Human cardiometabolic health

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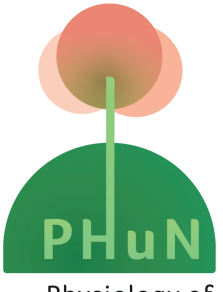
The role of genetic variants, diurnal rhythms, and intermittent energy restriction diets



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DISSERTATION

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CHAPTER 1

General introduction

Cholesterol homeostasis

Cholesterol is essential for cellular functioning and is an important precursor in the biosynthesis of various compounds, such as bile acids and vitamin D. The circulating cholesterol pool in the body is derived from two sources, which are *de novo* cholesterol biosynthesis and dietary cholesterol intake. Human cholesterol homeostasis is primarily maintained by a balance between endogenous cholesterol biosynthesis, intestinal cholesterol absorption, and the degradation or conversion of cholesterol into bile acids (1). Disturbance of the homeostatic state may lead to hypercholesterolemia, which is considered a major risk factor for the development of cardiovascular disease (2, 3).

Endogenous cholesterol synthesis

Cholesterol biosynthesis is a tightly regulated process which takes place in the endoplasmic reticulum (ER) within nearly all mammalian cells. Endogenous cholesterol synthesis begins with the conversion of acetyl coenzyme A (acetyl-CoA) into 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). HMG-CoA is converted into mevalonic acid by HMG-CoA reductase (HMGCR), which is a rate-limiting enzyme (4). Various cholesterol-lowering drugs target the upper part of this pathway thereby inhibiting endogenous cholesterol synthesis, which results in decreased concentrations of the atherogenic low-density lipoprotein cholesterol (LDL-C). For instance, statins inhibit HMGCR (5) and bempedoic acid blocks adenosine triphosphate-citrate (ATP) lyase, which is an enzyme needed for the generation of acetyl-CoA (6). The next step in the pathway is the conversion of mevalonic acid into squalene via farnesyl diphosphate, which is followed by the conversion of squalene into lanosterol. Cholesterol will ultimately be formed from this precursor. The conversion of lanosterol into cholesterol can take place via two different pathways: the Kandutsch-Russell pathway or the Bloch Pathway (**Fig. 1.1**) (7-10). The enzymes that regulate the conversions of the intermediates in these two pathways are similar. The difference between these pathways is characterized by the reduction of the double bond at C24 in the side chain of intermediates by 24-dehydrocholesterol reductase (DHCR24). In the Kandutsch-Russell pathway, the C24 double bond is reduced in the conversion from lanosterol to dihydrolanosterol. Then, via several intermediate steps, 7-dehydrocholesterol is formed which is converted into cholesterol via 7-dehydrocholesterol reductase (DHCR7). In contrast, the C24 double bond is reduced in the final step of the Bloch pathway, which is the conversion of desmosterol into cholesterol by DHCR24. Thus, the Bloch pathway proceeds via various reactions using intermediates with unsaturated side chains, whereas the Kandutsch-Russell pathway intermediates have a saturated side chain at the 24th position (7-10).

Cellular cholesterol levels are monitored by the sterol regulatory element binding protein (SREBP) pathway, in which SREBP cleavage-activating protein (SCAP) and INSIG-1 or -2 are involved (11). Three types of SREBPs exist – SREBP-1a, SREBP-1c, and SREBP-2 – of which SREBP-2 preferentially regulates the expression of genes involved in endogenous cholesterol synthesis (12). SREBPs are located in the ER of the cell, where they form pairs with SCAP. In case of high intracellular cholesterol concentrations, SCAP and INSIG-1 or -2 bind and, as a result, SREBP-2 remains in the ER (13, 14). When intracellular concentrations drop, the interaction between SCAP and INSIG cannot be maintained. This leads to the relocation of the SREBP/SCAP complex to the Golgi Apparatus.

SREBP-2 is then cleaved and becomes transcriptionally active. The cleaved SREBP then moves to the nucleus and binds to sterol response elements of various genes that are involved in endogenous cholesterol synthesis, thereby activating their transcription (11).

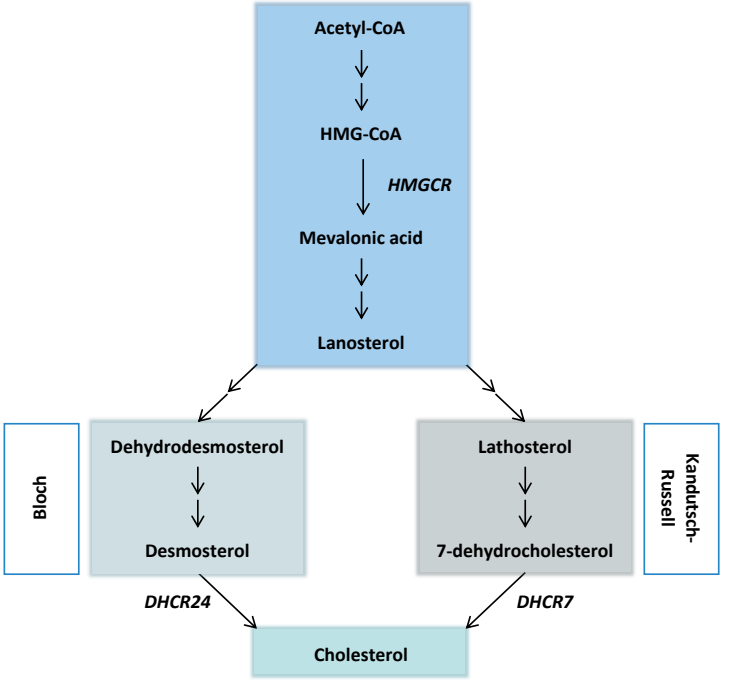

Figure 1.1. A simplified overview of the endogenous cholesterol synthesis pathway. A single arrow indicates that the product is formed in one step. Double arrows indicate that the product is formed via multiple steps.

Intestinal cholesterol absorption

Besides *de novo* cholesterol synthesis, the uptake of dietary cholesterol in the small intestine is essential in maintaining cholesterol homeostasis (**Fig. 1.2**). Intestinal cholesterol absorption is primarily mediated by Niemann-Pick type C1-like intracellular cholesterol transporter 1 (NPC1L1), which is located on the apical surface of enterocytes and facilitates the uptake of cholesterol and plant sterols from the intestinal lumen over the brush border membrane of the enterocyte (15). Together with triacylglycerol, the absorbed cholesterol is then packed into chylomicrons, which also contain proteins such as apolipoprotein B48. The chylomicron enters the circulation, a part of the triacylglycerol is hydrolyzed, and the chylomicron remnant ultimately reaches the liver (1). The cholesterol-lowering drug ezetimibe targets NPC1L1 and thereby inhibits intestinal cholesterol absorption (16, 17). Additionally, plant sterols and stanols (i.e., phytosterols) are known to lower LDL-C concentrations via inhibition of intestinal cholesterol absorption. The chemical structure of phytosterols and cholesterol is comparable, but the main difference is the length of the side chain. A variety of mechanisms has been proposed to explain the cholesterol-lowering effects of plant sterols and stanols, such as a decreased incorporation of cholesterol into micelles, a downregulation of the expression of genes that are relevant in the intestinal cholesterol uptake, and an increased removal of cholesterol from the body via transintestinal cholesterol excretion (18).

Two proteins, adenine triphosphate binding cassette subfamily G member 5 (ABCG5) and member 8 (ABCG8), are other important regulators in intestinal cholesterol absorption (19, 20). They function as heterodimer (ABCG5/G8) and are, just like NPC1L1, expressed on the apical membrane of enterocytes (21). The ABCG5/G8 heterodimer is responsible for the efflux of cholesterol and plant sterols from the enterocyte back into the intestinal lumen (21).

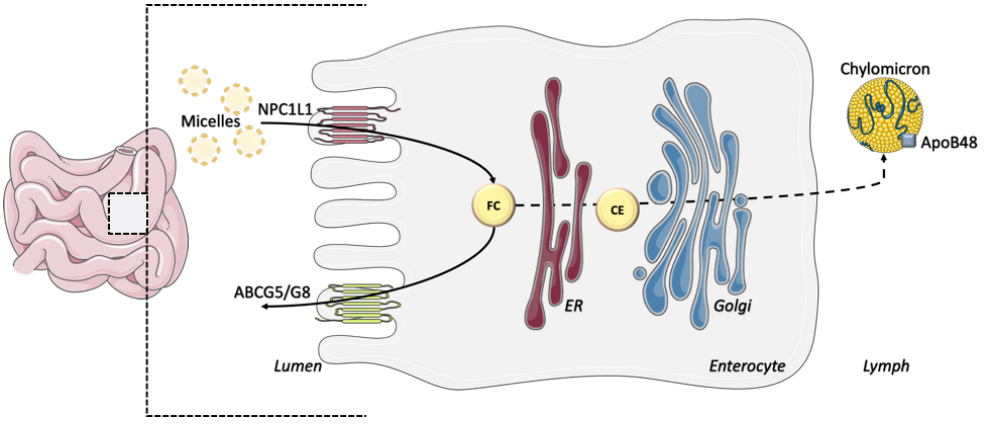

Figure 1.2. A simplified overview of intestinal cholesterol absorption. Free cholesterol is transported in micelles from the lumen into the enterocyte via Niemann-Pick C1-like 1 (NPC1L1). Excess free, non-esterified cholesterol is effluxed back from the enterocyte into the lumen via ATP-binding cassette (ABC) G5/G8. The free cholesterol in the enterocyte is esterified in the endoplasmic reticulum (ER). The cholesteryl ester (CE) is processed further in the Golgi Apparatus, together with triacylglycerol and apolipoprotein B48 (apoB48), which leads to the formation of chylomicrons. These chylomicrons are transferred to the lymphatic system and ultimately reach the liver. The image was designed using Servier Medical Art Images (https://smart.servier.com).

Bile acid synthesis

The conversion of cholesterol into bile acids is the major pathway for the elimination of cholesterol from the body and is therefore crucial for cholesterol homeostasis maintenance. *De novo* bile acid synthesis can occur through two different pathways in the liver: the classic (or neutral) pathway and the alternative (or acidic) pathway (**Fig. 1.3**). The first step in the classic pathway is performed by the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) which regulates the conversion of cholesterol into 7α-hydroxycholesterol. This intermediate is converted into 7α-hydroxy-4-cholesten-3-one (C4), which can be measured in serum and is used as a marker to reflect bile acid synthesis (22). C4 is then converted by sterol 12α-hydroxylase (CYP8B1), which is followed by side chain oxidation by sterol 27-hydroxylase (CYP27A1) and ultimately the production of the two primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) (23). The alternative pathway begins with the hydroxylation of the side chain of cholesterol by CYP27A1. This leads to the formation of oxysterols, which are then hydroxylated by oxysterol 7α-hydroxylase (CYP7B1) (24). These reactions ultimately lead to the production of CDCA (24). The formation of secondary bile acids takes place in the colon (25). Here, the gut microbiota promotes the deconjugation and biotransformation of the primary bile acids into the secondary bile acids. CA can be converted into dihydroxy deoxycholic acid, and CDCA into monohydroxy lithocholic acid (25).

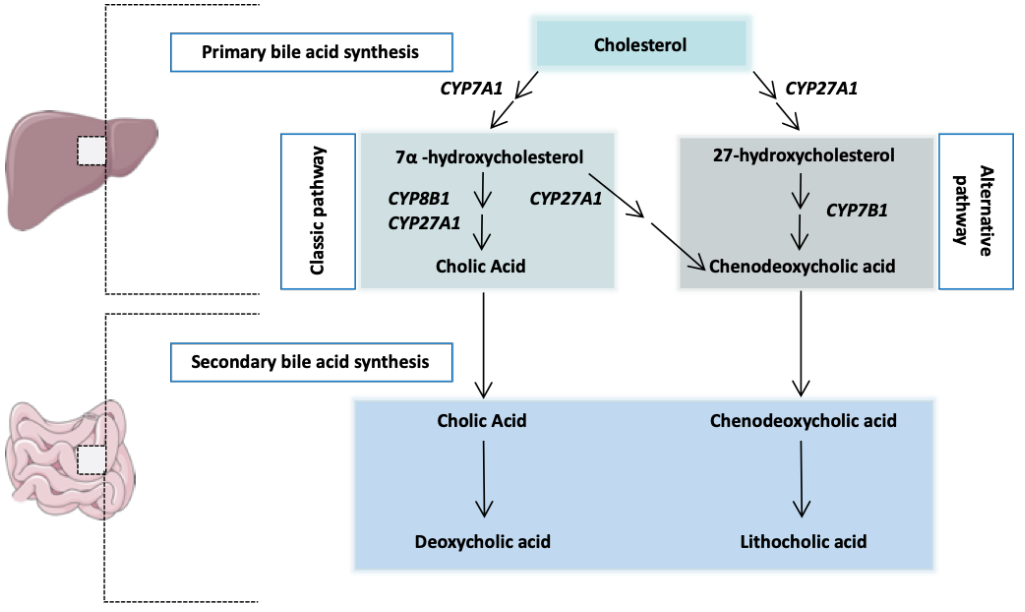

Figure 1.3. An overview of primary and secondary bile acid synthesis. Primary bile acids are formed in the liver via the classic or alternative pathway. Secondary bile acids are formed via the gut microbiota in the intestines.

Endogenous cholesterol synthesis and intestinal cholesterol absorption markers

Various methods have been developed to gain insight into cholesterol homeostasis by measuring absolute cholesterol synthesis and absorption, such as the sterol balance technique (26). However, these absolute methods are very laborious and not suitable for large sample sizes. Therefore, a commonly used method is the measurement of circulating serum non-cholesterol sterol concentrations, which have been validated as markers of endogenous cholesterol synthesis and intestinal cholesterol absorption. The Kandutsch-Russell pathway intermediate lathosterol correlated positively with the cholesterol balance technique (27, 28) and is therefore used as endogenous cholesterol synthesis marker. The Bloch pathway intermediate desmosterol has also been shown to reflect endogenous cholesterol synthesis (28). Cholestanol and the plant sterols campesterol and sitosterol have been validated as intestinal cholesterol absorption markers by comparing their serum concentrations to absolute measurements of intestinal cholesterol absorption (28, 29). To standardize for variations in cholesterol and lipoprotein levels, these non-cholesterol sterols should be expressed as ratios relative to total circulating cholesterol concentrations.

Factors that influence cholesterol homeostasis

Various internal factors are known to influence cholesterol homeostasis, including genetics and the circadian clock. Additionally, external factors, such as the diet or drug treatment, may affect cholesterol homeostasis. These internal and external factors frequently have an impact on cholesterol homeostasis by influencing either endogenous cholesterol synthesis or intestinal cholesterol absorption (30).

Genetic factors

Previous studies have established a large inter-individual variation in intestinal cholesterol absorption and endogenous cholesterol synthesis. Bosner et al. found a mean between-person variation in intestinal cholesterol absorption of approximately 56% (range 29% – 80%) (31), whereas the within-person variation was relatively small (32). For endogenous cholesterol synthesis, another study found a mean between-person variation of around 50% and a within-person variation of approximately 23% (33). These differences in intestinal cholesterol absorption and endogenous cholesterol synthesis rates among individuals have supported the idea that genetic factors are involved in the regulation of cholesterol homeostasis. Moreover, various single-nucleotide polymorphisms (SNPs) that are located in genes essential in cholesterol metabolism have been associated with endogenous cholesterol synthesis (34), intestinal cholesterol absorption (34-36), and total cholesterol concentrations (34, 35). The role of genetics in the regulation of endogenous cholesterol synthesis and intestinal cholesterol absorption has further been supported by studies in which SNPs in lipid related genes were associated with responses to cholesterol-lowering medication (37-39) and foods (40).

The circadian system

Circadian (*circa,* "about" and *diem,* "day") rhythms are physiological or behavioral cycles with a duration of approximately 24 hours (41). Examples are the cycles of hormone secretion, the daily body-temperature cycle, and the sleep-wake cycle. Circadian rhythms are generated endogenously and are thus maintained in the absence of external or environmental cues. These external cues are known as *zeitgebers* and include for instance light exposure, temperature, dietary intake, and physical activity. Biological 24-hour rhythms that are influenced by zeitgebers are called diurnal rhythms (41). The circadian system consists of a master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus and peripheral clocks in almost all other tissues in the body (41). The master and peripheral clocks work through a transcriptional-translational feedback loop, which is regulated by a variety of genes, including clock circadian regulator (*CLOCK*), aryl hydrocarbon receptor nuclear translocator like (*ARNTL* and *ARNTL2*), period circadian regulators (*PER*), and cryptochrome circadian regulators (*CRY1* and *CRY2*) (42). This loop takes approximately 24 hours to complete and turns the transcription of multiple downstream target genes on or off (42).

Regarding cholesterol homeostasis, both animal (43) and human studies (44, 45) have suggested that a diurnal rhythm in endogenous cholesterol synthesis exists. In humans, these rhythms were measured by the deuterium incorporation method (44) and by serum non-cholesterol sterol levels (45). Interestingly, a human study found that the 24-hour rhythm in cholesterol synthesis can be influenced by the timing of food intake (46). A delay in meal timing shifted the time of the peak and nadir with several hours (46), suggesting that this rhythm in endogenous cholesterol synthesis was influenced by an external factor. In contrast to endogenous cholesterol synthesis, little attention has been paid to a potential diurnal rhythm in intestinal cholesterol absorption.

Dietary macronutrient intake

Of the dietary macronutrients, most studies have focused on the impact of dietary fat intake on cholesterol concentrations. In humans, dietary saturated fat intake increased total cholesterol and

LDL-C concentrations, which may partially have been influenced by the chain length of the fatty acid (47). Replacement of saturated fat by polyunsaturated fatty acids, monounsaturated fatty acids, or carbohydrates all showed a decrease in total cholesterol and LDL-C concentrations (47). In addition to this, dietary macronutrient intake has been shown to target the intestinal cholesterol absorption or endogenous cholesterol synthesis pathway (48-50). For example, one study reported that a diet high in carbohydrates and low in fat increased fractional cholesterol synthesis rates compared to a diet low in carbohydrates and high in fat (48). Another study found that consumption of a high-fat low-cholesterol diet increased cholesterol-standardized lathosterol levels compared to a low-fat low-cholesterol diet, a low-fat high-cholesterol diet, and the habitual diet (50). Moreover, fractional cholesterol absorption was significantly decreased after intake of a high-cholesterol low-fat, a low-cholesterol high-fat or a low-cholesterol low-fat diet for 6 weeks compared to their habitual dietary habits that were studied at baseline (50). In another study, a 4-week high-fructose diet lowered endogenous cholesterol synthesis levels, expressed by serum cholesterol-standardized lathosterol and lanosterol levels, compared to a high-glucose diet (49).

The randomized controlled trials that are presented in this thesis both included an overweight and obese study population. Overweight and obese individuals have a median daily food intake window of around 14 to 15 hours (51, 52), and thus spend a large part of the day in the postprandial state. This makes it important to study acute effects of meal consumption on postprandial lipid responses. In two acute human studies, consumption of a high-fat meal lowered postprandial serum endogenous cholesterol synthesis markers (53, 54). In addition, small but significant increases in postprandial serum cholesterol absorption markers were observed in one study (53), while two other studies reported no acute changes in intestinal cholesterol absorption after consumption of a high-fat meal (54, 55). The acute effects of meals high in carbohydrates or proteins on serum non-cholesterol sterol concentrations has been studied less.

Intermittent energy restriction diets

Interest in intermittent energy restriction (IER) diets is rising in scientific literature and popular media. The general principle of IER diets is to restrict from energy intake for a period of time, alternated with a period of unrestricted energy intake (56). IER diets include a variety of diets, including alternate-day fasting (ADF), time-restricted eating (TRE), and the 5:2-diet (also called periodic fasting). In the ADF diet, individuals alternate between days of fasting and ad libitum energy intake days (56). TRE is a diet in which energy intake is reduced to a window of less than twelve hours per day (57). The 5:2-diet includes five days per week of ad libitum energy intake and two consecutive or non-consecutive days per week of fasting or reduced energy intake (58).

Previous meta-analyses in humans have reported that IER diets may have a beneficial influence on body weight (59-62), body mass index (60-62), waist circumference (60), fat mass (60-62), lean mass (61), fasting glucose concentrations (59, 60), fasting insulin concentrations (60), fasting total cholesterol concentrations (60-62), fasting LDL-C concentrations (61), fasting triacylglycerol concentrations (60, 61), and blood pressure (60, 61) compared to a habitual energy intake controlled schedule. These findings are promising but comparing these diets to other weight loss regimens would give more insight into their efficacy. More specifically, this would demonstrate

whether the beneficial effects of IER diets are likely to be due to meal timing and fasting duration or to the decrease in body weight.

Thesis outline

This thesis focusses on the involvement of genetics, diurnal rhythms, and dietary factors in the regulation of human cholesterol homeostasis. Furthermore, a comparison between IER diets and continuous energy restriction diets was made and the effect of alternating energy intake compared to regular energy intake on various components of human metabolic health was studied. **Chapter 2** studied whether genetic variants in genes essential in intestinal cholesterol absorption or endogenous cholesterol synthesis were associated with markers reflecting cholesterol absorption or cholesterol synthesis and may thereby partly explain the large variation that has been observed in intestinal cholesterol absorption and endogenous cholesterol synthesis between individuals. The review in **Chapter 3** provides an overview of the diurnal rhythms of intestinal cholesterol absorption, endogenous cholesterol synthesis, and bile acid synthesis as described in previous literature. Additionally, serum non-cholesterol sterol concentrations were analyzed to confirm the findings of the systematic review and to further study possible diurnal rhythms in cholesterol absorption and synthesis markers. The results showed a clear diurnal rhythm for endogenous cholesterol synthesis, whereas intestinal cholesterol absorption did not demonstrate a significant diurnal rhythm. To investigate if the composition of the diet may have had an influence on these diurnal patterns, we compared whether acute consumption of a high-fat, high-carbohydrate, or high-protein meal had different effects on intestinal cholesterol absorption and endogenous cholesterol synthesis markers in apparently healthy individuals with overweight or obesity (**Chapter 4**). Next, it was examined whether SNPs in genes involved in the regulation of the circadian clock were related to serum intestinal cholesterol absorption or endogenous cholesterol synthesis markers (**Chapter 5**). To provide an overview of the difference between IER diets and continuous energy restriction diets on body weight, body composition, and cardiometabolic risk markers, we have performed a systematic review and meta-analysis of randomized controlled trials which is presented in **Chapter 6**. Additionally, it was studied whether alternate-day energy intake compared to regular energy intake differentially impacted various fasting and postprandial cardiometabolic risk markers in apparently healthy individuals with abdominal obesity (**Chapter 7**). Finally, **Chapter 8** summarizes the main findings of the studies presented in this thesis, followed by an overall discussion, conclusions, and possible directions for future research.

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CHAPTER 2

Associations between SNPs in intestinal cholesterol absorption and endogenous cholesterol synthesis genes with cholesterol metabolism

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ABSTRACT

Single nucleotide polymorphisms (SNPs) have been associated with cholesterol metabolism and may partly explain large inter-individual variability in intestinal cholesterol absorption and endogenous cholesterol synthesis rates. This cross-sectional study therefore examined whether SNPs in genes encoding for proteins involved in intestinal cholesterol absorption (*ABCG5, ABCG8,* and *NPC1L1*) and endogenous cholesterol synthesis (*CYP51A1*, *DHCR7*, *DHCR24*, *HMGCR*, *HSD17B7*, *LBR,* and *MSMO1*) were associated with intestinal cholesterol absorption markers (total cholesterol [TC] standardized campesterol and sitosterol levels), an endogenous cholesterol synthesis marker (TC-standardized lathosterol levels), and serum low-density lipoprotein cholesterol (LDL-C) concentrations in a European cohort. *ABCG5* (rs4245786) and the tag SNP *ABCG8* (rs4245791) were significantly associated with serum campesterol and/or sitosterol levels. In contrast, *NPC1L1* (rs217429 and rs217416) were significantly associated with serum lathosterol levels. The tag SNP in *HMGCR* (rs12916) and a SNP in *LBR* (rs12141732) were significantly associated with serum LDL-C concentrations. SNPs in the cholesterol absorption genes were not associated with serum LDL-C concentrations. SNPs in *CYP51A1, DHCR24, HSD17B7,* and *MSMO1* were not associated with the serum non-cholesterol sterols and LDL-C concentrations. Given the variable efficiency of cholesterol-lowering interventions, the identification of SNPs associated with cholesterol metabolism could be a step forward towards personalized approaches.

INTRODUCTION

Cholesterol homeostasis is determined by the interaction between various complex processes including intestinal dietary and biliary cholesterol absorption, and endogenous cholesterol synthesis (1, 2). For the uptake of sterols into the enterocyte, the apical transporter Niemann-Pick C1-Like 1 (NPC1L1) plays a key role (3). After absorption, the sterol efflux pump ATP-binding cassette (ABC) transporters G5 and G8 secrete a fraction of these sterols back into the intestinal lumen, while the remaining part is incorporated into chylomicrons and secreted into the circulation (**Fig. S2.1**) (4). *De novo* cholesterol synthesis, which involves approximately 30 reactions and more than 20 different enzymes, mainly takes place in the liver (2). Other tissues, however, synthesize cholesterol as well (2). The endogenous cholesterol synthesis pathway starts with acetyl-CoA, which is converted into the intermediate lanosterol in a multistep process. Lanosterol is ultimately converted into cholesterol via either the Bloch or the Kandutsch-Russell pathway (**Fig. S2.2**). The intermediates in these two pathways differ, but the same enzymes are involved (5-8). To estimate fractional intestinal cholesterol absorption, cholesterol-standardized campesterol and sitosterol levels can be used, while those of the Kandutsch-Russell pathway intermediate lathosterol reflect endogenous cholesterol synthesis rates. The use of these markers has been validated by correlating their plasma levels to stable isotope tracer measurements (9).

A reciprocal relation exists between intestinal cholesterol absorption and endogenous cholesterol synthesis (10). For example, statin treatment decreases cholesterol synthesis but increases cholesterol absorption (11), while ezetimibe treatment results in the opposite effects (12). Furthermore, large inter-individual differences are present in relative intestinal cholesterol absorption and endogenous cholesterol synthesis rates. To illustrate, intestinal cholesterol absorption values ranged from approximately 29% to 80% in healthy adults. However, within subject-variability was small (13). For the cholesterol synthesis marker lathosterol, an intra-individual variation of around 23% and an inter-individual variation of more than 50% has been reported for healthy adults (14). Genetic variants, including single-nucleotide polymorphisms (SNPs), might at least partly explain these large inter-individual variations and the wide ranges between individuals in responses to lipid-lowering medications (15). In fact, some SNPs in intestinal cholesterol absorption genes have already been associated with fractional cholesterol absorption rates (16-19). Additionally, several studies have reported associations between SNPs in genes related to intestinal cholesterol absorption and endogenous cholesterol synthesis with lipid-lowering effects of both pharmacological (20-23) and dietary interventions (24, 25). However, whether these associations relate to differences in intestinal cholesterol absorption and endogenous cholesterol synthesis rates has unfortunately not been documented. Identification of SNPs associated with intestinal cholesterol absorption and endogenous cholesterol synthesis is important, as findings may contribute to the development of personalized interventions aimed at improving cholesterol metabolism. The present study therefore investigated in a European population the relation between a number of selected SNPs in genes essential in intestinal cholesterol absorption – *ABCG5, ABCG8,* and *NPC1L1* – and SNPs in genes involved in endogenous cholesterol synthesis – *CYP51A1*, *DHCR7*, *DHCR24*, *HMGCR*, *HSD17B7*, *LBR,* and *MSMO1* – with serum intestinal cholesterol absorption markers (total cholesterol [TC] standardized levels of campesterol and sitosterol), an endogenous cholesterol synthesis marker (TC-standardized levels

of lathosterol), and LDL-C concentrations.

METHODS

Study population

The present study included participants' baseline data from five human intervention studies (Study 1 to Study 5), performed between 1997 and 2012 at Maastricht University, The Netherlands. All participants were recruited from Maastricht and the surrounding area, and data from N = 456 were available for the present study. Overall, the study sample consisted of healthy adults aged ≥ 18 years old. The body mass index (BMI) was calculated for each participant by diving their body weight (kg) by the square of height (m). Most participants had a normal weight (N = 225; 49.3%) or were overweight (N = 179; 39.3%). BMI of few participants fell within the underweight (N = 7; 1.5%), obesity class I (N = 28, 6.1%) or obesity class II (N = 6; 1.3%) range (26). None of the participants used medication known to affect lipid metabolism. Details of the studies have been published (27-30), except for Study 4, which was a 6-week randomized, double-blinded, placebo-controlled parallel trial evaluating effects of plant-sterol ester supplementation as part of a combined lifestyle intervention. For the analysis of this project, we only used samples that were collected at baseline or at the end of a control period. All studies were approved by the Medical Ethics Committee of Maastricht University and were conducted according to the principles laid down in the Declaration of Helsinki. Written informed consent was obtained from all participants.

Blood sampling and biochemical measurements

Blood samples were drawn from participants after an overnight fast. At least one hour after venipuncture, serum was obtained by centrifugation at 2000 x g for 15–30 min at 4 °C and aliquots were stored at −80 °C. The concentrations of TC (CHOD/PAP method; Roche Diagnostics Systems Hoffmann-La Roche Ltd, Basel, Switzerland), high-density lipoprotein cholesterol (HDL-C) (precipitation method by adding phosphotungstic acid and magnesium ions, and CHOD/PAP method; Roche Diagnostics Systems Hoffmann-La Roche Ltd., Basel, Switzerland) and triacylglycerol (TAG) corrected for free glycerol (GPO-Trinder; Sigma Diagnostics, St Louis, USA) were determined in serum by using enzyme-based methods. LDL-C concentrations were calculated using the Friedewald equation (31). Serum concentrations of the intestinal cholesterol absorption markers campesterol and sitosterol, and the endogenous cholesterol synthesis marker lathosterol were analyzed using gas chromatography with flame-ionization detection (GC-FID) in Study 1 and Study 5, while GC-mass spectrometry (GC-MS) was used in the three other studies. Further details on the non-cholesterol sterol analysis have been presented in the article by Mackay et al. (32). Campesterol, sitosterol, and lathosterol concentrations are transported in plasma by cholesterol-rich lipoproteins, and therefore their concentrations were corrected for the differing number of lipoprotein particles by standardizing the concentrations of the markers to the TC concentrations ($10^2 \times$ μmol/mmol TC) as measured with the CHOD/PAP method.

DNA extraction, genotyping, and quality control

Genomic DNA was isolated from either full blood or buffy coats using the QIAamp genomic DNA isolation kit (Westburg BV, Leusden, The Netherlands) according to the instructions of the manufacturer. After isolation, the purity of the genomic DNA was checked by measuring the

260/280 nm and the 260/230 nm ratios (NanoDrop; ND-1000 spectrophotometer, Isogen Lifescience B.V., De Meern, The Netherlands). For all samples, ratios varied between 1.7 and 1.9 and around 2.0, respectively. DNA concentrations were calculated using the relationship that an A_{260} of 1.0 corresponds with 50 µg/ml DNA. All samples were stored at -80 °C after isolation. After thawing, the quality of about 5% of the samples was tested by evaluating the degradation of DNA on agarose gels before further analysis. Results indicated that the quality of these samples was sufficient for genotyping. In the end, 471 DNA samples were genotyped by using the Axiom™ Precision Medicine Research Array (PMRA) kit (Thermo Fisher Scientific, Waltham, MA, USA) (33).

After running the arrays, the software package PLINK (version 1.90 beta; www.cog-genomics.org/plink/1.9/) (34) was used to exclude SNPs: 1) with > 2% missing data, 2) located on sex chromosomes, 3) with a minor allele frequency (MAF) < 0.05, or 4) that deviated from Hardy-Weinberg Equilibrium (HWE) based on a p-value < 1×10^{-10}. Six subjects were removed, because they had a heterozygosity rate ± 3 standard deviations (SDs) from the mean heterozygosity rate. Nine subjects were excluded because there was a sex discrepancy between DNA results with clinical records. Ultimately, 456 samples and 306898 SNPs passed the quality-control criteria. Only SNPs in genes with a clear role in intestinal cholesterol absorption (*ABCG5, ABCG8,* and *NPC1L1*) or endogenous cholesterol synthesis (*CYP51A1, DHCR7, DHCR24, HMGCR, HSD17B7, LBR*, and *MSMO1*) that were present on the array and had passed the quality control steps were included in this study. An overview of the full gene names is provided in **Table S2.1**. The rs-numbers of the selected SNPs are presented, except for two SNPs in ABCG8 for which the rs-numbers were unknown. For these SNPs, their Affymetrix SNP ID (AX-number), i.e. their unique probe set identifier, is given. **Table S2.2** presents information about these two SNPs that was provided by the PMRA array.

Statistics

Continuous values are reported as mean ± SD and categorical values as N (%). Visual inspection of histograms and Q-Q plots of the residuals showed a skewed distribution for TAG and concentrations were therefore log-transformed. Analysis of variance (ANOVA) was used to examine whether continuous variables differed significantly between the five studies. A chi-square test was used for categorical variables.

Possible deviations of the genotype frequencies from those expected under HWE were assessed using chi-square tests in Microsoft Excel. Thereafter, SNPs with a genotype group with a frequency of < 12 participants, which equals < 2.5% of the sample size, were moved to the supplements. All SNPs in *DHCR7* were moved to the supplements due to this reason. Only for SNPs with a genotype group with a frequency of > 12 participants, linkage disequilibrium (LD) was estimated and reported as r^2-values for pairs of SNPs < 500 kB apart using the Haploview software package (version 4.1, Broad Institute of MIT and Harvard, Cambridge, MA, USA) (35). A threshold of $r^2 \geq 0.8$ was used to define SNPs in LD. Haplotype blocks were constructed in Haploview by using the default algorithm as defined by Gabriel et al. (36). In short, blocks were generated by this algorithm when at least 95% of the informative SNPs were in strong LD (36). Furthermore, the Tagger program in Haploview version 4.1 was used to select tag SNPs using the pairwise tagging approach (35). Selection criteria

were a r^2 threshold ≥ 0.8 and a log of the likelihood odds ratio (LOD) threshold of 3.0. Results of the statistical analysis of the tag SNPs are presented in the main text, whereas results for the captured SNPs have been placed in the supplemental information.

Linear regression analyses, corrected for the factor study, were used to examine associations among the TC-standardized non-cholesterol sterols and LDL-C concentrations. Additionally, the general linear model (GLM) was used to examine associations between the SNPs with serum non-cholesterol sterol levels, and LDL-C and TC concentrations. The analyses were adjusted for the factor study. In case of a statistically significant effect of a SNP, the differences in TC-standardized non-cholesterol sterol levels, serum LDL-C concentrations, or serum TC concentrations between the genotype groups were compared with a Bonferroni post-hoc test. The Benjamini-Hochberg multiple testing correction with a false discovery rate of 0.2 was applied to the GLM results for each gene separately. Only SNPs with genotype groups consisting of at least 12 individuals were included in the Benjamini-Hochberg correction. If the original p-value obtained from the general linear model analysis was smaller than the Benjamini-Hochberg critical value, the p-value was considered statistically significant. Next, for SNPs that were significantly associated with TC-standardized non-cholesterol sterols or LDL-C concentrations, an additive, dominant, or recessive multiple linear regression model was built with adjustment for the factor study. The additive model was used when the Bonferroni post-hoc test indicated that all three genotypes were significantly different or when the post-hoc test did not show which genotypes differed significantly. A dominant or recessive model was used when the Bonferroni post-hoc indicated a significant difference between only two genotypes. A dominant model was used if the least frequent homozygous genotype (e.g. aa) and the heterozygous genotype (e.g. aA) had a comparable relation with the outcome (i.e. the non-cholesterol sterols or LDL-C). The dominant model used the major homozygous group as reference, hence AA was compared with aa + aA. Moreover, a recessive model was used if the least frequent homozygous genotype and the heterozygous genotype did not have a comparable relation with the outcome. The recessive model thus compared AA + aA with aa. All analyses were carried out using SPSS for Mac OS X (version 26.0, SPSS Inc., Chicago, IL, USA).

RESULTS

Baseline characteristics for all participants and the five studies separately are shown in **Table S2.3**. Significant differences between the studies were reported for all characteristics of the participants (all $p < 0.05$), except for gender ($p=0.064$).

Associations between markers for cholesterol absorption and cholesterol synthesis, and serum LDL-C concentrations

Linear regression analyses showed that, after controlling for the factor study, sitosterol was positively associated with campesterol ($\beta = 1.39 \times 10^2$ µmol/mmol TC; $p < 0.001$) and inversely with lathosterol ($\beta = -0.09 \times 10^2$ µmol/mmol TC; $p=0.025$). In addition, campesterol showed a significant inverse association with lathosterol ($\beta = -0.10 \times 10^2$ µmol/mmol TC; $p < 0.001$). Campesterol, sitosterol, and lathosterol were not significantly associated with serum LDL-C concentrations (all $p > 0.05$) (**Table S2.4**).

The location and allele frequencies of the selected SNPs

Table S2.5 shows the location and allele frequencies of the selected SNPs. The majority of SNPs were located in an intron and all SNPs had a call rate of ≥ 98.2%. The reference and alternative allele frequencies of the SNPs in our cohort were comparable to those of the European population, which were obtained from the National Center for Biotechnology Information (NCBI) (37). Five of the twelve selected SNPs in the *ABCG8* gene (AX_11180448, rs41360247, rs4245791, rs4299376, rs6544713) deviated significantly from HWE ($p < 0.05$). All other SNPs were in HWE (all $p > 0.05$).

Linkage disequilibrium and tagging for SNPs in genes related to intestinal cholesterol absorption

SNPs in *ABCG8* (rs4299376, rs6544713, and rs4245791) were in high LD (all $r^2 > 0.90$) and consequently included in a haplotype block (**Fig. 2.1a**). Haplotype block 2 included *ABCG8* (rs13390041, rs4077440, and rs3795860). Of these SNPs, rs13390041 and rs3795860 showed a high LD ($r^2 = 0.98$). The tag SNP *ABCG8* (rs4245791) captured rs6544713 and rs4299376, while tag SNP *ABCG8* (rs3795860) captured rs13390041 (**Table 2.1**). For SNPs in *ABCG5* (**Fig. S2.3a**) and *NPC1L1* (**Fig. S2.3b**), no high LD was found (all $r^2 < 0.70$).

Linkage disequilibrium and tagging for SNPs in genes related to endogenous cholesterol synthesis

All SNPs in *HMGCR* were in (borderline) LD (all $r^2 \geq 0.75$) and consequently all SNPs were included in one single haplotype block (**Fig. 2.1b**). One tag SNP in *HMGCR* was selected (rs12916), which captured rs12654264, rs3846662, and rs3846663 (**Table 2.1**). For *DHCR24*, rs6676774 and rs7551288 were in high LD ($r^2 = 0.90$) and *DHCR24* (rs6676774) was selected as a tag SNP for rs7551288 (**Fig. S2.4c; Table 2.1**). None of the other SNPs in *DHCR24,* as well as the SNPs in *LBR* were in pairwise LD (all $r^2 < 0.80$) (**Fig. S2.4**).

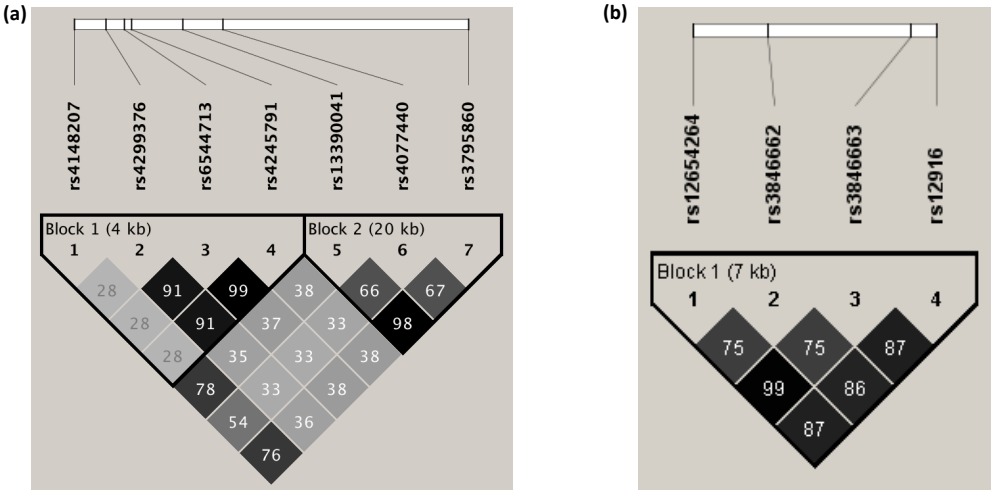

Figure 2.1. Pairwise LD among (a) 7 SNPs in *ABCG8* and (b) 4 SNPs in *HMGCR* is indicated in the diamond shapes. The triangles mark the two haplotype blocks within this region (based on the confidence interval of D'). The shading with a dark grey to white gradient indicates the level of higher to lower LD between each pair of SNPs based on the r^2-value. The LD plot was created by Haploview version 4.1 (35).

Table 2.1. Tag SNPs and their captured SNPs with their corresponding r^2-values

Gene	Tag SNP	Captured SNP	r^2-value
ABCG8	rs4245791	rs6544713	0.995
	rs4245791	rs4299376	0.919
	rs3795860	rs13390041	0.982
DHCR24	rs6676774	rs7551288	0.906
HMGCR	rs12916	rs12654264	0.872
	rs12916	rs3846662	0.862
	rs12916	rs3846663	0.879

Tag SNPs and their captured SNPs were selected using the Tagger program within Haploview version 4.1. (35).

Associations between SNPs in ABCG5, ABCG8, and NPC1L1 with TC-standardized serum non-cholesterol sterol levels and serum LDL-c concentrations

Significant associations were found for a SNP in ABCG8 (rs4245791; $p < 0.001$) with both TC-standardized serum campesterol and TC-standardized serum sitosterol levels. ABCG5 (rs4245786) was also significantly associated with TC-standardized sitosterol levels ($p=0.041$). In addition, two SNPs in NPC1L1 (rs217429 and rs217416) were significantly related with TC-standardized serum lathosterol levels ($p < 0.05$) (**Table 2.2**). After Benjamini-Hochberg multiple testing correction, all associations remained significant. Results for SNPs with a genotype group <12 participants are presented in **Table S2.6**. A recessive model was built for NPC1L1 (rs217429 and rs217416) with lathosterol levels (**Fig. S2.5**). The additive models for ABCG5 (rs4245786) with sitosterol, and for ABCG8 (rs4245791) with sitosterol and campesterol levels can be found in **Table S2.7**. No significant associations were observed between SNPs in ABCG5, ABCG8, or NPC1L1 with serum LDL-C concentrations (all $p > 0.05$) (**Table 2.2**) or TC concentrations (all $p > 0.05$) (**Table S2.8**).

Table 2.2. Associations between various SNPs in cholesterol absorption genes with serum TC-standardized campesterol, sitosterol and lathosterol levels (N = 455), and serum LDL-C concentrations (N = 456)

Gene	SNP	Genotype	N	Campesterol (10^2*µmol/mmol TC)		Sitosterol (10^2*µmol/mmol TC)		Lathosterol (10^2*µmol/mmol TC)		N	LDL-C (mmol/L)	
				Mean (95%CI)	p-value	Mean (95%CI)	p-value	Mean (95%CI)	p-value		Mean (95%CI)	p-value
ABCG5	rs4245786	AA	266	252 (236 – 267)		152 (142 – 162)		120 (112 – 129)		266	3.44 (3.30 – 3.57)	
		AG	160	230 (212 – 249)	0.074	136 (124 – 148)	**0.041**[$]	120 (110 – 131)	0.959	161	3.34 (3.18 – 3.50)	0.306
		GG	29	259 (222 – 296)		154 (130 – 178)		123 (103 – 144)		29	3.23 (2.90 – 3.55)	
	rs7599296	AA	15	261 (210 – 312)		164 (131 – 197)		109 (81 – 137)		15	3.40 (2.95 – 3.85)	
		AG	141	255 (236 – 274)	0.228	152 (140 – 165)	0.173	119 (108 – 130)	0.653	141	3.38 (3.21 – 3.54)	0.980
		GG	299	239 (224 – 254)		143 (133 – 152)		122 (113 – 130)		300	3.39 (3.26 – 3.52)	
	rs4148184	TT	74	232 (207 – 256)		142 (126 – 158)		117 (103 – 130)		74	3.30 (3.08 – 3.51)	
		TC	219	251 (235 – 268)	0.297	148 (137 – 159)	0.803	117 (108 – 126)	0.217	219	3.42 (3.28 – 2.57)	0.561
		CC	161	242 (223 – 260)		146 (134 – 158)		126 (116 – 137)		162	3.39 (3.23 – 3.55)	
	rs13396273	TT	53	236 (207 – 264)		144 (126 – 163)		116 (101 – 132)		53	3.36 (3.11 – 3.60)	
		TC	214	251 (234 – 267)	0.431	148 (138 – 159)	0.819	119 (109 – 128)	0.526	214	3.40 (3.26 – 3.55)	0.922
		CC	188	240 (222 – 257)		145 (133 – 156)		124 (114 – 134)		189	3.38 (3.22 – 3.53)	
ABCG8	rs4148207	TT	156	249 (231 – 268)		151 (139 – 163)		121 (111 – 131)		157	3.34 (3.18 – 3.50)	
		TC	227	243 (226 – 259)	0.757	145 (123 – 155)	0.364	121 (112 – 130)	0.713	227	3.43 (3.29 – 3.58)	0.530
		CC	72	241 (216 – 266)		139 (123 – 155)		116 (102 – 129)		72	3.35 (3.13 – 3.57)	
	rs3795860[+]	TT	128	253 (234 – 273)		154 (141 – 167)		120 (109 – 131)		129	3.32 (3.15 – 3.50)	
		TC	233	244 (228 – 260)	0.342	146 (135 – 156)	0.174	123 (114 – 131)	0.515	233	3.46 (3.32 – 3.60)	0.175
		CC	94	234 (211 – 257)		138 (123 – 152)		115 (102 – 127)		94	3.29 (3.09 – 3.49)	

Table 2.2. *Cont.*

Gene	SNP	Genotype	N	Campesterol (10^2*µmol/mmol TC)		Sitolserol (10^2*µmol/mmol TC)		Lathosterol (10^2*µmol/mmol TC)		N	LDL-C (mmol/L)	
				Mean (95%CI)	p-value	Mean (95%CI)	p-value	Mean (95%CI)	p-value		Mean (95%CI)	p-value
ABCG8	rs4077440	TT	92	256 (233 – 279)		154 (140 – 169)		120 (107 – 132)		92	3.38 (3.18 – 3.58)	
		TC	217	249 (232 – 266)	0.129	149 (138 – 159)	0.125	124 (115 – 133)	0.378	218	3.45 (3.31 – 1.60)	0.252
		CC	145	232 (213 – 251)		138 (126 – 150)		116 (105 – 126)		145	3.30 (3.13 – 3.46)	
	AX_82902928	--	197	248 (231 – 265)		151 (140 – 161)		120 (111 – 130)		197	3.40 (3.25 – 3.55)	
		-AC	192	240 (223 – 258)	0.752	141 (130 – 165)	0.334	120 (110 – 130)	0.955	193	3.43 (3.28 – 3.58)	0.145
		ACAC	66	246 (219 – 272)		147 (130 – 165)		122 (108 – 137)		66	3.19 (2.60 – 3.42)	
	rs4245791+	TT	206	221 (205 – 237)[A]		130 (120 – 141)[A]		123 (114 – 132)		206	3.32 (3.17 – 3.47)	
		TC	215	256 (239 – 272)[B]	**<0.001**[$]	153 (143 – 164)[B]	**<0.001**[$]	119 (109 – 128)	0.642	216	3.46 (3.31 – 3.61)	0.239
		CC	34	315 (282 – 349)[C]		180 (176 – 219)[C]		117 (97 – 136)		34	3.34 (3.04 – 3.65)	
NPC1L1	rs217429	AA	259	239 (223 – 254)		142 (132 – 152)		119 (110 – 128)[A]		259	3.37 (3.23 – 3.50)	
		AC	169	256 (238 – 275)	0.190	154 (142 – 166)	0.134	117 (107 – 127)[A]	**0.017**[#]	170	3.42 (3.26 – 3.58)	0.825
		CC	27	238 (200 – 276)		146 (121 – 170)		149 (128 – 170)[B]		27	3.39 (3.06 – 3.73)	
	rs217416	TT	239	240 (223 – 256)		143 (132 – 153)		119 (110 – 127)[A]		239	3.40 (3.26 – 3.54)	
		TC	189	254 (237 – 272)	0.208	153 (141 – 164)	0.236	118 (108 – 128)[A]	**0.020**[#]	190	3.38 (3.23 – 3.54)	0.922
		CC	25	228 (188 – 267)		140 (114 – 165)		149 (128 – 171)[B]		25	3.33 (2.98 – 3.67)	
	rs11763759	TT	208	244 (227 – 261)		145 (134 – 156)		120 (111 – 130)		209	3.42 (3.27 – 3.56)	
		TC	202	246 (229 – 263)	0.961	147 (136 – 158)	0.938	120 (111 – 129)	0.953	202	3.31 (3.16 – 3.46)	0.084
		CC	43	242 (211 – 273)		149 (128 – 169)		123 (106 – 140)		43	3.62 (3.35 – 3.89)	

Table 2.2. *Cont.*

Gene	SNP	Genotype	N	Campesterol (10^2*µmol/mmol TC)		Sitosterol (10^2*µmol/mmol TC)		Lathosterol (10^2*µmol/mmol TC)		N	LDL-C (mmol/L)	
				Mean (95%CI)	p-value	Mean (95%CI)	p-value	Mean (95%CI)	p-value		Mean (95%CI)	p-value
NPC1L1	rs2072183	CC	18	260 (213 – 307)		154 (123 – 184)		121 (95 – 147)		18	3.33 (2.91 – 3.75)	
		CG	173	254 (235 – 272)	0.314	152 (140 – 164)	0.361	122 (112 – 133)	0.862	174	3.40 (3.24 – 3.57)	0.930
		GG	263	240 (225 – 255)		143 (134 – 153)		119 (111 – 128)		263	3.38 (3.25 – 3.52)	

Abbreviations: LDL-C, low-density lipoprotein cholesterol; N/A, not applicable; SNP, single-nucleotide polymorphism; TC, total cholesterol.

Note: All analyses were adjusted for the factor study. Data are presented as estimated marginal means (95% CI). Non-cholesterol sterol levels were missing for N = 1. Different letters between genotypes within a SNP indicate significantly different non-cholesterol sterol levels between the genotypes based on a Bonferroni post-hoc test. Significant p-values remained significant after adjustment for multiple testing by calculating critical values for each p-value using the Benjamini-Hochberg principle.

[+]Indicates a tag SNP. [#] Recessive models are presented in the supplemental material (**Fig. S2.5**). [$] Additive models are presented in the supplemental material (**Table S2.7**).

Table 2.3. Associations between various SNPs in endogenous cholesterol synthesis genes with serum TC-standardized campesterol, sitosterol and lathosterol levels (N = 455), and serum LDL-C concentrations (N = 456)

Gene	SNP	Genotype	N	Campesterol (10^2*µmol/mmol TC)		Sitosterol (10^2*µmol/mmol TC)		Lathosterol (10^2*µmol/mmol TC)		N	LDL-C (mmol/L)	
				Mean (95%CI)	p-value	Mean (95%CI)	p-value	Mean (95%CI)	p-value		Mean (95%CI)	p-value
CYP51A1	rs35968894	AA	161	240 (222 – 258)		142 (131 – 154)		115 (104 – 124)		161	3.40 (3.24 – 3.56)	
		AG	223	241 (224 – 258)	0.239	146 (135 – 157)	0.334	127 (118 – 136)	0.066	224	3.38 (3.23 – 3.53)	0.976
		GG	71	262 (238 – 287)		156 (140 – 172)		117 (103 – 131)		71	3.39 (3.17 – 3.60)	
DHCR24	rs6676774[+]	AA	75	231 (207 – 256)		144 (128 – 160)		120 (106 – 134)		75	3.42 (3.20 – 3.63)	
		AG	208	246 (230 – 263)	0.436	146 (135 – 157)	0.887	123 (114 – 132)	0.535	208	3.30 (3.16 – 3.45)	0.122
		GG	172	249 (230 – 267)		148 (136 – 160)		117 (107 – 127)		173	3.48 (3.33 – 3.64)	
	rs718265	AA	43	231 (200 – 263)		143 (123 – 164)		117 (98 – 134)		43	3.35 (3.07 – 3.62)	
		AG	190	252 (235 – 269)	0.292	149 (138 – 160)	0.794	123 (114 – 133)	0.570	190	3.34 (3.19 – 3.49)	0.460
		GG	222	240 (223 – 257)		145 (134 – 156)		118 (109 – 127)		223	3.44 (3.29 – 3.59)	
HMGCR	rs12916[+]	TT	151	240 (221 – 260)		145 (133 – 158)		122 (112 – 133)		152	3.22 (3.05 – 3.39)[A]	
		TC	231	242 (226 – 259)	0.373	145 (134 – 155)	0.541	119 (110 – 128)	0.838	231	3.49 (3.35 – 3.63)[B]	**0.011[@]**
		CC	73	259 (234 – 284)		154 (138 – 170)		122 (108 – 135)		73	3.35 (3.13 – 3.56)	
HSD17B7	rs77482353	AA	156	241 (222 – 259)		142 (130 – 154)		121 (111 – 131)		156	3.40 (3.24 – 3.56)	
		AG	227	250 (233 – 266)	0.676	150 (139 – 160)	0.516	120 (111 – 130)	0.889	228	3.32 (3.18 – 3.47)	0.070
		GG	68	246 (220 – 272)		150 (133 – 167)		117 (103 – 132)		68	3.60 (3.72 – 3.83)	
LBR	rs6678087	TT	141	247 (228 – 267)		147 (134 – 160)		120 (109 – 131)		141	3.41 (3.24 – 3.58)	
		TC	223	248 (232 – 265)	0.367	147 (136 – 157)	0.988	121 (112 – 130)	0.997	223	3.39 (2.25 – 3.53)	0.970
		CC	90	232 (209 – 254)		146 (131 – 161)		120 (108 – 133)		91	3.39 (3.19 – 3.59)	

Table 2.3. *Cont.*

Gene	SNP	Genotype	N	Campesterol (10^2*µmol/mmol TC)		Sitosterol (10^2*µmol/mmol TC)		Lathosterol (10^2*µmol/mmol TC)		N	LDL-C (mmol/L)	
				Mean (95%CI)	p-value	Mean (95%CI)	p-value	Mean (95%CI)	p-value		Mean (95%CI)	p-value
LBR	rs12141732	TT	226	241 (224 – 258)		144 (133 – 155)		121 (111 – 130)		227	3.50 (3.35 – 3.65)^{A}	
		TC	194	248 (232 – 265)	0.706	147 (136 – 158)	0.453	121 (112 – 130)	0.799	194	3.28 (3.13 – 3.43)^{B}	**0.027**$^{@}$
		CC	34	251 (216 – 286)		159 (136 – 182)		114 (95 – 134)		34	3.50 (3.20 – 3.81)	
MSMO1	rs17046216	AA	53	237 (209 – 266)		147 (128 – 165)		113 (97 – 128)		53	3.63 (3.38 – 3.88)	
		AG	205	236 (219 – 253)	0.112	142 (131 - 153)	0.347	121 (112 – 131)	0.542	206	3.35 (3.21 – 3.50)	0.101
		GG	197	256 (239 – 273)		151 (140 – 162)		122 (112 – 131)		197	3.36 (3.21 – 3.51)	

Abbreviations: LDL-C, low-density lipoprotein cholesterol; N/A, not applicable; SNP, single-nucleotide polymorphism; TC, total cholesterol.

Note: All analyses were adjusted for the factor study. Data are presented as estimated marginal means (95% CI). Non-cholesterol sterol levels were missing for N = 1. Different letters between genotypes within a SNP indicate significantly different LDL-C concentrations between the genotypes based on a Bonferroni post-hoc test. Significant p-values remained significant after adjustment for multiple testing by calculating critical values for each p-value using the Benjamini-Hochberg principle.

$^{+}$ Indicates a tag SNP. $^{@}$ Dominant models are presented in the supplemental material (**Fig. S2.6**).

Associations between SNPs in *CYP51A1, DHCR24, HMGCR, HSD17B7, LBR,* and *MSMO1* with TC-standardized serum non-cholesterol sterol levels and serum LDL-C concentrations

None of the SNPs in genes essential in endogenous cholesterol synthesis showed a significant association with TC-standardized campesterol, sitosterol or lathosterol serum levels (all $p > 0.05$). Significant associations were reported for *HMGCR* (rs12916) and *LBR* (rs12141732) with serum LDL-C concentrations (all $p < 0.05$) (**Table 2.3**). Dominant models for these SNPs can be found in **Fig. S2.6**. SNPs in *CYP51A1, DHCR24, HSD17B7,* and *MSMO1* were not significantly associated with serum LDL-C concentrations (all $p > 0.05$). **Table S2.9** presents associations for SNPs with a genotype group <12 participants. Results for serum TC concentrations (**Table S2.10**) are comparable to these of serum LDL-C concentrations (**Table 2.3**).

DISCUSSION

Large inter-individual variation in intestinal cholesterol absorption and endogenous cholesterol synthesis exists, which may relate to differences in genetic background. Indeed, we found that SNPs in *ABCG5* and *ABCG8* were associated with intestinal cholesterol absorption, while SNPs in *NPC1L1* were significantly associated with endogenous cholesterol synthesis. However, none of the SNPs that were associated with intestinal cholesterol absorption or endogenous synthesis were associated with serum LDL-C concentrations, whereas SNPs in *HMGCR* and *LBR* did show such a relation. No associations were found for SNPs in *CYP51A1*, *DHCR24*, *HSD17B7*, and *MSMO1* with either one of the evaluated parameters.

ABCG5 (rs4245786) was significantly related with TC-standardized serum sitosterol levels, a marker for intestinal cholesterol absorption. To the best of our knowledge, this association has not been reported before. *ABCG8* (rs4245791) had tagged rs6544713 and rs4299376, which all showed significant associations with intestinal cholesterol absorption markers. A previous study in a European cohort has also reported that SNPs in *ABCG8* were associated with cholesterol absorption (19). In that study, the minor allele of rs41360247 was negatively related to cholesterol absorption and the minor allele of rs4245791 positively (19), which is in agreement with our findings.

For genes encoding enzymes of the endogenous cholesterol synthesis pathways, no significant associations with TC-standardized serum lathosterol levels were reported. Lathosterol is an intermediate in the Kandutsch-Russell pathway. To what extent the selected SNPs that are essential in endogenous cholesterol synthesis are associated with cholesterol synthesis rates in the Bloch pathway is not clear. For this, serum desmosterol should have been measured, which is specific for the Bloch pathway, whereas we analyzed lathosterol which is only part of the Kandutsch-Russell pathway. An explanation for the non-significant relations for the SNPs in the endogenous cholesterol synthesis genes that were selected in our study may be that other SNPs in these genes are associated with endogenous cholesterol synthesis, which were not included in the present study. Another explanation might be that the regulation of endogenous cholesterol synthesis is more complex and does not relate to one single SNP, as many enzymes are involved in the endogenous cholesterol synthesis pathway. In contrast to the absence of an association with lathosterol levels, SNPs in *LBR* (rs12141732) and *HMGCR* (rs12916) were significantly related with serum LDL-C concentrations. *HMGCR* (rs12916) was selected as tag SNP for *HMGCR* (rs12654264,

rs3846662, and rs3846663), which also showed significant associations with serum LDL-C concentrations. For *HMGCR* (rs12654264, rs3846662, rs3846663, and rs12916) these associations with LDL-C concentrations agree with previous studies in Asian and European populations (38-42). Although intestinal cholesterol absorption and endogenous cholesterol synthesis play a key role in the regulation of plasma LDL-C concentrations (2), they do not explain the significant associations between SNP in *HMGCR* and *LBR* with serum LDL-C concentrations. It is likely that other genes that are involved in cholesterol homeostasis have contributed to these findings.

Interestingly, SNPs in genes involved in intestinal cholesterol absorption were not exclusively associated with markers for their postulated physiological process. However, the cholesterol absorption genes *ABCG5*, *ABCG8,* and *NPC1L1* are not only expressed in the human intestine, but also in the liver (43, 44). On hepatocytes, ABCG5/G8 regulates the secretion of cholesterol into bile and NPC1L1 facilitates hepatic cholesterol re-uptake, thereby finetuning an otherwise potentially large biliary and fecal loss of cholesterol (45). In transgenic mice, overexpression of human *ABCG5* and *ABCG8* in the liver and small intestine reduced plasma plant sterol levels and fractional cholesterol absorption as measured by the fecal dual-isotope radio method (46). In contrast, plasma lathosterol and liver mRNA levels of HMGCR were increased. Additionally, in vivo cholesterol synthesis was increased in the liver, possibly to compensate for the elevated biliary cholesterol secretion rates in these transgenic mice (46). This animal study thus shows that ABCG5 and ABCG8 expression influences endogenous cholesterol synthesis which confirms our observations. Moreover, in our cohort, we noticed a similar association for an absorption gene, i.e two SNPs in *NPC1L1* (rs217429 and rs217416) were associated with endogenous cholesterol synthesis. The question remains whether these associations between SNPs in intestinal cholesterol absorption genes and lathosterol only show the reciprocal phenomenon or should also be interpreted as a possible direct effect of the SNP on hepatic cholesterol synthesis. Temei *et al*. have shown that hepatic NPC1L1 expression in transgenic mice increased hepatic cholesterol levels by enhancing the reuptake of cholesterol from the bile (47). It may be that SNPs in *NPC1L1* have increased the expression or activity of NPC1L1 in the liver, which in turn impacts serum lathosterol levels. Furthermore, the SNPs in *ABCG5* and *ABCG8* that showed an association with intestinal cholesterol absorption were not associated with serum LDL-C concentrations and also did not show an inverse association with endogenous cholesterol synthesis. This may suggest that the cholesterol has been eliminated from the body, via for example hepatobiliary cholesterol excretion involving ABCG5/G8 or transintestinal cholesterol efflux (2, 48).

There are some points that should be considered while interpreting our data. Firstly, it should be noted that almost all selected SNPs were located in intron regions. In general, SNPs in introns do not induce changes in protein-coding sequences, suggesting that they are potentially of less functional relevance than SNPs located in exons. However, SNPs in the intron regions can impact the protein via alternative regulation of splicing (49). This can lead to incorrectly spliced mRNA, which may ultimately affect mRNA translation and result in non-functional proteins and can also have clinical consequences (50). SNPs in introns could also serve as markers for other functionally relevant SNPs, as should be indicated by high LD between the SNPs. Secondly, significant differences were found between all baseline characteristics, except for gender distribution, between the five

different studies. This heterogeneity between study populations was taken into account by correcting for the factor study in our analyses. In addition, only European individuals were included, which has further minimized this heterogeneity. In four studies, only individuals with a stable body weight (weight gain or loss of <3 kg for studies 1, 2 and 3 and <2 kg for study 5) could participate. For study 4, a stable body weight was not an inclusion criterion. It is therefore possible that some of the participants lost or gained some weight in the months preceding the study. However, it is not expected that possible changes in weight were related to a specific genotype group and therefore biased the results. Thirdly, this study had a relatively small sample size. This suggests that the significant findings that we found reflect strong associations. Our results can therefore help to determine whether individuals with specific genotypes are more sensitive to specific nutritional and pharmacological interventions, such as foods enriched with plant sterols or stanols, or ezetimibe and statin treatment. To illustrate, 4-week statin treatment in women with familiar hypercholesterolemia resulted in a significantly smaller percentage reduction in LDL-C concentrations in women with the AA genotype of *HMGCR* (rs3846662) compared to women with the other genotypes. Moreover, statin efficacy was significantly decreased in the AA group compared with women with the other genotypes (51). This suggests that genotyping SNPs, even those located in the intron region, may play an important role in the development of more personalized treatment. Finally, an independent cohort in which we could replicate the positive findings was unavailable. Therefore, an additional study is needed to reach greater validity.

CONCLUSION

This study showed that several SNPs in genes that are essential in intestinal cholesterol absorption were associated with serum markers for intestinal cholesterol absorption and/or endogenous cholesterol synthesis. In addition, a number of SNPs in genes that are essential in endogenous cholesterol synthesis were associated with serum LDL-C concentrations in a European cohort.

SUPPLEMENTARY MATERIALS

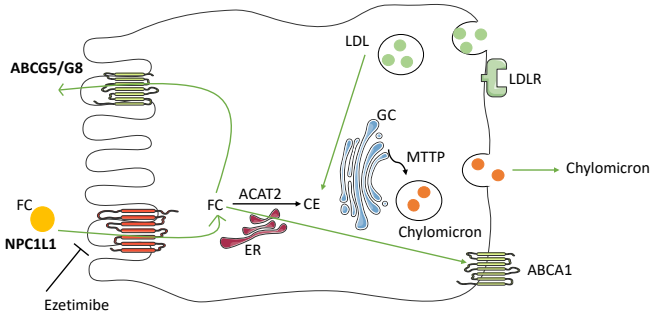

Figure S2.1. Schematic overview of the intestinal cholesterol absorption pathway. Free cholesterol (FC) enters the enterocyte via Niemann-Pick C1-like 1 (NPC1L1) and is esterified by Acetyl-CoA Acetyltransferase 2 (ACAT2) in the endoplasmic reticulum (ER). FC can also be transported back into the intestinal lumen via ATP-binding cassette member 5 and 8 (ABCG5/G8). Low-density lipoprotein (LDL) is taken up from the basolateral side via LDL receptor-mediated endocytosis. FC is also used by ATP-binding cassette A1 (ABCA1) to form high-density lipoprotein. Cholesterol ester (CE) is further processed in the Golgi complex (GC) with other components to form chylomicrons which are further transported to the lymphatic system. The drug ezetimibe is a NPC1L1 blocker and thus inhibits intestinal cholesterol absorption. *Note:* Single-nucleotide polymorphisms in genes in bold have been included in the present study.

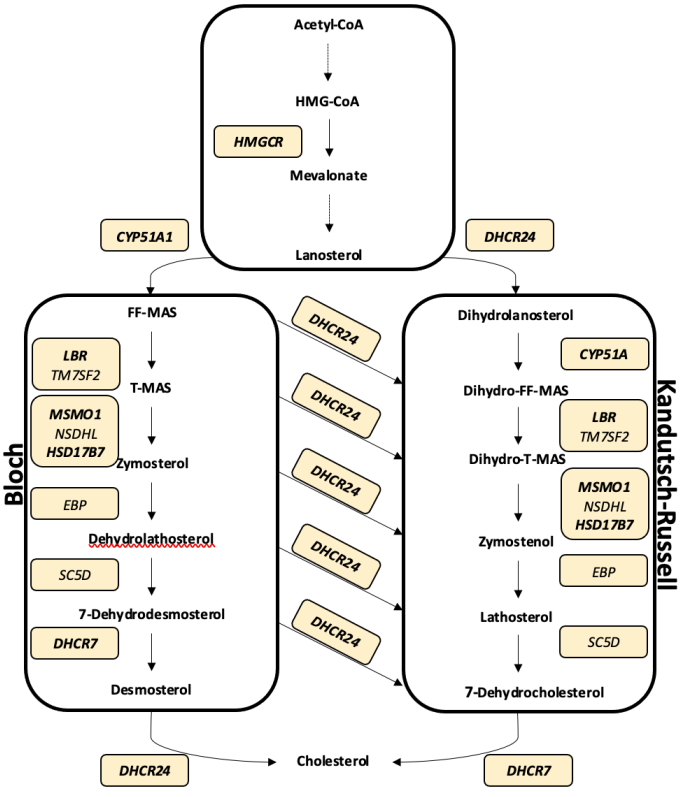

Figure S2.2. The endogenous cholesterol synthesis pathway. Cholesterol is synthesized via the Bloch and/or Kandutsch-Russell pathway. Similar enzymes are involved in these pathways, but intermediates differ. *Note:* Single-nucleotide polymorphisms in genes in bold have been included in the present study.

Table S2.1. List of full names of genes included in the present study

Gene symbol/ HGNC	Approved gene name in HGNC
Cholesterol absorption genes	
ABCG5	ATP binding cassette subfamily G member 5
ABCG8	ATP binding cassette subfamily G member8
NPC1L1	NPC1 like intracellular cholesterol transporter 1
Cholesterol synthesis genes	
CYP51A1	Cytochrome P450 family 51 subfamily A member 1
DHCR7	7-dehydrocholesterol reductase
DHCR24	24-dehydrocholesterol reductase
HMGCR	3 -hydroxy-3-methylglutaryl-CoA reductase
HSD17B7	Hydroxysteroid 17-beta dehyrogenase 7
LBR	Lamin B receptor
MSMO1	Methylsterol monooxygenase 1

Abbreviation: HGNC, Human Genome Organisation (HUGO) Gene Nomenclature Committee.

Table S2.2. Information given by the Precision Medicine Research Array for the two SNPs in *ABCG8* with an unknown rs-number

Affymetrix SNP ID	Transcript ID Ensembl	SNP Location	Accession Number Nucleotide Database NCBI	Gene		
				Full name	Abbreviation	NCBI Gene ID
AX_11180448	ENST00000272286	Missense	NM_022437	ATP-binding cassette, sub-family G (WHITE), member 8	ABCG8	64241
AX_82902928	ENST00000272286	Intron	NM_022437	ATP-binding cassette, sub-family G (WHITE), member 8	ABCG8	64241

Note: Accession Number Nucleotide Database NCBI = reference sequence of mRNA which links to the nucleotide database of NCBI; Affymetrix SNP ID = a unique Affymetrix identifier for the SNP; NCBI Gene ID = ID for a specific gene provided by NCBI; Transcript ID Ensembl = an identifier for the transcripts in the Ensembl database.

Table S2.3. Baseline characteristics for all participants and stratified by study

	All subjects (N = 456)	Study 1 (N = 108)	Study 2 (N = 34)	Study 3 (N = 39)	Study 4 (N = 257)	Study 5 (N = 18)	P-value
Age (years)	45.4 ± 15.3	33.0 ± 14.9	31.2 ± 13.8	50.0 ± 11.9	53.0 ± 10.1	33.0 ± 12.2	**<0.001**
Body mass index (kg/m²)*	25.1 ± 3.6	23.1 ± 2.9	22.8 ± 2.5	25.3 ± 3.0	26.3 ± 3.6	23.9 ± 2.8	**<0.001**
Underweight	7 (1.5)	3 (2.8)	1 (2.9)	0 (0.0)	3 (1.2)	0 (0.0)	
Normal weight	225 (49.3)	76 (70.4)	27 (79.4)	18 (46.2)	92 (35.8)	12 (66.7)	
Overweight	179 (39.3)	27 (25.0)	6 (17.6)	21 (53.8)	119 (46.3)	6 (33.3)	**< 0.001**
Obesity class I	28 (6.1)	2 (1.9)	0 (0.0)	0 (0.0)	26 (10.1)	0 (0.0)	
Obesity class II	6 (1.3)	0 (0.0)	0 (0.0)	0 (0.0)	6 (2.3)	0 (0.0)	
Obesity class III	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Females	254 (55.7)	69 (63.9)	23 (67.6)	23 (59.0)	128 (49.8)	11 (61.1)	0.064
Smoking**	55 (12.1)	21 (19.4)	N/A	4 (10.3)	30 (11.7)	0 (100)	**<0.001**
Lipids							
TC	5.50 ± 1.02	4.95 ± 0.79	4.97 ± 0.91	5.95 ± 0.84	5.73 ± 1.05	5.38 ± 0.83	**<0.001**
HDL-C	1.46 ± 0.41	1.60 ± 0.38	1.42 ± 0.34	1.46 ± 0.44	1.40 ± 0.43	1.59 ± 0.33	**<0.001**
LDL-C	3.54 ± 0.95	2.93 ± 0.76	3.05 ± 0.89	3.93 ± 0.83	3.82 ± 0.91	3.20 ± 0.76	**<0.001**
TAG	0.97 (0.93 – 1.01)	0.82 (0.75 – 0.90)	0.99 (0.86 – 1.15)	1.05 (0.88 – 1.26)	1.00 (0.94 – 1.06)	1.25 (1.06 – 1.48)	**<0.001**
Non-cholesterol sterols***							
Lathosterol	107 ± 55	104 ± 35	131 ± 40	113 ± 54	101 ± 61	153 ± 70	**<0.001**
Sitosterol	137 ± 65	121 ± 44	153 ± 66	166 ± 54	136 ± 71	155 ± 60	**<0.001**
Campesterol	214 ± 120	321 ± 100	241 ± 126	256 ± 96	156 ± 95	249 ± 92	**<0.001**

Abbreviations: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TAG, triacylglycerol; TC, total cholesterol.

Notes: Categorical data are presented as n (%), and continuous data as mean ± SD. For TAG, the mean and (95% CI) are presented after back-transformation of the log-transformed values. Lipids are presented in mmol/L and the non-cholesterol sterols in $10^2 \times$ µmol/mmol cholesterol.

* BMI data presented for N = 445, as data were unavailable for N = 11 in Study 4. BMI categories: underweight <18.5 kg/m², normal weight 18.5 – 24.9 kg/m², overweight 25.0 – 29.9 kg/m², obesity class I 30.0 – 34.9 kg/m², obesity class II 35.0 – 39.9 kg/m², and obesity class III ≥40 kg/m² (26).

** Smoking data presented for N = 410, because data were unavailable for N = 34 in S2 and for N = 12 in Study 4.

*** Non-cholesterol sterol levels presented for N = 455, because data were unavailable for N = 1 in Study 2.

Table S2.4. Associations between intestinal cholesterol absorption markers, an endogenous cholesterol synthesis marker and serum LDL-C concentrations

Independent variable	Dependent variable	t	β	95 % CI for β		P-value
				Lower Bound	Upper Bound	
Sitosterol	Campesterol	42.424	1.39	1.321	1.449	**<0.001**
Sitosterol	Lathosterol	−2.253	−0.09	−0.169	−0.012	**0.025**
Campesterol	Lathosterol	−3.733	−0.10	−0.146	−0.045	**<0.001**
Campesterol	LDL-C	0.335	0.00	−0.001	0.001	0.738
Sitosterol	LDL-C	0.277	0.00	−0.001	0.001	0.782
Lathosterol	LDL-C	−0.403	0.00	−0.002	0.001	0.687

Abbreviations: LDL-C, low-density lipoprotein cholesterol

Note: Non-cholesterol sterols are presented in $10^2 \times$ µmol/mmol cholesterol and LDL-C in mmol/L. All results were obtained from a linear regression analysis adjusted for the factor study.

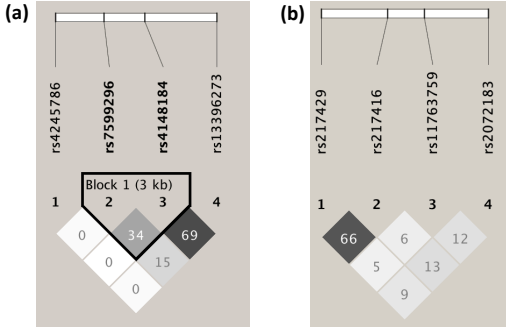

Figure. S2.3. Pairwise LD among SNPs in (a) *ABCG5* and (b) *NPC1L1* is indicated in the diamond shapes. The triangle marks the haplotype block within each region (based on the confidence interval of D'). The shading with a dark grey to white gradient indicates higher to lower LD between each pair of SNPs based on the r^2-value. The LD plots were created by Haploview version 4.1 (35).

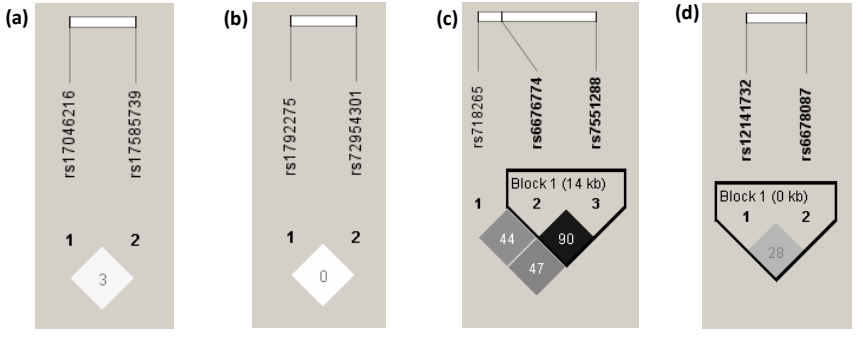

Figure. S2.4. Pairwise LD among SNPs in (a) *MSMO1*, (b) *DHCR7*, (c) *DHCR24*, and (d) *LBR* is indicated in the diamond shapes. The triangle marks the haplotype block within each region (based on the confidence interval of D'). The shading with a dark grey to white gradient indicates higher to lower LD between each pair of SNPs based on the r^2-value. The LD plots were created by Haploview version 4.1 (35).

Table S2.5. The location and allele frequencies for various SNPs in intestinal cholesterol absorption and endogenous cholesterol synthesis genes for 456 participants

Gene	SNP	Location	Call rate (%)	Alleles	Frequencies		HWE
					Our cohort	European Cohort*	
	rs-number			(Ref/Alt)	(Ref/Alt)	(Ref/Alt)	P-value
Cholesterol absorption							
ABCG5	rs10208987	Intron	99.8	T/G	0.938/0.062	0.928/0.072	0.065
	rs4148189	Intron	100	C/T	0.899/0.101	0.888/0.112	0.741
	rs4245786	Intron	100	G/A	0.240/0.760	0.236/0.764	0.487
	rs7599296	Intron	100	G/A	0.813/0.188	0.832/0.168	0.751
	rs4148184	Intron	99.8	C/T	0.597/0.403	0.619/0.381	0.999
	rs13396273	Intron	100	C/T	0.649/0.351	0.640/0.360	0.518
ABCG8	AX_11180448**	Missense	100	G/C	0.932/0.068	-	0.033
	rs4148207	Intron	100	T/C	0.593/0.407	0.610/0.390	0.532
	rs4299376	Intron	98.2	G/T	0.320/0.680	0.323/0.677	0.004
	rs41360247	Intron	100	T/C	0.939/0.061	0.937/0.063	0.008
	rs6544713	Intron	100	T/C	0.310/0.690	0.322/0.678	0.019
	rs4245791	Intron	100	C/T	0.311/0.689	0.327/0.673	0.021
	rs13390041	Intron	100	A/G	0.543/0.457	0.554/0.446	0.694
	rs6709904	Intron	100	A/G	0.902/0.098	0.884/0.116	0.158
	rs4077440	Intron	99.8	T/C	0.442/0.558	0.439/0.561	0.511
	rs3795860	Intron	100	T/C	0.538/0.462	0.559/0.441	0.581
	AX_82902928**	Intron	100	AC/-	0.356/0.644	-	0.089
	rs55924588	Intron	100	T/C	0.950/0.050	0.935/0.065	0.257
NPC1L1	rs217429	Intron	100	A/C	0.754/0.246	0.751/0.249	0.898
	rs217416	Intron	99.6	T/C	0.736/0.264	0.737/0.263	0.105
	rs11763759	Intron	99.6	T/C	0.683/0.317	0.697/0.303	0.562
	rs2072183	Synonymous	99.8	G/C	0.769/0.231	0.774/0.226	0.100
Cholesterol synthesis							
CYP51A1	rs35968894	Intron	100	A/G	0.599/0.401	0.626/0.374	0.634
DHCR7	rs1792275	Intron	99.8	C/T	0.053/0.947	0.054/0.946	0.235
	rs72954301	Upstream	100	G/T	0.894/0.106	0.913/0.087	0.288
DHCR24	rs77668549	Intron	99.8	A/G	0.866/0.134	0.884/0.116	0.379
	rs7553385	Intron	100	A/G	0.938/0.062	0.942/0.058	0.300
	rs7551288	Intron	99.8	A/G	0.403/0.597	0.430/0.570	0.243
	rs11206456	Intron	100	C/T	0.917/0.083	0.905/0.095	0.512
	rs111480286	Intron	100	ACAG/-	0.934/0.066	0.941/0.059	0.434
	rs6676774	Intron	100	G/A	0.607/0.393	0.609/0.391	0.865
	rs718265	Synonymous	100	A/G	0.303/0.697	0.310/0.690	0.784
HMGCR	rs12654264	Intron	100	A/T	0.620/0.380	0.617/0.383	0.232
	rs3846662	Intron	100	A/G	0.553/0.447	0.564/0.436	0.319
	rs3846663	Intron	99.8	C/T	0.619/0.381	0.618/0.382	0.155
	rs12916	Prime UTR	100	T/C	0.587/0.413	0.594/0.06	0.342
HSD17B7	rs77482353	Intron	99.1	A/G	0.597/0.403	0.649/0.351	0.302
LBR	rs6678087	Intron	99.8	T/C	0.163/0.837	0.585/0.415	0.868
	rs12141732	Intron	99.8	T/C	0.712/0.288	0.700/0.300	0.395
	rs4653635	Intron	100	A/G	0.163/0.837	0.151/0.849	0.688
	rs12410357	Intron	100	G/A	0.889/0.111	0.875/0.125	0.449
MSMO1	rs17585739	Synonymous	100	G/A	0.944/0.056	0.938/0.062	0.611
	rs17046216	Intron	100	T/A	0.658/0.342	0.671/0.329	0.939

Abbreviations: Alt, Alternative allele; HWE, Hardy-Weinberg Equilibrium; Ref, Reference allele; SNP, single-nucleotide polymorphism. * European cohort data (release version: 20201027095038) were obtained on January the 28th, 2021 from NCBI (37). *HSD17B7* (rs7748253) was merged into rs11590043 on July the 1st, 2015. ** SNPs with unknown rs-numbers and European cohort frequencies.

Table S2.6. Associations between various SNPs in cholesterol absorption genes, that were either captured by a tag SNP or contained a genotype group < 12 individuals, with serum TC-standardized campesterol, sitosterol and lathosterol levels (N = 455), and serum LDL-C concentrations (N = 456)

Gene	SNP	Genotype	N	Campesterol (10^2*µmol/mmol TC)		Sitosterol (10^2*µmol/mmol TC)		Lathosterol (10^2*µmol/mmol TC)		N	LDL-C (mmol/L)	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value		Mean (95% CI)	P-value
ABCG5	rs10208987	TT	403	249 (235 – 263)		149 (140 – 158)^{A}		119 (111 – 127)		403	3.39 (3.27 – 3.51)	
		TG	47	214 (184 – 244)	**0.041**	124 (104 – 143)^{B}	**0.018**	131 (115 – 148)	0.340	48	3.38 (3.12 – 3.64)	0.900
		GG	4	195 (98 – 292)		112 (50 – 175)		124 (70 – 178)		4	3.19 (2.33 – 4.05)	
	rs4148189	TT	4	290 (192 – 388)		158 (94 – 221)		114 (60 – 167)		4	2.94 (2.08 – 3.80)	
		TC	83	227 (204 – 251)	0.137	136 (121 – 151)	0.236	137 (125 – 150)^{A}	**0.005**	84	3.43 (3.22 – 3.63)	0.533
		CC	368	249 (234 – 263)		149 (140 – 158)		116 (108 – 124)^{B}		368	3.38 (3.25 – 3.51)	
ABCG8	AX_11180448	CC	5	193 (106 – 280)		126 (70 – 182)		150 (102 – 198)		5	3.05 (2.28 – 3.81)	
		CG	51	217 (189 – 245)	**0.040**	125 (107 – 143)^{A}	**0.022**	117 (101 – 132)	0.427	52	3.28 (3.03 – 3.52)	0.381
		GG	399	250 (236 – 264)		150 (141 – 159)^{B}		121 (113 – 129)		399	3.41 (3.29 – 3.54)	
	rs4299376*	TT	194	220 (204 – 237)^{A}		130 (119 – 140)^{A}		124 (114 – 133)		194	3.34 (3.19 – 3.49)	
		TG	220	256 (239 – 272)^{B}	**<0.001$^{\$}$**	153 (143 – 164)^{B}	**<0.001$^{\$}$**	118 (109 – 128)	0.577	221	3.41 (3.27 – 3.56)	0.662
		GG	33	320 (286 – 254)^{C}		201 (179 – 222)^{C}		117 (98 – 137)		33	3.36 (3.05 – 3.67)	
	rs41360247	TT	405	249 (235 – 264)		150 (141 – 159)		120 (113 – 128)		405	3.42 (3.29 – 3.54)	
		TC	45	216 (187 – 246)	0.052	124 (105 – 144)	**0.031**	119 (102 – 135)	0.468	46	3.22 (2.97 – 3.48)	0.239
		CC	5	193 (106 – 280)		126 (70 – 182)		150 (102 – 198)		5	3.05 (2.28 – 3.81)	
	rs6544713*	TT	33	316 (282 – 350)^{A}		198 (176 – 220)^{A}		117 (98 – 137)		33	3.38 (3.08 – 3.69)	
		TC	216	256 (239 – 272)^{B}	**<0.001$^{\$}$**	153 (143 – 164)^{B}	**<0.001$^{\$}$**	118 (109 – 128)	0.653	217	3.45 (3.31 – 3.60)	0.285
		CC	206	221 (205 – 237)^{C}		130 (120 – 141)^{C}		123 (114 – 132)		206	3.32 (3.17 – 3.47)	

Table S2.6. *Cont.*

Gene	SNP	Genotype	N	Campesterol (10^2*µmol/mmol TC)		Sitosterol (10^2*µmol/mmol TC)		Lathosterol (10^2*µmol/mmol TC)		N	LDL-C (mmol/L)	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value		Mean (95% CI)	P-value
ABCG8	rs6709904	AA	374	246 (231 – 260)		147 (137 – 156)		121 (113 – 129)		374	3.41 (3.28 – 3.54)	
		AG	74	243 (219 – 268)	0.443	145 (130 – 161)	0.948	116 (103 – 129)	0.576	75	3.33 (3.21 – 3.54)	0.306
		GG	7	198 (124 – 272)		139 (91 – 187)		135 (95 – 176)		7	2.94 (2.29 – 3.59)	
	rs55924588	TT	409	247 (232 – 261)		147 (138 – 156)		121 (113 – 128)		410	3.38 (3.26 – 3.50)	
		TC	46	223 (192 – 254)	0.124	136 (116 – 156)	0.250	117 (100 – 134)	0.676	46	3.45 (3.18 – 3.71)	0.632
		CC	0	N/A		N/A		N/A		0	N/A	
	rs13390041*	AA	131	252 (232 – 272)		153 (140 – 166)		120 (109 – 131)		132	3.33 (3.15 – 3.50)	
		AG	213	245 (229 – 261)	0.378	146 (136 – 157)	0.168	123 (114 – 132)	0.527	231	3.46 (3.32 – 3.60)	0.180
		GG	93	233 (210 – 256)		136 (122 – 151)		115 (103 – 128)		93	3.29 (3.09 – 3.49)	

Abbreviations: LDL-C, low-density lipoprotein cholesterol; N/A, not applicable; SNP, single-nucleotide polymorphism; TC, total cholesterol.

Note: All analyses were adjusted for the factor study. Data are presented as estimated marginal means (95% CI). Non-cholesterol sterol levels were missing for N = 1. Different letters within a SNP indicate significantly different non-cholesterol sterol levels between the genotypes based on a Bonferroni post-hoc test.

* Indicates a SNP captured by a tag SNP. $ Additive models are presented in the supplemental material (**Table S2.7**).

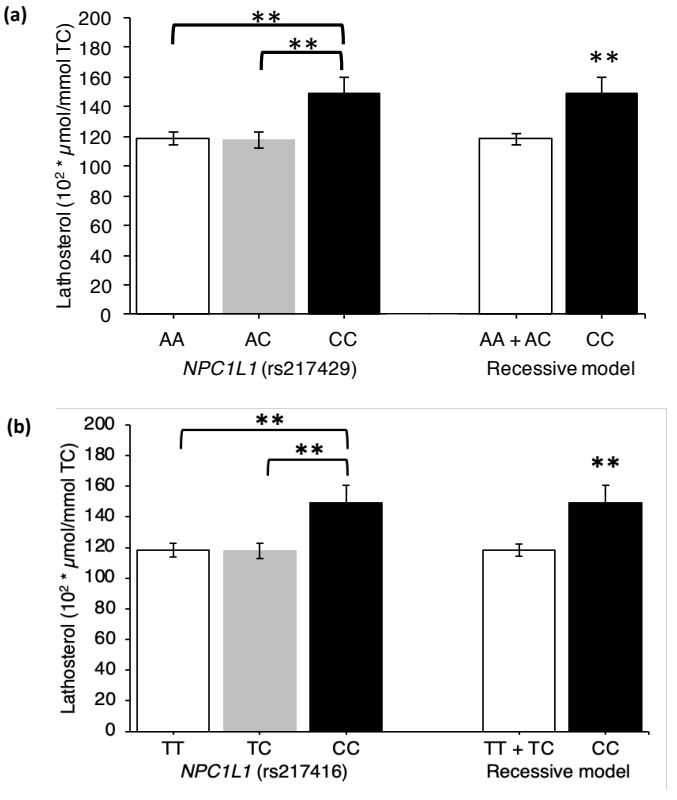

Figure. S2.5. Association between SNPs (a) *NPC1L1* (rs217429) and (b) *NPC1L1* (rs217416) with serum levels of cholesterol-standardized lathosterol using recessive models. All values were adjusted for the factor study and presented as estimated marginal means ± SE. The black bars refer to the least frequent homozygous genotype, and the white bars refer to the most frequent homozygous genotype. * $p \leq 0.05$, ** $p \leq 0.01$.

Table S2.7. Associations between SNPs in intestinal cholesterol absorption genes with TC-standardized non-cholesterol sterols using additive models (N = 455)

Gene	SNP	Alleles (Ref/Alt)	Marker	t	β	95 % CI for β		P-value
						Lower bound	Upper bound	
ABCG5	rs4245786	G/A	Sitosterol	-1.4	-6.9	-16.5	2.7	0.161
ABCG8	rs6544713	T/C	Campesterol	5.7	41.5	27.3	55.8	**<0.001**
ABCG8	rs6544713	T/C	Sitosterol	6.2	28.7	19.6	37.9	**<0.001**
ABCG8	rs4245791	C/T	Campesterol	5.8	41.4	27.2	55.5	**<0.001**
ABCG8	rs4245791	C/T	Sitosterol	6.2	28.6	19.5	37.7	**<0.001**
ABCG8	rs4299376	G/T	Campesterol	5.8	42.9	28.5	75.4	**<0.001**
ABCG8	rs4299376	G/T	Sitosterol	6.3	29.7	20.4	38.9	**<0.001**

Abbreviations: Alt, alternative allele; Ref, reference allele; SNP, single-nucleotide polymorphism

Notes: Non-cholesterol sterols are presented in $10^2 \times$ µmol/mmol total cholesterol. All results were obtained from a linear regression analysis adjusted for the factor study. The alternative allele was used as reference in the model; each copy of the reference allele changes the outcome parameter (marker) with β.

Table S2.8. Associations between various SNPs in genes involved in intestinal cholesterol absorption with serum total cholesterol concentrations (N = 456)

Gene	SNP	Genotype	N	Total cholesterol (mmol/L)	
				Mean (95% CI)	P-value
ABCG5	rs10208987	TT	403	5.40 (5.26 – 5.53)	
		TG	48	5.40 (5.11 – 5.69)	0.906
		GG	4	5.19 (4.24 – 6.14)	
	rs4148189	TT	4	5.13 (4.81 – 6.09)	
		TC	84	5.43 (5.21 – 5.66)	0.803
		CC	368	5.39 (5.25 – 5.53)	
	rs4245786	AA	266	5.45 (5.29 – 5.60)	
		AG	161	5.35 (5.18 – 5.53)	0.356
		GG	29	5.21 (4.85 – 5.58)	
	rs7599296	AA	15	5.16 (4.66 – 5.66)	
		AG	141	5.42 (5.23 – 5.60)	0.616
		GG	300	5.40 (5.26 – 5.55)	
	rs4148184	TT	74	5.22 (4.98 – 5.46)	
		TC	219	5.41 (5.24 – 5.57)	0.182
		CC	162	5.47 (5.29 – 5.65)	
	rs13396273	TT	53	5.25 (4.98 – 5.53)	
		TC	214	5.39 (5.23 – 5.55)	0.376
		CC	189	5.46 (5.29 – 5.63)	
ABCG8	AX_11180448	CC	5	5.16 (4.31 – 6.01)	
		CG	52	5.27 (5.00 – 5.54)	0.463
		GG	399	5.42 (5.29 – 5.56)	
	rs4148207	TT	157	5.43 (5.25 – 5.61)	
		TC	227	5.42 (5.26 – 5.58)	0.408
		CC	72	5.26 (5.02 – 5.50)	
	rs4299376	TT	194	5.33 (5.16 – 5.50)	
		TG	221	5.42 (5.25 – 5.58)	0.467
		GG	33	5.52 (5.18 – 5.86)	
	rs41360247	TT	405	5.44 (5.30 – 5.57)	
		TC	46	5.18 (4.90 – 5.47)	0.203
		CC	5	5.16 (4.31 – 6.01)	
	rs6544713	TT	33	5.54 (5.21 – 5.88)	
		TC	217	5.46 (5.30 – 5.63)	0.151
		CC	206	5.30 (5.14 – 5.47)	
	rs4245791	TT	206	5.31 (5.14 – 5.47)	
		TC	216	5.47 (5.31 – 5.63)	0.163
		CC	34	5.51 (5.18 – 5.85)	
	rs13390041	AA	132	5.41 (5.22 – 5.60)	
		AG	231	5.46 (5.30 – 5.61)	0.115
		GG	93	5.21 (4.99 – 5.44)	
	rs6709904	AA	374	5.42 (5.28 – 5.56)	
		AG	75	5.33 (5.10 – 5.56)	0.392
		GG	7	4.99 (4.27 – 5.71)	
	rs4077440	TT	92	5.49 (5.27 – 7.71)	
		TC	218	5.46 (5.30 – 5.62)	0.066
		CC	145	5.25 (5.06 – 5.43)	
	rs3795860	TT	129	5.41 (5.22 – 5.60)	
		TC	233	5.46 (5.30 – 5.61)	0.127
		CC	94	5.21 (5.00 – 5.44)	
	AX_82902928	--	197	5.45 (5.29 – 5.62)	
		-AC	193	5.41 (5.24 – 5.58)	0.057
		ACAC	66	5.13 (4.88 – 5.39)	
	rs55924588	TT	410	5.39 (5.26 – 5.53)	
		TC	46	5.46 (5.16 – 5.76)	0.645
		CC	0	N/A	
NPC1L1	rs217429	AA	259	5.39 (5.24 – 5.54)	
		AC	170	5.41 (5.23 – 5.59)	0.938
		CC	27	5.45 (5.08 – 5.83)	
	rs217416	TT	239	5.43 (5.28 – 5.59)	
		TC	190	5.36 (5.18 – 5.53)	0.698
		CC	25	5.37 (4.90 – 5.76)	
	rs11763759	TT	209	5.42 (5.26 – 5.58)	
		TC	202	5.31 (5.15 – 5.48)	**0.043**
		CC	43	5.71 (5.41 – 6.01)	
	rs2072183	CC	18	5.34 (4.88 – 5.80)	
		CG	174	5.41 (5.23 – 5.59)	0.956
		GG	263	5.40 (5.25 – 5.54)	

Abbreviations: N/A, not applicable; SNP, single-nucleotide polymorphism.

Note: All analyses were adjusted for the factor study. Data are presented as estimated marginal means (95% CI). Statistical significance was set as a p-value < 0.05.

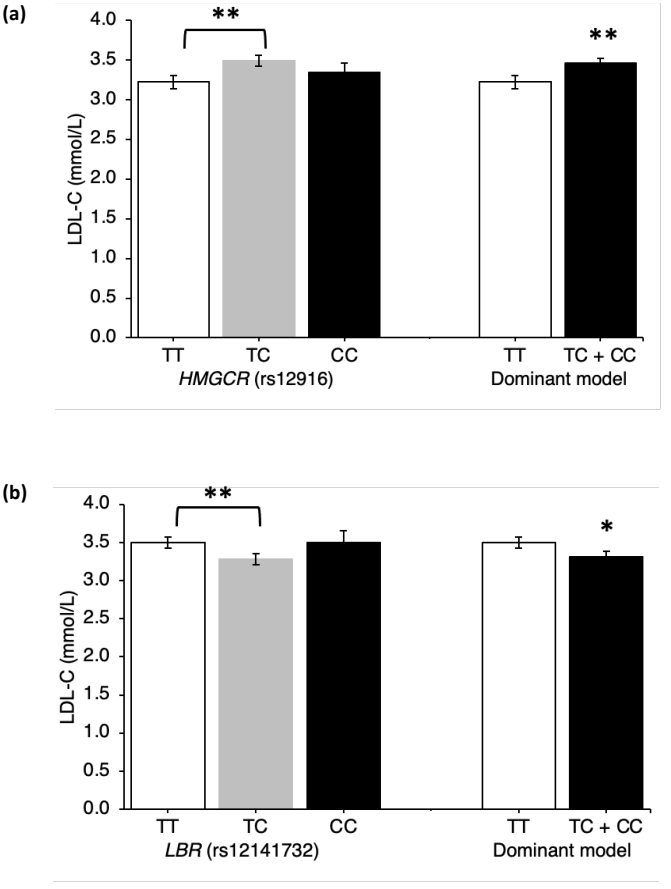

Figure. S2.6. Association between SNPs (a) *HMGCR* (rs12916) and (b) *LBR* (rs12141732) with serum LDL-C concentrations using dominant models. All values were adjusted for the factor study and presented as estimated marginal means ± SE. The black bars refer to the least frequent homozygous genotype, and the white bars refer to the most frequent homozygous genotype. * $p \leq 0.05$, ** $p \leq 0.01$.

Table S2.9. Associations between various SNPs in endogenous cholesterol synthesis genes, that were either captured by a tag SNP or contained a genotype group < 12 individuals, with serum TC-standardized campesterol, sitosterol and lathosterol levels (N = 455), and serum LDL-C concentrations (N = 456)

Gene	SNP	Genotype	N	Campesterol (10^2*µmol/mmol TC)		Sitosterol (10^2*µmol/mmol TC)		Lathosterol (10^2*µmol/mmol TC)		N	LDL-C (mmol/L)	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value		Mean (95% CI)	P-value
DHCR7	rs1792275	TT	406	243 (229 – 256)		145 (136 – 154)		123 (115 – 130)		407	3.37 (3.24 – 3.49)	
		TC	48	259 (230 – 289)	0.266	156 (137 – 175)	0.250	104 (88 – 120)	**0.024**	48	3.60 (3.34 – 3.86)	0.078
		CC	0	N/A		N/A		N/A		0	N/A	
	rs72954301	TT	3	207 (94 – 320)		121 (48 – 194)		137 (75 – 200)		3	3.35 (2.36 – 4.34)	
		TG	91	253 (230 – 276)	0.537	148 (133 – 163)	0.763	122 (109 – 135)	0.828	91	3.34 (3.14 – 3.54)	0.837
		GG	361	243 (229 – 258)		146 (137 – 155)		120 (112 – 128)		362	3.40 (3.27 – 3.52)	
DHCR24	rs77668549	AA	339	244 (229 – 258)		145 (136 – 155)		119 (111 – 127)		339	3.39 (3.26 – 3.52)	
		AG	109	252 (230 – 273)	0.354	152 (138 – 166)	0.461	125 (113 – 137)	0.654	110	3.36 (3.17 – 3.55)	0.765
		GG	6	194 (115 – 274)		124 (72 – 176)		134 (90 – 178)		6	3.62 (2.93 – 4.32)	
	rs7551288*	AA	80	232 (208 – 256)		144 (128 – 159)		119 (106 – 133)		80	3.40 (3.19 – 3.61)	
		AG	207	251 (234 – 267)	0.376	148 (138 – 159)	0.799	123 (114 – 133)	0.519	207	3.33 (3.18 – 3.48)	0.358
		GG	167	244 (225 – 262)		145 (133 – 157)		117 (107 – 127)		168	3.46 (3.30 – 3.62)	
	rs7553385	AA	401	246 (232 – 250)		147 (138 – 156)		121 (113 – 128)		402	3.39 (3.26 – 3.51)	
		AG	51	229 (200 – 259)	0.494	139 (121 – 158)	0.725	119 (103 – 135)	0.916	51	3.34 (3.09 – 3.60)	0.211
		GG	3	261 (149 – 374)		144 (72 – 217)		109 (46 – 171)		3	4.25 (3.26 – 5.23)	
	rs11206456	TT	2	224 (86 – 362)		137 (48 – 226)		140 (64 – 216)		2	3.88 (2.67 – 5.09)	
		TC	72	255 (230 – 280)	0.608	153 (137 – 170)	0.605	129 (115 – 143)	0.323	72	3.33 (3.11 – 3.55)	0.597
		CC	381	243 (229 – 257)		145 (136 – 154)		119 (111 – 127)		382	3.40 (3.27 – 3.52)	

Table S2.9. *Cont.*

Gene	SNP	Genotype	N	Campesterol (10^2*µmol/mmol TC)		Sitosterol (10^2*µmol/mmol TC)		Lathosterol (10^2*µmol/mmol TC)		N	LDL-C (mmol/L)	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value		Mean (95% CI)	P-value
DHCR24	rs111480286	--	3	261 (149 – 374)		145 (72 – 217)		109 (46 – 171)		3	4.25 (3.26 – 5.23)	
		-ACAG	54	230 (202 – 259)	0.512	140 (121 – 158)	0.730	118 (103 – 134)	0.891	54	3.29 (3.04 – 5.53)	0.153
		ACAGACAG	398	246 (232 – 260)		147 (138 – 156)		121 (113 – 129)		399	3.39 (3.27 – 3.52)	
	rs4653635	AA	11	244 (185 – 304)		148 (110 – 187)		100 (68 – 133)		11	3.05 (2.53 – 3.57)	
		AG	127	235 (214 – 255)	0.426	139 (126 – 152)	0.351	121 (109 – 132)	0.496	127	3.46 (3.28 – 3.63)	0.275
		GG	317	248 (233 – 263)		149 (139 – 158)		121 (113 – 129)		318	3.37 (3.24 – 3.50)	
	rs12410357	AA	4	284 (186 – 382)		138 (75 – 202)		125 (71 – 179)		4	4.10 (3.24 – 4.95)	
		AG	93	244 (221 – 267)	0.728	139 (124 – 154)	0.450	125 (112 – 138)	0.690	93	3.34 (3.13 – 3.54)	0.219
		GG	358	245 (230 – 259)		148 (139 – 157)		119 (112 – 127)		359	3.39 (3.27 – 3.52)	
HMGCR	rs12654264*	AA	168	240 (221 – 259)		144 (132 – 157)		112 (112 – 133)		169	3.24 (3.08 – 3.40)[A]	
		AT	227	245 (229 – 262)	0.699	147 (137 – 158)	0.906	119 (110 – 128)	0.820	227	3.48 (3.34 – 3.62)[B]	**0.021**
		TT	60	253 (226 – 279)		147 (130 – 165)		122 (107 – 136)		60	3.40 (3.17 – 3.63)	
	rs3846662*	AA	134	239 (218 – 259)		144 (131 – 157)		117 (106 – 129)		134	3.20 (3.02 – 3.38)[A]	
		AG	235	243 (227 – 258)	0.339	145 (134 – 155)	0.426	121 (112 – 130)	0.717	236	3.46 (3.32 – 3.59)[B]	**0.020**
		GG	86	258 (235 – 281)		154 (140 – 169)		123 (110 – 136)		86	3.43 (3.23 – 3.63)	
	rs3846663*	TT	59	245 (218 – 272)		143 (126 – 160)		122 (107 – 137)		59	3.39 (3.16 – 3.63)	
		TC	229	244 (228 – 260)	0.811	146 (136 – 157)	0.905	119 (110 – 128)	0.809	229	3.48 (3.34 – 3.62)[A]	**0.018**
		CC	166	238 (220 – 257)		144 (132 – 156)		122 (112 – 133)		167	3.23 (3.07 – 3.94)[B]	

Table S2.9. *Cont.*

Gene	SNP	Genotype	N	Campesterol (10^2*µmol/mmol TC)		Sitosterol (10^2*µmol/mmol TC)		Lathosterol (10^2*µmol/mmol TC)		N	LDL-C (mmol/L)	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value		Mean (95% CI)	P-value
LBR	rs4653635	AA	11	244 (185 – 304)		148 (110 – 187)		100 (68 – 133)		11	3.05 (2.53 – 3.57)	
		AG	127	235 (214 – 255)	0.426	139 (126 – 152)	0.351	121 (109 – 132)	0.496	127	3.46 (3.28 – 3.63)	0.275
		GG	317	248 (233 – 263)		149 (139 – 158)		121 (113 – 129)		318	3.37 (3.24 – 3.50)	
	rs12410357	AA	4	284 (186 – 382)		138 (75 – 202)		125 (71 – 179)		4	4.10 (3.24 – 4.95)	
		AG	93	244 (221 – 267)	0.728	139 (124 – 154)	0.450	125 (112 – 138)	0.690	93	3.34 (3.13 – 3.54)	0.219
		GG	358	245 (230 – 259)		148 (139 – 157)		119 (112 – 127)		359	3.39 (3.27 – 3.52)	
MSMO1	rs17585739	AA	2	231 (91 – 371)		140 (50 – 231)		91 (14 – 168)		2	3.23 (2.01 – 4.46)	
		AT	47	238 (231 – 260)	0.865	140 (120 – 159)	0.759	125 (109 – 142)	0.638	47	3.40 (3.14 – 3.67)	0.965
		TT	406	246 (231 – 250)		147 (138 – 156)		120 (112 – 128)		407	3.39 (3.26 – 3.51)	

Abbreviations: LDL-C, low-density lipoprotein cholesterol; N/A, not applicable; SNP, single-nucleotide polymorphism; TC, total cholesterol.

Note: All analyses were adjusted for the factor study. Data are presented as estimated marginal means (95% CI). Non-cholesterol sterol levels were missing for N = 1. Different letters within a SNP indicate significantly different non-cholesterol sterol levels or LDL-C concentrations between the genotypes based on a Bonferroni post-hoc test. * Indicates a SNP captured by a tag SNP.

Table S2.10. Associations between various SNPs in genes involved in endogenous cholesterol synthesis with serum total cholesterol concentrations (N = 456)

Gene	SNP	Genotype	N	Total cholesterol (mmol/L)	
				Mean (95% CI)	P-value
CYP51A1	rs35968894	AA	161	5.40 (5.22 – 5.57)	
		AG	224	5.39 (5.23 – 5.56)	0.993
		GG	71	5.41 (5.17 – 5.65)	
DHCR7	rs1792275	TT	407	5.37 (5.24 – 5.51)	
		TC	48	5.63 (5.35 – 5.92)	0.075
		CC	0	N/A	
	rs72954301	TT	3	5.50 (4.40 – 6.59)	
		TG	91	5.28 (5.06 – 5.51)	0.453
		GG	362	5.43 (5.29 – 5.56)	
DHCR24	rs77668549	AA	339	5.41 (5.27 – 5.55)	
		AG	110	5.36 (5.15 – 5.57)	0.738
		GG	6	5.64 (4.87 – 6.41)	
	rs7553385	AA	402	5.40 (5.26 – 5.53)	
		AG	51	5.38 (5.09 – 5.66)	0.347
		GG	3	6.20 (5.11 – 7.29)	
	rs7551288	AA	80	5.44 (5.21 – 5.67)	
		AG	207	5.36 (5.19 – 5.52)	0.700
		GG	168	5.43 (5.25 – 5.61)	
	rs11206456	TT	2	5.81 (4.47 – 7.15)	
		TC	72	5.34 (5.10 – 5.59)	0.725
		CC	382	5.41 (5.27 – 5.54)	
	rs111480286	--	3	6.20 (5.11 – 7.29)	
		-ACAG	54	5.31 (5.03 – 5.58)	0.276
		ACAGACAG	399	5.40 (5.27 – 5.54)	
	rs6676774	AA	75	5.45 (5.21 – 5.68)	
		AG	208	5.34 (5.18 – 5.50)	0.427
		GG	173	5.46 (5.28 – 5.63)	
	rs718265	AA	43	5.30 (5.00 – 5.61)	
		AG	190	5.36 (5.20 – 5.53)	0.437
		GG	223	5.45 (5.29 – 5.62)	
HMGCR	rs12654264	AA	169	5.26 (5.08 – 5.44)^{A}	
		AT	227	5.51 (5.35 – 5.66)^{B}	**0.037**
		TT	60	5.34 (5.08 – 5.60)	
	rs3846662	AA	134	5.24 (5.04 – 5.44)	
		AG	236	5.47 (5.31 – 5.62)	0.087
		GG	86	5.41 (5.18 – 5.63)	
	rs3846663	TT	59	5.34 (5.08 – 5.60)^{A}	
		TC	229	5.51 (5.35 – 5.66)^{B}	**0.034**
		CC	167	5.26 (5.08 – 5.44)	
	rs12916	TT	152	5.24 (5.06 – 5.43)^{A}	
		TC	231	5.51 (5.36 – 5.67)^{B}	**0.022**
		CC	73	5.32 (5.08 – 5.55)	
HSD17B7	rs77482353	AA	156	5.39 (5.21 – 5.57)	
		AG	228	5.34 (5.18 – 5.50)^{A}	0.103
		GG	68	5.62 (5.37 – 5.87)^{B}	
LBR	rs6678087	TT	141	5.41 (5.22 – 5.60)	
		TC	223	5.36 (5.20 – 5.52)	0.530
		CC	91	5.49 (5.27 – 5.71)	
	rs12141732	TT	227	5.52 (5.36 – 5.68)^{A}	
		TC	194	5.28 (5.12 – 5.44)^{B}	**0.032**
		CC	34	5.54 (5.20 – 5.88)	
	rs4653635	AA	11	5.11 (4.53 – 5.69)	
		AG	127	5.41 (5.21 – 5.60)	0.600
		GG	318	5.40 (5.26 – 5.55)	
	rs12410357	AA	4	6.34 (5.39 – 7.29)	
		AG	93	5.33 (5.10 – 5.55)	0.111
		GG	359	5.41 (5.27 – 5.54)	
MSMO1	rs17585739	AA	2	5.54 (4.18 – 6.90)	
		AT	47	5.43 (5.13 – 5.72)	0.956
		TT	407	5.39 (5.26 – 5.53)	
	rs17046216	AA	53	5.68 (5.41 – 5.96)	
		AG	206	5.39 (5.23 – 5.55)	0.060
		GG	197	5.33 (5.17 – 5.50)	

Abbreviations: N/A, not applicable; SNP, single-nucleotide polymorphism.

Note: All analyses were adjusted for the factor study. Data are presented as estimated marginal means (95% CI). Different letters within a SNP indicate significantly different TC concentrations between the genotypes based on a Bonferroni post-hoc test. Statistical significance was set as a p-value < 0.05.

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CHAPTER 3

Diurnal variation of markers for cholesterol synthesis, cholesterol absorption, and bile acid synthesis: a systematic review and the Bispebjerg study of diurnal variations

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ABSTRACT

Human studies have shown diurnal rhythms of cholesterol and bile acid synthesis, but a better understanding of the role of the circadian system in cholesterol homeostasis is needed for the development of targeted interventions to improve metabolic health. Therefore, we performed a systematic literature search on the diurnal rhythms of cholesterol synthesis and absorption markers and of bile acid synthesis markers. We also examined the diurnal rhythms of the cholesterol synthesis markers lathosterol and desmosterol, and of the cholesterol absorption markers cholestanol, campesterol, and sitosterol in serum samples from the Bispebjerg study. These samples were collected every three hours over a 24-h period in healthy males (n=24) who consumed low-fat meals. The systematic search identified sixteen papers that had examined the diurnal rhythms of the cholesterol synthesis markers lathosterol (n=3), mevalonate (n=9), squalene (n=2), or the bile acid synthesis marker 7α-hydroxy-4-cholesten-3-one (C4) (n=4). Results showed that lathosterol, mevalonate, and squalene had a diurnal rhythm with nocturnal peaks, while C4 had a diurnal rhythm with daytime peaks. Furthermore, cosinor analyses of the serum samples showed a significant diurnal rhythm for lathosterol (*cosinor p*<0.001), but not for desmosterol, campesterol, sitosterol, and cholestanol (*cosinor p* > 0.05). In conclusion, cholesterol synthesis and bile acid synthesis have a diurnal rhythm, though no evidence for a diurnal rhythm of cholesterol absorption was found under highly standardised conditions. More work is needed to further explore the influence of external factors on the diurnal rhythms regulating cholesterol homeostasis.

INTRODUCTION

The circadian system coordinates many physiological processes in the human body around a 24-hour diurnal rhythm. This system consists of a central clock located in the suprachiasmatic nucleus of the hypothalamus and multiple clocks in peripheral tissues, including the liver and intestines. To regulate these rhythms, peripheral clocks combine their own rhythms with signals from the central clock and with external factors, such as sleep and meal timing (1, 2). Evidence increasingly suggests that misalignment between the endogenous central clock and peripheral clocks negatively influences metabolic health. Circadian misalignment for example occurs when the sleep-wake cycle and the light-dark cycle are not synchronized (i.e. sleeping during the day), or when feeding rhythms and the light-dark cycle are not synchronized (i.e. eating during the night) (2). Reviews have indeed suggested that shift workers, who regularly experience circadian misalignment, have an increased risk of developing the metabolic syndrome and cardiovascular disease (3, 4). Thereby, studies in healthy non-shift workers have shown that circadian misalignment increased blood pressure, sleeping metabolic rate, glucose, insulin, fasting plasma free fatty acids, and inflammatory marker levels, while it decreased insulin sensitivity and leptin levels (5-8).

Cholesterol homeostasis, which is regulated by the interplay between endogenous cholesterol synthesis, intestinal dietary and biliary cholesterol absorption, and bile acid synthesis and excretion, is another important determinant of metabolic health (9). Various plasma markers reflect endogenous cholesterol synthesis (lathosterol, desmosterol, mevalonate, squalene), intestinal cholesterol absorption (sitosterol, campesterol, cholestanol), or bile acid synthesis (7α-hydroxy-4-cholesten-3-one (C4)) in both healthy and diseased people (10-14). Their use as markers has been validated by analysing relations between their plasma levels and absolute measurements of endogenous cholesterol synthesis, intestinal cholesterol absorption, or bile acid synthesis (10-14). It is known that cholesterol synthesis and absorption are reciprocally related. To illustrate, increased dietary cholesterol intake has been shown to increase cholesterol absorption, which was compensated for by a decreased endogenous cholesterol synthesis (15). Treatment with ezetimibe and consumption of plant sterols and stanols has been shown to decrease cholesterol absorption, which was compensated for by an increased cholesterol synthesis (16, 17). However, large inter-individual variation in this reciprocal relation exists and people can be classified as cholesterol synthesizers (elevated cholesterol synthesis), cholesterol absorbers (elevated cholesterol absorption), or intermediate (15, 16, 18).

Although previous studies have demonstrated a diurnal rhythm of cholesterol synthesis and bile acid synthesis (19, 20), a better understanding of the influence of the circadian system on cholesterol homeostasis is needed for targeted interventions. Knowledge of the diurnal regulation of cholesterol and bile acid metabolism may help determining when people should use specific dietary components and drugs to optimize treatment and maintain in circadian alignment. Therefore, this paper had two primary aims: 1. To give a systematic overview of the diurnal rhythms of markers reflecting endogenous cholesterol synthesis, intestinal cholesterol absorption and bile acid synthesis, and 2. To examine the diurnal rhythms of cholesterol synthesis markers (lathosterol and desmosterol) and cholesterol absorption markers (cholestanol, campesterol, and sitosterol) in serum samples from the Bispebjerg study (21). This study was specifically designed to examine in

healthy males diurnal rhythms of several metabolic and physiological parameters, such as LDL-cholesterol, triglycerides, and glucose.

METHODS - Systematic Review

Search methods

The systematic review was carried out using the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) checklist (22). The search was conducted to identify original research papers examining the diurnal rhythms of markers reflecting endogenous cholesterol synthesis, intestinal cholesterol absorption, or bile acid synthesis in humans. Potentially relevant studies were retrieved by searching the following databases: Medline, Embase, and the Cochrane Library. Databases were searched from inception until December 2018. The Medical Subject Heading (MeSH) terms used were: ((plant sterol OR plant sterols OR phytosterol OR phytosterols OR sitosterol OR campesterol OR brassicasterol OR stigmasterol OR avenasterol OR lathosterol OR desmosterol OR cholestanol OR lanosterol OR squalene OR mevalonic acid OR mevalonate OR cholesterol precursors OR cholic acid OR chenodeoxycholic acid OR deoxycholic acid OR ursodeoxycholic acid OR lithocholic acid OR C4 OR 7alphahydroxy4cholesten3one OR bile acid synthesis) AND (diurnal rhythm OR circadian rhythm) AND (humans OR human)).

Selection procedure

Criteria to be included were: (1) scientific papers (conference papers and posters were excluded); (2) original studies (reviews and studies based on previously published data were excluded); (3) human studies; (4) diurnal rhythms measured for ≥ 24 hours; (5) levels of validated markers of cholesterol synthesis, cholesterol absorption or bile acid synthesis measured; (6) markers measured in plasma or serum (e.g. markers measured in urine were excluded); (7) at least five samples collected over a 24-hour period; and (8) studies written in English. No specific publication period was considered.

The selection procedure consisted of two rounds. In the first round, two researchers (M.M.S. and R.P.M.) screened all the titles and abstracts of the papers obtained from the databases. Potentially relevant articles were selected. In the second round, the full-texts of all selected papers were read and papers that met the inclusion criteria were included. Papers that were identified through the reference lists of the selected articles were included as well. Any discrepancies between the researchers were resolved by discussion.

Data extraction

For each included study, the following data were extracted into an Excel spreadsheet: study information (reference number, first author, publication year), study characteristics (study design, subgroups, study setting, study duration, time between measurements), participant characteristics (*n*, age, sex, BMI, health status, medication use), marker collection (type of marker, analytical method, measurement in serum or plasma), dietary information (type of meals, time of consumption), sleep information (sleeping hours and conditions), and the main findings (mean marker concentrations at the measured time points, times of peak and nadir). In case a study presented the diurnal rhythms for each subject separately, the mean marker concentration at each

time point was calculated. If mean concentrations were not provided in a table, these were estimated from graphs using a pixel ruler. Both absolute and cholesterol-standardised data were collected. In the end, only cholesterol-standardised markers were used, because these have been validated to measure cholesterol absorption, cholesterol synthesis or bile acid synthesis. Mevalonate, however, is a water-soluble compound, and was not standardised for total cholesterol.

The marker levels measured during the first 24 hours of each study were presented in graphs. First, the 24-hour period was divided into the following time blocks: 06:30 – 09:00 h; 09:30 – 12:00 h; 12:30 – 15:00 h; 15:30 – 18:00 h; 18:30 – 21:00 h; 21:30 – 00:00 h; 00:30 – 03:00 h; 03:30 – 06:00 h; 06:30 – 09:00 h. Second, for each time block the median marker level measured in all studies was calculated, which was then put in the graph.

METHODS - Bispebjerg Study of Diurnal Variations

Subjects and study design

Serum samples from the Bispebjerg study were used for the analysis of cholesterol synthesis and absorption markers. A detailed description of the study design has been published (23). In summary, twenty-four Caucasian males, with a mean age of 26 years old (range 20–40 years) and mean BMI of 22.9 kg/m^2 (range 19.6–24.9 kg/m^2) participated in the study. Subjects spent 24 hours at the hospital ward, and standardised meals (low fat, no sugar) were provided at 09:30 h, 13:00 h, and 19:00 h. As both high-fat and high-sugar intake may influence the circadian system (24), it was decided to use meals that were low in fat and sugar to minimize effects on the primary outcomes of the Bispebjerg study. Subjects slept between 23:00 h and 08:00 h in total darkness. Blood samples were collected by cubital venepuncture every three hours from 09:00 h until 09:00 h the next day. The study was executed in accordance with the Helsinki Declaration and written informed consent was signed by all the subjects. Ethical approval was given by the Regional Scientific Ethical Committee of the Capital Region of Denmark (protocol number H-B-2008-011) and the Danish Data Protection agency (journal number 2008-41-1821).

Serum analyses

Serum concentrations of cholesterol, lathosterol, desmosterol, campesterol, sitosterol, and cholestanol were determined using a gas chromatography flame ionization detector (GC-FID) as described by Mackay et al (25). Lathosterol, desmosterol, campesterol, sitosterol, and cholestanol were standardised for cholesterol concentrations (µmol/ mmol cholesterol), because they are transported in serum by lipoproteins. The lathosterol/campesterol-ratio at 09:00 h on day 1 was also calculated to classify subjects as cholesterol absorbers (n = 8), cholesterol synthesizers (n = 8), or intermediate (n = 8).

Statistical analyses

Pearson correlations were calculated to examine associations between the cholesterol absorption and synthesis markers at 09:00 h on day 1. Cosinor analysis for populations was used to examine whether total cholesterol, lathosterol, desmosterol, campesterol, sitosterol, and cholestanol showed a diurnal rhythm (26, 27). A curve was fitted to the data using the following sine and cosine function:

$$\text{Analyte} = M + k1\cos\left(\frac{2\pi t}{24}\right) + k2\sin\left(\frac{2\pi t}{24}\right)$$

Substituting $\cos\left(\frac{2\pi t}{24}\right) = X$ and $\sin\left(\frac{2\pi t}{24}\right) = Z$

gave the final formula: $\text{Analyte} = M + k1X + k2Z$

Using the GLM procedure in SAS, the model fit was tested and M, k1, and k2 were calculated. Confidence bounds were also calculated and added to the fitted curves. The cosinor model further estimated the mesor, amplitude, and time of peak for total cholesterol and each marker. The mesor represents the rhythm-adjusted mean value of the cosinor curve, and the amplitude represents the difference between the mesor and peak or between the mesor and nadir. It was concluded that a diurnal rhythm was present when the cosinor curve was significant (*cosinor p* < 0.05). In addition, linear mixed models were used to examine whether the marker levels fluctuated significantly over time. Time was defined as fixed factor, subjects as random factor and the best model fit was based on the lowest Akaike Information Criterion. Random intercept models with the identity covariance structures were used. In case of significant time effects ($p < 0.05$), Bonferroni post hoc tests were used to make comparisons between the marker levels at 09:00 h on day 1 and the other time points. The cosinor analyses were conducted in SAS (SAS Institute Inc., Cary, NC, USA) and the other analyses in SPSS version 25 for Mac OS X (SPSS Inc., Chicago, IL, USA).

RESULTS - Systematic Review

Study selection

Fig. 3.1 provides an overview of the study selection. The search yielded 204 potentially relevant articles and 26 of these were assessed for full-text evaluation. Ultimately, 16 studies met the inclusion criteria and their characteristics are summarized in **Table 3.1**.

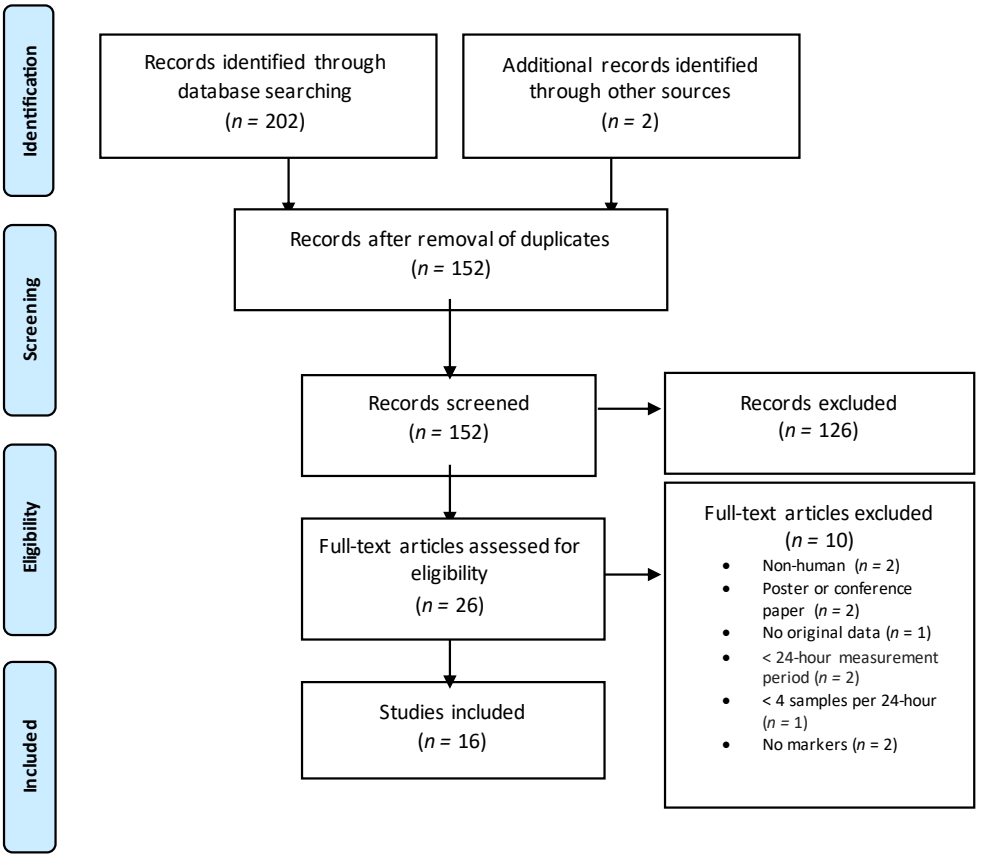

Figure 3.1. PRISMA flowchart of the study selection process

Lathosterol

Three papers, including five study-arms, have examined the diurnal rhythm of serum lathosterol (40, 41, 43). Two studies have examined the diurnal rhythms without treatment (40, 43), two studies following cholestyramine (CME) treatment (41, 43), and one study following CME plus atorvastatin treatment (43). See **Table S3.1**.

In non-treated subjects, cholesterol-standardised lathosterol levels were lowest during the day and highest during the night (**Fig. 3.2**) (40, 43). A comparable pattern was observed when subjects received CME with the meals, although cholesterol synthesis rates were increased (**Fig. 3.2**) (41, 43). In one study, lathosterol levels were almost twice as high on the morning after one-day treatment with CME compared to no treatment (43). In contrast, lathosterol levels were decreased at all time points following CME plus atorvastatin treatment (**Fig. 3.2**) (43). In this study, CME was given with the meals on the first day, and atorvastatin on the morning two days before the study and on the morning of the first and second study day (43). On the morning of the second day, lathosterol levels were approximately 34% lower compared to no treatment and 66% lower compared to CME alone (43).

Table 3.1. Characteristics of studies included in the systematic review

First Author (year)	Study Design	Population	Intervention	n	Age (years)	Male (%)	Marker	Study Duration (hours)	Dietary Information				
									Type of Meals	Breakfast	Lunch	Dinner	Other
Kopito (1982) (28)	Experimental, crossover	Healthy subjects	• One meal/ day • No meal	1	40	100	MVA	72	• *Day 1 and 2:* standardised dinner • *Day 3:* no meal			19:00	
Miettinen (1982) (29)	Longitudinal	Healthy subjects		7	Range: 16–49	N/A	Squalene + methyl sterols	24	Three meals and evening snacks. Fat intake provided approximately 35% of total calories	08:00	12:00	16:00	20:00
Parker (1982) (30)	Experimental, crossover	Subject with hyper-cholesterolemia and ischemic heart disease		1	55	100	MVA	72	Four daily liquid-formula meals in equal portions; caloric intakes adjusted to maintain BW within ± 1.5 kg over 3–5-weeks	08:00	13:00	19:00	10:00
		Subject with hypertri-glyceridemia, obesity, and maturity-onset diabetes	• No treatment • 12-day fast	1	66	100	MVA	48	• *No treatment:* liquid formula diet in five equal portions per day for three weeks • *12-day fast:* no dietary intake	08:00	13:00	19:00	10:00/17:00
		Subject with heterozygous familial hyper-cholesterolemia and ischemic heart disease	• Moderate cholesterol intake • High cholesterol intake	1	45	100	MVA	48	• *Moderate intake:* five liquid formula feedings, 550 mg cholesterol/ day, for three weeks • *High intake:* five liquid formula feedings, 1200 mg cholesterol/day, for three weeks	08:00	13:00	19:00	10:00/17:00

Table 3.1. *Cont.*

First Author (year)	Study Design	Population	Intervention	n	Age (years)	Male (%)	Marker	Study Duration (hours)	Dietary Information				
									Type of Meals	Breakfast	Lunch	Dinner	Other
Parker (1984) (31)	Experimental, crossover	Subject with hypertri-glyceridemia	• No treatment	1	68	100	MVA	72	• *No treatment:* eating ad libitum 3 times/day as outpatient	08:00	13:00	19:00	10:00/17:00
			• Moderate cholesterol intake						• Moderate intake: five liquid formula feedings, 207 mg cholesterol/day, for four weeks				
			• High cholesterol intake						• High intake: five liquid formula feedings, 972 mg cholesterol/ day, for four weeks				
Miettinen (1985) (32)	Longitudinal	Subjects with jejunoileal bypass		4	26 ± 8	N/A	Squalene + methyl sterols	24	Low-cholesterol diet, 125 mg cholesterol/ 2400 kcal, 100 g fat/day	08:00	12:00	16:00	20:00
		Subjects with ileal exclusion		4	38 ± 12	N/A							
Scoppola (1991) (33)	Longitudinal	Healthy subjects		1	N/A	N/A	MVA	24	Low fat (<5%), cholesterol-free meals	09:30	12:30	19:00	
Jones (1992) (34)	Longitudinal	Healthy subject		5	26 ± 4	100	MVA	48	Three self-selected, habitual meals/day for three days prior to and 48 hours during the study	08:00-09:00	12:00 – 13:00	18:00-19:00	
Pappu (1994) (35)	Longitudinal	Healthy subjects		6	30 ± 2	50	MVA	24	Three meals/day (40% fat, 25% CHO, 15% protein)	08:00	12:00	18:00	
		Patients with abetalipo-proteinemia		3	24 ± 10	66.6			Three meals/day (12-15% fat, 70-75% CHO, 13-17% protein)	08:00	12:00	18:00	

Table 3.1. *Cont.*

First Author (year)	Study Design	Population	Intervention	n	Age (years)	Male (%)	Marker	Study Duration (hours)	Dietary Information				
									Type of Meals	Breakfast	Lunch	Dinner	Other
Yoshida (1994) (36)	Longitudinal	Patients with cholelithiasis and patients with early cancer of the GI-tract		3	Range: 24 –28	N/A	C4	24	Normal hospital diets	08:00	12:30	17:30	
Nozaki (1996) (37)	Experimental, crossover	Subjects with heterozygous familial hyper-cholesterolemia	• No treatment • Morning pravastatin • Evening pravastatin	8	58 ± 9	37.5	MVA	24	Cholesterol intake ± 300 mg/day; 20% fat intake; ratio polyunsaturated to saturated FAs was 1.5; single dose pravastatin (20 mg) taken after breakfast or after dinner	08:00	12:00	18:00	
Pappu (2002) (38)	Experimental, crossover	Subjects with heterozygous familial hyper-cholesterolemia	• No treatment • Lovastatin • Simvastatin	9 5	41 ± 4 N/A	0	MVA	24	Low-cholesterol, low-fat diet conforming to phase I of the American Heart Association Diet; statins (40 mg) given after breakfast and dinner for eight weeks	08:00	12:00	18:00	
Martin (2002) (39)	Experimental, crossover	Healthy subjects	• Morning rovustatin • Evening rovustatin	21	N/A	N/A	MVA	24	Individual caloric and fat intake was stabilized; Rosuvastatin (10 mg) taken each morning (~07:00 h) or evening (~18:00 h) for 14 days	N/A	N/A	N/A	
Gälman (2005) (40)	Longitudinal	Healthy subjects (n = 5) and cholecystecto-mized subjects (n = 3)		8	Range: 25–58	50	C4, lathosterol	24	Standardised meals	09:00	12:00	18:00	

Table 3.1. *Cont.*

First Author (year)	Study Design	Population	Intervention	n	Age (years)	Male (%)	Marker	Study Duration (hours)	Dietary Information				
									Type of Meals	Breakfast	Lunch	Dinner	Other
Persson (2010) (41)	Experimental	Healthy subjects	• CME treatment	10	N/A	75	Lathosterol	33	Standardised meals; CME was taken with meals day 1 (4 × 4 g)	08:30	12:30	16:00	21:30
Steiner (2011) (42)	Longitudinal	Healthy subjects		4	Range: 27–29	50	C4	24	Subjects consumed identical meals	09:15 (day 1) 08:45 (day 2)	11:30	20:15	
Al-Khaifi (2018) (43)	Experimental, crossover	Healthy subjects	• No treatment • CME • CME + atorvastatin	8	Range: 20–45	100	C4, lathosterol	32	Standardised meals; CME (4 × 4 g) taken before meals day 1; atorvastatin (four daily doses of 40 mg) dose 1 and 2 taken on the morning two days before CME treatment, dose 3 and 4 on the morning of the first and second study day	08:30	13:00	18:00	20:30

Abbreviations: BW, body weight; C4, 7α-hydroxy-4-cholesten-3-one; CHO, carbohydrates; CME, cholestyramine; FA, fatty acids; GI, gastrointestinal; MVA, mevalonate; N/A, data not available.

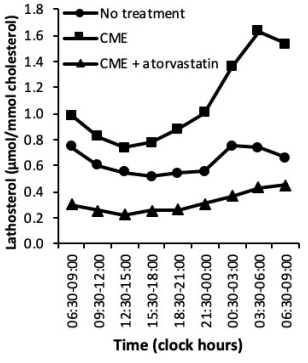

Figure 3.2. The diurnal rhythm of lathosterol levels in untreated subjects, in subjects treated with cholestyramine (CME) alone, and in subjects treated with CME plus atorvastatin (40, 41, 43). Data are presented as medians.

Mevalonate (MVA)

Nine papers, including 22 study-arms, have reported on the diurnal rhythm of plasma or serum MVA concentrations (28, 30, 31, 33-35, 37-39). All studies examined subjects who had not received any treatment (28, 30, 31, 33-35, 37-39), three studies examined the effects of different diets (i.e. high cholesterol intake and fasting) (28, 30, 31), and three studies examined the effects of statin treatment (37-39). See **Table S3.2**.

MVA concentrations were lowest during the day and highest during the night and early morning (**Fig. 3.3a**). However, two studies found lower MVA concentrations on the morning of the first day compared with the second day (30, 35) and another study did not report morning concentrations on the second day (38), which explains the difference in MVA concentrations between the first and second day (**Fig. 3.3a**).

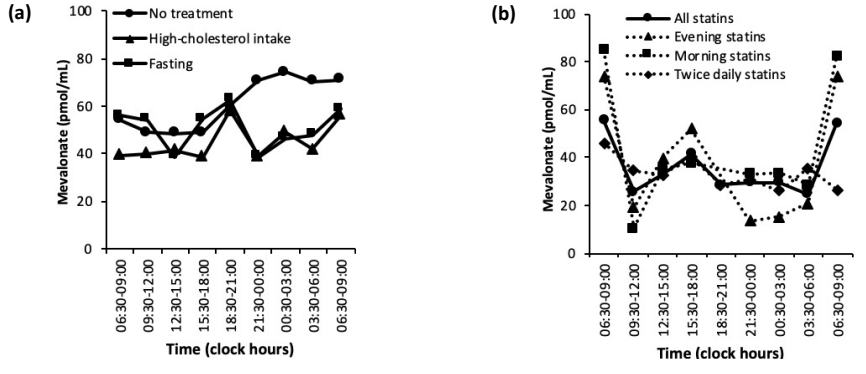

Figure 3.3. The diurnal rhythm of MVA concentrations: (a) in untreated subjects, in subjects who consumed high-cholesterol meals, and in subjects who fasted (28, 30, 31, 33-35, 37-39), and (b) in subjects who were treated with statins (37-39). Data are presented as medians.

Consumption of high-cholesterol meals decreased MVA concentrations and the nocturnal peak (**Fig. 3.3a**) (30, 31). The high-cholesterol graph shows lower MVA morning concentrations on the first than on the second day (**Fig. 3.3a**), which was caused by differences in the number of observations at each time period due to different sampling times between studies. The high-cholesterol graph

also shows increased MVA concentrations in the evening (**Fig. 3.3a**), which was due to a study that found slightly increased MVA concentrations in the evening when high-cholesterol meals (972 mg cholesterol/ day) had been consumed for four weeks (31). In another study, the 24-hour mean MVA concentrations were significantly decreased by 33% when high-cholesterol (1200 mg cholesterol/day) meals had been consumed for three weeks compared to lower-cholesterol meals (550 mg cholesterol/day) (30).

Fasting also decreased MVA concentrations and the nocturnal peak (**Fig. 3.3a**) (28, 30). In one study, the 24-hour mean MVA concentrations were significantly decreased by 55% following a 12-day fast compared to no treatment (30). One study (28) did not collect samples between 18:30 and 21:00 h and reported lower MVA concentrations than the other study that examined fasting (30), which explains the peak in the fasting graph (**Fig. 3.3a**). Moreover, fasting MVA concentrations were slightly decreased in the afternoon and at midnight in one study (30), as shown by the nadirs (**Fig. 3.3a**).

Statin treatment decreased MVA concentrations and the nocturnal MVA peak irrespective of the time of administration (**Fig. 3.3b**) (37-39). The reason for the high MVA concentrations between 06:30 and 09:00 h in the morning and evening statin group is that only one paper, that found relatively high MVA concentrations compared with other studies (See **Table S3.2**), sampled at these time points (37). In one study, the 24-hour mean MVA concentrations were significantly decreased by 33% and 44% following eight-week twice daily lovastatin and simvastatin treatment, respectively, compared to no treatment (38). Lovastatin decreased the nocturnal MVA peak, which was not present at all after simvastatin treatment, and the ratio between the mean MVA night-time (20:00 h – 07:00 h) to daytime (08:00 h – 19:00 h) concentration was significantly more reduced by simvastatin (58.3%) than by lovastatin (43.8%) (38). In another study, the 24-hour area under the curve for MVA decreased by approximately 30% and 33% following 14-day morning and evening rosuvastatin treatment, respectively, which was not significantly different (39). Moreover, the MVA peak concentration observed pre-treatment was reduced by approximately 26% following morning rosuvastatin treatment and by 31% following evening rosuvastatin treatment. These values were not tested for statistical difference (39). Another study showed that a single pravastatin dose in the morning non-significantly decreased MVA concentrations at 17:00 h, while a single pravastatin dose in the evening borderline significantly decreased MVA concentrations at 23:00 h (37).

Squalene

Two papers were identified that described the diurnal rhythm of squalene. In the first study healthy subjects were investigated, and the other studied subjects with jejunoileal bypass and subjects with ileal exclusion (29, 32). In healthy subjects, squalene levels were lowest during the day and highest during the night (**Fig. 3.4a**) (29), as shown by the lowest squalene levels in the VLDL and HDL+LDL fractions during the day and highest during the night. This pattern was not observed in subjects with jejunoileal bypass nor in subjects with ileal exclusion (**Fig. 3.4b**) (32). See **Table S3.3** and **S3.4**.

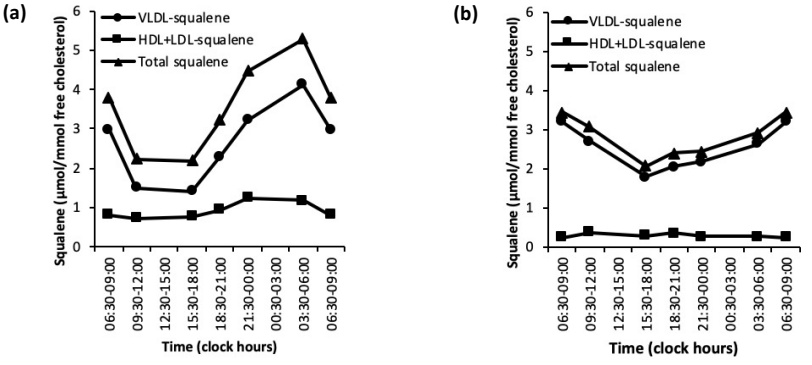

Figure 3.4. The diurnal rhythm of squalene levels: (a) in healthy subjects (29), and (b) in subjects with jejunoileal bypass and ileal exclusion (32). Data are presented as medians.

C4

Four papers, including seven study-arms, in which the diurnal rhythm of C4 was evaluated met the inclusion criteria (36, 40, 42, 43). All papers examined the diurnal rhythm of C4 without any interventions (36, 40, 42, 43). One study examined cholecystectomized subjects (40), and two other studies examined healthy subjects treated with CME alone and with CME plus atorvastatin (43). See **Table S3.5**.

In non-treated subjects, C4 levels were highest during the day and evening, and lowest during the night (**Fig. 3.5**) (40, 43). Peaks were reported at 13:00 h, 21:00 h, 22:00 h, and between 05:30 and 07:00 h (40, 43). One study measured relatively high baseline C4 levels compared to the other time points (43), which explains the difference in morning C4 levels between the first and second day (**Fig. 3.5**). Two papers only reported absolute C4 concentrations (36, 42). In one paper, C4 concentrations showed a peak around 05:30 h and nadir in the evening (36), while the other paper reported peaks at noon and during the evening and night, and nadirs in the morning and afternoon (42). In cholecystectomized subjects, cholesterol-standardised C4 levels peaked during the day and evening (around 12:00 h, 22:30 h, and 05:30 h) (40).

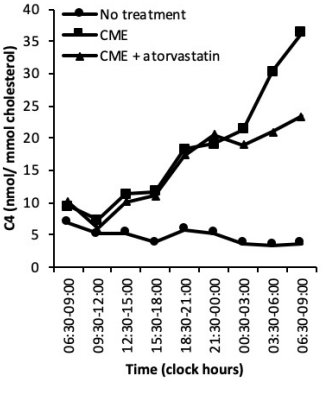

Figure 3.5. The diurnal rhythm of C4 levels in untreated subjects, in subjects treated with cholestyramine (CME) alone, and in subjects treated with CME plus atorvastatin (40, 43). Data are presented as medians.

CME treatment, given with meals on the first day, ended the rhythm that had been observed without any interventions and increased C4 levels (**Fig. 3.5**) (43). This increase had already been observed after the first CME dose in the morning, and levels continued to increase until noon on the second day (43). One-day CME treatment combined with atorvastatin, which was given on the morning two days before the study and on the morning of the first and second day, also ended the rhythm and increased C4 levels, which was less pronounced compared to CME treatment alone (43). The increase had already been observed after the first CME plus atorvastatin dose in the morning of the first study day and continued to increase until the afternoon on the second study day (**Fig. 3.5**) (43). On the morning of the second day, C4 levels were approximately seven times higher following CME treatment and four times higher following CME plus atorvastatin treatment, compared to no treatment (43).

RESULTS - Bispebjerg Study of Diurnal Variations

Table 3.2 shows the means ± SD and correlations between the cholesterol-standardised markers at 09:00 h on day 1. Significant negative correlations were found between lathosterol and campesterol, lathosterol and sitosterol, and desmosterol and cholestanol. Significant positive correlations were found between campesterol and sitosterol, campesterol and cholestanol, and sitosterol and cholestanol.

Table 3.2. Mean ± standard deviation (SD) and correlations between cholesterol-standardised markers reflecting endogenous cholesterol synthesis and intestinal cholesterol absorption at 09:00 h on day 1 in healthy males (*n* = 24)

Marker	Mean ± SD (µmol/mmol cholesterol)	Lathosterol	Desmosterol	Campesterol	Sitosterol
Lathosterol	1.5 ± 0.3				
Desmosterol	1.0 ± 0.2	0.190			
Campesterol	2.4 ± 0.8	-0.521*	-0.148		
Sitosterol	1.9 ± 0.4	-0.438*	-0.309	0.868**	
Cholestanol	1.6 ± 0.3	-0.368	-0.427*	0.755**	0.853**

*p<0.05; **p<0.001.

Table 3.3. Results of the cosinor analysis for total cholesterol, and markers reflecting endogenous cholesterol synthesis and intestinal cholesterol absorption in healthy males (*n* = 24)

Marker	Mesor (SE)	Amplitude (SE)	Peak time (clock hours)	Cosinor *p*
Cholesterol	4.18 (0.06)	0.19 (0.04)	14:08	0.070
Lathosterol	1.52 (0.03)	0.22 (0.02)	02:47	<0.001
Desmosterol	1.02 (0.01)	0.02 (0.01)	19:57	0.640
Campesterol	2.47 (0.06)	0.04 (0.04)	23:39	0.890
Sitosterol	1.97 (0.03)	0.05 (0.02)	01:57	0.460
Cholestanol	1.57 (0.02)	0.01 (0.01)	17:00	0.950

Abbreviations: SE, standard error. Data presented for cholesterol-standardised marker levels (µmol/mmol cholesterol).

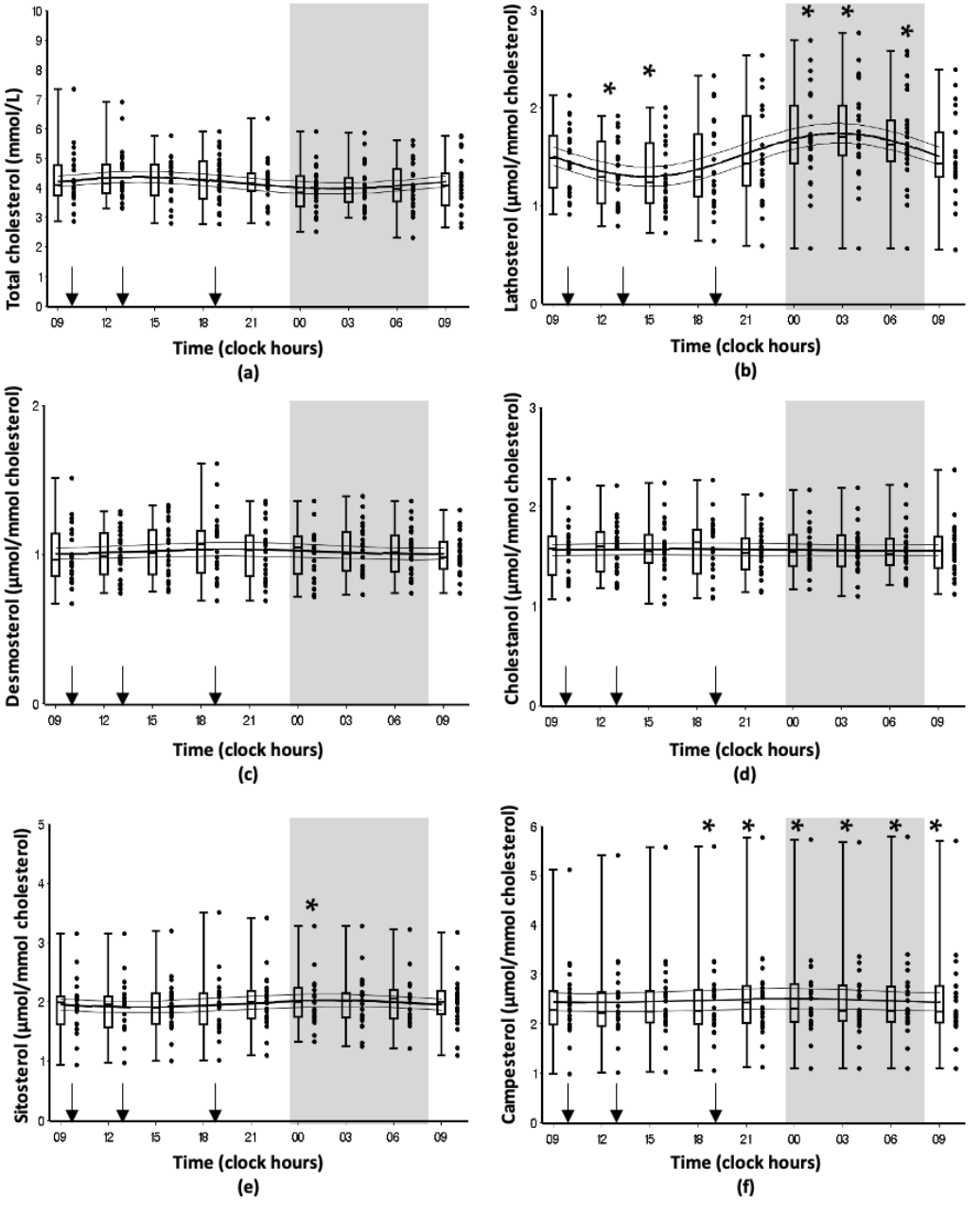

Figure 3.6. The diurnal rhythms of: (a) total cholesterol (*cosinor p*=0.070), (b) lathosterol (*cosinor p*<0.001), (c) desmosterol (*cosinor p*=0.640), (d) cholestanol (*cosinor p*=0.950), (e) sitosterol (*cosinor p*=0.460), and (f) campesterol (*cosinor p*=0.890) in healthy males (*n* = 24). Data are presented as cosinor curves with confidence bounds. The dots next to the boxplots indicate individual data points. The grey area indicates the sleeping period and the arrows indicate meal timing. * Indicates significant difference ($p < 0.05$) compared with 09:00 h on day 1 using Bonferroni post hoc tests following significant time-effects using a linear mixed model.

The regular day-night rhythms of the subjects were previously confirmed by analysing the 24-hour serum melatonin concentrations, which peaked at 03:34 h and were lowest in the afternoon (*cosinor p*<0.001) (23). Total cholesterol showed a borderline significant diurnal rhythm (*cosinor p*=0.070) and significant time-effect (p=0.017), with the highest values during the day and the lowest values during the night (amplitude (amp) = 0.19 mmol/L) (**Fig. 3.6a, Table 3.3**). Of the cholesterol synthesis markers, lathosterol has a significant diurnal rhythm (*cosinor p*<0.001) and a significant time-effect (*p*<0.001), with a nocturnal peak and nadir in the afternoon (amp = 0.22 µmol/mmol cholesterol) (**Fig. 3.6b, Table 3.3**). Desmosterol levels remained relatively stable over time and showed no significant diurnal rhythm (*cosinor p*=0.640) or significant time-effect (p=0.731) (**Fig. 3.6c, Table 3.3**). Of the cholesterol absorption markers, cosinor analysis revealed no significant diurnal rhythms for cholestanol, sitosterol, and campesterol (*cosinor p*>0.05) (**Fig. 3.6d, e, f, Table 3.3**). Moreover, no significant time-effect was found for cholestanol (p=0.896), but significant time-effects were found for sitosterol (*p*<0.001) and campesterol (*p*<0.001), which both showed small nocturnal peaks.

The lathosterol/campesterol-ratio at 09:00 h on day 1 was used to classify subjects as cholesterol absorbers (ratio ≤ 0.54), intermediate (ratio > 0.54 – < 0.73), or cholesterol synthesizers (ratio ≥ 0.73). Overall, the 24-hour patterns of lathosterol, desmosterol, cholestanol, sitosterol, and campesterol did not differ between the groups, whereas the mean levels measured at each time point did differ between the groups (data not shown).

DISCUSSION

Based on a systematic literature search and experimental data, we conclude that plasma markers for endogenous cholesterol synthesis and bile acid synthesis have a diurnal rhythm. No evidence was found for a diurnal rhythm in plasma markers reflecting intestinal cholesterol absorption. The 24-hour patterns of the cholesterol synthesis and cholesterol absorption markers were not different for cholesterol synthesizers, cholesterol absorbers, and intermediate subjects.

Endogenous cholesterol synthesis

Previous studies have clearly established the diurnal rhythms of plasma MVA, lathosterol, and squalene, which show that cholesterol synthesis is lowest during the day and highest during the night. In the Bispebjerg study, we observed a comparable diurnal rhythm of lathosterol. These findings are in line with studies that used the deuterium incorporation method to examine the diurnal rhythm of the cholesterol fractional synthesis rate (FSR) in healthy people (19, 44, 45). These studies reported that cholesterol FSR was lowest during the daytime and highest during the night-time. This agreement in findings also indicates that using non-cholesterol sterol markers to measure cholesterol synthesis is a valid method. Besides MVA plasma concentrations, urinary MVA excretion reflects HMG-CoA reductase activity as well (46). Interestingly, comparable diurnal patterns have been reported for MVA plasma concentrations and urinary MVA excretion, thereby confirming that both MVA plasma concentrations and urinary MVA excretion reflect endogenous cholesterol synthesis (28, 37).

The diurnal rhythm of cholesterol synthesis is mainly regulated by 3-hydroxy-3-methylglutaryl-CoA

(HMG-CoA) reductase, which is the rate-limiting enzyme in the cholesterol synthesis pathway (47). The diurnal pattern of this enzyme is influenced by circadian clock genes (e.g. *Clock*) and nutrition (47). Cholesterol synthesis rates were significantly decreased in people who consumed high-cholesterol meals compared to medium- and low-cholesterol meals, indicating a feedback mechanism between increased dietary cholesterol intake and HMG-CoA reductase activity (30, 31, 48). The observed decrease in cholesterol synthesis rates following a period of fasting may be attributed to a decreased expression of the sterol regulatory element-binding protein 2 (SREBP2). This is a transcription factor that activates many enzymes in the cholesterol synthesis pathway, including HMG-CoA reductase (49). Horton et al. has reported a decrease in nuclear forms of SREBP2 in mice liver following a 24-hour fast. Consequently, the mRNAs of several genes involved in the cholesterol synthesis pathway were decreased by 40 – 70% (50). Under normal circumstances, SREBP2 expression is regulated by the SCAP-INSIG complex. However, when intracellular sterol concentrations are low (e.g. during fasting), this SCAP-INSIG interaction gets disrupted (51). Previously, it has been shown that SREBP2 expression during fasting is also regulated by sirtuin 1 (SIRT1) (52). Activation of SIRT1 depends on the presence of NAD^+, which acts as co-factor in this activation. During a period of fasting, NAD^+ concentrations are increased and therefore SIRT1 activity is increased as well (53). In human cells, it was demonstrated that activation of SIRT1 resulted in significantly reduced expression of genes regulated by SREBP2, including HMG-CoA reductase and the LDL-receptor (52). These findings indicate that SIRT1 reduces SREBP2 expression during a period of fasting and can thereby lead to a decrease in cholesterol synthesis (52).

CME treatment increased lathosterol levels, indicating that HMG-CoA reductase activity was increased by the reduced hepatic cholesterol levels following CME treatment (41, 43). Moreover, no major differences between morning versus evening treatment with rosuvastatin and pravastatin in decreasing 24-hour cholesterol synthesis rates were observed, which is likely due to their relatively long half-life in plasma (37, 39, 54). The type of statin had a more profound impact on the diurnal rhythm of cholesterol synthesis, as greater reductions in 24-hour MVA concentrations have been reported following simvastatin compared to lovastatin treatment (38). Overall, these findings emphasize that external factors mainly influence the diurnal rhythms of cholesterol synthesis by altering the mean 24-hour concentrations and maximum peak value, but not by shifting the period of maximal cholesterol synthesis from night-time to daytime.

Surprisingly, we found no significant diurnal rhythm of desmosterol. The weak baseline correlation between desmosterol and lathosterol combined with the finding that lathosterol and HMG-CoA reductase activity are more strongly correlated than desmosterol and HMG-CoA reductase activity (55), could indicate that lathosterol is the preferred marker to assess cholesterol synthesis. Furthermore, although interventions aimed at reducing intestinal cholesterol absorption have been shown to increase cholesterol synthesis rates, a review has suggested that plant stanol or sterol consumption increased cholesterol-standardised lathosterol levels in 13 out of 17 interventions, while those of desmosterol were only increased in 8 out of 17 interventions (56). Thereby, in the studies in which lathosterol and desmosterol both significantly increased following plant stanol or sterol consumption, this increase in percentages was often higher in lathosterol than in desmosterol (56). Perhaps, desmosterol levels remain more stable over time, while lathosterol

levels fluctuate more and respond more strongly to interventions. Moreover, desmosterol is formed in the Bloch pathway, whereas lathosterol is formed in the Kandutsch-Russell pathway. Two important enzymes in the regulation of these pathways are 24-dehydrocholesterol reductase (DHCR-24) and 7-dehydrocholesterol reductase (DHCR-7). Of these, DHCR-24 mediates the shift from the Bloch pathway to the Kandutsch-Russell pathway, as well as the conversion of desmosterol to cholesterol, while DHCR-7 mediates the conversion of 7-dehydrodesmosterol to desmosterol, as well as the conversion of 7-dehydrocholesterol to cholesterol (57). These enzymes are thus involved in both pathways, but the intermediates in both pathways differ. In mice, 7-dehydrocholesterol and 24,25-dihydrolanosterol, both metabolites in the Kandutsch-Russell pathway, had a marker diurnal pattern (58). Thereby, a diurnal pattern of DHCR-24 was reported in rats fed with CME and with lovastatin (59). Although Mietinnen et al. has suggested that the absolute concentrations of certain free sterols involved in the conversion of lanosterol to cholesterol might fluctuate over a 24-hour period (29), more conclusive evidence is needed to confirm whether metabolites in the Kandutsch-Russell and Bloch pathway, as well as DHCR-24 or DHCR-7, are regulated by the circadian system in humans, and could thereby offer an explanation for the different findings for lathosterol and desmosterol.

Intestinal cholesterol absorption

Up to now, the influence of the circadian system on human cholesterol absorption has received little attention. In the Bispebjerg study, we found no significant diurnal rhythm of cholestanol, campesterol, and sitosterol levels. Nevertheless, significant time effects were found for campesterol and sitosterol, indicating that their levels did fluctuate over time, but not in a rhythmic 24-hour pattern. Animal research has shown that intestinal cells express circadian clock genes, which may control circadian expression of different proteins involved in cholesterol absorption (60). However, it is currently unknown whether the Niemann-Pick C1-Like 1 (NPC1L1) protein, essential for intestinal cholesterol absorption (61), or the ATP-binding cassette transporters ABCG5/G8, essential for intestinal cholesterol export (62), are under circadian regulation in humans.

A possible explanation for not finding a diurnal rhythm in cholesterol absorption may be that subjects of the Bispebjerg study consumed low-fat meals. Dietary intake of fat and cholesterol both influence bile acid secretion and cholesterol absorption. For example, high-fat diets increase bile acid excretion, whereas low-fat diets reduce cholesterol absorption in humans (63, 64). Thus, it is possible that other 24-hour patterns for cholesterol absorption would have been observed in case the subjects of the Bispebjerg study would have consumed high-fat or high-cholesterol meals.

Bile acid synthesis

Based on earlier studies, it is evident that C4 has a diurnal rhythm, with the lowest levels during the night and the highest levels during the day. These findings are partly in agreement with studies that have measured bile acid synthesis as function of CO_2 output from [26-^{14}C]cholesterol, which reported that bile acid synthesis had one peak in the morning and was lowest during the evening and night (20, 65). Possible reasons why only one peak has been reported in these studies could be the small sample sizes (n = 3) and relatively complicated method to measure bile acid synthesis. Another study in healthy males reported a mean C4 peak at noon, although C4 also peaked in the

morning in some of the subjects (66). However, this study only collected samples for a 15-hour period (07:00 – 22:00 h). Thus, the nocturnal C4 levels are unknown in these subjects (66).

Previous findings have suggested that the gallbladder has a minimal role in the diurnal regulation of bile acid synthesis, because the rhythm was comparable in cholecystectomized and healthy subjects, and C4 concentrations measured in gallbladder bile were too low to explain the peaks in C4 (40). Cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme in the bile acid synthesis pathway, and can be suppressed by the fibroblast growth factor 19 (FGF19) via the farnesoid X receptor (FXR) (67). Thus, FGF19 and FXR may be underlying mechanisms regulating the diurnal rhythm of C4. Studies found that postprandial increases in total and unconjugated bile acids were followed by peaks in FGF19, which indicates that FGF19 is secreted in response to the postprandial increases in bile acids (43, 68). Furthermore, comparable rhythms have been reported for FGF19 and C4, although peaks in FGF19 preceded the decline of C4 (68). A recent study has suggested that the rhythm of C4 can also be regulated via FXR, independent of FGF19 (69). In this study, subjects received a single dose of a nonsteroidal FXR agonist (Px-102) and the influence of Px-102 on the diurnal rhythms of serum C4, FXR, and FGF19 was investigated. The lowest dose caused C4 levels to decrease by 80% compared with baseline, while FGF19 concentrations only increased when higher Px-102 doses were given. Hence, the decrease in C4 following the lowest dose was not preceded by increased FGF19 levels, which indicates that FXR influenced the diurnal rhythm of C4 independent of FGF19 (69). In summary, these findings suggest that FXR and FGF19 are both involved in the regulation of the diurnal rhythm of C4, but the exact mechanism remains unclear.

Total cholesterol

We did not find a significant diurnal rhythm for total cholesterol. Previous studies on the diurnal rhythm of total cholesterol have reported conflicting findings. Some studies did not show a significant diurnal rhythm for total cholesterol concentrations (70, 71), whereas other studies did (72-74). However, these latter studies showed little agreement on the timing of peak concentrations, as acrophases were reported in the morning (72), afternoon (73), and at the beginning of the evening (74). LDL-cholesterol may have a diurnal rhythm with peak concentrations during the day (21, 74). For HDL-cholesterol, several studies have reported that it did not have a significant diurnal rhythm (21, 70, 71), while other studies did report a significant diurnal rhythm with peaks in the afternoon (73, 74).

Discrepancies between studies included in the systematic review

Although comparable diurnal patterns have been reported by the studies included in the review, there were some discrepancies with regard to the average marker concentrations and timing of peaks and nadirs. These may be explained by methodological differences between the studies, such as different time intervals between sample collection and analytical methods. For example, one study included in the review did not report a nocturnal MVA peak, which is probably a result of not collecting a nocturnal sample (37). Thereby, total cholesterol in samples from the Bispebjerg study were previously measured using the colorimetric slide technology (21), while we used the GC-FID. Even though the concentrations and rhythms of total cholesterol were comparable between both analytical methods, there were small differences in the mesor, amplitude, and times of peak and

nadir. Therefore, it is expected that the different analytical methods used to measure MVA (e.g. the radio-enzymatic method and gas chromatography-mass spectrometry (GCMS)), lathosterol (e.g. isotope dilution mass spectrometry and GCMS), and C4 (e.g. high-performance liquid chromatography, liquid chromatography-mass spectrometry, and gas chromatography-selected-ion monitoring mass spectrometry) may have resulted in different concentrations, and consequently different mesors, amplitudes and times of peak and nadir between the studies included in the review. Thereby, 14 out of 16 papers measured the diurnal rhythms in less than ten people, which is important considering the large inter-individual variations observed in the diurnal rhythms of cholesterol and bile acid synthesis (40, 42, 43, 66).

The control of external factors might offer an additional explanation for the differences between studies, e.g. meal composition, meal timing, and the number of meals per day differed between the included studies. As dietary fat and cholesterol intake are known to influence cholesterol absorption and cholesterol synthesis, differences in meal composition between the studies may have influenced the rhythms. Furthermore, the impact of meal timing on the diurnal rhythm of cholesterol synthesis has been demonstrated by Cella et al. (44). In this study, a 6.5-hour delay in meal time caused the maximum FSR to delay with six hours after the first day, and with 8.6 hours after the third day of delayed meal times (44). Moreover, a study in which subjects consumed meals with a similar fat and cholesterol content, but either as three or as six meals per day, showed a significantly lower 24-hour cholesterol FSR in the subjects who consumed six meals per day (75). This illustrates that both meal timing and the number of meals per day influenced cholesterol synthesis patterns and rates. In addition to nutrition, information on sleep timing and duration was missing in 12 out of 16 articles. However, sleep timing, sleep duration, and light exposure are known to influence diurnal rhythms, and should preferably be well aligned within and between studies to be able to make good comparisons between findings (2).

Strengths and limitations

We combined a systematic review with an experimental study, in which serum samples were collected under standardised conditions. This minimized the influence of between-subject variation in exposure to external factors on diurnal rhythms. Nonetheless, the present study does have certain limitations. Given that all serum samples in the experimental study were obtained from young and healthy males, it is unclear to what extent the findings can be extrapolated to other populations, especially since age- and sex-specific differences in the circadian system have been reported (76, 77). Furthermore, the cosinor analysis provided a curve in which the peak and nadir were always twelve hours apart and small fluctuations over time were no longer detectable (23). The additional mixed model analyses, however, enabled us to examine whether marker levels did fluctuate significantly over the 24-hour period, though not in a rhythmic manner. In contrast to most of the studies in the review, the Bispebjerg study had a large sample size, which is relevant considering the inter-individual variation often reported in diurnal studies. In addition, multiple markers reflecting cholesterol synthesis, cholesterol absorption, and bile acid synthesis were examined, thereby providing a complete overview of the influence of the circadian rhythms on cholesterol homeostasis.

CONCLUSIONS

The results of this systematic review show that both cholesterol synthesis and bile acid synthesis have a diurnal rhythm, with a reciprocal pattern. The experimental study confirmed the diurnal rhythm of cholesterol synthesis, which had a nocturnal peak. Although the campesterol and sitosterol levels did fluctuate over time, no diurnal rhythm for cholesterol absorption markers was found under the highly standardised conditions of the Bispebjerg study. This may be due to the low-fat meals consumed by the subjects during the 24-hour period. The diurnal rhythms involved in cholesterol homeostasis should be considered when people are exposed to dietary or therapeutic interventions to prevent diseases related to metabolic health. Future research should be carried out to further study the influence of external factors, especially the impact of meal timing and meal composition, on the diurnal rhythms regulating cholesterol homeostasis, as well as whether these rhythms differ for various age groups and health conditions.

Table S3.1. Lathosterol levels (µmol/mmol cholesterol) of the included studies

First author	Intervention	Day 1 (hours)																	
		06:30	07:00	07:30	08:00	08:30	09:00	09:30	10:00	10:30	11:00	11:30	12:00	12:30	13:00	13:30	14:00	14:30	15:00
Al-Khaifi (43)	No treatment					0.9			0.7			0.7			0.6			0.6	
	CME					0.9			0.8			0.7			0.6			0.6	
	CME+STAT					0.3			0.3			0.2			0.2			0.2	
Gälman (40)	No treatment						0.6			0.6			0.5			0.5			0.5
Persson (41)	CME					1.1			1.0			0.9			0.8			0.9	

First author	Intervention	Day 1 (hours)																	
		15:30	16:00	16:30	17:00	17:30	18:00	18:30	19:00	19:30	20:00	20:30	21:00	21:30	22:00	22:30	23:00	23:30	00:00
Al-Khaifi (43)	No treatment		0.6			0.6			0.5			0.6			0.6			0.6	
	CME		0.6			0.6			0.7			0.8			0.9			1.0	
	CME+STAT		0.3			0.3			0.3			0.3			0.3			0.3	
Gälman (40)	No treatment			0.5			0.5			0.5			0.5			0.6			0.7
Persson (41)	CME		1.1			0.9			1.0			1.1			1.0			1.3	

Table S3.1. *Cont.*

First author	Intervention	Day 1 (hours)																	Day 2
		00:30	01:00	01:30	02:00	02:30	03:00	03:30	04:00	04:30	05:00	05:30	06:00	06:30	07:00	07:30	08:00	08:30	09:00
Al-Khaifi (43)	No treatment		0.6			0.7			0.7			0.7			0.7			0.7	
	CME		1.1			1.2			1.5			1.3			1.3			1.3	
	CME+STAT		0.3			0.4			0.4			0.4			0.5			0.4	
Gälman (40)	No treatment			0.9			0.8			0.8			0.8			0.7			0.6
Persson (41)	CME		1.5			1.7			1.8			1.8			1.8			1.8	

First author	Intervention	Day 2 (hours)																	
		09:30	10:00	10:30	11:00	11:30	12:00	12:30	13:00	13:30	14:00	14:30	15:00	15:30	16:00	16:30	17:00	17:30	18:00
Al-Khaifi (43)	No treatment		0.6			0.6			0.6			0.6			0.7				
	CME		1.3			1.2			1.2			1.4			1.5				
	CME+STAT		0.4			0.4			0.4			0.4			0.4				
Gälman (40)	No treatment			0.5															
Persson (41)	CME		1.7			1.6			1.6			1.7			1.9			1.8	

Abbreviations: CME, cholestyramine; STAT, statins. N/A, data unknown.
Note: Values are means.

Table S3.2. Mevalonate concentrations (pmol/mL) of the included studies

First author	Intervention	Day 1 (hours)																	
		06:00	06:30	07:00	07:30	08:00	08:30	09:00	09:30	10:00	10:30	11:00	11:30	12:00	12:30	13:00	13:30	14:00	14:30	15:00
Scoppola (33)	No treatment							15.5		12.8				9.5				8.1		
Pappu (35)	No treatment					63.5		50.9		76.6		61.7		61.9		75.2		78.6		65.3
	No treatment[a]					57.0		57.4		57.2		55.6		45.9		55.9		57.9		65.3
Pappu (38)	No treatment					66.3		43.8		43.9		66.7		N/A		N/A		N/A		54.4
	Lovastatin					63.8		45.3		48.0		42.0		67.19		N/A		50.5		N/A
	Simvastatin					47.2		41.5		24.5		27.0		21.1		31.0		15.9		34.2
Parker (30)	No treatment							56.9						91.5						75.1
	No treatment							79.4		68.4		76.3		61.4		57.2		61.2		79.8
	No treatment							45.2		39.0		34.9		37.9		40.1		50.6		94.9
	Fasting							56.1		46.9		61.4		64.3		40.4		36.9		59.9
	HChol meals							62.7		55.3		49.3		27.4		33.6		47.8		44.5
Parker (31)	No treatment					31.0		27.8		11.6		14.3		16.9		18.8		24.9		34.3
	No treatment					29.9		34.9		40.9		37.6		36.1		39.9		35.1		45.3
	HChol meals					39.3		36.3		45.1		35.2		32.4		27.5		38.4		45.3
Jones(34)	No treatment									73.3								48.5		
Kopito (28)	One daily meal																			
	Fasting																			
Nozaki (37)	No treatment					77.8										57.4				
	Morning pravastatin					85.0										57.4				
	Evening pravastatin																			
	Evening pravastatin																			
Martin (39)	Pre-morning treatment	29.7								20.9								25.6		
	Pre-evening treatment	36.2								21.4								26.4		
	Morning rosuvastatin	27.1								10.3								12.2		
	Evening rosuvastatin	19.3								19.0								23.8		

Table S3.2. *Cont.*

First author	Intervention	Day 1 (hours)																
		15:30	16:00	16:30	17:00	17:30	18:00	18:30	19:00	19:30	20:00	20:30	21:00	21:30	22:00	22:30	23:00	23:30	00:00
Scoppola (33)	No treatment		6.1				8.1				10.8				10.8				14.2
Pappu (35)	No treatment		52.9		54.1		33.3		53.4		56.3		49.1		57.9		68.7		83.6
	No treatment[a]		59.0		65.8		45.3		66.7		67.8		66.2		68.0		87.2		86.5
Pappu (38)	No treatment		N/A		51.1		34.7		49.2		51.7		75.5		72.0		80.9		70.8
	Lovastatin		N/A		22.5		52.2		34.2		54.1		59.2		55.2		47.2		38.9
	Simvastatin		43.1		40.1		23.0		20.7		23.0		21.0		22.8		20.8		21.7
Parker (30)	No treatment						71.3						63.7						92.6
	No treatment		79.8		93.9		113.4		100.4		107.7		119.9		167.5		151.7		150.0
	No treatment		115.1		98.0		102.4		74.1		77.8		77.2		75.0		76.8		87.9
	Fasting		71.7		44.3		65.1		62.5		68.0		55.1		N/A		61.9		38.6
	HChol meals		41.4		51.5		61.8		69.7		66.5		84.4		N/A		91.0		51.7
Parker (31)	No treatment		40.0		36.5		38.1		37.9		40.0		50.2		63.6		59.7		76.0
	No treatment		56.9		62.3		63.4		70.9		70.9		75.3		83.5		70.4		63.4
	HChol meals		30.2		31.2		36.2		49.3		44.5		38.3		30.2		32.6		39.1
Jones (34)	No treatment						40.3								50.1				
Kopito (28)	One daily meal						10.9								10.3				
	Fasting						13.0								12.9				
Nozaki (37)	No treatment				89.2												84.0		
	Morning pravastatin				55.7												84.1		
	Evening pravastatin				71.4												50.7		
Martin (39)	Pre-morning treatment						35.8								38.5				47.9
	Pre-evening treatment						37.2								35.3				43.5
	Morning rosuvastatin						19.8								26.6				33.0
	Evening rosuvastatin						32.9								11.7				13.4

Table S3.2. *Cont.*

First author	Intervention	Day 1 (hours)																
		00:30	01:00	01:30	02:00	02:30	03:00	03:30	04:00	04:30	05:00	05:30	06:00	06:30	07:00	07:30	08:00	08:30	09:00
Scoppola (33)	No treatment				22.9				21.6						18.9				14.2
Pappu (35)	No treatment		129.3		101.8		93.2		84.7		84.5		66.0		94.0		71.0		
	No treatment[a]		87.8		92.1		85.4		101.8		87.4		79.1		81.3		83.6		
Pappu (38)	No treatment		89.1		59.1		81.7		80.9		87.5		56.3		N/A		N/A		
	Lovastatin		40.3		29.7		42.8		31.3		45.0		45.0		N/A		N/A		
	Simvastatin		22.3		22.4		17.5		23.9		33.5		37.9		26.6		N/A		
Parker (30)	No treatment						87.5						48.5						52.2
	No treatment		127.4		166.0		113.2		113.2		108.8		99.6		101.5		78.7		
	No treatment		147.1		196.0		129.8		117.3		95.2		104.2		94.5		79.6		79.2
	Fasting		56.1		47.8		45.4		57.9		80.3		56.4		55.3		61.9		
	HChol meals		63.2		61.8		71.3		65.8		57.9		49.6		55.7		56.8		73.5
Parker (31)	No treatment		68.2		62.6		62.4		56.3		55.3		45.2		N/A		N/A		
	No treatment		53.7		46.0		44.0		40.5		39.4		37.3		35.8		35.8		
	HChol meals		35.8		35.1		36.9		34.0		29.8		32.0		27.9		N/A		
Jones (34)	No treatment				66.6								68.4						
Kopito (28)	One daily meal				34.4								54.2						
	Fasting				10.6								8.1						
Nozaki (37)	No treatment																76.5		
	Morning pravastatin																82.4		
	Evening pravastatin																74.1		
Martin (39)	Pre-morning treatment				43.2								25.0						
	Pre-evening treatment				47.5								31.3						
	Morning rosuvastatin				33.4								30.1						
	Evening rosuvastatin				15.5								21.0						

Table S3.2. *Cont.*

First author	Intervention	Day 2 (hours)																	
		09:30	10:00	10:30	11:00	11:30	12:00	12:30	13:00	13:30	14:00	14:30	15:00	15:30	16:00	16:30	17:00	17:30	18:00
Parker (30)	No treatment						47.9						48.7						57.8
Jones (34)	No treatment		57.6								46.7								45.8
Kopito (28)	One daily meal		39.7								18.0								15.1
	Fasting		7.6								7.9								N/A
Nozaki (37)	No treatment																		
	Morning pravastatin																		
	Evening pravastatin								55.7								75.9		

First author	Intervention	Day 2 (hours)																	
		18:30	19:00	19:30	20:00	20:30	21:00	21:30	22:00	22:30	23:00	23:30	00:00	00:30	01:00	01:30	02:00	02:30	03:00
Parker (30)	No treatment						58.4						62.1						125.1
Jones (34)	No treatment								51.2								65.0		
Kopito (28)	One daily meal								12.2								32.2		

First author	Intervention	Day 2 (hours)																	
		03:30	04:00	04:30	05:00	05:30	06:00	06:30	07:00	07:30	08:00	08:30	09:00	09:30	10:00	10:30	11:00	11:30	12:00
Parker (30)	No treatment						56.5						65.7						87.8
Jones (34)	No treatment						58.4												
Kopito (28)	One daily meal						46.3								32.8				

First author	Intervention	Day 3 (hours)																	
		12:30	13:00	13:30	14:00	14:30	15:00	15:30	16:00	16:30	17:00	17:30	18:00	18:30	19:00	19:30	20:00	20:30	21:00
Parker (30)	No treatment						81.9						71.2						90.0
Kopito (28)	One daily meal				23.3														

First author	Intervention	Day 3 (hours)																	
		21:30	22:00	22:30	23:00	23:30	00:00	00:30	01:00	01:30	02:00	02:30	03:00	03:30	04:00	04:30	05:00	05:30	06:00
Parker (30)	No treatment						123.1						45.3						35.0

Abbreviations: CME, cholestyramine; STAT, statins. N/A, data unknown. *Note:* Values are means. [a]Cholecystectomized subjects.

Table S3.3. VLDL-squalene levels (µmol/mmol free cholesterol) of the included studies

First author	Subgroup	Day 1 (hours)																	
		06:30	07:00	07:30	08:00	08:30	09:00	09:30	10:00	10:30	11:00	11:30	12:00	12:30	13:00	13:30	14:00	14:30	15:00
Mietinnen (29)	Healthy subjects				3.0								1.5						
Mietinnen (32)	Jejunoileal bypass				4.7								3.8						
	Ileal exclusion				1.7								1.6						

First author	Subgroup	Day 1 (hours)																	
		15:30	16:00	16:30	17:00	17:30	18:00	18:30	19:00	19:30	20:00	20:30	21:00	21:30	22:00	22:30	23:00	23:30	00:00
Mietinnen (29)	Healthy subjects		1.4								2.3								3.2
Mietinnen (32)	Jejunoileal bypass		2.1								2.1								2.6
	Ileal exclusion		1.5								1.9								1.7

First author	Subgroup	Day 1 (hours)																	
		00:30	01:00	01:30	02:00	02:30	03:00	03:30	04:00	04:30	05:00	05:30	06:00	06:30	07:00	07:30	08:00	08:30	09:00
Mietinnen (29)	Healthy subjects								4.1										
Mietinnen (32)	Jejunoileal bypass								3.5										
	Ileal exclusion								1.8										

Note: Values are means.

Table S3.4. LDL+HDL-squalene levels (µmol/mmol free cholesterol) of the included studies

First author	Subgroup	Day 1 (hours)																	
		06:30	07:00	07:30	08:00	08:30	09:00	09:30	10:00	10:30	11:00	11:30	12:00	12:30	13:00	13:30	14:00	14:30	15:00
Mietinnen (29)	Healthy subjects				0.8								0.7						
Mietinnen (32)	Jejunoileal bypass				0.3								0.5						
	Ileal exclusion				0.1								0.3						

First author	Subgroup	Day 1 (hours)																	
		15:30	16:00	16:30	17:00	17:30	18:00	18:30	19:00	19:30	20:00	20:30	21:00	21:30	22:00	22:30	23:00	23:30	00:00
Mietinnen (29)	Healthy subjects		0.8								0.9								1.2
Mietinnen (32)	Jejunoileal bypass		0.4								0.4								0.3
	Ileal exclusion		0.2								0.2								0.2

First author	Subgroup	Day 1 (hours)																	
		00:30	01:00	01:30	02:00	02:30	03:00	03:30	04:00	04:30	05:00	05:30	06:00	06:30	07:00	07:30	08:00	08:30	09:00
Mietinnen (29)	Healthy subjects								1.2										
Mietinnen (32)	Jejunoileal bypass								0.3										
	Ileal exclusion								0.2										

Note: Values are means.

Table S3.5. C4 levels (nmol/mmol cholesterol) of the included studies

First author	Intervention	Day 1 (hours)																	
		06:30	07:00	07:30	08:00	08:30	09:00	09:30	10:00	10:30	11:00	11:30	12:00	12:30	13:00	13:30	14:00	14:30	15:00
Al-Khaifi (43)	No treatment					11.0			7.2			5.8			6.7			5.6	
	CME					9.4			5.9			8.6			10.4			12.2	
	CME + STAT					10.2			5.4			6.5			9.0			11.3	
Gälman (40)	No treatment						2.6			2.2			4.6			4.9			3.6
	No treatment[a]						3.4			2.7			8.3			5.3			4.5

First author	Intervention	Day 1 (hours)																	
		15:30	16:00	16:30	17:00	17:30	18:00	18:30	19:00	19:30	20:00	20:30	21:00	21:30	22:00	22:30	23:00	23:30	00:00
Al-Khaifi (43)	No treatment		4.8			5.9			6.4			6.1			7.2			5.3	
	CME		11.7			11.8			16.2			20.2			19.1			18.9	
	CME + STAT		10.3			11.9			15.1			19.4			19.0			22.0	
Gälman (40)	No treatment			2.8			2.4			3.8			5.6			5.4			3.5
	No treatment[a]			3.8			3.2			3.2			5.1			5.7			4.5

First author	Intervention	Day 1 (hours)																Day 2	
		00:30	01:00	01:30	02:00	02:30	03:00	03:30	04:00	04:30	05:00	05:30	06:00	06:30	07:00	07:30	08:00	08:30	09:00
Al-Khaifi (43)	No treatment		5.2			4.4			4.7			7.0			6.5			5.1	
	CME		19.7			23.0			24.2			36.5			37.6			35.1	
	CME + STAT		20.5			17.3			19.7			22.3			24.4			22.3	
Gälman (40)	No treatment			2.8			2.7			2.2			1.9			2.2			2.1
	No treatment[a]			5.3			4.6			7.4			5.2			5.0			3.2

First author	Intervention	Day 2 (hours)																	
		09:30	10:00	10:30	11:00	11:30	12:00	12:30	13:00	13:30	14:00	14:30	15:00	15:30	16:00	16:30	17:00	17:30	18:00
Al-Khaifi (43)	No treatment		3.9			5.6			6.8			6.2			4.2				
	CME		38.7			49.3			46.6			41.3			29.3				49.3
	CME + STAT		20.7			24.5			27.8			25.7			20.3				24.5
Gälman (40)	No treatment			1.8															
	No treatment[a]			2.6															

Abbreviations: CME, cholestyramine; STAT, statins. N/A, data unknown. *Note:* Values are means. [a]Cholecystectomized subjects.

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CHAPTER 4

Effect of dietary macronutrients on intestinal cholesterol absorption and endogenous cholesterol synthesis: a randomized crossover trial

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ABSTRACT

Extensive research showed a diurnal rhythm of endogenous cholesterol synthesis, whereas recent research reported no diurnal rhythm of intestinal cholesterol absorption in males who consumed low-fat meals. Little is known about the acute effect of macronutrient consumption on cholesterol metabolism, and hence if meal composition may explain this absence of rhythmicity in cholesterol absorption. Therefore, we examined the effect of a high-fat, high-carbohydrate, and high-protein meal on postprandial intestinal cholesterol absorption and endogenous cholesterol synthesis in apparently healthy overweight and slightly obese males. Eighteen males consumed in random order an isoenergetic high-fat, high-carbohydrate, and high-protein meal on three occasions. Serum total cholesterol concentrations, cholesterol absorption markers (campesterol, cholestanol, and sitosterol), and cholesterol synthesis intermediates (7-dehydrocholesterol, 7-dehydrodesmosterol, desmosterol, dihydrolanosterol, lanosterol, lathosterol, zymostenol, and zymosterol) were measured at baseline (T0) and 240 min postprandially (T240). Meal consumption did not significantly change total cholesterol concentrations and cholesterol absorption marker levels (all $p>0.05$). Serum levels of 7-dehydrocholesterol, lanosterol, lathosterol, zymostenol, and zymosterol decreased significantly between T0 and T240 (all $p<0.05$). These decreases were not significantly different between the three meals (all $p>0.05$), except for a larger decrease in dihydrolanosterol levels after the high-fat versus the high-carbohydrate meal ($p=0.009$). In conclusion, the high-fat, high-carbohydrate, and high-protein meal did not significantly influence postprandial intestinal cholesterol absorption. Several cholesterol synthesis intermediates decreased postprandially, but the individual macronutrients did not differentially affect these intermediates, except for a possible effect on dihydrolanosterol.

INTRODUCTION

Whole body cholesterol homeostasis is tightly regulated by endogenous *de novo* cholesterol synthesis, intestinal biliary and dietary cholesterol absorption, and bile acid synthesis and excretion (1). Intestinal cholesterol absorption and endogenous cholesterol synthesis rates can be estimated by the measurement of serum non-cholesterol sterol concentrations (1). Serum plant sterol concentrations, i.e. campesterol and sitosterol, and the cholesterol metabolite cholestanol reflect fractional intestinal cholesterol absorption, while the cholesterol precursors lathosterol and desmosterol reflect whole body endogenous cholesterol synthesis (2, 3). Although lathosterol is the only well-validated marker for endogenous cholesterol synthesis, the cholesterol synthesis pathway comprises numerous intermediates. Cholesterol synthesis starts with the formation of lanosterol from acetyl-CoA via a multistep process. Lanosterol can enter either the Bloch or the Kandutsch-Russell (K-R) pathway, which ultimately leads to the formation of cholesterol (**Fig. S4.1**) (4-7).

Cholesterol homeostasis is well-controlled within cells, mainly by balancing synthesis, uptake, esterification, and efflux of cholesterol (8). However, this balanced system is influenced by various factors, including diet. For example, long-term consumption of diets specifically enriched in fats, carbohydrates, or proteins affect intestinal cholesterol absorption and endogenous cholesterol synthesis in humans (9-12), which will ultimately lead to a new steady state.

Recently, we have confirmed that endogenous cholesterol synthesis has a diurnal rhythm in healthy subjects, as cholesterol-standardised lathosterol levels were lowest during the day and highest during the night. For intestinal cholesterol absorption, no diurnal rhythm was found (13). We hypothesized that the absence of a diurnal rhythm of intestinal cholesterol absorption in our study was related to the consumption of low-fat meals during the day. Hence, it would be interesting to examine whether acute consumption of a meal rich in dietary fat, in comparison to meals rich in the other two macronutrients, modifies cholesterol absorption and synthesis marker levels and may consequently affect the diurnal rhythm of cholesterol absorption and synthesis. It is known that meal consumption can influence the diurnal rhythm of cholesterol synthesis, as a delay in meal timing shifted the cholesterol synthesis peak time and increased the amplitude (14), but the influence of meal composition is less clear.

To date, only few studies have investigated the acute effects of high-fat meals on postprandial intestinal cholesterol absorption and endogenous cholesterol synthesis (15-17), and results suggest that endogenous cholesterol synthesis decreased (15, 16). In one study (16), but not in the other studies (15, 17), postprandial cholesterol absorption markers increased. Acute effects of high-carbohydrate or high-protein meals on postprandial intestinal cholesterol absorption and endogenous cholesterol synthesis have not been studied. Therefore, the objective of this study was to perform a side-by-side comparison of a single high-fat, high-carbohydrate, and high-protein meal on serum intestinal cholesterol absorption and endogenous cholesterol synthesis markers in apparently healthy overweight and slightly obese male volunteers.

METHODS

Participants, study design, and test meals

Recruitment, inclusion and exclusion criteria, and the study design have already been described in detail (18). Briefly, twenty apparently healthy overweight and slightly obese men were included in this randomized, double-blind, crossover trial. Two participants dropped out due to personal reasons and the remaining eighteen participants completed the study. These participants had a median (interquartile [IQR] range) age of 65 (50.8–67.0) years, a mean (±SD) body mass index (BMI) of 30.5±2.9 kg/m^2, fasting serum total cholesterol concentrations of 5.3±0.9 mmol/L, and fasting serum triacylglycerol concentrations of 1.3±0.5 mmol/L. The subjects received in random order three different meals at the Metabolic Research Unit Maastricht. The experimental days were scheduled with an interval of at least one week. At the beginning of each session, a fasting venous blood sample (T0) was drawn. This was followed by a postprandial test, during which participants consumed a high-fat meal (55.3 g (52.3 percent of energy (EN%)) fat; 93.5 g (39.2 EN%) carbohydrate; 19.2 g (8.0 EN%) protein), a high-carbohydrate meal (10.2 g (9.6 EN%) fat; 194.3 g (81.5 EN%) carbohydrate; 20.4 g (8.6 EN%) protein), or a high-protein meal (11.3 g (10.6 EN%) fat; 122.7 g (51.5 EN%) carbohydrate; 87.9 g (36.9 EN%) protein), of which one third had to be consumed within 1 min followed by 2-min breaks after which the following third had to be consumed. The high-fat meal provided 331 mg cholesterol, and the high-carbohydrate and high-protein meals both provided 334 mg cholesterol. The three meals were isocaloric (953 kilocalories [kcal] each) and participants received water to correct for differences in weight of the meals leading to an identical total volume of 730 mL (**Table 4.1**). A second venous blood sample was collected 240 min (T240) after meal consumption. Written informed consent was signed before the start of the study by all participants. The Medical Ethical Committee of the University Hospital Maastricht/ Maastricht University (METC azM/UM) has approved the study, which was registered at ClinicalTrials.gov in May 2017 as NCT03139890.

Table 4.1. The nutrient and energy composition of the high-fat, high-carbohydrate, and high-protein meal

	High-fat	High-carbohydrate	High-protein
Energy, kcal	953	953	953
Carbohydrates, g/En%	93.5/39.2	194.3/81.5	122.7/51.5
Protein, g/En%	19.2/8.0	20.4/8.6	87.9/36.9
Fat, g/En%	55.3/52.3	10.2/9.6	11.3/10.6
Saturated fat, g	33.1	3.4	4.0
Monounsaturated fat, g	16.0	4.0	4.0
Polyunsaturated fat, g	5.0	0.9	0.9
Cholesterol, mg	331	334	334
Water, g	297	262	115

Notes: EN%, energy percentage; g, gram; kcal, kilocalories; mg, milligram.

Biochemical analyses

Blood was collected at T0 and T240 in 6 mL serum STT-II advance collection tubes (Becton Dickinson, Erembodegem, Belgium), which were allowed to clot for a minimum of 30 minutes at 21 °C. Serum was obtained by centrifuging the tubes at 1300 x g for 15 minutes at 21 °C and stored in aliquots at -80 °C until further analysis. Serum concentrations of total cholesterol and the cholesterol

absorption marker cholestanol were quantitated using gas chromatography with flame ionization detector (GC-FID) as described previously (19). In addition, serum concentrations of the cholesterol absorption markers campesterol and sitosterol, as well as the serum concentrations of several cholesterol synthesis pathway intermediates 7-dehydrocholesterol, 7-dehydrodesmosterol, desmosterol, dihydrolanosterol, lanosterol, lathosterol, zymostenol, and zymosterol were determined using a gas chromatography triple quadrupole mass spectrometer (GC-MS/MS). Analytical aspects of the extraction and this GC-MS/MS methodology have been described by Mackay et al. (19), but we have now added the identification of several cholesterol precursors to the spectrum. The technical performance details (respectively limit of quantification (LOQ), within and between run variation coefficients) of this panel of non-cholesterol sterol analyses are: 6.47 nmol/L, 6.4% and 11.9% for 7-dehydrocholesterol; 6.02 nmol/L, 16.0% and 22.9% for 7-dehydrodesmosterol; 11.76 nmol/L, 8.1% and 8.1% for desmosterol; 0.77 nmol/L, 16.1% and 2.6% for dihydrolanosterol; 0.95 nmol/L, 7.2% and 10.2% for lanosterol; 9.53 nmol/L, 8.0% and 6.8% for lathosterol; 0.62 nmol/L, 11.3% and 11.1% for zymostenol; 0.94 nmol/L, 7.5% and 10.4% for zymosterol; 12.15 nmol/L, 3.8% and 5.2% for campesterol; 12.43 nmol/L, 4.3% and 8.6% for sitosterol; 64.80 nmol/L, 5.3% and 2.5% for cholestanol (GC-FID); 323 nmol/L, 3.3% and 2.8% for cholesterol (GC-FID). All non-cholesterol sterols were expressed as absolute concentrations (µmol/L) and standardized for total cholesterol (µmol/mmol cholesterol), as analysed with the GC-FID.

Statistical analyses

Data are presented as mean ± standard deviation (SD), unless indicated otherwise. A one-way univariate analysis of variance (ANOVA) was used to assess differences in total cholesterol concentrations and cholesterol-standardised non-cholesterol sterol levels at T0 between the three experimental sessions. In addition, the mean level of each cholesterol absorption marker and of each cholesterol synthesis intermediate for the three meals at T0 was calculated for each participant. Pearson correlation coefficients were calculated to analyse associations between these mean non-cholesterol sterol levels at T0.

The changes (T240–T0) in total cholesterol and non-cholesterol sterol levels were calculated. Linear mixed models with a random intercept were used to examine whether these changes differed from zero (time-effect), and were significantly different between the three experimental meals (meal-effect). Subjects were defined as random factor and meal and period as fixed factors. Bonferroni post hoc tests were carried out to make pairwise comparisons.

A subgroup analysis was performed to investigate whether postprandial effects were different between subjects with a relatively low lathosterol/campesterol-ratio at T0 versus those with a relatively high lathosterol/campesterol-ratio at T0 (20). First, the mean lathosterol/campesterol-ratio at T0 was calculated for each participant. Second, the median lathosterol/campesterol-ratio of the whole group was used to divide the study population in a lower-ratio group (n=9) and a higher-ratio group (n=9). To compare the lower-ratio and higher-ratio group, the linear mixed model analyses were repeated with group and a group*meal interaction added to the model. All

analyses were performed with SPSS version 25 for Mac OS X (SPSS Inc., Chicago, IL, USA) and *p*-values < 0.05 were considered statistically significant.

RESULTS

Serum total cholesterol concentrations and non-cholesterol sterol levels after the overnight fast (T0) were not significantly different between the three experimental sessions (all p>0.05). For cholesterol synthesis intermediates, the mean serum levels at T0 were lowest for dihydrolanosterol ($0.8 \pm 0.4 \times 10^{-2}$ µmol/mmol cholesterol) and highest for lathosterol ($227 \pm 71 \times 10^{-2}$ µmol/mmol cholesterol). For cholesterol absorption markers, the mean levels at T0 ranged from $134 \pm 24 \times 10^{-2}$ µmol/mmol cholesterol for cholestanol to $266 \pm 96 \times 10^{-2}$ µmol/mmol cholesterol for campesterol.

Table 4.2 shows the correlations between the markers reflecting intestinal cholesterol absorption and endogenous cholesterol synthesis at T0. Strong positive and significant correlations were found between the three cholesterol absorption markers campesterol, sitosterol, and cholestanol (range from r = 0.713 to r = 0.879; p<0.01). Moreover, the three Bloch pathway intermediates correlated positively (range from r = 0.544 to r = 0.715; p<0.05). Several, but not all, K-R pathway intermediates correlated positively (range from r = -0.185 (NS) to r = 0.930 (p<0.01)). In addition, most but not all, correlations between the Bloch and K-R pathway intermediates were positive (range from r = -0.020 (NS) to r = 0.864 (p<0.01)). Finally, although most correlations between cholesterol absorption and synthesis markers were negative, not all reached statistical significance (range from r = -0.497 (p<0.05) to r = 0.137 (NS)).

During the postprandial phase, i.e. after consumption of the three meals, no significant meal- and time-effects were found for serum total cholesterol concentrations and the cholesterol absorption markers campesterol, sitosterol, and cholestanol (p>0.05, **Table 4.3**). For markers reflecting endogenous cholesterol synthesis, meal consumption significantly decreased serum cholesterol-standardized levels of lanosterol (p=0.004), the Bloch pathway intermediate zymosterol (p=0.001), and the K-R pathway intermediates 7-dehydrocholesterol (p=0.032), lathosterol (p<0.001), and zymostenol (p=0.005). However, these reductions were not significantly different between the three meals (all p>0.05). The only exception was the K-R pathway intermediate dihydrolanosterol, which showed a significantly greater decrease after consumption of the high-fat meal as compared with the high-carbohydrate meal (p=0.009).

Based on the median lathosterol/campesterol-ratio, participants were divided into the lower-ratio group (ratio < 0.86) and the higher-ratio group (ratio ≥ 0.86). The three meals did not result in significantly different changes between the higher-ratio and lower-ratio group for any of the markers reflecting intestinal cholesterol absorption and endogenous cholesterol synthesis (all p>0.05). The higher-ratio group showed a significantly greater postprandial decrease in cholesterol-standardized serum lathosterol (p=0.004), zymostenol (p=0.008), and zymosterol (p=0.031) levels following meal consumption compared to the lower-ratio group. There were no significant differences in changes in cholesterol absorption markers between the lower-ratio and higher-ratio group following overall meal consumption (**Table S4.1**).

Table 4.2. Pearson correlation coefficients between serum intestinal cholesterol absorption markers and intermediates of the Bloch and K-R pathway (µmol/ mmol cholesterol) at T0

		CAMP	CHOL	SITO	LANO	7-DHD	DESM	ZYM-R	7-DHC	DHL	LATH
Cholesterol Absorption	**CAMP**	—									
	CHOL	0.879**	—								
	SITO	0.873**	0.713**	—							
Cholesterol Synthesis	**LANO**	-0.186	-0.294	0.137	—						
	Bloch Pathway										
	7-DHD	-0.140	-0.328	0.074	0.621**	—					
	DESM	-0.373	-0.462	-0.155	0.464	0.544*	—				
	ZYM-R	-0.398	-0.464	-0.194	0.722**	0.715**	0.698**	—			
	Kandutsch-Russell Pathway										
	7-DHC	-0.244	-0.263	-0.161	0.451	0.436	0.546*	0.705**	—		
	DHL	-0.175	-0.314	0.077	0.625**	0.320	-0.020	0.276	-0.185	—	
	LATH	-0.409	-0.470*	-0.132	0.744**	0.482*	0.501*	0.857**	0.432	0.544*	—
	ZYM-N	-0.497*	-0.475*	-0.314	0.606**	0.459	0.452	0.864**	0.404	0.474*	0.930**

Notes: *p < 0.05; **p < 0.01

Abbreviations: CAMP, campesterol; CHOL, cholestanol; 7-DHC, 7-dehydrocholesterol; 7-DHD, 7-dehydrodesmosterol; DESM, desmosterol; DHL, dihydrolanosterol; LANO, lanosterol; LATH, lathosterol; SITO, sitosterol; ZYM-N, zymostenol; ZYM-R, zymosterol.

Table 4.3. Serum total cholesterol concentrations and cholesterol-standardized absorption and synthesis marker levels at T0 and the changes between T0 and T240 following the high-fat, high-carbohydrate, and high-protein meal in apparently healthy overweight and slightly obese men (*n* = 18)

		High-fat		High-carbohydrate		High-protein		P-value	
		T0	Change	T0	Change	T0	Change	Meal	Time
	Total cholesterol	5.3 ± 1.0	0.1 (-0.03 – 0.2)	5.3 ± 0.9	-0.1 (-0.2 – 0.1)	5.2 ± 0.9	0.01 (-0.1 – 0.1)	0.279	0.548
Cholesterol absorption	Campesterol	277 ± 98	-1.8 (-9.7 – 6.1)	262 ± 97	1.4 (-6.5 – 9.3)	259 ± 101	0.9 (-7.0 – 8.8)	0.825	0.940
	Cholestanol	134 ± 27	-1.4 (-5.0 – 2.2)	137 ± 25	1.3 (-2.3 – 4.9)	133 ± 24	1.8 (-1.8 – 5.4)	0.414	0.577
	Sitosterol	157 ± 62	-11 (-21 – -1)	146 ± 47	-3 (-13 – 7)	144 ± 46	4 (-6 – 14)	0.109	0.264
Cholesterol synthesis	Lanosterol	12 ± 5	-3.0 (-4.5 – -1.1)	11 ± 4	-1.5 (-3.2 – -0.2)	11 ± 4	-2.0 (-3.7 – -0.3)	0.368	0.004
Bloch Pathway									
	7-Dehydrodesmosterol	6.5 ± 1.6	-0.1 (-0.7 – -0.5)	6.8 ± 2.1	-0.3 (-0.9 – -0.4)	6.7 ± 1.7	-0.6 (-1.2 – -0.03)	0.435	0.138
	Desmosterol	141 ± 26	-2 (-9 – -5)	141 ± 27	2 (-6 – 9)	146 ± 26	-3 (-11 – -4)	0.602	0.558
	Zymosterol	5.3 ± 1.1	-0.3 (-0.6 – -0.1)	5.3 ± 1.5	-0.4 (-0.7 – -0.1)	5.5 ± 1.4	-0.3 (-0.6 – -0.1)	0.864	0.001
Kandutsch-Russell Pathway									
	7-Dehydrocholesterol	98 ± 11	-3.9 (-7.7 – 0.02)	99 ± 14	-1.7 (-5.6 – 2.2)	100 ± 13	-4.1 (-8.0 – -0.3)	0.518	0.032
	Dihydrolanosterol	0.8 ± 0.4	-0.1 (-0.2 – -0.1)	0.8 ± 0.4	-0.01 (-0.1 – -0.1)	0.8 ± 0.4	-0.1 (-0.1 – -0.04)	0.009	0.080
	Lathosterol	223 ± 64	-17 (-32 – -2)	229 ± 89	-22 (-37 – -7)	228 ± 82	-27 (-42 – -11)	0.625	<0.001
	Zymostenol	32 ± 9	-1.3 (-3.2 – -0.6)	32 ± 10	1.6 (3.6 – -0.3)	33 ± 11	-2.7 (-4.7 – -0.8)	0.529	0.005

Notes: Data at T0 are presented as mean ± SD and changes as estimated marginal means with 95% confidence interval (CI) in $10^2 \times$ µmol/ mmol cholesterol, except for total cholesterol concentrations (mmol/L).

DISCUSSION

The aim of this study was to compare in apparently healthy overweight or slightly obese men the effects of high-fat, high-carbohydrate, and high-protein meals on markers reflecting intestinal cholesterol absorption and endogenous cholesterol synthesis. In a previous study, we did not observe a significant diurnal rhythm in intestinal cholesterol absorption (13). In that study, healthy men consumed low-fat diets throughout the study. Since it is well-known that external factors, including the diet, may influence these fluctuations over time (21), we questioned whether the low-fat diet might have been the reason for the lack of rhythmicity in intestinal cholesterol absorption. The results of the present randomized, double-blind, cross-over study showed no statistically significant effects of the three macronutrients on changes in postprandial 4-hour markers reflecting fractional intestinal cholesterol absorption. Strictly, rhythmicity was not investigated, but the results do not suggest that high intakes of the specific macronutrients influence postprandial changes in cholesterol absorption over time. Moreover, this may indicate that the consumption of a low-fat diet by participants in our previous study is unlikely to be an explanation for the lack of rhythmicity in intestinal cholesterol absorption. The present study also showed that serum levels of several intermediates in the Bloch pathway and the K-R pathway were significantly decreased following meal consumption, suggesting a postprandial decrease in endogenous cholesterol synthesis. Effects on cholesterol synthesis, however, did not differ between the three meals, except for the K-R pathway intermediate dihydrolanosterol. This postprandial decrease in endogenous cholesterol synthesis most likely reflects the diurnal rhythm, with a decrease in cholesterol synthesis during daytime. Altogether, these results suggest that the reciprocal relation between endogenous cholesterol synthesis and intestinal cholesterol absorption, which has often been observed in the fasting state, was no longer present in the postprandial state.

To the best of our knowledge, the effect of a high-protein or high-carbohydrate meal on intestinal cholesterol absorption has not been studied before. Two previous studies also found no significant effect of a single high-fat meal on fractional intestinal cholesterol absorption (15, 17). In contrast, Hallikainen et al. found small, but significant, increases in campesterol, sitosterol, and cholestanol levels during an 8-h postprandial period (16). Therefore, it can be suggested that the 4-h postprandial period of our study was not long enough. This is, however, not supported by the findings of Relas et al. who also reported no significant changes in cholesterol absorption as measured several times during a 12-h postprandial period (15). In the present study, the high-fat meal provided approximately 55 g fat. Hallikainen and colleagues used 35 g fat/m^2 body surface (16). Assuming a body surface of approximately 2.0 m^2 (22), it is likely that the amount of fat in their meal was higher than the amount used in the present study. However, differences in fat intake also cannot explain the difference in findings between the study of Hallikainen et al. and our study, as the meal in the study of Relas and colleagues provided 90 g of fat (15).

Previous studies have suggested that consumption of a second meal plays a role in the process of intestinal cholesterol absorption, as the dietary cholesterol from the first meal is then incorporated into chylomicrons and subsequently secreted into plasma after consumption of one or multiple subsequent meals (17, 23). A possible explanation for the finding that cholesterol absorption markers were unaffected by single meal consumption may therefore be that subjects were not

provided with a second meal. Baumgartner et al. provided subjects with a second meal without cholesterol 4 h after the first meal, which resulted in a non-significant increase in campesterol concentrations, while sitosterol concentrations remained stable for the next 4 h (17). Relas et al. also provided the subjects with a low-fat, low-cholesterol meal 9 h after the first meal, and reported no significant changes in cholesterol absorption marker levels over the complete postprandial period (15). These previous findings make it unlikely that a second meal would have led to significant changes in intestinal cholesterol absorption in the present study.

The subgroup analysis for the higher-ratio group, who have relatively high cholesterol synthesis rates, compared to the lower-ratio group, who have relatively lower cholesterol synthesis rates, suggested that overall meal consumption did not significantly affect changes in cholesterol absorption within both groups. These findings suggest that single-meal consumption does not differentially affect intestinal cholesterol absorption in the study population when divided into a higher-ratio and lower-ratio group. However, the results of this exploratory post-hoc analysis must be interpreted with caution.

The present study showed that cholesterol-standardized levels of several cholesterol synthesis intermediates were decreased after meal consumption, but the effects did not differ between the macronutrients. Although no studies were found which investigated the impact of a high-carbohydrate or high-protein meal on endogenous cholesterol synthesis, the present findings are in line with two previous studies that investigated the acute effect of a high-fat meal (15, 16). Hallikainen et al. showed that consumption of a single high-fat meal significantly decreased cholesterol-standardised lathosterol, but not desmosterol levels over an 8-hour postprandial period (16). Relas et al. found that a single high-fat meal significantly decreased the lathosterol/campesterol-ratio – which was used as indicator of endogenous cholesterol synthesis – up to 12 h postprandially in healthy males (15).

A likely explanation for the postprandial decrease in endogenous cholesterol synthesis would be an inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity. Although a single high-fat meal did not significantly affect HMG-CoA reductase mRNA activity in mononuclear human blood cells during a 10-h postprandial period (24), which may suggest no effect on cholesterol synthesis, a study in obese subjects reported that HMG-CoA reductase mRNA levels were not significantly correlated with HMG-CoA reductase activity (25). The decrease in endogenous cholesterol synthesis may also be related to postprandial changes in insulin, which increased after consumption of all three meals (18). Previously, plasma mevalonate levels – an intermediate in the endogenous cholesterol biosynthesis pathway – have been measured during a 3-h hyperinsulinemic euglycemic clamp (26, 27). Results showed that insulin treatment significantly decreased 2- and 3-h mevalonate concentrations compared with baseline, suggesting that postprandial increases in insulin decreased endogenous cholesterol synthesis (26, 27). However, it remains uncertain if this was the underlying mechanism, as insulin peaks were observed one to 2 h postprandially (18).

The subgroup analysis we performed revealed a significantly greater decrease in serum lathosterol,

zymostenol, and zymosterol in the higher-ratio group compared with the lower-ratio group, suggesting that meal consumption resulted in a stronger decrease in endogenous cholesterol synthesis in subjects with relatively high fasting cholesterol synthesis rates. Various metabolic disorders have been linked to being a high cholesterol synthesizer, including type II diabetes and the metabolic syndrome (28), and the findings may be of relevance for this subgroup. However, studies that examine whether health benefits of lowering cholesterol synthesis in this so-called synthesizer subgroup, either by diet or via other interventions, is larger as compared to the benefit in absorbers, are warranted.

The present study has investigated the acute effects of macronutrient consumption on intestinal cholesterol absorption and endogenous cholesterol synthesis. Long-term intakes of the three macronutrients, however, may differently affect intestinal cholesterol absorption and endogenous cholesterol synthesis. Vidon et al. have reported that in healthy humans, endogenous cholesterol synthesis increased after 3-week consumption of a diet moderately high in carbohydrates compared to a 3-week diet high in fat (9). In another study, replacement of protein with mainly fat suggested a decreased cholesterol synthesis in healthy subjects after a one-month low-protein-high-fat diet compared to a one-month high-protein-low-fat diet (29). No study has investigated the effect of longer-term high-carbohydrate compared to high-protein diets on intestinal cholesterol absorption and endogenous cholesterol synthesis.

As mentioned before, we previously found a significant diurnal rhythm for the K-R pathway intermediate lathosterol, but not for the Bloch pathway intermediate desmosterol (13). In agreement, we here found that meal consumption decreased postprandial lathosterol, but not desmosterol, levels. In fact, three out of the four measured K-R pathway intermediates showed a significant postprandial decrease, whereas only one of the measured Bloch intermediates decreased. Based on the findings, it is tempting to hypothesize that the K-R pathway may be more responsive to external influences, such as cholesterol-lowering treatments or dietary intake, than the Bloch pathway. Several studies have indeed reported that dietary interventions significantly affected lathosterol, but not desmosterol, levels (12, 16, 30), although not all studies support this (15, 31). It should be noted that recent research suggested that desmosterol has several functions other than cholesterol synthesis alone (32), which may explain the stable desmosterol production.

In the present study, cholesterol precursors were measured in serum and do not necessarily reflect cholesterol synthesis in a single tissue but is rather a mixture of several tissues. Recently, Mitsche et al. have reported that the use of endogenous synthesis pathways is highly variable between tissues (33). Several tissues used the Bloch pathway for cholesterol synthesis, whereas a cross-over from the Bloch to the K-R pathway was detected in other tissues (33). The regulation of the pathways in humans warrants further study, because it is not completely clear to what extent the two pathways are differentially regulated by external factors, including diet, and how this regulation differs among various tissues.

In conclusion, this study shows that consumption of a single high-fat, high-carbohydrate, or high-protein meal did not significantly affect intestinal cholesterol absorption levels or total cholesterol

concentrations. Meal consumption decreased endogenous cholesterol synthesis over a 4-h postprandial period, as indicated by decreased serum levels of cholesterol-standardised lanosterol and several intermediates in the Bloch (zymosterol) and K-R pathway (7-dehydrocholesterol, lathosterol, and zymostenol). The separate macronutrients did not differentially affect these serum levels, except for dihydrolanosterol. Overall, these findings suggest that the reciprocal relation between endogenous cholesterol synthesis and intestinal cholesterol absorption is not present in a postprandial state.

SUPPLEMENTARY MATERIALS

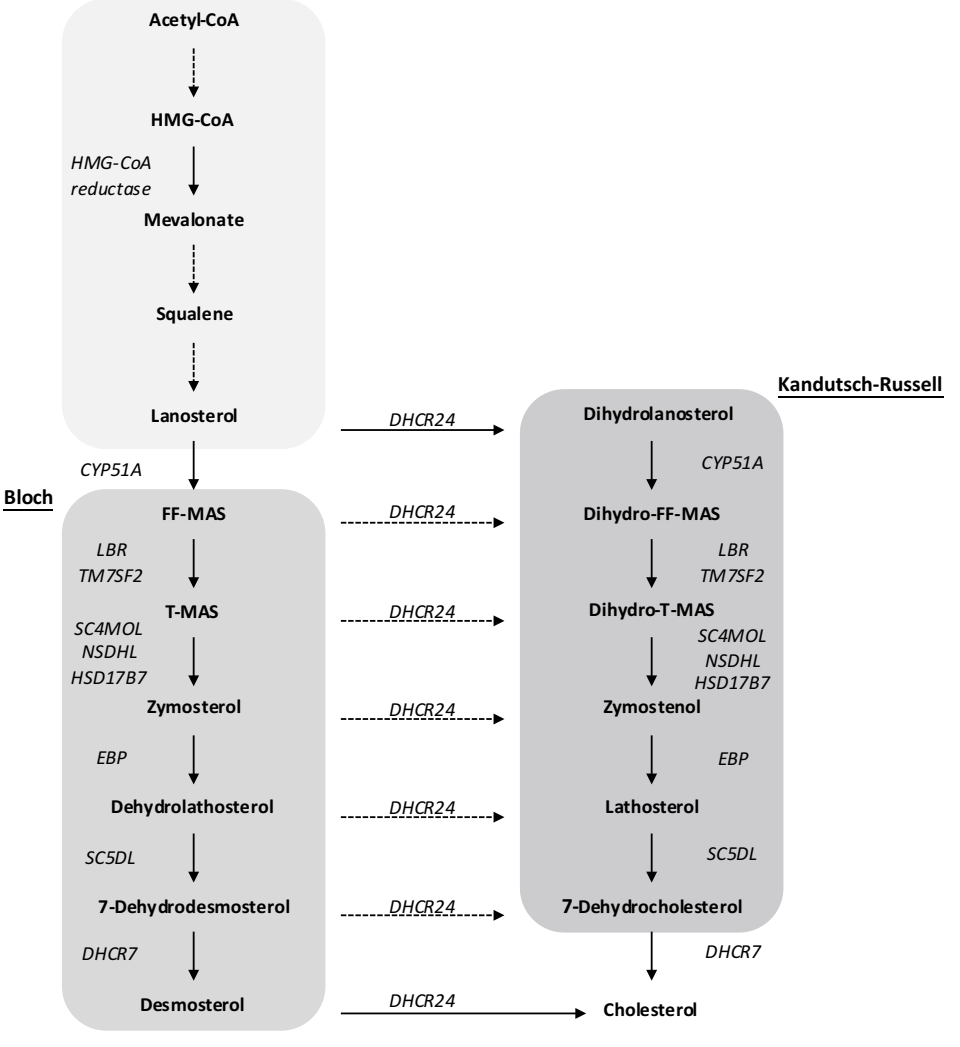

Figure S4.1. An overview of the endogenous cholesterol synthesis pathway, including the Bloch and Kandutsch-Russell pathway

Table S4.1. Serum total cholesterol concentrations and cholesterol-standardized absorption and synthesis marker levels at T0 and the changes between T0 and T240 following the high-fat, high-carbohydrate, and high-protein meal in apparently healthy overweight and slightly obese men with a relatively low (*n* = 9) and high (*n* = 9) fasting lathosterol/campesterol-ratio

		High-fat				High-carbohydrate				High-protein			
		Lower-ratio group		Higher-ratio group		Lower-ratio group		Higher-ratio group		Lower-ratio group		Higher-ratio group	
		T0	Change	T0	Change	T0	Change	T0	Change	T0	Change	T0	Change
	TC	5.5 ± 1.1	-0.02 (-0.2 – 0.2)	5.1 ± 0.8	0.2 (0.03 – 0.4)	5.5 ± 1.0	-0.03 (-0.2 – 0.1)	5.1 ± 0.9	-0.1 (-0.2 – 0.1)	5.3 ± 1.0	0.1 (-0.1 – 0.2)	5.1 ± 0.9	-0.03 (-0.2 – 0.2)
Cholesterol Absorption	CAMP	328 ± 100	-2 (-13 – 10)	226 ± 67	-2 (-13 – 10)	313 ± 105	2 (-9 – 14)	211 ± 56	1 (-11 – 12)	311 ± 109	-0.1 (-12 – 11)	206 ± 61	3 (-9 – 14)
	CHOL	147 ± 30	-0.1 (-5.1 – 4.9)	120 ± 15	-2.7 (-7.7 – 2.3)	153 ± 23	-2.3 (-7.3 – 2.7)	120 ± 13	4.9 (-0.1 – 9.9)	142 ± 29	3.0 (-2.0 – 8.0)	123 ± 14	0.7 (-4.3 – 5.8)
	SITO	167 ± 59	-9 (-24 – 5)	147 ± 66	-13 (-27 – 2)	163 ± 52	-5 (-19 – 10)	129 ± 37	-1 (-16 – 13)	158 ± 29	4 (-11 – 19)	130 ± 40	5 (-10 – 19)
Cholesterol Synthesis	LAN	10 ± 2	-2 (-4 – 1)	14 ± 7	-4 (-6 – -2)	9 ± 3	-1 (-3 – 2)	13 ± 4	-2 (-5 – 0.01)	9 ± 2	-1 (-4 – 1)	14 ± 4	-3 (-5 – -0.4)
Bloch Pathway	7-DHD	6.3 ± 1.7	-0.4 (-1.3 – 0.5)	6.7 ± 1.5	0.3 (-0.6 – 1.1)	6.0 ± 1.4	0.2 (-0.6 – 1.1)	7.5 ± 2.5	-0.8 (-1.6 – 0.1)	6.1 ± 1.1	-0.6 (-1.4 – 0.3)	7.4 ± 1.9	-0.7 (-1.6 – 0.2)
	DESM	132 ± 24	-2 (-13 – 8)	150 ± 27	-2 (-12 – 9)	133 ± 26	2 (-8 – 12)	149 ± 26	1 (-9 – 11)	133 ± 21	-9 (19 – 2)	156 ± 25	2 (-8 – 13)
	ZYM-R	4.7 ± 0.7	-0.2 (-0.6 – 0.2)	5.9 ± 1.1	-0.4 (-0.8 – 0.02)	4.8 ± 0.9	-0.2 (-0.6 – 0.2)	6.3 ± 1.7	-0.6 (-1.0 – -0.2)	4.5 ± 0.7	-0.1 (-0.5 – 0.3)	6.4 ± 1.2	-0.6 (-1.0 – -0.2)
Kandutsch-Russell Pathway	7-DHC	94 ± 8	-3 (-9 – 2)	103 ± 12	-4 (-10 – 1)	95 ± 11	-2 (-8 – 3)	103 ± 15	-1 (-7 – 4)	93 ± 6	-1 (-6 – 5)	106 ± 14	-8 (-13 – -2)
	DHL	0.6 ± 0.2	-0.1 (-0.2 – 0.05)	1.0 ± 0.5	-0.2 (-0.3 – -0.1)	0.6 ± 0.2	0.5 (-0.1 – 0.2)	1.0 ± 0.5	-0.1 (-0.2 – 0.05)	0.6 ± 0.2	-0.1 (-0.2 – 0.1)	1.0 ± 0.4	-0.04 (-0.2 – 0.1)
	LATH	178 ± 40	-9 (-29 – 11)	268 ± 50	-24 (-44 – -4)	181 ± 44	-11 (-31 – 9)	277 ± 99	-33 (-53 – -13)	168 ± 28	-9 (-29 – 11)	289 ± 71	-45 (-65 – -24)
	ZYM-N	26 ± 4	-0.1 (-2.7 – 2.5)	38 ± 8	-2.5 (-5.1 – 0.1)	26 ± 6	0.3 (-2.3 – 2.9)	38 ± 11	-3.5 (-6.1– -0.9)	26 ± 5	-1.5 (-4.1 – 1.1)	40 ± 11	-4.1 (-6.7 – 1.4)

Abbreviations: CAMP, campesterol; CHOL, cholestanol; 7-DHC, 7-dehydrocholesterol; 7-DHD, 7-dehydrodesmosterol; DESM, desmosterol; DHL, dihydrolanosterol; LANO, lanosterol; LATH, lathosterol; SITO, sitosterol; ZYM-N, zymostenol; ZYM-R, zymosterol. *Notes:* Data at T0 are presented as mean ± SD and changes as estimated marginal means with 95% confidence interval (CI) in $10^2 \times$ µmol/ mmol cholesterol, except for total cholesterol (mmol/

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CHAPTER 5

Relation between single nucleotide polymorphisms in circadian clock relevant genes and cholesterol metabolism

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ABSTRACT

Single nucleotide polymorphisms (SNPs) in circadian clock relevant genes are associated with several metabolic health variables, but little is known about their associations with human cholesterol metabolism. Therefore, this study examined associations between SNPs in A*RNTL, ARNTL2, CLOCK, CRY1, CRY2, PER2,* and *PER3* with the intestinal cholesterol absorption markers campesterol and sitosterol, the endogenous cholesterol synthesis marker lathosterol, and total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) concentrations in 456 healthy individuals from Western European descent. One SNP in *ARNTL2* (rs1037924) showed a significant association with lathosterol. Several SNPs in *ARNTL* (rs4146388, rs58901760, rs6486121), *ARNTL2* (rs73075788), *CLOCK* (rs13113518, rs35115774, rs6832769), and *CRY1* (rs2078074) were significantly associated with intestinal cholesterol absorption. Genetic variants in *CRY2, PER2,* and *PER3* were not significantly associated with intestinal cholesterol absorption or endogenous cholesterol synthesis. None of the SNPs were associated with TC or LDL-C, except for one SNP in *PER2* (rs11894491) with serum LDL-C concentrations. The findings suggest that various SNPs in *ARNTL, ARNTL2, CLOCK* and *CRY1* play a role in intestinal cholesterol absorption and endogenous cholesterol synthesis, which was not reflected in TC and LDL-C concentrations. The significant associations between SNPs and intestinal cholesterol absorption and endogenous cholesterol synthesis should be validated in other cohorts.

INTRODUCTION

Diurnal rhythms are biological rhythms that are influenced by extrinsic and intrinsic factors. Numerous physiological processes follow a diurnal rhythm and are thus repeated approximately every 24 h (1). The master clock located in the hypothalamic suprachiasmatic nucleus mainly regulates these rhythms. Peripheral clocks located within tissues combine signals from the master clock with their own autonomous rhythms and with external factors to rhythmically regulate metabolism (2). Disruptions of these rhythms, such as misalignment between the sleep-wake cycle and the biological night or between the light-dark cycle and dietary intake, have been associated with features of metabolic syndrome and may increase the risk of developing cardiovascular diseases (3, 4). Diurnal variations of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) concentrations have been examined before and findings were mixed. Various studies have reported a significant diurnal rhythm of total cholesterol and LDL-C concentrations (5-7) with TC peaks in the morning (6), afternoon (5), and evening (7), and LDL-C peaks in the morning (5, 6) and afternoon (7). Another study however found no evidence for a diurnal rhythm of total cholesterol and LDL-C concentrations (8). For cholesterol metabolism, a diurnal rhythm has been observed in serum levels of lathosterol (9), which is a cholesterol precursor that reflects endogenous cholesterol synthesis rates (10). In contrast, no diurnal pattern has been found for serum levels of the plant sterols campesterol and sitosterol (9), which both reflect fractional intestinal cholesterol absorption (10). Endogenous cholesterol synthesis primarily takes place in the liver and starts with acetyl-CoA which is finally converted into cholesterol via a number of intermediate steps that involve over twenty different enzymes (11). The absorption of cholesterol mainly takes place in the intestine. Niemann-Pick type C1-like intracellular cholesterol transporter 1 (NPC1L1) facilitates the uptake of cholesterol from the intestinal lumen into the enterocyte (12). The proteins adenine triphosphate binding cassette subfamily G member 5 (ABCG5) and member 8 (ABCG8) efflux part of the cholesterol back from the enterocyte into the intestinal lumen (13, 14).

The master and peripheral clocks work through a transcriptional-translational feedback loop (TTFL), which is regulated by different genes and proteins. The proteins encoded by clock circadian regulator (*CLOCK*) and aryl hydrocarbon receptor nuclear translocator like (*ARNTL* and *ARNTL2*) form a heterodimer in the nucleus that promotes the transcription of period circadian regulators (*PER1*, *PER2*, and *PER3*) and cryptochrome circadian regulators (*CRY1* and *CRY2*) in de cytoplasm (2, 15). PER and CRY then dimerize and enter the nucleus, where they inhibit the function of the CLOCK/ARNTL heterodimer, thereby repressing their own transcription (15). The degradation of the PER and CRY proteins releases the CLOCK/ARNTL heterodimer from suppression, which restarts the transcription of PER and CRY. This TTFL takes approximately 24 h to complete and is present in almost all mammalian cells (2).

Various single nucleotide polymorphisms (SNPs) in these genes essential in the TTFL have been associated with human metabolic health. For instance, associations between these SNPs and fasting serum lipid and lipoprotein concentrations, and individual components and presence of metabolic syndrome, have been reported (16-20). Much less is known about the relations of SNPs in genes essential in circadian regulation with intestinal cholesterol absorption and endogenous cholesterol synthesis, and whether these relations may underlie possible associations with serum TC and LDL-

C concentrations. We have therefore examined associations between selected SNPs in *ARNTL, ARNTL2, CLOCK, CRY1, CRY2, PER1, PER2,* and *PER3* with fasting serum lathosterol, campesterol, sitosterol, TC, and LDL-C concentrations in healthy individuals of Western European descent.

METHODS

Study design

This cross-sectional study used baseline data from individuals of Western European descent that were enrolled in one of five human intervention studies, which were carried out between 1997 and 2012 at Maastricht University, The Netherlands. Four of these studies have been published (21-24). For all studies, only samples that were collected at baseline or at the end of a control period were used (for further details see (25)). All five studies have been conducted according to the Declaration of Helsinki.

Study subjects

An overview of subject characteristics for each separate study and the full study sample can be found in **Table S5.1**. In brief, the pooled study sample consisted of 456 apparently healthy adults (53.7% women) with a body mass index (BMI) that ranged from 17.3 to 39.9 kg/m^2. Participants did not suffer from diabetes or coronary heart disease and also did not take lipid-lowering medication. The protocols of the five studies were approved by the Medical Ethical Committee of Maastricht University Medical Centre (Study 1 approval number: MEC 96-181; Study 2 approval number: MEC 97-204; Study 3 approval number: MEC 99-206; Study 4 approval number: MEC 07-3-027; Study 5 approval number: METC 12-3-005). All participants provided written informed consent before the start of the studies.

Serum analyses

In each study, blood samples from individuals who had fasted overnight were collected in the early morning between 08:00 and 11:00 h. Serum was obtained by centrifuging the samples at 2000 g for 15 to 30 min at 4 °C at least an hour after sample collection. The aliquots were stored at -80 °C until further analysis. Enzymatic methods were used to measure serum TC (CHOD-PAP method; Roche Diagnostics Systems, Hoffmann-La Roche Ltd, Basel, Switzerland), high-density lipoprotein cholesterol (HDL-C) (precipitation method by adding phosphotungstic acid and magnesium ions and CHOD-PAP method; Roche Diagnostics Systems, Hoffmann-La Roche Ltd., Basel, Switzerland) and triacylglycerol (TAG) with correction for free glycerol (GPO-Trinder; Sigma Diagnostics, St. Louis, USA) concentrations. Serum LDL-C concentrations were calculated according to Friedewald's formula (26). Gas chromatography-mass spectrometry was used to determine serum campesterol, sitosterol, and lathosterol concentrations in Study 2, 3, and 4 (24, 27), whereas gas chromatography with flame-ionization detection was used to measure these serum non-cholesterol sterol concentrations in Study 1 and 5 (21, 23). Non-cholesterol sterol concentrations were adjusted for TC concentrations and expressed as $10^2 \times$ µmol/mmol TC, because these sterols are transported by cholesterol-rich lipoproteins in serum. Moreover, these TC-standardized concentrations have been validated as markers for endogenous cholesterol synthesis (lathosterol) and intestinal cholesterol absorption (campesterol and sitosterol) (10). Non-cholesterol sterol concentrations were unavailable for N=1.

Genotyping and quality control

A detailed description of the procedures for deoxyribonucleic acid (DNA) extraction, genotyping, and quality control has been published before (25). In summary, the QIAamp genomic DNA isolation kit (Westburg BV, Leusden, the Netherlands) was used to isolate genomic DNA from full blood or buffy coats. Purity of the genomic DNA was checked and samples were then stored at -80 ◦C. The quality of the samples for genotyping was adequate, which was shown by the absence of DNA degradation products on agarose gel. The AxiomTM Precision Medicine Research Array (Thermo Fisher Scientific, Waltham, MA, USA) was used to genotype a total of 471 samples (28). We excluded SNPs that had a call rate < 98%, had a minor allele frequency (MAF) < 5%, were located on sex chromosomes, or had a Hardy-Weinberg Equilibrium (HWE) test p-value < 1e-10 by using PLINK version 1.90 beta software (www.cog-genomics.org/plink/1.9/) (29). In addition, heterozygosity outliers (± 3 standard deviations (SD) from the mean), and sex discrepancies between DNA samples and clinical records were removed. Finally, 456 samples passed the genotyping quality control. The present study only included SNPs that were present on the AxiomTM Precision Medicine Research Array (Thermo Fisher Scientific, Waltham, MA, USA) and located in *ARNTL, ARNTL2, CLOCK, CRY1, CRY2, PER1, PER2*, or *PER3*.

Statistical analysis

The allele frequencies of each SNP were calculated and compared with data from another European cohort obtained from the National Center for Biotechnology Information (NCBI) (30). Distributions of genotypes were tested for deviation from HWE by chi-square tests in Microsoft Excel. Then, pairwise linkage disequilibrium (LD) was calculated in Haploview (version 4.2; Broad Institute of MIT and Harvard, Cambridge, MA, USA) (31). In addition, haplotype blocks were built between the selected SNPs in each gene based on the algorithm described by Gabriel et al. (32). Each block included at least 95% of informative SNPs that were in strong LD (32). Insertion and deletion variants were not included in the LD analysis, which were *ARNTL* (rs58901760), *CLOCK* (rs35115774), and *PER2* (rs3217472). Next, tag SNPs were selected using the Tagger feature implemented in Haploview version 4.2 (31). The pairwise tagging option and r^2-values of at least 0.8 were used to select tag SNPs.

The General Linear Model (GLM) procedure, adjusted for the factor study, was performed to examine associations between the SNPs and serum non-cholesterol sterols (i.e., TC-standardized campesterol, sitosterol, lathosterol, and the lathosterol/campesterol-ratio), TC concentrations, and LDL-C concentrations. SNPs with genotype groups that contained < 2.5% of the individuals of the complete cohort (i.e., less than N = 12) were excluded from analyses in the main text. For this reason, results for SNPs in *PER1* are only presented in the supplemental information and should be interpreted with caution. Bonferroni post-hoc tests were performed to make pairwise comparisons between genotypes of SNPs that were significantly associated with the non-cholesterol sterols or lipids. The Benjamini-Hochberg adjustment was applied to the results of the GLM analysis to control the false discovery rate (FDR), which was set at 0.2 (33). This adjustment was done within each individual gene and outcome parameter and only for SNPs with genotype groups of at least 12

people. FDR adjusted p-values, called q-values, were estimated for each nominal p-value. These q-values are a measure of the proportion of nominal significant results (i.e., a p-value < 0.05), which were actually false.

In addition, either additive, recessive, or dominant multiple linear regression models were made for each SNP that was significantly associated with one or more of the non-cholesterol sterols, serum TC concentrations, or serum LDL-C concentrations. These models were adjusted for the factor study. In case the most frequent homozygous genotype (e.g. MM) and the heterozygous genotype (e.g. mM) had a comparable relation with the outcome, a recessive model was built. This model compared MM + mM with mm. In contrast, the dominant model compared MM with mM + mm. An additive model was made in case the Bonferroni post-hoc test did not indicate which genotypes within a SNP were significantly different or in case the two homozygous groups differed significantly from each other. In this model, each additional copy of an allele increased the dependent variable by the same amount. Statistical analyses were performed using SPSS software version 26.0 for Mac OS X (SPSS Inc., Chicago, IL, USA) and p-values < 0.05 indicated nominal statistical significance. We have reported the results of the statistical analyses for the tag SNPs in the main text. Results for their associated SNPs have been reported in the supplements.

RESULTS

Table 5.1 presents the location, MAF, and HWE for each SNP. Comparable minor and major allele frequencies were reported for our cohort and another European cohort obtained from NCBI (30). Genotype distributions were in accordance with HWE for all SNPs ($p > 0.05$), except for one SNP in *ARNTL2* (rs7300833), one SNP in *CLOCK* (rs12648271), and two SNPs in *CRY1* (rs2078074 and rs7967939) ($p < 0.05$).

Linkage disequilibrium

Fig. 5.1 presents LD structures for *ARNTL, ARNTL2, CLOCK, CRY1, CRY2, PER2,* and *PER3*. *ARNTL* (rs11824092, rs6486121, and rs4146388), *CLOCK* (rs6832769 and rs62303722), *CRY1* (rs2078074), and *PER3* (rs875994) were included as tag SNPs due to high pairwise LD with their captured SNPs ($r^2 > 0.8$) (**Table 5.2**). No high LD was reported between SNPs in *ARNTL2, CRY2*, and *PER2*, and hence no tag SNPs were selected in these genes (**Fig. 5.1b, e, f**).

Associations with intestinal cholesterol absorption and endogenous cholesterol synthesis

Three SNPs in *ARNTL* were significantly associated with serum intestinal cholesterol absorption markers, of which two with sitosterol (rs58901760, p=0.039, q-value=0.240; rs4146388, p=0.048, q-value=0.240) and one with campesterol (rs6486121, p=0.018, q-value=0.180). No significant relations with lathosterol levels were found for SNPs in *ARNTL* (p>0.05) (**Table 5.3** and **S5.2; Fig. S5.1**). One SNP in *ARNTL2* (rs73075788) was significantly associated with sitosterol levels (p=0.038, q-value=0.152) and the SNP *ARNTL2* (rs1037924) with lathosterol levels (p=0.031, q-value=0.124) (**Table 5.3, S5.2** and **S5.3; Fig. S5.2**). The associations between SNPs in *ARNTL* (rs58901760 and rs4146388) with sitosterol levels did not remain significant after correction for multiple comparisons, whereas the other associations did. Three SNPs in *CLOCK* were significantly associated with campesterol levels (rs13113518, p=0.026, q-value=0.052; rs35115774, p=0.017,

q-value=0.051; rs6832769, p=0.017, q-value=0.051), and two with sitosterol levels (rs35115774, p=0.013, q-value=0.039; rs6832769, p=0.013, q-value=0.039) (**Table 5.3** and **S5.4; Fig. S5.3**). These associations remained significant after correction for multiple testing. None of the SNPs in *CLOCK* showed a significant association with serum lathosterol levels (p>0.05). *CLOCK* (rs13113518) was significantly associated with the lathosterol/campesterol-ratio (p=0.009) (**Table S5.5**). Individuals with the TT-genotype had a significantly lower ratio compared to the TC-genotype (p=0.018) (**Fig. S5.4**). No other significant associations were reported between the selected SNPs with genotype groups of at least 2.5% of the population and this ratio (p>0.05).

One SNP in *CRY1* (rs2078074) was significantly related to sitosterol levels (p=0.025, q-value=0.050), also after correction for multiple comparisons (**Table 5.4; Fig. S5.5**). None of the other SNPs in *CRY1, CRY2, PER2*, and *PER3* were associated with lathosterol, campesterol, or sitosterol levels (p>0.05) (**Table 5.4**). One significant association between a SNP in *PER2* (rs11894491) with serum LDL-C concentrations was found (p=0.036, q-value=0.072), which did persist after correction of multiple comparisons (**Table S5.6; Fig. S5.6**). SNPs in *ARNTL, ARNTL2, CLOCK, CRY1, CRY2*, and *PER3* were not significantly associated with serum LDL-C or TC concentrations (**Table S5.6**). All results for SNPs that had a genotype group of <12 individuals or that were tagged by a tag SNP are presented in **Table S5.7- S5.10.**

Stratification for men and women

The analyses were repeated for men and women separately (**Table S5.11–S5.14**). For men, significant associations with campesterol levels were observed for *ARNTL* (rs56051850 [p=0.004] and rs6486121 [p<0.001]), *CLOCK* (rs13113518 [p=0.013]), and *PER3* (rs875994 [p=0.035]). In addition, significant associations were reported for *ARNTL* (rs11022778 [p=0.043] and rs6486121 [p=0.003]), *CLOCK* (rs13113518 [p=0.040], rs35115774 [p=0.026], and rs6832769 [p=0.026]), and *PER3 (*rs875994 [p=0.034]) with sitosterol levels in men. No significant relations were found between the selected SNPs and lathosterol levels, LDL-C concentrations or TC concentrations in male subjects. For women, only a SNP in *CLOCK* (rs12648271 [p=0.040]) was significantly associated with lathosterol levels. Furthermore, significant associations were found between *CRY1* (rs2078074) and *PER2* (rs76784767) with both campesterol and sitosterol levels (p<0.05). Finally, *ARNTL* (rs4757142) was associated with serum LDL-C concentrations (p=0.028) and *ARNTL2* (rs1037924) with serum TC concentrations (p=0.041) in women.

Table 5.1. Description of selected single nucleotide polymorphisms in *ARNTL, ARNTL2, CLOCK, CRY1, CRY2, PER1, PER2,* and *PER3*

Gene	SNP ID	Location	Alleles		MAF		HWE
			Major allele	Minor allele	Present Cohort	European Cohort	P-value
ARNTL	rs10832020	Intron	T	C	0.152	0.161	0.383
	rs11022756	Intron	C	A	0.299	0.302	0.799
	rs11022776	Intron	C	G	0.073	0.035	0.675
	rs11022778	Intron	T	G	0.373	0.327	0.406
	rs11824092	Intron	C	T	0.379	0.402	0.087
	rs1562437	Intron	C	T	0.051	0.046	0.256
	rs17452044	Intron	G	A	0.171	0.179	0.836
	rs34834014	Intron	T	C	0.091	0.090	0.660
	rs4146388	Intron	C	T	0.274	0.211	0.470
	rs4757142	Intron	A	G	0.404	0.407	0.871
	rs4757144	Intron	A	G	0.416	0.419	0.052
	rs56051850	Intron	G	A	0.229	0.163	0.952
	rs58901760	Intron	GG	-	0.258	0.279	0.248
	rs6486121	Intron	T	C	0.401	0.371	0.502
	rs6486122	Intron	T	C	0.355	0.421	0.692
	rs7107287	Intron	G	T	0.273	0.258	0.478
	rs7130064	Intron	C	T	0.139	0.134	0.951
	rs72867447	Intron	C	G	0.439	0.443	0.744
	rs9633835	Intron	G	A	0.365	0.379	0.145
ARNTL2	rs1037924	Intron	A	G	0.420	0.452	0.935
	rs3751222	Intron	G	C	0.141	0.116	0.267
	rs4931075	Intron	G	A	0.209	0.194	0.396
	rs4964055	Intron	G	T	0.110	0.100	0.817
	rs7300833	Intron	A	G	0.095	0.089	0.031
	rs73075788	Intron	G	A	0.180	0.111	0.579
CLOCK	rs12648271	Intron	G	C	0.235	0.179	0.020
	rs13113518	Intron	T	C	0.340	0.361	0.188
	rs1522108	Intron	C	T	0.361	0.335	0.344
	rs35115774	Indel	C	-	0.360	0.336	0.414
	rs57826934	Intron	C	T	0.361	0.350	0.344
	rs62303689	Prime UTR	C	A	0.138	0.118	0.811
	rs62303722	Intron	A	C	0.302	0.300	0.554
	rs6554283	Intron	G	T	0.300	0.381	0.527
	rs6832769	Intron	A	G	0.360	0.352	0.414
CRY1	rs10861688	Intron	C	T	0.172	0.188	0.619
	rs2078074	Intron	T	C	0.423	0.468	0.017
	rs7967939	Intron	G	A	0.452	0.500	0.036
CRY2	rs11605924	Intron	C	A	0.490	0.473	0.645
	rs72902436	Intron	G	A	0.250	0.241	0.532
PER1	rs2518023	Intron	G	T	0.092	0.099	0.627
	AX83126559	N.A.	N.A.	N.A.	0.159	N.A.	0.593
PER2	rs11894491	Intron	G	A	0.337	0.312	0.262
	rs3217472	Intron	CAC	CACAC	0.075	0.096	0.094
	rs76784767	Intron	C	A	0.149	0.095	0.291
PER3	rs170631	Intron	C	G	0.112	0.096	0.894
	rs228654	Intron	G	A	0.113	0.104	0.932
	rs228669	Synonymous	C	T	0.080	0.070	0.190
	rs228690	Intron	C	T	0.095	0.085	0.935
	rs2797685	Intron	C	T	0.164	0.183	0.798
	rs4908482	Intron	G	A	0.368	0.371	0.153
	rs61773390	Intron	G	T	0.175	0.195	0.199
	rs875994	Intron	T	C	0.165	0.171	0.056

Abbreviations: HWE, Hardy-Weinberg Equilibrium; MAF, minor allele frequency; N.A., not available; SNP, single-nucleotide polymorphism. *Note:* MAFs European cohort were derived from dbSNP build 155 (release version: 20201027095038) (30).

Table 5.2. List of tag SNPs in *ARNTL, CLOCK, CRY1,* and *PER3*

Gene	Tag SNP	Tagged SNP	r^2-value
ARNTL	rs11824092	rs4757144	0.808
		rs9633835	0.801
ARNTL	rs4146388	rs7107287	1.000
		rs11022756	0.879
ARNTL	rs6486121	rs6486122	0.822
CLOCK	rs6832769	rs1522108	0.995
CLOCK	rs62303722	rs6554283	1.000
CRY1	rs2078074	rs7967939	0.890
PER3	rs875994	rs61773390	0.895

The Tagger software within Haploview version 4.2 was used to select tag SNPs (31).

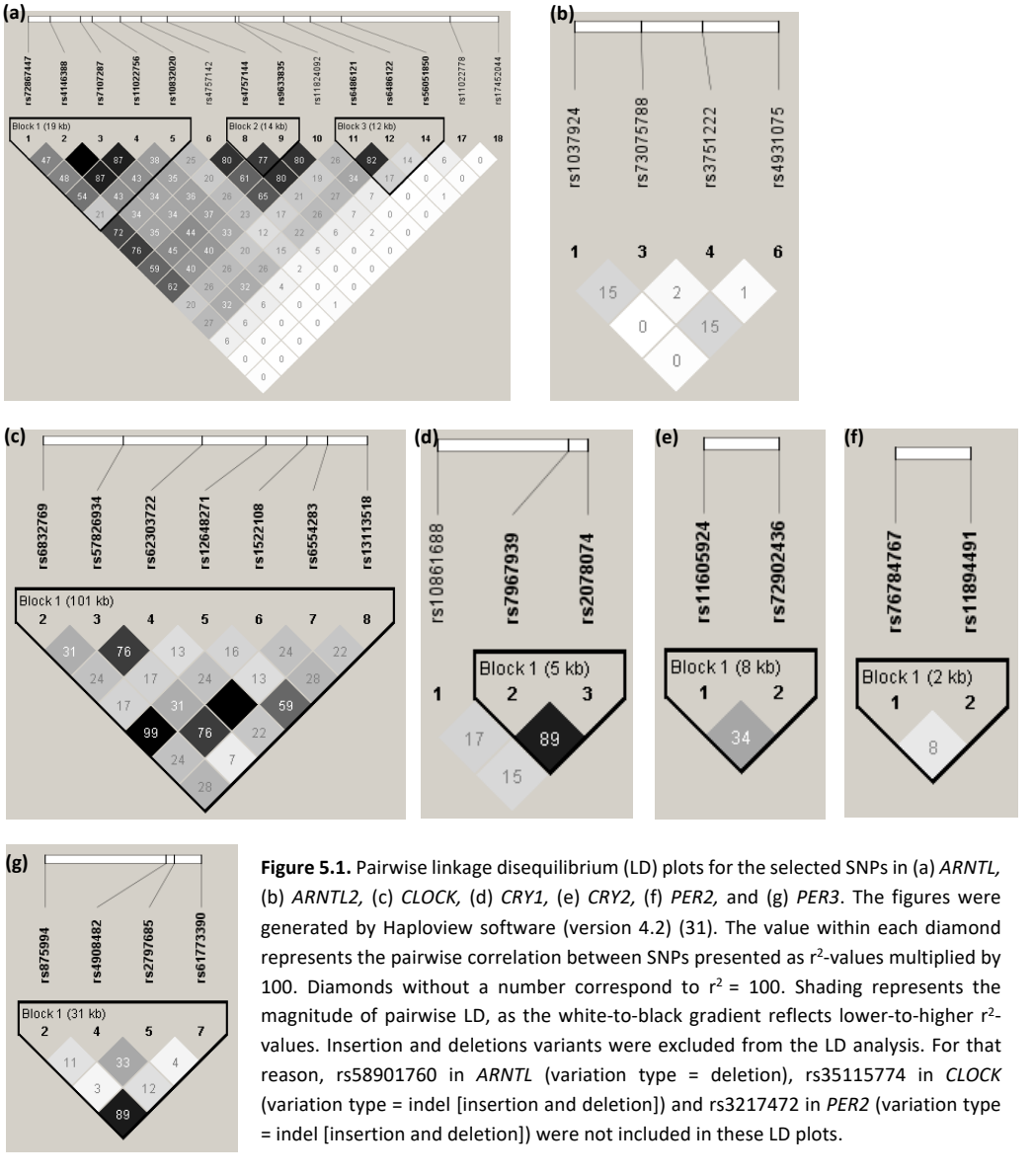

Figure 5.1. Pairwise linkage disequilibrium (LD) plots for the selected SNPs in (a) *ARNTL,* (b) *ARNTL2,* (c) *CLOCK,* (d) *CRY1,* (e) *CRY2,* (f) *PER2,* and (g) *PER3*. The figures were generated by Haploview software (version 4.2) (31). The value within each diamond represents the pairwise correlation between SNPs presented as r^2-values multiplied by 100. Diamonds without a number correspond to $r^2 = 100$. Shading represents the magnitude of pairwise LD, as the white-to-black gradient reflects lower-to-higher r^2-values. Insertion and deletions variants were excluded from the LD analysis. For that reason, rs58901760 in *ARNTL* (variation type = deletion), rs35115774 in *CLOCK* (variation type = indel [insertion and deletion]) and rs3217472 in *PER2* (variation type = indel [insertion and deletion]) were not included in these LD plots.

Table 5.3. Relation between selected SNPs in *ARNTL, ARNTL2,* and *CLOCK* (i.e., the positive regulators in the transcriptional-translational feedback loop) with serum lathosterol, campesterol, and sitosterol levels in 455 individuals of Western European descent

Gene	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
ARNTL	rs10832020	TT	330	121 (113 – 129)		243 (228 – 258)		145 (135 – 155)	
		TC	112	118 (107 – 130)	0.890	252 (231 – 272)	0.584	150 (137 – 164)	0.741
		CC	13	121 (92 – 152)		227 (172 – 282)		142 (107 – 177)	
	rs11022778	TT	175	116 (106 – 125)		253 (235 – 270)		153 (142 – 164)	
		TG	221	125 (115 – 134)	0.459	238 (221 – 255)	0.371	140 (130 – 151)	0.180
		GG	58	121 (106 – 137)		242 (213 – 270)		145 (127 – 164)	
	rs11824092	TT	74	113 (100 – 126)		249 (225 – 274)		148 (132 – 163)	
		TC	196	123 (114 – 133)	0.374	253 (236 – 270)	0.111	151 (140 – 163)	0.168
		CC	184	121 (111 – 131)		232 (214 – 250)		139 (128 – 151)	
	rs17452044	AA	14	113 (84 – 143)		257 (204 – 311)		144 (109 – 178)	
		AG	128	114 (103 – 125)	0.291	248 (228 – 269)	0.779	145 (132 – 158)	0.975
		GG	312	123 (115 – 131)		243 (228 – 258)		147 (137 – 156)	
	rs4146388	TT	37	112 (94 – 130)		265 (232 – 298)		154 (133 – 175)	
		TC	173	120 (110 – 130)	0.501	252 (234 – 269)	0.085	154 (143 – 165) [A]	**0.048*** **
		CC	242	123 (114 – 132)		235 (218 – 251)		139 (128 – 150) [B]	
	rs4757142	AA	162	121 (111 – 132)		231 (212 – 250)		138 (126 – 150)	
		AG	216	121 (112 – 130)	0.820	253 (236 – 269)	0.104	153 (142 – 163)	0.082
		GG	75	117 (104 – 130)		246 (222 – 270)		143 (128 – 159)	
	rs56051850	AA	24	137 (115 – 160)		210 (168 – 251)		137 (110 – 164)	
		AG	160	124 (114 – 134)	0.113	241 (223 – 259)	0.140	143 (132 – 155)	0.513
		GG	270	117 (108 – 125)		250 (234 – 265)		149 (139 – 159)	
	rs58901760	--	35	112 (94 – 131)		268 (234 – 302)		159 (137 – 181)	
		-GG	164	121 (111 – 131)	0.631	251 (234 – 269)	0.082	153 (141 – 165)	**0.039***
		GGGG	256	122 (113 – 131)		235 (219 – 251)		139 (128 – 149)	

Table 5.3. *Cont.*

Gene	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
ARNTL	rs6486121	TT	160	123 (113 – 134)		226 (207 – 245) [A]		136 (125 – 148)	
		TC	225	120 (111 – 129)	0.474	255 (239 – 271) [B]	**0.018*****	152 (142 – 163)	0.052
		CC	70	114 (100 – 128)		249 (224 – 275) [B]		147 (131 – 164)	
	rs72867447	GG	86	115 (103 – 128)		248 (226 – 271)		147 (133 – 162)	
		GC	227	121 (111 – 130)	0.440	253 (236 – 269)	0.055	151 (140 – 162)	0.130
		CC	142	125 (114 – 135)		228 (208 – 247)		137 (125 – 150)	
ARNTL2	rs1037924	AA	153	113 (103 – 123) [A]		245 (226 – 264)		149 (136 – 161)	
		AG	223	121 (112 – 130)	**0.031***	247 (230 – 263)	0.812	143 (132 – 153)	0.455
		GG	79	133 (120 – 146) [B]		238 (214 – 262)		152 (137 – 168)	
	rs3751222	CC	12	119 (87 – 150)		222 (164 – 279)		140 (103 – 177)	
		CG	105	117 (105 – 128)	0.714	237 (216 – 258)	0.443	143 (129 – 157)	0.739
		GG	338	122 (114 – 130)		248 (233 – 263)		148 (138 – 157)	
	rs4931075	AA	23	125 (102 – 148)		232 (190 – 274)		143 (116 – 170)	
		AG	145	117 (107 – 128)	0.656	251 (232 – 270)	0.550	147 (134 – 159)	0.961
		GG	287	122 (113 – 130)		242 (227 – 258)		146 (137 – 156)	
	rs73075788	AA	13	118 (88 – 149)		267 (212 – 322)		172 (137 – 208)	
		AG	138	122 (111 – 133)	0.906	234 (214 – 254)	0.259	136 (124 – 149)	**0.038***
		GG	304	120 (112 – 128)		248 (233 – 263)		150 (140 – 159)	
CLOCK	rs12648271	CC	34	133 (114 – 152)		224 (189 – 258)		136 (114 – 158)	
		CG	145	121 (111 – 132)	0.330	237 (218 – 257)	0.194	144 (131 – 156)	0.454
		GG	276	118 (110 – 127)		251 (235 – 266)		149 (139 – 159)	
	rs13113518	TT	205	117 (108 – 126)		257 (240 – 273)		153 (142 – 163)	
		TC	191	123 (113 – 132)	0.254	235 (218 – 253)	**0.026***	142 (130 – 153)	0.099
		CC	59	129 (114 – 144)		224 (197 – 251)		135 (118 – 153)	

Table 5.3. *Cont.*

Gene	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
CLOCK	rs35115774	--	63	120 (105 – 135)		276 (249 – 303)		167 (149 – 184) [A]	
		-C	202	120 (111 – 129)	0.979	246 (229 – 263)	**0.017***	148 (137 – 158)	**0.013***
		CC	190	121 (111 – 131)		235 (218 – 252)		140 (128 – 151) [B]	
	rs57826934	TT	64	112 (97.7 – 126)		242 (216 – 268)		143 (126 – 159)	
		TC	200	122 (113 – 132)	0.381	241 (224 – 258)	0.588	143 (132 – 154)	0.265
		CC	191	122 (112 – 132)		251 (233 – 269)		153 (141 – 164)	
	rs62303722	AA	224	123 (114 – 133)		246 (228 – 263)		149 (138 – 160)	
		AC	185	122 (112 – 131)	0.169	242 (225 – 259)	0.880	144 (133 – 155)	0.713
		CC	44	106 (90 – 123)		250 (219 – 280)		145 (125 – 165)	
	rs6832769	AA	190	121 (111 – 131)		235 (218 – 252)		140 (128 – 151) [A]	
		AG	202	120 (111 – 129)	0.979	246 (229 – 263)	**0.017***	148 (137 – 158)	**0.013***
		GG	63	120 (105 – 135)		276 (249 – 303)		167 (149 – 184) [B]	

Abbreviation: SNP, single-nucleotide polymorphism. *Notes:* P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. Non-cholesterol sterol levels were presented in 10^2 * µmol/mmol TC. Different superscript letters between genotypes indicate statistical significance ($p < 0.05$, Bonferroni correction). * An additive model was made. ** A recessive model was made. *** A dominant model was made.

Table 5.4. Relation between selected SNPs in *CRY1, CRY2, PER2,* and *PER3* (i.e., the negative regulators in the transcriptional-translational feedback loop) with serum lathosterol, campesterol, and sitosterol levels in 455 individuals of Western European descent

Gene	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
CRY1	rs2078074	TT	139	125 (114 – 136)		237 (217 – 257)		144 (132 – 157)	
		TC	246	120 (111 – 129)	0.425	243 (227 – 259)	0.125	142 (132 – 152) [A]	**0.025****
		CC	69	114 (100 – 128)		266 (241 – 291)		165 (149 – 182) [B]	
	rs10861688	TT	12	116 (84 – 147)		221 (164 – 278)		143 (107 – 180)	
		TC	132	125 (114 – 136)	0.479	253 (233 – 273)	0.386	153 (140 – 166)	0.396
		CC	311	119 (110 – 127)		242 (227 – 257)		144 (134 – 153)	
CRY2	rs11605924	AA	112	118 (107 – 130)		243 (222 – 264)		143 (129 – 156)	
		AC	223	119 (110 – 128)	0.603	239 (222 – 256)	0.388	144 (133 – 155)	0.363
		CC	120	125 (114 – 136)		255 (235 – 275)		153 (140 – 166)	
	rs72902436	AA	26	123 (101 – 145)		211 (171 – 250)		129 (104 – 155)	
		AG	176	119 (109 – 129)	0.877	249 (232 – 267)	0.181	146 (135 – 157)	0.343
		GG	253	121 (112 – 130)		245 (228 – 261)		148 (138 – 159)	
PER2	rs11894491	AA	53	118 (102 – 134)		238 (209 – 268)		142 (123 – 161)	
		AG	198	117 (108 – 127)	0.568	242 (225 – 260)	0.797	145 (134 – 157)	0.832
		GG	202	123 (114 – 132)		247 (231 – 264)		148 (137 – 158)	
	rs76784767	AA	13	104 (74 – 134)		254 (200 – 308)		149 (114 – 184)	
		AC	110	120 (108 – 131)	0.504	261 (240 – 282)	0.112	157 (143 – 171)	0.121
		CC	332	122 (114 – 130)		239 (224 – 254)		143 (133 – 152)	
PER3	rs2797685	TT	13	110 (79 – 140)		250 (196 – 305)		133 (98 – 168)	
		TC	123	123 (112 – 134)	0.686	247 (227 – 266)	0.838	148 (135 – 161)	0.710
		CC	316	121 (112 – 129)		241 (226 – 256)		145 (135 – 155)	
	rs4908482	AA	69	120 (106 – 134)		245 (220 – 271)		139 (122 – 155)	
		AG	198	121 (112 – 131)	0.948	244 (227 – 261)	0.982	148 (137 – 159)	0.553
		GG	188	120 (110 – 130)		246 (227 – 264)		147 (136 – 159)	
	rs875994	TT	322	123 (114 – 131)		242 (227 – 257)		143 (134 – 153)	
		TC	113	117 (105 – 128)	0.608	256 (235 – 276)	0.202	156 (143 – 170)	0.118
		CC	18	119 (94 – 145)		216 (169 – 262)		133 (103 – 163)	

Abbreviation: SNP, single-nucleotide polymorphism. *Notes:* P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. Non-cholesterol sterol levels were presented in 10^2 * µmol/mmol TC. Different superscript letters between genotypes indicate statistical significance ($p < 0.05$, Bonferroni correction). ** A recessive model was made.

DISCUSSION

This study demonstrated that various SNPs in *ARNTL, ARNTL2, CLOCK*, and *CRY1* were associated with intestinal cholesterol absorption and endogenous cholesterol synthesis in a cohort of healthy individuals of Western European descent. Of these, *ARNTL2* (rs1037924) was the only SNP that was significantly associated with lathosterol, whereas several SNPs in *ARNTL, CLOCK*, and *CRY1* showed associations with serum markers for intestinal cholesterol absorption. None of the SNPs in *CRY2, PER2*, and *PER3* were significantly associated with the cholesterol metabolism markers. Furthermore, none of the SNPs were associated with fasting serum TC concentrations, and one SNP in *PER2* (rs11894491) was associated with fasting serum LDL-C concentrations. Finally, although it was not the primary aim of the study, we performed the analyses for men and women separately. The results of these analyses may suggest that differences between sexes exist, which should be examined further in future studies.

SNPs that were significantly associated with the non-cholesterol sterols, reflecting either endogenous cholesterol synthesis or intestinal cholesterol absorption, were in our study not significantly associated with fasting serum TC or LDL-C concentrations. This agrees with previous cross-sectional studies, which also did not find a relation between TC-standardized campesterol, sitosterol, or lathosterol levels with serum TC and LDL-C concentrations (10, 34). For example, no significant associations were observed in cross-sectional studies between cholesterol-adjusted campesterol and lathosterol levels with serum LDL-C concentrations in healthy individuals (34). In contrast, human intervention studies have shown that changing intestinal cholesterol absorption or endogenous cholesterol synthesis affects serum TC and LDL-C concentrations. For instance, statin-induced decreases in lathosterol levels were related to decreases in serum TC and LDL-C concentrations in hypercholesterolemic men (35). Additionally, changing intestinal cholesterol absorption, e.g. by ezetimibe treatment, also influences serum TC and LDL-C concentrations (36). Therefore, it would be of interest to perform a human intervention study, specifically designed to examine the SNPs that we here identified for their cross-sectional associations, with changes in TC and LDL-C concentrations after treatment with compounds that influence either intestinal cholesterol absorption or endogenous cholesterol synthesis.

In the present study, most SNPs that were significantly associated with intestinal cholesterol absorption showed a significant association with TC-standardized campesterol or sitosterol alone, but not with both. Campesterol and sitosterol are both used as markers for intestinal cholesterol absorption, and therefore significant associations with both markers were expected. However, p-values for campesterol and sitosterol were mostly comparable within the same SNP and therefore the non-significant association with one of the two markers was most likely due to chance.

We have earlier reported a significant diurnal rhythm for the endogenous cholesterol synthesis marker lathosterol, whereas no significant rhythms were found for the cholesterol absorption markers campesterol and sitosterol (9). Surprisingly, most significant associations in the present cross-sectional study were found between SNPs and the cholesterol absorption markers, and only one significant association was reported with cholesterol synthesis. These significant findings between SNPs with intestinal cholesterol absorption may suggest that these SNPs are not involved

in regulating possible diurnal rhythms in cholesterol absorption, although that remains speculation and cannot be concluded with certainty.

Previous studies have already demonstrated associations between SNPs in genes essential in the TTFL and cardiometabolic health parameters, such as frequency of being overweight, hypertension, and plasma glucose concentrations (18, 19, 37, 38). Moreover, SNPs in the *CLOCK* gene (rs4580707 and rs1801260) have been related to serum LDL-C and TAG concentrations before (19, 20), but these SNPs were not present on the array used in the current study. These earlier findings could therefore unfortunately not be evaluated. It would also have been interesting to examine combinations of SNPs with markers of cholesterol metabolism, but a larger sample size would be needed to investigate this into detail.

Most SNPs that were associated with non-cholesterol sterols or serum TC or LDL-C concentrations in the present study were located in intron regions. Introns are removed during splicing and therefore do not code for amino acids within proteins. However, genetic variants located in intron regions can disrupt the normal splicing process, which may lead to human diseases (39). It has not been reported in literature whether the intron variant SNPs that were associated with endogenous cholesterol synthesis, intestinal cholesterol absorption or serum TC or LDL-C concentrations in our study are also known to disrupt the normal splicing process and may, in that way, have an impact on human health.

The validity of using non-cholesterol sterols as a measure of intestinal cholesterol absorption and endogenous cholesterol synthesis has been assessed by comparing their serum levels to stable isotope tracer measures of cholesterol metabolism (10). The between-person variation in intestinal cholesterol absorption and endogenous cholesterol synthesis is relatively high compared to the within-person variation. For intestinal cholesterol absorption, previous research reported a between-person variation with a mean of around 56% and range between 29 – 80% (40). The within-person variation in intestinal cholesterol absorption variation was very small, as repeated measures within the same individuals showed a SD of difference between tests of around 3% (41). The endogenous cholesterol synthesis marker lathosterol showed a mean between-person variation of around 50% and a within-person variation of around 23% (42). TC-standardized serum non-cholesterol sterol levels are therefore relevant to classify individuals as high-cholesterol absorbers, high-cholesterol synthesizers, or intermediates. In a recent systematic review, non-cholesterol sterol levels in individuals with various metabolic disorders were compared with those of healthy controls (43). It was suggested that either intestinal cholesterol absorption or endogenous cholesterol synthesis was higher in specific disorders compared with healthy controls. For example, increased intestinal cholesterol absorption was reported in individuals with type I diabetes, non-familial hypercholesterolemia, and cardiovascular diseases (43). Increased endogenous cholesterol synthesis was reported in individuals with overweight or obesity, type II diabetes, familial combined hyperlipidemia, metabolic syndrome, and hepatic steatosis and cholestasis (43). For future research, it would be interesting to examine whether genotypes of SNPs that were associated with intestinal cholesterol absorption or endogenous cholesterol synthesis in our study are also more prevalent in individuals with specific metabolic conditions that have been

linked to intestinal cholesterol absorption or endogenous cholesterol synthesis.

In conclusion, several SNPs in *ARNTL, ARNTL2, CLOCK*, and *CRY1* were associated with cholesterol metabolism in a cohort of healthy individuals from Western European descent. Most associations were reported between the SNPs and intestinal cholesterol absorption, as only one significant association with endogenous cholesterol synthesis was reported. SNPs in *CRY2, PER2*, and *PER3* were not significantly associated with cholesterol metabolism. Finally, no significant associations were found between SNPs in *ARNTL, ARNTL2, CLOCK, CRY1, CRY2*, and *PER3* with fasting serum TC and LDL-C concentrations, and only one association was found between a SNP in *PER2* with LDL-C concentrations. This study can be considered as a first step towards a better understanding of SNPs located in circadian rhythm relevant genes and endogenous cholesterol synthesis and intestinal cholesterol absorption. Future studies should be carried out to reproduce these findings in individual cohorts. In addition to studying associations between individual SNPs and intestinal cholesterol absorption and endogenous cholesterol synthesis, it would also be interesting to study combinations of SNPs. The results may be used in the future to provide individuals with optimal dietary or pharmacological treatments that are known to lower elevated endogenous cholesterol synthesis or intestinal cholesterol absorption.

Table S5.1. An overview of subject characteristics for each separate study and the complete study sample

Variable	Complete sample[1]	Study 1	Study 2	Study 3	Study 4	Study 5
Sample size, n	456	108	34	39	257	18
Males, n (%)	202 (44.3%)	39 (36.1%)	11 (32.4%)	16 (41.0%)	129 (50.2%)	7 (38.9%)
Non-smoker, n (%)[2]	355 (77.9%)	87 (80.6%)	Unknown	35 (89.7%)	215 (83.7%)	18 (100%)
BMI (kg/m^2)[3]	25.1 ± 3.6	23.1 ± 2.9	22.8 ± 2.5	25.3 ± 3.0	26.3 ± 3.6	23.9 ± 2.8
Age (years)	45.4 ± 15.3	33.0 ± 14.9	31.2 ± 13.8	50.0 ± 11.9	53.0 ± 10.1	33.0 ± 12.2
Campesterol (10^2 × µmol/mmol cholesterol)[4]	214 ± 120	321 ± 100	241 ± 126	256 ± 96	156 ± 95	249 ± 92
Sitosterol (10^2 × µmol/mmol cholesterol)[4]	137 ± 65	121 ± 44	153 ± 66	166 ± 54	136 ± 71	155 ± 60
Lathosterol (10^2 × µmol/mmol cholesterol)[4]	107 ± 55	104 ± 35	131 ± 40	113 ± 54	101 ± 61	153 ± 70
Total cholesterol (mmol/L)	5.5 ± 1.0	5.0 ± 0.8	5.0 ± 0.9	6.0 ± 0.8	5.7 ± 1.05	5.4 ± 0.8
LDL-cholesterol (mmol/L)	3.5 ± 1.0	2.9 ± 0.8	3.1 ± 0.9	3.9 ± 0.8	3.8 ± 0.9	3.2 ± 0.8
HDL-cholesterol (mmol/L)	1.5 ± 0.4	1.6 ± 0.4	1.4 ± 0.3	1.5 ± 0.4	1.4 ± 0.4	1.6 ± 0.3
Triacylglycerol (mmol/L)	1.0 (0.9-1.0)	0.8 (0.8-0.9)	1.0 (0.9-1.2)	1.1 (0.9-1.3)	1.0 (0.9-1.1)	1.3 (1.1-1.5)

Notes: Baseline characteristics are presented as mean ± standard deviation, unless stated otherwise. Fasting triacylglycerol concentrations were not normally distributed and therefore a log-transformation was applied. Triacylglycerol concentrations in the table represent the back-transformed data (median [interquartile range]).

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

[1] Statistical comparison of the five separate studies using ANOVA (continuous) or chi-square (categorical) tests showed that all characteristics differed significantly between the studies ($p < 0.001$) except for sex ($p = 0.064$).

[2] N = 410 due to missing data for N = 46.

[3] N = 445 due to missing data for N = 11.

[4] N = 455 due to missing data for N = 1.

Table S5.2. Additive models for SNPs in *ARNTL, ARNTL2,* and *CLOCK* with TC-standardized sitosterol levels

Gene	SNP ID	Alleles	Sitosterol		
			β	95% CI for β	P-value
ARNTL	rs58901760	GG>-	11.79	2.54 – 21.04	0.013
ARNTL2	rs73075788	G>A	-5.45	-16.43 – 5.23	0.329
CLOCK	rs1522108	C>T	12.27	3.84 – 20.70	0.004
	rs35115774	C>-	12.17	3.71 – 20.64	0.005
	rs6832769	A>G	12.17	3.71 – 20.64	0.005

Notes: Alleles are presented as major allele>minor allele. Sitosterol concentrations were normalized to total cholesterol concentrations and presented in $10^2 \times$ µmol/mmol TC. P-values were obtained from a multiple linear regression analysis adjusted for the covariate study. The beta-coefficient (β) represents the change in sitosterol levels for each copy of the minor allele relative to the major allele.

Table S5.3. Additive model for *ARNTL2* (rs1037924) with TC-standardized lathosterol levels

Gene	SNP ID	Alleles	Lathosterol		
			β	95% CI for β	P-value
ARNTL2	rs1037924	A>G	9.51	2.34 – 16.69	0.009

Notes: Alleles are presented as major allele>minor allele. Lathosterol concentrations were normalized to total cholesterol concentrations and presented in $10^2 \times$ µmol/mmol TC. P-values were obtained from a multiple linear regression analysis adjusted for the covariate study. The beta-coefficient (β) represents the change in lathosterol levels for each copy of the minor allele relative to the major allele.

Table S5.4. Additive models for three SNPs in the *CLOCK* gene with TC-standardized campesterol levels

Gene	SNP ID	Alleles	Campesterol		
			β	95% CI for β	P-value
CLOCK	rs13113518	T>C	-17.91	-31.04 – -4.77	0.008
	rs35115774	C>-	18.03	4.92 – 31.15	0.007
	rs6832769	A>G	18.03	4.92 – 31.15	0.007

Notes: Alleles are presented as major allele>minor allele. Campesterol concentrations were normalized to total cholesterol concentrations and presented in $10^2 \times$ µmol/mmol TC. P-values were obtained from a multiple linear regression analysis adjusted for the covariate study. The beta-coefficient (β) represents the change in campesterol levels for each copy of the minor allele relative to the major allele.

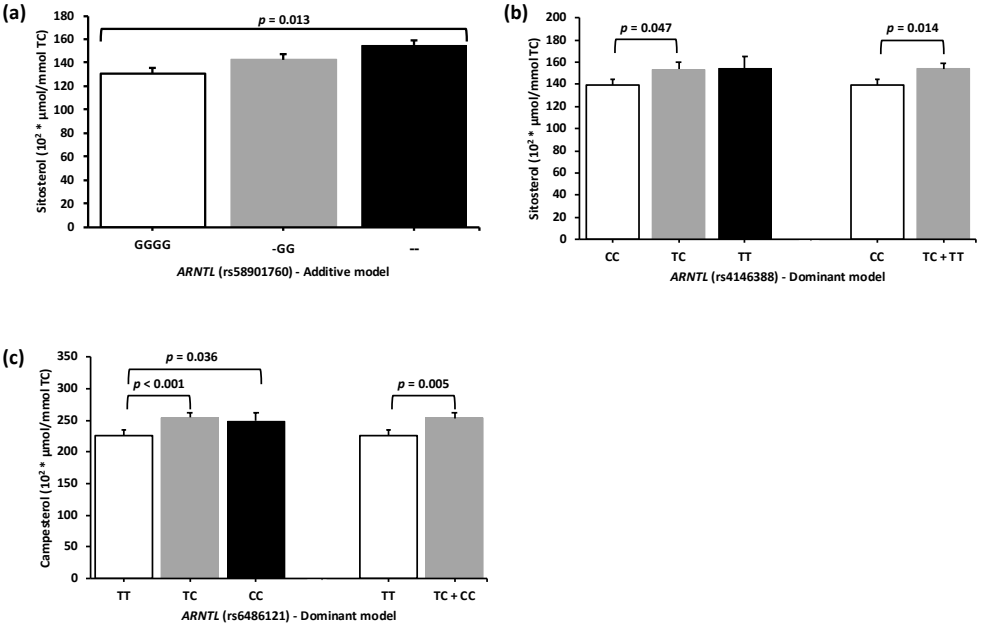

Figure S5.1. Additive model for the relationship between (a) *ARNTL* (rs58901760) and TC-standardized sitosterol levels, and dominant models for (b) *ARNTL* (rs4146388) with TC-standardized sitosterol levels and (c) *ARNTL* (rs6486121) with TC-standardized campesterol levels. The white bar refers to the most frequent homozygous genotype.

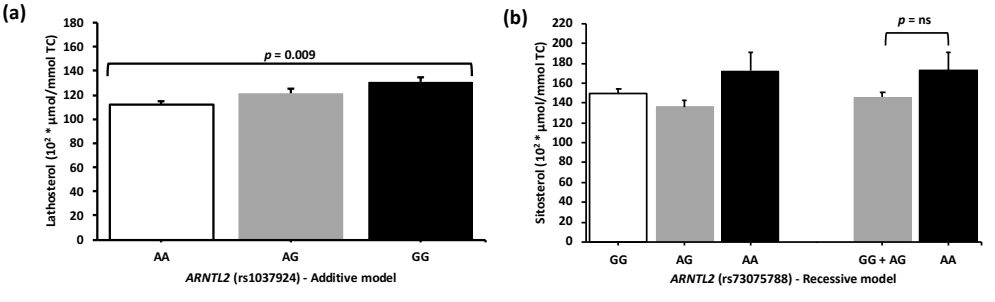

Figure S5.2. Additive model for the relationship between (a) *ARNTL2* (rs1037924) with TC-standardized lathosterol levels, and a recessive model for (b) *ARNTL2* (rs73075788) with TC-standardized sitosterol levels. The white bars refer to the most frequent homozygous genotype.

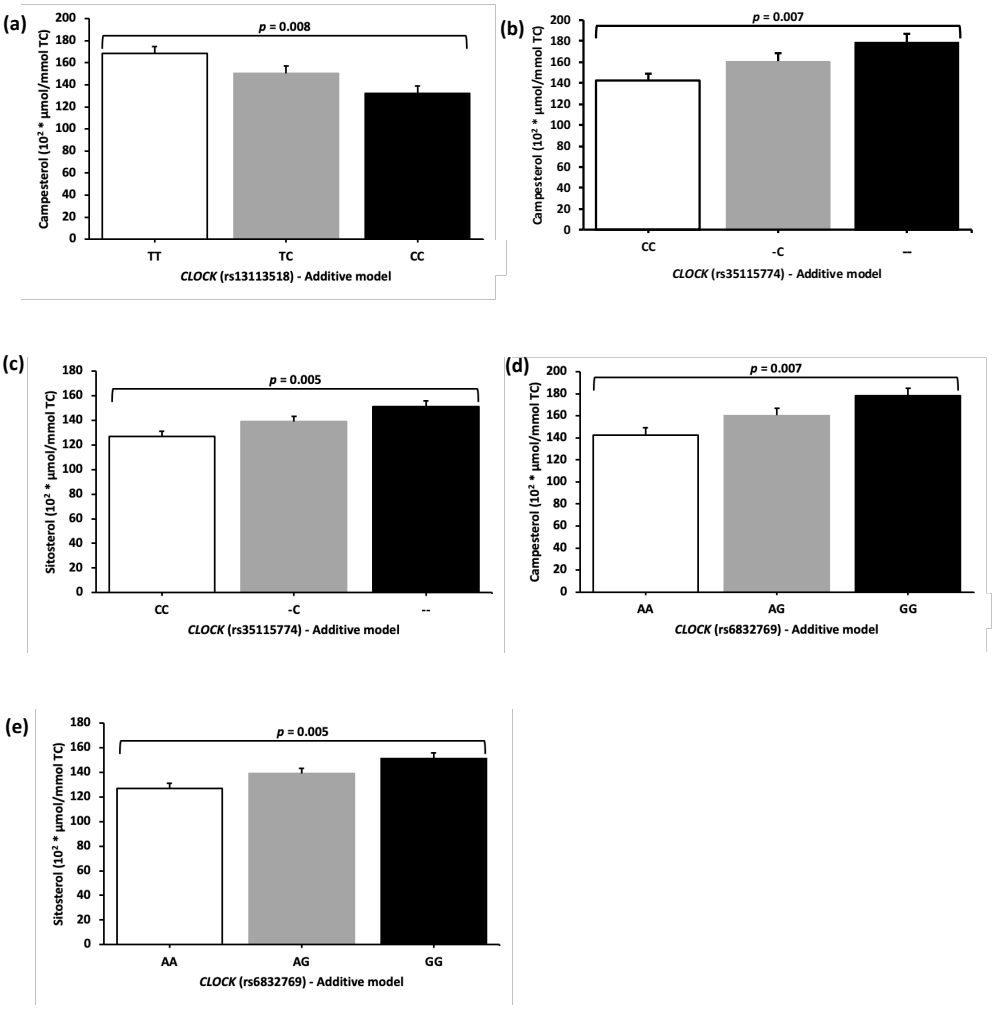

Figure S5.3. Additive models for the relationship between (a) *CLOCK* (rs13113518) with TC-standardized campesterol levels, for *CLOCK* (rs35115774) with (b) TC-standardized campesterol levels and (c) TC-standardized sitosterol levels, and for *CLOCK* (rs6832769) with (d) TC-standardized campesterol levels and (e) TC-standardized sitosterol levels. The white bar refers to the most frequent homozygous genotype.

Table S5.5. Relations between selected SNPs in *ARNTL, ARNTL2, CLOCK, CRY1, CRY2, PER2,* and *PER3* with the serum lathosterol/campesterol-ratio in 455 individuals of Western European descent

Gene	SNP ID	Genotype	N	Lathosterol/campesterol	
				Mean (95% CI)	P-value
ARNTL	rs10832020	TT	330	0.64 (0.55 – 0.72)	
		TC	112	0.67 (0.55 – 0.79)	0.797
		CC	13	0.57 (0.25 – 0.88)	
	rs11022778	TT	175	0.64 (0.54 – 0.74)	
		TG	221	0.66 (0.56 – 0.75)	0.422
		GG	58	0.58 (0.42 – 0.74)	
	rs11824092	TT	74	0.56 (0.42 – 0.70)	
		TC	196	0.67 (0.57 – 0.76)	0.395
		CC	184	0.65 (0.55 – 0.76)	
	rs17452044	AA	14	0.54 (0.23 – 0.85)	
		AG	128	0.58 (0.46 – 0.69)	0.242
		GG	312	0.67 (0.58 – 0.75)	
	rs4146388	TT	37	0.61 (0.42 – 0.80)	
		TC	173	0.60 (0.50 – 0.70)	0.322
		CC	242	0.68 (0.59 – 0.78)	
	rs4757142	AA	162	0.68 (0.57 – 0.79)	
		AG	216	0.63 (0.54 – 0.73)	0.614
		GG	75	0.61 (0.47 – 0.75)	
	rs56051850	AA	24	0.82 (0.58 – 1.10)	
		AG	160	0.68 (0.57 – 0.78)	0.123
		GG	270	0.61 (0.51 – 0.70)	
	rs58901760	--	35	0.48 (0.28 – 0.67)	
		-GG	164	0.69 (0.56 – 0.76)	0.201
		GGGG	256	0.66 (0.56 – 0.75)	
	rs6486121	TT	160	0.69 (0.58 – 0.80)	
		TC	225	0.62 (0.53 – 0.72)	0.408
		CC	70	0.59 (0.45 – 0.74)	
	rs72867447	GG	86	0.61 (0.48 – 0.74)	
		GC	227	0.62 (0.52 – 0.71)	0.264
		CC	142	0.71 (0.60 – 0.82)	
ARNTL2	rs1037924	AA	153	0.56 (0.45 – 0.67)	
		AG	223	0.70 (0.60 – 0.79)	0.061
		GG	79	0.63 (0.49 – 0.77)	
	rs3751222	CC	12	0.71 (0.38 – 1.04)	
		CG	105	0.59 (0.46 – 0.71)	0.475
		GG	338	0.66 (0.57 – 0.74)	
	rs4931075	AA	23	0.69 (0.45 – 0.93)	
		AG	145	0.59 (0.48 – 0.70)	0.474
		GG	287	0.66 (0.57 – 0.75)	
	rs73075788	AA	13	0.51 (0.19 – 0.83)	
		AG	138	0.71 (0.59 – 0.82)	0.223
		GG	304	0.62 (0.53 – 0.70)	
CLOCK	rs12648271	CC	34	0.75 (0.55 – 0.95)	
		CG	145	0.71 (0.60 – 0.83)	0.074
		GG	276	0.60 (0.51 – 0.68)	
	rs13113518	TT	205	0.56 (0.47 – 0.65) [A]	
		TC	191	0.73 (0.62 – 0.84) [B]	0.009***
		CC	59	0.69 (0.53 – 0.85)	
	rs35115774	--	63	0.50 (0.34 – 0.65)	
		-C	202	0.63 (0.54 – 0.73)	0.069
		CC	190	0.69 (0.59 – 0.79)	

Table S5.5. *Cont.*

Gene	SNP ID	Genotype	N	Lathosterol/campesterol	
				Mean (95% CI)	P-value
CLOCK	rs57826934	TT	64	0.57 (0.42 – 0.72)	
		TC	200	0.68 (0.59 – 0.78)	0.293
		CC	191	0.62 (0.52 – 0.82)	
	rs62303722	AA	224	0.63 (0.53 – 0.73)	
		AC	185	0.69 (0.59 – 0.79)	0.170
		CC	44	0.52 (0.34 – 0.69)	
	rs6832769	AA	190	0.69 (0.59 – 0.79)	
		AG	202	0.63 (0.54 – 0.73)	0.069
		GG	63	0.50 (0.34 – 0.65)	
CRY1	rs2078074	TT	139	0.65 (0.54 – 0.77)	
		TC	246	0.66 (0.57 – 0.75)	0.401
		CC	69	0.56 (0.41 – 0.70)	
	rs10861688	TT	12	0.54 (0.21 – 0.87)	
		TC	132	0.68 (0.56 – 0.79)	0.612
		CC	311	0.63 (0.55 – 0.72)	
CRY2	rs11605924	AA	112	0.68 (0.56 – 0.80)	
		AC	223	0.64 (0.54 – 0.74)	0.629
		CC	120	0.61 (0.49 – 0.72)	
	rs72902436	AA	26	0.89 (0.66 – 1.12)	
		AG	176	0.62 (0.52 – 0.72)	0.072
		GG	253	0.63 (0.54 – 0.72)	
PER2	rs11894491	AA	53	0.58 (0.41 – 0.75)	
		AG	198	0.62 (0.52 – 0.72)	0.513
		GG	202	0.67 (0.58 – 0.77)	
	rs76784767	AA	13	0.63 (0.32 – 0.95)	
		AC	110	0.63 (0.51 – 0.75)	0.960
		CC	332	0.65 (0.56 – 0.73)	
PER3	rs2797685	TT	13	0.41 (0.10 – 0.73)	
		TC	123	0.63 (0.51 – 0.74)	0.260
		CC	316	0.67 (0.58 – 0.76)	
	rs4908482	AA	69	0.57 (0.43 – 0.72)	
		AG	198	0.64 (0.54 – 0.74)	0.450
		GG	188	0.68 (0.57 – 0.78)	
	rs875994	TT	322	0.66 (0.57 – 0.74)	
		TC	113	0.57 (0.45 – 0.69)	0.080
		CC	18	0.88 (0.61 – 1.15)	

Abbreviation: SNP, single-nucleotide polymorphism.

Notes: P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. *** A dominant model was made.

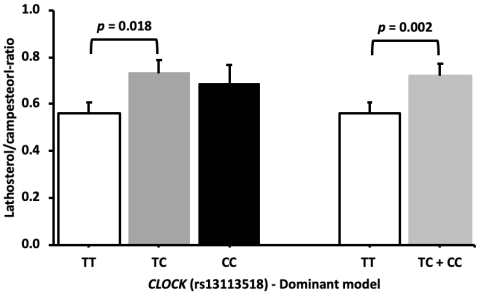

Figure S5.4. Dominant model for the relation between *CLOCK* (rs13113518) with the lathosterol/campesterol-ratio. The white bar refers to the most frequent homozygous genotype.

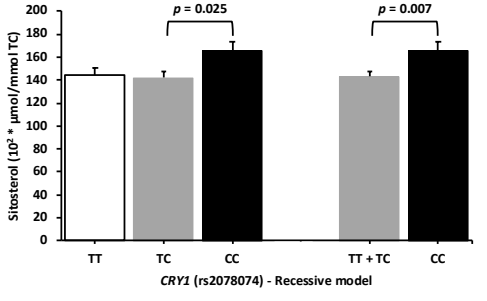

Figure S5.5. Recessive model for the relationship between *CRY1* (rs20780744) with TC-standardized sitosterol levels. The white bar refers to the most frequent homozygous genotype.

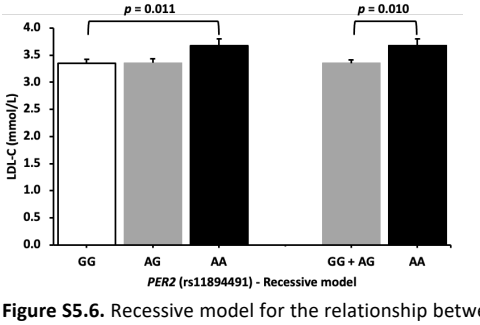

Figure S5.6. Recessive model for the relationship between *PER2* (rs11894491) with serum LDL-C concentrations. The white bar refers to the most frequent homozygous genotype.

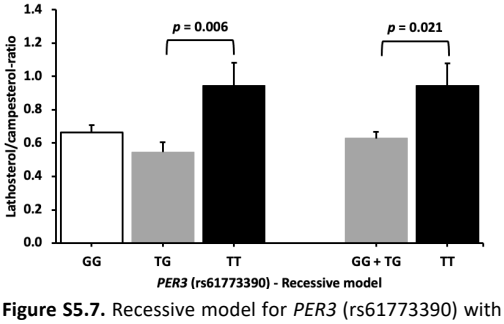

Figure S5.7. Recessive model for *PER3* (rs61773390) with the lathosterol/campesterol-ratio. The white bar refers to the most frequent homozygous genotype.

Table S5.6. Relations between selected SNPs in *ARNTL, ARNTL2, CLOCK, CRY1, CRY2, PER2,* and *PER3* with the serum TC or LDL-C concentrations in 456 individuals of Western European descent

Gene	SNP ID	Genotype	N	LDL-cholesterol		Total cholesterol	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value
ARNTL	rs10832020	TT	330	3.39 (3.26 – 3.52)		5.42 (5.28 – 5.57)	
		TC	113	3.36 (3.12 – 3.54)	0.773	5.33 (5.13 – 5.53)	0.616
		CC	13	3.53 (3.06 – 4.01)		5.49 (4.96 – 6.02)	
	rs11022778	TT	175	3.32 (3.17 – 3.47)		5.34 (5.17 – 5.51)	
		TG	221	3.45 (3.30 – 3.59)	0.478	5.45 (5.28 – 5.61)	0.529
		GG	59	3.40 (3.16 – 3.64)		5.42 (5.15 – 5.69)	
	rs11824092	TT	74	3.28 (3.07 – 3.49)		5.30 (5.07 – 5.54)	
		TC	197	3.46 (3.31 – 3.61)	0.274	5.47 (5.30 – 5.63)	0.362
		CC	184	3.36 (3.20 – 3.52)		5.37 (5.19 – 5.54)	
	rs17452044	AA	14	3.55 (3.08 – 4.02)		5.43 (4.91 – 5.95)	
		AG	128	3.31 (3.13 – 3.38)	0.396	5.29 (5.10 – 5.49)	0.372
		GG	313	3.41 (3.28 – 3.54)		5.44 (5.29 – 5.58)	
	rs4146388	TT	37	3.44 (3.15 – 3.73)		5.42 (5.10 – 5.74)	
		TC	174	3.41 (3.26 – 3.57)	0.887	5.43 (5.26 – 5.60)	0.812
		CC	242	3.38 (3.24 – 3.53)		5.37 (5.21 – 5.54)	
	rs4757142	AA	162	3.31 (3.15 – 3.48)		5.33 (5.15 – 5.52)	
		AG	217	3.46 (3.32 – 3.60	0.190	5.46 (5.30 – 5.62)	0.367
		GG	75	3.30 (3.09 – 3.51)		5.33 (5.09 – 5.56)	
	rs56051850	AA	24	3.63 (3.27 – 3.99)		5.66 (5.26 – 6.06)	
		AG	160	3.35 (3.19 – 3.51)	0.322	5.37 (5.20 – 5.55)	0.379
		GG	271	3.40 (3.26 – 3.53)		5.39 (5.24 – 5.55)	
	rs58901760	--	35	3.39 (3.09 – 3.68)		5.47 (5.14 – 5.80)	
		-GG	165	3.43 (3.27 – 3.59)	0.696	5.42 (5.25 – 5.59)	0.768
		GGGG	256	3.36 (3.21 – 3.50)		5.37 (5.21 – 5.53)	
	rs6486121	TT	160	3.39 (3.23 – 3.56)		5.43 (5.25 – 5.61)	
		TC	226	3.37 (3.23 – 3.51)	0.805	5.35 (5.20 – 5.51)	0.464
		CC	70	3.45 (3.22 – 2.67)		5.50 (5.25 – 5.75)	
	rs72867447	GG	86	3.35 (3.15 – 3.55)		5.40 (5.18 – 5.62)	
		GC	228	3.42 (3.28 – 3.56)	0.762	5.42 (5.26 – 5.58)	0.855
		CC	142	3.36 (3.19 – 3.53)		5.36 (5.17 – 5.55)	
ARNTL2	rs1037924	AA	153	3.49 (3.33 – 3.36)		5.53 (5.35 – 5.71)	
		AG	223	3.32 (3.18 – 3.46)	0.162	5.32 (5.16 – 5.48)	0.112
		GG	80	3.38 (3.18 – 3.59)		5.37 (5.14 – 5.60)	
	rs3751222	CC	12	3.22 (2.72 – 3.72)		5.22 (4.66 – 5.77)	
		CG	105	3.45 (3.26 – 3.63)	0.605	5.43 (5.22 – 5.63)	0.768
		GG	339	3.37 (3.24 – 3.50)		5.39 (5.25 – 5.54)	
	rs4931075	AA	23	3.59 (3.22 – 3.95)		5.52 (5.11 – 5.93)	
		AG	145	3.41 (3.24 – 3.57)	0.497	5.38 (5.19 – 5.56)	0.811
		GG	288	3.37 (3.23 – 3.50)		5.40 (5.25 – 5.55)	
	rs73075788	AA	13	3.55 (3.07 – 4.03)		5.43 (4.89 – 5.96)	
		AG	138	3.34 (3.17 – 3.52)	0.648	5.37 (5.17 – 5.56)	0.889
		GG	305	3.40 (3.27 – 3.53)		5.41 (5.37 – 5.56)	
CLOCK	rs12648271	CC	34	3.35 (3.05 – 3.65)		5.35 (5.02 – 5.69)	
		CG	146	3.44 (3.27 – 3.61)	0.696	5.45 (5.26 – 5.64)	0.735
		GG	276	3.37 (3.24 – 3.50)		5.38 (5.23 – 5.53)	
	rs13113518	TT	205	3.34 (3.20 – 3.49)		5.35 (5.19 – 5.51)	
		TC	191	3.45 (3.30 – 3.61)	0.448	5.48 (5.31 – 5.65)	0.354
		CC	59	3.36 (3.12 – 3.60)		5.35 (5.08 – 5.61)	
	rs35115774	--	63	3.28 (3.04 – 3.51)		5.26 (5.00 – 5.53)	
		-C	202	3.38 (3.24 – 3.53)	0.522	5.41 (5.24 – 5.57)	0.500
		CC	191	3.42 (3.27 – 3.57)		5.42 (5.26 – 5.59)	
	rs57826934	TT	64	3.58 (3.35 – 3.80)		5.57 (5.32 – 5.82)	
		TC	201	3.32 (3.18 – 3.47)	0.121	5.34 (5.18 – 5.50)	0.229
		CC	191	3.39 (3.23 – 3.55)		5.40 (5.23 – 5.58)	
	rs62303722	AA	224	3.38 (3.23 – 3.53)		5.39 (5.22 – 5.55)	
		AC	186	3.35 (3.21 – 3.51)	0.652	5.38 (5.21 – 5.55)	0.753
		CC	44	3.50 (3.23 – 3.76)		5.50 (5.20 – 5.80)	
	rs6832769	AA	191	3.42 (3.27 – 3.57)		5.42 (5.26 – 5.59)	
		AG	202	3.38 (3.24 – 3.53)	0.522	5.41 (5.24 – 5.57)	0.500
		GG	63	3.28 (3.04 – 3.51)		5.26 (5.00 – 5.53)	

Table S5.6. *Cont.*

Gene	SNP ID	Genotype	N	LDL-cholesterol		Total cholesterol	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value
CRY1	rs2078074	TT	139	3.39 (3.21 – 3.56)		5.41 (5.22 – 5.61)	
		TC	247	3.38 (3.24 – 3.52)	0.994	5.39 (5.24 – 5.55)	0.915
		CC	69	3.37 (3.15 – 3.59)		5.36 (5.11 – 5.60)	
	rs10861688	TT	12	3.77 (3.27 – 4.27)		5.78 (5.26 – 6.33)	
		TC	133	3.36 (3.19 – 3.54)	0.289	5.37 (5.18 – 5.56)	0.373
		CC	311	3.38 (3.25 – 3.51)		5.39 (5.25 – 5.54)	
CRY2	rs11605924	AA	112	3.33 (3.14 – 3.51)		5.37 (5.16 – 5.57)	
		AC	223	3.34 (3.19 – 3.48)	0.127	5.35 (5.19 – 5.52)	0.396
		CC	121	3.52 (3.35 – 3.69)		5.50 (5.30 – 5.69)	
	rs72902436	AA	26	3.31 (2.96 – 3.65)		5.37 (4.99 – 5.75)	
		AG	176	3.42 (3.27 – 3.57)	0.761	5.46 (5.29 – 5.63)	0.528
		GG	254	3.37 (3.23 – 3.51)		5.35 (5.20 – 5.51)	
PER2	rs11894491	AA	54	3.68 (3.43 – 3.93) [A]		5.68 (5.41 – 5.96)	
		AG	198	3.36 (3.20 – 3.51)	0.036**	5.38 (5.21 – 5.55)	0.067
		GG	202	3.35 (3.21 – 3.49) [B]		5.35 (5.20 – 5.51)	
	rs76784767	AA	13	3.48 (3.00 – 3.95)		5.53 (5.01 – 6.06)	
		AC	110	3.48 (3.29 – 3.66)	0.396	5.46 (5.26 – 5.67)	0.580
		CC	333	3.35 (3.22 – 3.48)		5.37 (5.23 – 5.51)	
PER3	rs2797685	TT	13	3.32 (2.84 – 3.80)		5.16 (4.63 – 5.69)	
		TC	123	3.35 (3.18 – 3.53)	0.894	5.36 (5.17 – 5.55)	0.617
		CC	316	3.39 (3.25 – 3.52)		5.41 (5.26 – 5.56)	
	rs4908482	AA	69	3.39 (3.17 – 3.61)		5.36 (5.11 – 5.60)	
		AG	198	3.38 (3.23 – 3.52)	0.963	5.40 (5.23 – 5.56)	0.899
		GG	189	3.40 (3.24 – 3.56)		5.42 (5.24 – 5.59)	
	rs875994	TT	322	3.40 (3.27 – 3.52)		5.42 (5.27 – 5.56)	
		TC	114	3.38 (3.20 – 3.56)	0.950	5.40 (5.20 – 5.60)	0.727
		CC	18	3.35 (2.94 – 3.76)		5.23 (4.78 – 5.68)	

Notes: P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. TC and LDL-C concentrations were presented in mmol/L. Different superscript letters between genotypes indicate statistical significance ($p < 0.05$, Bonferroni correction).
* An additive model was made. ** A recessive model was made. *** A dominant model was made.

Table S5.7. Relations between selected SNPs with a genotype group with less than 12 individuals or that were tagged by a tag SNP in *ARNTL, ARNTL2,* and *CLOCK* (i.e., the positive regulators in the transcriptional-translational feedback loop) with serum lathosterol, campesterol, and sitosterol levels in 455 individuals of Western European descent

Gene	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
ARNTL	rs11022756	AA	42	112 (95 – 130)		260 (229 – 291)		154 (134 – 174) [A]	
		AC	188	120 (110 – 129)	0.503	253 (236 – 270)	0.062	154 (143 – 165)	0.015
		CC	225	123 (114 – 132)		233 (216 – 250)		137 (126 – 148) [B]	
	rs11022776	CC	390	121 (114 – 129)		244 (230 – 258)		146 (137 – 155)	
		CG	60	117 (102 – 133)	0.644	237 (210 – 265)	0.050	140 (123 – 158)	0.007
		GG	3	96 (34 – 158)		380 (268 – 493)		259 (186 – 331)	
	rs1562437	TT	0	N/A		N/A		N/A	
		TC	46	115 (98 – 132)	0.442	244 (213 – 275)	0.975	136 (116 – 156)	0.274
		CC	408	121 (113 – 129)		244 (230 – 258)		147 (138 – 156)	
	rs34834014	TT	375	121 (113 – 129)		244 (230 – 258)		146 (137 – 155) [A]	
		TC	77	119 (105 – 132)	0.696	247 (222 – 273)	0.054	149 (133 – 165) [A]	0.008
		CC	3	96 (33 – 158)		381 (269 – 494)		260 (187 – 322) [B]	
	rs4757144	AA	165	122 (112 – 132)		231 (212 – 250)		138 (126 – 150)	
		AG	200	123 (114 – 133)	0.395	250 (233 – 267)	0.111	150 (139 – 161)	0.121
		GG	89	114 (102 – 126)		254 (232 – 276)		151 (137 – 166)	
	rs6486122	TT	187	121 (111 – 131)		227 (209 – 244) [A]		137 (126 – 148) [A]	
		TC	213	122 (113 – 131)	0.302	260 (243 – 276) [B]	0.004	155 (144 – 165) [B]	0.016
		CC	55	110 (94 – 125)		240 (212 – 268)		141 (122 – 159)	
	rs7107287	TT	37	112 (94 – 130)		265 (232 – 298)		154 (133 – 175)	
		TG	174	120 (110 – 129)	0.509	252 (234 – 269)	0.089	154 (142 – 165)	**0.048*****
		GG	244	123 (114 – 132)		235 (218 – 251)		139 (128 – 149)	
	rs7130064	TT	9	125 (89 – 161)		230 (194 – 296)		135 (93 – 178)	
		TC	109	118 (106 – 130)	0.849	242 (221 – 263)	0.836	141 (127 – 155)	0.508
		CC	337	121 (113 – 129)		246 (231 – 261)		148 (139 – 158)	

Table S5.7. *Cont.*

Gene	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
ARNTL	rs9633835	AA	68	113 (99 – 127)		257 (232 – 282)		150 (134 – 166)	
		AG	196	123 (114 – 133)	0.404	248 (231 – 266)	0.204	150 (139 – 162)	0.214
		GG	191	121 (111 – 131)		235 (217 – 252)		140 (129 – 151)	
ARNTL2	rs4964055	TT	5	120 (72 – 168)		262 (174 – 349)		137 (81 – 194)	
		TG	90	114 (101 – 127)	0.476	240 (217 – 263)	0.825	145 (130 – 160)	0.917
		GG	360	122 (114 – 130)		245 (231 – 260)		147 (138 – 156)	
	rs7300833	AA	377	120 (112 – 128)		246 (232 – 261)		147 (137 – 156)	
		AG	69	124 (110 – 138)	0.807	236 (211 – 262)	0.716	145 (129 – 162)	0.984
		GG	8	114 (75 – 152)		239 (168 – 308)		148 (103 – 193)	
CLOCK	rs1522108	TT	64	120 (105 – 135)		277 (250 – 304) [A]		167 (150 – 185) [A]	
		TC	201	120 (111 – 129)	0.977	246 (229 – 262) [B]	0.013	148 (137 – 158)	**0.011**
		CC	190	121 (111 – 131)		235 (218 – 253)		140 (128 – 151) [B]	
	rs62303689	AA	8	102 (63.4 – 140)		324 (255 – 294)		183 (138 – 228)	
		AC	108	120 (108 – 132)	0.614	248 (227 – 268)	0.064	148 (134 – 161)	0.705
		CC	337	121 (113 – 129)		242 (227 – 257)		145 (136 – 154)	
	rs6554283	TT	226	123 (114 – 133)		246 (229 – 263)		149 (138 – 160)	
		TG	185	121 (112 – 131)	0.167	242 (225 – 259)	0.873	144 (133 – 155)	0.244
		GG	44	106 (89.7 – 123)		250 (219 – 280)		145 (125 – 164)	

Abbreviations: N/A, not applicable; SNP, single-nucleotide polymorphism.

Notes: P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. Non-cholesterol sterol levels were presented in 10^2 * µmol/mmol TC. Different superscript letters between genotypes indicate statistical significance ($p < 0.05$, Bonferroni correction).

Table S5.8. Relation between selected SNPs with a genotype group with less than 12 individuals or that were tagged by a tag SNP in *CRY2, PER2,* and *PER3* (i.e., the negative regulators in the transcriptional-translational feedback loop) with serum lathosterol, campesterol, and sitosterol levels in 455 individuals of Western European descent

Gene	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
CRY1	rs7967939	GG	126	125 (113 – 136)		237 (217 – 258)		144 (131 – 157)	
		GA	247	120 (111 – 129)	0.573	243 (227 – 259)	0.259	143 (133 – 153)	0.152
		AA	82	117 (104 – 130)		260 (236 – 283)		158 (143 – 174)	
PER1	rs2518023	TT	3	81 (19 – 143)		294 (181 – 407)		173 (100 – 246)	
		TG	78	119 (106 – 132)	0.425	253 (229 – 276)	0.478	149 (133 – 164)	0.712
		GG	374	121 (113 – 129)		242 (228 – 257)		146 (136 – 155)	
	AX83126559	?	10	118 (84 – 153)		267 (205 – 330)		154 (113 – 194)	
		?	125	117 (106 – 127)	0.616	250 (231 – 270)	0.528	148 (136 – 161)	0.838
		?	320	122 (114 – 131)		241 (226 – 257)		145 (135 – 155)	
PER2	rs3217472	CACCAC	392	118 (110 – 126)		225 (230 – 260)		147 (138 – 157)	
		CACCACAC	58	131 (117 – 146)	0.245	254 (227 – 280)	0.147	148 (131 – 165)	0.434
		CACACCACAC	5	119 (71 – 167)		163 (77 – 250)		110 (54 – 166)	
PER3	rs170631	CC	358	119 (112 – 127)		243 (229 – 258)		145 (136 – 154)	
		CG	90	126 (113 – 139)	0.496	254 (230 – 277)	0.269	154 (139 – 169)	0.315
		GG	6	111 (66 – 155)		190 (110 – 271)		121 (69 – 173)	
	rs228654	AA	6	111 (66 – 155)		190 (110 – 270)		121 (69 – 173)	
		AG	91	126 (113 – 139)	0.517	254 (230 – 277)	0.268	154 (139 – 169)	0.317
		GG	358	119 (112 – 127)		243 (229 – 258)		145 (136 – 154)	
	rs228669	TT	5	98 (49 – 146)		302 (214 – 390)		162 (105 – 218)	
		TC	63	120 (106 – 135)	0.640	224 (197 – 251)	0.098	136 (119 – 154)	0.358
		CC	385	121 (113 – 129)		247 (233 – 261)		148 (139 – 157)	
	rs228690	TT	4	94 (40 – 147)		197 (100 – 295)		129 (66 – 192)	
		TC	79	126 (112 – 139)	0.428	251 (227 – 276)	0.529	155 (139 – 171)	0.411
		CC	372	120 (112 – 128)		244 (230 – 258)		145 (136 – 154)	
	rs61773390	TT	18	125 (99 – 150)		211 (164 – 258)		128 (98 – 159)	
		TG	123	114 (103 – 125)	0.280	256 (236 – 276)	0.131	156 (144 – 169)	0.071
		GG	314	123 (115 – 131)		242 (226 – 257)		143 (133 – 153)	

Abbreviations: N/A, not applicable; SNP, single-nucleotide polymorphism. *Notes:* P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. Non-cholesterol sterol levels were presented in 10^2 * µmol/mmol TC. Different superscript letters between genotypes indicate statistical significance ($p < 0.05$, Bonferroni correction).

Table S5.9. Relations between selected SNPs with a genotype group with less than 12 individuals or that were tagged by a tag SNP in *ARNTL, ARNTL2, CLOCK, CRY1, CRY2, PER2,* and *PER3* with the serum lathosterol/campesterol-ratio in 455 individuals of Western European descent

Gene	SNP ID	Genotype	N	Lathosterol/campesterol	
				Mean (95% CI)	P-value
ARNTL	rs11022756	AA	42	0.60 (0.42 – 0.78)	
		AC	188	0.61 (0.51 – 0.70)	0.314
		CC	225	0.69 (0.59 – 0.78)	
	rs11022776	CC	390	0.66 (0.58 – 0.74)	
		CG	60	0.55 (0.39 – 0.70)	0.177
		GG	3	0.23 (-0.42 – 0.88)	
	rs1562437	TT	0	N/A	
		TC	46	0.59 (0.41 – 0.77)	0.522
		CC	408	0.65 (0.57 – 0.73)	
	rs34834014	TT	375	0.66 (0.57 – 0.74)	
		TC	77	0.56 (0.41 – 0.70)	0.176
		CC	3	0.23 (-0.42 – 0.88)	
	rs4757144	AA	165	0.68 (0.57 – 0.79)	
		AG	200	0.66 (0.56 – 0.76)	0.266
		GG	89	0.56 (0.43 – 0.69)	
	rs6486122	TT	187	0.68 (0.58 – 0.78)	
		TC	213	0.62 (0.53 – 0.72)	0.482
		CC	55	0.60 (0.43 – 0.76)	
	rs7107287	TT	37	0.61 (0.42 – 0.80)	
		TG	174	0.60 (0.50 – 0.70)	0.347
		GG	244	0.68 (0.59 – 0.78)	
	rs7130064	TT	9	0.63 (0.25 – 1.01)	
		TC	109	0.63 (0.51 – 0.75)	0.965
		CC	337	0.65 (0.56 – 0.73)	
	rs9633835	AA	68	0.51 (0.37 – 0.65)	
		AG	196	0.69 (0.59 – 0.79)	0.077
		GG	191	0.65 (0.59 – 0.79)	
ARNTL2	rs4964055	TT	5	0.62 (0.12 – 1.12)	
		TG	90	0.61 (0.47 – 0.74)	0.821
		GG	360	0.65 (0.57 – 0.73)	
	rs7300833	AA	377	0.63 (0.55 – 0.72)	
		AG	69	0.67 (0.53 – 0.82)	0.789
		GG	8	0.73 (0.32 – 1.13)	
CLOCK	rs1522108	TT	64	0.50 (0.34 – 0.65)	
		TC	201	0.63 (0.54 – 0.73)	0.066
		CC	190	0.69 (0.59 – 0.79)	
	rs62303689	AA	8	0.35 (-0.05 – 0.75)	
		AC	108	0.67 (0.55 – 0.79)	0.302
		CC	337	0.64 (0.55 – 0.72)	
	rs6554283	TT	226	0.63 (0.53 – 0.72)	
		TG	185	0.69 (0.59 – 0.79)	0.166
		GG	44	0.52 (0.34 – 0.69)	
CRY1	rs7967939	GG	126	0.65 (0.53 – 0.77)	
		GA	247	0.65 (0.56 – 0.74)	0.741
		AA	82	0.60 (0.46 – 0.73)	
PER1	rs2518023	TT	3	0.21 (-0.44 – 0.86)	
		TG	78	0.67 (0.54 – 0.81)	0.373
		GG	374	0.64 (0.55 – 0.72)	
	AX83126559	?	10	0.45 (0.09 – 0.81)	
		?	125	0.63 (0.52 – 0.74)	0.523
		?	320	0.65 (0.56 – 0.74)	
PER2	rs3217472	CACCAC	392	0.63 (0.54 – 0.71)	
		CACCACAC	58	0.68 (0.53 – 0.83)	0.756
		CACACCACAC	5	0.74 (0.23 – 1.24)	
PER3	rs170631	CC	358	0.63 (0.55 – 0.72)	
		CG	90	0.68 (0.55 – 0.82)	0.768
		GG	6	0.67 (0.20 – 1.23)	
	rs228654	AA	6	0.67 (0.20 – 1.13)	
		AG	91	0.68 (0.54 – 0.81)	0.802
		GG	358	0.63 (0.55 – 0.72)	
	rs228669	TT	5	0.35 (-0.16 – 0.86)	
		TC	63	0.74 (0.59 – 0.90)	0.186
		CC	385	0.63 (0.55 – 0.71)	
	rs228690	TT	4	0.41 (-0.15 – 1.00)	
		TC	79	0.70 (0.56 – 0.84)	0.463
		CC	372	0.63 (0.55 – 0.72)	
	rs61773390	TT	18	0.95 (0.68 – 1.21) [A]	
		TG	123	0.55 (0.43 – 0.66) [B]	**0.010****
		GG	314	0.66 (0.58 – 0.75)	

Abbreviation: SNP, single-nucleotide polymorphism. *Notes:* P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. Different superscript letters between genotypes indicate statistical significance ($p < 0.05$, Bonferroni correction). ** A recessive model was made.

Table S5.10. Relations between selected SNPs with a genotype group with less than 12 individuals or that were tagged by a tag SNP in *ARNTL, ARNTL2, CLOCK, CRY1, CRY2, PER1, PER2,* and *PER3* with the serum TC or LDL-C concentrations in 456 individuals of Western European descent

Gene	SNP ID	Genotype	N	LDL-cholesterol		Total cholesterol	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value
ARNTL	rs11022756	AA	42	3.34 (3.07 – 3.61)		5.36 (5.06 – 5.66)	
		AC	189	3.43 (3.28 – 3.57)	0.711	5.44 (5.28 – 5.61)	0.667
		CC	225	3.36 (3.21 – 3.51)		5.36 (5.20 – 5.53)	
	rs11022776	CC	391	3.39 (3.26 – 3.51)		5.41 (5.27 – 5.54)	
		CG	60	3.38 (3.14 – 3.62)	0.957	5.35 (5.09 – 5.63)	0.927
		GG	3	3.24 (2.25 – 4.23)		5.29 (4.19 – 6.39)	
	rs1562437	TT	0	-		-	
		TC	46	3.24 (2.97 – 3.51)	0.243	5.24 (4.94 – 5.54)	0.258
		CC	409	3.40 (3.28 – 3.52)		5.41 (5.28 – 5.55)	
	rs34834014	TT	376	3.39 (3.26 – 3.51)		5.40 (5.27 – 5.54)	
		TC	77	3.39 (3.17 – 3.61)	0.959	5.28 (5.14 – 5.63)	0.972
		CC	3	3.24 (2.25 – 4.24)		5.30 (4.20 – 6.39)	
	rs4757144	AA	165	3.37 (3.20 – 3.53)		5.37 (5.18 – 5.55)	
		AG	201	3.44 (3.29 – 3.59)	0.477	5.45 (5.28 – 5.61)	0.630
		GG	89	3.32 (3.12 – 3.51)		5.35 (5.14 – 5.57)	
	rs6486122	TT	187	3.34 (3.18 – 3.50)		5.38 (5.21 – 5.55)	
		TC	214	3.42 (3.27 – 3.56)	0.658	5.39 (5.23 – 5.55)	0.766
		CC	55	3.41 (3.16 – 3.66)		5.49 (5.21 – 5.76)	
	rs7107287	TT	37	3.43 (3.14 – 3.72)		5.42 (5.10 – 5.74)	
		TG	175	3.41 (3.26 – 3.57)	0.763	5.44 (5.27 – 5.61)	0.720
		GG	244	3.36 (3.21 – 3.50)		5.36 (5.20 – 5.52)	
	rs7130064	TT	9	3.21 (2.63 – 3.78)		5.16 (4.52 – 5.80)	
		TC	109	3.39 (3.21 – 3.57)	0.821	5.42 (5.21 – 5.62)	0.751
		CC	338	3.39 (3.26 – 3.52)		5.40 (5.26 – 5.54)	
	rs9633835	AA	68	3.36 (3.14 – 3.58)		5.37 (5.13 – 5.61)	
		AG	197	3.43 (3.28 – 3.58)	0.675	5.45 (5.29 – 5.62)	0.564
		GG	191	3.36 (3.20 – 3.51)		5.35 (5.18 – 5.52)	
ARNTL2	rs4964055	TT	5	3.08 (2.32 – 3.84)		5.17 (4.32 – 6.02)	
		TG	90	3.31 (3.10 – 3.51)	0.426	5.37 (5.14 – 5.59)	0.810
		GG	361	3.41 (3.29 – 3.54)		5.41 (5.27 – 5.55)	
	rs7300833	AA	377	3.42 (3.29 – 3.54)		5.42 (5.28 – 5.55)	
		AG	70	3.25 (3.04 – 3.48)	0.180	5.35 (5.10 – 5.59)	0.361
		GG	8	3.03 (2.42 – 3.65)		4.95 (4.27 – 5.63)	
CLOCK	rs1522108	TT	64	3.28 (3.04 – 3.52)		5.26 (5.00 – 5.53)	
		TC	201	3.38 (3.24 – 3.53)	0.545	5.41 (5.24 – 5.57)	0.500
		CC	191	3.42 (3.27 – 3.57)		5.42 (5.25 – 5.59)	
	rs62303689	AA	8	3.25 (2.64 – 3.86)		5.65 (4.97 – 6.32)	
		AC	109	3.44 (3.25 – 3.62)	0.736	5.39 (5.18 – 5.59)	0.762
		CC	337	3.38 (3.25 – 3.51)		5.40 (5.26 – 5.54)	
	rs6554283	TT	226	3.38 (3.23 – 3.53)		5.39 (5.22 – 5.55)	
		TG	186	3.36 (3.21 – 3.51)	0.651	5.38 (5.21 – 5.55)	0.754
		GG	44	3.50 (3.23 – 3.76)		5.50 (5.20 – 5.79)	
CRY1	rs7967939	GG	126	3.40 (3.23 – 3.58)		5.43 (5.24 – 5.63)	
		GA	248	3.37 (3.23 – 3.51)	0.871	5.39 (5.23 – 5.54)	0.894
		AA	82	3.42 (3.21 – 3.62)		5.38 (5.15 – 5.61)	
PER1	rs2518023	TT	3	3.12 (2.13 – 4.10)		5.37 (4.28 – 6.47)	
		TG	78	3.32 (3.12 – 3.53)	0.642	5.37 (5.15 – 5.60)	0.967
		GG	375	3.41 (3.28 – 3.53)		5.40 (5.26 – 5.55)	
	AX83126559	?	10	3.39 (2.84 – 3.94)		5.54 (4.93 – 6.14)	
		?	125	3.41 (3.24 – 3.58)	0.923	5.46 (5.27 – 5.65)	0.586
		?	321	3.38 (3.24 – 3.51)		5.37 (5.22 – 5.51)	
PER2	rs3217472	CACCAC	393	3.42 (3.29 – 3.54) [A]		5.40 (5.26 – 5.55)	
		CACCACAC	58	3.11 (2.89 – 3.34) [B]	<0.001	5.25 (5.00 – 5.50)	0.021
		CACACCACAC	5	4.61 (3.86 – 5.36) [A]		6.47 (5.63 – 7.31)	
PER3	rs170631	CC	359	3.38 (3.25 – 3.50)		5.37 (5.24 – 5.51)	
		CG	90	3.43 (3.22 – 3.63)	0.709	5.51 (5.28 – 5.74)	0.389
		GG	6	3.63 (2.93 – 4.33)		5.68 (4.91 – 6.46)	
	rs228654	AA	6	3.63 (2.93 – 4.33)		5.68 (4.91 – 6.46)	
		AG	91	3.42 (3.21 – 3.62)	0.731	5.49 (5.27 – 5.72)	0.449
		GG	359	3.38 (3.25 – 3.50)		5.38 (5.24 – 5.51)	

Table S5.10. *Cont.*

Gene	SNP ID	Genotype	N	LDL-cholesterol		Total cholesterol	
				Mean (95% CI)	P-value	Mean (95% CI)	P-value
PER3	rs228669	TT	5	2.94 (2.16 – 3.71)		4.86 (4.01 – 5.71)	
		TC	63	3.35 (3.12 – 3.59)	0.475	5.34 (5.08 – 5.61)	0.410
		CC	386	3.40 (3.27 – 3.52)		5.41 (5.27 – 5.54)	
	rs228690	TT	4	3.80 (2.94 – 4.65)		5.91 (4.97 – 6.86)	
		TC	79	3.36 (3.14 – 3.58)	0.611	5.41 (5.17 – 5.65)	0.533
		CC	373	3.39 (3.26 – 3.51)		5.39 (5.25 – 5.53)	
	rs61773390	TT	18	3.46 (3.05 – 3.88)		5.27 (4.81 – 5.73)	
		TG	124	3.36 (3.19 – 3.53)	0.864	5.38 (5.19 – 5.57)	0.796
		GG	314	3.40 (3.26 – 3.53)		5.41 (5.27 – 5.56)	

Abbreviation: SNP, single-nucleotide polymorphism.

Notes: P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. TC and LDL-C concentrations were presented in mmol/L. Different superscript letters between genotypes indicate statistical significance ($p < 0.05$, Bonferroni correction).

* An additive model was made. ** A recessive model was made. *** A dominant model was made.

Table S5.11. Relation between selected SNPs in *ARNTL, ARNTL2,* and *CLOCK* (i.e., the positive regulators in the transcriptional-translational feedback loop) with serum lathosterol, campesterol, and sitosterol levels for men and women separately

Gene	Sex	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
					Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
ARNTL	Women	rs10832020	TT	187	122 (114 – 130)		232 (213 – 251)		146 (133 – 159)	
			TC	61	119 (108 – 131)	0.814	249 (222 – 277)	0.331	152 (133 – 171)	0.782
			CC	6	129 (95 – 162)		201 (121 – 281)		138 (84 – 193)	
	Men	rs10832020	TT	143	118 (101 – 136)		265 (240 – 289)		144 (129 – 160)	
			TC	51	116 (94 – 138)	0.966	260 (229 – 292)	0.925	149 (129 – 168)	0.890
			CC	7	114 (62 – 166)		252 (177 – 327)		141 (95 – 187)	
	Women	rs11022778[$]	TT	175	115 (105 – 124)		238 (216 – 260)		150 (135 – 165)	
			TG	221	127 (118 – 136)	0.131	232 (210 – 254)	0.738	144 (129 – 159)	0.677
			GG	58	127 (112 – 143)		240 (203 – 278)		151 (125 – 176)	
	Men	rs11022778	TT	71	115 (96 – 135)		285 (256 – 313)		159 (142 – 177)[A]	
			TG	104	120 (102 – 138)	0.856	249 (223 – 275)	0.050	136 (120 – 152)[B]	**0.043**
			GG	26	113 (83 – 142)		245 (203 – 288)		139 (113 – 165)	
	Women	rs11824092	TT	44	117 (104 – 130)		228 (197 – 260)		141 (120 – 162)	
			TC	100	129 (119 – 138)	0.069	242 (219 – 265)	0.648	154 (139 – 170)	0.384
			CC	109	116 (107 – 126)		231 (209 – 253)		143 (128 – 158)	
	Men	rs11824092	TT	30	107 (81 – 134)		285 (247 – 323)		159 (135 – 182)	
			TC	96	116 (98 – 134)	0.409	268 (242 – 294)	0.068	147 (131 – 164)	0.126
			CC	75	126 (105 – 146)		240 (211 – 270)		134 (116 – 152)	
	Women	rs17452044	AA	6	131 (97 – 165)		230 (150 – 311)		129 (75 – 184)	
			AG	70	123 (111 – 134)	0.818	237 (210 – 264)	0.981	148 (130 – 166)	0.803
			GG	178	121 (113 – 129)		235 (216 – 254)		148 (135 – 160)	
	Men	rs17452044	AA	8	94 (45 – 144)		264 (232 – 295)		155 (111 – 199)	
			AG	58	103 (81 – 125)	0.102	261 (237 – 285)	0.753	142 (123 – 161)	0.811
			GG	134	124 (107 – 140)		264 (232 – 295)		146 (132 – 161)	
	Women	rs4146388	TT	16	121 (100 – 142)		223 (173 – 273)		144 (111 – 178)	
			TC	99	122 (112 – 131)	0.984	248 (226 – 270)	0.193	156 (141 – 171)	0.157
			CC	137	122 (113 – 131)		225 (203 – 246)		139 (124 – 154)	

Table S5.11. *Cont.*

Gene	Sex	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
					Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
ARNTL	Men	rs4146388	TT	21	103 (73 – 134)		299 (255 – 343)		159 (132 – 187)	
			TC	74	115 (95 – 135)	0.457	262 (233 – 291)	0.156	150 (132 – 169)	0.286
			CC	105	123 (104 – 141)		254 (227 – 280)		140 (123 – 156)	
	Women	rs4757142	AA	95	118 (107 – 128)		224 (199 – 248)		138 (122 – 155)	
			AG	117	125 (116 – 134)	0.418	247 (226 – 268)	0.149	157 (143 – 171)	0.077
			GG	41	119 (105 – 133)		221 (188 – 253)		136 (114 – 158)	
	Men	rs4757142	AA	67	125 (104 – 147)		248 (217 – 279)		138 (119 – 156)	
			AG	99	114 (96 – 133)	0.566	265 (238 – 291)	0.357	147 (131 – 164)	0.505
			GG	34	114 (89 – 139)		275 (240 – 311)		150 (128 – 172)	
	Women	rs56051850	AA	12	128 (103 – 152)		241 (184 – 300)		159 (120 – 199)	
			AG	80	122 (111 – 132)	0.867	235 (210 – 259)	0.975	147 (130 – 163)	0.820
			GG	161	121 (113 – 130)		235 (216 – 255)		147 (134 – 160)	
	Men	rs56051850	AA	12	146 (106 – 186)		180 (123 – 238)[A]		115 (77 – 150)	
			AG	80	123 (104 – 142)	0.104	258 (231 – 285)[A]	**0.004**	141 (124 – 158)	0.070
			GG	109	109 (91 – 127)		277 (251 – 302)[B]		153 (137 – 169)	
	Women	rs58901760	--	18	120 (100 – 140)		241 (194 – 288)		155 (123 – 186)	
			-GG	90	125 (115 – 135)	0.584	246 (223 – 269)	0.345	155 (140 – 171)	0.223
			GGGG	146	119 (111 – 128)		227 (206 – 248)		140 (126 – 155)	
	Men	rs58901760	--	17	100 (66 – 135)		307 (257 – 356)		165 (134 – 195)	
			-GG	74	114 (95 – 133)	0.340	262 (235 – 290)	0.118	150 (132 – 167)	0.161
			GGGG	110	124 (105 – 142)		254 (228 – 280)		138 (122 – 154)	
	Women	rs6486121	TT	90	117 (107 – 128)		235 (210 – 259)		145 (128 – 162)	
			TC	126	124 (115 – 132)	0.507	240 (220 – 260)	0.477	152 (138 – 165)	0.348
			CC	38	121 (107 – 135)		218 (183 – 252)		134 (111 – 157)	
	Men	rs6486121	TT	70	130 (110 – 150)		224 (196 – 253)[A]		126 (108 – 144)[A]	
			TC	99	113 (95 – 131)	0.124	280 (255 – 306)[B]	**<0.001**	154 (138 – 170)[B]	**0.003**
			CC	32	103 (76 – 129)		294 (256 – 331)[B]		163 (140 – 187)[B]	

Table S5.11. *Cont.*

Gene	Sex	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
					Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
ARNTL	Women	rs72867447	GG	45	117 (104 – 130)		228 (197 – 259)		143 (123 – 164)	
			GC	127	125 (116 – 133)	0.499	243 (222 – 264)	0.441	153 (139 – 167)	0.345
			CC	82	119 (109 – 130)		227 (201 – 252)		140 (123 – 157)	
	Men	rs72867447	GG	41	111 (88 – 134)		273 (240 – 306)		151 (131 – 172)	
			GC	100	113 (95 – 132)	0.242	271 (244 – 298)	0.072	149 (132 – 165)	0.268
			CC	60	130 (109 – 152)		237 (206 – 268)		134 (115 – 154)	
ARNTL2	Women	rs1037924	AA	87	115 (104 – 125)		242 (217 – 267)		151 (134 – 168)	
			AG	129	121 (113 – 130)	0.050	236 (215 – 256)	0.613	144 (130 – 159)	0.754
			GG	38	134 (121 – 148)		223 (190 – 256)		150 (127 – 172)	
	Men	rs1037924	AA	66	111 (90 – 131)		254 (224 – 283)		146 (128 – 164)	
			AG	94	118 (99 – 137)	0.399	271 (244 – 299)	0.513	142 (125 – 159)	0.658
			GG	41	129 (104 – 154)		259 (223 – 295)		153 (130 – 175)	
	Women	rs3751222	CC	5	121 (84 – 157)		182 (95 – 269)		125 (65 – 184)	
			CG	59	122 (110 – 134)	0.993	218 (190 – 245)	0.110	137 (118 – 156)	0.252
			GG	190	121 (114 – 129)		243 (224 – 261)		151 (139 – 164)	
	Men	rs3751222	CC	7	114 (62 – 167)		255 (179 – 331)		150 (103 – 197)	
			CG	46	108 (85 – 131)	0.566	268 (235 – 302)	0.896	151 (131 – 172)	0.715
			GG	148	120 (104 – 137)		261 (237 – 285)		143 (128 – 158)	
	Women	rs4931075	AA	13	129 (106 – 152)		224 (168 – 279)		137 (100 – 175)	
			AG	74	120 (109 – 131)	0.767	239 (213 – 264)	0.872	148 (131 – 165)	0.854
			GG	167	122 (114 – 130)		235 (215 – 254)		148 (135 – 161)	
	Men	rs4931075	AA	10	118 (73 – 163)		249 (185 – 314)		150 (111 – 190)	
			AG	71	111 (91 – 132)	0.675	270 (240 – 299)	0.715	145 (127 – 163)	0.964
			GG	120	120 (103 – 138)		260 (235 – 285)		145 (130 – 161)	
	Women	rs73075788	AA	8	108 (78 – 137)		266 (196 – 336)		166 (119 – 213)	
			AG	80	127 (116 – 137)	0.279	226 (201 – 251)	0.437	139 (122 – 156)	0.309
			GG	166	120 (112 – 128)		238 (219 – 258)		151 (138 – 164)	

Table S5.11. *Cont.*

Gene	Sex	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
					Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
ARNTL2	Men	rs73075788	AA	5	135 (74 – 196)		277 (188 – 365)		183 (129 – 237)	
			AG	58	114 (92 – 135)	0.775	253 (222 – 285)	0.691	134 (115 – 153)	0.120
			GG	138	118 (101 – 135)		266 (242 – 290)		149 (134 – 163)	
CLOCK	Women	rs12648271	CC	20	143 (124 – 161)[A]		214 (169 – 258)		138 (108 – 168)	
			CG	74	123 (112 – 134)[B]	**0.040**	240 (213 – 266)	0.568	147 (129 – 165)	0.803
			GG	160	118 (110 – 126)[B]		237 (218 – 257)		149 (136 – 162)	
	Men	rs12648271	CC	14	115 (76 – 153)		248 (193 – 303)		132 (98 – 166)	
			CG	71	117 (97 – 138)	0.988	243 (213 – 273)	0.098	140 (121 – 158)	0.411
			GG	116	118 (100 – 135)		274 (249 – 298)		149 (134 – 165)	
	Women	rs13113518	TT	119	117 (108 – 126)		241 (220 – 262)		150 (136 – 165)	
			TC	102	124 (114 – 134)	0.117	233 (210 – 257)	0.581	148 (132 – 163)	0.595
			CC	33	133 (118 – 147)		221 (186 – 257)		137 (113 – 161)	
	Men	rs13113518	TT	86	116 (97 – 134)		283 (257 – 309)[A]		156 (140 – 172)[A]	
			TC	89	118 (99 – 138)	0.924	244 (217 – 272)[B]	**0.013**	135 (118 – 152)[B]	**0.040**
			CC	26	121 (91 – 151)		234 (192 – 276)[B]		132 (106 – 158)	
	Women	rs35115774	--	34	112 (97 – 128)		266 (230 – 303)		164 (140 – 189)	
			-C	117	124 (115 – 133)	0.379	236 (215 – 258)	0.119	147 (133 – 162)	0.273
			CC	103	122 (112 – 131)		226 (204 – 248)		143 (128 – 158)	
	Men	rs35115774	--	29	128 (99 – 157)		290 (249 – 332)		169 (143 – 194)	
			-C	85	114 (95 – 132)	0.618	268 (241 – 295)	0.156	150 (133 – 166)	**0.026**
			CC	87	119 (100 – 138)		251 (224 – 278)		135 (119 – 152)	
	Women	rs57826934	TT	35	113 (98 – 127)		230 (196 – 265)		148 (125 – 171)	
			TC	116	123 (114 – 133)	0.379	229 (207 – 250)	0.421	142 (127 – 157)	0.439
			CC	103	123 (113 – 133)		245 (223 – 268)		154 (138 – 169)	
	Men	rs57826934	TT	29	110 (82 – 137)		257 (218 – 297)		135 (110 – 159)	
			TC	84	120 (102 – 138)	0.781	263 (237 – 290)	0.937	145 (129 – 162)	0.426
			CC	88	117 (97 – 138)		265 (235 – 294)		152 (133 – 170)	

Table S5.11. *Cont.*

Gene	Sex	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
					Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
CLOCK	Women	rs62303722	AA	122	124 (115 – 134)		243 (221 – 265)		151 (136 – 166)	
			AC	105	121 (112 – 131)	0.304	226 (203 – 249)	0.452	142 (127 – 158)	0.585
			CC	25	110 (94 – 127)		237 (197 – 276)		151 (124 – 178)	
	Men	rs62303722	AA	102	119 (99 – 138)		255 (226 – 283)		146 (128 – 163)	
			AC	80	120 (102 – 139)	0.472	268 (241 – 295)	0.646	148 (131 – 164)	0.677
			CC	19	100 (67 – 132)		267 (220 – 314)		134 (105 – 163)	
	Women	rs6832769	AA	103	122 (112 – 131)		226 (204 – 248)		143 (128 – 158)	
			AG	117	124 (115 – 133)	0.379	236 (215 – 258)	0.119	147 (133 – 162)	0.273
			GG	34	112 (97 – 128)		266 (230 – 303)		164 (140 – 189)	
	Men	rs6832769	AA	87	119 (100 – 138)		251 (224 – 278)		135 (119 – 152)	
			AG	85	114 (95 – 132)	0.618	268 (241 – 295)	0.156	150 (133 – 166)^{A}	**0.026**
			GG	29	128 (99 – 157)		290 (249 – 332)		169 (143 – 194)^{B}	

Abbreviation: SNP, single-nucleotide polymorphism.

Notes: P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. Non-cholesterol sterol levels were presented in 10^2 * µmol/mmol TC. Different superscript letters between genotypes indicate statistical significance ($p < 0.05$, Bonferroni correction).

$^{\$}$ genotype unknown for n=1.

Table S5.12. Relation between selected SNPs in *ARNTL, ARNTL2,* and *CLOCK* (i.e., the positive regulators in the transcriptional-translational feedback loop) with serum TC or LDL-C concentrations for men and women separately

Gene	Sex	SNP ID	Genotype	N	LDL-cholesterol		Total cholesterol	
					Mean (95% CI)	P–value	Mean (95% CI)	P-value
ARNTL	Women	rs10832020	TT	187	3.27 (3.11 – 3.42)		5.37 (5.20 – 5.54)	
			TC	61	3.15 (2.93 – 3.37)	0.420	5.20 (4.94 – 5.44)	0.232
			CC	6	2.95 (2.31 – 3.59)		4.93 (4.21 – 5.64)	
	Men	rs10832020	TT	143	3.63 (3.39 – 3.86)		5.54 (5.27 – 5.80)	
			TC	52	3.65 (3.36 – 3.95)	0.455	5.51 (5.18 – 5.84)	0.467
			CC	7	4.09 (3.38 – 4.80)		6.02 (5.22 – 6.82)	
	Women	rs11022778[$]	TT	104	3.24 (3.06 – 3.41)		5.30 (5.10 – 5.49)	
			TG	117	3.24 (3.06 – 3.41)	0.808	5.33 (5.13 – 5.53)	0.781
			GG	32	3.14 (2.84 – 3.44)		5.33 (4.99 – 5.67)	
	Men	rs11022778	TT	71	3.51 (3.24 – 3.78)		5.46 (5.15 – 5.76)	
			TG	104	3.75 (3.50 – 4.00)	0.242	5.63 (5.35 – 5.91)	0.571
			GG	27	3.75 (3.35 – 4.14)		5.56 (5.11 – 6.00)	
	Women	rs11824092	TT	44	3.12 (2.87 – 3.37)		5.23 (4.94 – 5.51)	
			TC	100	3.37 (3.19 – 3.55)	0.071	5.47 (5.27 – 5.67)	0.073
			CC	109	3.14 (2.96 – 3.32)		5.21 (5.01 – 5.41)	
	Men	rs11824092	TT	30	3.55 (3.18 – 3.91)		5.43 (5.02 – 5.84)	
			TC	97	3.64 (3.39 – 3.89)	0.502	5.54 (5.26 – 5.81)	0.559
			CC	75	3.77 (3.48 – 4.05)		5.66 (5.34 – 5.98)	
	Women	rs17452044	AA	6	3.53 (2.88 – 4.17)		5.46 (4.74 – 6.18)	
			AG	70	3.13 (2.91 – 3.34)	0.333	5.23 (4.99 – 5.47)	0.621
			GG	178	3.26 (3.11 – 3.41)		5.34 (5.17 – 5.51)	
	Men	rs17452044	AA	8	3.72 (3.03 – 4.41)		5.55 (4.78 – 6.32)	
			AG	58	3.57 (3.27 – 3.87)	0.696	5.40 (5.06 – 5.73)	0.427
			GG	135	3.69 (3.46 – 3.92)		5.61 (5.36 – 5.87)	
	Women	rs4146388	TT	16	3.03 (2.64 – 3.43)		5.07 (4.62 – 5.51)	
			TC	99	3.25 (3.08 – 3.43)	0.576	5.37 (5.17 – 5.57)	0.437
			CC	137	3.24 (3.07 – 3.41)		5.30 (5.11 – 5.50)	
	Men	rs4146388	TT	21	3.80 (3.38 – 4.22)		5.73 (5.26 – 6.21)	
			TC	75	3.71 (3.44 – 3.99)	0.687	5.58 (5.27 – 5.89)	0.627
			CC	105	3.63 (3.38 – 3.88)		5.50 (5.22 – 5.78)	
	Women	rs4757142	AA	95	3.10 (2.91 – 3.29)[A]		5.19 (4.97 – 5.40)	
			AG	117	3.35 (3.19 – 3.52)[B]	**0.028**	5.44 (5.25 – 5.62)	0.070
			GG	41	3.06 (2.81 – 3.32)		5.17 (4.88 – 5.46)	
	Men	rs4757142	AA	67	3.69 (3.39 – 3.98)		5.59 (5.26 – 5.92)	
			AG	100	3.67 (3.41 – 3.92)	0.925	5.55 (5.27 – 5.83)	0.948
			GG	34	3.61 (3.27 – 3.95)		5.51 (5.13 – 5.90)	
	Women	rs56051850	AA	12	2.99 (2.52 – 3.45)		5.23 (4.71 – 5.75)	
			AG	80	3.18 (2.98 – 3.37)	0.397	5.31 (5.09 – 5.53)	0.942
			GG	161	3.27 (3.11 – 3.42)		5.32 (5.14 – 5.50)	
	Men	rs56051850	AA	12	4.31 (3.76 – 4.86)		6.11 (5.48 – 6.73)	
			AG	80	3.62 (3.36 – 3.33)	0.046	5.52 (5.23 – 5.18)	0.169
			GG	110	3.62 (3.38 – 3.37)		5.52 (5.24 – 5.80)	
	Women	rs58901760	--	18	3.14 (2.76 – 3.51)		5.29 (4.87 – 5.71)	
			-GG	90	3.31 (3.12 – 3.49)	0.476	5.38 (5.17 – 5.59)	0.677
			GGGG	146	3.19 (3.02 – 3.35)		5.28 (5.09 – 5.46)	
	Men	rs58901760	--	17	3.77 (3.29 – 4.24)		5.78 (5.25 – 6.31)	
			-GG	75	3.64 (3.37 – 3.90)	0.877	5.51 (5.22 – 5.81)	0.646
			GGGG	110	3.66 (3.41 – 3.90)		5.54 (5.26 – 5.83)	
	Women	rs6486121	TT	90	3.17 (2.97 – 3.37)		5.28 (5.06 – 5.51)	
			TC	126	3.25 (3.09 – 3.41)	0.750	5.31 (5.13 – 5.49)	0.690
			CC	38	3.26 (2.98 – 3.53)		5.43 (5.12 – 5.74)	
	Men	rs6486121	TT	70	3.75 (3.47 – 4.03)		5.67 (5.36 – 5.98)	
			TC	100	3.57 (3.32 – 3.82)	0.439	5.45 (5.17 – 5.73)	0.366
			CC	32	3.73 (3.36 – 4.10)		5.64 (5.22 – 6.05)	
	Women	rs72867447	GG	45	3.31 (3.15 – 3.48)		5.24 (4.96 – 5.51)	
			GC	127	3.15 (2.95 – 3.36)	0.203	5.40 (5.22 – 5.59)	0.241
			CC	82	3.31 (3.15 – 3.48)		5.21 (4.98 – 5.43)	
	Men	rs72867447	GG	41	3.66 (3.34 – 3.98)		5.60 (5.24 – 5.95)	
			GC	101	3.63 (3.37 – 3.88)	0.869	5.49 (5.21 – 5.78)	0.723
			CC	60	3.71 (3.41 – 4.01)		5.62 (5.28 – 5.95)	

Table S5.12. *Cont.*

Gene	Sex	SNP ID	Genotype	N	LDL-cholesterol		Total cholesterol	
					Mean (95% CI)	P–value	Mean (95% CI)	P-value
ARNTL2	Women	rs1037924	AA	87	3.37 (3.17 – 3.57)		5.50 (5.28 – 5.72)	
			AG	129	3.20 (3.03 – 3.36)	0.112	5.28 (5.09 – 5.46)	**0.041**
			GG	38	3.07 (2.81 – 3.34)		5.10 (4.81 – 5.39)	
	Men	rs1037924	AA	66	3.70 (3.42 – 3.98)		5.59 (5.28 – 5.90)	
			AG	94	3.58 (3.32 – 3.83)	0.495	5.45 (5.16 – 5.74)	0.433
			GG	42	3.77 (3.43 – 4.10)		5.70 (5.32 – 6.07)	
	Women	rs3751222	CC	5	3.38 (2.67 – 4.08)		5.49 (4.70 – 6.28)	
			CG	59	3.29 (3.06 – 3.51)	0.717	5.34 (5.09 – 5.59)	0.874
			GG	190	3.20 (3.05 – 3.36)		5.30 (5.13 – 5.47)	
	Men	rs3751222	CC	7	3.20 (2.48 – 3.93)		5.11 (4.30 – 5.91)	
			CG	46	3.72 (3.40 – 4.03)	0.401	5.59 (5.23 – 5.94)	0.515
			GG	149	3.66 (3.43 – 3.88)		5.56 (5.31 – 5.81)	
	Women	rs4931075	AA	13	3.57 (3.13 – 4.01)		5.67 (5.17 – 6.16)	
			AG	74	3.15 (2.95 – 3.36)	0.211	5.21 (4.99 – 5.44)	0.210
			GG	167	3.24 (3.08 – 3.39)		5.34 (5.16 – 5.51)	
	Men	rs4931075	AA	10	3.70 (3.08 – 4.31)		5.40 (4.71 – 6.09)	
			AG	71	3.76 (3.48 – 4.04)	0.566	5.62 (5.31 – 5.93)	0.751
			GG	121	3.61 (3.37 – 3.84)		5.53 (5.27 – 5.79)	
	Women	rs73075788	AA	8	3.36 (2.79 – 3.92)		5.39 (4.76 – 6.02)	
			AG	80	3.19 (2.99 – 3.40)	0.824	5.27 (5.05 – 5.50)	0.860
			GG	166	3.24 (3.08 – 3.39)		5.33 (5.16 – 5.50)	
	Men	rs73075788	AA	5	3.91 (3.07 – 4.76)		5.51 (4.56 – 6.46)	
			AG	58	3.63 (3.33 – 3.93)	0.809	5.55 (5.22 – 5.89)	0.996
			GG	139	3.66 (3.44 – 3.89)		5.56 (5.30 – 5.81)	
CLOCK	Women	rs12648271	CC	20	3.38 (3.02 – 3.73)		5.47 (5.08 – 5.87)	
			CG	74	3.19 (2.98 – 3.41)	0.654	5.25 (5.01 – 5.48)	0.583
			GG	160	3.22 (3.06 – 3.37)		5.32 (5.14 – 5.49)	
	Men	rs12648271	CC	14	3.44 (2.92 – 3.97)		5.29 (4.71 – 5.88)	
			CG	72	3.78 (3.50 – 4.06)	0.340	5.73 (5.42 – 6.04)	0.180
			GG	116	3.62 (3.38 – 3.85)		5.48 (5.22 – 5.74)	
	Women	rs13113518	TT	119	3.23 (3.06 – 3.41)		5.32 (5.13 – 5.51)	
			TC	102	3.23 (3.04 – 3.42)	0.947	5.33 (5.12 – 5.54)	0.884
			CC	33	3.19 (2.90 – 3.47)		5.25 (4.93 – 5.57)	
	Men	rs13113518	TT	86	3.54 (3.29 – 3.79)		5.41 (5.13 – 5.69)	
			TC	90	3.79 (3.53 – 4.05)	0.224	5.72 (5.42 – 6.01)	0.159
			CC	26	3.71 (3.30 – 4.12)		5.58 (5.12 – 6.04)	
	Women	rs35115774	—	34	3.28 (2.99 – 3.57)		5.37 (5.04 – 5.70)	
			–C	117	3.16 (2.98 – 3.33)	0.383	5.24 (5.05 – 5.43)	0.435
			CC	103	3.30 (3.12 – 3.48)		5.39 (5.19 – 5.59)	
	Men	rs35115774	—	29	3.31 (2.92 – 3.71)		5.15 (4.71 – 5.60)	
			–C	85	3.77 (3.52 – 4.03)	0.076	5.68 (5.40 – 5.97)	0.068
			CC	88	3.62 (3.36 – 3.87)		5.51 (5.22 – 5.79)	
	Women	rs57826934	TT	35	3.47 (3.20 – 3.74)		5.53 (5.23 – 5.84)	
			TC	116	3.11 (2.94 – 3.29)	0.052	5.21 (5.02 – 5.41)	0.151
			CC	103	3.26 (3.08 – 3.44)		5.35 (5.14 – 5.55)	
	Men	rs57826934	TT	29	3.74 (3.37 – 4.12)		5.65 (5.23 – 6.07)	
			TC	85	3.64 (3.39 – 3.89)	0.875	5.52 (5.24 – 5.80)	0.854
			CC	88	3.65 (3.37 – 3.93)		5.55 (5.23 – 5.86)	
	Women	rs62303722	AA	122	3.21 (3.04 – 3.39)		5.29 (5.10 – 5.49)	
			AC	105	3.16 (2.98 – 3.34)	0.160	5.26 (5.06 – 5.46)	0.260
			CC	25	3.50 (3.18 – 3.82)		5.58 (5.22 – 5.94)	
	Men	rs62303722	AA	102	3.68 (3.42 – 3.95)		5.58 (5.28 – 5.88)	
			AC	81	3.67 (3.42 – 3.92)	0.844	5.56 (5.28 – 5.84)	0.847
			CC	19	3.55 (3.10 – 4.00)		5.43 (4.93 – 5.93)	
	Women	rs6832769	AA	103	3.30 (3.12 – 3.48)		5.39 (5.19 – 5.59)	
			AG	117	3.16 (2.98 – 3.33)	0.383	5.24 (5.05 – 5.43)	0.435
			GG	34	3.28 (2.99 – 3.57)		5.37 (5.04 – 5.70)	
	Men	rs6832769	AA	88	3.62 (3.36 – 3.87)		5.51 (5.22 – 5.79)	
			AG	85	3.77 (3.52 – 4.03)	0.076	5.68 (5.40 – 5.97)	0.068
			GG	29	3.31 (2.92 – 3.71)		5.15 (4.71 – 5.60)	

Notes: P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. TC and LDL-C concentrations were presented in mmol/L. Different superscript letters between genotypes indicate statistical significance ($p < 0.05$, Bonferroni correction). $^{\$}$ genotype unknown for n=1.

Table S5.13. Relation between selected SNPs in *CRY1, CRY2, PER2,* and *PER3* (i.e., the negative regulators in the transcriptional-translational feedback loop) with serum lathosterol, campesterol, and sitosterol levels for men and women separately

Gene	Sex	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
					Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
CRY1	Women	rs2078074	TT	86	123 (113 – 133)		237 (213 – 261)[A]		148 (132 – 164)	
			TC	133	124 (115 – 133)	0.191	223 (202 – 244)[B]	**0.026**	137 (123 – 151)[A]	**0.005**
			CC	35	110 (96 – 124)		273 (239 – 307)[B]		178 (156 – 201)[B]	
	Men	rs2078074	TT	53	126 (102 – 150)		243 (209 – 278)		137 (115 – 158)	
			TC	113	115 (98 – 132)	0.621	271 (246 – 296)	0.261	148 (133 – 164)	0.478
			CC	34	117 (91 – 143)		262 (225 – 300)		150 (127 – 173)	
	Women	rs10861688	TT	6	119 (86 – 153)		204 (124 – 284)		137 (83 – 192)	
			TC	69	131 (120 – 142)	0.089	236 (209 – 263)	0.726	149 (131 – 167)	0.914
			CC	179	118 (110 – 126)		236 (218 – 255)		147 (134 – 160)	
	Men	rs10861688	TT	6	108 (51 – 165)		245 (163 – 327)		144 (93 – 194)	
			TC	63	118 (97 – 139)	0.941	275 (245 – 306)	0.436	156 (137 – 174)	0.256
			CC	132	117 (100 – 135)		257 (232 – 282)		140 (125 – 155)	
CRY2	Women	rs11605924	AA	48	126 (113 – 138)		224 (193 – 254)		139 (118 – 160)	
			AC	129	121 (112 – 129)	0.758	226 (205 – 247)	0.076	142 (128 – 156)	0.110
			CC	77	121 (110 – 131)		256 (231 – 281)		160 (143 – 177)	
	Men	rs11605924	AA	64	110 (90 – 131)		263 (233 – 292)		145 (127 – 164)	
			AC	94	115 (96 – 135)	0.255	265 (237 – 293)	0.920	147 (130 – 165)	0.888
			CC	43	132 (108 – 156)		258 (224 – 292)		142 (121 – 163)	
	Women	rs72902436	AA	11	118 (93 – 143)		193 (134 – 252)		123 (83 – 164)	
			AG	96	123 (114 – 133)	0.846	229 (206 – 253)	0.202	141 (125 – 157)	0.201
			GG	147	121 (112 – 129)		243 (223 – 263)		153 (140 – 167)	
	Men	rs72902436	AA	15	123 (85 – 160)		227 (173 – 281)		132 (98 – 165)	
			AG	80	113 (95 – 132)	0.709	276 (250 – 302)	0.100	151 (135 – 167)	0.374
			GG	106	121 (102 – 141)		251 (224 – 279)		141 (124 – 158)	
PER2	Women	rs11894491	AA	23	110 (92 – 128)		242 (199 – 285)		152 (123 – 181)	
			AG	117	121 (112 – 130)	0.348	237 (215 – 259)	0.907	148 (133 – 163)	0.920
			GG	114	124 (115 – 133)		233 (212 – 254)		146 (132 – 161)	

Table S5.13. *Cont.*

Gene	Sex	SNP ID	Genotype	N	Lathosterol		Campesterol		Sitosterol	
					Mean (95% CI)	P-value	Mean (95% CI)	P-value	Mean (95% CI)	P-value
PER2	Men	rs11894491	AA	30	121 (93 – 149)		241 (201 – 282)		132 (107 – 157)	
			AG	81	110 (90 – 130)	0.560	256 (227 – 286)	0.322	142 (124 – 161)	0.362
			GG	88	121 (103 – 139)		271 (245 – 297)		150 (134 – 166)	
	Women	rs76784767	AA	6	96 (62 – 129)		285 (207 – 364)		181 (128 – 234)	
			AC	53	119 (107 – 132)	0.245	264 (234 – 293)^{A}	**0.024**	167 (147 – 187)	**0.021**
			CC	195	123 (116 – 131)		227 (209 – 245)^{B}		141 (129 – 154)	
	Men	rs76784767	AA	7	110 (59 – 162)		232 (157 – 306)		122 (76 – 168)	
			AC	57	117 (96 – 139)	0.958	269 (238 – 300)	0.628	150 (130 – 169)	0.526
			CC	137	118 (101 – 136)		263 (237 – 288)		146 (130 – 161)	
PER3	Women	rs2797685	TT	6	119 (85 – 154)		222 (142 – 303)		113 (59 – 167)	
			TC	68	121 (110 – 132)	0.928	241 (216 – 267)	0.654	149 (132 – 166)	0.439
			CC	179	123 (115 – 131)		229 (210 – 248)		145 (132 – 157)	
	Men	rs2797685	TT	7	104 (52 – 156)		284 (209 – 358)		158 (112 – 205)	
			TC	55	125 (103 – 147)	0.618	258 (226 – 289)	0.805	146 (127 – 166)	0.838
			CC	137	116 (98 – 135)		263 (236 – 289)		144 (127 – 161)	
	Women	rs4908482	AA	39	121 (107 – 135)		246 (213 – 279)		141 (118 – 164)	
			AG	116	123 (114 – 132)	0.912	233 (212 – 255)	0.769	150 (135 – 165)	0.773
			GG	99	120 (110 – 130)		234 (210 – 257)		147 (131 – 163)	
	Men	rs4908482	AA	30	118 (91 – 144)		250 (212 – 289)		138 (114 – 161)	
			AG	82	119 (100 – 137)	0.951	264 (237 – 291)	0.696	147 (131 – 164)	0.725
			GG	89	115 (95 – 136)		269 (239 – 298)		147 (129 – 166)	
	Women	rs875994	TT	190	122 (114 – 130)		234 (215 – 253)		145 (132 – 158)	
			TC	57	124 (112 – 136)	0.558	235 (206 – 263)	0.833	152 (133 – 172)	0.649
			CC	7	106 (75 – 137)		257 (184 – 331)		162 (113 – 212)	
	Men	rs875994	TT	132	123 (106 – 141)		259 (234 – 284)^{A}		141 (125 – 156)	
			TC	56	109 (87 – 130)	0.412	278 (247 – 308)^{B}	**0.035**	158 (139 – 177)^{A}	**0.034**
			CC	11	123 (80 – 166)		194 (132 – 255)^{B}		109 (72 – 147)^{B}	

Abbreviation: SNP, single-nucleotide polymorphism. *Notes:* P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. Non-cholesterol sterol levels were presented in 10^2 * µmol/mmol TC. Different superscript letters between genotypes indicate statistical significance ($p < 0.05$, Bonferroni correction).

Table S5.14. Relation between selected SNPs in *CRY1, CRY2, PER2,* and *PER3* (i.e., the negative regulators in the transcriptional-translational feedback loop) with serum TC or LDL-C concentrations for men and women separately

Gene	Sex	SNP ID	Genotype	N	LDL-cholesterol		Total cholesterol	
					Mean (95% CI)	P-value	Mean (95% CI)	P-value
CRY1	Women	rs2078074	TT	86	3.22 (3.03 – 3.42)		5.33 (5.12 – 5.55)	
			TC	133	3.23 (3.06 – 3.40)	0.997	5.33 (5.14 – 5.52)	0.871
			CC	35	3.24 (2.96 – 3.51)		5.25 (4.94 – 5.55)	
	Men	rs2078074	TT	53	3.76 (3.43 – 4.09)		5.62 (5.25 – 5.99)	
			TC	114	3.62 (3.39 – 3.85)	0.651	5.51 (5.25 – 5.78)	0.837
			CC	34	3.61 (3.25 – 3.96)		5.55 (5.15 – 5.95)	
	Women	rs10861688	TT	6	3.51 (2.87 – 4.15)		5.64 (4.93 – 6.36)	
			TC	69	3.10 (2.89 – 3.31)	0.243	5.19 (4.96 – 5.43)	0.312
			CC	179	3.26 (3.11 – 3.41)		5.35 (5.18 – 5.52)	
	Men	rs10861688	TT	6	4.19 (3.41 – 4.97)		6.04 (5.17 – 6.92)	
			TC	64	3.71 (3.42 – 3.99)	0.314	5.60 (5.29 – 5.92)	0.444
			CC	132	3.62 (3.39 – 3.85)		5.51 (5.25 – 5.77)	
CRY2	Women	rs11605924	AA	48	3.07 (2.83 – 3.32)		5.24 (4.97 – 5.52)	
			AC	129	3.18 (3.01 – 3.34)	0.055	5.27 (5.08 – 5.46)	0.397
			CC	77	3.39 (3.20 – 3.59)		5.43 (5.20 – 5.65)	
	Men	rs11605924	AA	64	3.60 (3.32 – 3.88)		5.53 (5.22 – 5.85)	
			AC	94	3.64 (3.38 – 3.91)	0.618	5.53 (5.23 – 5.82)	0.862
			CC	44	3.78 (3.46 – 4.10)		5.63 (5.27 – 5.99)	
	Women	rs72902436	AA	11	3.10 (2.62 – 3.58)		5.37 (4.84 – 5.90)	
			AG	96	3.21 (3.02 – 3.40)	0.812	5.33 (5.11 – 5.54)	0.962
			GG	147	3.25 (3.09 – 3.41)		5.30 (5.12 – 5.48)	
	Men	rs72902436	AA	15	3.56 (3.04 – 4.08)		5.47 (4.89 – 6.05)	
			AG	80	3.71 (3.46 – 3.96)	0.710	5.64 (5.36 – 5.92)	0.501
			GG	107	3.61 (3.35 – 3.87)		5.46 (5.17 – 5.75)	
PER2	Women	rs11894491	AA	23	3.48 (3.14 – 3.83)		5.57 (5.19 – 5.96)	
			AG	117	3.24 (3.06 – 3.41)	0.247	5.30 (5.10 – 5.50)	0.348
			GG	114	3.18 (3.01 – 3.35)		5.29 (5.10 – 5.48)	
	Men	rs11894491	AA	31	3.91 (3.54 – 4.28)		5.85 (5.43 – 6.26)	
			AG	81	3.61 (3.33 – 3.88)	0.274	5.54 (5.23 – 5.85)	0.224
			GG	88	3.62 (3.38 – 3.87)		5.47 (5.20 – 5.74)	
	Women	rs76784767	AA	6	3.25 (2.61 – 3.90)		5.23 (4.52 – 5.95)	
			AC	53	3.34 (3.10 – 3.58)	0.514	5.42 (5.15 – 5.69)	0.631
			CC	195	3.20 (3.05 – 3.35)		5.29 (5.13 – 5.46)	
	Men	rs76784767	AA	7	3.70 (2.99 – 4.41)		5.80 (5.01 – 6.60)	
			AC	57	3.70 (3.41 – 4.00)	0.894	5.58 (5.25 – 5.91)	0.752
			CC	138	3.63 (3.40 – 3.87)		5.52 (5.25 – 5.78)	
PER3	Women	rs2797685	TT	6	3.23 (2.57 – 3.89)		5.14 (4.40 – 5.87)	
			TC	68	3.22 (3.01 – 3.43)	0.998	5.33 (5.10 – 5.56)	0.881
			CC	179	3.23 (3.07 – 3.39)		5.32 (5.14 – 5.49)	
	Men	rs2797685	TT	7	3.38 (2.67 – 4.09)		5.13 (4.34 – 5.93)	
			TC	55	3.58 (3.28 – 3.88)	0.600	5.45 (5.12 – 5.78)	0.417
			CC	138	3.69 (3.44 – 3.94)		5.60 (5.32 – 5.88)	
	Women	rs4908482	AA	39	3.15 (2.88 – 3.41)		5.18 (4.88 – 5.48)	
			AG	116	3.31 (3.13 – 3.48)	0.329	5.39 (5.19 – 5.58)	0.411
			GG	99	3.16 (2.97 – 3.35)		5.28 (5.07 – 5.49)	
	Men	rs4908482	AA	30	3.76 (3.40 – 4.13)		5.61 (5.20 – 6.02)	
			AG	82	3.52 (3.27 – 3.78)	0.164	5.44 (5.16 – 5.73)	0.357
			GG	90	3.79 (3.51 – 4.06)		5.67 (5.37 – 5.98)	
	Women	rs875994	TT	190	3.26 (3.11 – 3.41)		5.35 (5.18 – 5.52)	
			TC	57	3.26 (3.03 – 3.49)	0.097	5.34 (5.09 – 5.60)	0.117
			CC	7	2.60 (2.02 – 3.19)		4.65 (3.99 – 5.30)	
	Men	rs875994	TT	132	3.73 (3.49 – 3.97)		5.60 (5.33 – 5.87)	
			TC	57	3.58 (3.29 – 3.86)	0.308	5.50 (5.18 – 5.83)	0.695
			CC	11	4.02 (3.43 – 4.60)		5.78 (5.12 – 6.44)	

Notes: P-values were obtained from a general linear model analysis adjusted for the factor study. Estimated marginal means and the 95% confidence interval were reported. TC and LDL-C concentrations were presented in mmol/L. Different superscript letters between genotypes indicate statistical significance ($p < 0.05$, Bonferroni correction).

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CHAPTER 6

Effects of intermittent energy restriction compared to those of continuous energy restriction on body composition and cardiometabolic risk markers: a systematic review and meta-analysis of randomized controlled trials in adults

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Submitted

CHAPTER 7

Effects of alternating energy intake compared to regular energy intake on fasting and postprandial cardiometabolic risk markers in individuals with abdominal obesity: a randomized crossover trial

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In preparation

CHAPTER 8

General discussion

APPENDIX

SUMMARY

A balance between endogenous cholesterol biosynthesis, intestinal absorption of dietary and biliary cholesterol, and bile acid synthesis and excretion is essential to maintain healthy serum low-density lipoprotein cholesterol (LDL-C) concentrations and hence for cardiovascular disease risk prevention. Previous research has reported a large between-person variation in endogenous cholesterol synthesis and intestinal cholesterol absorption, which may be due to differences in genetic background. Furthermore, various biological processes follow a diurnal pattern, but little is known about the link between the circadian system and human cholesterol homeostasis, especially intestinal cholesterol absorption. In addition, individuals spend most of the day in the postprandial state, but data on the acute effects of macronutrient consumption on endogenous cholesterol synthesis and intestinal cholesterol absorption is limited. To maintain healthy serum LDL-C concentrations it is relevant to examine these abovementioned factors that may influence cholesterol homeostasis.

As mentioned above, the circadian system regulates daily oscillations of many physiological processes. Over the last few decades, the interest in chrononutrition, which studies the interplay between circadian biology, nutrition, and metabolism, has risen. The focus of nutrition research is no longer only on what, but also on when people eat. Common intermittent energy restriction (IER) protocols include time-restricted eating, alternate day fasting, and the 5:2 diet. Obesity, mainly abdominal obesity, is an important modifiable cardiovascular disease risk marker. To reduce the number of people living with (abdominal) obesity, it is important to study which weight loss approach is most beneficial to lower body weight and consequently improve cardiometabolic risk markers.

This thesis therefore aimed to study (1) the involvement of genetic variants, diurnal rhythms, and macronutrients in the regulation of endogenous cholesterol synthesis and intestinal cholesterol absorption, and (2) the effects of different IER diets with and without weight loss on body weight and cardiometabolic risk factors in apparently healthy individuals with and without overweight.

In **Chapter 2**, cross-sectional associations between single nucleotide polymorphisms (SNPs) in genes that encode for proteins involved in cholesterol metabolism with endogenous cholesterol synthesis and intestinal cholesterol absorption markers and LDL-C concentrations were studied. Pre-selected SNPs in *ABCG5, ABCG8, CYP51A1, DHCR7, DHCR24, HMGCR, HSD17B7, LBR, MSMO1,* and *NPC1L1* were studied in serum samples obtained from 456 Western European individuals. Two SNPs in *NPC1L1* (rs217429 and rs217416) were associated with the endogenous cholesterol synthesis marker lathosterol and two SNPs in *ABCG5* (rs4245786) and *ABCG8* (rs4245791) with intestinal cholesterol absorption markers. Furthermore, SNPs in *HMGCR* (rs12916) and *LBR* (rs12141732) were associated with serum LDL-C concentrations. Selected SNPs in *CYP51A1, DHCR24, HSD17B7,* and *MSMO1* were not associated with non-cholesterol sterols and LDL-C concentrations. In **Chapter 5**, comparable associations in the same population were studied, but now with selected SNPs located in circadian clock genes (*ARNTL, ARNTL2, CLOCK, CRY1, CRY2, PER1, PER2,* and *PER3*). One SNP in *ARNTL2* (rs1037924) was associated with cholesterol synthesis. Multiple SNPs in *ARNTL*

(rs4146388, rs58901760, rs6486121), *ARNTL2* (rs73075788), *CLOCK* (rs13113518, rs35115774, rs6832769), and *CRY1* (rs2078074) were associated with campesterol or sitosterol levels, which reflect cholesterol absorption. Only *PER2* (rs11894491) was associated with serum LDL-C concentrations. SNPs in *CRY2*, *PER2*, and *PER3* were not related to non-cholesterol sterol levels. In both **Chapter 2** and **5**, associations between SNPs and cholesterol synthesis or cholesterol absorption did not translate into significant associations with LDL-C concentrations. The findings suggest that genetic variants indeed partly explain the high inter-individual variation in intestinal cholesterol absorption and endogenous cholesterol synthesis.

The systematic review and single-arm study in **Chapter 3** studied the diurnal rhythms of bile acid synthesis, endogenous cholesterol synthesis, and intestinal cholesterol absorption markers. The systematic review indicated that the bile acid synthesis marker 7α-hydroxy-4-cholesten-3-one had a diurnal rhythm with peaks during the day and that cholesterol synthesis markers had a diurnal rhythm with a peak at night. The single-arm study confirmed the nocturnal endogenous cholesterol synthesis peak and found no significant diurnal rhythm for intestinal cholesterol absorption markers. Bile acid synthesis markers were not analyzed. A reciprocal relationship between endogenous cholesterol synthesis and intestinal cholesterol absorption, as frequently observed in the fasted situation, was not confirmed. We further hypothesized that the non-significant diurnal rhythm of cholesterol absorption may have been due to the consumption of low-fat meals during the study period.

To study the possible role of the low fat meals as explanation for the results of **Chapter 3**, the randomized cross-over trial in **Chapter 4** compared the effects of three isoenergetic meals high in fat (fat [f], carbohydrates [c], protein [p]: 55.2 g/52.3 EN% f, 93.5 g/39.2 EN% c, 19.2 g/8.0 EN% p), carbohydrates (10.2 g/9.6 EN% f, 194.3 g/81.5 EN% c, 20.4 g/8.6 EN% p), and proteins (11.3 g/10.6 EN% f, 122.7 g/51.5 EN% c, 87.9 g/36.9 EN% p) on intestinal cholesterol absorption and endogenous cholesterol synthesis markers over a four-hour period in overweight/obese men. Acute meal consumption did not change serum total cholesterol concentrations and cholesterol-standardized campesterol, sitosterol, and cholestanol levels. This suggests that meal consumption did not explain the absence of a diurnal rhythm of cholesterol absorption. The cholesterol synthesis intermediates 7-dehydrocholesterol, lanosterol, lathosterol, zymostenol, and zymosterol all decreased significantly over time, but no significant differences between the meals were found for these intermediates.

The systematic review and meta-analysis in **Chapter 6** compared IER diets with continuous energy restriction (CER) in healthy individuals. No different changes in anthropometrics (body weight, body mass index, and fat mass) and cardiometabolic risk markers (fasting total cholesterol, high-density lipoprotein cholesterol [HDL-C], LDL-C, triacylglycerol, glucose and insulin concentrations, homeostatic model assessment for insulin resistance [HOMA-IR] and blood pressure) were found for IER compared to CER. However, larger reductions in fat free mass (weighted mean difference [WMD]: -0.20 kg; 95% CI: -0.39 to -0.01; p=0.044) and waist circumference (WMD: -0.91 cm; 95% CI -1.76 to -0.06; p=0.036) were observed for IER diets. Further, body weight, fat mass, and fat free mass were more reduced in time-restricted eating, HOMA-IR decreased more in alternate-day

fasting, and body mass index was more decreased after CER compared with the 5:2 diet. It remains uncertain whether the findings of the meta-analysis were completely due to the type of diet or also to differences in energy intake between groups within the studies. The findings further suggest that weight loss may be more important than the type of diet to improve cardiometabolic risk markers.

To further study the benefit of IER for cardiometabolic health in absence of weight loss, we compared a 4-week alternating energy intake schedule to a regular energy intake schedule in individuals with abdominal obesity (**Chapter 7**). No differences between the two dietary patterns were reported for anthropometrics and fasting glucose, insulin, total cholesterol, HDL-C, LDL-C, triacylglycerol, and high-sensitivity C-reactive protein concentrations. A high-fat mixed meal was consumed at the end of both four-week periods, and no significant between-group differences in postprandial triacylglycerol, glucose, and insulin concentrations were found. Overall, the results of this study suggest that an IER approach without weight loss was not superior in improving anthropometrics and cardiovascular risk markers compared with a regular energy intake schedule. It may therefore be suggested that beneficial effects of intermittent energy restriction diets on cardiometabolic health are primarily due to the loss in body weight instead of to the eating pattern.

In conclusion, the major findings of the studies included in this these were:

1. Genetic variants in endogenous cholesterol synthesis, intestinal cholesterol absorption, and circadian clock genes are partly responsible for differences in endogenous cholesterol synthesis and intestinal cholesterol absorption among individuals.
2. Endogenous cholesterol synthesis depicts a clear diurnal pattern with a nocturnal peak, whereas no diurnal rhythm was found for intestinal cholesterol absorption markers.
3. Acute high-fat, high-carbohydrate or high-protein consumption had no effect on postprandial intestinal cholesterol markers and thereby also did not explain the absence of a diurnal rhythm in intestinal cholesterol absorption. Various intermediates in the endogenous cholesterol synthesis pathway decreased after meal consumption.
4. In apparently healthy individuals, the effects of IER diets on weight loss and cardiovascular risk markers were not significantly better compared to the CER diet.
5. An alternating energy intake approach without a net reduction in energy intake did not improve anthropometrics and cardiometabolic risk markers compared to a regular energy intake schedule.

SAMENVATTING

De balans tussen de aanmaak van cholesterol door het lichaam, de opname in de darm van cholesterol afkomstig uit voeding en gal, en de galzuursynthese en galuitscheiding is essentieel voor het handhaven van gezonde serumcholesterolconcentraties in de lage-dichtheid lipoproteïnen (LDL-C), een belangrijke risicofactor voor het krijgen van hart- en vaatziekten. Uit eerder onderzoek is gebleken dat er tussen personen grote verschillen bestaan in de aanmaak en opname van cholesterol. Dit duidt erop dat de genetische achtergrond van een persoon een belangrijke rol speelt bij deze processen. Bovendien zorgt het circadiaanse systeem ervoor dat verschillende biologische processen een diurnaal ritme volgen, hetgeen betekent dat deze processen iedere 24 uur in dit ritme herhaald worden. Er is weinig bekend over het verband tussen het circadiaanse systeem en de opname van cholesterol in de darm. Verder brengen mensen het grootste deel van de dag door in de postprandiale toestand, wat de periode na een maaltijd is. Het effect van een acute inname van maaltijden hoog in vetten, koolhydraten of eiwitten op de aanmaak en opname van cholesterol in het lichaam is nog niet eerder onderzocht. Om gezonde serum LDL-C concentraties te behouden is het van belang om deze factoren, die mogelijk de cholesterolhomeostase kunnen beïnvloeden, te onderzoeken.

Zoals hierboven benoemd, zorgt het zogenaamde circadiaanse systeem ervoor dat veel biologische processen zich iedere 24 uur herhalen. De afgelopen decennia is de belangstelling voor chrono-nutrition, waarbij de wisselwerking tussen het circadiaanse systeem, voeding en stofwisseling wordt bestudeerd, toegenomen. De focus van voedingsonderzoek ligt niet langer alleen op wat, maar ook op wanneer mensen eten. Daarom worden bijvoorbeeld gezondheidskundige effecten onderzocht van voedingspatronen waarbij de nadruk ligt op het tijdstip van voedingsinname, de zogenaamde ‘intermittent energy restriction’ diëten. Voorbeelden hiervan zijn ‘time-restricted eating’, ‘alternate day fasting’ en het 5:2 dieet. Obesitas, met name rondom de buik, is een belangrijke risicofactor voor hart- en vaatziekten. Het is daarom belangrijk om na te gaan of deze ‘intermittent energy restriction’ diëten een gunstig effect hebben op het lichaamsgewicht, zodat het risico op het krijgen van hart- en vaatziekten wordt verlaagd.

Het doel van dit proefschrift was dan ook het bestuderen van (1) de invloed van genetische varianten, diurnale ritmes en acute voedingsinname op de cholesterolproductie en -opname en (2) de effecten van verschillende typen ‘intermittent energy restriction’ met en zonder gewichtsverlies op het lichaamsgewicht en risicofactoren voor hart- en vaatziekten bij gezonde personen met en zonder overgewicht.

In **Hoofdstuk 2** werden relaties onderzocht tussen bepaalde variaties in genen — deze variaties worden ook wel ‘single-nucleotide polymorphisms’ (SNPs) genoemd — die betrokken zijn bij de stofwisseling van cholesterol met nuchtere LDL-C concentraties en met markers die een maat zijn voor de aanmaak en opname van cholesterol. Vooraf geselecteerde SNPs in *ABCG5, ABCG8, CYP51A1, DHCR7, DHCR24, HMGCR, HSD17B7, LBR, MSMO1* en *NPC1L1* werden onderzocht in monsters verkregen van 456 West-Europese personen. Twee SNPs in *NPC1L1* (rs217429 en rs217416) waren geassocieerd met lathosterol, een marker voor de aanmaak van cholesterol. Ook

waren twee SNPs in *ABCG5* (rs4245786) en *ABCG8* (rs4245791) geassocieerd met de opname van cholesterol in de darm. Verder waren SNPs in *HMGCR* (rs12916) en *LBR* (rs12141732) geassocieerd met serum LDL-C concentraties. SNPs in *CYP51A1, DHCR24, HSD17B7* en *MSMO1* waren niet geassocieerd met de markers en LDL-C concentraties. In **Hoofdstuk 5** werden vergelijkbare relaties in dezelfde onderzoekspopulatie bestudeerd, maar nu met SNPs in genen die een rol spelen in circadiaanse ritmes (*ARNTL, ARNTL2, CLOCK, CRY1, CRY2, PER1, PER2* en *PER3*). Een SNP in *ARNTL2* (rs1037924) was geassocieerd met de aanmaak van cholesterol. Meerdere SNPs in *ARNTL* (rs4146388, rs58901760, rs6486121), *ARNTL2* (rs73075788), *CLOCK* (rs13113518, rs35115774, rs6832769) en *CRY1* (rs2078074) waren geassocieerd met campesterol- of sitosterolwaarden, die de cholesterolopname reflecteren. Alleen *PER2* (rs11894491) was geassocieerd met serum LDL-C concentraties. SNPs in *CRY2*, *PER2* en *PER3* waren niet gerelateerd aan de markers. In zowel **Hoofdstuk 2** als **5** vertaalden relaties tussen SNPs met de aanmaak of opname van cholesterol zich niet in verschillen in LDL-C concentraties. Deze resultaten suggereren dat de genetische achtergrond de variatie in de aanmaak of opname van cholesterol tussen personen inderdaad gedeeltelijk verklaart.

De systematische review en studie in **Hoofdstuk 3** bestudeerden of de aanmaak van galzuren, de aanmaak van cholesterol en de opname van cholesterol in de darm diurnale ritmes vertoonden. Uit de review bleek dat gedurende de dag de aanmaak van galzuren het hoogste was, terwijl de aanmaak van cholesterol tijdens de nacht het hoogst was. De studie bevestigde het diurnale ritme voor de aanmaak van cholesterol, maar vond geen diurnaal ritme voor de opname van cholesterol. Galzuren werden niet onderzocht. Een inverse relatie tussen de aanmaak en opname van cholesterol, zoals vaak aanwezig in de nuchtere situatie, werd niet gevonden. Op basis van de resultaten stelden wij de hypothese op dat de afwezigheid van een diurnaal ritme in de opname van cholesterol mogelijk verklaard kon worden door de consumptie van de vetarme maaltijden tijdens de studieperiode.

Om verder onderzoek te doen naar de mogelijke rol van de maaltijden laag in vet als verklaring voor de bevindingen van **Hoofdstuk 3**, werden in **Hoofdstuk 4** de effecten van drie verschillende maaltijden op de aanmaak en opname van cholesterol onderzocht. De maaltijden hadden een vergelijkbare hoeveelheid energie, maar verschillende hoeveelheden van de macronutriënten (vetten, koolhydraten en eiwitten). Mannen met overgewicht of obesitas kregen in willekeurige volgorde een maaltijd hoog in vet (vet [v], koolhydraten [k], eiwitten [e]: 55.2 g/52.3 EN% v, 93.5 g/39.2 EN% k, 19.2 g/8.0 EN% e), koolhydraten (10.2 g/9.6 EN% v, 194.3 g/81.5 EN% k, 20.4 g/8.6 EN% e) en eiwitten (11.3 g/10.6 EN% v, 122.7 g/51.5 EN% k, 87.9 g/36.9 EN% e). Vier uur na de maaltijdinname waren de totale serumcholesterolconcentraties en de opname van cholesterol in de darm onveranderd. Dit suggereert dat de samenstelling van de maaltijd geen verklaring was voor de afwezigheid van een diurnaal ritme in de cholesterolopname. De resultaten lieten verder zien dat de aanmaak van cholesterol was verlaagd na maaltijdinname, maar deze afname was vergelijkbaar voor de drie maaltijden. De systematische review en meta-analyse in **Hoofdstuk 6** vergeleken in gezonde mensen ‘intermittent energy restriction’ met dagelijks minder eten waarbij geen rekening gehouden werd met het tijdstip van eten. Veranderingen in lichaamsgewicht, vetmassa, bloeddruk en vetten en suikers in het bloed verschilden niet tussen deze twee

eetschema's. De afnames in vetvrije massa en de buikomvang waren groter in 'intermittent energy restriction' dan in dagelijks minder eten. Daarnaast waren de afnames in lichaamsgewicht, vetmassa en vetvrije massa groter na 'time-restricted eating', was de insulineresistentie verbeterd na 'alternate day fasting' en de body mass index meer afgenomen na dagelijks minder eten vergeleken met het 5:2 dieet. Het is niet zeker of deze resultaten alleen te wijten waren aan het type dieet of dat verschillen in de hoeveelheid energie die gegeten werd tussen de diëten binnen de studies ook een rol heeft gespeeld. De resultaten suggereren verder dat gewichtsverlies belangrijker is dan het type energiebeperkt dieet voor het verbeteren van risicofactoren voor hart- en vaatziekten.

Om het voordeel van 'intermittent energy restriction' zonder gewichtsverlies voor de gezondheid verder te onderzoeken, vergeleken we afwisselende energie-inname met regelmatige energie-inname voor vier weken in mensen met een grote buikomvang (**Hoofdstuk 7**). Veranderingen over tijd in het lichaamsgewicht, de buikomvang, de bloeddruk en vetten en suikers in het bloed verschilden niet tussen de twee voedingspatronen. Aan het einde van de vier weken aten de deelnemers een vetrijke maaltijd, en er werden geen verschillen gevonden in de vetten en suikers in het bloed gedurende de vier uur na het eten van deze maaltijd. Deze resultaten suggereren dat 'intermittent energy restriction' zonder gewichtsverlies geen gunstigere invloed heeft op de gezondheid in gezonde volwassenen met een grote buikomvang vergeleken met een schema van regelmatige energie-inname.

Samenvattend waren de voornaamste bevindingen van de studies in dit proefschrift:

1. Variaties in genen essentieel in de aanmaak en opname van cholesterol en circadiaanse ritmes kunnen de verschillen in de aanmaak en opname van cholesterol tussen mensen deels verklaren.
2. De aanmaak van cholesterol vertoonde een diurnaal ritme met een piek tijdens de nacht, terwijl de opname van cholesterol in de darm geen diurnaal ritme vertoonde.
3. Acute inname van een vetrijke, koolhydraatrijke of eiwitrijke maaltijd had geen effect op de opname van cholesterol na de maaltijd en was dus geen verklaring voor de afwezigheid van een diurnaal ritme in de opname van cholesterol in de darm. De aanmaak van cholesterol nam af na het eten van de maaltijden.
4. In gezonde mensen waren de effecten op gewichtsverlies en risicofactoren voor hart- en vaatziekten niet beduidend beter na 'intermittent energy restriction' vergeleken met dagelijks minder eten.
5. Een afwisselende energie-inname zonder gewichtsverlies vergeleken met een regelmatige energie-inname leidde niet tot een verbetering van de risicofactoren voor hart- en vaatziekten.

IMPACT

The human studies described in this thesis were centered around two aims: 1) to examine the influence of genetic factors, the circadian system, and macronutrient intakes on endogenous cholesterol synthesis and intestinal cholesterol absorption, and 2) to study the cardiometabolic health effects of various intermittent energy restriction diets. In summary, results showed that multiple genetic variants are associated with endogenous cholesterol synthesis or intestinal cholesterol absorption. Furthermore, we observed that endogenous cholesterol synthesis peaked at night, which agreed with previous findings. We were the first to examine the possible diurnal rhythm of cholesterol absorption, which however was not present. We also showed that this absence of a cholesterol-absorption rhythm was not related to the type of macronutrient that was mainly consumed, at least not in the acute situation. Regarding longer-term dietary interventions, this thesis did not find that intermittent energy restriction diets were superior to continuous energy restriction diets for human cardiometabolic health. This chapter will discuss the potential impact of these findings in terms of societal, economic, and scientific relevance. Then, implications for the translation of the findings into practice will be discussed.

Societal and economic impact

Global disability and death from cardiovascular diseases (CVDs) increased in the previous thirty years (1). The prevalent cases of total CVD increased from 271 million in 1990 to 523 million in 2019, and the number of CVD deaths from 12.1 million to 18.6 million (1). This represented 32% of all global deaths in 2019 (2). In addition, the worldwide disability-adjusted life years (DALYs), years lived with disability (YLDs), and years of life lost (YLLs) due to CVD all increased from 1990 to 2019 (1). The European Society of Cardiology published that high-income countries spent on average four times more money on healthcare than middle-income countries in 2018 (3). Most healthcare costs were due to CVD and accounted for around 16% of the total costs in a selection of high-income European countries in 2016 (3). To add, it was estimated that CVD cost the European economy 210 billion euros in 2015, of which 53% was due to healthcare costs, 26% to loss of productivity, and 21% to the informal care of CVD patients (3). These data highlight the importance of targeting CVD risk factors at early stages to prevent the development of CVD and thereby lower death, disability, and healthcare costs due to this type of disease. Important modifiable CVD risk factors include increased low-density lipoprotein cholesterol (LDL-C) concentrations and overweight or obesity (1, 2).

Various interventions have been developed to lower serum LDL-C concentrations, thereby reducing CVD risk. Next to LDL-C concentrations, attention is paid to other novel risk markers for CVD. High intestinal cholesterol absorption, for example, has been positively associated with CVD (4-6). In addition, a high cholesterol synthesis or a high cholesterol absorption has been related to other diseases and metabolic disturbances (6). It may thus also be interesting to examine other approaches that further reduce CVD risk, including cholesterol synthesis and absorption. To optimize interventions, it is of relevance to clearly understand how various factors influence or are related to cholesterol homeostasis. In this thesis, a relation between different single-nucleotide polymorphisms (SNPs) with cholesterol synthesis and absorption was found, which may be a first

step towards more personalized cholesterol-lowering interventions. In addition, this thesis demonstrates that endogenous cholesterol synthesis has a diurnal rhythm with a nocturnal peak. Information regarding the timing of the endogenous cholesterol synthesis peak may be used for timed administration of cholesterol-lowering diets or drugs to have the largest impact on serum LDL-C lowering. The initial treatment for Dutch individuals with hypercholesterolemia is either atorvastatin, rosuvastatin or simvastatin (7). Of these, the latter has a short half-life time whereas the first two have a long half-life time (8). A recent meta-analysis of randomized controlled trials observed that statins taken in the evening led to a greater reduction in total cholesterol and LDL-C concentrations compared to statins taken in the morning (9). Comparison of short half-life time versus long half-life time statins, however, showed that this benefit of evening intake was only significant for statins with a short half-life time (9). The diurnal rhythm of endogenous cholesterol synthesis may thus be relevant when prescribing statins with a short half-life time but is less important for statins with a long half-life time.

Another important CVD risk factor is overweight or obesity. The global obesity prevalence has almost tripled between 1975 and 2016 and around 40% of adults was overweight and 13% was obese in 2016 (10). In the Netherlands, half of the adult population was overweight of which slightly more than 14% was obese in 2021 (11). Between 1990 and 2019, global deaths due to high body mass index (BMI) increased with 4.9%, DALYs with 18.0%, YLDs with 60.2%, and YLLs with 8.3%, after correction for population growth and aging (1). Results of a cross-sectional analysis showed that the health-related quality of life decreased with an increasing BMI (12). Moreover, loss of work productivity in people who were employed full-time increased with increasing BMI (12). Another study assessed the impact of obesity on life expectancy for 26 European populations and the United states over 1975 to 2012 and reported that the age-standardized obesity-attributable mortality fraction (i.e., the share of mortality caused by obesity) was 11% among men and 10% among women in European countries in 2012 (13). This obesity-attributable mortality fraction increased over time for all countries, but not to the same extent. Furthermore, the estimated potential gain in life expectancy if obesity was eliminated increased in all countries between 1975 and 2012 (13). Overweight or obesity is traditionally based on BMI, but a shift towards alternative measures which take abdominal adiposity into account, such as waist circumference, is observed (14). An increase in abdominal obesity was associated with an increased risk of future CVD events in adult men and women (15). Additionally, waist circumference showed a strong and significant association with the risk of death with and without adjustment for BMI in a large European cohort (16). In agreement, another study that pooled data from 11 prospective cohort studies that included over 650.000 individuals, observed that waist circumference was positively associated with all-cause mortality in men and women with and without adjustment for BMI (17). Each 5 cm increase in waist circumference was associated with a 9% higher mortality risk in women and 7% greater mortality risk in men (17). Finally, intensive care unit patients with abdominal obesity had a significantly higher mortality rate compared to patients without abdominal obesity (18). That study also reported that abdominal obesity was a significantly better predictor of mortality in an intensive care unit setting than a BMI higher than 30 kg/m^2 (18).

These previous paragraphs demonstrate that it is of societal and economic relevance to reduce the prevalence of CVD and overweight or obesity, especially abdominal obesity. It is therefore important that intervention studies are designed that target overweight and obesity and thereby reduce disability and premature death due to CVDs. It is essential to make lifestyle changes to effectively lower body weight, which is often achieved by reducing total energy intake. In this thesis, various types of intermittent energy restriction diets were examined, and results showed that effects of these types of diets on anthropometric and cardiometabolic risk parameters did not substantially differ from those of continuous energy restriction. It can therefore be suggested that intermittent energy restriction diets may be advised as an alternative to continuous energy restriction diets for individuals who are unable to adhere to continuous energy restriction diets on the long-term.

Scientific relevance

The studies included in this thesis provide novel information that is of scientific relevance. First of all, we identified new associations between genetic variants with endogenous cholesterol synthesis and intestinal cholesterol absorption. Most of these associations with SNPs in cholesterol metabolism and circadian clock genes had not been reported in literature before and other associations confirmed findings from previous research. The relation between SNPs in circadian relevant genes with cholesterol synthesis and absorption provides further knowledge on the link between the circadian system and cholesterol homeostasis. Second, this thesis increases our understanding of the diurnal variation in endogenous cholesterol synthesis and intestinal cholesterol absorption. The reciprocal relation between cholesterol synthesis and cholesterol absorption that has been reported before in the fasted state was not observed over 24 hours. Third, a comparison between acute consumption of a meal high in fat, protein, or carbohydrates on endogenous cholesterol synthesis and intestinal cholesterol absorption had not been performed before. The fact that people spend most of the day in the postprandial state instead of the fasted state makes it essential to understand how meals influence cholesterol homeostasis on the short term. The non-significant changes in intestinal cholesterol absorption following meal consumption further suggest that the absence of a diurnal rhythm of cholesterol absorption could not be explained by dietary macronutrient intake during the study. Next to cholesterol homeostasis, intermittent energy restriction, which has become increasingly popular among the public and in science over the previous years, was investigated to better understand the potential health effects of meal timing and a prolonged fasting duration. So far, a large amount of scientific evidence describing the benefits of intermittent energy restriction diets is based on animal studies. The meta-analysis presented in this thesis not only compared intermittent energy restriction diets to continuous energy restriction diets in humans but focused on different types of intermittent energy restriction diets as well. Previous results from animal studies are hopeful, but the results obtained from the meta-analysis based on human intervention studies indicate that intermittent energy restriction is not superior to continuous energy restriction with regards to changes in anthropometric and cardiovascular measures. This suggests that perhaps not the duration of fasting itself produced the health benefits, but that these may have resulted from an overall reduction in energy intake. More human intervention studies should be performed to distinguish between the influence of weight loss and meal timing on the health effects of intermittent energy restriction.

Translation into practice

This thesis includes two studies that presented associations between genetic variants in genes essential in cholesterol metabolism and the circadian clock with cholesterol synthesis and absorption. It has been observed that having a relatively high endogenous cholesterol synthesis or intestinal cholesterol absorption is associated with various health conditions, including CVD. Thus, classifying individuals as high synthesizer or high absorber based on genetic variants may be a promising step towards personalized cholesterol-lowering therapies. It remains, however, important to first replicate those findings in other independent cohorts before these genetic variants can be used for personalized treatment in practice. The information regarding the timing of the endogenous cholesterol synthesis peak is relevant for health care professionals who prescribe cholesterol synthesis inhibitors with a short half-life time to their patients, but does not have to be taken into account for statins with a longer half-life time. In animal studies, intermittent energy restriction diets seemed very promising for improving cardiometabolic health, but it is important to confirm findings from animal studies in human clinical trials before recommendations can be made to the general population. The findings of our meta-analysis, which included trials in healthy overweight/obese individuals, may be relevant for dietitians and other health care workers who want to provide overweight or obese people with advice on possible weight loss diets. In the future, more studies should be performed that measure compliance to different types of intermittent energy restriction diets on the longer term. All studies presented in this thesis have been or will be published in international peer-reviewed scientific journals. Furthermore, the findings have been or will be presented via poster presentations and oral presentations. The knowledge obtained from these studies is therefore available for interested scientists, dietitians, policymakers, and other (para)medical health professionals.

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