased risk for developing the metabolic syndrome and CVD. This relationship may not be restricted to low-fat dairy products alone. However, intervention studies are scarce and have mostly been performed in the fasted state. As most time of the day is spent in a postprandial state and postprandial lipemia is related to CVD-risk, intervention studies should also focus on postprandial responses. **Chapter 2** reviews the effects of the different macronutrients on postprandial lipemic, inflammatory, and endothelial responses and the possible mechanisms involved. Dietary fatty acids are a prerequisite for a postprandial lipemic response. Fat-specific determinants include the amount of fat and the fatty acid composition, e.g. chain length, degree of saturation, triacylglycerol (TAG) structure and solid fat content. Dietary protein and carbohydrates also affect postprandial metabolism of dietary lipids at various steps of digestion, absorption and clearance. Important effects may be found on fatty acid handling, mainly through insulinotropic effects, hereby decreasing the hepatic output of very low-density lipoproteins (VLDL). Regarding the inflammatory and endothelial responses, meal consumption may result in activated endothelium and inflammatory signaling. However, data on macronutrient-specific differences is limited and contrasting.
Although an increased consumption of high-fat dairy products may be related to a decreased incidence of the metabolic syndrome, these products contain relatively large amounts of cholesterol and saturated fatty acids (SFA). As no data was available on the most important sites of uptake of cholesterol and fatty acids in the human intestine, we measured protein levels of the most important transporter proteins involved along the human digestive tract (**chapter 3)**. We found for the transport proteins NPC1L1, ABCA1, ABCG8, FAT/CD36 and FABPpm the highest protein levels in the ileum, whereas for ABCG5 and FATP-4, levels were comparable in all segments. Noteworthy, we also found significant amounts of transporters in the colon. Our findings therefore indicate that the ileum plays an important role in transporter-mediated uptake of both cholesterol and long-chain fatty acids, (LCFA) especially when considering its large surface area as compared to that of the duodenum and colon. It should be noted however, that results of our study are

limited to active uptake, and that protein activity and functionality were not studied.

After absorption, fatty acids are incorporated into chylomicrons and released into the circulation, eliciting a lipemic response. Because of the proposed relation of dairy consumption with the metabolic syndrome and CVD, it is important to know how lipemic responses of dairy fat compare to those of other fats and oils. Therefore, in **chapter 4**, we compared postprandial lipemic, inflammatory and endothelial responses in thirteen overweight men, consuming at different occasions muffins rich in saturated butterfat and muffins rich in (n-6) polyunsaturated fatty acids (PUFA). The TAG iAUCs were comparable, but after butterfat peak time was significantly delayed and the late iAUC was increased. As lipemia is a resultant of absorption and clearance, it was not possible to identify which of these two processes was affected. The comparable apoB-48 responses suggested that the number of chylomicrons released was comparable between the fats, but that chylomicron size may have differed, resulting in a higher clearance rate after (n-6) PUFA intake. Exchanging SFA for (n-6) PUFA resulted in decreased interleukin (IL)-6, tumor necrosis factor (TNF)α, soluble TNF receptor 2 and sVCAM-1 concentrations.

Because of the possible beneficial effects of dairy consumption on CVD and the metabolic syndrome, it may be hypothesized that dairy products contain constituents that negate the potential deleterious effects of SFA and cholesterol. To investigate the dairy constituents involved, **chapter 5** reports on postprandial inflammatory and endothelial responses in sixteen overweight men, consuming at different occasions butter cake with 500 mL of either water, water with calcium, water with calcium and milk protein, or milk. Both the milk meal and the protein meal reduced sICAM-1 and sVCAM-1 concentrations as compared to the other meals, whereas TNFα concentrations were decreased after the control meal as compared to the other meals. Milk protein however, was ingested together with calcium, so the effects of milk protein may be dependent on the presence of calcium. Since milk tended to induce even larger reductions in adhesion molecules, other nutrients may be involved as well. Arguably, the complex dairy matrix may also have modulated the effects of the individual constituents.

Several studies have compared postprandial responses to different fats or dairy products, without adequately accounting for differences in the food matrix. **Chapter 6** therefore reports on an intervention study, in which eighteen overweight men consumed in random order homogenized (H) milk, non-homogenized (NH) milk or butterfat. As compared to NH-milk, H-milk contains smaller fat globules and an altered milk fat globule membrane (MFGM) structure. Except for differences in the food matrix, the meals were

comparable. No differences in postprandial TAG concentrations or peak times between H- and NH- milk were however found. Based on changes in TAG, apoB-48 and non-esterified fatty acid concentrations, we found no evidence for faster digestion, absorption or intestinal lipolysis rates. The structural differences between NH-milk and H-milk increased the iAUC and total count of white blood cells. Markers of inflammation and endothelial activation, and measures of the retinal vasculature were not affected by homogenization. After butterfat, TAG concentrations were increased as compared to NH-milk, which was accompanied by a trend for delayed apoB-48 peak concentrations, suggesting a delayed absorption and incorporation of fat from butter into chylomicrons. Moreover, consistently lower concentrations of IL-6 and TNFα were found, when fat was presented as butter, suggesting that the structure of fat in the meal is important.

The studies described do not unambiguously indicate major alterations in postprandial responses after dairy consumption. Future studies however, should investigate whether the findings reported in this thesis are also valid over a longer period of time, and which other dairy constituents may be involved. Also, studies should be performed in subjects who already have developed the metabolic syndrome or CVD, to determine if dairy products may slow down the progression or even regress the clinical manifestations of these conditions.

SAMENVATTING

Wereldwijd neemt de incidentie van het metabool syndroom, een belangrijke risicofactor voor hart- en vaatziekten (HVZ), met een alarmerende snelheid toe. Daarom is het noodzakelijk om onze kennis over de oorzaken en progressie van deze stofwisselingsaandoening te vergroten, alsmede om voedingsinterventies te ontwikkelen om deze aandoening te voorkomen of te genezen. Binnen deze benadering vervullen zuivelproducten mogelijk een belangrijke rol.

Verschillende observationele studies laten zien dat een verhoogde zuivelconsumptie gerelateerd is aan een verlaagd risico op het ontwikkelen van het metabool syndroom en HVZ; een relatie die zich mogelijk niet enkel beperkt tot magere zuivelproducten. Desalniettemin zijn er maar weinig interventiestudies met zuivelproducten uitgevoerd. In deze interventiestudies werd bloed - om risicofactoren voor HVZ te meten - veelal in de gevaste staat afgenomen. Echter, het grootste deel van de dag wordt doorgebracht in een postprandiale staat en postprandiale lipemie is gerelateerd aan het risico op HVZ.
Hoofdstuk 2 geeft een overzicht van de effecten van de verschillende macronutriënten op postprandiale lipemie, inflammatiemarkers en endotheelmarkers, waarbij ook mogelijke mechanismen zijn beschreven. Voedingsvetzuren zijn een vereiste voor postprandiale lipemie. Vet-gerelateerde factoren zijn onder andere de hoeveelheid vet in de voeding en de vetzuursamenstelling, waaronder bijvoorbeeld ketenlengte, mate van verzadiging, triglyceride (TG) structuur en de hoeveelheid vast vet. Tevens kunnen zowel eiwit als koolhydraten in de voeding het postprandiale vetmetabolisme beïnvloeden door effecten op vertering, opname en klaring. Ook zijn er aanzienlijke effecten op de verwerking van vrije vetzuren, voornamelijk door verhoogde insulinewaarden, waardoor de output van very low-density lipoproteins (VLDL) in de lever daalt. Tenslotte kan het nuttigen van een maaltijd leiden tot endotheelactivatie en een verhoging van ontstekingsparameters. Het is niet duidelijk of er in dit opzicht grote verschillen tussen de diverse macronutriënten zijn.
Een verhoogde consumptie van volle zuivelproducten is mogelijk gerelateerd aan een verlaagde incidentie van het metabool syndroom, ondanks het feit dat deze producten relatief veel cholesterol en verzadigd vet bevatten. Aangezien er geen gegevens beschikbaar waren over de belangrijkste locaties van cholesterol- en vetzuuropname in de darm, hebben we de hoeveelheden van de belangrijkste betrokken transporteiwitten bepaald in de darm van de mens (**hoofdstuk 3**). De grootste hoeveelheden NPC1L1, ABCA1, ABCG8, FAT/CD36 en FABPpm bevonden zich in het ileum, terwijl voor ABCG5 en FATP-4 de hoeveelheden gelijk waren in alle

darmsegmenten. Opvallend was ook de aanwezigheid van transporters in het colon. Uit de resultaten blijkt dat het ileum een belangrijke rol speelt in transporter-gemedieerde opname van cholesterol en lange keten vetzuren, mede vanwege de grotere oppervlakte van het ileum ten opzichte van het duodenum en colon. Echter, in deze studie is alleen gekeken naar eiwitten betrokken bij de actieve opname van nutriënten, terwijl eiwitactiviteit en functionaliteit niet zijn gemeten.

Na absorptie en inbouw van vetzuren in chylomicronen worden deze vrijgegeven in de circulatie, waardoor een lipemische respons optreedt. Vanwege het mogelijke verband tussen zuivelconsumptie en het metabool syndroom en HVZ is het van belang om de lipemische respons na het eten van zuivelvet te vergelijken met die van andere vetten en oliën. Daarom hebben we in **hoofdstuk 4** bij dertien mannen met overgewicht de postprandiale lipemische, inflammatoire en endotheelresponsen vergeleken van muffins rijk aan botervet of (n-6) meervoudig onverzadigde vetten (MOV). De TG iAUC was gelijk, maar de serum TG piek trad later op en na die piek was de iAUC verhoogd na botervet. Het was echter onmogelijk om na te gaan of de absorptie of de klaring beïnvloed was, aangezien lipemie de resultante is van beide processen. De vergelijkbare apoB-48 respons suggereerde dat het aantal chylomicronen gelijk was na de twee maaltijden, maar dat de grootte verschillend was, waardoor de klaring na (n-6) MOV mogelijk verhoogd was. (n-6) MOV verlaagde interleukine (IL)-6, tumor necrosis factor (TNF)α, oplosbare TNF receptor 2, en sVCAM-1 concentraties.

Vanwege de mogelijk positieve effecten van zuivelconsumptie op HVZ en het metabool syndroom, is het interessant om na te gaan of zuivelproducten bestanddelen bevatten die de nadelige effecten van verzadigd vet en cholesterol teniet doen. Om dit te onderzoeken, werden in **hoofdstuk 5** de postprandiale inflammatoire en endotheelresponsen vergeleken bij zestien mannen met overgewicht, die op verschillende dagen boterkoek met ofwel water (controle), water met calcium, water met calcium en melkeiwit, of melk aten. Consumptie van zowel melk als melkeiwit verlaagde sICAM-1 en sVCAM-1 concentraties, terwijl TNFα verlaagd was na de controlemaaltijd ten opzichte van de andere maaltijden. Echter, aangezien melkeiwit samen met calcium werd gegeten, kunnen de effecten van melkeiwit afhankelijk zijn van calcium. Aangezien melk gunstigere effecten leek te hebben op de adhesiemoleculen, is het mogelijk dat melk nog andere bestanddelen bevat die de respons beïnvloeden. Bovendien is het mogelijk dat de complexe zuivelmatrix de effecten van de afzonderlijke bestanddelen heeft beïnvloed.

Hoewel in meerdere studies de postprandiale responsen na de inname van verschillende vetten of zuivelproducten met elkaar zijn vergeleken, werd hierbij vaak geen rekening gehouden met verschillen in de voedingsmatrix.

Hoofdstuk 6 beschrijft de resultaten van een interventiestudie waarin achttien mannen met overgewicht ofwel gehomogeniseerde melk (H), niet-gehomogeniseerde melk (NH) of botervet consumeerden. In vergelijking met H-melk bevat NH-melk grotere vetdruppels, met een andere structuur en bouw van het melkvetmembraan. Voor de rest waren de maaltijden vergelijkbaar. Postprandiale TGs en piektijden waren vergelijkbaar tussen H- en NH-melk en er waren geen aanwijzingen voor een snellere vertering, absorptie of lipolyse van het vet in de darm. De veranderde structuur tussen NH- en H-melk verhoogde de iAUC en het aantal witte bloedcellen, maar markers van inflammatie en endotheelactivatie waren vergelijkbaar tussen H- en NH-melk. Na botervet daarentegen, waren de TG-concentraties hoger dan na NH-melk, met een trend voor een vertraagde apoB-48 piek. Dit suggereert een vertraagde absorptie en inbouw van vet uit boter in chylomicronen. Daarbij vonden we ook lagere IL-6 en TNFα concentraties na boter, hetgeen suggereert dat de vetstructuur een belangrijke rol speelt.

Uit deze studies komt niet éénduidig naar voren dat de postprandiale respons door het eten van zuivelproducten sterk verandert. In toekomstige studies moet echter worden bekeken of de resultaten, zoals beschreven in dit proefschrift, geëxtrapoleerd kunnen worden naar effecten op de lange termijn en welke andere zuivelbestanddelen verder betrokken kunnen zijn. Verder dienen er studies uitgevoerd te worden bij mensen die reeds het metabool syndroom of HVZ hebben ontwikkeld om vast te stellen of zuivelproducten mogelijk de progressie kunnen vertragen of zelfs de klinische manifestaties van deze aandoeningen kunnen verminderen.

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LIST OF PUBLICATIONS

Masson CJ, Plat J, Mensink RP, Namiot A, Kisielewski W, Namiot Z, Fuellekrug J, Ehehalt R, Glatz JFC, Pelsers MMAL. Fatty acid- and cholesterol transporter protein expression along the human intestinal tract. *PLoS One. 2010 Apr 29;5(4):e10380.*

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CURRICULUM VITAE

Christiaan Josef Masson was born on November 18th 1983 in Heerlen, the Netherlands. After he completed secondary school at the Bernardinus College in Heerlen in 2002 he started his study Health Sciences at Maastricht University in 2003, for which he obtained his bachelor's degree in 2006. In 2007 he obtained his master's degree for the European master of Metabolism and Nutrition at Maastricht University. In December of the same year he started his PhD research project at the Human Biology department of Maastricht University under supervision of prof. dr. ir. R.P. Mensink. In 2010 he was awarded with a travel grant for his oral and poster presentations at the International Symposium on Chylomicrons in Disease (ISCD). His research, described in this thesis, was supported by the Dutch Dairy Association. He conducted human studies to investigate the effects of constituents and the food matrix of dairy products on postprandial metabolism in overweight subjects, who are at risk for developing the metabolic syndrome and cardiovascular disease.