Human cholesterol metabolism

HUMAN CHOLESTEROL METABOLISM:

Effects of aerobic exercise training, nutrition and inflammation

SULTAN QASSEM MASHNAFI

The research presented in this dissertation was conducted within the NUTRIM School of Nutrition and Translational Research in Metabolism and was financially supported by Al-Baha University, Saudi Arabia.

ISBN: 978-94-6469-171-9

Cover & Lay-out: Stefanie van den Herik || HerikMedia || herikmedia.nl
Printing: ProefschriftMaken || proefschriftmaken.nl

HUMAN CHOLESTEROL METABOLISM:

Effects of aerobic exercise training, nutrition and inflammation

DISSERTATION

to obtain the degree of Doctor at the Maastricht University,
on the authority of the Rector Magnificus,
Prof. dr. Pamela Habibović
in accordance with the decision of the Board of Deans,
to be defended in public
on Thursday, January 12th 2023, at 16.00 hours

by

Sultan Qassem Mashnafi

Born on October 4th 1985 in Makkah, Saudi Arabia

Supervisors

Prof. dr. J. Plat
Prof. dr. ir. R.P. Mensink

Co-supervisor

Dr. S. Baumgartner

Assessment Committee

Prof. dr. M.K.C Hesselink (chair)
Dr. T.C.M. Adam
Prof. dr. J.M. Geleijnse (Wageningen University and Research, the Netherlands)
Prof. dr. M. Poeze
Prof. dr. E.A. Trautwein (Christian Albrechts University, Germany)

Table of contents

CHAPTER

GENERAL INTRODUCTION

Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of mortality worldwide and the prevalence is still rising annually [1]. The main underlying determinant to develop CVD is atherosclerosis [2] and several factors such as cigarette smoking, hypertension, physical inactivity, dyslipidemia, obesity and type 2 diabetes are known to increase the risk of atherosclerosis development [3]. Dyslipidemia represents an abnormality in the serum lipoprotein profile that is characterized by reduced high-density lipoprotein (HDL) cholesterol concentrations, and/or elevated low-density lipoprotein (LDL) cholesterol and triacylglycerol concentrations [4]. Hypercholesterolemia, defined as high serum cholesterol concentrations, especially in the LDL fraction, is considered a well-defined risk factor for atherosclerosis development [5, 6]. Moreover, many lines of evidence suggest a positive causal relationship between LDL cholesterol concentrations and CVD events [7, 8]. Exploring cholesterol metabolism in more detail, it seems that especially a high intestinal cholesterol absorption is atherogenic [9, 10]. It is estimated that approximately 30% of the population is characterized by a high intestinal cholesterol absorption in which CVD risk is elevated 2-fold [11]. Therefore, it is of interest to examine characteristics of cholesterol metabolism into more detail. Finally, inflammation is involved in nearly all stages of atherogenesis processes [12] revealing its crucial role in initiation and progression of CVD [13-15]. Recent European guidelines recommend lifestyle and dietary changes for CVD prevention [16]. The changes include amongst others, increasing the amount of physical activity and aiming to lose weight. Therefore, in this thesis we aim to assess the effect of several factors such as physical activity, diet-induced weight loss and using lipopolysaccharide (LPS) as a well-known pro-inflammatory trigger on characteristics of cholesterol metabolism.

Cholesterol and non-cholesterol sterols

Cholesterol consists of a steroid nucleus with four cycloalkane rings, a 3-hydroxyl group and an alkyl side chain. The main sources of cholesterol in our diet are beef, poultry, egg yolk, butter and cheese, which altogether contribute to an average daily intake of 200-300 mg/day [17]. However, there are no clear guidelines for the amount of dietary cholesterol in European policies [17]. Non-cholesterol sterols encompass plant sterols, cholesterol precursors and degradation products of cholesterol **(Table 1)**. Plant sterols have a similar structure to cholesterol with a different side chain and are abundant in plant-based diets. For example, they are present in vegetable oils, cereals, nuts, fruits and vegetables. Generally, the average intake of plant sterols in the population is around 300 mg per day [18-21]. Sitosterol, campesterol and stigmasterol are the most abundant plant sterols in our diet [20]. Since non-cholesterol sterols are transported in lipoproteins, the circulating

concentrations of non-cholesterol sterols are frequently standardized to those of cholesterol [22]. Circulating cholesterol-standardized levels of campesterol and sitosterol serve as surrogate markers for measuring cholesterol absorption [23]. Furthermore, the cholesterol metabolite cholestanol is used as a measure for cholesterol absorption [24]. Lathosterol and desmosterol are two intermediates of the endogenous cholesterol synthesis pathway, and these metabolites reflect the level of cholesterol synthesis when standardized for cholesterol concentrations [25, 26].

Table 1. Non-cholesterol sterol products that can serve as markers for cholesterol metabolism.

Cholesterol synthesis biomarkers	**Cholesterol absorption biomarkers**	
Cholesterol precursors	Plant sterols	Cholesterol degradation metabolite
Lanosterol Lathosterol Desmosterol Cholestenol	Campesterol Sitosterol Stigmasterol	Cholestanol

Cholesterol and plant sterol absorption

As shown in **Figure 1**, cholesterol and plant sterols, either derived from the diet or biliary secretion, are dissolved in mixed micelles before they become available for absorption into the enterocytes. Cholesterol and plant sterols are transported into the enterocyte via the Niemann-Pick C1-Like 1 (NPC1L1) transporter, located in the enterocyte brush borders membranes. Within the enterocyte, the majority of plant sterols are secreted back into the intestinal lumen by the heterodimer ATP-binding cassette (ABC) transporters ABCG5/G8, while cholesterol is re-excreted via this route to lesser extent. One of the assumed reasons for this is that plant sterols are poor substrates for the enzyme Acyl-CoA cholesterol acyltransferase-2 (ACAT), an enzyme that esterifies cholesterol in the enterocyte before they are integrated into chylomicrons and transferred into the circulation via the lymphatic system. Since plant sterols remain in its free form mainly, they are preferentially secreted back into the lumen instead of being incorporated into chylomicrons, meaning that only small amounts are actually entering the circulation. Unlike cholesterol, humans are unable to synthesize plant sterols [27].

Several approaches using fractional and absolute parameters have been used in humans to study the process of intestinal cholesterol absorption including radioisotope tracer or stable isotope tracer methods. However, these methods are laborious, complex and expensive and need a steady-state condition. Therefore, an alternative approach via measuring circulating levels of plant sterols and cholestanol has been suggested for

estimating intestinal cholesterol absorption. The use of plant sterols and cholestanol (degradation product of cholesterol) as surrogate markers for cholesterol absorption has been validated by comparing their cholesterol standardized levels with measurements for intestinal cholesterol absorption using tracer techniques. Plasma cholesterol standardized levels of campesterol, sitosterol and cholestanol in a random selected population were correlated with the percent cholesterol absorption rates obtained by using the dual-isotope continuous feeding method [23, 28].

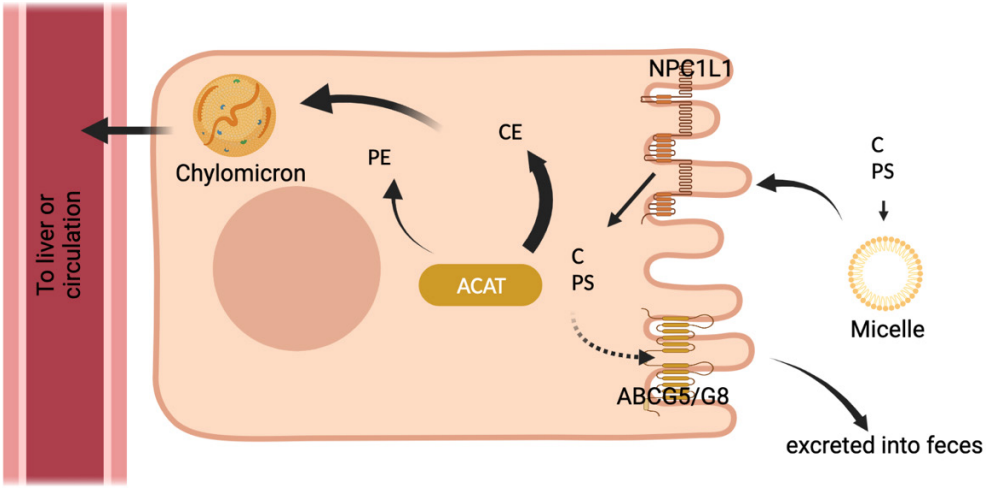


Figure 1. Overview of intestinal absorption of cholesterol and plant sterols (modified based on Ryan et al., 2009). Figure was created with BioRender.com.

C: cholesterol; PS: plant sterols (phytosterols); NPC1L1: Niemann-Pick C1-Like 1; ABCG5/G8: ATP-binding cassette sub-family G member 5 and 8; ACAT; Acyl-CoA cholesterol acyltransferase-2 enzyme; CE; cholesterol ester; PE; plant sterol ester.

Cholesterol biosynthesis pathways

The important steps of the endogenous cholesterol synthesis process are illustrated in **Figure 2** [29]. Hydroxymethyl-glutaryl (HMG)-CoA synthase converts acetate into 3-hydroxy-3-methyl-glutaryl-CoA before it converts to mevalonate via HMG-CoA reductase. Mevalonate is processed into squalene, which is then converted to lanosterol. The Kandutsch-Russell pathway and Bloch pathway shuttle lanosterol through the last steps of cholesterol synthesis. In the Kandutsch-Russell pathway, the reduction of the double bond at C24 in the sterol side chain occurs early leading to production of lathosterol, then 7-dehydrocholesterol and lastly cholesterol. In the Bloch pathway, the double bond at C24 is reduced at the last step when cholesterol is produced from desmosterol [30, 31].

Different methods are used to measure endogenous whole-body cholesterol synthesis such as the cholesterol balance technique, the fractional conversion of squalene, or mass isotopomer analysis and deuterium incorporation. Due to methodological drawbacks such as being laborious, time consuming and costly, using plasma levels of the cholesterol precursors are used as an alternative to reflect endogenous cholesterol biosynthesis. The cholesterol metabolite lathosterol serves as the best validated marker for endogenous cholesterol synthesis [25]. However, the validity of other cholesterol precursors has also been demonstrated since cholesterol standardized levels of desmosterol and cholestenol were positively correlated with direct measurements for endogenous cholesterol synthesis using the sterol balance method [23, 32, 33]. Moreover, plasma squalene, lanosterol, lathosterol, and desmosterol correlated positively with values obtained from the deuterium incorporation method in hypercholesterolemic women [32].

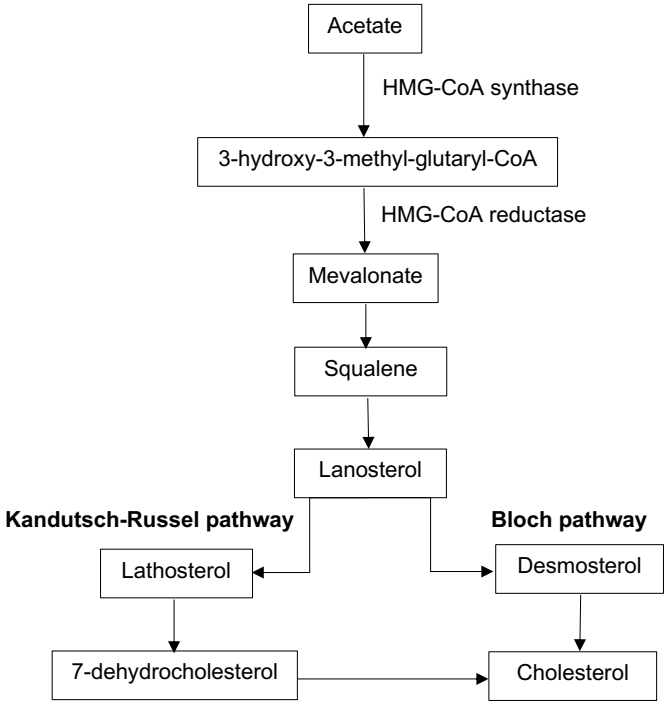


Figure 2. Simplified scheme of endogenous cholesterol biosynthesis pathways.

Method of measuring cholesterol and non-cholesterol sterols

Measuring cholesterol and non-cholesterol sterols from plasma and serum samples involves lipid extraction followed by quantifying their concentrations using gas chromatography methods such as GCFID or GCMS as described by McKay et al. [34]. Analyzing these non-cholesterol sterols is accepted to assess intestinal cholesterol absorption and endogenous cholesterol synthesis in large population studies [23, 28, 35, 36]. To quantify the total amount of plant sterols (i.e., the sum of free and esterified sterol molecules), plasma or serum samples are first saponified using alkaline hydrolysis to convert esterified sterols into their free forms. Next, tetramethylsilane (TMS) reagent is used to derivatize the extracted molecules before being injected into the gas chromatography apparatus. Finally, the data analysis involves detecting, integration, and quantifying these non-cholesterol sterol concentrations followed by standardizing these concentrations to cholesterol concentrations, as analyzed in the same run.

Phenotyping and targeting cholesterol metabolism characteristics

Cholesterol homeostasis is regulated by balancing different processes like intestinal cholesterol absorption, endogenous cholesterol synthesis, and bile acid synthesis and excretion [37-39]. Cholesterol homeostasis plays an important role in maintaining metabolic health [40]. It is generally accepted that a reciprocal relationship between intestinal cholesterol absorption and endogenous cholesterol synthesis exists [37]. For example, a dietary study has shown that an increase in cholesterol absorption following high dietary cholesterol was compensated for by a reduction in endogenous cholesterol synthesis [41]. In contrary, consumption of plant sterols / stanols or treatment with ezetimibe, which are all known to decrease intestinal cholesterol absorption, resulted in increased cholesterol synthesis [42, 43]. However, significant inter-individual variation in this reciprocal relationship has been found [41].

Interestingly, subjects can be identified either by having a higher cholesterol absorption or a higher cholesterol synthesis, phenotyping them as so-called preferential cholesterol absorbers or cholesterol synthesizers, respectively [43]. These characteristics can theoretically be applied in targeting personalized dietary and pharmacological interventions. A statin drug binds to the rate-limiting enzyme HMG-CoA reductase, and as such inhibits cholesterol synthesis [44]. On other hand, ezetimibe and plant sterol or stanol consumption inhibit intestinal cholesterol absorption via lowering dietary and biliary cholesterol uptake by the enterocyte [45]. Thus, subjects with a cholesterol

synthesizer phenotype should respond more effectively to statin treatment while those with a cholesterol absorber phenotype should benefit more from ezetimibe or plant sterols/stanols treatment. Since cholesterol homeostasis is assumed to be important for maintaining metabolic health, there is a need to explore associations between characteristics of cholesterol metabolism and a variety of diseased conditions.

Outline thesis

The aim of this thesis was to examine the effects of physical activity, diet-induced weight loss and LPS infusion used as an inflammatory trigger on characteristics of cholesterol metabolism. In addition, we evaluated the effects of different lipid emulsions with various amounts of plant sterols on plasma plant sterols, liver function and inflammatory markers. To understand the characteristics of cholesterol absorption and synthesis in health and diseased conditions, we first extensively reviewed the potential relevant literature on the relationship between plasma non-cholesterol sterols as biomarkers for intestinal absorption and endogenous cholesterol synthesis with different metabolic disorders (**chapter 2**). Assumptions of validity of these markers in dietary and pharmacological studies were also discussed in this chapter. Next, we described the effects of an 8-week aerobic physical activity program on plasma markers for cholesterol absorption and synthesis in older men with obesity (**chapter 3**). The effect of diet-induced weight loss on plasma cholesterol absorption and synthesis markers were studied in apparently healthy men with abdominal obesity (**chapter 4**). **Chapter 5** describes the effects of LPS infusion on non-cholesterol sterol concentrations and inflammatory markers in healthy young volunteers. Furthermore, in a human intervention study carried out in adult patients with intestinal failure receiving home parental nutrition with different lipid emulsions, the effects of plant sterols content of these emulsions on plasma non-cholesterol sterol concentrations and the link with liver function and inflammation markers were investigated (**chapter 6**). Finally, the main findings, conclusions and future perspectives in this thesis are reviewed and discussed in **chapter 7**.

References

1. World Health Organization (WHO). Cardiovascular diseases (CVDs) fact sheet. June 2021. Available from: https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).
2. Frostegard J. Immunity, atherosclerosis and cardiovascular disease. BMC Med. 2013;11:117.
3. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med. 2011;17(11):1410-22.
4. Badimon L, Vilahur G. LDL-cholesterol versus HDL-cholesterol in the atherosclerotic plaque: inflammatory resolution versus thrombotic chaos. Ann N Y Acad Sci. 2012;1254:18-32.
5. Gidding SS, Allen NB. Cholesterol and Atherosclerotic Cardiovascular Disease: A Lifelong Problem. J Am Heart Assoc. 2019;8(11):e012924.
6. Zarate A, Manuel-Apolinar L, Saucedo R, Hernandez-Valencia M, Basurto L. Hypercholesterolemia As a Risk Factor for Cardiovascular Disease: Current Controversial Therapeutic Management. Arch Med Res. 2016;47(7):491-5.
7. Ference BA, Ginsberg HN, Graham I, Ray KK, Packard CJ, Bruckert E, et al. Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. Eur Heart J. 2017;38(32):2459-72.
8. Stein EA. Low-density lipoprotein cholesterol reduction and prevention of cardiovascular disease. Mayo Clin Proc. 2009;84(4):307-9.
9. Zhong VW, Van Horn L, Cornelis MC, Wilkins JT, Ning H, Carnethon MR, et al. Associations of Dietary Cholesterol or Egg Consumption With Incident Cardiovascular Disease and Mortality. JAMA. 2019;321(11):1081-95.
10. Zhuang P, Wu F, Mao L, Zhu F, Zhang Y, Chen X, et al. Egg and cholesterol consumption and mortality from cardiovascular and different causes in the United States: A population-based cohort study. PLoS Med. 2021;18(2):e1003508.
11. Helgadottir A, Thorleifsson G, Alexandersson KF, Tragante V, Thorsteinsdottir M, Eiriksson FF, et al. Genetic variability in the absorption of dietary sterols affects the risk of coronary artery disease. Eur Heart J. 2020;41(28):2618-28.
12. Ridker PM, Luscher TF. Anti-inflammatory therapies for cardiovascular disease. Eur Heart J. 2014;35(27):1782-91.
13. Hansson GK, Robertson AK, Soderberg-Naucler C. Inflammation and atherosclerosis. Annu Rev Pathol. 2006;1:297-329.
14. Libby P. Inflammation and cardiovascular disease mechanisms. Am J Clin Nutr. 2006;83(2):456S-60S.
15. Rocha VZ, Libby P. Obesity, inflammation, and atherosclerosis. Nat Rev Cardiol. 2009;6(6):399-409.
16. Visseren FLJ, Mach F, Smulders YM, Carballo D, Koskinas KC, Back M, et al. 2021 ESC Guidelines on cardiovascular disease prevention in clinical practice. Eur Heart J. 2021;42(34):3227-337.

17. Graham I, Atar D, Borch-Johnsen K, Boysen G, Burell G, Cifkova R, et al. European guidelines on cardiovascular disease prevention in clinical practice: executive summary. Fourth Joint Task Force of the European Society of Cardiology and other societies on cardiovascular disease prevention in clinical practice (constituted by representatives of nine societies and by invited experts). Eur J Cardiovasc Prev Rehabil. 2007;14 Suppl 2:E1-40.
18. Escurriol V, Mari-Dell'Olmo M, Rohlfs I, Borrell C, Chirlaque MD, Buckland G, et al. Plant sterol intake and education level in the Spanish EPIC cohort. Nutrition. 2009;25(7-8):769-73.
19. Klingberg S, Andersson H, Mulligan A, Bhaniani A, Welch A, Bingham S, et al. Food sources of plant sterols in the EPIC Norfolk population. Eur J Clin Nutr. 2008;62(6):695-703.
20. Ostlund RE, Jr. Phytosterols in human nutrition. Annu Rev Nutr. 2002;22:533-49.
21. Sioen I, Matthys C, Huybrechts I, Van Camp J, De Henauw S. Consumption of plant sterols in Belgium: estimated intakes and sources of naturally occurring plant sterols and beta-carotene. Br J Nutr. 2011;105(6):960-6.
22. MacKay DS, Jones PJH. Plasma noncholesterol sterols: current uses, potential and need for standardization. Curr Opin Lipidol. 2012;23(3):241-7.
23. Miettinen TA, Tilvis RS, Kesaniemi YA. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. Am J Epidemiol. 1990;131(1):20-31.
24. Seyama Y. Cholestanol metabolism, molecular pathology, and nutritional implications. J Med Food. 2003;6(3):217-24.
25. Kempen HJ, Glatz JF, Gevers Leuven JA, van der Voort HA, Katan MB. Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans. J Lipid Res. 1988;29(9):1149-55.
26. Miettinen TA. Detection of changes in human cholesterol metabolism. Ann Clin Res. 1970;2(4):300-20.
27. Chan YM, Varady KA, Lin Y, Trautwein E, Mensink RP, Plat J, et al. Plasma concentrations of plant sterols: physiology and relationship with coronary heart disease. Nutr Rev. 2006;64(9):385-402.
28. Miettinen TA, Tilvis RS, Kesaniemi YA. Serum cholestanol and plant sterol levels in relation to cholesterol metabolism in middle-aged men. Metabolism. 1989;38(2):136-40.
29. Wu ANB. Biomarkers for Cholesterol Absorption and Synthesis in Hyperlipidemic Patients: Role for Therapeutic Selection. Clin Lab Med. 2014;34(1):157-+.
30. Clayton PT. Disorders of cholesterol biosynthesis. Arch Dis Child. 1998;78(2):185-9.
31. Vainio S, Jansen M, Koivusalo M, Rog T, Karttunen M, Vattulainen I, et al. Significance of sterol structural specificity. Desmosterol cannot replace cholesterol in lipid rafts. J Biol Chem. 2006;281(1):348-55.
32. Matthan NR, Raeini-Sarjaz M, Lichtenstein AH, Ausman LM, Jones PJ. Deuterium uptake and plasma cholesterol precursor levels correspond as methods for measurement of endogenous cholesterol synthesis in hypercholesterolemic women. Lipids. 2000;35(9):1037-44.

33. Miettinen TA, Gylling H, Nissinen MJ. The role of serum non-cholesterol sterols as surrogate markers of absolute cholesterol synthesis and absorption. Nutr Metab Cardiovasc Dis. 2011;21(10):765-9.
34. Mackay DS, Jones PJ, Myrie SB, Plat J, Lutjohann D. Methodological considerations for the harmonization of non-cholesterol sterol bio-analysis. J Chromatogr B Analyt Technol Biomed Life Sci. 2014;957:116-22.
35. Gylling H. Clinical utility of serum markers of cholesterol absorption and synthesis. Curr Opin Lipidol. 2014;25(3):207-12.
36. Matthan NR, Lichtenstein AH. Approaches to measuring cholesterol absorption in humans. Atherosclerosis. 2004;174(2):197-205.
37. Cohen DE. Balancing cholesterol synthesis and absorption in the gastrointestinal tract. J Clin Lipidol. 2008;2(2):S1-3.
38. Trapani L, Segatto M, Pallottini V. Regulation and deregulation of cholesterol homeostasis: The liver as a metabolic "power station". World J Hepatol. 2012;4(6):184-90.
39. Yeagle PL. Cholesterol and the cell membrane. Biochim Biophys Acta. 1985;822(3-4):267-87.
40. Kruit JK, Groen AK, van Berkel TJ, Kuipers F. Emerging roles of the intestine in control of cholesterol metabolism. World J Gastroenterol. 2006;12(40):6429-39.
41. Quintao E, Grundy SM, Ahrens EH, Jr. Effects of dietary cholesterol on the regulation of total body cholesterol in man. J Lipid Res. 1971;12(2):233-47.
42. Santosa S, Varady KA, AbuMweis S, Jones PJ. Physiological and therapeutic factors affecting cholesterol metabolism: does a reciprocal relationship between cholesterol absorption and synthesis really exist? Life Sci. 2007;80(6):505-14.
43. Thuluva SC, Igel M, Giesa U, Lutjohann D, Sudhop T, von Bergmann K. Ratio of lathosterol to campesterol in serum predicts the cholesterol-lowering effect of sitostanol-supplemented margarine. Int J Clin Pharmacol Ther. 2005;43(7):305-10.
44. Nissinen MJ, Miettinen TE, Gylling H, Miettinen TA. Applicability of non-cholesterol sterols in predicting response in cholesterol metabolism to simvastatin and fluvastatin treatment among hypercholesterolemic men. Nutr Metab Cardiovasc Dis. 2010;20(5):308-16.
45. Stellaard F, Lutjohann D. The Interpretation of Cholesterol Balance Derived Synthesis Data and Surrogate Noncholesterol Plasma Markers for Cholesterol Synthesis under Lipid Lowering Therapies. Cholesterol. 2017;2017:5046294.

CHAPTER

2

NON-CHOLESTEROL STEROL CONCENTRATIONS AS BIOMARKERS FOR CHOLESTEROL ABSORPTION AND SYNTHESIS IN DIFFERENT METABOLIC DISORDERS:

a systematic review

Sultan Mashnafi, Jogchum Plat,
Ronald P. Mensink, Sabine Baumgartner

Nutrients. 2019;11(1),124

Abstract

Non-cholesterol sterols are validated biomarkers for intestinal cholesterol absorption and endogenous cholesterol synthesis. However, their use in metabolic disturbances has not been systematically explored. Therefore, we conducted a systematic review to provide an overview of non-cholesterol sterols as markers for cholesterol metabolism in different metabolic disorders. Potentially relevant studies were retrieved by a systematic search of three databases in July 2018 and ninety-four human studies were included. Cholesterol-standardized levels of campesterol, sitosterol and cholestanol were collected to reflect cholesterol absorption and those of lathosterol and desmosterol to reflect cholesterol synthesis. Their use as biomarkers was examined in the following metabolic disorders: overweight/obesity (n = 16), diabetes mellitus (n = 15), metabolic syndrome (n = 5), hyperlipidemia (n = 11), cardiovascular disease (n = 17), and diseases related to intestine (n = 16), liver (n = 22) or kidney (n = 2). In general, markers for cholesterol absorption and synthesis displayed reciprocal patterns, showing that cholesterol metabolism is tightly regulated by the interplay of intestinal absorption and endogenous synthesis. Distinctive patterns for cholesterol absorption or cholesterol synthesis could be identified, suggesting that metabolic disorders can be classified as 'cholesterol absorbers or cholesterol synthesizers'. Future studies should be performed to confirm or refute these findings and to examine whether this information can be used for targeted (dietary) interventions.

Keywords

non-cholesterol sterols; plant sterols; BMI; diabetes mellitus; metabolic syndrome; hyperlipidemia; cardiovascular disease; intestinal disease; liver disease; kidney disease

Abstract

1. Introduction

Cholesterol metabolism is tightly regulated, since cholesterol plays an essential role in many physiological processes [1]. Cells have different possibilities to regulate cellular free cholesterol concentrations such as changing low-density lipoprotein-receptor expression, endogenous synthesis, intracellular esterification and excretion [2]. Cholesterol can not only be obtained from de novo endogenous cholesterol synthesis, which occurs virtually in every single cell [3], but also from intestinal absorption of dietary and biliary cholesterol. Interestingly, an inverse relationship exists between cholesterol absorption and synthesis, whereby low intestinal cholesterol absorption is compensated by upregulation of cholesterol synthesis and vice versa [4]. To study the processes of intestinal cholesterol absorption and endogenous cholesterol synthesis in humans, several approaches can be used. Intestinal cholesterol absorption can be measured by using radioisotope tracer or stable isotope tracer methods. Radioisotope methods can be used to quantify cholesterol absorption via both direct and indirect procedures [5]. The only direct method to measure intestinal cholesterol absorption in humans is the intestinal perfusion technique using radioisotopes [6], whereas cholesterol balance and isotope ratio methods can be used to indirectly calculate cholesterol absorption. Cholesterol absorption using stable isotopes can be estimated using the dual plasma isotope ratio method, continuous isotope feeding, or single stable isotopes [5]. For quantifying endogenous cholesterol synthesis, other techniques such as cholesterol balance, fractional conversion of squalene, mass isotopomer distribution analysis (MIDA), and deuterium incorporation (DI) are used. Overall, the cholesterol balance technique, dual plasma isotope ratio, and continuous isotope feeding are the gold standard methods to quantify respectively cholesterol synthesis and absorption. These methods were developed and validated many years ago [7–10]. However, although these techniques are very precise, they are also complex, laborious, expensive and require a steady-state condition. Thus, these techniques are only suitable for small-scale in-depth studies, but not for large-scale intervention studies [11]. Consequently, there is a clear need for alternative approaches to monitor intestinal cholesterol absorption and endogenous cholesterol synthesis. In the early 90s, serum non-cholesterol sterols were introduced as validated biomarkers for assessing intestinal cholesterol absorption and endogenous synthesis [12]. The cholesterol precursors squalene, desmosterol and lathosterol reflect cholesterol synthesis, and the non-cholesterol sterols sitosterol, campesterol and cholestanol reflect fractional intestinal cholesterol absorption [13].

The use of non-cholesterol sterols as biomarkers to estimate intestinal cholesterol absorption and endogenous cholesterol synthesis has been validated by comparing plasma non-cholesterol sterols with absolute measurements for intestinal cholesterol absorption and whole-body cholesterol synthesis [12]. In more detail, Miettinen and

colleagues measured intestinal cholesterol absorption via the dual isotope continuous feeding approach and by determining the ratio of plasma campesterol, sitosterol and cholestanol to total cholesterol (TC) in a randomly selected healthy normocholesterolemic population [12]. It was shown that the ratio of these plasma non-cholesterol sterols to cholesterol highly correlated with the absorption values obtained by using the dual isotope method. In another study, including middle-aged men, plasma cholestanol-to-cholesterol and plant sterol-to-cholesterol ratios were associated with fractional and absolute absorption of dietary cholesterol. From this second study, it was concluded that the serum ratio of cholestanol-to-cholesterol is a sensitive indicator to detect changes in intestinal sterol absorption [14]. Regarding endogenous whole-body cholesterol synthesis, the cholesterol precursors lathosterol, desmosterol and cholestenol, calculated as their ratios to cholesterol, are often used as alternative markers. Their validity was demonstrated by showing positive correlations with endogenous cholesterol synthesis measured by the sterol balance method [12,14,15]. Furthermore, Matthan and colleagues validated the plasma cholesterol precursors squalene, lanosterol, desmosterol and lathosterol by measuring the rate of uptake of deuterium into plasma free cholesterol in a small study with hypercholesterolemic women [16]. The deuterium incorporation method correlated positively with cholesterol synthesis as estimated by the plasma biomarkers. It should be noted that the quality of the methodology used to measure non-cholesterol sterol levels is important for the validity of these biomarkers to reflect cholesterol metabolism [17]. In addition, since cholesterol and non-cholesterol sterol absorption in the intestinal lumen is regulated by the Niemann-Pick C1-like 1 sterol transporter (NPC1L1) and the ABCG5/G8 transporters, genetic variation in these protein transporters may impact their activity, which could possibly have an effect on selectivity of cholesterol absorption biomarkers.

Another approach to address the validity of these biomarkers is by evaluating a change in their levels during drug therapies such as statin and ezetimibe, which have known and accepted effects on cholesterol metabolism [18]. Briefly, statins lower endogenous cholesterol synthesis by inhibiting the rate-limiting enzyme, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, leading to reduced production of cholesterol. Ezetimibe inhibits the NPC1L1 sterol transporter in the enterocyte resulting in decreased absorption of dietary and biliary cholesterol [19]. Nissinen and co-workers conducted a clinical trial to evaluate the change in whole-body cholesterol metabolism among hypercholesterolemic men during two different statin treatments and showed that ratios of lathosterol-to-cholesterol and desmosterol-to-cholesterol correlated with absolute cholesterol synthesis measured using isotope tracer techniques at baseline, as well as on statin treatment [20]. This study also demonstrated inverse correlations at baseline between cholesterol synthesis markers (ratios of plasma lathosterol-to-cholesterol and desmosterol-to-cholesterol) and cholesterol absorption markers (ratios of plasma sitosterol-to-cholesterol, campesterol-to-cholesterol and cholestanol-to-cholesterol). In

a study by Stellaard et al. [21], ezetimibe treatment of a population with normal daily cholesterol intake, serum ratios of sitosterol-to-cholesterol, campesterol-to-cholesterol and cholestanol-to-cholesterol were shown to reflect changes in fractional cholesterol absorption. In the same study, serum lathosterol-to-cholesterol ratios correlated with absolute cholesterol synthesis. Taking together, findings from validation studies comparing non-cholesterol sterols with radio or stable isotope tracer techniques together with changes in non-cholesterol sterols during pharmacological intervention studies indicate that the use of serum non-cholesterol sterols as biomarkers for cholesterol metabolism are valid under various conditions.

As outlined above, non-cholesterol sterols are used as markers for intestinal cholesterol absorption and endogenous synthesis in healthy subjects under normal metabolic conditions [12,14]. However, there is a need to explore in more detail what we can learn from these non-cholesterol sterols as biomarkers of cholesterol metabolism in various metabolic conditions. Therefore, the aim of this paper is to provide a systematic overview of the use of non-cholesterol sterols as biomarkers to reflect cholesterol metabolism in different metabolic disorders.

2. Methods

2.1. Search Strategy

Potentially relevant studies were retrieved by a systematic search of three databases (Medline, Embase, and the Cochrane Central Register of Clinical Trials) in July 2018. Search terms consisted of keywords that were related to plant sterols and metabolic conditions. The following search terms were used: plant sterols (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 cholestenol) combined with metabolic syndrome, diabetes, heart disease (cardiovascular disease or cardiovascular diseases or CVD or coronary heart disease or coronary heart diseases or CHD or carotid artery disease or coronary artery disease or coronary artery diseases or CAD or atherosclerosis), hyperlipidemia (hypercholesterolemia or hypercholesterolaemia or hyperlipidemia or hyperlipidaemia or hyperlipoproteinemia or hyperlipoproteinaemia) and organ-related diseases (liver or kidney or kidneys or intestine or intestines or intestinal or small bowel or ileum or ileal or jejunum or duodenum or duodenal or colon or colonic) and plasma (plasma or serum or blood).

2.2. Selection of Studies

Human studies investigating cholesterol metabolism using the surrogate non-cholesterol sterol markers in different metabolic conditions were selected. The selection procedures

were divided into two stages, and for the first stage, titles and abstracts were screened and papers were included if they met the following criteria: (1) studies performed in human subjects, (2) measurement of plasma (or serum) non-cholesterol sterol concentrations, (3) original research (i.e., no case reports, conference proceedings or reviews), (4) written in English language, and (5) no duplicates. For the second selection stage, full papers were read to assess their eligibility and studies were excluded when they lacked a control group or when plasma TC-standardized non-cholesterol sterols were not reported or could not be calculated. When inconclusive, the eligibility of the studies was discussed with the authors to reach consensus.

2.3. Data Collection and Transformation

Data were collected using a spreadsheet that included the following information: publication characteristics (reference number, first author and year of publication), study characteristics (design, specification of subgroups and sample size), subject characteristics (health status, mean age, mean BMI and gender distribution), measurement characteristics (plasma/serum and type of analytical method), and variable characteristics (sitosterol, campesterol, cholestanol, lathosterol, desmosterol and cholesterol). For all variables, mean and variance measures were collected at baseline in the metabolic condition and the control group. If non-cholesterol sterol or TC concentrations were expressed in µg/dL, µg/mL, mg/dL, mg/L, mg/mL, µg/L, or ng/mL, these units were converted to µmol/L and mmol/L based on their molecular weight (sitosterol: 414.7, campesterol: 400.7, lathosterol and cholesterol: 386.7, cholestanol: 388.7, desmosterol: 384.6 g/mol). These conversions applied for means and variance measures. Absolute as well as TC-standardized data were collected, and TC standardized values were calculated if these were not reported. If TC-standardized values were not reported or could not be calculated, the study was excluded. Data reported as median (interquartile range) values were transformed to means and standard deviation (SD) based on the method of Wan et al. [22] and data displayed in graphs were estimated manually.

3. Results

The systematic search retrieved 1953 potentially relevant papers and after two selection rounds, 94 studies were included in the systematic review. A flowchart of the study selection process is presented in Figure 1.

3.1. Serum Non-Cholesterol Sterol Markers in Overweight and Obese Subjects

Sixteen articles carried out in overweight or obese subjects met the inclusion criteria (Table 1). In these 16 papers, eight studies were reported that compared cross-sectionally

obese subjects with normal weight subjects [23–30], while two intervention studies compared subjects before and after surgery [31,32], and six intervention studies before and after diet-induced weight loss [33–38]. The mean BMI (Body Mass Index) in the cross-sectional studies of the cases was >30 kg/m^2 in six comparisons, between 25–30 kg/m^2 in one comparison [24], and not reported in another comparison [25]. Control subjects had a mean BMI in the normal-weight range (20.9–25.1 kg/m^2).

In general, the cross-sectional studies suggested that an increased BMI was associated with a lower cholesterol absorption and higher cholesterol synthesis. Surprisingly, TC-standardized levels of sitosterol and campesterol and of lathosterol both increased in subjects after sleeve gastrectomy [31] or gastric bypass [32]. In contrast, cholesterol absorption was higher and synthesis lower before weight loss induced by gastric banding surgery. The intervention studies also showed that diet-induced weight loss increased cholesterol absorption and decreased cholesterol synthesis.

Overall, these findings suggest cholesterol absorption is decreased and synthesis increased in obese subjects. These associations are reversed after diet-induced weight loss. Effects of the different types of surgical interventions are not clear.

3.2. Serum Non-Cholesterol Sterol Markers in Subjects with Diabetes Mellitus

Fifteen studies were identified, of which four studies were performed in patients with type 1 diabetes mellitus (T1DM) [39–42], and nine studies in patients with type 2 diabetes mellitus (T2DM) of which one paper yielded two data points [43–51], while in one study, both T1DM and T2DM patients were included [52] (Table 2). In one study, women with gestational diabetes (GDM) were examined [53].

Three studies in subjects with T1DM suggested that cholesterol absorption was higher in T1DM patients [39,40,42], and four studies reported that cholesterol synthesis was lower in T1DM patients compared to non-diabetic controls [39,40,42,52]. A study performed in children [41] did not find any differences in cholesterol absorption and synthesis markers between cases and controls. The opposite was found for T2DM, i.e., four studies [43–45,50] suggested lower cholesterol absorption and higher cholesterol synthesis in T2DM patients. Two of these studies [43,50] were performed in T2DM subjects on statin therapy, but cholesterol absorption and synthesis patterns were comparable to those of T2DM patients not using statins [44,45]. In two studies, no differences in cholesterol absorption markers were observed [47,51], and three studies did not report differences in synthesis markers in T2DM patients compared to non-diabetics [49,51,52]. Finally, pregnant women with gestational diabetes showed comparable levels of cholesterol absorption and

Table 1. Serum non-cholesterol sterol markers in overweight and obese subjects.

		Sitosterol		Campesterol		Cholestanol		Lathosterol		Desmosterol	
Cross-Sectional											
Chan, 2002 [23]	Cases (*n* = 48)							1.87			
	Controls (*n* = 10)							1.54			
Lupattelli, 2012 [24]	Cases (*n* = 63)	0.87 ± 0.43	↓	0.40 ± 0.33	↓			1.04 ± 0.45	↑		
	Controls (*n* = 63)	1.09 ± 0.49		0.56 ± 0.29				0.81 ± 0.35			
Matthan, 2013 [25]	Cases (*n* = 352) [a]	1.35 ± 0.03		1.87 ± 0.05		1.23 ± 0.02		1.18 ± 0.03		0.57 ± 0.02	
	Cases (*n* = 504) [b]	1.53 ± 0.03		2.04 ± 0.04		1.21 ± 0.02		1.10 ± 0.02		0.55 ± 0.01	
	Controls (*n* = 603)	1.82 ± 0.03		2.40 ± 0.04		1.19 ± 0.02		1.06 ± 0.02		0.51 ± 0.01	
	Cases (*n* = 331) [c]	1.45 ± 0.03		1.99 ± 0.05		1.30 ± 0.03		1.15 ± 0.03		0.59 ± 0.02	
	Cases (*n* = 567) [d]	1.74 ± 0.03		2.34 ± 0.05		1.24 ± 0.02		1.14 ± 0.02		0.59 ± 0.01	
	Controls (*n* = 251)	1.35 ± 0.03		2.39 ± 0.06		1.27 ± 0.03		1.14 ± 0.03		0.62 ± 0.02	
Miettinen, 2000 [26]	Cases (*n* = 10)	0.72 ± 0.04	↓	1.14 ± 0.11	↓	0.64 ± 0.07	↓	1.67 ± 0.18	↑	0.64 ± 0.03	=
	Controls (*n* = 10)	1.29 ± 0.14		1.95 ± 0.18		0.88 ± 0.08		1.17 ± 0.15		0.55 ± 0.01	
Paramsothy, 2011 [27]	Cases (*n* = 37)	1.08 ± 0.43	=	1.68 ± 0.65	=	0.97 ± 0.23	=	1.29 ± 0.55	↑		
	Controls (*n* = 37)	1.27 ± 0.42		1.92 ± 0.64		1.06 ± 0.23		0.95 ± 0.47			
Riches, 1998 [28]	Cases (*n* = 16)							6.47 ± 4.30	↑		
	Controls (*n* = 16)							1.51 ± 1.52			
Simonen, 2002a [29]	Cases (*n* = 44)	0.96 ± 0.05	↓	1.83 ± 0.11	↓	0.89 ± 0.04	↓	2.34 ± 0.12	=	1.16 ± 0.12	↑
	Controls (*n* = 20)	1.22 ± 0.09		2.24 ± 0.17		1.08 ± 0.05		1.96 ± 0.18		0.85 ± 0.05	
Simonen, 2007 [30]	Cases (*n* = 23)	1.02 ± 0.06	↓	1.93 ± 0.16	↓	0.92 ± 0.06	↓	2.51 ± 0.21	↑	1.06 ± 0.10	↑
	Controls (*n* = 10)	1.42 ± 0.13		2.55 ± 0.23		1.17 ± 0.06		1.73 ± 0.09		0.76 ± 0.07	
Surgery											
De Vuono, 2017 [31]	Before (*n* = 42)	0.47 ± 0.35	↑	0.49 ± 0.33	=			2.05 ± 0.91	↑		
	After (*n* = 42)	0.42 ± 0.27		0.39 ± 0.33				1.01 ± 0.56			
Pihlajamaki, 2010a [32]	Before (*n* = 29)	0.87 ± 0.33	↑	1.72 ± 0.66	↑	1.32 ± 0.26	=	1.95 ± 0.60	↑	0.97 ± 0.17	↑
	After (*n* = 29)	0.64 ± 0.26		1.24 ± 0.62		1.36 ± 0.27		1.40 ± 0.53		0.80 ± 0.15	
Pihlajamaki, 2010b [32]	Before (*n* = 26)	0.63 ± 0.20	↓	1.25 ± 0.47	↓	1.36 ± 0.36	↓	2.41 ± 0.66	↑	1.33 ± 0.42	↑
	After (*n* = 26)	0.73 ± 0.28		1.56 ± 0.63		1.46 ± 0.36		1.78 ± 0.60		1.12 ± 0.34	

Table 1. Cont.

Diet-induced												
Chan, 2008 [34]	Before (n = 20)							2.90				
	After (n = 20)							2.29				
Chan, 2010 [33]	Before (n = 10)			0.93				2.07				
	After (n = 10)			0.89				1.82				
Mateo-Gallego, 2014 [35]	Before (n = 16) [e]	1.45 ± 0.59	↓	1.56 ± 0.59	=	0.44 ± 0.16	=	1.29 ± 0.45	=	1.50 ± 0.33	=
	After (n = 16)	1.62 ± 0.65		1.80 ± 0.69		0.35 ± 0.07		1.31 ± 0.65		1.49 ± 0.31	
	Before (n = 34) [f]	1.18 ± 0.67	↓	1.37 ± 0.59	=	0.45 ± 0.15	=	1.76 ± 0.61	↑	1.84 ± 0.48	=
	After (n = 34)	1.25 ± 0.43		1.48 ± 0.43		0.45 ± 0.18		1.34 ± 0.49		1.68 ± 0.40	
Riches, 1999 [36]	Before (n = 14)							7.78				
	After (n = 14)							6.02				
Simonen, 2000 [37]	Before (n = 16)	0.87 ± 0.05	↓	1.62 ± 0.14	↓	0.85 ± 0.04	=	2.26 ± 0.08	=	1.36 ± 0.29	=
	After (n = 16)	1.03 ± 0.08		1.97 ± 0.14		0.95 ± 0.05		2.18 ± 0.10		1.09 ± 0.15	
Simonen, 2002b [38]	Before (n = 10)	0.84 ± 0.09	=	1.55 ± 0.16	=	0.96 ± 0.04	↓	2.08 ± 0.15	↑	0.82 ± 0.05	=
	After (n = 10)	0.79 ± 0.07		1.26 ± 0.11		1.25 ± 0.05		1.59 ± 0.10		0.66 ± 0.04	

Values are mean ± SD and expressed in µmol/mmoL cholesterol. Non-cholesterol markers are significantly lower (↓), higher (↑) or not-significantly different (=) between cases and controls, or not statistically tested (blank). [a] Women with BMI >30 or [b] between 25–30 kg/m^2 and [c] men with BMI >30 or [d] between 25–30 kg/m^2. [e] Subjects with familial hyperlipidemia or with [f] familial combined hyperlipidemia.

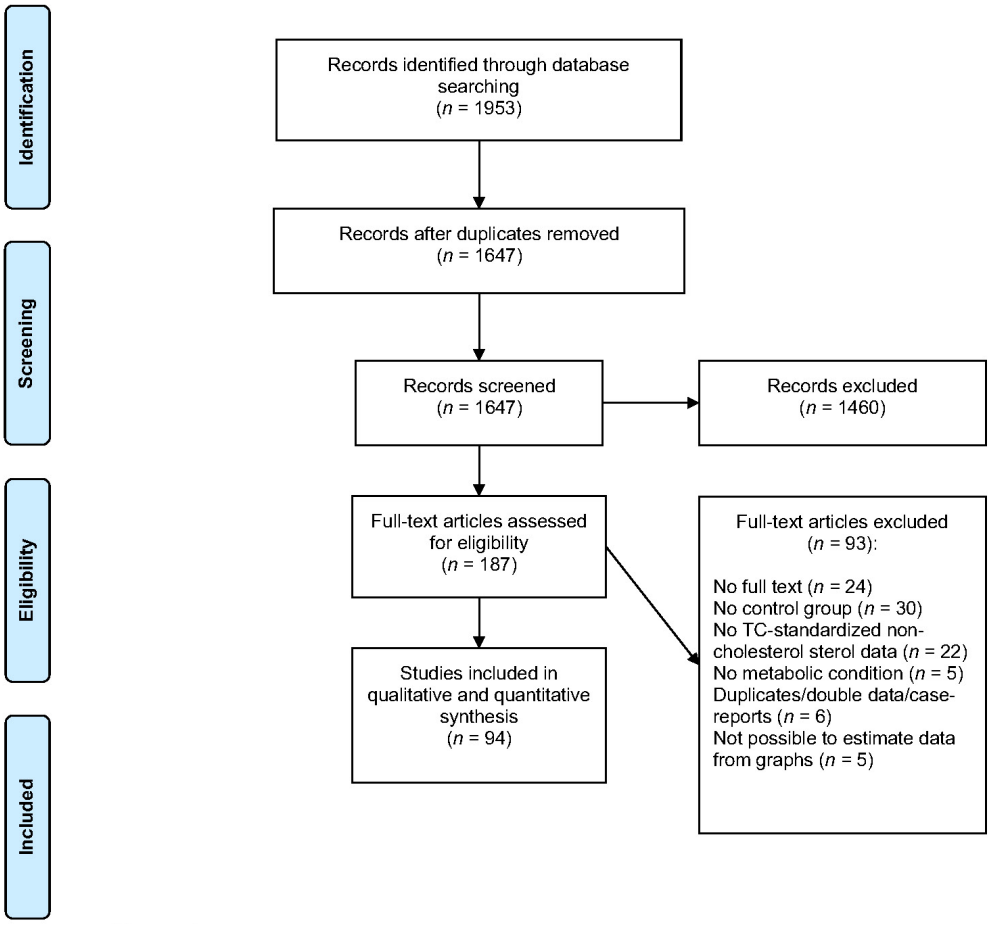


Figure 1. Flowchart of the study selection process.

synthesis markers compared to non-diabetic pregnant women (Data tabulated in Table S1) [53].

To summarize, most studies in T1DM patients suggested that cholesterol absorption is higher and cholesterol synthesis lower compared to non-diabetic control subjects. For T2DM patients, the opposite was found. No effects of GDM were reported.

3.3. Serum Non-Cholesterol Sterol Markers in Hyperlipidemic Subjects

Eleven studies met the inclusion criteria (Table 3). Three studies were performed in patients with Familial Combined Hyperlipidemia (FCH) [54–56], one study in patients with Familial Hypercholesterolemia (FH) [57], and four studies included patients with FCH, FH or another (non-FH) form of hypercholesterolemia [58–61]. Patients with hypercholesterolemia other than FCH of FH were evaluated in three studies [62–64].

In patients with FCH, cholesterol absorption markers were significantly lower compared to control subjects in four studies [55,56,58,60], comparable in one study [61], and not measured in one study [54]. At least one of the cholesterol synthesis markers was significantly increased in five studies [54–56,60,61], and were comparable between FCH patients and control subjects in one study [58]. Two studies in FH patients, of which one was performed in children, reported comparable cholesterol absorption markers between cases and controls subjects, while cholesterol synthesis was lower [58] or comparable between patients with or without FH [57].

In patients diagnosed with hypercholesterolemia other than FCH or FH, at least one of the cholesterol absorption markers increased in three comparisons [58,60,61], was comparable in two comparisons [64], and was not tested or measured in three comparisons [59,62,63]. One study reported higher cholesterol absorption in subjects diagnosed with heterogeneous autosomal dominant hypercholesterolemia compared to normolipidemic control subjects [61]. For cholesterol synthesis, most comparisons did not find a difference between hypercholesterolemic cases and their controls [60–62,64], while cholesterol synthesis was suggested to be higher in one study [61] and lower in another study [58] in cases compared to controls. Two studies measured cholesterol synthesis, but did report differences between cases and control subjects, although it seems that cholesterol synthesis was lower in subjects with hypercholesterolemia [59,63].

Overall, studies in patients with FCH suggested that cholesterol absorption was lower and cholesterol synthesis higher compared to control subjects, while patients with FH showed a comparable cholesterol absorption and synthesis pattern as control subjects. Studies in patients with hypercholesterolemia other than FCH or FH suggested a pattern

of increased cholesterol absorption, while cholesterol synthesis seems comparable or decreased compared to normolipidemic subjects.

3.4. Serum Non-Cholesterol Sterol Markers in Subjects with the Metabolic Syndrome

Five studies met the inclusion criteria for the metabolic syndrome (Table 4). Findings suggested that cholesterol absorption was lower and synthesis higher in subjects with the metabolic syndrome. It should be noted, however, that in four studies [65–68], cases were obese and had a BMI >30 kg/m^2, while controls had a BMI around 25 kg/m^2. It remains to be elucidated whether the metabolic syndrome per se affects cholesterol absorption or synthesis, or if relations can be explained by one of the underlying characteristics of the metabolic syndrome.

3.5. Serum Non-Cholesterol Sterol Markers in Subjects with Cardiovascular Diseases

Seventeen studies matched the inclusion criteria (Table 5). The studies varied widely in the selected patient groups. For many studies, it was reported that at least a part of the population had comorbidities such as T2DM [70–78], T1DM [79] or hypertension, dyslipidemia, or the metabolic syndrome [73,76–79]. In general, controls were to some extent matched for these comorbidities.

One or more of the cholesterol absorption markers were significantly increased in seven of the comparisons, decreased in two comparisons, not statistically different in eleven comparisons, and not tested in the two remaining comparisons. In one study, no cholesterol absorption markers were measured [76]. At least one of the cholesterol synthesis markers was significantly increased in two comparisons, decreased in five comparisons, not statistically different in nine comparisons, not tested in three comparisons. In two studies, opposite findings were reported, as cholesterol-standardized lathosterol levels increased and those of desmosterol decreased [80,81]. Surprisingly, in these two studies, only postmenopausal women were included. Also, in another study with only postmenopausal women, only a significant increase in desmosterol was reported [82].

Overall, studies in patients with cardiovascular suggested a pattern of increased cholesterol absorption. Relationships with cholesterol synthesis markers were less clear. In the majority of studies, however, decreases or no differences were reported. Whether effects on lathosterol and desmosterol are gender-specific warrant further study.

Table 2. Serum non-cholesterol sterol markers in subjects with diabetes mellitus type 1 or diabetes mellitus type 2.

		Sitosterol		Campesterol		Cholestanol		Lathosterol		Desmosterol	
T1DM											
Feillet, 1994 [52]	Cases (n = 10)							0.86 ± 0.11	↓		
	Controls (n = 10)							1.16 ± 0.07			
Gylling, 2004 [40]	Cases (n = 7)	1.94 ± 0.36	=	3.48 ± 0.58	=	1.55 ± 0.15	↑	0.56 ± 0.07	↓		
	Controls (n = 5)	1.24 ± 0.14		2.20 ± 0.29		0.92 ± 0.09		0.86 ± 0.12			
Gylling, 2007 [39]	Cases (n = 56)	1.88 ± 0.08	=	3.96 ± 0.20	↑	1.64 ± 0.04	=	1.57 ± 0.07	=	0.76 ± 0.02	=
	Controls (n = 18)	1.66 ± 0.14		3.10 ± 0.25		1.45 ± 0.09		1.71 ± 0.14		0.84 ± 0.04	
Jarvisalo, 2006 [41]	Cases (n = 48)	2.07 ± 0.10	=	4.45± 0.27	=	1.80 ± 0.05	=	1.19 ± 0.05	=	0.74 ± 0.01	=
	Controls (n = 79)	1.91 ± 0.08		3.73 ± 0.18		1.77 ± 0.04		1.29 ± 0.05		0.72 ± 0.01	
Kojima, 1999 [42]	Cases (n = 12) [a]	2.33 ± 0.25	↑	3.84 ± 0.44	↑						
	Cases (n = 10) [b]	1.44 ± 0.14		2.63 ± 0.15							
	Controls (n = 10)	1.38 ± 0.06		2.02 ± 0.20							
T2DM											
Blaha, 2006 [43]	Cases (n = 30)	0.67 ± 0.26	↓	0.83 ± 0.66	↓			1.45 ± 0.62	↑		
	Controls (n = 30)	1.92 ± 1.49		2.10 ± 1.31				0.88 ± 0.58			
Feillet, 1994 [52]	Cases (n = 9)							0.98 ± 0.10	=		
	Controls (n = 10)							1.16 ± 0.07			
Gylling, 2010 [44]	Cases (n = 76)	1.13 ± 0.07	↓	2.32 ± 0.13	↓	1.37 ± 0.03	↓	1.52 ± 0.08	↑	0.99 ± 0.03	↑
	Controls (n = 549)	1.15 ± 0.07		2.78 ± 0.05		1.43 ± 0.01		1.46 ± 0.02		0.90 ± 0.01	
Gylling, 1997 [45]	Cases (n = 13)	1.12 ± 0.11	↓	2.04 ± 0.23	↓	0.92 ± 0.05	↓	2.0 ± 0.10	=	1.08 ± 0.05	↑
	Controls (n = 18)	1.78 ± 0.11		3.89 ± 0.23		1.17 ± 0.04		1.80 ± 0.11		0.73 ± 0.01	
Kurano, 2018 [46]	Cases (n = 46)	3.02		1.82							
	Controls (n = 178)	2.17		1.59							
Lau, 2005 [c] [47]	Cases (n = 14)	0.45 ± 0.10	=	1.31 ± 0.30	=						
	Cases (n = 14)	0.45 ± 0.10	=	1.10 ± 0.20	=						
	Controls (n = 15)	0.71 ± 0.30		1.29 ± 0.30							
	Controls (n = 15)	0.55 ± 0.10		1.43 ± 0.30							

Okada, 2010 [48]	Cases (n = 42)	1.16		2.09		1.38		1.52			
	Controls (n = 21)	1.13		2.09		1.41		1.25			
Smahelova, 2005 [50]	Cases (n = 63)	1.00 ± 0.57	↓	1.44 ± 1.56	↓			1.50 ± 1.19	↑		
	Controls (n = 72)	1.70 ± 1.10		1.88 ± 1.13				0.95 ± 0.53			
Smahelova, 2007 [49]	Cases (n = 63)	0.94		1.31				1.53			
	Controls (n = 72)	1.75		1.84				0.96			
Yoshida, 2006 [51]	Cases (n = 13)	0.82 ± 0.13	=					0.91 ± 0.11	=		
	Controls (n = 16)	0.73 ± 0.10						0.73 ± 0.07			

Values are mean ± SD and expressed in µmol/mmoL cholesterol. Non-cholesterol markers are significantly lower (↓), higher (↑) or not-significantly different (=) between cases and controls, or not statistically tested (blank). T1DM: diabetes mellitus type 1, T2DM: diabetes mellitus type 2. [a] Subjects on conventional insulin therapy or [b] intensive insulin therapy. [c] Baseline levels of a randomized clinical trial with 4 arms.

Table 3. Serum non-cholesterol sterol markers in hyperlipidemic subjects.

		Sitosterol		Campesterol		Cholestanol		Lathosterol		Desmosterol	
FCH											
Baila-Rueda, 2014 [54]	Cases (n = 107)									6.65	↑
	Controls (n = 126)									4.14	
Brouwers, 2013 [55]	Cases (n = 103)	1.14 ± 0.53	↓	1.82 ± 0.80	↓	1.34 ± 0.48	↓	1.79 ± 0.61	↑	0.38 ± 0.18	=
	Controls (n = 240)	1.30 ± 0.53		2.70 ± 0.81		1.60 ± 0.53		1.50 ± 0.55		0.35 ± 0.16	
Garcia-Otin, 2007 [58]	Cases (n = 38)	2.17 ± 0.51	↓	2.86 ± 0.62	↓			1.58 ± 0.45	=		
	Controls (n = 45)	2.68 ± 0.57		3.37 ± 0.67				1.68 ± 0.44			
Lupattelli, 2012 [60]	Cases (n = 38)	0.81 ± 0.33	=	0.46 ± 0.58	↓			1.25 ± 0.61	↑		
	Controls (n = 19)	0.95 ± 0.37		0.82 ± 0.59				0.88 ± 0.49			
Noto, 2010 [61]	Cases (n = 29)	1.80 ± 0.80	=	1.98 ± 0.90	=			0.69 ± 0.30	↑		
	Controls (n = 79)	1.50 ± 0.70		1.82 ± 0.80				0.50 ± 0.30			
Van Himbergen, 2010 [56]	Cases (n = 103)	1.14 ± 0.53	↓	1.82 ± 0.80	↓	1.34 ± 0.48	↓	1.79 ± 0.61	↑	0.38 ± 0.19	=
	Controls (n = 204)	1.30 ± 0.50		2.07 ± 0.81		1.60 ± 0.53		1.50 ± 0.55		0.35 ± 0.16	

Table 3. Cont.

FH											
Hirayama, 2013 [59]	Cases (*n* = 47)	0.50		1.79				1.08		0.19	
	Controls (*n* = 32)	0.50		1.55				1.34		0.38	
Ketomaki, 2003 [57]	Cases (*n* = 18)	1.76 ± 0.14	=	3.30 ± 0.35	=	1.57 ± 0.05	=	1.02 ± 0.07	=	0.60 ± 0.02	=
	Controls (*n* = 29)	1.77 ± 0.11		3.58 ± 0.19		1.51 ± 0.05		1.08 ± 0.06		0.55 ± 0.02	
Garcia-Otin, 2007 [58]	Cases (*n* = 31)	3.08 ± 0.70	=	4.00 ± 0.88	=			1.19 ± 0.33	↓		
	Controls (*n* = 45)	2.68 ± 0.57		3.37 ± 0.67				1.68 ± 0.44			
Non-FH											
Baila-Rueda, 2017 [62]	Cases (*n* = 200)									2.53	=
	Controls (*n* = 100)									2.41	
Garcia-Otin, 2007 [58]	Cases (*n* = 21)	3.93 ± 1.14	↑	5.10 ± 1.40	↑			1.00 ± 0.36	↓		
	Controls (*n* = 45)	2.68 ± 0.57		3.37 ± 0.67				1.68 ± 0.44			
Hirayama, 2013 [59]	Cases (*n* = 47)	0.50		1.79				1.08		0.19	
	Controls (*n* = 32)	0.50		1.55				1.34		0.38	
Lupattelli, 2012 [60]	Cases (*n* = 53)	1.30 ± 0.81	↑	0.79 ± 0.81	=			0.95 ± 0.68	=		
	Controls (*n* = 19)	0.95 ± 0.37		0.82 ± 0.59				0.88 ± 0.49			
Nagy, 2006 [63]	Cases (*n* = 6)	0.81				3.09		0.52		0.14	
	Controls (*n* = 6)	0.68				3.20		0.62		0.21	
Noto, 2010 [61]	Cases (*n* = 19) [a]	1.20 ± 0.70	↓	0.83 ± 0.50	↓			1.04 ± 0.50	↑		
	Cases (*n* = 2) [b]	1.54 ± 2.01	=	0.66 ± 1.15	=			0.95 ± 3.40	=		
	Cases (*n* = 63) [c]	1.97 ± 0.70	↑	2.04 ± 0.70	↑			0.49 ± 0.30	=		
	Controls (*n* = 79)	1.50 ± 0.70		1.82 ± 0.80				0.50 ± 0.30			
Von Bergmann, 2003 [64]	Cases (*n* = 8) [d]			1.97 ± 0.29	=	2.88 ± 0.80	=	1.64 ± 0.24	=		
	Cases (*n* = 6) [e]			1.92 ± 0.21	=	2.72 ± 0.40	=	2.03 ± 0.40	=		
	Controls (*n* = 6)			2.41 ± 0.18		2.74 ± 0.40		2.39 ± 0.58			

Values are mean ± SD and expressed in µmol/mmol cholesterol. In case absolute concentrations were reported, only TC-standardized means were calculated. Non-cholesterol markers are lower (↓) or higher (↑) in cases compared to control, comparable between cases and controls (=) or not statistically tested (blank). FCH: Familial Combined Hyperlipidemia, FH: Familial Hypercholesterolemia. [a] Subjects with heterozygous or [b] homozygous autosomal dominant hypercholesterolemia or [c] polygenic hypercholesterolemia. [d] Subjects carrier for apolipoprotein E2/2 or for [e] apolipoprotein E4/4.

Table 4. Serum non-cholesterol sterol markers in subjects with the metabolic syndrome.

		Sitosterol		Campesterol		Cholestanol		Lathosterol		Desmosterol	
Chan, 2003 [65]	Cases (*n* = 35)			2.63 ± 0.19	↓			2.58 ± 0.15	↑		
	Controls (*n* = 9)			3.28 ± 0.32				1.90 ± 0.31			
Chan, 2003 [66]	Cases (*n* = 35)			2.60	↓			2.60	↑		
	Controls (*n* = 10)			3.40				1.90			
Gylling, 2007 [67]	Cases (*n* = 74)	0.97 ± 0.05	↓	1.84 ± 0.09	↓	1.19 ± 0.03	↓	2.02 ± 0.07	↑	0.79 ± 0.04	↑
	Controls (*n* = 74)	1.25 ± 0.06		1.84 ± 0.12		1.58 ± 0.04		1.33 ± 0.05		0.82 ± 0.02	
Hernandez-Mijares, 2011 [69]	Cases (*n* = 24)	1.14		0.81							
	Controls (*n* = 24)	1.60		1.15							
Ooi, 2009 [68]	Cases (*n* = 140)			1.59 ± 0.09	↓			2.13 ± 0.08	↑		
	Controls (*n* = 10)			3.17 ± 0.30				1.51 ±0.22			

Values are mean ± SD and expressed in µmol/mmol cholesterol. Non-cholesterol markers are significantly lower (↓), higher (↑) or not-significantly different (=) between cases and controls, or not statistically tested (blank).

3.6. Serum Non-Cholesterol Sterol Markers in Subjects with Intestinal Diseases

Six studies were identified that matched our inclusion criteria, of which one had three study arms, providing eight comparisons versus controls in total (Table 6). Three of these studies evaluated conditions where the length of the small intestine was reduced, i.e., two studies in patients with short bowel syndrome [87,88], and one study in Familial Hypercholesterolemia (FH) patients who underwent ileal bypass surgery [89]. Another study evaluated three conditions where the colon was removed, i.e., ileostomy, ileorectal anastomosis and ileal pouch anastomosis [90]. This latter condition was also studied by Hakala et al. [91]. Finally, there was one study in patients with gastric bleeding [92]. Two studies in subjects with a shortened small intestine suggested that cholesterol synthesis increased [87,88], whereas only one study showed a lower cholesterol absorption [88] as compared to controls. In contrast to the first study by Ellegard et al. [87], the study showing the effects on cholesterol absorption was conducted in children [88]. The third study in subjects with an ileal bypass plus FH did not show any differences versus controls [89]. In two studies including subjects without a colon cholesterol synthesis increased, i.e., in ileostomy patients and patients with ileal pouch anastomosis [90], whereas two other studies did not show a difference in cholesterol synthesis, i.e., in patients with ileorectal anastomosis [90] and ileal pouch anastomosis [91]. In all four comparisons, cholesterol absorption increased [90,91], although this conclusion was not consistent for all absorption markers. Finally, data from subjects with gastric bleeding did not suggest differences in cholesterol synthesis or absorption.

To summarize, studies in patients with shorter small intestines suggested that cholesterol synthesis is higher and cholesterol absorption lower compared to control subjects. For patients without a colon, both cholesterol synthesis and absorption seem higher.

3.7. Serum Non-Cholesterol Sterol Markers in Subjects with Kidney Disease

Two studies were identified, of which one study was performed in hyperlipidemic patients with nephrotic proteinuria [93] and one in hemodialysis subjects with diabetes type 2 plus manifestations of CVD [94] (Table 7). Both studies suggested that endogenous synthesis is lower in patients with kidney disease as compared to controls without kidney disease. The study in hemodialysis patients showed a higher cholesterol absorption in patients as compared to controls [94], whereas absorption markers were not reported in the study including patients with nephrotic proteinuria [93]. To summarize, these two studies suggested that cholesterol absorption is higher and cholesterol synthesis lower compared to controls, even in the presence of T2DM.

Table 5. Serum non-cholesterol sterol markers in subjects with cardiovascular diseases.

		Sitosterol		Campesterol		Cholestanol		Lathosterol		Desmosterol	
Assmann, 2006 [83]	Cases (n = 159)	0.75		1.53				0.68			
	Controls (n = 318)	0.72		1.53				0.72			
Escurriol, 2010 [84]	Cases (n = 299)	1.32 ± 0.52	=	1.53 ± 0.57	=			1.61 ± 0.61	=		
	Controls (n = 584)	1.39 ± 0.55		1.56 ± 0.58				1.59 ± 0.68			
Fassbender, 2008 [70]	Cases (n = 58)	1.27 ± 0.720	=	1.39 ± 0.86	=			1.33 ± 0.86	=	1.17 ± 1.73	=
	Controls (n = 957)	1.42 ± 0.66		1.54 ± 0.77				1.17 ± 0.50		0.84 ± 0.36	
Gylling, 2006 [82]	Cases (n = 22)	1.47 ± 0.13	=	3.01 ± 0.31	=	1.27 ± 0.09	=	1.70 ± 0.12	=	0.91 ± 0.07	↑
	Controls (n = 14)	1.44 ± 0.09		2.74 ± 0.21		1.19 ± 0.06		1.75 ± 0.12		0.62 ± 0.03	
Gylling, 2009 [80]	Cases (n = 47)	1.56 ± 0.10	↑	2.87 ± 0.12	↑	1.30 ± 0.06	=	1.68 ± 0.08	↓	0.96 ± 0.08	↑
	Controls (n = 62)	1.24 ± 0.06		2.27 ± 0.11		1.29 ± 0.04		1.96 ± 0.07		0.76 ± 0.02	
Gylling, 1996 [71]	Cases (n = 7)	1.39 ± 0.10	↑	3.00 ± 0.21	↑	1.02 ± 0.07	=	2.05 ± 0.22	=	1.01 ± 0.07	=
	Controls (n = 6)	0.80 ± 0.09		1.63 ± 0.14		0.83 ± 0.08		2.00 ± 0.26		1.16 ± 0.07	
Matthan, 2009 [72]	Cases (n = 155)	1.69 ± 0.06	↑	2.29 ± 0.07	↑	1.44 ± 0.05	↑	1.16 ± 0.04	↓	0.73 ± 0.03	↓
	Controls (n = 414)	1.49 ± 0.03		1.96 ± 0.04		1.35 ± 0.03		1.38 ± 0.03		0.75 ± 0.02	
Mori, 2017 [73]	Cases (n = 103) [a]			3.48 ± 1.25	↑			0.61 ± 0.33	↑		
	Controls (n = 40)			2.44 ± 1.42				0.44 ± 0.16			
	Cases (n = 42) [b]			2.89 ± 0.94	=			1.28 ± 0.87	=		
	Controls (n = 42)			2.83 ± 0.97				1.45 ± 0.53			
Nasu, 2013 [74]	Cases (n = 42)	1.46 ± 0.43	↑	2.00 ± 0.37	↑			0.61 ± 0.18	↓		
	Controls (n = 38)	1.13 ± 0.32		1.44 ± 0.52				0.97 ± 0.66			
Pinedo, 2007 [75]	Cases (n = 373)	1.22 ± 0.47	↓	1.86 ± 0.79	=			1.21 ± 0.47	=		
	Controls (n = 758)	1.31 ± 0.46		1.94 ± 0.80				1.18 ± 0.48			
Rajaratnam, 2000 [81]	Cases (n = 48)	1.52 ± 0.09	↑	2.78 ± 0.19	↑	1.30 ± 0.06	=	1.71 ± 0.08	↓	0.98 ± 0.09	↑
	Controls (n = 61)	1.25 ± 0.06		2.28 ± 0.11		1.30 ± 0.04		1.96 ± 0.07		0.76 ± 0.02	
Shay, 2009 [79]	Cases (n = 82)	1.35 ± 0.67	=	2.13 ± 1.15	=			0.47 ± 0.30	↓	0.34 ± 0.20	↓
	Controls (n = 213)	1.45 ± 0.76		2.29 ± 1.12				0.54 ± 0.38		0.42 ± 0.32	
Sonoda, 1992 [76]	Cases (n = 22)							2.64			

	Controls (n = 33)					2.13	
Weingartner, 2009 [77]	Cases (n = 8) [c]			1.47 ± 0.66	=	0.60 ± 0.23	↓
	Cases (n = 4) [d]			1.51 ± 0.93	=	0.77 ± 0.24	=
	Cases (*n* = 6) [e]			1.47 ± 0.69	=	0.87 ± 0.24	=
	Controls (*n* = 22)			1.39 ± 0.61		1.12 ± 0.47	
Weingartner, 2011 [85]	Cases (*n* = 66)	1.35 ± 0.41	=	1.82 ± 1.04	↑	1.26 ± 0.62	↓
	Controls (*n* = 111)	1.21 ± 0.03		1.50 ± 0.69		1.38 ± 0.63	
Wilund, 2004 [86]	Cases (*n* = 323) [f]	0.76 ± 0.54	=	1.27 ± 0.77	=		
	Controls (*n* = 808)	0.78 ± 0.50		1.39 ± 0.78			
	Cases (*n* = 209) [g]	0.73 ± 0.58	=	1.20 ± 0.81	=		
	Controls (*n* = 1202)	0.76 ± 0.51		1.28 ± 0.74			
Windler, 2009 [78]	Cases (*n* = 186)	0.63		1.04		0.58	
	Controls (*n* = 231)	0.68		1.13		0.80	

Values are mean ± SD and expressed in µmol/mmol cholesterol. Non-cholesterol markers are significantly lower (↓), higher (↑) or not-significantly different (=) between cases and controls, or not statistically tested (blank). [a] Subjects with or [b] without statin therapy. [c] Subjects with three-vessel disease, [d] two-vessel disease or [e] single-vessel disease. [f] Study performed in men or [g] women.

Table 6. Serum non-cholesterol sterol markers in subjects with intestinal diseases.

		Sitosterol		Campesterol		Cholestanol		Lathosterol		Desmosterol	
DSI											
Ellegard, 2005 [87]	Cases (*n* = 16)							8.08 ± 2.73	↑		
	Controls (*n* = 21)							2.28 ± 0.70			
Pakarinen, 2010 [c] [88]	Cases (*n* = 12)	1.95 ± 1.24	=	2.54 ± 2.00	↓			3.53 ± 1.18	↑	1.41 ± 0.20	↑
	Controls (*n* = 80)	1.96 ± 0.50		3.95 ± 0.95				1.15 ± 0.19		0.69 ± 0.14	
Vanhanen, 1992 [89]	Cases (*n* = 6)	1.52	=	2.30	=	1.00	=	1.27	=	1.00	=
	Controls (*n* = 7)	1.40		2.10		1.15		1.23		0.55	
DLI											
Hakala, 1997 [91]	Cases (*n* = 12) [a]	1.83 ± 0.12	=	3.16 ± 0.26	↑	1.49 ± 0.08	=	1.90 ± 0.19	=	0.70 ± 0.05	=
	Controls (*n* = 10)	1.44 ± 0.20		1.80 ± 0.29		1.48 ± 0.10		2.03 ± 0.13		0.76 ± 0.08	
Nissinen, 2004 [90]	Cases (*n* = 34) [a]	1.56 ± 0.09	↑	3.51 ± 0.17	↑	1.40 ± 0.05	=	2.30 ± 0.14	↑	0.91 ± 0.06	=
	Cases (*n* = 8) [b]	1.39 ± 0.18	=	2.66 ± 0.33	↑	1.18 ± 0.05	↓	3.37 ± 0.75	↑	1.04 ± 0.21	=
	Cases (*n* = 6) [c]	1.98 ± 0.19	↑	4.03 ± 0.48	↑	1.35 ± 0.09	=	1.69 ± 0.22	=	0.75 ± 0.05	=
	Controls (*n* = 29)	1.27 ± 0.09		1.85 ± 0.16		1.45 ± 0.06		1.88 ± 0.09		0.74 ± 0.03	
Bleeding											
Hrabovsky, 2012 [92]	Cases (*n* = 24)	1.13		2.19				1.11			
	Controls (*n* = 100)	0.99		2.04				1.37			

Values are mean ± SD and expressed in µmol/mmol cholesterol. In case absolute concentrations were reported, only TC-standardized means were calculated. Non-cholesterol markers are lower (↓) or higher (↑) in cases compared to control, comparable between cases and controls (=) or not statistically tested (blank). DSI: Deprived Small Intestine, DLI: Deprived Large Intestine. [a] Subjects underwent ileal pouch anastomosis, [b] conventional ileostomy or [c] ileorectal anastomosis.

Table 7. Serum non-cholesterol sterol markers in subjects with kidney disease.

		Sitosterol	Campesterol	Cholestanol		Lathosterol		Desmosterol
Dullaart, 1996 [93]	Cases (*n* = 11)					0.99 ± 0.43	↓	
	Controls (*n* = 22)					1.29 ± 0.41		
Rogacev, 2012 [94]	Cases (*n* = 113)			2.40	↑	1.25	↓	
	Controls (*n* = 229)			1.25		1.40		

Values are mean ± SD and expressed in µmol/mmoL cholesterol. In case absolute concentrations were reported, only TC-standardized means were calculated. Non-cholesterol markers are lower (↓) or higher (↑) in cases compared to control, comparable between cases and controls (=) or not statistically tested (blank).

3.8. Serum Non-Cholesterol Sterol Markers in Subjects with Liver Diseases

Twenty-two studies were identified, of which five studies were performed in patients with steatosis [95–99], eleven studies in patients with cholestasis [100–110], and six studies included patients with liver diseases related to cirrhosis or necrosis [111–116] (Table 8).

In patients with a fatty liver (steatosis), cholesterol absorption markers were lower in one study [96], comparable to control subjects in another study [97] and not significantly tested or measured in three studies [95,98,99]. At least one of the cholesterol synthesis markers increased in two studies (three comparisons) [95,97], which was independent of statin use, comparable in one study [96] and not significantly tested in two studies [98,99].

In nine comparisons in cholestasis patients with gallstones, at least one of the cholesterol absorption markers decreased in three comparisons [106,108] comparable in one [105] and not tested or measured in five comparisons [100,102,104,109]. On the other hand, cholesterol synthesis markers increased in five comparisons [102,107,108] comparable in two [106,109] and not tested or measured in the remaining comparisons. In children with gallstones, results are inconsistent. In patients with black pigment stones, cholesterol absorption was comparable, while cholesterol synthesis increased compared to controls. On the other hand, patients with cholesterol stones had significantly lower cholesterol absorption and higher cholesterol synthesis markers. In cholecystectomized patients, cholesterol absorption was comparable to controls, while cholesterol synthesis increased [101], and in children after successful surgery for biliary atresia, cholesterol absorption markers were inconsistent, while cholesterol synthesis was lower in cases compared with healthy controls [110]. Markers for cholesterol absorption and synthesis were also measured in pregnant women with cholestasis, but differences compared to control subjects were not significantly tested [103].

In patients with primary biliary cirrhosis, markers for cholesterol absorption are inconsistent, i.e., increased concentrations in two comparisons [111,112], reduced concentrations in one comparison [114], and not tested in two comparisons [111,115]. Patients with acute necrosis have a higher cholesterol absorption compared with controls [114], while cholesterol absorption was comparable in children with Intestinal Failure Associated Liver Disease (IFALD) [113]. At least one cholesterol synthesis marker was reduced in primary biliary cirrhosis, Hepatitis C-related cirrhosis and acute necrosis [111,114,116], while cholesterol synthesis increased in IFALD patients [113].

To summarize, studies in patients with steatosis or cholestasis suggested a pattern of reduced cholesterol absorption and increased cholesterol synthesis, while it appears that patients with cirrhosis have increased cholesterol absorption and reduced cholesterol

synthesis. Studies performed in children with cholestasis and IFALD are inconsistent and warrant further investigation.

3.9. Summary of the Results

An overview of non-cholesterol sterol concentrations as biomarkers for cholesterol absorption and synthesis in different metabolic disorders is presented in Table 9.

Table 8. Serum non-cholesterol sterol markers in subjects with liver diseases.

		Sitosterol		Campesterol		Cholestanol	Lathosterol		Desmosterol	
Steatosis										
Brindisi, 2012 [95]	Cases (n = 74) [a]	1.26		1.60			1.81 ± 1.30	↑		
	Controls (n = 63)	1.30		1.65			1.31 ± 0.80			
	Cases (n = 91) [b]	0.90		1.29			3.50 ± 1.49	↑		
	Controls (n = 35)	1.05		1.34			2.72 ± 1.24			
Ikegami, 2012 [96]	Cases (n = 15)	0.73 ± 0.06	↓	0.75 ± 0.07	↓		3.03 ± 0.31	=		
	Controls (n = 36)	1.90 ± 0.08		2.34 ± 0.11			3.29 ± 0.17			
Min, 2012 [97]	Cases (n = 20)	0.85	=						0.98	↑
	Controls (n = 6)	1.35							0.52	
Simonen, 2011 [99]	Cases (n = 114)	0.81		1.68		1.19	1.77		0.95	
	Controls (n = 128)	1.05		2.22		1.32	1.46		0.83	
Simonen, 2013 [98]	Cases (n = 17) [c]						1.81		0.85	
	Cases (n = 23) [d]						1.55		0.94	
	Controls (n = 32)						1.53		0.71	
Cholestasis										
Castro, 2007 [100]	Cases (n = 18)						0.80			
	Controls (n = 9)						0.74			
Galman, 2004 [e] [102]	Cases (n = 45)						3.00 ± 1.10	↑		
	Controls (n = 80)						2.40 ± 0.90			
	Cases (n = 20)						2.80 ± 1.20			
	Controls (n = 20)						3.00 ± 1.10			
Galman, 2011 [101]	Cases (n = 18)			6.90 ± 5.02	=		3.09 ± 1.33	↑		
	Controls (n = 222)			8.04 ± 4.23			2.54 ± 0.94			
Gylling, 1998 [f] [103]	Cases (n = 20)					3.52 ± 0.23	1.95		0.80	
	Cases (n = 19)					3.84 ± 0.33	2.30		0.82	
	Controls (n = 20)					2.82 ± 0.11	1.90		0.87	
Hillebrant, 2002 [104]	Cases (n = 19)						3.2 ± 0.40			

	Controls (n = 20)							2.8 ± 0.40			
Jiang, 2009 [105]	Cases (n = 12)	4.10	=	1.90	=						
	Controls (n = 31)	1.80		2.20							
Kakela, 2017 [106]	Cases (n = 95)	0.59 ± 0.24	↓	1.25 ± 0.54	↓	1.50 ± 0.73	=	1.94 ± 0.79	=	1.11 ± 1.01	=
	Controls (n = 147)	0.85 ± 0.45		1.78 ± 0.85		1.54 ± 0.34		1.73 ± 0.82		0.86 ± 0.32	
Koivusalo, 2015 [107]	Cases (n = 17) [g]	1.70 ± 0.18	=	3.12 ± 0.34	=	1.86 ± 0.09	↑	1.37 ± 0.17	↑	1.07 ± 0.07	↑
	Cases (n = 11) [h]	1.17 ± 0.22	↓	2.07 ± 0.41	↓	1.26 ± 0.11	↓	1.68 ± 0.21	↑	1.04 ± 0.08	=
	Controls (n = 82)	1.75 ± 0.08		3.10 ± 0.15		1.68 ± 0.04		0.87 ± 0.08		0.86 ± 0.03	
Krawczyk, 2012 [108]	Cases (n = 112) [e]	1.07 ± 0.50	↓	1.43 ± 0.80	=			1.28 ± 0.70	↑	0.87 ± 0.50	=
	Controls (n = 152)	1.20 ± 0.60		1.58 ± 0.90				1.11 ± 0.78		0.87 ± 0.50	
	Cases (n = 100)	0.69 ± 0.38	↓	1.03 ± 0.39	=			1.33 ± 0.52	↑	0.56 ± 0.27	↑
	Controls (n = 100)	0.83 ± 0.54		1.01 ± 0.57				1.05 ± 0.43		0.46 ± 0.21	
Muhrbeck, 2017 [109]	Cases (n = 41)							0.80 ± 0.08	=		
	Controls (n = 72)							0.70 ± 0.05			
Pakarinen, 2010 [110]	Cases (n = 17)	1.95 ± 0.77	=	3.00 ± 1.41	↓	2.75 ± 0.81	↑	0.81 ± 0.40	↓		
	Controls (n = 129)	1.96 ± 0.51		3.95 ± 0.95		1.60 ± 0.35		1.21 ± 0.32			
Cirrhosis-Necrosis											
Del Puppo, 1998 [111]	Cases (n = 12) [i]	5.15 ± 1.17		2.20 ± 0.52				0.64 ± 0.09			
	Controls (n = 10)	1.86 ± 0.44		1.34 ± 0.24				0.70 ± 0.08		0.14	
	Cases (n = 13) [j]	5.49 ± 0.92	↑	2.69 ± 0.79	↑			0.67 ± 0.07	↓	0.21	
	Controls (n = 10)	1.66 ± 0.44		0.88 ± 0.06				1.05 ± 0.17			
Gylling, 1996 [112]	Cases (n = 16)	3.72 ± 0.95	↑	4.62 ± 0.88	↑	4.53 ± 0.79	↑	1.11 ± 0.12	=	0.61 ± 0.08	=
	Controls (n = 36) [k]	1.03 ± 0.06		1.58 ± 0.09		0.82 ± 0.05		1.32 ± 0.08		0.55 ± 0.02	
	Controls (n = 8) [l]	1.05 ± 0.14		1.69 ± 0.31		0.80 ± 0.05		1.40 ± 0.16		0.57 ± 0.10	
Mutanen, 2014 [113]	Cases (n = 34)	1.55 ± 1.11	=	2.73 ± 1.83	=	1.79 ± 0.39	=	2.90 ± 2.22	↑	1.22± 0.44	↑
	Controls (n = 86)	1.68 ± 0.55		3.06 ± 0.79		1.64 ± 0.29		0.83 ± 0.37		0.85 ± 0.14	
Nikkila, 2005 [115]	Cases (n = 67)	10.52 ± 19.34		7.63 ± 13.43		12.95 ± 15.20		1.15 ± 1.63			
	Controls (n = 59)	1.96 ± 1.66		3.38 ± 3.57		1.34 ± 0.85		1.96 ± 1.66			

Nikkila, 1992 [114]	Cases (n = 8) [m]	3.20		1.50	↓		0.49	↓	0.50	=
	Cases (n = 3) [n]	2.50	↑	2.80	=		0.62	↓	0.60	=
	Controls (n = 27)	1.20		2.10			1.25		0.50	
Ydreborg, 2014 [o] [116]	Cases (n = 36)						0.94 ± 0.54	↓		
	Controls (n = 242)						1.18 ± 0.47			
	Cases (n = 8)						0.80 ± 0.63	↓		
	Controls (n = 75)						1.19 ± 0.53			

Values are mean ± SD and expressed in µmol/mmol cholesterol. In case absolute concentrations were reported, only TC-standardized means were calculated. Non-cholesterol markers are lower (↓) or higher (↑) in cases compared to control, comparable between cases and controls (=) or not statistically tested (blank). [a] Subjects with or [b] without statin therapy. [c] Subjects with NAFLD or [d] NASH. [e] Study performed in different ethnicities. [f] Randomized clinical trial with three arms in pregnant women with cholestasis. [g] Subjects with black pigment stones or [h] cholesterol stones. [i] Hypercholesterolemic or [j] normocholesterolemic subjects. [k] Study performed in men or [l] women. [m] Subjects with liver cirrhosis or [n] acute liver necrosis. [o] Study performed in two different groups of patients with liver cirrhosis.

Table 9. Serum non-cholesterol sterol markers in different metabolic disorders.

	Cholesterol Absorption Compared to Control	Cholesterol Synthesis Compared to Control
BMI	↓	↑
Diabetes mellitus		
T1DM	↑	↓
T2DM	↓	↑
Hyperlipidemia		
FCH	↓	↑
FH	-	-
Non-FH hyperlipemia	↑	↓
Metabolic Syndrome	↓	↑
CVD	↑	↓
Intestine		
Deprived small intestine	↓	↑
Deprived large intestine	↑	↑
Liver		
Steatosis	↓	↑
Cholestasis	↓	↑
Cirrhosis-necrosis	↑	↓
Kidney	↑	↓

BMI: body mass index, T1DM: diabetes mellitus type 1, T2DM: diabetes mellitus type 2, FCH: familial combined hyperlipidemia, FH: familial hypercholesterolemia, CVD: cardiovascular disease ↓ lower compared to control ↑ higher compared to control.

4. Conclusions

To better understand cholesterol metabolism in a wide variety of metabolic disorders, we here present the first extensive systematic overview of plasma non-cholesterol sterols levels, which are validated biomarkers for intestinal cholesterol absorption and endogenous cholesterol synthesis, in different metabolic disorders.

In the vast majority of studies, TC-standardized levels of campesterol, sitosterol and cholestanol on the one hand, and TC-standardized levels of lathosterol and desmosterol on the other hand, showed comparable patterns. This underlines their use as biomarkers for cholesterol absorption and cholesterol synthesis, respectively. Moreover, non-cholesterol biomarkers displayed reciprocal patterns, indicating that cholesterol metabolism is tightly regulated by the balance of intestinal absorption and endogenous synthesis. Furthermore, we identified that certain metabolic disorders are characterized by either higher cholesterol absorption or by higher cholesterol synthesis, classifying them as 'cholesterol absorbers or cholesterol synthesizers'. The identification of metabolic disorders as cholesterol absorbers or synthesizers is important for future (dietary) interventions, since pharmacological and dietary treatments have different underlying mechanisms by which they affect cholesterol metabolism [117,118]. In more detail, cholesterol absorbers would benefit from ezetimibe treatment and plant sterol or stanol consumption, since these interventions inhibit intestinal cholesterol absorption [119,120]. On the other hand, subjects with higher cholesterol synthesis would achieve better cholesterol target levels by using statins, i.e., drugs that reduce endogenous cholesterol synthesis rates [121].

In general, cholesterol absorption is decreased and synthesis is increased in overweight and obese subjects, in T2DM patients, and in metabolic syndrome subjects. Since the metabolic syndrome is a constellation of at least three of the following features: visceral obesity, impaired fasting glucose, high triacylglycerol, low high-density lipoprotein cholesterol, and hypertension, it could be speculated that low absorption/high synthesis patterns are (partly) explained by insulin resistance, obesity status, or disturbed lipid profile. However, differences in BMI are most likely not the only factor to explain differences in cholesterol metabolism between metabolic syndrome subjects and controls, since Hernandez-Mijares et al. [69] showed comparable results in weight-matched metabolic syndrome subjects. Interestingly, differences between obese and control subjects in cholesterol absorption and/or synthesis are reversed after diet-induced weight loss, while the effect on cholesterol metabolism after weight loss by different types of surgical interventions are inconsistent. It might be possible that energy balance (or the lack thereof) plays a role in the inconsistent effects on cholesterol homeostasis after surgery-induced weight loss. Future research on the effects of weight loss on cholesterol absorption and synthesis is therefore warranted. As stated, T2DM patients also display a low cholesterol absorption/

high cholesterol synthesis pattern, while the opposite is true for T1DM patients, where cholesterol absorption is higher and synthesis is lower than in non-diabetic controls. Insulin resistance might be a contributory factor in the lower cholesterol absorption/higher cholesterol synthesis pattern, since both metabolic syndrome and T2DM patients showed a comparable low absorption/high synthesis pattern, which is in contrast to T1DM patients who are insulin-dependent but can still be insulin sensitive [122].

We also investigated the interplay in cholesterol absorption and synthesis in hyperlipidemias and cardiovascular diseases. Both FCH and FH are inherited disorders of cholesterol metabolism. However, FH patients are characterized by high serum cholesterol concentrations, while patients with FCH also exhibit high serum triacylglycerol concentrations. Although studies in FH patients suggested that cholesterol absorption and synthesis pattern are comparable to those of normolipidemic subjects, FCH patients are characterized by a pattern of low cholesterol absorption and high cholesterol synthesis. It has been postulated that patients with FCH have an altered cholesterol synthesis pathway, resulting in higher cholesterol synthesis and thus lower cholesterol absorption [56]. In contrast to FCH, hyperlipidemic patients with a cause other than FH or FCH were characterized by higher cholesterol absorption and lower cholesterol synthesis. Patients with CVD also displayed this reciprocal pattern of high absorption/low synthesis. Indeed, most studies have suggested that higher cholesterol absorption and lower cholesterol synthesis is more atherogenic [72,123,124]. Nevertheless, this is difficult to reconcile with the low absorption/high synthesis pattern in T2DM and FCH patients, who are also at increased risk to develop CVD. The relationship between cholesterol metabolism and CVD risk and the underlying mechanisms should therefore be investigated in more detail in future studies.

In organ-specific diseases (intestine, liver and kidney), the relationship between cholesterol absorption and synthesis was more heterogeneous. In subjects with a deprived small intestine, cholesterol synthesis increased, while cholesterol absorption decreased or comparable to control subjects. Since cholesterol is mainly absorbed at the duodenum and proximal jejunum, the upregulation of cholesterol synthesis is most likely a counter-reaction in patients with a shorter small intestine. On the other hand, in patients without a colon, both cholesterol synthesis and absorption appeared to be higher. This finding does not concur with the notion of reciprocal changes in cholesterol homeostasis and might suggest that absence of microbiota could play a role in cholesterol metabolism in patients with a deprived large intestine. In patients with kidney-related diseases (nephrotic proteinuria or hemodialysis), cholesterol absorption was higher and synthesis was lower. These patients were hyperlipidemic or at risk to develop CVD, which might partly explain the high cholesterol absorption/low cholesterol synthesis pattern. Patients with chronic kidney disease also have a high CVD risk [125] and it might be of interest to

investigate whether changes in cholesterol metabolism could play a role in the CVD risk of kidney patients. In patients with steatosis (fatty liver), cholesterol absorption was lower and cholesterol synthesis higher compared with control subjects. Simonen et al. [99] indeed demonstrated that biomarkers of cholesterol synthesis correlated positively with liver fat—independent of BMI—suggesting that cholesterol synthesis increases when the liver contains more fat. In patients with cholestasis, the same pattern is seen, i.e., reduced cholesterol absorption and increased cholesterol synthesis. For patients suffering from a cirrhotic or necrotic liver, it appears that the liver can no longer maintain its cholesterol synthesis, which most likely results in an increased cholesterol absorption. Few studies were performed in children with liver diseases, and with inconsistent results, warranting further investigation.

Overall, distinctive patterns for cholesterol absorption or cholesterol synthesis could be identified, suggesting that metabolic disorders can be classified as 'cholesterol absorbers or cholesterol synthesizers'. It should be noted that our conclusions are mainly based on cross-sectional studies, which makes it impossible to draw any conclusions on causal relationships. Therefore, future research should confirm or refute our findings. Ultimately, the classification of a metabolic disorder as cholesterol absorber or synthesizer based on non-cholesterol sterol biomarkers can be used for targeted (dietary) interventions.

Author Contributions: S.B., J.P. and R.P.M. designed the study; S.M. and S.B. performed the literature search; S.M., R.P.M, and S.B. screened the articles, which were tabulated by S.M. and S.B. All authors interpreted the data, wrote the paper, and have read and approved the final manuscript.

Funding: This research received no external funding,

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Yeagle, P.L. Cholesterol and the cell membrane. *Biochim. Biophys. Acta* **1985**, *822*, 267–287.
2. Ikonen, E. Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 125–138, doi:10.1038/nrm2336.
3. Dietschy, J.M.; Turley, S.D.; Spady, D.K. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J. Lipid Res.* **1993**, *34*, 1637–1659.
4. Cohen, D.E. Balancing cholesterol synthesis and absorption in the gastrointestinal tract. *J. Clin. Lipidol.* **2008**, *2*, S1–S3, doi:10.1016/j.jacl.2008.01.004.
5. Mackay, D.; Jones, P.J. Evaluation of methods for the determination of cholesterol absorption and synthesis in humans. *Atherosclerosis* **2011**, *218*, 253–262, doi:10.1016/j.atherosclerosis.2010.04.022.
6. Grundy, S.M.; Mok, H.Y. Determination of cholesterol absorption in man by intestinal perfusion. *J. Lipid Res.* **1977**, *18*, 263–271.
7. Crouse, J.R.; Grundy, S.M. Evaluation of a continuous isotope feeding method for measurement of cholesterol absorption in man. *J. Lipid Res.* **1978**, *19*, 967–971.
8. Grundy, S.M.; Ahrens, E.H., Jr.; Miettinen, T.A. Quantitative Isolation and Gas—Liquid Chromatographic Analysis of Total Fecal Bile Acids. *J. Lipid Res.* **1965**, *6*, 397–410.
9. Lutjohann, D.; Meese, C.O.; Crouse, J.R., 3rd; von Bergmann, K. Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in humans. *J. Lipid Res.* **1993**, *34*, 1039–1046.
10. Miettinen, T.A.; Ahrens, E.H., Jr.; Grundy, S.M. Quantitative Isolation and Gas—Liquid Chromatographic Analysis of Total Dietary and Fecal Neutral Steroids. *J. Lipid Res.* **1965**, *6*, 411–424.
11. Mackay, D.S.; Jones, P.J. Plasma noncholesterol sterols: Current uses, potential and need for standardization. *Curr. Opin. Lipidol.* **2012**, *23*, 241–247, doi:10.1097/MOL.0b013e328353292e.
12. Miettinen, T.A.; Tilvis, R.S.; Kesaniemi, Y.A. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am. J. Epidemiol.* **1990**, *131*, 20–31.
13. Miettinen, T.A.; Gylling, H.; Nissinen, M.J. The role of serum non-cholesterol sterols as surrogate markers of absolute cholesterol synthesis and absorption. *Nutr. Metab. Cardiovasc. Dis.* **2011**, *21*, 765–769, doi:10.1016/j.numecd.2011.05.005.
14. Miettinen, T.A.; Tilvis, R.S.; Kesaniemi, Y.A. Serum cholestanol and plant sterol levels in relation to cholesterol metabolism in middle-aged men. *Metabolism* **1989**, *38*, 136–140.
15. Tilvis, R.S.; Miettinen, T.A. Serum plant sterols and their relation to cholesterol absorption. *Am. J. Clin. Nutr.* **1986**, *43*, 92–97, doi:10.1093/ajcn/43.1.92.
16. Matthan, N.R.; Raeini-Sarjaz, M.; Lichtenstein, A.H.; Ausman, L.M.; Jones, P.J. Deuterium uptake and plasma cholesterol precursor levels correspond as methods for measurement of endogenous cholesterol synthesis in hypercholesterolemic women. *Lipids* **2000**, *35*, 1037–1044.

17. Mackay, D.S.; Jones, P.J.; Myrie, S.B.; Plat, J.; Lutjohann, D. Methodological considerations for the harmonization of non-cholesterol sterol bio-analysis. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2014**, *957*, 116–122, doi:10.1016/j.jchromb.2014.02.052.
18. Clarenbach, J.J.; Reber, M.; Lutjohann, D.; von Bergmann, K.; Sudhop, T. The lipid-lowering effect of ezetimibe in pure vegetarians. *J. Lipid Res.* **2006**, *47*, 2820–2824, doi:10.1194/jlr.P600009-JLR200.
19. Lupattelli, G.; De Vuono, S.; Mannarino, E. Patterns of cholesterol metabolism: Pathophysiological and therapeutic implications for dyslipidemias and the metabolic syndrome. *Nutr. Metab. Cardiovasc. Dis.* **2011**, *21*, 620–627, doi:10.1016/j.numecd.2011.04.010.
20. Nissinen, M.J.; Miettinen, T.E.; Gylling, H.; Miettinen, T.A. Applicability of non-cholesterol sterols in predicting response in cholesterol metabolism to simvastatin and fluvastatin treatment among hypercholesterolemic men. *Nutr. Metab. Cardiovasc. Dis.* **2010**, *20*, 308–316, doi:10.1016/j.numecd.2009.04.014.
21. Stellaard, F.; Lutjohann, D. The Interpretation of Cholesterol Balance Derived Synthesis Data and Surrogate Noncholesterol Plasma Markers for Cholesterol Synthesis under Lipid Lowering Therapies. *Cholesterol* **2017**, *2017*, 5046294, doi:10.1155/2017/5046294.
22. Wan, X.; Wang, W.; Liu, J.; Tong, T. Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. *BMC Med. Res. Methodol.* **2014**, *14*, 135, doi:10.1186/1471-2288-14-135.
23. Chan, D.C.; Watts, G.F.; Redgrave, T.G.; Mori, T.A.; Barrett, P.H. Apolipoprotein B-100 kinetics in visceral obesity: Associations with plasma apolipoprotein C-III concentration. *Metab. Clin. Exp.* **2002**, *51*, 1041–1046.
24. Lupattelli, G.; Pirro, M.; Mannarino, M.R.; Siepi, D.; Roscini, A.R.; Schillaci, G.; Mannarino, E. Visceral fat positively correlates with cholesterol synthesis in dyslipidaemic patients. *Eur. J. Clin. Investig.* **2012**, *42*, 164–170.
25. Matthan, N.R.; Zhu, L.; Pencina, M.; D'Agostino, R.B.; Schaefer, E.J.; Lichtenstein, A.H. Sex-specific differences in the predictive value of cholesterol homeostasis markers and 10-year cardiovascular disease event rate in Framingham Offspring Study participants. *J. Am. Heart Assoc.* **2013**, *2*, e005066.
26. Miettinen, T.A.; Gylling, H. Cholesterol absorption efficiency and sterol metabolism in obesity. *Atherosclerosis* **2000**, *153*, 241–248.
27. Paramsothy, P.; Knopp, R.H.; Kahn, S.E.; Retzlaff, B.M.; Fish, B.; Ma, L.; Ostlund, R.E., Jr. Plasma sterol evidence for decreased absorption and increased synthesis of cholesterol in insulin resistance and obesity. *Am. J. Clin. Nutr.* **2011**, *94*, 1182–1188.
28. Riches, F.M.; Watts, G.F.; Naoumova, R.P.; Kelly, J.M.; Croft, K.D.; Thompson, G.R. Hepatic secretion of very-low-density lipoprotein apolipoprotein B-100 studied with a stable isotope technique in men with visceral obesity. *Int. J. Obes. Relat. Metab. Disord. J. Int. Assoc. Study Obes.* **1998**, *22*, 414–423.
29. Simonen, P.P.; Gylling, H.; Miettinen, T.A. Body weight modulates cholesterol metabolism in non-insulin dependent type 2 diabetics. *Obes. Res.* **2002**, *10*, 328–335.

30. Simonen, P.P.; Gylling, H.; Miettinen, T.A. The distribution of squalene and non-cholesterol sterols in lipoproteins in type 2 diabetes. *Atherosclerosis* **2007**, *194*, 222–229.
31. De Vuono, S.; Ricci, M.A.; Siepi, D.; Boni, M.; Gentili, A.; Scavizzi, M.; Daviddi, G.; Labate, P.; Roscini, A.R.; Lupattelli, G. Laparoscopic sleeve gastrectomy modifies cholesterol synthesis but not cholesterol absorption. *Obes. Res. Clin. Pract.* **2017**, *11*, 118–122.
32. Pihlajamaki, J.; Gronlund, S.; Simonen, M.; Kakela, P.; Moilanen, L.; Paakkonen, M.; Pirinen, E.; Kolehmainen, M.; Karja, V.; Kainulainen, S.; et al. Cholesterol absorption decreases after Roux-en-Y gastric bypass but not after gastric banding. *Metab. Clin. Exp.* **2010**, *59*, 866–872.
33. Chan, D.C.; Watts, G.F.; Gan, S.K.; Ooi, E.M.; Barrett, P.H. Effect of ezetimibe on hepatic fat, inflammatory markers, and apolipoprotein B-100 kinetics in insulin-resistant obese subjects on a weight loss diet. *Diabetes Care* **2010**, *33*, 1134–1139.
34. Chan, D.C.; Watts, G.F.; Ng, T.W.; Yamashita, S.; Barrett, P.H. Effect of weight loss on markers of triglyceride-rich lipoprotein metabolism in the metabolic syndrome. *Eur. J. Clin. Investig.* **2008**, *38*, 743–751.
35. Mateo-Gallego, R.; Perez-Calahorra, S.; Cofan, M.; Baila-Rueda, L.; Cenarro, A.; Ros, E.; Puzo, J.; Civeira, F. Serum lipid responses to weight loss differ between overweight adults with familial hypercholesterolemia and those with familial combined hyperlipidemia. *J. Nutr.* **2014**, *144*, 1219–1226.
36. Riches, F.M.; Watts, G.F.; Hua, J.; Stewart, G.R.; Naoumova, R.P.; Barrett, P.H. Reduction in visceral adipose tissue is associated with improvement in apolipoprotein B-100 metabolism in obese men. *J. Clin. Endocrinol. Metab.* **1999**, *84*, 2854–2861.
37. Simonen, P.; Gylling, H.; Howard, A.N.; Miettinen, T.A. Introducing a new component of the metabolic syndrome: Low cholesterol absorption. *Am. J. Clin. Nutr.* **2000**, *72*, 82–88.
38. Simonen, P.; Gylling, H.; Miettinen, T.A. Acute effects of weight reduction on cholesterol metabolism in obese type 2 diabetes. *Clin. Chim. Acta* **2002**, *316*, 55–61.
39. Gylling, H.; Laaksonen, D.E.; Atalay, M.; Hallikainen, M.; Niskanen, L.; Miettinen, T.A. Markers of absorption and synthesis of cholesterol in men with type 1 diabetes. *Diabetes/Metab. Res. Rev.* **2007**, *23*, 372–377.
40. Gylling, H.; Tuominen, J.A.; Koivisto, V.A.; Miettinen, T.A. Cholesterol metabolism in type 1 diabetes. *Diabetes* **2004**, *53*, 2217–2222.
41. Jarvisalo, M.; Raitakari, O.; Gylling, H.; Miettinen, T.A. Cholesterol absorption and synthesis in children with type 1 diabetes. *Diabetes Care* **2006**, *29*, 2300–2304.
42. Kojima, H.; Hidaka, H.; Matsumura, K.; Fujita, Y.; Yamada, S.; Haneda, M.; Yasuda, H.; Kikkawa, R.; Kashiwagi, A. Effect of glycemic control on plasma plant sterol levels and post-heparin diamine oxidase activity in type 1 diabetic patients. *Atherosclerosis* **1999**, *145*, 389–397.
43. Blaha, V.; Andrys, C.; Smahelova, A.; Knizek, J.; Hyspler, R.; Solichova, D.; Blaha, M.; Zadak, Z. Effect of atorvastatin on soluble CD14, CD40 Ligand, sE- and sP-selectins and MCP-1 in patients with type 2 diabetes mellitus: Relationship to cholesterol turnover. *Pharmacol. Res.* **2006**, *54*, 421–428.

44. Gylling, H.; Hallikainen, M.; Pihlajamaki, J.; Simonen, P.; Kuusisto, J.; Laakso, M.; Miettinen, T.A. Insulin sensitivity regulates cholesterol metabolism to a greater extent than obesity: Lessons from the METSIM Study. *J. Lipid Res.* **2010**, *51*, 2422–2427.
45. Gylling, H.; Miettinen, T.A. Cholesterol absorption, synthesis, and LDL metabolism in NIDDM. *Diabetes Care* **1997**, *20*, 90–95.
46. Kurano, M.; Hasegawa, K.; Kunimi, M.; Hara, M.; Yatomi, Y.; Teramoto, T.; Tsukamoto, K. Sitosterol prevents obesity-related chronic inflammation. *Biochim. Biophys. Acta* **2018**, *1863*, 191–198.
47. Lau, V.W.; Journoud, M.; Jones, P.J. Plant sterols are efficacious in lowering plasma LDL and non-HDL cholesterol in hypercholesterolemic type 2 diabetic and nondiabetic persons. *Am. J. Clin. Nutr.* **2005**, *81*, 1351–1358.
48. Okada, K.; Yagyu, H.; Kotani, K.; Miyamoto, M.; Osuga, J.; Nagasaka, S.; Ishibashi, S. Lipid-lowering effects of ezetimibe for hypercholesterolemic patients with and without type 2 diabetes mellitus. *Endocr. J.* **2010**, *57*, 903–908.
49. Smahelova, A.; Hyspler, R.; Haas, T. Relation of cholesterol metabolism and non-cholesterol sterols to insulin resistance. *Physiol. Res.* **2007**, *56*, 749–755.
50. Smahelova, A.; Hyspler, R.; Haas, T.; Ticha, A.; Blaha, V.; Zadak, Z. Effect of atorvastatin on non-cholesterol sterols in patients with type 2 diabetes mellitus and cardiovascular disease. *Pharmacol. Res.* **2005**, *51*, 31–36.
51. Yoshida, M.; Vanstone, C.A.; Parsons, W.D.; Zawistowski, J.; Jones, P.J. Effect of plant sterols and glucomannan on lipids in individuals with and without type II diabetes. *Eur. J. Clin. Nutr.* **2006**, *60*, 529–537.
52. Feillet, C.; Monnier, L.H.; Barjon, J.N.; Perrin, M.; Descomps, B.; Crastes de Paulet, A. Evidence for a short-term stimulatory effect of insulin on cholesterol synthesis in newly insulin-treated diabetic patients. *Metab. Clin. Exp.* **1994**, *43*, 1233–1240.
53. Miettinen, H.E.; Rono, K.; Koivusalo, S.; Stach-Lempinen, B.; Poyhonen-Alho, M.; Eriksson, J.G.; Hiltunen, T.P.; Gylling, H. Elevated serum squalene and cholesterol synthesis markers in pregnant obese women with gestational diabetes mellitus. *J. Lipid Res.* **2014**, *55*, 2644–2654.
54. Baila-Rueda, L.; Mateo-Gallego, R.; Jarauta, E.; de Castro-Oros, I.; Bea, A.M.; Cenarro, A.; Civeira, F. Bile acid synthesis precursors in familial combined hyperlipidemia: The oxysterols 24S-hydroxycholesterol and 27-hydroxycholesterol. *Biochem. Biophys. Res. Commun.* **2014**, *446*, 731–735.
55. Brouwers, M.C.; Konrad, R.J.; van Himbergen, T.M.; Isaacs, A.; Otokozawa, S.; Troutt, J.S.; Schaefer, E.J.; van Greevenbroek, M.M.; Stalenhoef, A.F.; de Graaf, J. Plasma proprotein convertase subtilisin kexin type 9 levels are related to markers of cholesterol synthesis in familial combined hyperlipidemia. *Nutr. Metab. Cardiovasc. Dis.* **2013**, *23*, 1115–1121.
56. van Himbergen, T.M.; Otokozawa, S.; Matthan, N.R.; Schaefer, E.J.; Buchsbaum, A.; Ai, M.; van Tits, L.J.; de Graaf, J.; Stalenhoef, A.F. Familial combined hyperlipidemia is associated with alterations in the cholesterol synthesis pathway. *Arterioscler. Thromb. Vasc. Biol.* **2010**, *30*, 113–120.

57. Ketomaki, A.; Gylling, H.; Siimes, M.A.; Vuorio, A.; Miettinen, T.A. Squalene and noncholesterol sterols in serum and lipoproteins of children with and without familial hypercholesterolemia. *Pediatr. Res.* **2003**, *53*, 648–653.
58. Garcia-Otin, A.L.; Cofan, M.; Junyent, M.; Recalde, D.; Cenarro, A.; Pocovi, M.; Ros, E.; Civeira, F. Increased intestinal cholesterol absorption in autosomal dominant hypercholesterolemia and no mutations in the low-density lipoprotein receptor or apolipoprotein B genes. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 3667–3673.
59. Hirayama, S.; Nakagawa, S.; Soda, S.; Kamimura, Y.; Nishioka, E.; Ueno, T.; Fukushima, Y.; Higuchi, K.; Inoue, M.; Seino, U.; et al. Ezetimibe decreases serum oxidized cholesterol without impairing bile acid synthesis in Japanese hypercholesterolemic patients. *Atherosclerosis* **2013**, *230*, 48–51.
60. Lupattelli, G.; Pirro, M.; Siepi, D.; Mannarino, M.R.; Roscini, A.R.; Vaudo, G.; Pasqualini, L.; Schillaci, G.; Mannarino, E. Non-cholesterol sterols in different forms of primary hyperlipemias. *Nutr. Metab. Cardiovasc. Dis.* **2012**, *22*, 231–236.
61. Noto, D.; Cefalu, A.B.; Barraco, G.; Martino, E.; Fayer, F.; Mina, M.; Montali, A.; Arca, M.; Averna, M.; Martino, F. Plasma non-cholesterol sterols: A useful diagnostic tool in pediatric hypercholesterolemia. *Pediatr. Res.* **2010**, *67*, 200–204.
62. Baila-Rueda, L.; Cenarro, A.; Lamiquiz-Moneo, I.; Mateo-Gallego, R.; Bea, A.M.; Perez-Calahorra, S.; Marco-Benedi, V.; Civeira, F. Bile acid synthesis precursors in subjects with genetic hypercholesterolemia negative for LDLR/APOB/PCSK9/APOE mutations. Association with lipids and carotid atherosclerosis. *J. Steroid Biochem. Mol. Biol.* **2017**, *169*, 226–233.
63. Nagy, K.; Jakab, A.; Pollreisz, F.; Bongiorno, D.; Ceraulo, L.; Averna, M.R.; Noto, D.; Vekey, K. Analysis of sterols by high-performance liquid chromatography/mass spectrometry combined with chemometrics. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2433–2440.
64. Von Bergmann, K.; Lutjohann, D.; Lindenthal, B.; Steinmetz, A. Efficiency of intestinal cholesterol absorption in humans is not related to apoE phenotype. *J. Lipid Res.* **2003**, *44*, 193–197.
65. Chan, D.C.; Watts, G.F.; Barrett, P.H.; O'Neill, F.H.; Redgrave, T.G.; Thompson, G.R. Relationships between cholesterol homoeostasis and triacylglycerol-rich lipoprotein remnant metabolism in the metabolic syndrome. *Clin. Sci.* **2003**, *104*, 383–388.
66. Chan, D.C.; Watts, G.F.; Barrett, P.H.; O'Neill, F.H.; Thompson, G.R. Plasma markers of cholesterol homeostasis and apolipoprotein B-100 kinetics in the metabolic syndrome. *Obes. Res.* **2003**, *11*, 591–596.
67. Gylling, H.; Hallikainen, M.; Kolehmainen, M.; Toppinen, L.; Pihlajamaki, J.; Mykkanen, H.; Agren, J.J.; Rauramaa, R.; Laakso, M.; Miettinen, T.A. Cholesterol synthesis prevails over absorption in metabolic syndrome. *Transl. Res. J. Lab. Clin. Med.* **2007**, *149*, 310–316.
68. Ooi, E.M.; Ng, T.W.; Chan, D.C.; Watts, G.F. Plasma markers of cholesterol homeostasis in metabolic syndrome subjects with or without type-2 diabetes. *Diabetes Res. Clin. Pract.* **2009**, *85*, 310–316.
69. Hernandez-Mijares, A.; Banuls, C.; Jover, A.; Sola, E.; Bellod, L.; Martinez-Triguero, M.L.; Lagarda, M.J.; Victor, V.M.; Rocha, M. Low intestinal cholesterol absorption is associated with a reduced

efficacy of phytosterol esters as hypolipemic agents in patients with metabolic syndrome. *Clin. Nutr.* **2011**, *30*, 604–609.

70. Fassbender, K.; Lutjohann, D.; Dik, M.G.; Bremmer, M.; Konig, J.; Walter, S.; Liu, Y.; Letiembre, M.; von Bergmann, K.; Jonker, C. Moderately elevated plant sterol levels are associated with reduced cardiovascular risk—The LASA study. *Atherosclerosis* **2008**, *196*, 283–288.
71. Gylling, H.; Miettinen, T.A. Cholesterol absorption and lipoprotein metabolism in type II diabetes mellitus with and without coronary artery disease. *Atherosclerosis* **1996**, *126*, 325–332.
72. Matthan, N.R.; Pencina, M.; LaRocque, J.M.; Jacques, P.F.; D'Agostino, R.B.; Schaefer, E.J.; Lichtenstein, A.H. Alterations in cholesterol absorption/synthesis markers characterize Framingham offspring study participants with CHD. *J. Lipid Res.* **2009**, *50*, 1927–1935.
73. Mori, K.; Ishida, T.; Tsuda, S.; Oshita, T.; Shinohara, M.; Hara, T.; Irino, Y.; Toh, R.; Hirata, K.I. Enhanced Impact of Cholesterol Absorption Marker on New Atherosclerotic Lesion Progression After Coronary Intervention During Statin Therapy. *J. Atheroscler. Thromb.* **2017**, *24*, 123–132.
74. Nasu, K.; Terashima, M.; Habara, M.; Ko, E.; Ito, T.; Yokota, D.; Ishizuka, S.; Kurita, T.; Kimura, M.; Kinoshita, Y.; et al. Impact of cholesterol metabolism on coronary plaque vulnerability of target vessels: A combined analysis of virtual histology intravascular ultrasound and optical coherence tomography. *JACC Cardiovasc. Interv.* **2013**, *6*, 746–755.
75. Pinedo, S.; Vissers, M.N.; von Bergmann, K.; Elharchaoui, K.; Lutjohann, D.; Luben, R.; Wareham, N.J.; Kastelein, J.J.; Khaw, K.T.; Boekholdt, S.M. Plasma levels of plant sterols and the risk of coronary artery disease: The prospective EPIC-Norfolk Population Study. *J. Lipid Res.* **2007**, *48*, 139–144.
76. Sonoda, M.; Sakamoto, K.; Miyauchi, T.; Sanada, J.; Nakamura, K.; Arima, T.; Kuriyama, M.; Nagata, K.; Osame, M.; Miyahara, K. Risk factors in normolipidemic male patients with coronary artery disease in Japanese. *Jpn. Circ. J.* **1992**, *56*, 829–836.
77. Weingartner, O.; Weingartner, N.; Scheller, B.; Lutjohann, D.; Graber, S.; Schafers, H.J.; Bohm, M.; Laufs, U. Alterations in cholesterol homeostasis are associated with coronary heart disease in patients with aortic stenosis. *Coron. Artery Dis.* **2009**, *20*, 376–382.
78. Windler, E.; Zyriax, B.C.; Kuipers, F.; Linseisen, J.; Boeing, H. Association of plasma phytosterol concentrations with incident coronary heart disease Data from the CORA study, a case-control study of coronary artery disease in women. *Atherosclerosis* **2009**, *203*, 284–290.
79. Shay, C.M.; Evans, R.W.; Orchard, T.J. Do plant sterol concentrations correlate with coronary artery disease in type 1 diabetes? A report from the Pittsburgh Epidemiology of Diabetes Complications Study. *J. Diabetes* **2009**, *1*, 112–117.
80. Gylling, H.; Hallikainen, M.; Rajaratnam, R.A.; Simonen, P.; Pihlajamaki, J.; Laakso, M.; Miettinen, T.A. The metabolism of plant sterols is disturbed in postmenopausal women with coronary artery disease. *Metab. Clin. Exp.* **2009**, *58*, 401–407.
81. Rajaratnam, R.A.; Gylling, H.; Miettinen, T.A. Independent association of serum squalene and noncholesterol sterols with coronary artery disease in postmenopausal women. *J. Am. Coll. Cardiol.* **2000**, *35*, 1185–1191.

82. Gylling, H.; Rajaratnam, R.A.; Vartiainen, E.; Puska, P.; Miettinen, T.A. Changes in serum level and metabolism of cholesterol with plant stanol esters in postmenopausal women with and without coronary artery disease. *Menopause* **2006**, *13*, 286–293.
83. Assmann, G.; Cullen, P.; Erbey, J.; Ramey, D.R.; Kannenberg, F.; Schulte, H. Plasma sitosterol elevations are associated with an increased incidence of coronary events in men: Results of a nested case-control analysis of the Prospective Cardiovascular Munster (PROCAM) study. *Nutr. Metab. Cardiovasc. Dis.* **2006**, *16*, 13–21.
84. Escurriol, V.; Cofan, M.; Moreno-Iribas, C.; Larranaga, N.; Martinez, C.; Navarro, C.; Rodriguez, L.; Gonzalez, C.A.; Corella, D.; Ros, E. Phytosterol plasma concentrations and coronary heart disease in the prospective Spanish EPIC cohort. *J. Lipid Res.* **2010**, *51*, 618–624.
85. Weingartner, O.; Lutjohann, D.; Vanmierlo, T.; Muller, S.; Gunther, L.; Herrmann, W.; Bohm, M.; Laufs, U.; Herrmann, M. Markers of enhanced cholesterol absorption are a strong predictor for cardiovascular diseases in patients without diabetes mellitus. *Chem. Phys. Lipids* **2011**, *164*, 451–456.
86. Wilund, K.R.; Yu, L.; Xu, F.; Vega, G.L.; Grundy, S.M.; Cohen, J.C.; Hobbs, H.H. No association between plasma levels of plant sterols and atherosclerosis in mice and men. *Arterioscler. Thromb. Vasc. Biol.* **2004**, *24*, 2326–2332.
87. Ellegard, L.; Sunesson, A.; Bosaeus, I. High serum phytosterol levels in short bowel patients on parenteral nutrition support. *Clin. Nutr.* **2005**, *24*, 415–420.
88. Pakarinen, M.P.; Kurvinen, A.; Gylling, H.; Miettinen, T.A.; Pesonen, M.; Kallio, M.; Koivusalo, A.I.; Nissinen, M.J. Cholesterol metabolism in pediatric short bowel syndrome after weaning off parenteral nutrition. *Dig. Liver Dis.* **2010**, *42*, 554–559.
89. Vanhanen, H.; Kesaniemi, Y.A.; Miettinen, T.A. Pravastatin lowers serum cholesterol, cholesterol-precursor sterols, fecal steroids, and cholesterol absorption in man. *Metab. Clin. Exp.* **1992**, *41*, 588–595.
90. Nissinen, M.J.; Gylling, H.; Jarvinen, H.J.; Miettinen, T.A. Ileal pouch-anal anastomosis, conventional ileostomy and ileorectal anastomosis modify cholesterol metabolism. *Dig. Dis. Sci.* **2004**, *49*, 1444–1453.
91. Hakala, K.; Luukkonen, P.; Vuoristo, M.; Jarvinen, H.; Miettinen, T.A. Cholesterol metabolism and non-cholesterol sterols in patients with ileal pouch anastomosis. *J. Hepatol.* **1997**, *26*, 1306–1312.
92. Hrabovsky, V.; Mendlova, A.; Zadak, Z.; Blaha, V.; Hyspler, R.; Ticha, A.; Svagera, Z. Cholesterol metabolism in acute upper gastrointestinal bleeding, preliminary observations. *Wien. Klin. Wochenschr.* **2012**, *124*, 815–821.
93. Dullaart, R.P.; Gansevoort, R.T.; Sluiter, W.J.; de Zeeuw, D.; de Jong, P.E. The serum lathosterol to cholesterol ratio, an index of cholesterol synthesis, is not elevated in patients with glomerular proteinuria and is not associated with improvement of hyperlipidemia in response to antiproteinuric treatment. *Metab. Clin. Exp.* **1996**, *45*, 723–730.

94. Rogacev, K.S.; Pinsdorf, T.; Weingartner, O.; Gerhart, M.K.; Welzel, E.; van Bentum, K.; Popp, J.; Menzner, A.; Fliser, D.; Lutjohann, D.; et al. Cholesterol synthesis, cholesterol absorption, and mortality in hemodialysis patients. *Clin. J. Am. Soc. Nephrol.* **2012**, *7*, 943–948.
95. Brindisi, M.C.; Guiu, B.; Duvillard, L.; Athias, A.; Rollot, F.; Bouillet, B.; Beacco, M.; Hillon, P.; Cercueil, J.P.; Verges, B., et al. Liver fat content is associated with an increase in cholesterol synthesis independent of statin therapy use in patients with type 2 diabetes. *Atherosclerosis* **2012**, *224*, 465–468.
96. Ikegami, T.; Hyogo, H.; Honda, A.; Miyazaki, T.; Tokushige, K.; Hashimoto, E.; Inui, K.; Matsuzaki, Y.; Tazuma, S. Increased serum liver X receptor ligand oxysterols in patients with non-alcoholic fatty liver disease. *J. Gastroenterol.* **2012**, *47*, 1257–1266.
97. Min, H.K.; Kapoor, A.; Fuchs, M.; Mirshahi, F.; Zhou, H.; Maher, J.; Kellum, J.; Warnick, R.; Contos, M.J.; Sanyal, A.J. Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease. *Cell Metab.* **2012**, *15*, 665–674.
98. Simonen, M.; Mannisto, V.; Leppanen, J.; Kaminska, D.; Karja, V.; Venesmaa, S.; Kakela, P.; Kuusisto, J.; Gylling, H.; Laakso, M., et al. Desmosterol in human nonalcoholic steatohepatitis. *Hepatology* **2013**, *58*, 976–982.
99. Simonen, P.; Kotronen, A.; Hallikainen, M.; Sevastianova, K.; Makkonen, J.; Hakkarainen, A.; Lundbom, N.; Miettinen, T.A.; Gylling, H.; Yki-Jarvinen, H. Cholesterol synthesis is increased and absorption decreased in non-alcoholic fatty liver disease independent of obesity. *J. Hepatol.* **2011**, *54*, 153–159.
100. Castro, J.; Amigo, L.; Miquel, J.F.; Galman, C.; Crovari, F.; Raddatz, A.; Zanlungo, S.; Jalil, R.; Rudling, M.; Nervi, F. Increased activity of hepatic microsomal triglyceride transfer protein and bile acid synthesis in gallstone disease. *Hepatology* **2007**, *45*, 1261–1266.
101. Galman, C.; Angelin, B.; Rudling, M. Pronounced variation in bile acid synthesis in humans is related to gender, hypertriglyceridaemia and circulating levels of fibroblast growth factor 19. *J. Intern. Med.* **2011**, *270*, 580–588.
102. Galman, C.; Miquel, J.F.; Perez, R.M.; Einarsson, C.; Stahle, L.; Marshall, G.; Nervi, F.; Rudling, M. Bile acid synthesis is increased in Chilean Hispanics with gallstones and in gallstone high-risk Mapuche Indians. *Gastroenterology* **2004**, *126*, 741–748.
103. Gylling, H.; Riikonen, S.; Nikkila, K.; Savonius, H.; Miettinen, T.A. Oral guar gum treatment of intrahepatic cholestasis and pruritus in pregnant women: Effects on serum cholestanol and other non-cholesterol sterols. *Eur. J. Clin. Investig.* **1998**, *28*, 359–363.
104. Hillebrant, C.G.; Nyberg, B.; Gustafsson, U.; Sahlin, S.; Bjorkhem, I.; Rudling, M.; Einarsson, C. Effects of combined treatment with pravastatin and ursodeoxycholic acid on hepatic cholesterol metabolism. *Eur. J. Clin. Investig.* **2002**, *32*, 528–534.
105. Jiang, Z.Y.; Jiang, C.Y.; Wang, L.; Wang, J.C.; Zhang, S.D.; Einarsson, C.; Eriksson, M.; Han, T.Q.; Parini, P.; Eggertsen, G. Increased NPC1L1 and ACAT2 expression in the jejunal mucosa from Chinese gallstone patients. *Biochem. Biophys. Res. Commun.* **2009**, *379*, 49–54.

106. Kakela, P.; Mannisto, V.; Ilves, I.; Vaittinen, M.; Tauriainen, M.M.; Eskelinen, M.; Gylling, H.; Paajanen, H.; Pihlajamaki, J. Serum Plant Sterols Associate with Gallstone Disease Independent of Weight Loss and Non-Alcoholic Fatty Liver Disease. *Obes. Surg.* **2017**, *27*, 1284–1291.
107. Koivusalo, A.; Pakarinen, M.; Gylling, H.; Nissinen, M.J. Relation of cholesterol metabolism to pediatric gallstone disease: A retrospective controlled study. *Bmc Gastroenterol.* **2015**, *15*, 74.
108. Krawczyk, M.; Lutjohann, D.; Schirin-Sokhan, R.; Villarroel, L.; Nervi, F.; Pimentel, F.; Lammert, F.; Miquel, J.F. Phytosterol and cholesterol precursor levels indicate increased cholesterol excretion and biosynthesis in gallstone disease. *Hepatology* **2012**, *55*, 1507–1517.
109. Muhrbeck, O.; Wang, F.H.; Bjorkhem, I.; Axelson, M.; Einarsson, K. Circulating markers for biosynthesis of cholesterol and bile acids are not depressed in asymptomatic gallstone subjects. *J. Hepatol.* **1997**, *27*, 150–155.
110. Pakarinen, M.P.; Lampela, H.; Gylling, H.; Miettinen, T.A.; Koivusalo, A.I.; Nissinen, M.J. Surrogate markers of cholesterol metabolism in children with native liver after successful portoenterostomy for biliary atresia. *J. Pediatric Surg.* **2010**, *45*, 1659–1664.
111. Del Puppo, M.; Kienle, M.G.; Petroni, M.L.; Crosignani, A.; Podda, M. Serum 27-hydroxycholesterol in patients with primary biliary cirrhosis suggests alteration of cholesterol catabolism to bile acids via the acidic pathway. *J. Lipid Res.* **1998**, *39*, 2477–2482.
112. Gylling, H.; Vuoristo, M.; Farkkila, M.; Miettinen, T.A. The metabolism of cholestanol in primary biliary cirrhosis. *J. Hepatol.* **1996**, *24*, 444–451.
113. Mutanen, A.; Nissinen, M.J.; Lohi, J.; Heikkila, P.; Gylling, H.; Pakarinen, M.P. Serum plant sterols, cholestanol, and cholesterol precursors associate with histological liver injury in pediatric onset intestinal failure. *Am. J. Clin. Nutr.* **2014**, *100*, 1085–1094.
114. Nikkila, K.; Hockerstedt, K.; Miettinen, T.A. Liver transplantation modifies serum cholestanol, cholesterol precursor and plant sterol levels. *Clin. Chim. Acta* **1992**, *208*, 205–218.
115. Nikkila, K.; Miettinen, T.A.; Hockerstedt, K.V.; Isoniemi, H. Sterol parameters as markers of liver function in primary biliary cirrhosis before and after liver transplantation. *Transpl. Int.* **2005**, *18*, 221–225.
116. Ydreborg, M.; Lisovskaja, V.; Lagging, M.; Brehm Christensen, P.; Langeland, N.; Buhl, M.R.; Pedersen, C.; Morch, K.; Wejstal, R.; Norkrans, G., et al. A novel fibrosis index comprising a non-cholesterol sterol accurately predicts HCV-related liver cirrhosis. *PLoS ONE* **2014**, *9*, e93601.
117. Scicchitano, P.; Cameli, M.; Maiello, M.; Modesti, P.A.; Muiesan, M.L.; Novo, S.; Palmiero, P.; Saba, P.S.; Pedrinelli, R.; Ciccone, M.M., et al. Nutraceuticals and dyslipidaemia: Beyond the common therapeutics. *J. Funct. Foods* **2014**, *6*, 11–32, doi:10.1016/j.jff.2013.12.006.
118. Barter, P.J.; Rye, K.A. New Era of Lipid-Lowering Drugs. *Pharm. Rev.* **2016**, *68*, 458–475, doi:10.1124/pr.115.012203.
119. Garcia-Calvo, M.; Lisnock, J.M.; Bull, H.G.; Hawes, B.E.; Burnett, D.A.; Braun, M.P.; Crona, J.H.; Davis, H.R.; Dean, D.C.; Detmers, P.A., et al. The target of ezetimibe is Niemann-Pick Cl-Like 1 (NPC1L1). *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 8132–8137, doi:10.1073/pnas.0500269102.

120. De Smet, E.; Mensink, R.P.; Plat, J. Effects of plant sterols and stanols on intestinal cholesterol metabolism: Suggested mechanisms from past to present. *Mol. Nutr. Food Res.* **2012**, *56*, 1058–1072, doi:10.1002/mnfr.201100722.
121. Matthan, N.R.; Resteghini, N.; Robertson, M.; Ford, I.; Shepherd, J.; Packard, C.; Buckley, B.M.; Jukema, J.W.; Lichtenstein, A.H.; Schaefer, E.J.; et al. Cholesterol absorption and synthesis markers in individuals with and without a CHD event during pravastatin therapy: Insights from the PROSPER trial. *J. Lipid Res.* **2010**, *51*, 202–209, doi:10.1194/jlr.M900032-JLR200.
122. Zaccardi, F.; Webb, D.R.; Yates, T.; Davies, M.J. Pathophysiology of type 1 and type 2 diabetes mellitus: A 90-year perspective. *Postgrad Med. J.* **2016**, *92*, 63–69, doi:10.1136/postgradmedj-2015-133281.
123. Silbernagel, G.; Fauler, G.; Hoffmann, M.M.; Lutjohann, D.; Winkelmann, B.R.; Boehm, B.O.; Marz, W. The associations of cholesterol metabolism and plasma plant sterols with all-cause and cardiovascular mortality. *J. Lipid Res.* **2010**, *51*, 2384–2393, doi:10.1194/jlr.P002899.
124. Silbernagel, G.; Fauler, G.; Renner, W.; Landl, E.M.; Hoffmann, M.M.; Winkelmann, B.R.; Boehm, B.O.; Marz, W. The relationships of cholesterol metabolism and plasma plant sterols with the severity of coronary artery disease. *J. Lipid Res.* **2009**, *50*, 334–341, doi:10.1194/jlr.P800013-JLR200.
125. Cedeno Mora, S.; Goicoechea, M.; Torres, E.; Verdalles, U.; Perez de Jose, A.; Verde, E.; Garcia de Vinuesa, S.; Luno, J. Cardiovascular risk prediction in chronic kidney disease patients. *Nefrologia* **2017**, *37*, 293–300, doi:10.1016/j.nefro.2016.10.002.

Supplementary Table. Serum non-cholesterol sterol markers in subjects with gestational diabetes

		Sitosterol		Campesterol		Cholestanol		Lathosterol		Desmosterol	
Miettinen, 2014 [53]	1st trimester Cases (n=22)	0.78	=	1.59	=	1.39	=	1.54	=	0.72	=
	Controls (n=30)	0.90		1.74		1.47		1.30		0.76	
	2nd trimester Cases (n=22)	0.86	=	1.74	=	1.80	=	1.44	=	0.75	=
	Controls (n=30)	0.97		1.89		1.81		1.32		0.75	
	3rd trimester Cases (n=22)	0.85	=	1.67	=	2.62	=	1.33	=	0.82	=
	Controls (n=30)	0.92		1.75		2.51		1.33		0.90	
	6 weeks PP Cases (n=22)	0.85	=	1.82	=	1.49	=	1.27	=	1.10	=
	Controls (n=30)	1.00		1.92		1.54		1.29		1.13	
	6 months PP Cases (n=22)	0.91	=	1.87	=	1.40	=	1.55	=	0.94	=
	Controls (n=30)	0.99		1.93		1.41		1.42		0.89	
	12 weeks PP Cases (n=22)	0.91	=	1.86	=	1.38	=	1.37	=	0.85	=
	Controls (n=30)	0.96		1.92		1.45		1.21		0.74	

Values are mean and expressed in µmol/mmoL cholesterol. Non-cholesterol markers are not-significantly different (=) between cases and controls

CHAPTER

3

EFFECTS OF AN 8-WEEK AEROBIC EXERCISE PROGRAM ON PLASMA MARKERS FOR CHOLESTEROL ABSORPTION AND SYNTHESIS IN OLDER OVERWEIGHT AND OBESE MEN

Mashnafi S, Plat J, Mensink RP, Joris PJ,
Kleinloog JPD, Baumgartner S

Lipids in Health and Disease. 2021;20(112)

Background

Increased physical activity is inversely related to the risk to develop cardiovascular disease (CVD). In a recent systematic review, it was reported that CVD patients had an increased cholesterol absorption and a decreased synthesis as compared with control participants. As increased physical activity levels reduce CVD risk, we hypothesized that exercise training will reduce cholesterol absorption and increase endogenous cholesterol synthesis in older overweight and obese men.

Methods

A randomized, controlled, crossover trial was performed. Seventeen apparently healthy older overweight and obese men were randomized to start with an aerobic exercise or no-exercise control period for 8 weeks, separated by 12 weeks washout. Fasting serum total cholesterol (TC) and non-cholesterol sterol concentrations were measured at baseline, and after 4 and 8 weeks.

Results

The aerobic exercise program did not affect serum TC concentrations. In addition, exercise did not affect TC-standardized serum concentrations of sitosterol and cholestanol that are markers for cholesterol absorption. However, a trend for reduced TC-standardized campesterol concentrations, which is another validated marker for cholesterol absorption, was observed as compared with control. Lathosterol concentrations, reflecting cholesterol synthesis, did not differ between both periods.

Conclusions

Aerobic exercise training for 8 weeks did not lower serum TC concentrations in older overweight and obese men, but a trend towards a decrease in the cholesterol absorption marker campesterol was found. The cholesterol synthesis marker lathosterol did not change.

Keywords

Aerobic exercise, cholesterol metabolism, cholesterol absorption, cholesterol synthesis, non-cholesterol sterols, plant sterols, cholesterol precursors.

Trial registry name and URL, registration number

posted on
www.clinicaltrials.gov as NCT03272061 on 7 September 2017.

Abstract

Introduction

Increased physical activity is inversely related to the risk to develop cardiovascular disease (CVD) [1]. Indeed, aerobic exercise improves several CVD risk markers such as body composition, blood pressure, low-grade systematic inflammation and immune function [2-5]. In addition, some training intervention studies have reported increased serum high-density lipoprotein cholesterol (HDL-C) [6], and decreased serum low-density lipoprotein cholesterol (LDL-C) and triglyceride (TG) concentrations [7]. However, the number of involved study participants in these studies was in general small and a meta-analysis including six randomized controlled trials and data from 192 men and women with intervention periods ranging from 10 to 104 weeks concluded that serum total cholesterol (TC), LDL-C and HDL-C concentrations were not improved after aerobic exercise training [8].

Even without affecting serum TC concentrations, aerobic exercise may still affect processes underlying cholesterol homeostasis. These processes e.g., intestinal cholesterol absorption and endogenous cholesterol synthesis do not only determine serum TC concentrations, but may also be related to certain metabolic diseases. Non-cholesterol sterols have been validated as markers for cholesterol absorption and cholesterol synthesis [9]. Circulating levels of campesterol, sitosterol and cholestanol reflect intestinal cholesterol absorption, while lathosterol and desmosterol levels are markers of endogenous cholesterol synthesis [10]. Using these markers, patients can be classified as cholesterol absorber or synthesizer [11-14]. For instance, chronic kidney disease (CKD) patients on hemodialysis are characterized by higher cholesterol absorption and lower cholesterol synthesis compared to controls [15, 16]. Furthermore, mortality rates in hemodialysis patients with a high cholesterol absorption are higher compared to those of patients with a low cholesterol absorption [16]. Further, in a recent systematic review, it was also reported that CVD patients have an increased cholesterol absorption and decreased cholesterol synthesis as compared with control participants [17]. It can be speculated that aerobic exercise does not lower serum TC concentrations, but may lower the risk to develop CVD by reducing intestinal cholesterol absorption and increasing cholesterol synthesis.

So far, only a few trials have examined the effects of exercise training on cholesterol absorption and synthesis, but results were inconsistent. Using stable isotopes, Varady et al. [18] showed that endurance exercise for 8 weeks did not affect cholesterol absorption and synthesis in hypercholesteremic subjects. However, Wilund et al. [19] observed, after an endurance exercise program for 6 months, an increase in TC-standardized campesterol levels, but no significant change in TC-standardized lathosterol levels. However, a no-exercise control group was lacking. In contrast, Cho and coworkers reported in ten overweight participants that an 8-week intervention of combined resistance and aerobic

exercise reduced cholesterol synthesis without changing cholesterol absorption [20]. These discrepant findings might be explained by various factors, such as type of exercise training program, sample size, lack of control group and the methodology to measure cholesterol metabolism markers. Therefore, the purpose of this study was to examine the effect of an 8-week aerobic exercise training period as compared to a control period on markers for cholesterol metabolism in older, overweight and obese sedentary men. The population group of the study was deliberately chosen, as they have an increased risk to develop CVD [21-24]. The hypothesis of the study is that exercise reduces cholesterol absorption and increases cholesterol synthesis, a phenotype that may be associated with a decreased CVD risk [17].

Methods

Study participants

Details of this study have been published before [25]. Briefly, nineteen Caucasian men aged between 60-70 years were recruited. Subjects were eligible to participate based on the following inclusion criteria: sedentary lifestyle (classified as low physical activity according to the guidelines for International Physical Activity Questionnaire (IPAQ)); body mass index (BMI) between 25-35 (kg/m^2); no smoking; no diabetes; no active CVD; no drug or alcohol abuse; no use of dietary supplements known to interfere with the study outcomes; no use of medication known to affect lipid or glucose metabolism; fasting plasma glucose concentrations < 7.0 mmol/L; fasting serum TC concentrations < 8.0 mmol/L and fasting serum TG concentrations < 4.5 mmol/L. This study was conducted in accordance with the declaration of Helsinki and written informed consent was obtained from all participants before the start of the study.

Study design

This 8-week, randomized, controlled, cross-over trial was conducted at Maastricht University in Maastricht, the Netherlands from January 2018 to December 2018. Participants were randomized to start with either the exercise period or the control period using a computer-generated, randomization scheme. The protocol was approved by the Medical Ethics Committee of Maastricht University Medical Center (METC173025), and the study was registered on September 7th, 2017 at ClinicalTrials.gov (NCT03272061).

Exercise protocol

The intervention period consisted of a supervised, progressive, aerobic exercise training 3 times a week for 8 weeks performed on a cycling ergometer. The training intensity was determined for each individual based on their maximal power (Pmax), which was reassessed every two weeks during the exercise period. Each exercise session started

with 10 min of warming-up and ended with 10 min of cooling down corresponding to 45% of participant's Pmax. In-between, the participant cycled for 30 min at 70% Pmax. Participants' attendance was recorded to assess exercise compliance. At the end of each period, energy and nutrient intakes were calculated for each participant using a validated food frequency questionnaire. During the control period, participants were requested to maintain their habitual activity levels. A wearable accelerometer (activPAL3; PAL Technologies Ltd., Glasgow, United Kingdom) was used to assess physical activity and sedentary behavior for a median duration of 5 days at the end of each period. During this period, no exercise training was performed [25]. The exercise and control periods were separated by 12 weeks washout period, in which participants returned to their habitual activity levels. As previously described [25], peak oxygen consumption (VO_2 peak) was measured during a maximal exercise test using an incremental step-wise protocol. Measurements were performed every two weeks during the exercise period and three times during the control period.

Serum lipid analysis

Twelve-hour fasting venous blood samples were drawn at baseline, week 4 and at two follow up days in week 8. The first follow up sample was collected 43 hours (median: range 19-72 hours) and the second follow up sample 117 hours (70-118 hours) after an exercise training. Blood samples were centrifuged at 1300x g for 15 min at 21 °C to collect serum samples that were stored at -80 °C until analyzed at the end of the study. Serum TC (CHOD-PAP method; Roche Diagnostic, Mannheim, Germany), HDL-C (CHOD-PAP method; Roche Diagnostic, Mannheim, Germany), and TG concentrations were analyzed with enzymatic methods (GPO-Tinder; Sigma-Aldrich Corp., St. Louis, MO, USA).

Serum non-cholesterol sterol analysis

Serum non-cholesterol sterol concentrations, campesterol, sitosterol, cholestanol and lathosterol, were measured by gas chromatography (GC) equipped with a flame ionization detector (GC-FID) (Hewlett Packard 6890 plus), using a capillary column (DB-XLB 30 m x 0.25 mm i.d. x0.25 um; Agilent Technologies, Amstelveen, Netherlands). Extraction of cholesterol and non-cholesterol sterols was performed as described by Mackay et al. [26]. Briefly, 100ul serum sample was saponified with 1mL of 90% ethanolic sodium hydroxide for 1hr at 60 °C. 5α-cholestane and epicoprostanol were used as internal standards. After two rounds of cyclohexane extraction, samples were derivatized with 30ul of TMS reagent [pyridine, hexamethyldisilazane and trimethylchlorosilane (9:3:1, v/v/v)]. Samples were injected into the GC-FID, and cholesterol and non-cholesterol sterol peaks were integrated (OpenLab CDS ChemStation Edition; Agilent Technologies, Santa Clara, USA), and their concentrations were calculated relative to the internal standard 5α-cholestane concentration. Non-cholesterol sterol concentrations were standardized for cholesterol

concentrations, as determined during the same GC run and expressed as umol/mmol cholesterol.

Statistical analysis

Data are presented as means ± standard deviations (SD), unless indicated otherwise. Normality of distribution was assessed using the Kolmogorov-Smirnov test. Baseline differences in cholesterol and non-cholesterol sterols concentrations between control and intervention periods were analyzed by a paired sample t-test. Changes from baseline over time between the control and intervention periods were analyzed using linear mixed models with treatment and time as within-subject fixed factors and with treatment*time interaction. If the interaction term was not statistically significant, it was omitted from the model. Bonferroni's correction for multiple comparisons was used, as appropriate. Additional analyses were also performed using linear mixed model to evaluate the difference in changes from baseline in all variables between absorbers and synthesizers subgroups. A *P*-value < 0.05 was considered statistically significant. All data were analyzed using SPSS 24.0 for windows (SPSS Inc., Chicago, IL, USA).

Results

Of the nineteen men that started the study, two dropped out due to personal reasons. A full consort flow chart is shown in **Supplemental figure 1**. Baseline characteristics of the seventeen men who completed the study are shown in **Table 1**. The VO_2 peak values were significantly increased during the exercise period from 2716 ± 454 mL at baseline to 2978 ± 473 mL at week 8, while it was 2713 ± 478 mL at baseline and remained nearly unchanged (2708 ± 459 mL) at the end of control period [25]. These results indicated that cardiorespiratory fitness was increased during the exercise period. BMI and body weight were comparable at the start of the control and intervention periods, and did not change during these periods (data not shown).

Table 1. Baseline characteristics (N=17)

Characteristic	Mean ± SD
Age (years)	66.8 ± 1.7
Weight (kg)	94.8 ± 11.7
BMI (kg/m^2)	30.3 ± 2.8
Glucose (mmol/L)	5.8 ± 0.4
Total cholesterol (mmol/L)	5.3 ± 1.1
Triglycerides (mmol/L)	1.4 ± 0.5

Physical activity levels, sedentary times and dietary intake between the intervention and control periods were comparable (unpublished data).

Serum TC concentrations and TC-standardized serum non-cholesterol sterol concentrations (campesterol, sitosterol, cholestanol and lathosterol) were comparable at the start of the intervention and control periods (**Supplemental table 1**). No effects of the aerobic exercise program on serum TC concentrations were observed (**Supplemental table 1)**. Changes in TC-standardized non-cholesterol levels are shown in **Figure 1**. For cholesterol absorption markers, no significant treatment or time effects were observed (**Figure 1, A-C**), although TC-standardized campesterol levels tended to be decreased after the exercise period (P=0.060). Levels of the cholesterol synthesis marker lathosterol were comparable between both intervention periods (**Figure 1, D**).

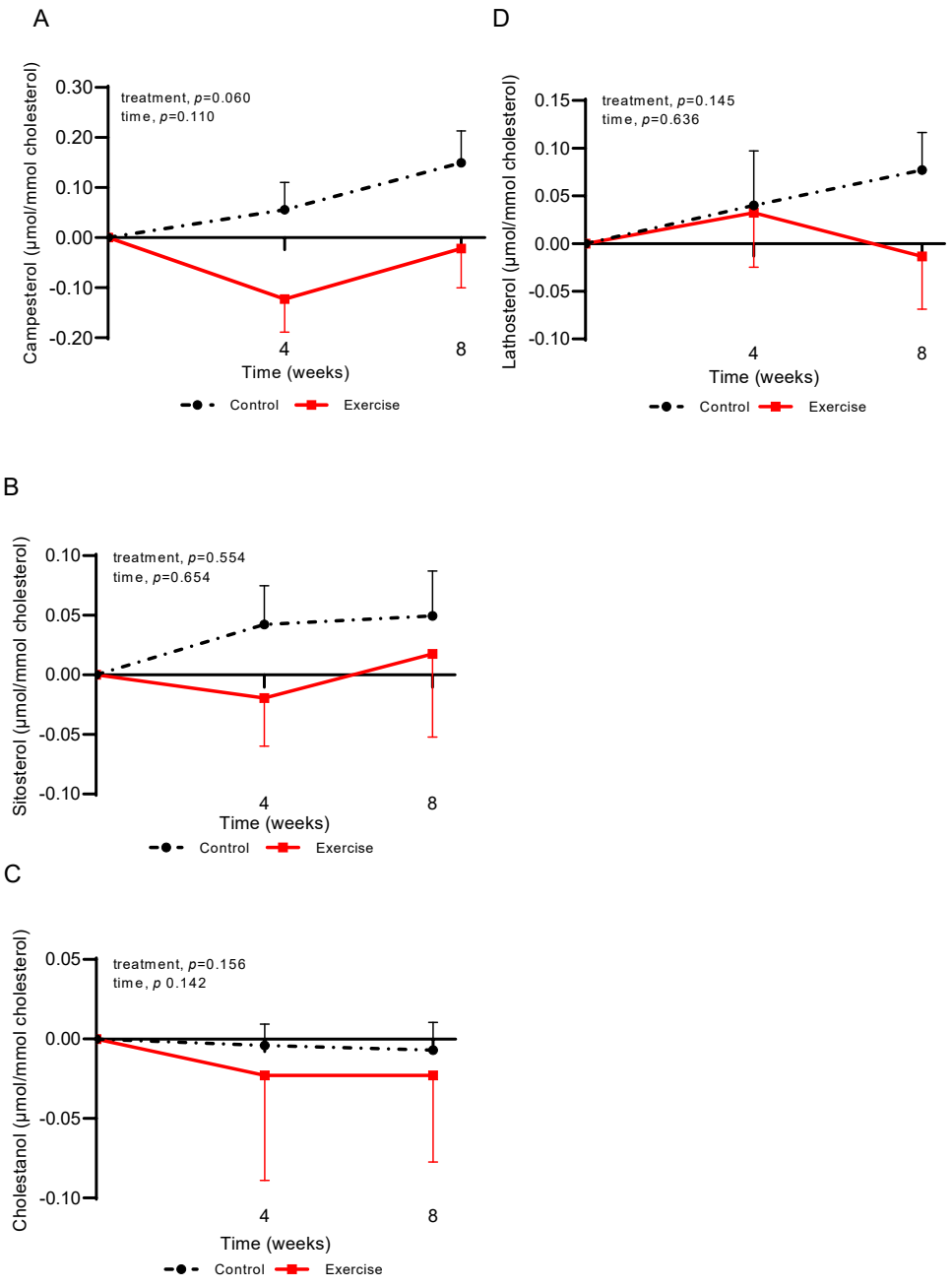


Figure 1. Changes in cholesterol standardized levels of cholesterol absorption markers (A, B, C) and cholesterol synthesis marker (D) during an 8-week intervention period. Data are presented as mean ± SEM.

As exploratory analyses, subjects were divided into cholesterol-absorbers and cholesterol-synthesizers based on the median lathosterol-to-campesterol ratio of the group at baseline to investigate differences in responses between these two subgroups after exercise. At baseline, the range in lathosterol-to-campesterol ratio was 0.21 – 0.69 for cholesterol-absorbers (n=9) and 0.84 – 1.77 for cholesterol-synthesizers (n=8). Even though changes in serum TC concentrations after aerobic exercise in the cholesterol-synthesizers appeared to be more pronounced than in the cholesterol-absorbers, there were no significant treatment or time effects for TC changes in absorbers or synthesizers (**Figure 2**). Also, for changes in TC-standardized campesterol levels, no significant treatment*time effect was observed (*P*=0.093). However, these levels tended to be lower after exercise in the cholesterol absorbers, but not in cholesterol synthesizers. Changes in other markers showed neither treatment nor time effects in absorbers and synthesizers after exercise (**Supplemental figure 2**).

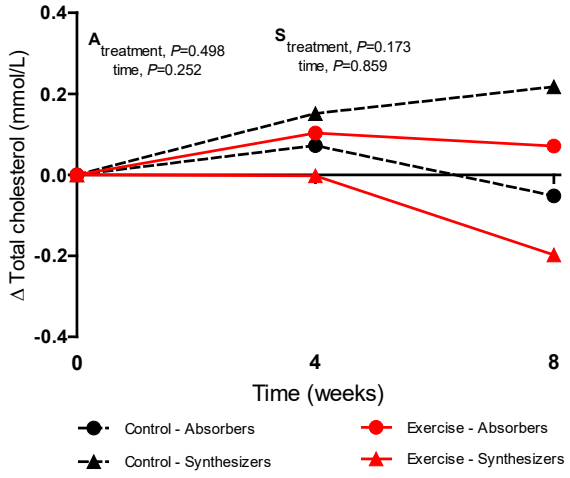


Figure 2. Changes in TC concentrations during intervention periods for cholesterol absorbers (n= 9) and cholesterol synthesizers (n=8) subgroups.

[A]*P* values for factor effects in cholesterol absorbers.
[S]*P* values for factor effects in cholesterol synthesizers.

Discussion

The present study shows that an 8-week aerobic exercise program did not affect validated plasma markers of cholesterol absorption or synthesis in older overweight and obese men. Based on a recent systematic review [17], it was hypothesized that aerobic exercise

training would reduce cholesterol absorption and increase cholesterol synthesis. Although campesterol levels tended to be lower after aerobic exercise, this difference did not reach significance. The two previous studies investigating the effect of exercise on plasma markers of cholesterol absorption and synthesis showed inconsistent findings. In one study, the effect of a long-term aerobic exercise program for 6 months increased plasma campesterol levels, reflecting a higher cholesterol absorption, but did not change plasma markers for cholesterol synthesis in men and women at high risk of developing metabolic syndrome [19]. In contrast, in the current study cholesterol absorption tended to decrease. However, Wilund et al. did not include a no-exercise control group. In the other study with overweight men and women, the effect of alternate day fasting and exercise, composed of combined aerobic and resistance training, was examined [20]. In this randomized controlled trial, no difference in cholesterol absorption markers after 8 weeks intervention between the exercise and control groups was found. However, a significant reduction in cholesterol synthesis in the exercise group was reported, which could also have been due to the alternate day fasting protocol that was followed at the same time [20]. Moreover, only desmosterol levels were decreased, while no changes were observed in lathosterol levels [20]. Desmosterol is an intermediate of the Bloch pathway for cholesterol synthesis and lathosterol of the Kandutsch-Russell pathway [27]. However, this does not necessarily mean that effects of the intervention on cholesterol synthesis were pathway-specific, also because lathosterol is the only validated marker for cholesterol synthesis [9]. In addition, in the recent systematic review, no evidence was found that metabolic aberrations were specifically related to desmosterol and not to lathosterol [17]. Differences in participants characteristics, duration of intervention, methods of dietary control and lack of control group could explain the inconsistency in the effects observed on plasma markers for cholesterol absorption and synthesis. Using stable isotopes, Varady et al. found no effects on cholesterol absorption and synthesis in previously sedentary hypercholesteremic subjects after 6 weeks of endurance exercise [18]. These results agree with the current findings and show that results can be extended to non-hypercholesterolemic sedentary older men using a different approach to estimate fractional cholesterol absorption and synthesis. Despite the 8-week aerobic exercise program, body weight did not change in the exercise period. Dietary habits were comparable between the intervention and control periods, suggesting that any potential loss of fat mass could have been compensated by an increase in fat free mass [28]. Furthermore, factors such as age, sedentary behavior and body composition might affect the exercise intervention, explaining the discrepancy in the current and other studies investigating the effect of exercise on cholesterol absorption and synthesis [29].

Participants can be classified as cholesterol absorbers or cholesterol synthesizers based on their baseline ratios of campesterol to lathosterol [30]. Although not the primary outcome of this study, an exploratory analysis was performed to examine whether the effects of

exercise on TC concentrations were different between absorbers and synthesizers. The effect of exercise on TC concentrations did not significantly differ between the subgroups, although we postulated that participants with a higher cholesterol synthesis at baseline (synthesizers) would respond more pronounced to exercise in terms of TC-lowering than those with a higher cholesterol absorption at baseline (absorbers). However, changes in markers for cholesterol metabolism were comparable between cholesterol absorbers and synthesizers and could not explain the slight, non-significant reduction in TC concentrations observed in cholesterol synthesizers, but not in absorbers.

Study strengths and limitations

A strength of this current study was the inclusion of a sedentary population at a high risk to develop CVD as well as the addition of a no-exercise control period. Furthermore, due to the crossover design, each participant acted as his own control thereby eliminating between-subject variability. There are several limitations in this study. First of all, intake of dietary plant sterols was not known. However, serum plant sterol concentrations were comparable between intervention periods at baseline, suggesting no change in the habitual consumption of plant sterols. Secondly, only male participants were included in the current study but so far there is no indication that non-cholesterol sterols are only valid as markers for cholesterol synthesis or absorption in men and not in women [31]. Lastly, since the current study was not originally designed to investigate the effect of exercise on markers of cholesterol and absorption and synthesis, power calculations were not performed a priori.

Conclusions

In summary, the 8-week aerobic exercise did not lower serum TC concentrations in older overweight and obese men, but a trend towards a decrease in the cholesterol absorption marker campesterol was found. The cholesterol synthesis marker lathosterol did not change. In future studies, the added value of using non-cholesterol sterol concentrations to classify participants as cholesterol absorbers or synthesizers to increase responsiveness to lifestyle interventions or lipid-lowering therapies needs to be addressed. Nonetheless, increasing physical activity levels is an important approach to reduce CVD risk [32].

Declaration

Ethics approval and consent to participate

The protocol was approved by the Medical Ethics Committee of Maastricht University Medical Center (METC173025). Written informed consent was obtained from all participants before the start of the study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this article and its supplementary files.

Competing interests

The authors declare that they have no competing interests.

Funding

The authors disclosed receipt of the following financial support for the research: The study was supported by the Netherlands Organization for Scientific Research [grant number (ALWTF.2016.012)] and it was organized by and executed under the auspices of TiFN, a public-private partnership on precompetitive research in food and nutrition. The authors have declared that no competing interests exist in the writing of this publication.

Author Contributions

SM performed the GC-MS analysis, performed the statistical analyses, interpreted the data, and wrote the manuscript. JP interpreted the data and reviewed the manuscript. RM designed the study, interpreted the data, had overall responsibility for the study, and reviewed the manuscript. PJ designed the study, had overall responsibility for the study and reviewed the manuscript. JK designed and conducted the study and performed the statistical analyses. SB interpreted the data and reviewed the manuscript.

Acknowledgements

We would like to thank E. T. H. C. Smeets and N. C. C. Thijssen for their assistance during the experimental phase of the trial and we would like to thank M.C.J.M Konings for his technical assistance. Finally, we thank our participants for their cooperation and enthusiasm.

References

1. Mora S, Cook N, Buring JE, Ridker PM, Lee IM: **Physical activity and reduced risk of cardiovascular events: potential mediating mechanisms.** *Circulation* 2007, **116:**2110-2118.
2. Diez-Fernandez A, Martinez-Vizcaino V, Torres-Costoso A, Canete Garcia-Prieto J, Franquelo-Morales P, Sanchez-Lopez M: **Strength and cardiometabolic risk in young adults: The mediator role of aerobic fitness and waist circumference.** *Scand J Med Sci Sports* 2018, **28:**1801-1807.
3. Fiuza-Luces C, Santos-Lozano A, Joyner M, Carrera-Bastos P, Picazo O, Zugaza JL, Izquierdo M, Ruilope LM, Lucia A: **Exercise benefits in cardiovascular disease: beyond attenuation of traditional risk factors.** *Nat Rev Cardiol* 2018, **15:**731-743.
4. Ostman C, Smart NA, Morcos D, Duller A, Ridley W, Jewiss D: **The effect of exercise training on clinical outcomes in patients with the metabolic syndrome: a systematic review and meta-analysis.** *Cardiovasc Diabetol* 2017, **16:**110.
5. Whelton SP, Chin A, Xin X, He J: **Effect of aerobic exercise on blood pressure: a meta-analysis of randomized, controlled trials.** *Ann Intern Med* 2002, **136:**493-503.
6. Halverstadt A, Phares DA, Wilund KR, Goldberg AP, Hagberg JM: **Endurance exercise training raises high-density lipoprotein cholesterol and lowers small low-density lipoprotein and very low-density lipoprotein independent of body fat phenotypes in older men and women.** *Metabolism* 2007, **56:**444-450.
7. Durstine JL, Grandjean PW, Cox CA, Thompson PD: **Lipids, lipoproteins, and exercise.** *J Cardiopulm Rehabil* 2002, **22:**385-398.
8. Kelley GA, Kelley KS, Roberts S, Haskell W: **Comparison of aerobic exercise, diet or both on lipids and lipoproteins in adults: a meta-analysis of randomized controlled trials.** *Clin Nutr* 2012, **31:**156-167.
9. Miettinen TA, Tilvis RS, Kesaniemi YA: **Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population.** *Am J Epidemiol* 1990, **131:**20-31.
10. Miettinen TA, Gylling H, Nissinen MJ: **The role of serum non-cholesterol sterols as surrogate markers of absolute cholesterol synthesis and absorption.** *Nutr Metab Cardiovasc Dis* 2011, **21:**765-769.
11. De Smet E, Mensink RP, Plat J: **Effects of plant sterols and stanols on intestinal cholesterol metabolism: suggested mechanisms from past to present.** *Mol Nutr Food Res* 2012, **56:**1058-1072.
12. Garcia-Calvo M, Lisnock J, Bull HG, Hawes BE, Burnett DA, Braun MP, Crona JH, Davis HR, Jr., Dean DC, Detmers PA, et al: **The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1).** *Proc Natl Acad Sci U S A* 2005, **102:**8132-8137.
13. Hoenig MR, Rolfe BE, Campbell JH: **Cholestanol: a serum marker to guide LDL cholesterol-lowering therapy.** *Atherosclerosis* 2006, **184:**247-254.

14. Matthan NR, Resteghini N, Robertson M, Ford I, Shepherd J, Packard C, Buckley BM, Jukema JW, Lichtenstein AH, Schaefer EJ, Group P: **Cholesterol absorption and synthesis markers in individuals with and without a CHD event during pravastatin therapy: insights from the PROSPER trial.** *J Lipid Res* 2010, **51:**202-209.
15. Fukushima M, Miura S, Mitsutake R, Fukushima T, Fukushima K, Saku K: **Cholesterol metabolism in patients with hemodialysis in the presence or absence of coronary artery disease.** *Circ J* 2012, **76:**1980-1986.
16. Rogacev KS, Pinsdorf T, Weingartner O, Gerhart MK, Welzel E, van Bentum K, Popp J, Menzner A, Fliser D, Lutjohann D, Heine GH: **Cholesterol synthesis, cholesterol absorption, and mortality in hemodialysis patients.** *Clin J Am Soc Nephrol* 2012, **7:**943-948.
17. Mashnafi S, Plat J, Mensink RP, Baumgartner S: **Non-Cholesterol Sterol Concentrations as Biomarkers for Cholesterol Absorption and Synthesis in Different Metabolic Disorders: A Systematic Review.** *Nutrients* 2019, **11**.
18. Varady KA, Houweling AH, Jones PJ: **Effect of plant sterols and exercise training on cholesterol absorption and synthesis in previously sedentary hypercholesterolemic subjects.** *Transl Res* 2007, **149:**22-30.
19. Wilund KR, Feeney LA, Tomayko EJ, Weiss EP, Hagberg JM: **Effects of endurance exercise training on markers of cholesterol absorption and synthesis.** *Physiol Res* 2009, **58:**545-552.
20. Cho AR, Moon JY, Kim S, An KY, Oh M, Jeon JY, Jung DH, Choi MH, Lee JW: **Effects of alternate day fasting and exercise on cholesterol metabolism in overweight or obese adults: A pilot randomized controlled trial.** *Metabolism* 2019, **93:**52-60.
21. Dhana K, Berghout MA, Peeters A, Ikram MA, Tiemeier H, Hofman A, Nusselder W, Kavousi M, Franco OH: **Obesity in older adults and life expectancy with and without cardiovascular disease.** *Int J Obes (Lond)* 2016, **40:**1535-1540.
22. Hajduk AM, Chaudhry SI: **Sedentary Behavior and Cardiovascular Risk in Older Adults: a Scoping Review.** *Curr Cardiovasc Risk Rep* 2016, **10**.
23. Kim K, Choi S, Hwang SE, Son JS, Lee JK, Oh J, Park SM: **Changes in exercise frequency and cardiovascular outcomes in older adults.** *Eur Heart J* 2020, **41:**1490-1499.
24. Wannamethee SG, Shaper AG, Walker M: **Changes in physical activity, mortality, and incidence of coronary heart disease in older men.** *Lancet* 1998, **351:**1603-1608.
25. Kleinloog JPD, Mensink RP, Ivanov D, Adam JJ, Uludag K, Joris PJ: **Aerobic Exercise Training Improves Cerebral Blood Flow and Executive Function: A Randomized, Controlled Cross-Over Trial in Sedentary Older Men.** *Front Aging Neurosci* 2019, **11:**333.
26. Mackay DS, Jones PJ, Myrie SB, Plat J, Lutjohann D: **Methodological considerations for the harmonization of non-cholesterol sterol bio-analysis.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2014, **957:**116-122.
27. Luu W, Hart-Smith G, Sharpe LJ, Brown AJ: **The terminal enzymes of cholesterol synthesis, DHCR24 and DHCR7, interact physically and functionally.** *J Lipid Res* 2015, **56:**888-897.
28. Goran MI, Poehlman ET: **Endurance training does not enhance total energy expenditure in healthy elderly persons.** *Am J Physiol* 1992, **263:**E950-957.

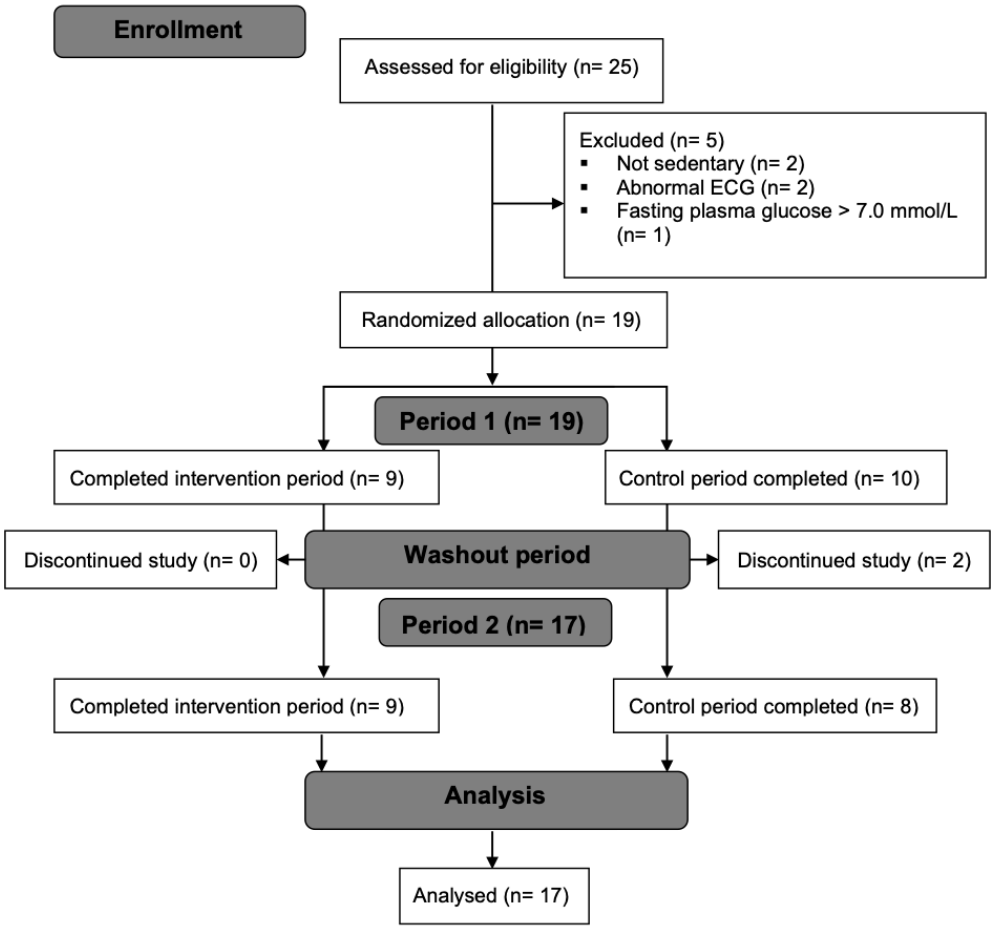
29. Gomez-Bruton A, Navarrete-Villanueva D, Perez-Gomez J, Vila-Maldonado S, Gesteiro E, Gusi N, Villa-Vicente JG, Espino L, Gonzalez-Gross M, Casajus JA, et al: **The effects of Age, Organized Physical Activity and Sedentarism on Fitness in Older Adults: An 8-Year Longitudinal Study.** *Int J Environ Res Public Health* 2020, **17**.
30. Thuluva SC, Igel M, Giesa U, Lutjohann D, Sudhop T, von Bergmann K: **Ratio of lathosterol to campesterol in serum predicts the cholesterol-lowering effect of sitostanol-supplemented margarine.** *Int J Clin Pharmacol Ther* 2005, **43:**305-310.
31. Trautwein EA, Vermeer MA, Hiemstra H, Ras RT: **LDL-Cholesterol Lowering of Plant Sterols and Stanols-Which Factors Influence Their Efficacy?** *Nutrients* 2018, **10**.
32. Li J, Siegrist J: **Physical activity and risk of cardiovascular disease--a meta-analysis of prospective cohort studies.** *Int J Environ Res Public Health* 2012, **9:**391-407.

Supplementary Materials

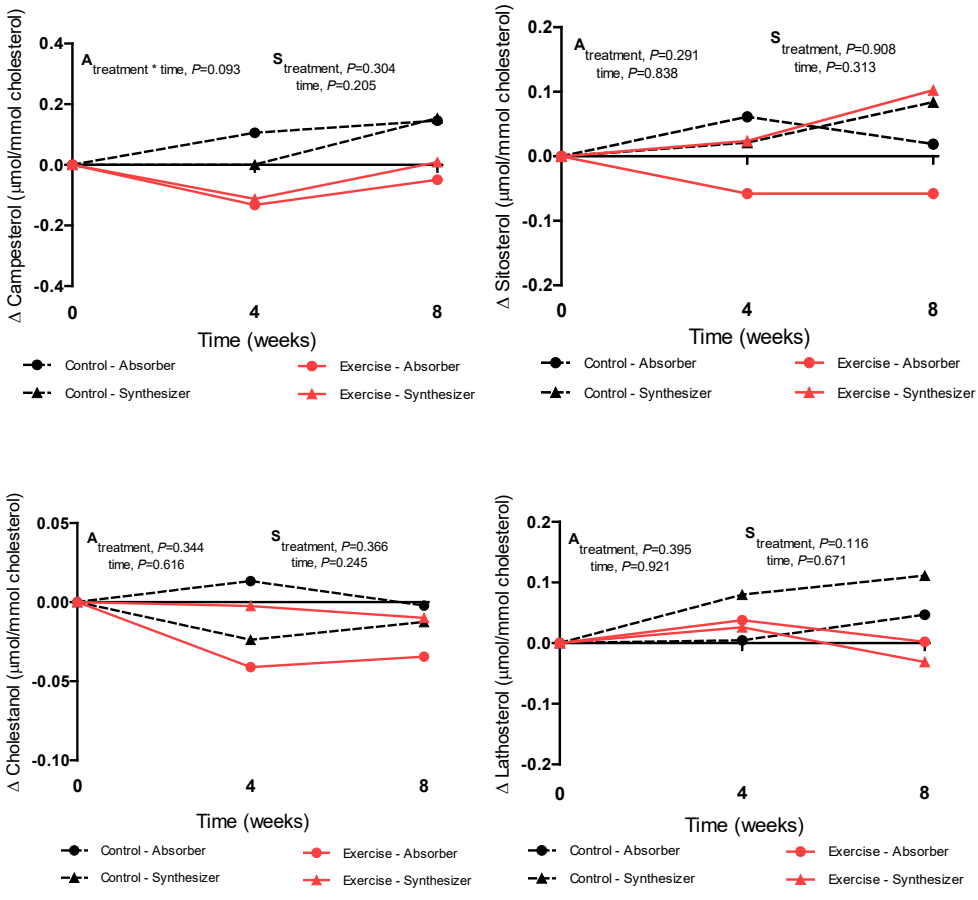
Supplemental table 1. Cholesterol and non-cholesterol sterol concentrations (n=17) at baseline, week 4 and week 8 in control and exercise periods.

	Control	Exercise	P values
Total cholesterol (mmol/l)			
Baseline	5.22 ± 1.10	5.36 ± 1.03	0.102
Week 4	5.33 ± 1.29	5.42 ± 1.16	
Week 8	5.29 ± 1.09	5.31 ± 1.27	
TC-Campesterol*			
Baseline	1.97 ± 0.78	2.03 ± 0.74	0.494
Week 4	2.02 ± 0.78	1.90 ± 0.70	
Week 8	2.11 ± 0.82	2.00 ± 0.67	
TC-Sitosterol*			
Baseline	1.43 ± 0.59	1.46 ± 0.55	0.498
Week 4	1.47 ± 0.56	1.44 ± 0.55	
Week 8	1.48 ± 0.54	1.48 ± 0.57	
TC-Cholestanol*			
Baseline	1.39 ± 0.25	1.37 ± 0.23	0.391
Week 4	1.39 ± 0.25	1.35 ± 0.23	
Week 8	1.39 ± 0.26	1.35 ± 0.23	
TC-Lathosterol*			
Baseline	1.26 ± 0.38	1.38 ± 0.42	0.120
Week 4	1.30 ± 0.41	1.41 ± 0.44	
Week 8	1.34 ± 0.39	1.36 ± 0.39	

* Expressed as µmol/mmol cholesterol. Data are presented as mean ± SD



Supplemental figure 1. CONSORT flow diagram of the randomised, controlled crossover study.



Supplemental figure 2. Changes in markers of cholesterol absorption and synthesis during intervention periods for cholesterol absorbers (n= 9) and cholesterol synthesizers (n=8) subgroups.

[A]*P* values for factor effects in cholesterol absorbers.

[S]*P* values for factor effects in cholesterol synthesizers.

CHAPTER

4

EFFECTS OF DIET-INDUCED WEIGHT LOSS ON PLASMA MARKERS FOR CHOLESTEROL ABSORPTION AND SYNTHESIS:

secondary analysis of a randomized trial in abdominally obese men

Mashnafi S, Plat J, Mensink RP, Joris PJ, Kusters YHAM, Houben AJHM, Stehouwer CDA, Schalkwijk CG, Baumgartner S

Nutrients. 2022;14(8),1546

Cross-sectional studies have shown that obesity is associated with a lower intestinal cholesterol absorption and a higher endogenous cholesterol synthesis. These metabolic characteristics have also been observed in patients with type 2 diabetes, the metabolic syndrome, steatosis or cholestasis. The number of intervention studies evaluating the effect of weight-loss on these metabolic characteristics is however limited, while the role of the different fat compartments has not been studied into detail. In a randomised trial, abdominally obese men (N=54) followed a 6-week very low caloric (VLCD) diet, followed by a 2-week of weight maintenance period. Non-cholesterol sterols were measured at baseline and after 8 weeks, and compared to levels in lean participants (N=25)**.** After weight loss, total cholesterol (TC)-standardized cholestanol levels increased by 0.18 µmol/mmol (p <0.001), while those of campesterol and lathosterol decreased by 0.25 µmol/mmol (p <0.05) and 0.39 µmol/mmol *(*p <0.001), respectively. Moreover, after weight-loss, TC-standardized lathosterol and cholestanol levels were comparable to those of lean men. Increases in TC-standardized cholestanol after weight loss were significantly associated with changes in waist circumference (p <0.01), weight (p <0.001), BMI (p <0.001) and visceral fat (p <0.01), but not with subcutaneous and intrahepatic lipid. In addition, cross-sectional analysis showed that visceral fat fully mediated the association between BMI and TC-standardized cholestanol levels. Intrahepatic lipid content was a partial mediator for the association between BMI and TC-standardized lathosterol levels. In conclusion, diet-induced weight loss decreased cholesterol synthesis and increased cholesterol absorption. The increase in TC-standardized cholestanol levels was not only related to weight loss, but also to a decrease in visceral fat volume. Whether these metabolic changes ameliorate other metabolic risk factors needs further study.

Keywords

Diet-induced weight loss, cholesterol absorption, cholesterol synthesis, non-cholesterol sterols, visceral fat, subcutaneous fat, intrahepatic lipid, cholesterol precursors, plant sterols

Abstract

1. Introduction

Obesity and its associated comorbidities are a major health problem worldwide. An increased visceral fat content, a characteristic of people with abdominal obesity, is clinically the most important form of obesity [1]. Abdominal obesity is strongly associated with insulin resistance, dyslipidaemia and hypertension [2], which all contribute to an increased cardiovascular disease risk [1,3]. Recently, we have suggested that overweight and obesity are associated with a lower intestinal cholesterol absorption and a higher endogenous cholesterol synthesis [4]. These metabolic characteristics have also been observed in patients with type 2 diabetes, the metabolic syndrome, steatosis or cholestasis [4]. However, these reported cross-sectional associations do not necessarily imply that weight loss will lead to an increase in cholesterol absorption and a decrease in cholesterol synthesis. To assess whether there is a causal association between weight loss with cholesterol absorption and synthesis, well-controlled intervention studies are needed.

To evaluate changes in cholesterol absorption and synthesis in humans, serum non-cholesterol sterols are frequently used as markers [5]. The cholesterol precursors desmosterol and lathosterol reflect endogenous cholesterol synthesis, while the non-cholesterol sterols sitosterol, campesterol, and cholestanol reflect fractional intestinal cholesterol absorption [6]. Using these markers, earlier intervention studies in obese individuals with type 2 diabetes [7,8] or the metabolic syndrome [9-11] have indeed suggested that diet-induced weight loss increased cholesterol absorption and decreased cholesterol synthesis. However, relations with fat distribution or the different fat compartments, which behave metabolically different [12-14], were not studied.

So far, studies evaluating the effects of diet-induced weight loss on cholesterol metabolism in apparently healthy individuals with abdominal obesity are limited. In addition, in most studies that did evaluate these effects, a no-weight loss control group was not included [7-9,11]. Furthermore, results have not been compared to those of normal-weight volunteers as a reference population in all previous studies. Finally, in some studies body weight had not reached a new steady state and participants were still in negative energy balance when serum non-cholesterol sterol concentrations were analyzed after weight-loss [8]. Therefore, the aim of this study was to examine the effect of a 6-week diet induced weight loss program, followed by a 2-week weight stable period, on markers of cholesterol absorption and synthesis in apparently healthy individuals with abdominal obesity. Results before and after the weight loss in the new steady energy balance were compared to those of normal-weight men. In addition, we examined the relations between changes in markers for cholesterol absorption and synthesis with changes in fat distribution and different fat compartments (visceral fat, subcutaneous fat, and intrahepatic lipid) to assess

whether changes in aforementioned compartments play a role in cholesterol metabolism characteristics after weight loss. Finally, we used cross-sectional mediation analysis to examine the mediating role of each fat compartments on the relationship between body mass index (BMI) and markers for cholesterol absorption and synthesis.

2. Materials and Methods

2.1. Participants and study design

Details of this study have been published before [15]. Briefly, Caucasian apparently healthy male subjects, aged between 18-56 years, were eligible to participate when they met the following inclusion criteria: stable body weight (± 3 kg in the last 3 months); no diabetes; no active cardiovascular diseases; no inflammatory diseases; no use of antihypertensive medication; no drug or alcohol abuse; no use of medication known to affect lipid or glucose metabolism and no participation in another biomedical trial in the previous 30 days. Both normal-weight men and abdominally obese men participated in this study. Normal-weight subjects had a waist circumference below 94 cm, while this was between 102 and 110 cm in the abdominal obese group. Upon inclusion, the abdominally obese men were randomized to the diet-induced weight loss group or the no-weight loss control group as described previously [15]. The participants in the weight loss group consumed, under strict guidance, a very-low caloric diet (VLCD; Modifast; Nutrition et Santé Benelux, Breda, The Netherlands) for 4 to 5 weeks. The aim was to achieve a waist circumference below 102 cm, which is the cut-off value used for the diagnosis of the metabolic syndrome [16]. Daily caloric intake of the VLCD was 2.1 MJ (500 kcal) and the content of minerals and vitamins met the Dutch dietary guidelines. Participants in the weight loss group consumed daily three VLCD formulas that had to be dissolved in water. Hereafter, in weeks 5 and 6, participants consumed daily three meals of a mixed solid caloric-restricted diet providing 4.2 MJ/day (1000 kcal) for one to two weeks. The composition of this diet again met the Dutch dietary guidelines. In week 7 and 8, weight maintenance was achieved by providing weekly menus which were adjusted to individual energy requirements. Men allocated to the no-weight loss group were asked to maintain their habitual diet, physical activity level and alcohol consumption throughout the entire study duration. A total of 79 men were included, 25 men had a normal weight (waist circumference <94 cm) and 54 men were abdominally obese (waist circumference 102-110 cm). One man dropped out before randomization and thus, 53 of the abdominally obese men were assigned to the weight loss group (N=26) or no-weight loss control group (N=27). Written informed consent was obtained from all participants before start of the study. The study protocol was approved by the Medical Ethical Committee of Maastricht University Medical Center (METC 12-30-40), and registered at ClinicalTrials.gov (NCT01675401).

2.2. Anthropometrics, fat distribution and compartments

Information about overall and abdominal obesity was obtained by measurements of weight, body mass index, waist circumference, hip circumference, and waist to hip ratio as previously described [15]. The volume of the visceral fat, and subcutaneous fat compartments as well as the intrahepatic lipid content were measured by magnetic resonance imaging (MRI) [17].

2.3. Blood sampling

Venous blood samples were drawn after an overnight fast at baseline and in week 8. Heparin vacutainer tubes were centrifuged at 1300x g for 15 min at 4 °C to collect plasma samples. Serum tubes were centrifuged at 1300x g for 15 min at 21 °C to collect serum samples. Aliquots were stored at -80 °C until analyzed at the end of the study.

2.4. Serum lipid analysis

Serum total cholesterol (TC) (CHOD-PAP method; Roche Diagnostic, Mannheim, Germany), high-density lipoprotein cholesterol (HDL-C) (CHOD-PAP method; Roche Diagnostic, Mannheim, Germany), and triglyceride (TG) concentrations – corrected for glycerol levels – were analyzed enzymatically (GPO-Tinder; Sigma-Aldrich Corp., St. Louis, MO, USA). Serum low-density lipoprotein cholesterol (LDL-C) concentrations were calculated using the Friedewald equation [18].

2.5. Non-cholesterol sterol analysis

Sterols were measured by gas-chromatography equipped with a flame ionization detector (GC-FID) (Hewlett Packard 6890 plus), and with a capillary column (DB-XLB 30 m x 0.25 mm i.d. x0.25 μm; Agilent Technologies, Amstelveen, Netherlands). Extraction of cholesterol and non-cholesterol sterols was performed based on Mackay et al.[19]. Briefly, 100μl plasma sample was saponified with 1ml of 90% ethanolic sodium hydroxide for 1hr at 60 °C. 5α-cholestane and epicoprostanol were used as internal standards. After two rounds of cyclohexane extraction, samples were derivatized with 30μl of TMS reagent [pyridine, hexamethyldisilazane and trimethylchlorosilane (9:3:1, v/v/v)]. Samples were injected into GC-FID, cholesterol and non-cholesterol sterol peaks were integrated (OpenLab CDS ChemStation Edition; Agilent Technologies, Santa Clara, USA), and their concentrations were calculated relative to the internal standard 5α-cholestane. Non-cholesterol sterol concentrations were standardized for cholesterol concentrations, as determined within the same GC run and expressed as μmol/mmol cholesterol.

2.6. Statistics analyses

Data are presented as means ± standard deviations (SD) unless indicated otherwise. Normality of the data was assessed using Kolmogorov-Smirnov test. The differences at baseline between normal weight and abdominally obese men were compared with an

independent t-test. A one-way ANCOVA using baseline concentrations as a covariate was used to examine differences in changes between the diet-induced weight loss and no-weight loss control treatments. An independent t-test was also used to compare differences between the normal weight men and the abdominally obese men after weight loss. Linear regression analysis was used to examine cross-sectional relations between cholesterol absorption or synthesis markers with anthropometric measures at baseline and with changes after weight loss. Cross-sectionally, we examined whether relationships between BMI (independent variable) with cholesterol absorption or synthesis markers (dependent variables) was mediated by visceral fat, subcutaneous fat or intrahepatic lipids (potential mediators). For this, the PROGRESS plug-in for SPSS version 4.0 (A.F. Hayes, Ohio State University, Columbus, Ohio, USA) was used (model 4). A p-value < 0.05 was considered statistically significant. All data were analyzed using SPSS versions 25.0 and 27.0 for Mac (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Clinical characteristics of study participants

A full consort flow diagram is shown in **Figure S1**. Plasma samples of 25 normal weight and 53 abdominally obese men were used for measurements at baseline. One participant in the weight loss group was excluded due to study protocol violations and three participants dropped out for reasons as indicated before [15]. In the end, 23 men in the diet-induced weight loss group and 26 men in the no-weight loss group completed the study. Characteristics of all participants at baseline have been described previously [15]. Briefly, as shown in **Table 1**, the median age was comparable between normal weight and abdominally obese men. As expected, BMI, waist circumference, waist to hip ratio, subcutaneous fat, visceral fat, and intrahepatic lipid contents were higher in the abdominally obese men as compared to those with normal weight. At baseline, serum LDL-C concentrations and plasma TC-standardized levels of the cholesterol synthesis marker lathosterol were higher in the abdominally obese men as compared to normal weight men. On the other hand, TC-standardized levels of all three cholesterol absorption markers campesterol, sitosterol and cholestanol were lower in the abdominally obese men (all p <0.05).

3.2. Effect of weight loss

In the abdominally obese participants allocated to the diet-induced weight loss group, mean body weight decreased by 10.3 kg (95% CI: -11.4, -9.2 kg; p <0.001), waist circumference by 11.0 cm (-9.9, -12.1 cm; p <0.001), subcutaneous fat by 0.81 L (-0.93, -0.69 L; p <0.001), visceral fat by 0.85 L (-1.0, -0.67 L; p <0.001), and intrahepatic lipid content by -5.80% (-6.58, -5.02%; p <0.001), compared with the no-weight loss control group.

Serum LDL-C and triglycerides concentrations were significantly reduced (all p <0.001) in abdominally obese men after 8 weeks of diet-induced weight loss as compared with the no-weight loss control treatment group as shown in **Table 1**. HDL concentrations did not differ between two treatment groups after 8 weeks. Compared with the normal-weight group, abdominally obese men had comparable values for serum LDL-C and triglycerides and HDL concentrations, at the end of the dietary weight loss period.

TC-standardized plasma campesterol levels were significantly reduced after weight loss (-0.25 µmol/mmol cholesterol (95% CI: -0.43, -0.07 µmol/mmol cholesterol; p <0.05)), while TC-standardized sitosterol levels remained unchanged. In contrast to campesterol, TC-standardized plasma cholestanol levels were significantly increased by 0.18 µmol/mmol cholesterol (95% CI: 0.19, 0.25 µmol/mmol cholesterol; p <0.001). After 8-weeks, TC-standardized campesterol and sitosterol levels remained lower in abdominally obese that lost weight as compared to normal weight subjects, (p <0.001 and p <0.05, respectively), while TC-standardized cholestanol levels were comparable between normal weight and obese participants after attaining weight loss. Diet-induced weight loss significantly reduced TC-standardized lathosterol levels (-0.39 µmol/mmol cholesterol (95% CI: -0.55, -0.24 µmol/mmol cholesterol; p <0.001)). After weight loss, TC-standardized lathosterol levels were comparable between the normal-weight and obese participants.

3.3. Associations between anthropometrics, fat distribution, and fat compartments with cholesterol absorption and synthesis markers

Cross-sectional analysis including abdominally obese and normal weight men at baseline showed significant relationships between markers for cholesterol absorption and synthesis with all anthropometric markers, fat distribution and fat compartments (weight, body mass index, waist circumference, hip circumference, waist to hip ratio, visceral fat, subcutaneous fat as-and intrahepatic lipid content; all p <0.05) (**Table S1**). The relation between changes in markers for cholesterol absorption and synthesis with changes in these variables is shown in **Table 2**. Changes in TC-standardized cholestanol levels after diet-induced weight loss were significantly associated with changes in waist circumference (p <0.01), weight (p <0.001), BMI (p <0.001) and visceral fat (p <0.01). Changes in TC-standardized sitosterol levels were only significantly related to changes in body weight (p <0.05). Changes in TC-standardized campesterol and lathosterol levels with changes in anthropometric measures or intrahepatic lipid were not significantly related.

Table 1. Anthropometric characteristics and plasma cholesterol and non-cholesterol concentrations of normal weight and abdominally obese men at baseline and after 8 weeks with diet induced weight loss or no weight loss control treatment.

	Normal-weight group (n=25)	Weight-loss group[1] (n=23)		Non-weight loss group[1] (n=26)		
	Baseline[1,2]	Baseline	After 8 weeks	Baseline	After 8 weeks	Treatment effect[3]
Age (y)	53.7 (25.0-61.6)	52.4 (46.8-61.7)		52.0 (45.4-61.1)		
Body weight (kg)	74.9 ± 8.3$^{\#\#\#}$	98.2 ± 8.1	88.2 ± 7.6	95.9 ± 8.9	96.4 ± 9.2	-10.3 (-11.4, -9.2)***
BMI (kg/m^2)	23.3 ± 1.8$^{\#\#\#}$	30.2 ± 1.5	27.1 ± 1.3	29.9 ± 2.5	30.0 ± 2.5	-3.1 (-3.4, -2.8)***
Waist circumference (cm)	84.9 ± 6.3$^{\#\#\#}$	106.8 ± 3.4	95.9 ± 4.2	106.2 ± 3.8	106.3 ± 4.2	-11.0 (-12.1, - 9.9)***
Hip circumference (cm)	96.6 ± 4.2	108.1 ± 4.4	102.3 ± 4.0	107.2 ± 5.9	107.2 ± 6.4	-5.8 (-6.5, -5.0)***
Waist-to-hip ratio	0.88 ± 0.05	0.99 ± 0.03	0.94 ± 0.04	0.99 ± 0.05	0.99 ± 0.05	- 0.05 (-0.06, -0.04)***
Visceral fat (L)[4]	0.89 ± 0.42	2.17 ± 0.64	1.44 ± 0.51	2.53 ± 0.75	2.62 ± 0.85	-0.85 (-1.0, -0.67)***
Subcutaneous fat (L)[4]	1.45 ± 0.51	3.23 ± 0.64	2.44 ± 0.54	2.92 ± 0.81	2.98 ± 0.81	-0.81 (-0.93, -0.69)***
Intrahepatic lipid (%)[4,5]	3.43 (3.14-3.69)	4.21 (3.59-6.53)	3.54 (3.08-4.19)	5.34 (4.33-8.31)	6.31 (4.56-9.45)	-0.18 (-0.25, -0.12)***
LDL-cholesterol (mmol/L)	2.80 ± 0.71$^{\#\#\#}$	3.67 ± 1.03	3.04 ± 0.88	3.70 ± 0.89	3.48 ± 0.77	-0.51 (-0.76, -0.25)***
HDL-cholesterol (mmol/L)	1.26 ± 0.27$^{\#}$	1.14 ± 0.16	1.13 ± 0.21	1.09 ± 0.24	1.11 ± 0.26	-0.02 (-0.11, 0.06)
Triglycerides (mmol/L)	1.01 ± 0.48$^{\#\#\#}$	1.63 ± 0.87	1.19 ± 0.54	1.87 ± 0.77	1.92 ± 0.79	-0.60 (-0.89, -0.30)***
Total cholesterol (mmol/L)[ǂ]	4.02 ± 0.69$^{\#\#\#}$	4.89 ± 0.99	4.15 ± 0.86	5.03 ± 0.78	4.87 ± 0.67	-0.62 (-0.90, -0.35)***
Campesterol (µmol/mmol cholesterol)	2.39 ± 1.02$^{\#\#}$	1.70 ± 0.56	1.54 ± 0.38‡‡	1.74 ± 0.64	1.83 ± 0.61	-0.25 (-0.43, -0.07)**
Sitosterol (µmol/mmol cholesterol)	1.55 ± 0.70$^{\#\#}$	1.08 ± 0.27	1.06 ± 0.19‡	1.12 ± 0.40	1.13 ± 0.35	-0.03 (-0.12, 0.04)
Cholestanol (µmol/mmol cholesterol)	1.53 ± 0.27$^{\#\#\#}$	1.27 ± 0.21	1.45 ± 0.24	1.27 ± 0.27	1.27 ± 0.27	0.18 (0.19, 0.25)***
Lathosterol (µmol/mmol cholesterol)	1.13 ± 0.46$^{\#\#}$	1.47 ± 0.26	1.19 ± 0.24	1.46 ± 0.39	1.59 ± 0.49	-0.39 (-0.55, -0.24)***

[1]Values expressed as means ± SD or medians (25-75 percentiles). [2]Values are significantly different from abdominally obese participants (n=49) (independent t-test): $^{\#}p$ <0.05, $^{\#\#}p$ <0.01, $^{\#\#\#}p$ <0.001. Significantly different from normal weight group (independent t-test): $^{\ddagger}p$ <0.05, $^{\ddagger\ddagger}p$ <0.01. [3]Values are differences in changes (95% CIs) between treatment groups obtained from one factor ANCOVA with baseline values as a covariate: ** p <0.05, *** p <0.001. [4] Data available from normal weight participants (n=24). [5]Log-transformed data. [ǂ]Obtained from GC-FID run.

Table 2. Results of linear regression analyses to investigate the relation between changes in cholesterol absorption and synthesis markers with changes in anthropometric measures, fat distribution and IHL after weight loss intervention (n= 23).

	Cholesterol absorption						Cholesterol synthesis	
	ΔCholestanol		ΔCampesterol		ΔSitosterol		ΔLathosterol	
	B	95% CIs	B	95% CI	B	95% CI	B	95% CI
ΔBW	-0.047	(-0.068,-0.025)***	0.063	(-0.004,0.130)	0.030	(0.001,0.058)*	0.011	(-0.039,0.060)
ΔBMI	-0.149	(-0.223,-0.074)***	0.203	(-0.020,0.427)	0.087	(-0.010,0.184)	0.044	(-0.119,0.207)
ΔWaist	-0.036	(-0.069,-0.002)*	0.020	(-0.069, 0.109)	0.009	(-0.029, 0.048)	0.027	(-0.032,0.086)
ΔHip	-0.043	(-0.085,0.000)*	0.070	(-0.037,0.177)	0.029	(-0.018,0.075)	0.005	(-0.070,0.081)
ΔWaist:Hip	-1.329	(-5.557,2.899)	-2.827	(-13.046,7.391)	-0.999	(-5.437,3.438)	3.867	(-2.900,10.634)
ΔVF	-0.246	(-0.422,-0.069)**	0.083	(-0.418,0.585)	-0.032	(-0.250,0.185)	0.074	(-0.266,0.414)
ΔSF	-0.066	(-0.362,0.229)	-0.194	(-0.906,0.517)	-0.049	(-0.359,0.260)	0.101	(-0.383, 0.585)
ΔIHL†	0.252	(-0.252,0.755)	0.376	(-0.857,1.609)	0.314	(-0.206,0.834)	-0.280	(-1.115,0.555)

ΔBW=changes in body weight; ΔBMI=changes in body mass index; ΔWaist=changes in waist circumference; ΔHip=changes in hip circumference; ΔWaist:Hip=changes in waist to hip ratio; ΔVT=changes in visceral fat; ΔST=changes in subcutaneous fat; ΔIHL=changes in intrahepatic lipid content. Significant relationships: * p <0.05, ** p <0.01, *** p <0.001. †Log transformed.

The effect of BMI on TC-standardized cholestanol levels was fully mediated by visceral fat (percentage of mediated effect: -52.9%; bootstrapped 95% CI: -74.0% to -5.5%) and the direct effect of BMI on TC-standardized cholestanol levels was no longer significant (p >0.05) (**Figure 1**). In addition, effect of BMI on TC-standardized lathosterol levels was partially mediated by intrahepatic lipid content (34.9%; bootstrapped 95% CI: 10.0% to 44.1%) and BMI had still a significant effect on TC-standardized lathosterol levels (p <0.05). Subcutaneous fat neither mediated fully nor partially the associations between BMI and markers of cholesterol absorption and synthesis.

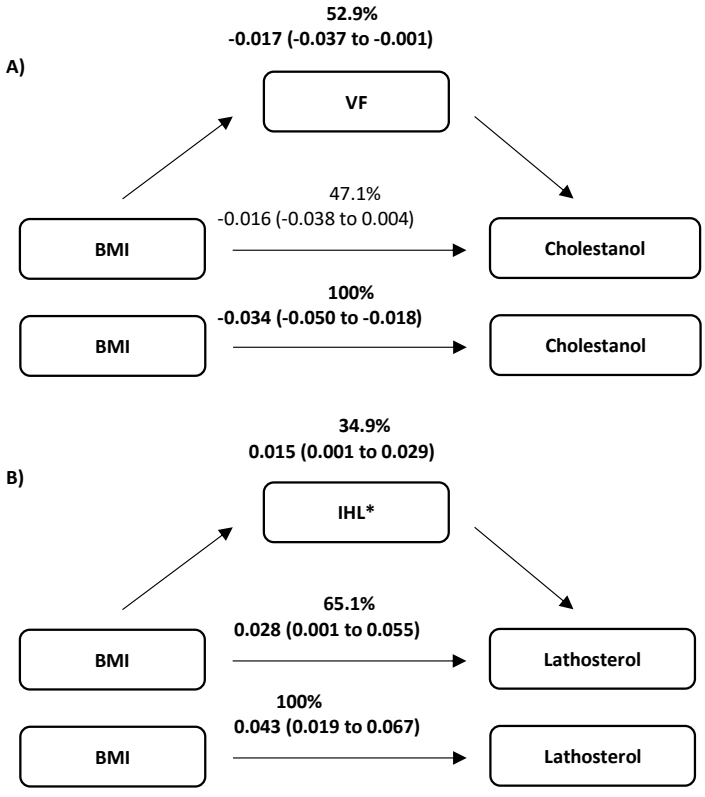


Figure 1. Mediation models of cross-sectional analyses at baseline (n=73) for effects of each mediator on the relationships between BMI (kg/m^2) and markers of cholesterol absorption (A) and synthesis (B), expressed in μmol/mmol cholesterol. Data is presented as B (bootstrapped 95% CI). Bold figures indicated for significant effects. VT=visceral fat; IHL=intrahepatic lipid content. *****Log transformed data.

4. Discussion

Diet-induced weight loss reduced levels of TC-standardized campesterol and lathosterol and increased those of TC-standardized cholestanol. After weight loss, TC-standardized lathosterol and cholestanol levels of the (previously) abdominally obese men were comparable to those of normal-weight men. Interestingly, increases in TC-standardized cholestanol levels after weight loss were associated with decreases in waist circumference, BMI, body weight, hip circumference and visceral fat but not intrahepatic fat and subcutaneous fat volume. Cross-sectionally, visceral fat was a full mediator for the association between BMI with TC-standardized cholestanol levels, while intrahepatic lipid content was a partial mediator for the association between BMI and TC-standardized lathosterol levels.

Our finding of a reduction in endogenous cholesterol synthesis after weight loss (10.3 kg) is in line with earlier studies. A decrease in cholesterol synthesis after weight loss of 6 kg was also observed in a study with apparently healthy obese men, who consumed a hypocaloric diet for 14 weeks followed by a 2 weeks isocaloric diet period [20]. In three studies in obese subjects with the metabolic syndrome, cholesterol synthesis also decreased after dietary weight loss of 13 kg, 6 kg, 10 kg, respectively [9-11]. Simonen et al., conducted two weight loss studies in type 2 diabetic obese patients. Lathosterol levels tended to decrease after a diet-induced weight loss of 15 kg in 3 months [8] and a weight loss of 6 kg resulted in a significant decrease in lathosterol levels after a comparable period immediately followed by a weight stable period up to 2 years [7].

For cholesterol absorption markers, we observed that after weight loss TC-standardized cholestanol levels increased, TC-standardized campesterol levels decreased and TC-standardized sitosterol levels did not change. The question is how these apparent discrepancies for the three different non-cholesterol sterol markers reflecting intestinal cholesterol absorption can be explained. The major diet-derived plant sterols are campesterol and sitosterol [21]. As the diet of the participants in the weight-loss program was different before and after the intervention period, plasma plant sterol levels may also have changed due to different dietary habits and not only due to changes in intestinal cholesterol absorption. Therefore, it can be debated whether TC-standardized plasma campesterol and sitosterol levels truly reflect intestinal cholesterol absorption when major dietary changes are evident. In this particular situation, TC-standardized plasma cholestanol levels may be a better marker for intestinal cholesterol absorption, as cholestanol levels in the diet are very low [22]. We therefore conclude – based on the increase in TC-standardized cholestanol levels – that diet-induced weight loss increased intestinal cholesterol absorption. This conclusion is in line with the study by Simonen

et al. [8] that measured cholestanol concentrations after weight loss in type 2 diabetic subjects.

So far, only a few studies have reported effects of diet-induced weight loss on TC-standardized campesterol levels and findings are inconsistent. In two studies with type 2 diabetic patients, a decrease of about 6 kg induced by 3 months of very-low energy diet or low-energy diet increased TC-standardized campesterol levels [7], while a trend for a decrease in these levels was found after a reduction of 15 kg induced by very-low energy diet virtually free of cholesterol, cholestanol and plant sterols for 3 months [8]. In a third study, weight loss of nearly 10 kg induced by 20 weeks of free-living diet with a 500-kcal deficiency in daily energy intake, followed by 5 weeks of Mediterranean diet under isoenergetic, weight stabilizing period tended to increase total plant sterols levels (campesterol + sitosterol) in obese men with metabolic syndrome compared with a Mediterranean diet in absence of weight reduction [11]. Chan et al. found that campesterol levels were decreased in obese men with insulin resistance after consumption of hypocaloric diet for 16 weeks followed by a 6-week weight maintaining period [9]. Taken together, studies on campesterol levels after diet-induced weight loss are conflicting. As discussed above, changes in TC-standardized campesterol levels may have been confounded by changes in dietary composition and therefore not truly reflect changes in intestinal cholesterol absorption. Information about dietary intake of plant sterols was only reported in two studies; one reported the total plant sterols content in the Mediterranean diet was higher than North American control diet [11] while the other study used a diet formula free of cholesterol, cholestanol and plant sterols [8]. The total plant sterol level tended to increase in the former study, while a trend of decreased campesterol and sitosterol levels was demonstrated in the latter study. These observations suggest that circulating sitosterol and campesterol concentrations reflect dietary intake and – in contrast to cholestanol levels – are not valid markers for intestinal cholesterol absorption during weight loss programs.

To the best of our knowledge, this is the first study in apparently healthy abdominally obese men that examined relationships between changes in cholesterol absorption and synthesis with changes in anthropometric measures, fat distribution as well as the size of different fat compartments after diet-induced weight loss. The relation between changes in TC-standardized cholestanol levels with changes in most anthropometric parameters were consistent, i.e. improvements were seen with increased cholesterol absorption. However, for the different fat compartments, changes in cholestanol were related with changes in visceral fat volume, but not with changes in subcutaneous and intrahepatic lipids. Visceral fat is a metabolically active fat depot, and is stronger associated with CVD risk than subcutaneous fat and intrahepatic lipids [12,23,24]. In addition, the amount of visceral fat is positively associated with cholesterol synthesis in obese subjects

[25,26], which has been explained by an increased flux of fatty acids from the visceral fat depot via the portal vein to the liver, thereby stimulating hepatic cholesterol synthesis. However, this current study did not find an association between cholesterol synthesis and intrahepatic fat. In the present study, we demonstrated a positive association between visceral fat and TC-standardized lathosterol levels but we could not find an association between the changes in visceral fat and cholesterol synthesis. This finding agrees with another controlled dietary intervention study in 26 obese men, where also no association was found between changes in visceral fat and cholesterol synthesis [20].

To examine the associations between BMI and markers of cholesterol absorption and synthesis in more detail, we used mediation analysis to investigate cross-sectionally the impact of several potential mediators (visceral fat, subcutaneous fat or intrahepatic lipid) on the direct association between BMI with cholesterol absorption and synthesis markers. Apparently, visceral fat mediated the link between BMI and cholesterol absorption marker cholestanol, while intrahepatic lipid mediated the link between BMI and cholesterol synthesis marker lathosterol. Due to the altered fatty acid flux from visceral fat to the liver, it can be speculated that there is a link between visceral fat volume with endogenous cholesterol synthesis. However, our findings showed a relation between visceral fat and cholesterol absorption, suggesting that fatty acid fluxes might influence intestinal cholesterol absorption. Although we found significant roles for some fat compartments on the associations between BMI and markers of cholesterol absorption and synthesis in the cross-sectional model, this does not eliminate any other mediators related to determinants or metabolic effects of these fat compartments.

Cholesterol synthesis and absorption clearly show a reciprocal pattern [27-29], which was also evident in the current study as intestinal cholesterol absorption increased and endogenous cholesterol synthesis decreased after weight loss. An important question arises whether changes in cholesterol absorption and synthesis after weight loss may reduce the risk for metabolic diseases. Circulating concentrations of desmosterol, a surrogate marker for cholesterol synthesis involved in the Bloch pathway, were associated with the development of nonalcoholic steatohepatitis (NASH) [30]. These findings were confirmed by Plat et al. describing increased serum desmosterol and lathosterol concentrations in patients with NASH [31]. Moreover, a plant sterol and stanol intervention in rodents showed a reduction in hepatic inflammation, which could be linked to changes in cholesterol synthesis and absorption [31]. In the current study, decreased cholesterol absorption and increased cholesterol synthesis in apparently healthy obese men (without diabetes or the metabolic syndrome) were reversed after diet-induced weight loss intervention. Whether this also suggests a lower risk of developing type 2 diabetes and metabolic syndrome after weight loss cannot be deduced from this data but definitely deserves further attention.

5. Conclusions

In summary, a 6-week diet-induced weight loss period followed by a 2-week weight stable period increases cholesterol absorption and lowers cholesterol synthesis and resulted in a normalization of cholesterol metabolism characteristics in abdominally obese men as compared to normal-weight men. Moreover, we also showed that changes in cholestanol levels were related not only to weight loss, but also to a decrease in visceral fat volume. Furthermore, mediation analysis results suggest that visceral fat and intrahepatic content play a role on the relationships between BMI and cholesterol absorption and synthesis. Whether this reflects a possible relation with the amelioration of metabolic risk factors needs further study.

Supplementary Materials

The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Consort flow diagram of the study participation; Table S1: Cross-sectional regression analyses to investigate the relations between cholesterol absorption and synthesis markers with anthropometric measures, fat distribution and IHL at baseline in all participants (n=73).

Author Contributions

SM**:** performed the GC-FID analysis, performed the statistical analyses, interpreted the data, and wrote the manuscript. SB: interpreted the data. JP and RM: designed the study, interpreted the data. PJ and YHAMK: designed and conducted the study. All authors reviewed and approved the manuscript.

Funding

The work was supported by a research grant (CH001) from the Top Institute Food and Nutrition, a public-private partnership on pre-competitive research in food and nutrition. The public partners were responsible for the study design, data collection and analysis, decision to publish and preparation of the manuscript. The private partners contributed to the project through regular discussions. Sterol analyses were funded by a scholarship from AlBaha University, Saudi Arabia.

Institutional Review Board Statement

The study was conducted according to the guidelines of the declaration of Helsinki, and approved by the Medical Ethical Committee of Maastricht University Medical Center (METC 12-30-40), and registered at ClinicalTrials.gov (NCT01675401).

Informed Consent Statement

Informed consent was obtained from all participants prior to the study.

Data Availability Statement

Data presented in this work are fully available without restriction.

Acknowledgments

We would like to thank M.C.J.M Konings for his technical assistance and our participants for taking part in the study.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Despres, J.P.; Moorjani, S.; Lupien, P.J.; Tremblay, A.; Nadeau, A.; Bouchard, C. Regional distribution of body fat, plasma lipoproteins, and cardiovascular disease. *Arteriosclerosis* **1990**, *10*, 497-511, doi:10.1161/01.atv.10.4.497.
2. Despres, J.P.; Lemieux, I. Abdominal obesity and metabolic syndrome. *Nature* **2006**, *444*, 881-887, doi:10.1038/nature05488.
3. Ritchie, S.A.; Connell, J.M. The link between abdominal obesity, metabolic syndrome and cardiovascular disease. *Nutr Metab Cardiovasc Dis* **2007**, *17*, 319-326, doi:10.1016/j.numecd.2006.07.005.
4. Mashnafi, S.; Plat, J.; Mensink, R.P.; Baumgartner, S. Non-Cholesterol Sterol Concentrations as Biomarkers for Cholesterol Absorption and Synthesis in Different Metabolic Disorders: A Systematic Review. *Nutrients* **2019**, *11*, doi:10.3390/nu11010124.
5. Miettinen, T.A.; Tilvis, R.S.; Kesaniemi, Y.A. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am J Epidemiol* **1990**, *131*, 20-31, doi:10.1093/oxfordjournals.aje.a115479.
6. Miettinen, T.A.; Gylling, H.; Nissinen, M.J. The role of serum non-cholesterol sterols as surrogate markers of absolute cholesterol synthesis and absorption. *Nutr Metab Cardiovasc Dis* **2011**, *21*, 765-769, doi:10.1016/j.numecd.2011.05.005.
7. Simonen, P.; Gylling, H.; Howard, A.N.; Miettinen, T.A. Introducing a new component of the metabolic syndrome: low cholesterol absorption. *Am J Clin Nutr* **2000**, *72*, 82-88, doi:10.1093/ajcn/72.1.82.
8. Simonen, P.; Gylling, H.; Miettinen, T.A. Acute effects of weight reduction on cholesterol metabolism in obese type 2 diabetes. *Clin Chim Acta* **2002**, *316*, 55-61, doi:10.1016/s0009-8981(01)00734-3.
9. Chan, D.C.; Watts, G.F.; Gan, S.K.; Ooi, E.M.; Barrett, P.H. Effect of ezetimibe on hepatic fat, inflammatory markers, and apolipoprotein B-100 kinetics in insulin-resistant obese subjects on a weight loss diet. *Diabetes Care* **2010**, *33*, 1134-1139, doi:10.2337/dc09-1765.
10. Chan, D.C.; Watts, G.F.; Ng, T.W.; Yamashita, S.; Barrett, P.H. Effect of weight loss on markers of triglyceride-rich lipoprotein metabolism in the metabolic syndrome. *Eur J Clin Invest* **2008**, *38*, 743-751, doi:10.1111/j.1365-2362.2008.02019.
11. Richard, C.; Couture, P.; Desroches, S.; Benjannet, S.; Seidah, N.G.; Lichtenstein, A.H.; Lamarche, B. Effect of the Mediterranean diet with and without weight loss on surrogate markers of cholesterol homeostasis in men with the metabolic syndrome. *Br J Nutr* **2012**, *107*, 705-711, doi:10.1017/S0007114511003436.
12. Fox, C.S.; Massaro, J.M.; Hoffmann, U.; Pou, K.M.; Maurovich-Horvat, P.; Liu, C.Y.; Vasan, R.S.; Murabito, J.M.; Meigs, J.B.; Cupples, L.A., et al. Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. *Circulation* **2007**, *116*, 39-48, doi:10.1161/CIRCULATIONAHA.106.675355.

13. Kershaw, E.E.; Flier, J.S. Adipose tissue as an endocrine organ. *J Clin Endocr Metab* **2004**, *89*, 2548-2556, doi:10.1210/jc.2004-0395.
14. Tchernof, A.; Despres, J.P. Pathophysiology of human visceral obesity: an update. *Physiol Rev* **2013**, *93*, 359-404, doi:10.1152/physrev.00033.2011.
15. Joris, P.J.; Plat, J.; Kusters, Y.H.; Houben, A.J.; Stehouwer, C.D.; Schalkwijk, C.G.; Mensink, R.P. Diet-induced weight loss improves not only cardiometabolic risk markers but also markers of vascular function: a randomized controlled trial in abdominally obese men. *Am J Clin Nutr* **2017**, *105*, 23-31, doi:10.3945/ajcn.116.143552.
16. Grundy, S.M.; Becker, D.; Clark, L.T.; Cooper, R.S.; Denke, M.A.; Howard, W.J.; Hunninghake, D.B.; Illingworth, R.; Luepker, R.V.; McBride, P., et al. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) Final Report. *Circulation* **2002**, *106*, 3143-3421, doi:DOI 10.1161/circ.106.25.3143.
17. Kusters, Y.H.; Schalkwijk, C.G.; Houben, A.J.; Kooi, M.E.; Lindeboom, L.; Op 't Roodt, J.; Joris, P.J.; Plat, J.; Mensink, R.P.; Barrett, E.J., et al. Independent tissue contributors to obesity-associated insulin resistance. *JCI Insight* **2017**, *2*, doi:10.1172/jci.insight.89695.
18. Friedewald, W.T.; Levy, R.I.; Fredrickson, D.S. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* **1972**, *18*, 499-502.
19. Mackay, D.S.; Jones, P.J.; Myrie, S.B.; Plat, J.; Lutjohann, D. Methodological considerations for the harmonization of non-cholesterol sterol bio-analysis. *J Chromatogr B Analyt Technol Biomed Life Sci* **2014**, *957*, 116-122, doi:10.1016/j.jchromb.2014.02.052.
20. Riches, F.M.; Watts, G.F.; Hua, J.; Stewart, G.R.; Naoumova, R.P.; Barrett, P.H. Reduction in visceral adipose tissue is associated with improvement in apolipoprotein B-100 metabolism in obese men. *J Clin Endocrinol Metab* **1999**, *84*, 2854-2861, doi:10.1210/jcem.84.8.5925.
21. Ostlund, R.E., Jr. Phytosterols in human nutrition. *Annu Rev Nutr* **2002**, *22*, 533-549, doi:10.1146/annurev.nutr.22.020702.075220.
22. Miettinen, T.A.; Tilvis, R.S.; Kesaniemi, Y.A. Serum cholestanol and plant sterol levels in relation to cholesterol metabolism in middle-aged men. *Metabolism* **1989**, *38*, 136-140, doi:10.1016/0026-0495(89)90252-7.
23. Despres, J.P. Body fat distribution and risk of cardiovascular disease: an update. *Circulation* **2012**, *126*, 1301-1313, doi:10.1161/CIRCULATIONAHA.111.067264.
24. Liu, J.; Fox, C.S.; Hickson, D.; Bidulescu, A.; Carr, J.J.; Taylor, H.A. Fatty liver, abdominal visceral fat, and cardiometabolic risk factors: the Jackson Heart Study. *Arterioscler Thromb Vasc Biol* **2011**, *31*, 2715-2722, doi:10.1161/ATVBAHA.111.234062.
25. Adeli, K.; Taghibiglou, C.; Van Iderstine, S.C.; Lewis, G.F. Mechanisms of hepatic very low-density lipoprotein overproduction in insulin resistance. *Trends Cardiovasc Med* **2001**, *11*, 170-176, doi:10.1016/s1050-1738(01)00084-6.

26. Tobin, K.A.; Ulven, S.M.; Schuster, G.U.; Steineger, H.H.; Andresen, S.M.; Gustafsson, J.A.; Nebb, H.I. Liver X receptors as insulin-mediating factors in fatty acid and cholesterol biosynthesis. *J Biol Chem* **2002**, *277*, 10691-10697, doi:10.1074/jbc.M109771200.
27. Alphonse, P.A.; Jones, P.J. Revisiting Human Cholesterol Synthesis and Absorption: The Reciprocity Paradigm and its Key Regulators. *Lipids* **2016**, *51*, 519-536, doi:10.1007/s11745-015-4096-7.
28. Quintao, E.; Grundy, S.M.; Ahrens, E.H., Jr. Effects of dietary cholesterol on the regulation of total body cholesterol in man. *J Lipid Res* **1971**, *12*, 233-247.
29. Santosa, S.; Varady, K.A.; AbuMweis, S.; Jones, P.J. Physiological and therapeutic factors affecting cholesterol metabolism: does a reciprocal relationship between cholesterol absorption and synthesis really exist? *Life Sci* **2007**, *80*, 505-514, doi:10.1016/j.lfs.2006.10.006.
30. Simonen, M.; Mannisto, V.; Leppanen, J.; Kaminska, D.; Karja, V.; Venesmaa, S.; Kakela, P.; Kuusisto, J.; Gylling, H.; Laakso, M., et al. Desmosterol in human nonalcoholic steatohepatitis. *Hepatology* **2013**, *58*, 976-982, doi:10.1002/hep.26342.
31. Plat, J.; Hendrikx, T.; Bieghs, V.; Jeurissen, M.L.; Walenbergh, S.M.; van Gorp, P.J.; De Smet, E.; Konings, M.; Vreugdenhil, A.C.; Guichot, Y.D., et al. Protective role of plant sterol and stanol esters in liver inflammation: insights from mice and humans. *PLoS One* **2014**, *9*, e110758, doi:10.1371/journal.pone.0110758.

Supplementary Materials

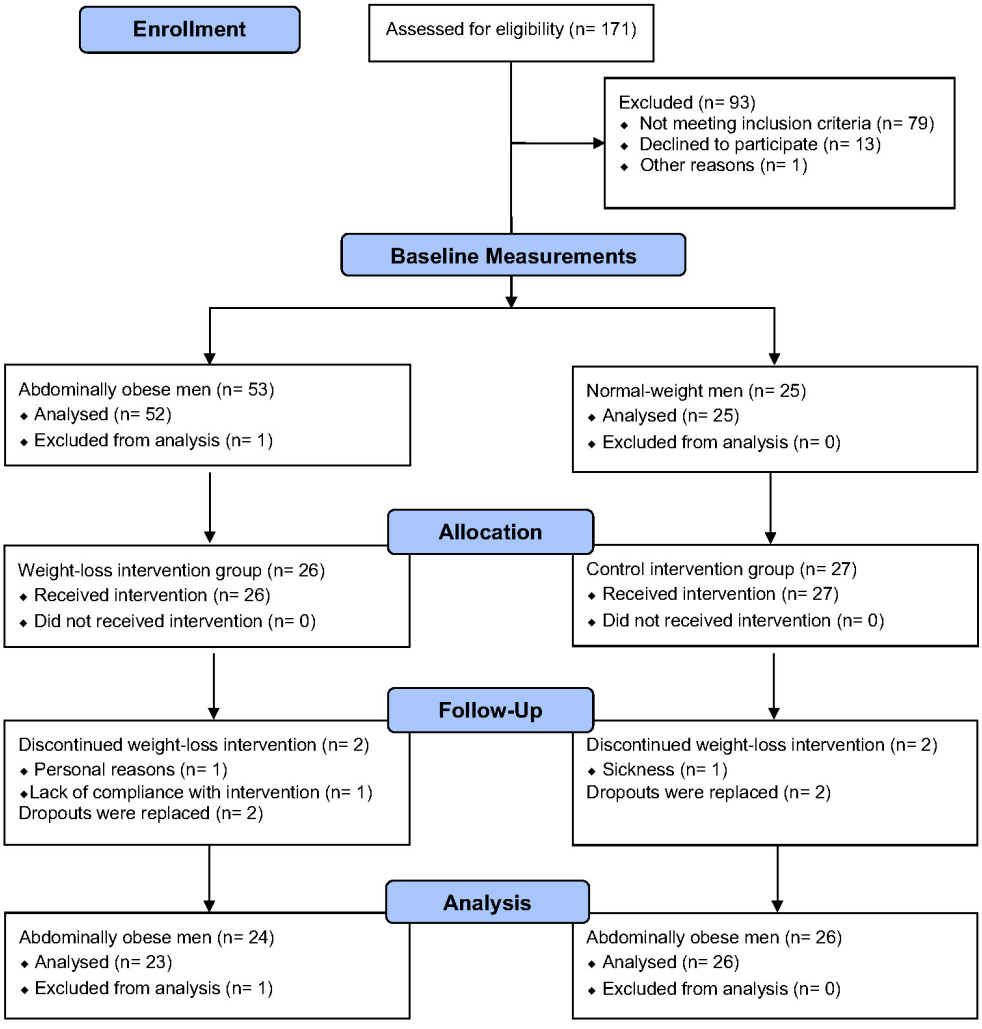


Figure S1

Table S1

	Cholesterol absorption									Cholesterol synthesis		
	Cholestanol			Campesterol			Sitosterol			Lathosterol		
	B	SE(B)	P	B	SE(B)	P	B	SE(B)	P	B	SE(B)	P
Body weight	-0.007	0.002	**0.001**	-0.018	0.007	**0.012**	-0.014	0.004	**0.002**	0.012	0.003	**0.001**
BMI	-0.032	0.008	**<0.001**	-0.087	0.024	**<0.001**	-0.064	0.015	**<0.001**	0.046	0.012	**<0.001**
Waist	-0.011	0.003	**<0.001**	-0.026	0.008	**0.002**	-0.018	0.005	**0.001**	0.015	0.004	**<0.001**
Hip	-0.009	0.004	**0.047**	-0.025	0.013	0.064	-0.022	0.008	**0.009**	0.019	0.006	**0.004**
Waist:Hip	-2.000	0.412	**<0.001**	-4.492	1.311	**0.001**	-2.635	0.862	**0.003**	2.120	0.655	**0.002**
VF	-0.121	0.32	**<0.001**	-0.241	0.101	**0.019**	-0.181	0.064	**0.006**	0.116	0.050	**0.023**
SF	-0.069	0.031	**0.031**	-0.242	0.092	**0.011**	-0.184	0.059	**0.003**	0.112	0.046	**0.017**
IHL*	-0.452	0.152	**0.004**	-1.199	0.458	**0.011**	-0.873	0.293	**0.004**	0.788	0.215	**<0.001**

CHAPTER

5

EFFECTS OF A TRANSIENT SYSTEMIC INFLAMMATORY RESPONSE VIA LIPOPOLYSACCHARIDE (LPS) INFUSION ON MARKERS OF CHOLESTEROL METABOLISM IN HEALTHY NORMOCHOLESTEROLEMIC YOUNG MEN

Mashnafi S, Plat J,Mensink RP, Perlee D,van Vught LA,
Lutjohann D,Baumgartner S

Submitted

Background

Inflammation is associated with changes in lipid and lipoprotein concentrations and cholesterol efflux capacity (CEC). Whether changes in lipids and lipoproteins are related to changes in cholesterol absorption, synthesis and bile acid synthesis is unknown.

Objective

To examine the effects of acute LPS-induced transient systemic inflammation on lipid and lipoprotein concentrations, CEC and markers of cholesterol metabolism. In addition, we evaluated whether markers for cholesterol metabolism at baseline predict the intensity of the LPS-induced transient inflammatory response.

Methods

Eight healthy young subjects received LPS and blood was sampled over the course of 24 hours following LPS infusion. Besides lipids, lipoproteins and CEC, markers for cholesterol absorption and synthesis, bile acid synthesis as well as inflammatory responses were measured.

Results

Compared to baseline, serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and CEC decreased, while triglycerides increased 24 hours following LPS infusion. TC-standardized levels of cholesterol synthesis markers (lathosterol, lanosterol and desmosterol) and a bile acid synthesis marker (7α-OH-cholesterol) also decreased, with no change in cholesterol absorption markers (campesterol, sitosterol and cholestanol). Baseline TC-standardized levels of desmosterol and 7α-OH-cholesterol were positively correlated with concentrations of various inflammatory markers. Changes in TC-standardized desmosterol and 7α-OH-cholesterol were negatively correlated with concentrations of inflammatory markers.

Conclusion

LPS infusion reduced endogenous cholesterol synthesis and bile acid synthesis in healthy young men. The decrease in cholesterol synthesis may explain in particular the observed reduction in serum TC and LDL-C concentrations. The relation between desmosterol and 7α-OH-cholesterol concentrations at baseline with the intensity of an inflammatory response after LPS exposure warrants further study.

Abstract

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide [1], of which atherosclerosis is one of the underlying pathogenic processes [2]. Lipid abnormalities and inflammation are the main drivers of atherosclerosis [3], and evidence highlighting the importance of inflammation in initiation and progression of atherosclerosis is expanding rapidly [4]. For example, lowering inflammation by targeting interleukin-1beta (IL-1β) reduced the occurrence of CVD events, even when lipid profiles were not affected [5]. In addition to direct effects of inflammation on the vasculature [6], there are also clear indications that inflammation may affect serum total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) and triglyceride (TG) concentrations [7,8]. Moreover, not only circulating levels but also alterations in HDL composition, size and functionality have been observed during inflammation [9-11], but studies are not conclusive [12]. Although these associations between inflammation and circulating lipoproteins are nowadays acknowledged, it is unclear whether changes in serum lipid and lipoprotein concentrations, and decreases in HDL functionality during inflammation are related to changes in intestinal cholesterol absorption, endogenous cholesterol synthesis and/or bile acid synthesis, which are main processes regulating cholesterol homeostasis. However, the etiology of inflammatory diseases is very different, which makes it difficult to compare studies. Therefore, infusion of lipopolysaccharide (LPS), a toxin derived from Gram negative bacteria, has been proposed as a controlled model of a transient systemic inflammatory response [13]. Indeed, a clear inflammatory response and endothelial cell activation upon LPS infusion have frequently been observed [14,15]. Others have shown changes in serum lipid and lipoproteins concentrations such as reductions in TC, and increases in TG after LPS administration in humans [7,16]. In addition, inflammation was associated with alterations in HDL composition and size and with a decreased functionality following 24 hours LPS infusion [17], while effects of LPS-induced inflammation on key processes regulating cholesterol homeostasis are unknown.

Analyzing intestinal cholesterol absorption, endogenous cholesterol synthesis and bile acid synthesis usually requires laborious stable isotope tracer methodology [18-20]. However, serum non-cholesterol sterols are frequently used as markers for evaluating changes in cholesterol metabolism [21]. The cholesterol precursors desmosterol and lathosterol reflect endogenous cholesterol synthesis, while the non-cholesterol sterols sitosterol, campesterol, and cholestanol reflect fractional intestinal cholesterol absorption [22]. Finally, 7α-OH-cholesterol and 27-OH-cholesterol can be used as markers for bile acid formation [23]. Interestingly, some of these non-cholesterol sterols might also affect inflammatory responses, i.e., for the cholesterol precursor desmosterol as well as for several oxysterols, anti-inflammatory effects have been described via activating

liver X receptors (LXR) [24]. Therefore, we decided to evaluate the effects of acute LPS-induced transient systemic inflammation on serum lipid and lipoprotein concentrations, HDL functionality, and markers reflecting cholesterol metabolism (absorption, synthesis, and bile acid formation). In addition, it was examined whether baseline characteristics of these markers were able to predict the LPS-induced transient inflammatory response.

Materials and methods

Subjects and study design

The study had a randomized, placebo-controlled, single-blind, parallel design and was carried out at the Center of Experimental & Molecular Medicine, Academic Medical Center, University of Amsterdam, The Netherlands. Details of this study have been described elsewhere [14]. In brief, to investigate the use of mesenchymal stem cells (MSCs) in sepsis, the response to intravenous LPS infusion was investigated in 32 healthy men that were divided over four treatment arms: infusion of placebo (Ringer's lactate solution) or allogeneic adipose MSCs intravenously at three doses. Next, all 32 participants received over a one-minute period one single dose of LPS intravenously (2 ng/kg from *Escherichia coli*, U.S. standard reference endotoxin; kindly provided by Anthony Suffredini, National Institute of Health, Bethesda, MD) one hour after placebo or MSC infusion. For the present paper, only the samples of the subjects from the placebo arm (n=8) before and 24h after LPS infusion have been included. All participants were apparently healthy young men and had a normal medical history, physical examination, haematological and biochemical screening values, and electrocardiograms [14]. The study was approved by the Dutch Central Committee on Research involving Human Subjects (CCMO) and the Medical Ethical Committee of the Academic Medical Center (AMC), Amsterdam (the Netherlands), and registered at ClinicalTrials.gov as NCT02328612. Written informed consent was obtained from all participants before start of the study.

Blood sampling

Blood samples were collected in EDTA tubes at baseline (T0), as well as 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 24 hours after LPS infusion. Plasma samples were obtained by centrifugation at 1,750 g for 10 min at 4 °C and stored in small aliquots at – 80 °C until use.

Biochemical analysis

In the samples collected at baseline and after 24 hours, plasma TC (CHOD-PAP method; Roche Diagnostic, Mannheim, Germany), HDL-C (CHOD-PAP method; Roche Diagnostic, Mannheim, Germany), and TG concentrations were analyzed enzymatically (GPO-Tinder; Sigma-Aldrich Corp., St. Louis, MO, USA). In these samples, plasma LDL-C concentrations were calculated using the Friedewald equation [25]. HDL functionality, defined as

the capacity of radioactive cholesterol efflux from cultured J774 macrophages using liquid scintillation counting was determined as described elsewhere [26]. Markers for inflammation were analyzed in plasma samples collected at all time points as described [14].

Non-cholesterol sterol and oxysterol concentrations

In the samples collected at baseline and after 24 hours, plasma non-cholesterol sterol and oxysterol concentrations were analyzed by gas-liquid chromatography-mass spectroscopy (GC-MS) as described before [21,27]. Concentrations of non-cholesterol sterols and oxysterols were standardized for TC concentrations and expressed as μmol/mmol and nmol/mmol cholesterol, respectively. The measured non-cholesterol sterols were campesterol, sitosterol, cholestanol, lathosterol, lanosterol and desmosterol. TC-standardized sitosterol, campesterol and cholestanol values are considered as markers for fractional intestinal cholesterol absorption and TC-standardized lathosterol and desmosterol values as markers for endogenous cholesterol synthesis. The measured oxysterols were 24-OH-, 27-OH-, and 7α-OH-cholesterol. TC-standardized 7α-OH-cholesterol and 27-OH-cholesterol values are considered as markers for bile acid formation [23].

Statistical analyses

Data are presented as means ± SD unless otherwise indicated. Normality of the data was assessed using the Kolmogorov-Smirnov test. In case of not normally distributed data, the median and ranges are presented. A paired two-tailed Student's T-Test was used to examine differences between lipid and lipoprotein concentrations, HDL functionality, non-cholesterol sterols, oxysterol and cytokine concentrations at baseline and 24 hours after LPS infusion. To evaluate the overall inflammatory responses, the incremental area under the curves (iAUC) for 24 hours after LPS infusion were analyzed using GraphPad Prism version 9.00 for Windows (GraphPad Software, San Diego, CA). Maximal changes (iMAX) for a parameter were calculated by subtracting baseline (T0) concentrations from its maximal concentration. The associations between baseline concentrations as well as changes in non-cholesterol sterol and oxysterol concentrations with iMAX or iAUC of the cytokines were statistically evaluated by calculating Pearson correlation coefficients. A p-value <0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS 27.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Lipids and lipoproteins

Eight healthy Caucasian men with median age of 23 years (range: 19 – 25) and a body mass index of 24 kg/m^2 (22 – 26) were included in the placebo arm. Plasma TC and LDL-C concentrations were significantly decreased 24 hours after LPS infusion ((-0.21 mmol/L; 95% CI: -0.36, -0.05; *p <0.05*), and (-0.49 mmol/L; 95% CI: -0.72, -0.26; *p <0.01*), respectively) compared with baseline (**Figure 1**). However, plasma TG concentrations were significantly increased by 0.34 mmol/L (95% CI: 0.07, 0.61; *p <0.05*) 24 hours following LPS exposure compared with baseline. There was no change in plasma HDL-C concentrations 24 hours after LPS infusion. However, despite unchanged HDL-C concentrations, cholesterol efflux capacity as a measure of HDL functionality, was decreased 24 hours following LPS compared with baseline (-8.2%; 95% CI: -15.25, -1.26; *p <0.05*) (**Figure 2**).

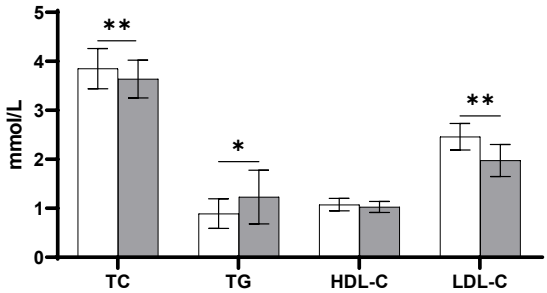


Figure 1. Fasting plasma lipid and lipoprotein concentrations at baseline (white bars) and 24 hours following LPS infusion (grey bars) (n=8). Data are presented as mean ± SEM. Significantly different from baseline: *p <0.05; **p <0.01. TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol.

Non-cholesterol sterols and oxysterols

Plasma cholesterol-standardized concentrations of non-cholesterol sterols at baseline and 24 hours after LPS infusion are shown in **Figure 3**. Values for the intestinal cholesterol absorption markers, TC-standardized levels of campesterol, sitosterol, and cholestanol were comparable at baseline and 24 hours after LPS exposure (**Figure 3A**). However, TC-standardized levels of the endogenous cholesterol synthesis markers lathosterol, lanosterol and desmosterol were all significantly lower at 24 hours after LPS infusion compared with baseline (**Figure 3B**). For lathosterol, levels were -0.21 μmol/mmol (95% CI: -0.36, -0.07; *p <0.01*) lower 24 hours after LPS infusion compared with baseline, for

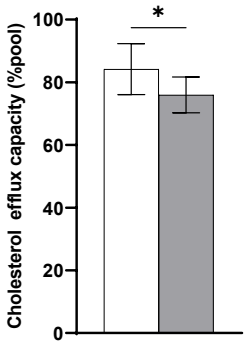


Figure 2. Percentage of cholesterol capacity efflux at baseline (white bar) and 24 hours following LPS infusion (grey bar) (n=8). Data are presented as mean ± SEM. Significantly different from baseline (p <0.05). Values were expressed relative to those of a serum pool of healthy volunteers, which was set at 100%.

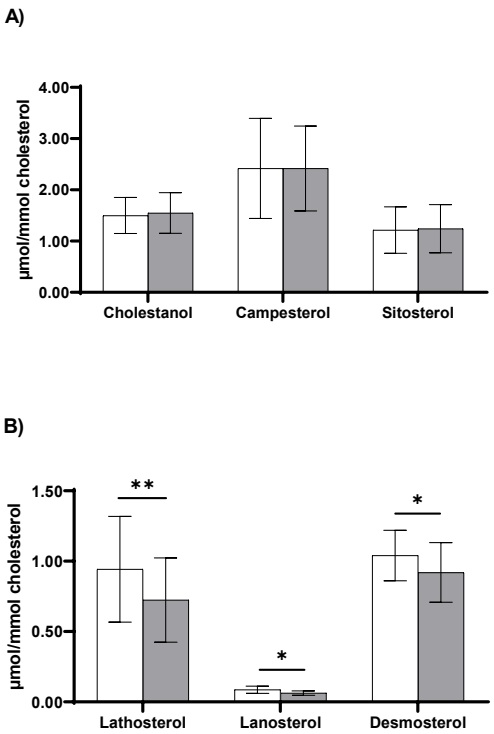


Figure 3. Cholesterol-standardized levels of cholesterol absorption markers (panel A) and cholesterol synthesis markers (panel B) at baseline (white bars) and 24 hours after LPS infusion (grey bars) (n=8). Data are presented as mean ± SEM. Significantly different compared to baseline: *p <0.05; **p <0.01.

lanosterol -0.23 μmol/mmol (95% CI: -0.05, 0.00; *p <0.05*) and for desmosterol -0.12 μmol/mmol (95% CI: -0.20, -0.03; *p <0.05*).

As shown in **Figure 4,** TC-standardized levels of oxysterols were comparable at baseline and 24 hours after LPS infusion. For 7α-OH-cholesterol, there was a borderline significant decrease of -8.07 nmol/mmol (95% CI: -16.14, 0.01; *p* =0.050) 24 hours after LPS infusion compared with baseline values. Absolute concentrations of non-cholesterol sterols and oxysterols are shown in **Supplemental table 1.** Results were comparable to those observed for cholesterol-standardized levels.

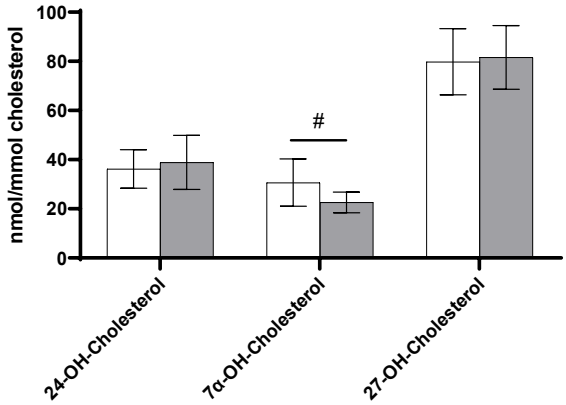


Figure 4. Values at baseline (white bars) and 24 hours after LPS infusion (grey bars) for cholesterol-standardized oxysterols (n=8). Data are presented as mean ± SEM. $^{\#}p$ =0.050, trend compared to baseline.

Inflammatory responses

Plasma concentrations of a panel inflammatory markers at baseline and 24 hours after LPS exposure, as well as the changes are shown in **Table 1**. Concentrations of the acute phase proteins albumin, C-reactive protein (CRP) and serum amyloid A (SAA) increased 24 hours following LPS infusion compared with baseline (all p <0.05). For the proinflammatory cytokines, only tumor necrosis factor (TNFα) concentrations increased 24 hours after LPS infusion, whereas IL-8 and IL12p40 concentrations remained unchanged. In addition, also concentrations of the anti-inflammatory cytokine IL-10 remained unchanged 24 hours after LPS infusion. Myeloperoxidase (MPO) concentrations, an enzyme released upon neutrophil activation, were also increased in response to LPS (p <0.001).

Correlations

We also questioned whether parameters related to cholesterol metabolism at baseline were predictive for the intensity of the LPS induced systemic inflammatory response. As shown in **Table 2**, positive correlations were found between baseline TC-standardized desmosterol levels and CRP concentrations at 24 hours (r=0.849; p <0.001) as well as with changes in CRP concentrations (r=0.829, p <0.05), and iMAX TNFα concentrations (r=0.917, p <0.05). Moreover, also positive correlations were found between baseline TC-standardized 7α-OH-cholesterol levels and iMAX IL-6 (r=0.763, p <0.05), iMAX IL-8 (r=0.766, p <0.05), iMAX TNFα (r=0.814, p <0.05) concentrations, and iAUC IL-6 (r=0.869, p <0.01). Furthermore, positive correlations were found between baseline TC-standardized 27-OH-cholesterol levels with 24 hours IL-8 concentrations (r=0.771, p <0.05) and iAUC TNFα (r=0.765, p <0.05). Finally, positive correlations were found between baseline TC-standardized cholestanol levels with iMAX MPO (r=0.758, p <0.05).

For changes, we found that changes in TC-standardized desmosterol and TC-standardized 7α-OH-cholesterol were both negatively correlated with iMAX IL-8 (r=-0.761; p <0.05, and r=-0.856, p <0.01, respectively). Moreover, there were also negative correlations between changes in TC-standardized 7α-OH-cholesterol and iMAX IL-6 (r=-0.751; p <0.05), iMAX TNFα (r=-0.821, p <0.05) and iAUC IL-6 (r=-904, p <0.01).

Table 1. Plasma concentrations for inflammatory markers before and after 24 hours following LPS infusion in all participants (n=8).

		Mean ± SD	***p*-value**
Albumin (g/L)	baseline	38.43 ± 1.60	**0.033**
	24hrs	40.59 ± 2.32	
	change	2.16 ± 2.31	
CRP (mg/L)	baseline	3.01 ± 4.42	**<0.001**
	24hrs	24.50 ± 6.83	
	change	21.49 ± 2.21	
TNFα (pg/mL)	baseline	3.02 ± 1.30	**0.002**
	24hrs	3.76 ± 1.37	
	change	0.74 ± 0.45	
IL-8 (pg/mL)	baseline	0.32 ± 0.22	0.110
	24hrs	0.60 ± 0.46	
	change	0.29 ± 0.45	
IL-10 (pg/mL)	baseline	0.19 ± 0.09	0.457
	24hrs	0.23 ± 0.13	
	change	0.04 ± 0.15	
IL12p40 (pg/mL)	baseline	1.86 ± 1.13	0.355
	24hrs	2.25 ± 2.12	
	change	0.39 ± 1.12	
SAA (mg/L)	baseline	5.40 ± 1.89	**0.017**
	24hrs	6.64 ± 1.95	
	change	1.24 ± 1.13	
MPO (ng/mL)	baseline	2.96± 1.05	**<0.001**
	24hrs	5.70 ± 1.56	
	change	2.73 ± 1.33	

CRP: C-reactive protein; TNFα: tumor necrosis factor; IL-8: interleukin 8; IL-10: interleukin 10; IL12p40: interleukin 12p40; SAA: serum amyloid A; MPO: myeloperoxidase.

Table 2. Correlations between plasma non-cholesterol sterols, oxysterols and inflammation responses‡.

Variable	Variable	Correlation	*p*-value
Baseline desmosterol	24hrs CRP	0.849	0.008
Baseline desmosterol	ΔCRP	0.829	0.011
Baseline 27-OH-cholesterol	24hrs IL8	0.771	0.025
Baseline cholestanol	iMAX MPO	0.758	0.029
Baseline desmosterol	iMAX TNFα	0.917	0.010
Baseline 7α-OH-cholesterol	iMAX IL-8	0.766	0.027
Baseline 7α-OH-cholesterol	iMAX IL-6	0.763	0.028
Baseline 7α-OH-cholesterol	iMAX TNFα	0.814	0.049
Δdesmosterol	iMAX IL-8	-0.761	0.028
Δ7α-OH-cholesterol	iMAX IL-8	-0.856	0.007
Δ7α-OH-cholesterol	iMAX IL-6	-0.751	0.032
Δ7α-OH-cholesterol	iMAX TNFα	-0.821	0.045
Baseline 27-OH-cholesterol	iAUC-TNFα	0.765	0.027
Baseline 7α-OH-cholesterol	iAUC-IL6	0.869	0.005
Δ7α-OH-cholesterol	iAUC-IL6	-0.904	0.002

‡ Only significant Pearson coefficients are reported.

Discussion

This study demonstrates that in healthy young male subjects, a transient LPS induced inflammatory response lowers serum TC and LDL-C concentrations as well as HDL functionality measured as cholesterol efflux capacity, while plasma TG concentrations increased. Moreover, endogenous cholesterol synthesis as well as bile acid production were reduced, while intestinal cholesterol absorption did not change. Finally, we found positive correlations between baseline TC-standardized desmosterol and 7α-OH-cholesterol levels with various markers for the inflammatory response and negative correlations between changes in TC-standardized desmosterol and 7α-OH-cholesterol and markers for the inflammatory response. This suggests that an acute LPS-induced transient inflammatory response affects cholesterol metabolism.

Several in vitro, animal and human studies already reported possible effects of inflammation on serum lipid and lipoprotein profiles, as well as on composition, structure and functionality of HDL particles. Our results on TC and LDL-C concentrations, and on TG and HDL-C concentrations are largely in line with earlier studies. Already in the nineties, two studies in rodents observed induction of hypertriglyceridemia upon LPS, TNF or IL-1β exposure [28, 29]. In humans, Hudgins et al. demonstrated reductions in serum TC and LDL-C with no effect on HDL-C concentrations in six normal volunteers who were provided with a small dose of endotoxin versus saline [16]. Also, another study in healthy volunteers including 10 males and 10 females reported no change in serum HDL-C concentrations after LPS infusion [30]. In a more recent study, Zimmetti et al. compared 59 subjects with infections, carcinomas or autoimmune diseases to 39 controls without infections. Although this study also reported lower serum TC and LDL-C concentrations in patients with inflammation as compared to controls, serum TG and HDL-C concentrations were lower [12]. It should however be realized that this was a very heterogenous patient population which could explain the observed differences as compared to our and other studies. During inflammation, the effects on lipoprotein metabolism are not limited to changes in circulating concentrations, but also in HDL particles in size, structure and functionality, at least in rodents [8]. In general, inflammation in humans seems associated with increases in the HDL component SAA [10, 11, 30], which is consistent with our observation. Unfortunately, we did not analyze HDL size, but did evaluate changes in CEC which is one of the postulated protective functions of HDL and negatively related with CVD development [31]. The decrease in CEC after LPS exposure was in line with other studies in humans upon LPS exposure [17, 30, 32] and in patients with inflammatory diseases [33-36].

The question is how these changes in serum lipid and lipoprotein concentrations can be explained. To the best of our knowledge, this is the first study that examined the effects

of a transient LPS-induced inflammation on plasma markers for intestinal cholesterol absorption, endogenous cholesterol synthesis and bile acid formation. In one cross-sectional study, no differences in plasma non-cholesterol sterols between subjects with infections, oncologic causes carcinomas or autoimmune diseases and controls were reported [12]. However, as already mentioned, the patient population in that study was highly variable which might have influenced the results. In contrast, we showed that cholesterol synthesis was significantly reduced following LPS infusion, while cholesterol absorption remained unchanged. Our data for cholesterol synthesis is in line with studies in human cell lines, but not in animals. For example, adding IL-1 to HepG2 cells inhibited cholesterol synthesis [37]. However, administrating the inflammatory cytokines TNFα, TNFβ and interferon gamma to mice stimulated hepatic cholesterol synthesis [38-40]. This latter finding was in line with a recent study by Liebergall et al., who reported that proinflammatory stimuli upregulated in macrophages from mice all enzymes involved in cholesterol synthesis, except 24-dehydrocholesterol reductase (DHCR24) [41]. We cannot explain the discrepancy in findings in animals as compared to the in vitro human cell data. Of course, results from animal studies cannot always be extrapolated to humans. Alternatively, it could relate that the way of inducing inflammation was different in all three settings.

With respect to the bile acid formation, the LPS induced inflammatory response resulted in decreased bile acid formation, as suggested by a reduction in plasma 7α-OH-cholesterol, which is a precursor in the classic pathway of bile acid synthesis. The 7α-hydroxylase, a rate limiting enzyme in the classical pathway of bile acid synthesis, converts cholesterol to 7α-OH-cholesterol and in series of steps by different enzymes ultimately to bile acids [42]. Two earlier studies in rodents already examined the effects of inflammation on mRNA and protein levels of 7α-hydroxylase after LPS infusion. One study infused Syrian hamsters with LPS, TNFα or IL-1, while the other study infused rats and mice with LPS. The study in hamsters reported a reduction in the mRNA levels of 7α-hydroxylase [43], whereas the study in rats and mice reported a decrease in the protein levels of 7α-hydroxylase [44]. Both findings are in line with the reduction in 7α-OH-cholesterol that we observed in humans.

Besides the effects of inflammation on circulating non-cholesterol sterols and oxysterols, it is interesting to note that these sterols also influence inflammation. For example, desmosterol and oxysterols such as 24S, 25 and 27-OH-cholesterol have anti-inflammatory properties via activating LXR [45-48]. Interestingly, these receptors are also known to mediate CEC in vivo and in vitro and the possible mechanism involves the activity of ABCA1 and ABCG1[49, 50]. In fact, desmosterol has shown to be the dominant LXR ligand in human atherosclerotic plaques and macrophage foam cells of murine [24], suggesting a reduction in desmosterol is linked with lower activation for LXR. This might explain the

reduction in CEC we observed here after LPS exposure. In a functional way, the reduction in HDL functionality may result in cellular cholesterol accumulation, which may enhance the inflammatory response to remove the infectious agents from host [51].

Finally, we found unexpected positive associations between baseline TC-standardized desmosterol and 7α-OH-cholesterol levels with the intensity of the inflammatory response. This suggests that higher desmosterol concentrations translate into higher inflammatory responses, which is in contrast with results from a recent study [52]. In that study, depletion of desmosterol by overexpressing DHCR24 in macrophage foam cells was associated with the activation of inflammatory responses. Moreover, Spann et al. found that activation of macrophage foam cells in the peritoneal cavities of mice was associated with suppression of hemostatic and anti-inflammatory properties of desmosterol [24]. We can only speculate that these associations are different between animals versus humans, which requires further study.

The present study has some limitations. First, the sample size for this study is small and information about dietary intake is lacking. Second, due to limited sample availability, data for non-cholesterol sterols could only be retrieved at baseline and 24 hours after LPS infusion and not in the samples at the timepoints in between as reported for inflammatory responses. The strength is that a transient LPS model was used in this study, which is a highly controlled and reproducible model for studying the effects of a systematic inflammatory responses.

Conclusions

To conclude, we here demonstrated that an LPS induced transient inflammation reduced endogenous cholesterol synthesis and bile acid formation in healthy young men. We speculate that mainly the reduction in cholesterol synthesis explains the observed reduction in serum TC and LDL-C concentrations. Furthermore, understanding the relation between circulating desmosterol and 7α-OH-cholesterol concentrations at baseline with the intensity of an inflammatory response after LPS exposure warrants further study.

References

1. World Health Organization (WHO). Cardiovascular diseases (CVDs) fact sheet. June 2021. Available from: https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).
2. Frostegard J. Immunity, atherosclerosis and cardiovascular disease. BMC Med. 2013;11:117.
3. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med. 2011;17(11):1410-22.
4. Ridker PM, Luscher TF. Anti-inflammatory therapies for cardiovascular disease. Eur Heart J. 2014;35(27):1782-91.
5. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. N Engl J Med. 2017;377(12):1119-31.
6. Zanoli L, Briet M, Empana JP, Cunha PG, Maki-Petaja KM, Protogerou AD, et al. Vascular consequences of inflammation: a position statement from the ESH Working Group on Vascular Structure and Function and the ARTERY Society. J Hypertens. 2020;38(9):1682-98.
7. Carpentier YA, Scruel O. Changes in the concentration and composition of plasma lipoproteins during the acute phase response. Curr Opin Clin Nutr Metab Care. 2002;5(2):153-8.
8. Khovidhunkit W, Kim MS, Memon RA, Shigenaga JK, Moser AH, Feingold KR, et al. Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. J Lipid Res. 2004;45(7):1169-96.
9. Feingold KR, Grunfeld C. The acute phase response inhibits reverse cholesterol transport. J Lipid Res. 2010;51(4):682-4.
10. Feingold KR, Grunfeld C. Effect of inflammation on HDL structure and function. Curr Opin Lipidol. 2016;27(5):521-30.
11. Jahangiri A, de Beer MC, Noffsinger V, Tannock LR, Ramaiah C, Webb NR, et al. HDL remodeling during the acute phase response. Arterioscler Thromb Vasc Biol. 2009;29(2):261-7.
12. Zimetti F, De Vuono S, Gomaraschi M, Adorni MP, Favari E, Ronda N, et al. Plasma cholesterol homeostasis, HDL remodeling and function during the acute phase reaction. J Lipid Res. 2017;58(10):2051-60.
13. van Lier D, Geven C, Leijte GP, Pickkers P. Experimental human endotoxemia as a model of systemic inflammation. Biochimie. 2019;159:99-106.
14. Perlee D, van Vught LA, Scicluna BP, Maag A, Lutter R, Kemper EM, et al. Intravenous Infusion of Human Adipose Mesenchymal Stem Cells Modifies the Host Response to Lipopolysaccharide in Humans: A Randomized, Single-Blind, Parallel Group, Placebo Controlled Trial. Stem Cells. 2018;36(11):1778-88.
15. Wilson M, Blum R, Dandona P, Mousa S. Effects in humans of intravenously administered endotoxin on soluble cell-adhesion molecule and inflammatory markers: a model of human diseases. Clin Exp Pharmacol Physiol. 2001;28(5-6):376-80.

16. Hudgins LC, Parker TS, Levine DM, Gordon BR, Saal SD, Jiang XC, et al. A single intravenous dose of endotoxin rapidly alters serum lipoproteins and lipid transfer proteins in normal volunteers. J Lipid Res. 2003;44(8):1489-98.
17. de la Llera Moya M, McGillicuddy FC, Hinkle CC, Byrne M, Joshi MR, Nguyen V, et al. Inflammation modulates human HDL composition and function in vivo. Atherosclerosis. 2012;222(2):390-4.
18. Czubayko F, Beumers B, Lammsfuss S, Lutjohann D, von Bergmann K. A simplified micro-method for quantification of fecal excretion of neutral and acidic sterols for outpatient studies in humans. J Lipid Res. 1991;32(11):1861-7.
19. Lutjohann D, Meese CO, Crouse JR, 3rd, von Bergmann K. Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in humans. J Lipid Res. 1993;34(6):1039-46.
20. Stellaard F, Lutjohann D. The Interpretation of Cholesterol Balance Derived Synthesis Data and Surrogate Noncholesterol Plasma Markers for Cholesterol Synthesis under Lipid Lowering Therapies. Cholesterol. 2017;2017:5046294.
21. Mackay DS, Jones PJ, Myrie SB, Plat J, Lutjohann D. Methodological considerations for the harmonization of non-cholesterol sterol bio-analysis. J Chromatogr B Analyt Technol Biomed Life Sci. 2014;957:116-22.
22. Lutjohann D, Bjorkhem I, Friedrichs S, Kerksiek A, Lovgren-Sandblom A, Geilenkeuser WJ, et al. First international descriptive and interventional survey for cholesterol and non-cholesterol sterol determination by gas- and liquid-chromatography-Urgent need for harmonisation of analytical methods. J Steroid Biochem Mol Biol. 2019;190:115-25.
23. Lutjohann D, Bjorkhem I, Friedrichs S, Kerksiek A, Geilenkeuser WJ, Lovgren-Sandblom A, et al. International descriptive and interventional survey for oxycholesterol determination by gas- and liquid-chromatographic methods. Biochimie. 2018;153:26-32.
24. Spann NJ, Garmire LX, McDonald JG, Myers DS, Milne SB, Shibata N, et al. Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. Cell. 2012;151(1):138-52.
25. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem. 1972;18(6):499-502.
26. Scharnagl H, Heuschneider C, Sailer S, Kleber ME, Marz W, Ritsch A. Decreased cholesterol efflux capacity in patients with low cholesteryl ester transfer protein plasma levels. Eur J Clin Invest. 2014;44(4):395-401.
27. Sosic-Jurjevic B, Lutjohann D, Renko K, Filipovic B, Radulovic N, Ajdzanovic V, et al. The isoflavones genistein and daidzein increase hepatic concentration of thyroid hormones and affect cholesterol metabolism in middle-aged male rats. J Steroid Biochem Mol Biol. 2019;190:1-10.
28. Feingold KR, Staprans I, Memon RA, Moser AH, Shigenaga JK, Doerrler W, et al. Endotoxin rapidly induces changes in lipid metabolism that produce hypertriglyceridemia: low doses

stimulate hepatic triglyceride production while high doses inhibit clearance. J Lipid Res. 1992;33(12):1765-76.

29. Hardardottir I, Moser AH, Memon R, Grunfeld C, Feingold KR. Effects of TNF, IL-1, and the combination of both cytokines on cholesterol metabolism in Syrian hamsters. Lymphokine Cytokine Res. 1994;13(3):161-6.
30. McGillicuddy FC, de la Llera Moya M, Hinkle CC, Joshi MR, Chiquoine EH, Billheimer JT, et al. Inflammation impairs reverse cholesterol transport in vivo. Circulation. 2009;119(8):1135-45.
31. Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, et al. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. N Engl J Med. 2011;364(2):127-35.
32. Annema W, Nijstad N, Tolle M, de Boer JF, Buijs RV, Heeringa P, et al. Myeloperoxidase and serum amyloid A contribute to impaired in vivo reverse cholesterol transport during the acute phase response but not group IIA secretory phospholipase A(2). J Lipid Res. 2010;51(4):743-54.
33. Holzer M, Wolf P, Curcic S, Birner-Gruenberger R, Weger W, Inzinger M, et al. Psoriasis alters HDL composition and cholesterol efflux capacity. J Lipid Res. 2012;53(8):1618-24.
34. Pussinen PJ, Jauhiainen M, Vilkuna-Rautiainen T, Sundvall J, Vesanen M, Mattila K, et al. Periodontitis decreases the antiatherogenic potency of high density lipoprotein. J Lipid Res. 2004;45(1):139-47.
35. Ronda N, Favari E, Borghi MO, Ingegnoli F, Gerosa M, Chighizola C, et al. Impaired serum cholesterol efflux capacity in rheumatoid arthritis and systemic lupus erythematosus. Ann Rheum Dis. 2014;73(3):609-15.
36. Siegel MO, Borkowska AG, Dubrovsky L, Roth M, Welti R, Roberts AD, et al. HIV infection induces structural and functional changes in high density lipoproteins. Atherosclerosis. 2015;243(1):19-29.
37. Ettinger WH, Varma VK, Sorci-Thomas M, Parks JS, Sigmon RC, Smith TK, et al. Cytokines decrease apolipoprotein accumulation in medium from Hep G2 cells. Arterioscler Thromb. 1994;14(1):8-13.
38. Feingold KR, Serio MK, Adi S, Moser AH, Grunfeld C. Tumor necrosis factor stimulates hepatic lipid synthesis and secretion. Endocrinology. 1989;124(5):2336-42.
39. Feingold KR, Soued M, Serio MK, Moser AH, Dinarello CA, Grunfeld C. Multiple cytokines stimulate hepatic lipid synthesis in vivo. Endocrinology. 1989;125(1):267-74.
40. Feingold KR, Soued M, Staprans I, Gavin LA, Donahue ME, Huang BJ, et al. Effect of tumor necrosis factor (TNF) on lipid metabolism in the diabetic rat. Evidence that inhibition of adipose tissue lipoprotein lipase activity is not required for TNF-induced hyperlipidemia. J Clin Invest. 1989;83(4):1116-21.
41. Liebergall SR, Angdisen J, Chan SH, Chang Y, Osborne TF, Koeppel AF, et al. Inflammation Triggers Liver X Receptor-Dependent Lipogenesis. Mol Cell Biol. 2020;40(2).
42. Jelinek DF, Andersson S, Slaughter CA, Russell DW. Cloning and regulation of cholesterol 7 alpha-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis. J Biol Chem. 1990;265(14):8190-7.

43. Feingold KR, Spady DK, Pollock AS, Moser AH, Grunfeld C. Endotoxin, TNF, and IL-1 decrease cholesterol 7 alpha-hydroxylase mRNA levels and activity. J Lipid Res. 1996;37(2):223-8.
44. Dikopoulos N, Weidenbach H, Adler G, Schmid RM. Lipopolysaccharide represses cholesterol 7-alpha hydroxylase and induces binding activity to the bile acid response element II. Eur J Clin Invest. 2003;33(1):58-64.
45. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature. 1996;383(6602):728-31.
46. Lehmann JM, Kliewer SA, Moore LB, Smith-Oliver TA, Oliver BB, Su JL, et al. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. J Biol Chem. 1997;272(6):3137-40.
47. Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE, et al. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. Cell. 1998;93(5):693-704.
48. Yang C, McDonald JG, Patel A, Zhang Y, Umetani M, Xu F, et al. Sterol intermediates from cholesterol biosynthetic pathway as liver X receptor ligands. J Biol Chem. 2006;281(38):27816-26.
49. Lund EG, Peterson LB, Adams AD, Lam MH, Burton CA, Chin J, et al. Different roles of liver X receptor alpha and beta in lipid metabolism: effects of an alpha-selective and a dual agonist in mice deficient in each subtype. Biochem Pharmacol. 2006;71(4):453-63.
50. Tall AR. Protease variants, LDL, and coronary heart disease. N Engl J Med. 2006;354(12):1310-2.
51. Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. Nat Rev Immunol. 2015;15(2):104-16.
52. Zhang X, McDonald JG, Aryal B, Canfran-Duque A, Goldberg EL, Araldi E, et al. Desmosterol suppresses macrophage inflammasome activation and protects against vascular inflammation and atherosclerosis. Proc Natl Acad Sci U S A. 2021;118(47).

Supplementary Materials

Supplemental table 1. Absolute values of non-cholesterol sterol and oxysterols at baseline and 24 hours after LPS infusion (n=8).

Variable	Baseline	24 hours	p-value
Cholestanol[#]	5.72 ± 1.16	5.54 ± 1.03	0.631
Lathosterol[#]	3.64 ± 1.52	2.67 ± 1.21	**0.004**
Campesterol[#]	9.23 ± 3.66	8.74 ± 3.11	0.350
Sitosterol[#]	4.68 ± 1.82	4.49 ± 1.73	0.294
Lanosterol[#]	0.32 ± 0.09	0.23 ± 0.07	**0.021**
Desmosterol[#]	4.03 ± 0.93	3.38 ± 1.02	**0.003**
24-OH-cholesterol[*]	139.24 ± 33.27	142.13 ± 46.67	0.839
27-OH-cholesterol[*]	307.18 ± 61.42	296.96 ± 61.02	0.421
7α-OH-cholesterol[*]	117.44 ± 36.43	81.41 ± 13.31	**0.022**

Data are presented as means ± SD. Values are in [#]μmol/L or *nmol/L. Significant differences between baseline and 24 hours samples (paired two-tailed Student's T-Test) are depicted in bold**.**

CHAPTER

6

DO OMEGA-3 FATTY ACIDS PROTECT AGAINST THE ADVERSE EFFECT OF PHYTOSTEROLS?

A pilot study comparing three different lipid emulsions in adult patients on home parenteral nutrition

Sylwia Osowska, Marek Kunecki, Jacek Sobocki,
Joanna Tokarczyk, Krystyna Majewska, Magdalena Burkacka,
Marek Radkowski, Helena L. Fisk, Sultan Mashnafi,
Sabine Baumgartner, Jogchum Plat, Philip C. Calder

Biology 2022;11(12),1699

Background

The effect of the different content of phytosterols in lipid emulsions (LEs) used in the parenteral nutrition (PN) regimen of adult home PN (HPN) patients is not clear.

Methods

Plasma sterol and cytokine concentrations, fatty acid composition, and liver function markers and triglycerides were measured in 58 adult HPN patients receiving one of three different LEs (soybean oil based: Intralipid; olive oil based: ClinOleic; containing fish oil: SMOFLipid).

Results

Patients receiving Intralipid had higher plasma campesterol and stigmasterol concentrations than those receiving ClinOleic or SMOFLipid. Plasma sterol concentrations were not different between patients receiving ClinOleic and SMOFLipid. Differences in plasma fatty acids reflected the fatty acid composition of the LEs. Markers of liver function did not differ among the three groups. Blood triglycerides were higher with ClinOleic than with Intralipid or SMOFLipid. Over half of patients in the SMOFLipid group had values for all plasma liver function markers and triglycerides in the normal range compared to one-third in the Intralipid group and one quarter in the ClinOleic group. Total bilirubin, ALT, AST and GGT each correlated positively with the concentrations of two, one, one and three plasma sterols, respectively.

Conclusions

Liver function markers correlate with plasma plant sterol concentrations in adult HPN patients. Adult HPN patients receiving SMOFLipid are more likely to have liver function markers and triglycerides within the normal range than those receiving ClinOleic or Intralipid. The omega-3 fatty acids in SMOFLipid may act to mitigate the adverse effects of plant sterols on liver function.

Abstract

Clinical Relevancy Statement

The choice of lipid emulsions (LEs) used in parenteral nutrition is based mainly on the fatty acids composition, However, there are other components of LEs that influence clinical outcome of the PN patients. The level of phytosterols is closely associated with the base on which the lipid emulsion was produced. Their effect, which is still subject to many studies, proves to be detrimental.

In our pilot study we show that the adverse effects of phytosterols delivered to patients in parenteral mixtures was mitigated by long chain omega-3 fatty acids.

Introduction

In parenteral nutrition (PN), lipid emulsions (LEs) are an important source of energy and the only source of essential fatty acids [1]. Depending on the oil from which they are produced, LEs differ in the amount and type of fatty acids [2]. The latter have a direct impact on metabolism, immune and inflammatory processes, and cell function [3]. Most of the LEs that are used in PN contain one or more vegetable oils. These oils contain plant sterols (phytosterols) [4,5]. Home parenteral nutrition (HPN) is an established therapy that aims to provide adequate amounts of all nutrients and water in order to prevent malnutrition in patients requiring long-term PN due to prolonged gastrointestinal tract failure [1,6,7]. One of the complications of the long-term PN is liver damage [8]. Its etiology, which is believed to be multifactorial, is not yet fully understood [8]. However, the literature suggests that there may be two important LE-related factors: the presence of phytosterols which have a detrimental effect and the presence of different fatty acids, with a view that omega-6 fatty acids are detrimental and omega-3 fatty acids are protective [9,10,11,12]. Fish oil is a source of the bioactive omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [13]. In the pediatric population, unlike adults, many studies describe the prevention or even the reversal of liver damage by using fish oil-based LEs [14,15,16,17].

Different LEs may be used as part of the nutrition support of adult HPN patients. As mentioned above, these LEs differ in content and composition of sterols, including plant sterols, and in composition of fatty acids. These differences between LEs might affect inflammation, lipid metabolism and liver function. The aim of this study was to compare plasma sterol concentrations in adult HPN patients receiving one of three different LEs (soybean oil based: Intralipid; olive oil based: ClinOleic; containing fish oil: SMOFLipid) and their relationship with markers of liver function.

Material and methods

Study design and patients

This was a cross-sectional comparative study with 3 groups of patients from two Polish parenteral nutrition centres (Department of Clinical Nutrition and Surgery, Orlowski Hospital in Warsaw and Center of Clinical Nutrition, Pirogov Hospital in Lodz). The study protocol was approved by the Bioethical Committee of Warsaw Medical University. 58 stable patients with intestinal failure supported by HPN (33 women and 25 men; mean age 58 years) were recruited. Patient inclusion criteria were: age > 18 years; being part of the hospital's HPN programme; duration of HPN for a minimum of 2 years prior to the study on the same lipid emulsion; PN provided as 7 infusions per week; oral feeding and

drug therapy unchanged during the 2 months prior to inclusion in study, clinical stability. Exclusion criteria were: active infection; liver or renal failure or both; pregnancy. The etiology of intestinal failure included: mesenteric ischemia (n = 11; 19%), Crohn's Disease (n = 10; 17%), obstruction (n = 6; 10%), malabsorption syndrome (n = 5; 9%), surgical complications (n = 12; 21%), radiation enteropathy (n = 8; 14%), adhesion ileus (n = 6; 10%). The clinical heterogeneity of the patients studied reflects the clinical reality of patients for whom HPN is indicated. Each patient was prescribed indexed amounts of energy, macronutrients, fluids, and electrolytes in relation to their weight, biochemical results and standard recommendations. Except for the type of lipid, each patient was prescribed the same type of macronutrients (glucose and amino acid mix (Aminomel)) and micronutrients (vitamins and trace elements). Patients typically received approx. 20 g of lipid emulsion daily. PN was administered by central catheter (Broviac) over 16-18 hours per 24 hours. Patients were receiving ClinOleic (80:20 olive oil:soybean oil; Baxter Healthcare, Maurepas, France), SMOFLipid (30:30:25:15 soybean oil:medium chain triglycerides:olive oil:fish oil; Fresenius-Kabi, Bad-Homburg, Germany) or Intralipid (soybean oil; Fresenius-Kabi, Bad Homberg, Germany) as part of their routine nutrition support. The characteristics of the three groups are summarized in table 1.

Blood processing and overview of analyses performed

Blood was collected into disodium-EDTA as anti-coagulant, 2-3 hours after completing infusion of PN (lasting for 16-hours). An aliquot was used for routine biochemical analyses. The following were measured: total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltranspeptidase (GGT), and total triglycerides. An aliquot of blood was immediately centrifuged and plasma was isolated; this was stored at -80°C until analysis. The following were measured in plasma: cholesterol, cholestanol, lathosterol, campesterol, stigmasterol, sitosterol, cytokines including interleukin (IL)-6, IL-8, IL-10, tumour necrosis factor (TNF)-α and interferon (IFN)-γ and fatty acids. The concentrations of cholesterol, cholestanol, lathosterol, campesterol, stigmasterol and sitosterol were also measured in original bottles of ClinOleic, SMOFLipid and Intralipid.

Measurement of fatty acids in plasma

Lipid was extracted from plasma using 5 ml of chloroform:methanol (2:1; vol/vol) containing 0.2 M butylated hydroxytoluene as antioxidant. Sodium chloride (1 M; 1 mL) was added and the sample vortexed and then centrifuged. The lower solvent phase containing the lipid was aspirated and evaporated to dryness under nitrogen at 40°C. Fatty acids were removed from complex lipids and simultaneously derivatized to methyl esters by incubation with 1 mL 2% H_2SO_4 (vol/vol) in methanol for a minimum of 2 hours at 50°C to form fatty acid methyl esters. The samples were then neutralized and fatty acid methyl esters transferred into hexane for analysis by gas chromatography. Fatty acid methyl esters were separated on a BPX-70 fused silica capillary column (30 m x 0.2 mm

Table 1. Characteristics of the patients according to lipid emulsion received.

	ClinOleic	SMOFLipid	Intralipid
Number of patients	21	17	20
Age range, years (mean)	19-91 (60.3)	27-84 (54.5)	25-89 (59.0)
Male	8	7	10
Female	13	10	10
Etiology of intestinal failure (n):			
Bowel obstruction	2	2	2
Mesenteric ischemia	5	3	3
Surgical complications	3	4	5
Crohn's Disease	3	3	4
Adhesion ileus	3	1	2
Radiation entheropathy	4	2	2
Malabsorption	1	2	2

x 0.25 µm, manufactured by SGE) in a HP6890 gas chromatograph fitted with a flame ionization detector. Gas chromatography run conditions were as described elsewhere [18]. A Supelco® 37 Component FAME Mix was used as a calibration reference standard (Sigma-Aldrich, Irvine, UK). FAME peaks were identified and integrated using Chem Station software (Agilent) and fatty acid data are expressed as % of total fatty acids present.

Measurement of plasma cytokine concentrations

The concentrations of TNF-α, IL-1β, IL-6, IL-8, IL-10 and IFN-γ were measured in plasma using a high sensitivity Bio-Techne multiplex immunoassay (R&D Systems, Abingdon, UK). Reagents were brought to room temperature before use and dilutions were prepared immediately before use according to the manufacturer's instructions. Samples were read using a Bio-Rad-plex Luminex Analyzer. Data are expressed as pg/mL plasma.

Measurement of sterol concentrations

5α-cholestane and epicoprostanol were added to plasma (or lipid emulsion) samples as internal standards, and these samples plus standards were saponified with 90% ethanolic sodium hydroxide for 1 hr at 60°C. After two rounds of cyclohexane extraction, samples were derivatized with TMS reagent (pyridine, hexamethyldisilazane and trimethylchlorosilane (9:3:1, vol/vol/vol)). Derivatized sterols were separated on a DB-XLB capillary column (30 m x 0.25 mm a 0.25 µm; Agilent Technologies, Amstelveen, Netherlands) in an HP6890 plus gas chromatograph fitted with a flame ionization detector. Gas chromatography run conditions were as described elsewhere [19]. Peaks were identified and integrated using Open Lab CDS Chem Station software (Agilent) and sterol concentrations were calculated relative to the internal standard 5α-cholestane concentration.

Statistical analysis

Data were checked for normality using the Kolmogorov-Smirnov test. Much of the data were skewed and therefore all data are expressed as median and interquartile range. Comparisons were made across treatment groups using the Kruskal Wallis test. Where the Kruskal Wallis test was significant, pairwise comparisons between groups were conducted and P values were Bonferroni adjusted for multiple comparisons. Correlations were investigated as Spearman rank correlations and are reported as Spearman's ρ. Percentages were compared between groups using the Chi-squared test. Statistical analyses were performed using SPSS version 21. In all cases a value for $P < 0.05$ was taken to indicate a statistically significant difference.

Results

Sterol and stanol concentrations in the lipid emulsions and in plasma

The sterol concentrations in the three lipid emulsions are shown in Table 2. The emulsions differed in total sterol (the sum of cholesterol, cholestanol, lathosterol, campesterol, stigmasterol and sitosterol) content (ClinOleic 27.65 mg/dL, Intralipid 68.34 mg/dL; SMOFLipid 61.21 mg/dL); thus patients in the ClinOleic group received less total sterols than those in the other two groups. Plant sterols (i.e. excluding cholesterol, cholestanol and lathosterol) were higher in Intralipid (40.22 mg/dL) than in ClinOleic (22.14 mg/dL) and SMOFLipid (18.63 mg/dL); thus patients in the ClinOleic and SMOFLipid groups received similar amounts of phytosterols and these were less than received by patients in the Intralipid group. Furthermore, the content of the different sterols differed across the emulsions. The most common sterol in ClinOleic was sitosterol followed by cholesterol. In Intralipid the most common sterols were cholesterol followed by sitosterol; there were also significant concentrations of stigmasterol and campesterol in Intralipid. In SMOFLipid, cholesterol was the most common sterol present and there was also a high content of sitosterol.

Table 2. Sterol and stanol concentrations (mg/dL) in the three lipid emulsions.

Sterol or stanol	ClinOleic	Intralipid	SMOFLipid
Cholesterol	5.37 ± 0.67	27.65 ± 1.14	42.00 ± 1.88
Cholestanol	0.06 ± 0.02	0.22 ± 0.01	0.35 ± 0.02
Lathosterol	0.08 ± 0.02	0.25 ± 0.01	0.23 ± 0.01
Campesterol	1.88 ± 0.22	7.05 ± 0.33	2.89 ± 0.10
Sitosterol	18.31 ± 2.16	24.08 ± 0.76	12.46 ± 0.25
Campestanol	0.06 ± 0.01	0.16 ± 0.02	0.07 ± 0.01
Stigmasterol	1.12 ± 0.15	7.44 ± 0.30	2.67 ± 0.07
Sitostanol	0.77 ± 0.09	1.49 ± 0.03	0.54 ± 0.04

Data are mean ± SD from three replicates.

Table 3 shows the sterol concentrations in the plasma of patients receiving the different lipid emulsions. Cholesterol concentrations were much higher than the concentrations of other sterols measured (Table 3). Cholestanol and lathosterol are markers of cholesterol absorption and endogenous cholesterol synthesis, respectively. Campesterol, stigmasterol and sitosterol are plant sterols. Patients in the Intralipid group had higher plasma concentrations of campesterol and stigmasterol than those in the ClinOleic and SMOFLipid groups (Table 3); this is consistent with Intralipid containing higher amounts of these two phytosterols (Table 2). Furthermore, patients in the Intralipid group tended to have had higher plasma concentrations of sitosterol than those in the ClinOleic and SMOFLipid groups (Table 3). Plasma sterol concentrations were not different between the ClinOleic and SMOFLipid groups; this is consistent with the similar phytosterol content and composition of these two LEs.

Table 3. Plasma sterol concentrations in patients according to the lipid emulsion being received. Data are median (interquartile range). Median values across a row not sharing superscript letters are significantly different after adjustment for multiple comparisons.

Sterol	ClinOleic	Intralipid	SMOFLipid
Cholesterol (mmol/L)	3.40 (2.65, 3.95)	2.94 (2.59, 3.33)	2.89 (2.36, 3.88)
Cholestanol (µmol/L)	5.45 (4.63, 6.51)	6.14 (4.87, 8.80)	6.44 (5.5, 8.22)
Lathosterol (µmol/L)	10.85 (7.51, 16.28)	11.64 (3.69, 14.81)	12.39 (6.59, 19.94)
Campesterol (µmol/L)	4.95[a] (3.19, 6.80)	15.17[b] (9.99, 17.94)	7.13[a] (6.33, 9.68)
Sitosterol (µmol/L)	23.18 (13.5, 48.6)	34.2 (19.0, 42.2)	21.8 (15.0, 27.6)
Stigmasterol (µmol/L)	0.52[a] (0.31, 0.87)	3.55[b] (2.13, 4.40)	1.58[a] (1.09, 1.76)

Plasma fatty acids

Being based solely on soybean oil, Intralipid is rich in linoleic acid (18:2n-6) which comprises about 53% of fatty acids present. Intralipid also contains about 8% α-linolenic acid (18:3n-3). ClinOleic is rich in oleic acid (18:1n-9) and contains about 19% linoleic acid and about 2% α-linoleic acid. SMOFLipid also contains about 19% linoleic acid and 2% α-linolenic acid, but it also contains EPA (about 3%) and DHA (about 2%). Table 4 shows the plasma fatty acid composition according to lipid emulsion received. There were a number of significant differences between the groups. Plasma oleic acid was higher in the ClinOleic group than in the other two groups and was lower in the Intralipid group than the other two groups. Plasma linoleic and α-linolenic acids were higher in the Intralipid group than in the other two groups. Plasma arachidonic acid was lower in the SMOFLipid

group than in the ClinOleic and Intralipid groups. Plasma EPA and DHA were both higher in the SMOFLipid group than in the other two groups. In general, these findings reflect the fatty acid composition of the emulsions themselves.

Table 4. Plasma fatty acid composition (% of total fatty acids) in patients receiving different lipid emulsions. Data are median (interquartile range). Median values across a row not sharing superscript letters are significantly different after adjustment for multiple comparisons.

Fatty acid	ClinOleic	Intralipid	SMOFLipid
Myristic (14:0)	1.04 (0.84, 1.32)	1.12 (0.92, 1.42)	1.13 (1.02, 1.40)
Palmitic (16:0)	25.97 (24.46, 27.35)	24.66 (23.85, 25.63)	25.31 (24.38, 28.73)
Palmitoleic (16:1n-7)	4.27 (2.27, 5.11)	3.79 (2.90, 4.23)	3.74 (3.03, 4.61)
Stearic (18:0)	7.34 (6.84, 8.08)	7.96 (6.88, 9.24)	7.67 (6.91, 8.80)
Oleic (18:1n-9)	31.34^{a} (27.64, 33.38)	21.78^{b} (20.8, 23.51)	25.27^{c} (23.84, 29.7)
Vaccenic (18:1n-7)	2.55^{a} (2.13, 2.80)	2.15^{b} (1.96, 2.28)	2.46^{ab} (1.90, 2.67)
Linoleic (18:2n-6)	14.25^{a} (12.01, 19.00)	22.67^{b} (21.21, 26.08)	16.06^{a} (12.99, 19.87)
α-Linolenic (18:3n-3)	0.42^{a} (0.35, 0.50)	0.91^{b} (0.73, 1.10)	0.60^{a} (0.48, 0.71)
Dihomo-γ-linolenic (20:3n-6)	1.69 (1.37, 2.03)	1.86 (1.51, 2.16)	1.51 (1.17, 1.86)
Arachidonic (20:4n-6)	6.86^{a} (6.07, 8.33)	7.03^{a} (5.85, 7.68)	5.77^{b} (5.13, 6.18)
Eicosapentaenoic (20:5n-3)	0.65^{a} (0.45, 0.75)	0.95^{b} (0.69, 1.22)	2.21^{c} (1.62, 2.41)
Docosapentaenoic (22:5n-3)	0.54^{a} (0.45, 0.64)	0.55^{a} (0.45, 0.64)	0.88^{b} (0.69, 1.21)
Docosahexaenoic (22:6n-3)	1.61^{a} (1.20, 2.11)	1.78^{a} (1.41, 2.42)	3.52^{b} (3.04, 4.18)

Plasma liver function markers and triglycerides

Table 5 shows the plasma liver function markers and triglycerides in the three groups. Liver function markers did not differ among groups. Triglycerides were higher in the ClinOleic group than in the other two groups.

The % of patients with values for liver function markers and plasma triglycerides above the normal range is shown in Table 6, while Table 7 shows the % of patients in each group with all values within the normal range. The % of patients with elevated ALT was highest in the ClinOleic and SMOFLipid groups, while the % with elevated AST was highest in the ClinOleic and Intralipid groups. The % of patients with elevated GGT was highest in the Intralipid group. The % of patients with elevated triglycerides was significantly higher in the ClinOleic group than in the other two groups. Over half of patients in the SMOFLipid group had values for all plasma liver function markers and triglycerides in the normal

range compared to one-third in the Intralipid group and one quarter in the ClinOleic group (Table 7).

Table 5. Plasma liver function markers and triglycerides in patients receiving different lipid emulsions. Data are median (interquartile range). Median values across a row not sharing superscript letters are significantly different after adjustment for multiple comparisons. Reference values are: total bilirubin: 0.2-1.3 mg/dL; ALT: 14-59 U/L; AST: 14-36 U/L; GGT:12-43 U/L; triglycerides < 150 mg/dL.

Marker	ClinOleic	Intralipid	SMOFLipid
Total bilirubin (mg/dL)	0.6 (0.4, 0.8)	0.6 (0.4, 0.9)	0.4 (0.3, 0.7)
ALT (U/L)	46 (27, 61)	36 (29, 59)	34 (26, 60)
AST (U/L)	28 (21, 43)	26 (21, 36)	25 (18, 32)
GGT (U/L)	50 (25, 101)	80 (35, 150)	61 (38, 75)
Triglycerides (mg/dL)	178[a] (114, 236)	94[b] (83, 146)	111[b] (70, 148)

Table 6. Percentage of patients in each group with plasma liver function markers and triglycerides above the normal range. Values across a row not sharing superscript letters are significantly different.

Marker	ClinOleic (% of patients)	Intralipid (% of patients)	SMOFLipid (% of patients)
Total bilirubin (mg/dL)	9.5	10.0	5.8
ALT (U/L)	28.6	15.0	29.4
AST (U/L)	23.8	25.0	11.8
GGT (U/L)	29.0	55.0	23.5
Triglycerides (mg/dL)	52.4[a]	15.0[b]	11.8[b]

Table 7. Percentage of patients in each group with all values for plasma liver function markers and triglycerides within the normal range.

Lipid emulsion	% of patients with ALL values within the normal range
ClinOleic	24
Intralipid	35
SMOFLipid	53

Plasma markers of inflammation

Table 8 shows the plasma markers of inflammation in the three groups. CRP was lower in the ClinOleic group than in the other two groups, while IL-8 was higher in the ClinOleic than the Intralipid group.

Table 8. Plasma inflammatory markers in patients receiving different lipid emulsions. Data are median (interquartile range). Median values across a row not sharing superscript letters are significantly different after adjustment for multiple comparisons.

Marker	ClinOleic	Intralipid	SMOFLipid
CRP (mg/dL)	4.10^{a} (0.60, 5.95)	6.36^{b} (5.57, 10.00)	5.49^{b} (5.01, 10.09)
IL-1β (pg/mL)	1.00 (0.54, 1.39)	0.96 (0.63, 1.39)	0.80 (0.43, 1.51)
IL-6 (pg/mL)	5.07 (1.94, 5.80)	2.99 (2.36, 4.93)	3.09 (2.16, 5.12)
IL-8 (pg/mL)	36.4^{a} (10.2, 34.8)	9.6^{b} (4.6, 12.3)	10.6^{ab} (5.3, 26.8)
IL-10 (pg/mL)	1.92 (0.88, 1.86)	1.90 (1.02, 2.70)	1.85 (1.32, 2.36)
IFN-γ (pg/mL)	2.59 (0.13, 3.88)	1.26 (0.66, 6.00)	1.12 (0.32, 2.23)
TNF-α (pg/mL)	19.6 (16.3, 21.9)	14.6 (12.5, 20.3)	16.0 (13.7, 18.9)

Correlations between liver function markers and plasma sterols and stanols

Using data from all patients irrespective of the type of LE they were receiving, bilirubin was positively correlated with plasma stigmasterol and sitosterol (ρ = 0.264, P = 0.032 and ρ = 0.290, P = 0.020, respectively) with a trend to a positive correlation with plasma campesterol (ρ = 0.236, P = 0.061). ALT and AST were both positively correlated with plasma sitosterol (ρ = 0.356, P = 0.004 and ρ = 0.412, P = 0.001, respectively). There was also a trend towards a positive correlation between AST and plasma stigmasterol (ρ = 0.233, P = 0.064). GGT was positively correlated with plasma cholestanol (ρ = 0.325, P = 0.009), campesterol (ρ = 0.42, P = 0.001) and sitosterol (ρ = 0.502, P < 0.001).

When correlations between liver function markers and plasma sterols were investigated within each LE group, there were no significant correlations in either the Intralipid or SMOFLipid groups. However, in the ClinOleic group, bilirubin, ALT, AST and GGT were all positively correlated with plasma stigmasterol and sitosterol, while ALT and GGT were positively correlated with campesterol.

Discussion

The main findings of our study suggest that provision of bioactive omega-3 polyunsaturated fatty acids (EPA and DHA) might attenuate the deleterious effects on liver health of phytosterols present in plant-based LEs used in patients on long-term PN. Patients in the ClinOleic and SMOFLipid groups received similar amounts of the different phytosterols and had plasma sterol concentrations that did not differ, yet in the ClinOleic group only 24% of patients had values for all liver function markers and triglycerides in the normal range compared with 53% in the SMOFLipid group. In the ClinOleic group there were significant correlations between plasma phytosterol concentrations and all of the liver function markers; these correlations were not seen in patients in the SMOFLipid group. This suggests that the adverse relation between phytosterols and liver function is attenuated by SMOFLipid. It is important to note that there were also no significant correlations between plasma phytosterols and liver function markers in the Intralipid group, despite Intralipid containing more phytosterols than the other LEs and despite patients receiving Intralipid having the highest plasma phytosterol concentrations.

Several studies have shown the reversal of cholestasis in infants receiving PN either by decreasing the dose of soybean oil based LEs [20,21] or by administration of pure fish oil based LEs or mixture of different lipids that included fish oil [22]. The mechanisms of liver injury during long-term PN that are currently receiving the most attention include the deleterious effect of plant sterols present in plant-based LEs and the pro-inflammatory effect of omega-6 polyunsaturated fatty acids. The concentrations of cholesterol, campesterol, stigmasterol and sitosterol we report for Intralipid and ClinOleic are consistent with the concentrations reported by Forcielli et al. [5] while the total concentrations of phytosterols we report for Intralipid (22.2 vs 20.8 mg/dL) and ClinOleic (40.2 vs 42.2 mg/dL) are consistent with the report of Llop Talaverón et al. [6] but our value for SMOFLipid is higher than theirs (18.6 vs 12.4 mg/dL). However, Llop Talaverón et al. [6] do report different total phytosterol concentrations in different batches of all three of these LEs. Nevertheless, in the present study we confirmed the higher concentration of phytosterols in 100 % soybean based Intralipid than in ClinOleic and SMOFLipid, as described by Llop Talaverón et al. [6]. ClinOleic had a similar phytosterol content as SMOFLipid but a much lower total sterol content, because of the differing cholesterol content. The plasma concentrations of phytosterols reflected the phytosterol content of the LEs, as might be expected: plasma campesterol and stigmasterol were higher in patients receiving Intralipid. In a study with mouse hepatocytes, out of three phytosterols tested (stigmasterol, campesterol and sitosterol), stigmasterol proved to have the greatest potential in promoting cholestasis through antagonism of multipurpose fanesoid X receptor (FXR) function and reduction in canalicular bile acid transporters (ABCB11) expression [23]. Increased serum stigmasterol was correlated with liver inflammation and

cholestasis in children receiving PN [24]. In the present study, sitosterol was positively correlated with plasma levels of bilirubin, ALT, AST and GGT. Bilirubin was also positively correlated with stigmasterol with a trend to positive correlation with plasma campesterol. GGT was positively correlated to cholestanol and campesterol. These correlations were seen only in patients receiving ClinOleic. This suggests that the fatty acid composition of LEs influences the effects of phytosterols on liver function. This might relate to the differential effects of fatty acids on inflammation.

Proinflammatory cytokines lead to suppression of nuclear receptor-mediated gene expression in liver, including FXR-dependent pathways which as a consequence lead to cholestasis [25,26,27]. In the current study a significantly higher plasma concentration of IL-8 was observed in the ClinOleic group in comparison to the other two groups. The mechanism behind this is not clear. However, IL-8 production has been shown to be enhanced by omega-6 fatty acids and by arachidonic acid metabolites [28,29]. Intralipid contains more omega-6 fatty acids (as linoleic acid) than ClinOleic and so might be expected to result in higher IL-8 concentrations, but this was not seen. ClinOleic contains the highest concentration of oleic acid which was reflected in the plasma of the patients. This emulsion contains 20% soybean oil in comparison to 30% soybean oil present in SMOFLipid. This difference in soybean oil content did not result in a different plasma concentration of linoleic acid. Plasma arachidonic acid was not different between the ClinOleic and Intralipid groups, but was higher than in the SMOFLipid group. Furthermore, the ClinOleic group had lower plasma EPA than both the Intralipid and SMOFLipid groups. The ratio of EPA to arachidonic acid was lowest in the ClinOleic group (0.094) compared with the Intralipid (0.135) and SMOFLipid (0.383) groups. This fatty acid ratio may be the link between the different LEs and inflammation.

Patients in the ClinOleic group had significantly higher plasma level of triglycerides than in the other two groups and these were more likely to be above the reference value. This could be due to ClinOleic having the lowest content of polyunsaturated fatty acids. Polyunsaturated fatty acids are strong activators of peroxisome proliferator activated receptors (PPARS), especially PPAR-α, with DHA being the strongest fatty acid activator [30]. PPAR-α plays a key role in the regulation of hepatic fatty acid oxidation by increasing the expression of the fatty acid transport protein, fatty acid translocase, acyl-CoA oxidase and carnitine palmitoyltransferase [31]. These effects act to partition fatty acids towards oxidation and away from triglyceride synthesis [32,33]. Furthermore, PPAR-α amplifies the expression of lipoprotein lipase and inhibits apolipoprotein C-III synthesis [34]. These mechanisms together result in decreased hepatic accumulation and secretion of triglycerides and decreased blood triglyceride concentrations.

Conclusions

We conclude that phytosterol content and composition and fatty acid composition are important in determining the physiological impact of LEs used in HPN. Phytosterols are linked to impaired liver function, but we show here that this relationship seems to be attenuated by bioactive omega-3 fatty acids (EPA and DHA) most likely through their effects on inflammation and hepatic fatty acid and triglyceride metabolism.

Footnotes

Financial disclosure

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest

SO, MK, JT, KM, MB, MR, HLF, SM, SB and JP have no conflict of interest to declare. JS gave lectures as part of educational grants for Fresenius-Kabi, B. Braun Melsungen and Baxter Healthcare. PCC acts as an ad-hoc advisor to Fresenius-Kabi, B. Braun Melsungen and Baxter Healthcare.

Statement of Authorship

Sylwia Osowska contributed to conception and design of the research. Marek Kunecki, Jacek Sobocki, Marek Radkowski, Joanna Tokarczyk, Krystyna Majewska contributed to acquisition of the data. Helena L. Fisk, Sultan Mashnafi, Sabine Baumgartner contributed to analysis of the data. Sylwia Osowska, Jogchum Plat, Philip Calder contributed to interpretation of the data. Sylwia Osowska and Philip Calder drafted the manuscript. Marek Kunecki, Jacek Sobocki, Jogchum Plat critically revised the manuscript. All authors had access to the study data, reviewed and approved the final manuscript, and agree to be fully accountable for ensuring the integrity and accuracy of the work.

References

1. Wanten, G., Calder, P.C. and Forbes, A. Managing adult patients who need home parenteral nutrition. *BMJ* 2011;342:696-701.
2. Calder, P.C., Adolph, M., Deutz, N.E., Grau, T., Innes, J.K., Klek, S., et al. Lipids in the intensive care unit: Recommendations from the ESPEN Expert Group. *Clin Nutr* 2018;37:1-18.
3. Calder, P.C. Functional roles of fatty acids and their effects on human health. *JPEN J Parent Ent Nutr* 2015;39 (1 Suppl):18S-32S.
4. Forchielli ML, Bersani G, Tala S, Grossi G, Puggioli C, Masi M. The spectrum of plant and animal sterols in different oil-derived intravenous emulsions. *Lipids* 2010;45:63-71.
5. Llop Talaverón JM, Novak A, Suñé Negre JM, Badia Tahull M, Leiva Badosa E, Ticó Grau JR. Phytosterol determination in lipid emulsions for parenteral nutrition. *Farm Hospit* 2018;42:116-119.
6. Pironi L, Arends J, Bozzetti F, Cuerda C, Gillanders L, Jeppesen PB, et al. ESPEN guidelines on chronic intestinal failure in adults. *Clin Nutr* 2016;35:247-307.
7. Ahmed, S., Innes, J.K. and Calder, P.C. Influence of different intravenous lipid emulsions on fatty acid status and laboratory and clinical outcomes in adult patients receiving home parenteral nutrition: A systematic review. *Clin Nutr* 2021;40:1115-1122.
8. Beath SV, Kelly DA. Total parenteral nutrition-induced cholestasis: prevention and management. *Clin Liver Dis* 2016;20:159-176.
9. Hukkinen M, Mutanen A, Nissinen M, Merras-Salmio L, Gylling H, Pakarinen MP. Parenteral plant sterols accumulate in the liver reflecting their increased serum levels and portal inflammation in children with intestinal failure. *JPEN J Parent Ent Nutr* 2017;41:1014-1022.
10. Premkumar MH, Carter BA, Hawthorne KM, King K, Abrams SA. High rates of resolution of cholestasis in parenteral nutrition associated liver disease with fish oil-based lipid emulsion monotherapy. *J Ped* 2013;162:793-798.
11. Muhammed R, Bremner R, Protheroe S, Johnson T, Holden C, Murphy MS. Resolution of parenteral nutrition-associated jaundice on changing from a soybean oil emulsion to a complex mixed-ipid emulsion. *J Ped Gastroenterol Nutr* 2012;54:797-802.
12. Prince E, Lazare FB, Treem WR, Xu J, Iqbal J, Pan X, et al. Omega-3 fatty acids prevent hepatic steatosis independent of PPAR-alpha activity, in a murine model of parenteral nutrition-associated liver disease. *JPEN J Parent Ent Nutr* 2014;38:608-616.
13. Calder, P.C. Very long-chain n-3 fatty acids and human health: fact, fiction and the future. *Proc Nutr Soc* 2018;77:52-72.
14. Kotiya P, Zhao X, Cheng P, Zhu X, Xiao Z, Wang J. Fish-oil and soy oil-based lipid emulsions in neonatal parenteral nutrition; a systematic review and meta-analysis. *Eur J Clin Nutr* 2016;70:1106-1115.
15. Nandivada P, Fell GL, Gura KM, Puder M. Lipid emulsions in the treatment and prevention of parenteral nutrition-associated liver disease in infants and children. *Am J Clin Nutr* 2016;103:629S-634S.

16. Edward R-R, Innes JK, Marino LV, Calder PC. Influence of different intravenous lipid emulsions on growth, development and laboratory and clinical outcomes in hospitalised paediatric patients: A systematic review. *Clin Nutr* 2018;37:765-783.
17. Gura KM, Calkins KL, Premkumar MH, Puder M. Use of intravenous soybean and fish oil emulsions in pediatric intestinal failure-associated liver disease: a multicentre integrated analysis report on extrahepatic adverse events. *J Ped* 2022;241:173-180.
18. Fisk HL, West AL, Childs CE, Burdge GC, Calder PC. The use of gas chromatography to analyze compositional changes of fatty acids in rat liver tissue during pregnancy. *J Vis Exp* 2014;13:51445.
19. Mackay DS, Jones PJ, Myrie SB, Plat J, Lütjohann D. Methodological considerations for the harmonization of non-cholesterol sterol bio-analysis. *J Chromatog B* 2014;957:116-122.
20. Clayton PT, Bowron A, Mills KA, Massoud A, Casteels M, Milla P. Phytosterolemia in children with parenteral nutrition-associated cholestatic liver disease. *J Gastroenterol* 1993;105:1806-1813.
21. Sanchez SE, Braun LP, Mercer LD, Sherrill M, Stevens J, Javid PJ. The effect of lipid restriction on the prevention of parenteral nutrition-associated cholestasis in surgical infants. *J Ped Surg* 2013;48:573-578.
22. Nandivada P, Cowan E, Carlson SJ, Chang M, Gura KM, Puder M. Mechanisms for the effects of fish oil lipid emulsions in the management of parenteral nutrition-associated liver disease. *Prostagland Leukotr Essent Fatty Acids* 2013;89:153-158.
23. Carter BA, Taylor OA, Prendergast DR, Zimmerman TL, Von Furstenberg R, Moore DD, Karpen SJ. Stigmasterol, a soy lipid–derived phytosterol, is an antagonist of the bile acid nuclear receptor FXR. *Ped Res* 2007;62:301-306.
24. Mutanen A, Nissinen MJ, Lohi J, Heikkilä P, Gylling H, Pakarinen MP. Serum plant sterols, cholestanol and cholesterol precursors associate with histological liver injury in pediatric onset intestinal failure. *Am J Clin Nutr* 2014;100:1085-1094.
25. Bhogal HK, Sanyal AJ. The molecular pathogenesis of cholestasis in sepsis. *Front Biosci* 2013;5:87-96.
26. Kosters A, Karpen SJ. The role of inflammation in cholestasis: Clinical and basic aspects. *Sem Liver Dis* 2010;30:186-194.
27. Geier A, Fickert P, Trauner M. Mechanisms of disease: Mechanisms and clinical implications of cholestasis in sepsis. *Nat Clin Pract Gastroenterol Hepatol* 2006;3:574-585.
28. Alzoghaibi MA, Walsh SW, Willey A, Yager DR, Fowler AA 3rd, Graham MF. Linoleic acid induces interleukin-8 production by Crohn's human intestinal smooth muscle cells via arachidonic acid metabolites. *Am J Physiol* 2004;286:G528-G537.
29. Leik CE, Walsh SW. Linoleic acid but not oleic acid upregulates production of interleukin-8 by human vascular smooth muscle cells via arachidonic acid metabolites under conditions of oxidative stress. *J Soc Gynecol Invest* 2005;12:593-598.
30. Keller H, Dreyer C, Medin J, Mahfoudi A, Ozato K, Wahli W. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc Natl Acad Sci USA* 1993;90:2160-2164.

31. Botta M, Audano M, Sahebkar A, Sirtori CR, Mitro N, Ruscica M. PPAR agonists and metabolic syndrome: an established role? *Int J Mol Sci* 2018;19:1197.
32. Hodson L, Gunn PJ. The regulation of hepatic fatty acid synthesis and partitioning: the effect of nutritional state. *Nat Rev Endocrinol* 2019;15:689-700.
33. Calder PC. Omega-3 fatty acids and metabolic partitioning of fatty acids within the liver in the context of non-alcoholic fatty liver disease. *Curr Opin Clin Nutr Metab Care* 2022;25:248-255.
34. Kota BP, Huang TH, Roufogalis BD. An overview on biological mechanisms of PPARs. *Pharmacol Res* 2005;51:85-94.

CHAPTER

7

GENERAL DISCUSSION

Cholesterol metabolism and CVD risk

Cardiovascular disease (CVD) is still the major cause of diseases and deaths worldwide, and atherosclerosis is an underlying process in the development of CVD [1, 2]. The role of elevated serum cholesterol concentrations, especially in the low-density lipoproteins (LDL-C), in the atherogenic process is well recognized [3-5].

Cholesterol homeostasis is maintained by the interplay between intestinal cholesterol absorption, endogenous cholesterol synthesis, and bile acid synthesis. Thus, it is important to investigate these processes as part of whole-body cholesterol metabolism into more detail to better understand how to improve cardiovascular health. Nowadays, cholesterol metabolism characteristics receive more and more attention. For example, it has been described that individuals with a relatively high intestinal cholesterol absorption (so-called cholesterol absorbers) have a higher risk of developing CVD [6, 7]. This suggests that targeting cholesterol absorption is an attractive approach in CVD risk management, but it is unknown which lifestyle interventions are suitable for this. Public guidelines in general recommend to increase physical activity and to promote weight loss. In addition, it is postulated that inflammation should be reduced since it plays an important role in atherosclerosis. However, effects of physical activity, diet-induced weight loss and inflammation on cholesterol metabolism characteristics are largely unknown.

Therefore, the main objective of this thesis is to understand changes in cholesterol metabolism characteristics underlying the effects of physical activity and diet-induced weight loss, as well as the pro-inflammatory effects of lipopolysaccharides (LPS) on cholesterol metabolism. In addition, we also evaluated the effect of plant sterol content in three different lipid emulsions used for home parenteral nutrition (HPN) by assessing effects on concentrations of plasma plant sterols, liver function, and inflammatory markers.

An overview of the main results described in this thesis is presented in **Table 1**. First, we carried out a systematic review to summarize the possible use of non-cholesterol sterol concentrations as biomarkers in different metabolic conditions. The main results were that non-cholesterol markers for intestinal cholesterol absorption and endogenous cholesterol synthesis displayed in general a reciprocal relationship. Moreover, distinctive patterns for markers of cholesterol metabolism suggested that various metabolic disorders can be characterized as being associated with either a cholesterol absorber or cholesterol synthesizer phenotype, which theoretically opens possibilities for targeted interventions to improve cholesterol metabolism (**chapter 2**). In the next three chapters, we examined changes in cholesterol metabolism characteristics after aerobic exercise training, diet-induced weight loss, and by triggering a pro-inflammatory condition by LPS infusion

(**chapters 3, 4 and 5**). The randomized crossover trial focusing on the effects of an 8-week aerobic exercise training showed a trend towards a decrease in the cholesterol absorption marker campesterol with no changes in serum TC concentrations or the cholesterol synthesis marker lathosterol. The randomized trial with a parallel design focusing on consumption of a caloric restricted diet to induce weight loss revealed an increase in the cholesterol absorption marker cholestanol and a decrease in the cholesterol synthesis marker lathosterol. Furthermore, after weight loss these markers became comparable to those of normal weight controls. With respect to different fat compartments, changes in cholestanol were positively related to changes in visceral fat volume but not to those in subcutaneous fat volume and intrahepatic lipid content. In the trial evaluating the effects of acute LPS induced transient systemic inflammation, we showed a decrease in various cholesterol synthesis markers, a trend toward a decrease in bile acid formation, while there was no change in the cholesterol absorption markers. Finally, a pilot study comparing three different lipid emulsions in patients with intestinal failure showed that the higher plant sterol content in the Intralipid emulsion was reflected by higher concentrations of plasma plant sterols in patients receiving this emulsion compared to those receiving ClinOleic or SMOFLipid emulsions (**chapter 6**). In addition, plasma concentrations of plant sterols were positively correlated with concentrations of liver function markers. Concentrations of triglycerides and liver function markers were apparently within normal ranges in patients receiving SMOFLipid compared to those receiving ClinOleic or Intralipid emulsions.

In this general discussion, we will discuss amongst others, 1) issues regarding the methodology for assessing characteristics of cholesterol metabolism, 2) possibilities to modify cholesterol metabolism, 3) the effects of physical activity, weight loss and LPS on cholesterol metabolism based on the data from our studies, and 4) the (future) implications of our findings for CVD prevention or treatment.

Table 1. Overview of main findings of the studies included in this thesis

Chapter	Research type	Exposure	Main results
2	Systematic review.	Non-cholesterol sterols concentrations as biomarkers for cholesterol absorption and synthesis in different metabolic disorders.	Non-cholesterol markers for intestinal cholesterol absorption and endogenous cholesterol synthesis displayed in general a reciprocal relationship. Metabolic disorders can be categorized as being related to cholesterol absorption or cholesterol synthesis.
3	Randomized, controlled, crossover in 17 apparently healthy older overweight and obese men.	Effects of an 8-week aerobic exercise program on plasma markers for cholesterol absorption and synthesis.	Aerobic exercise program for 8 weeks tended to decrease serum cholesterol absorption marker campesterol. An 8-week aerobic exercise did not change serum TC concentrations and cholesterol synthesis marker lathosterol.
4	Randomized parallel study with 54 abdominally obese men.	Effects of diet-induced weight loss on plasma markers for cholesterol absorption and synthesis.	Diet induced weight loss for 6 weeks followed by a 2-week weight stabilizing period significantly increased cholesterol absorption marker cholestanol and decreased cholesterol synthesis marker lathosterol. After weight loss, cholesterol absorption marker cholestanol and cholesterol synthesis marker lathosterol in previously abdominally obese men were comparable to those observed in normal weight men. Changes in cholestanol were positively related to changes in visceral fat volume but not to subcutaneous fat volume and intrahepatic lipids content.
5	Data analysis of a previous study for a placebo arm with 8 healthy young men.	Effect of LPS infusion on plasma markers for cholesterol absorption and synthesis.	LPS infusion decreased several cholesterol synthesis markers, but did not affect cholesterol absorption markers. Plasma markers of cholesterol synthesis desmosterol and bile acid synthesis marker 7α-OH-cholesterol were associated with various inflammatory responses.
6	Data analysis from a pilot study with 58 stable adult patients with intestinal failure supported by home parenteral nutrition (HPN).	Effects of plant sterols content in three different lipid emulsions used for HPN on markers of inflammation and liver functions.	Higher plasma plant sterol concentrations were found in adult HPN patients receiving Intralipid compared to those receiving ClinOleic or SMOFLipid emulsions. Plasma plant sterol concentrations were positively correlated with liver function markers.

TC: total cholesterol; LPS: lipopolysaccharides; Intralipid: soybean oil-based emulsion; ClinOleic: olive oil-based emulsion; SMOFLipid: emulsion containing fish oil.

Methodology and phenotyping of cholesterol metabolism characteristics

As discussed in chapter 2 of this thesis, several methods have been developed over the past decades to assess intestinal cholesterol absorption and endogenous cholesterol synthesis [8-12]. These characteristics of cholesterol metabolism can be expressed in absolute or fractional rates. Both radio-active or stable isotope tracers are used [13]. In general, these methods are laborious, expensive, highly invasive and require a new steady state. Fortunately, less laborious alternative approaches using non-cholesterol sterol concentrations for assessing intestinal cholesterol absorption and endogenous cholesterol synthesis have been developed. Using these surrogate markers avoids the complexity and administration of tracers, require only a small blood sample and is easily applicable for large-scale intervention and population studies. The validity of this approach has been shown by positive correlations after analyzing and calculating the ratios of non-cholesterol sterol to cholesterol ratios with the absolute/fractional measurements for intestinal cholesterol absorption and endogenous cholesterol synthesis using tracers [14-16]. The cholesterol-standardized levels of campesterol, sitosterol and cholestanol can be used as markers for intestinal cholesterol absorption[13, 17], while the cholesterol-standardized levels of cholesterol synthesis precursors desmosterol and lathosterol can be used as markers for endogenous synthesis [14, 16, 18, 19]. The validity of these surrogate markers to reflect cholesterol absorption and synthesis was also shown during serum cholesterol-lowering intervention studies [19-21]. Ratios of campesterol to cholesterol, sitosterol to cholesterol, and cholestanol to cholesterol decreased in a population with a normal daily dietary cholesterol intake after treatment with ezetimibe, a drug known to reduce cholesterol absorption [21]. Moreover, ratios of lathosterol to cholesterol and desmosterol to cholesterol decreased in hypercholesteremic men during treatments with two different statins, known to reduce cholesterol synthesis [19]. In addition, the ratio of lathosterol to campesterol has been used to reflect the whole-body cholesterol metabolism [22, 23]. This ratio can be used to characterize individuals into two distinctive cholesterol metabolism phenotypes: a higher lathosterol/campesterol ratio identifies individuals as cholesterol synthesizer, while a lower lathosterol/campesterol ratio is a characteristic linked to the so-called cholesterol absorbers.

By using these non-cholesterol sterols as markers for cholesterol metabolism in various larger studies, it becomes more and more clear that intestinal cholesterol absorption and endogenous cholesterol synthesis are tightly regulated to maintain whole-body cholesterol homeostasis. Moreover, it has been suggested that absorption and synthesis follow a reciprocal relationship [24]. In our systemic literature review (**chapter 2**), we also found evidence for this relationship. For example, obese individuals are characterized by a low intestinal cholesterol absorption and a high endogenous cholesterol synthesis.

Moreover, our diet-induced weight loss intervention resulted in a downregulation of endogenous cholesterol synthesis and an upregulation of intestinal cholesterol absorption (**chapter 4**). Also, other interventions support evidence for this reciprocal relationship [25]. Consumption of foods enriched in either plant sterol or stanol esters demonstrated a reduction in intestinal cholesterol absorption with a compensatory increase in endogenous cholesterol synthesis [26-29]. The net effect of the reduced absorption and increased synthesis is a reduction in serum LDL-C concentrations [30]. In line with these effects, also a study evaluating the effects of ezetimibe, a drug that lowers intestinal cholesterol absorption by inhibiting the cholesterol transporter Niemann-Pick C1-Like 1 (NPC1L1), showed a compensatory increase in cholesterol synthesis [31]. In contrast, treatment with Hydroxymethyl-glutaryl (HMG)-CoA reductase inhibitors or the so-called statins, drugs used to inhibit endogenous cholesterol synthesis, showed in the majority of the studies a compensatory increase in intestinal cholesterol absorption [32-34]. Taken together, the findings from these studies and **chapters 2 and 4** suggest indeed a reciprocal relationship between cholesterol absorption and synthesis in order to maintain whole body cholesterol homeostasis.

Even though non-cholesterol sterol concentrations are widely applied in ongoing intervention and prospective cohort studies to reflect (changes in) cholesterol metabolism characteristics, there are also limitations of these measurements. A recent survey by Lütjohann et al. reported unacceptable high variations for analyzing both cholesterol and non-cholesterol concentrations between several laboratories [35]. This illustrates that it is difficult to work with “normal values” for non-cholesterol sterol concentrations, which also hampers defining clear cut-off values to identify cholesterol absorbers and synthesizers.

Possibilities to modify characteristics of cholesterol metabolism

Inhibition of cholesterol absorption

As described above, having a high intestinal cholesterol absorption has been associated with an increased CVD risk [36]. This may suggest the need for approaches that interfere with intestinal cholesterol absorption to prevent CVD. Several possibilities have shown to reduce intestinal cholesterol absorption such as foods enriched with plant sterol and stanol esters as well as ezetimibe treatment. The transporters involved in intestinal cholesterol metabolism, i.e., heterodimer ATP-binding cassette (ABC) transporters ABCG5/G8 and NPC1L1, also control intestinal plant sterol metabolism [31, 37]. Due to the comparable structure of plant sterols and stanols to cholesterol, these plant-based sterols compete with cholesterol for incorporation into mixed micelles at the small intestinal

leading to displacement of micellar cholesterol [29, 38, 39]. This means that there is a lower bioavailability of micellar cholesterol for absorption into the enterocytes. Numerous intervention studies have indeed demonstrated that both plant sterol or stanol esters lower intestinal cholesterol absorption [40-43], which translates into lower serum LDL-C concentrations. Similar effects have been shown for the pharmaceutical drug ezetimibe, which is known to inhibit intestinal cholesterol absorption via inhibiting the function of the NPC1L1 transporter at the apical side of the enterocyte [44, 45]. Several studies have indeed shown that ezetimibe treatment also translates into lower serum LDL-C concentrations [46-49]. In general, plant sterols and stanols lower LDL-C up to 12% [30] whereas ezetimibe treatment lowers serum LD-C by around 19% [50]. Interestingly the combination of sterols and ezetimibe showed additive effects since LDL-C concentrations were further reduced when sterols were added to ezetimibe treatment [51]. This suggests that plant sterols can be used as add-on interventions, and that plant sterol and ezetimibe interventions target different underlying mechanisms.

Inhibition of cholesterol synthesis

Elevated serum LDL-C concentrations can also be lowered by statins that inhibit the function of HMG-CoA reductase, which is the rate-limiting enzyme for endogenous cholesterol synthesis. Studies have shown that statin therapy indeed reduced endogenous cholesterol synthesis [34, 52, 53]. However, due to low effectiveness of further increasing statin dose in those not reaching target LDL-C values, combination therapies that modulate simultaneously cholesterol synthesis and absorption seem more favorable [54, 55]. In fact, as statin treatment will slightly elevate intestinal cholesterol absorption, using plant sterols or stanols as combination strategy to counteract this increase in cholesterol absorption maybe helpful. Indeed, in statin users, additional cholesterol-lowering effects on top of those of statins after long-term plant sterol or stanol ester consumption of 8.7% and 13.1%, respectively were found [56]. In randomized placebo-controlled studies, the additive effect of plant sterol or stanols on reduction in LDL-C has been shown, which ranged between 7% to 11% [30, 57-60].

Other “novel” treatment options

Besides plant sterols and stanols, also other dietary ingredients and supplements may have beneficial effects on serum LDL-C concentrations, amongst others red yeast rice, soluble fibers, soy protein, probiotics, berberine, bergamot and policosanols [61]. However, the magnitude of their LDL-C lowering effects and evidence levels for their efficacy are different [61]. When effective, these functional foods and supplements can also be used as an add-on therapy to drug interventions, i.e., either ezetimibe or statins, to achieve LDL-C goals. However, this has not been explored into detail for most ingredients. Furthermore, novel pharmacological therapies such as proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors and bempedoic acid have proven benefits on serum LDL-C

lowering by upregulating the expression of hepatic LDL receptors [62]. Using these two drugs either alone or in combination to statin and/or ezetimibe treatment resulted in significant reductions in LDL-C levels [63-67], which can be explained by the fact that other enzymes are targeted. Since bempedoic acid lowers endogenous cholesterol synthesis at the level of adenosine triphosphate (ATP) citrate synthase [68], which is one step earlier as compared to the action of statins, it can be expected that cholesterol absorption will be increased as is known for the statins. However, bempedoic acid treatment has not been explored yet using non-cholesterol sterols biomarkers for cholesterol absorption and synthesis. A treatment with a PCSK9 inhibitor has shown significant reductions in absolute concentrations for cholesterol absorption and synthesis markers while the effects on their cholesterol-standardized levels were not consistent [69]. In another study, significant reductions in absolute values for cholesterol absorption markers were reported after PCSK9 treatment, but cholesterol-standardized levels of these markers did not reach significance [70]. Thus, the effects of inhibiting PCSK9 (which results in overexpression hepatic LDL receptors) on cholesterol absorption or synthesis is not conclusive.

Effects of physical activity, weight loss and LPS infusion on cholesterol metabolism

We evaluated the effects of A) aerobic exercise training, B) diet-induced weight loss, and C) LPS infusion on markers for cholesterol metabolism, i.e., absorption and synthesis. It is well known that increased physical activity translates into a decreased risk to develop CVD [71]. In **chapter 3** of this thesis, we explored whether this relation may be mediated through effects on characteristics of cholesterol metabolism. An earlier small-scale study using stable isotope methodology showed that 8 weeks of aerobic exercise had no effects on cholesterol absorption and cholesterol synthesis in hypercholesteremic participants [72]. However, an endurance exercise program for 6 months showed an increase in the intestinal cholesterol absorption marker campesterol, with no change in the cholesterol synthesis marker lathosterol [73]. Finally, 8 weeks of combined resistance and aerobic exercise resulted in a reduction of the cholesterol synthesis marker desmosterol without changing cholesterol absorption [74]. We proposed that factors such as sample size, lack of control group, type of exercise intervention and method used to quantify cholesterol metabolism markers might explain the discrepant findings in these earlier studies. Therefore, we decided to conduct a more controlled intervention study taking in consideration the above-mentioned attention points. **In chapter 3** of this thesis, we presented the data of 17 apparently healthy older overweight and obese men that were randomized to start with aerobic exercise or no-exercise control period for 8 weeks. We showed a trend towards a decrease in the cholesterol absorption marker campesterol with no changes in serum TC concentrations and the cholesterol synthesis marker lathosterol

(**Table 1**). We did not observe a change in body weight and dietary habits after the 8-weeks aerobic exercise program. Sedentary time and physical activity levels of the subjects was also monitored, but did not differ between the intervention and control periods. This may suggest that our study has a better estimate regarding the effects of aerobic exercise training itself on cholesterol metabolism characteristics as compared with other studies [72-74]. In **chapter 2** we showed that individuals with obesity were characterized with a lower intestinal cholesterol absorption and higher endogenous cholesterol synthesis compared to controls. As not much was known about the effect of weight loss and the role of different fat compartments on cholesterol metabolism, we investigated the effects of diet-induced weight loss on markers of cholesterol metabolism in 54 men with abdominal obesity (**chapter 4**). Subjects followed a 6-week very low caloric diet (VCLD), immediately followed by a 2-weeks weight maintenance period. We found that cholesterol absorption marker cholestanol increased and cholesterol synthesis marker lathosterol decreased after 8 weeks, suggesting a normalization of cholesterol metabolism characteristics in these previous abdominally obese subjects. The validity of other cholesterol absorption markers including campesterol and sitosterol can be questioned in this particular study design. Due to the abundance of campesterol and sitosterol in the plant-based diet and the change in habitual dietary intake throughout this study, their concentrations may not be truly reflecting intestinal cholesterol absorption. In addition, in this population the possible relationship between cholesterol absorption and synthesis with fat distribution was examined. We observed a positive association between cholestanol changes with weight loss as well as with a decrease in visceral fat volume. We then performed a mediation analysis to evaluate in detail the mediating role of different fat compartments on the relationships between body mass index (BMI) and cholesterol absorption and synthesis. The results of mediation analysis suggested roles of visceral fat and intrahepatic fat in mediating the relationships between BMI and cholesterol absorption and synthesis. This may indicate that it is likely that the effect of weight loss on cholesterol metabolism can be attributed to changes in fat compartments. These findings are highly relevant, because visceral fat and intrahepatic fat depots are associated with CVD risk [75, 76]. In **chapter 5**, the effect of LPS infusion on characteristics of cholesterol metabolism was investigated in 8 healthy young men. Levels of all three cholesterol synthesis markers (lathosterol, desmosterol and lanosterol) as well as the bile acid formation marker 7 alpha hydroxycholesterol (7α-OH-cholesterol) both decreased 24 hours after LPS infusion, whereas the three cholesterol absorption markers (campesterol, sitosterol, and cholestanol) that were analyzed did not change. This means that acute inflammation was associated with a reduction in cholesterol synthesis, which is likely explaining the reduction in total and LDL-C observed in the subjects. Information about the effect of different content of phytosterols in lipid emulsions used in home parental nutrition in adult patients is lacking. We measured the plasma plant sterol concentrations in patients with intestinal failure receiving one of three different lipid emulsions (soybean oil based:

Intralipid; olive oil based: ClinOleic; containing fish oil: SMOFLipid) (**chapter 6**). We also examined the relationship between plasma plant sterol levels with those of inflammatory cytokines and liver function markers. Due to its higher content of plant sterols, plasma plant sterol levels were higher in patients receiving Intralipid compared to those receiving ClinOleic or SMOFLipid. In addition, plasma plant sterol levels were positively related with liver function markers. The effects of long-term use of parental nutrition on liver function in patients with intestinal failure are well known [77]. However, the mechanism behind the effects of plant sterols (particularly stigmasterol) on liver function is not clear. Intravenous lipid infusion may cause hepatic accumulation of stigmasterol, leading to reduction of bile flow [78, 79]. This accumulation might particularly occur during infusion of free sterols as found in the TPN. The physiology of this route of administration is markedly different from that after consuming plant sterols via the diet, as plant sterols then enter the circulation at much lower levels and as part of the chylomicrons. Liver function markers were apparently within normal ranges in patients receiving SMOFLipid compared to those receiving ClinOleic or Intralipid emulsions. This protection could possibly be explained by the presence of the omega-3 fatty acids in SMOFLipid emulsion, which are known for their anti-inflammatory effects [80].

Possible implications of our findings for CVD prevention or treatment

Recent studies have suggested that a high intestinal cholesterol absorption may be atherogenic [81, 82]. In addition, it is estimated that approximately 30% of the population is characterized by a high intestinal cholesterol absorption, which is associated with a 2-fold increased CVD risk [6]. This suggests that interventions through lowering cholesterol absorption may have the potential to reduce CVD risk. The data from our systematic review indicated that we could classify individuals with different metabolic disturbance as having the so-called preferential cholesterol absorber or cholesterol synthesizer phenotype. Individuals with obesity, type 2 diabetes, metabolic syndrome, intestinal or liver disease can be categorized as being mainly a cholesterol synthesizer, while those with type 1 diabetes, non-hereditary hyperlipidemia and kidney diseases as being mainly a cholesterol absorber (**chapter 2**). Based on these observations, various personalized or targeted interventions might be advised to the cholesterol synthesizers phenotype populations, such as statins. For example, data from a subgroup of the Scandinavian Simvastatin Survival Study (4S) in coronary patients indicated that subjects with a high baseline cholesterol synthesis markers were more responsive for statin treatment compared to those with a high baseline cholesterol absorption [7]. Moreover, cholesterol absorbers might benefit more from personalized (dietary) interventions affecting cholesterol absorption such as plant sterol or stanol ester enriched functional foods,

viscous fibers or pharmacological drugs like ezetimibe. The need for combination therapy for subjects with low cholesterol synthesis and high cholesterol absorption has also been suggested. In fact, for hypercholesterolemic subjects who are poor statin responders, and characterized as high cholesterol absorbers and low cholesterol synthesizer, a combination drug therapy (statin and ezetimibe) was most effective [83, 84]. Hemodialysis patients, who are characterized as high cholesterol absorbers, benefit less from statin treatment compared to other patients with cardiovascular risk [85-88]. However, it was shown that hemodialysis patients from the lowest cholesterol absorption tertile appeared to benefit from statin treatment and had a reduction in all-cause mortality and all cardiac events risk [36]. In addition, hypercholesterolemic patients with high cholesterol absorption and low cholesterol synthesis showed the largest LDL-C reductions after plant sterols and stanols supplementation[23].

As indicated in our systemic review, cholesterol absorption is decreased and synthesis is increased in overweight and obese subjects, in type 2 diabetes patients, and in metabolic syndrome subjects, while most studies suggest that patients with CVD have higher cholesterol absorption and lower cholesterol synthesis (**chapter 2**). This concept remains difficult to reconcile with the low absorption/high synthesis pattern in subjects with obesity and/or type 2 diabetes, who are also at increased risk to develop CVD. We aimed to elucidate this notion in our weight loss trial, where we demonstrated that obese subjects have a lower cholesterol absorption, which normalized compared to the lean population after weight loss (**chapter 4**). However, in terms in CVD risk, an increase in cholesterol absorption would translate into a higher CVD risk, which is obviously not the case after weight loss. This demonstrates that, although cholesterol metabolism is important in assessing CVD risk, more factors should be taken into account, such as improvements in flow-mediated dilation and blood pressure as shown in the weight loss trial. Ultimately, non-cholesterol sterols can be used to classify individuals as a cholesterol absorber or synthesizer to determine a targeted cholesterol-lowering (dietary) strategy, while the assessment of CVD risk involves more factors than only cholesterol metabolism.

Main conclusions and future directions

The studies described in this thesis focused on effects of aerobic exercise training, diet-induced weight loss and LPS infusion on markers reflecting cholesterol metabolism. In particular attention was paid to well-validated markers reflecting cholesterol absorption and synthesis. In the systematic review, there was a clear inverse relationship between non-cholesterol sterol biomarkers for intestinal cholesterol absorption and endogenous cholesterol synthesis in different metabolic disturbances (**chapter 2**). This distinctive pattern of cholesterol metabolism characteristics in these metabolic disorders could be

a potential lead for targeted dietary or pharmacological interventions. Evaluating effects of aerobic exercise training for 8 weeks tended to decrease cholesterol absorption and had no effect on cholesterol synthesis (**chapter 3**). Diet-induced weight reduction after 6 weeks of caloric restrict diet followed by 2 weeks of weight maintenance increased cholesterol absorption and decreased cholesterol synthesis. In addition, cholesterol absorption marker cholestanol was not only related to weight loss parameters but also to visceral fat volume (**chapter 4**). LPS infusion decreased cholesterol synthesis and bile acid formation, while no effect was found on cholesterol absorption (**chapter 5**). Finally, the plant sterol content of different lipid emulsions used for home adult parental nutrition was reflected in plasma plant sterol concentrations, which correlated with inflammatory cytokines and liver function markers (**chapter 6**). This indicates that evaluating markers for cholesterol absorption and synthesis add relevant knowledge to discussions around CVD risk lowering interventions. However, the variability in measuring techniques between labs warrants attention.

Although the evidence for the relationship between cholesterol metabolism characteristics and CVD risk is accumulating, causality has not been proven. Future studies should be carried out to assess whether alterations in non-cholesterol sterols relate to CVD risk. In addition, non-cholesterol sterols were only determined in male subjects in the aerobic exercise and weight loss interventions of this thesis. Analyzing these sterols in female subjects would expand our knowledge on the role of gender in cholesterol metabolism characteristics. Furthermore, sample size in the exercise intervention study was relatively small and the effect on cholesterol absorption did not reach significance. Larger trials should also investigate the effects of different types of exercise on changes in cholesterol metabolism characteristics. Moreover, the exercise and weight loss studies in this thesis have only included a population of older apparently healthy adults and it remains to be determined to what extent our findings can be extended to other populations. In addition, variations in race and gender have been associated with different inflammatory responses during an inflammatory trigger [89]. Future studies need to investigate the effects of other populations and gender in relation to the changes in cholesterol metabolism characteristics in response to inflammatory triggers. It has been shown that desmosterol is a strong activator for liver X receptors in animals and humans in in vitro experiments [90, 91], which integrates the interplay between cholesterol homeostasis and immune responses [92]. Development of LPS tolerance, which is defined as a reduction in response to a subsequent LPS trigger, was associated with attenuation in pro and anti-inflammatory cytokines responses after 5 consecutive days of LPS infusion in humans [93]. It would therefore be interesting to investigate the effects of longer-term trials for LPS infusion on cholesterol metabolism characteristics and whether the conclusion might differ. In **chapter 6**, the content of phytosterols in Intralipid emulsion, that was higher compared to those sterols in ClinOleic or SMOFLipid, related to higher liver function

markers. Future research investigating the effects of different types of emulsion with low versus high phytosterol content on liver and inflammatory markers is warranted to confirm the adverse effects of phytosterols.

To summarize, in this thesis the effects of lifestyle, nutrition and inflammation on cholesterol metabolism characteristics have been studied. These findings add information to the possibility to develop more targeted interventions for CVD prevention or treatment.

References

1. Frostegard J. Immunity, atherosclerosis and cardiovascular disease. BMC Med. 2013;11:117.
2. World Health Organization (WHO). Cardiovascular diseases (CVDs) fact sheet. June 2021. Available from: https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).
3. Boren J, Chapman MJ, Krauss RM, Packard CJ, Bentzon JF, Binder CJ, et al. Low-density lipoproteins cause atherosclerotic cardiovascular disease: pathophysiological, genetic, and therapeutic insights: a consensus statement from the European Atherosclerosis Society Consensus Panel. Eur Heart J. 2020;41(24):2313-30.
4. Gidding SS, Allen NB. Cholesterol and Atherosclerotic Cardiovascular Disease: A Lifelong Problem. J Am Heart Assoc. 2019;8(11):e012924.
5. Zarate A, Manuel-Apolinar L, Saucedo R, Hernandez-Valencia M, Basurto L. Hypercholesterolemia As a Risk Factor for Cardiovascular Disease: Current Controversial Therapeutic Management. Arch Med Res. 2016;47(7):491-5.
6. Helgadottir A, Thorleifsson G, Alexandersson KF, Tragante V, Thorsteinsdottir M, Eiriksson FF, et al. Genetic variability in the absorption of dietary sterols affects the risk of coronary artery disease. Eur Heart J. 2020;41(28):2618-28.
7. Miettinen TA, Gylling H, Strandberg T, Sarna S. Baseline serum cholestanol as predictor of recurrent coronary events in subgroup of Scandinavian simvastatin survival study. Finnish 4S Investigators. BMJ. 1998;316(7138):1127-30.
8. Crouse JR, Grundy SM. Evaluation of a continuous isotope feeding method for measurement of cholesterol absorption in man. J Lipid Res. 1978;19(8):967-71.
9. Grundy SM, Ahrens EH, Jr., Miettinen TA. Quantitative Isolation and Gas--Liquid Chromatographic Analysis of Total Fecal Bile Acids. J Lipid Res. 1965;6:397-410.
10. Grundy SM, Mok HY. Determination of cholesterol absorption in man by intestinal perfusion. J Lipid Res. 1977;18(2):263-71.
11. Lutjohann D, Meese CO, Crouse JR, 3rd, von Bergmann K. Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in humans. J Lipid Res. 1993;34(6):1039-46.
12. Miettinen TA, Ahrens EH, Jr., Grundy SM. Quantitative Isolation and Gas--Liquid Chromatographic Analysis of Total Dietary and Fecal Neutral Steroids. J Lipid Res. 1965;6:411-24.
13. Mackay D, Jones PJ. Evaluation of methods for the determination of cholesterol absorption and synthesis in humans. Atherosclerosis. 2011;218(2):253-62.
14. Matthan NR, Raeini-Sarjaz M, Lichtenstein AH, Ausman LM, Jones PJ. Deuterium uptake and plasma cholesterol precursor levels correspond as methods for measurement of endogenous cholesterol synthesis in hypercholesterolemic women. Lipids. 2000;35(9):1037-44.
15. Miettinen TA, Tilvis RS, Kesaniemi YA. Serum cholestanol and plant sterol levels in relation to cholesterol metabolism in middle-aged men. Metabolism. 1989;38(2):136-40.

16. Miettinen TA, Tilvis RS, Kesaniemi YA. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. Am J Epidemiol. 1990;131(1):20-31.
17. Miettinen TA, Gylling H, Nissinen MJ. The role of serum non-cholesterol sterols as surrogate markers of absolute cholesterol synthesis and absorption. Nutr Metab Cardiovasc Dis. 2011;21(10):765-9.
18. Kempen HJ, Glatz JF, Gevers Leuven JA, van der Voort HA, Katan MB. Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans. J Lipid Res. 1988;29(9):1149-55.
19. Nissinen MJ, Miettinen TE, Gylling H, Miettinen TA. Applicability of non-cholesterol sterols in predicting response in cholesterol metabolism to simvastatin and fluvastatin treatment among hypercholesterolemic men. Nutr Metab Cardiovasc Dis. 2010;20(5):308-16.
20. Clarenbach JJ, Reber M, Lutjohann D, von Bergmann K, Sudhop T. The lipid-lowering effect of ezetimibe in pure vegetarians. J Lipid Res. 2006;47(12):2820-4.
21. Stellaard F, Lutjohann D. The Interpretation of Cholesterol Balance Derived Synthesis Data and Surrogate Noncholesterol Plasma Markers for Cholesterol Synthesis under Lipid Lowering Therapies. Cholesterol. 2017;2017:5046294.
22. Matthan NR, Pencina M, LaRocque JM, Jacques PF, D'Agostino RB, Schaefer EJ, et al. Alterations in cholesterol absorption/synthesis markers characterize Framingham offspring study participants with CHD. J Lipid Res. 2009;50(9):1927-35.
23. Thuluva SC, Igel M, Giesa U, Lutjohann D, Sudhop T, von Bergmann K. Ratio of lathosterol to campesterol in serum predicts the cholesterol-lowering effect of sitostanol-supplemented margarine. Int J Clin Pharmacol Ther. 2005;43(7):305-10.
24. Alphonse PA, Jones PJ. Revisiting Human Cholesterol Synthesis and Absorption: The Reciprocity Paradigm and its Key Regulators. Lipids. 2016;51(5):519-36.
25. Simonen P, Gylling H, Miettinen TA. Acute effects of weight reduction on cholesterol metabolism in obese type 2 diabetes. Clinica Chimica Acta. 2002;316(1-2):55-61.
26. Gylling H, Radhakrishnan R, Miettinen TA. Reduction of serum cholesterol in postmenopausal women with previous myocardial infarction and cholesterol malabsorption induced by dietary sitostanol ester margarine: women and dietary sitostanol. Circulation. 1997;96(12):4226-31.
27. Jakulj L, Trip MD, Sudhop T, von Bergmann K, Kastelein JJ, Vissers MN. Inhibition of cholesterol absorption by the combination of dietary plant sterols and ezetimibe: effects on plasma lipid levels. J Lipid Res. 2005;46(12):2692-8.
28. Jones PJ, Raeini-Sarjaz M, Ntanios FY, Vanstone CA, Feng JY, Parsons WE. Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. J Lipid Res. 2000;41(5):697-705.
29. Vanstone CA, Raeini-Sarjaz M, Parsons WE, Jones PJ. Unesterified plant sterols and stanols lower LDL-cholesterol concentrations equivalently in hypercholesterolemic persons. Am J Clin Nutr. 2002;76(6):1272-8.

30. Gylling H, Plat J, Turley S, Ginsberg HN, Ellegard L, Jessup W, et al. Plant sterols and plant stanols in the management of dyslipidaemia and prevention of cardiovascular disease. Atherosclerosis. 2014;232(2):346-60.
31. Sudhop T, Lutjohann D, Kodal A, Igel M, Tribble DL, Shah S, et al. Inhibition of intestinal cholesterol absorption by ezetimibe in humans. Circulation. 2002;106(15):1943-8.
32. Gylling H, Miettinen TA. Baseline intestinal absorption and synthesis of cholesterol regulate its response to hypolipidaemic treatments in coronary patients. Atherosclerosis. 2002;160(2):477-81.
33. Miettinen TA, Gylling H. Synthesis and absorption markers of cholesterol in serum and lipoproteins during a large dose of statin treatment. Eur J Clin Invest. 2003;33(11):976-82.
34. Miettinen TA, Strandberg TE, Gylling H. Noncholesterol sterols and cholesterol lowering by long-term simvastatin treatment in coronary patients: relation to basal serum cholestanol. Arterioscler Thromb Vasc Biol. 2000;20(5):1340-6.
35. Lutjohann D, Bjorkhem I, Friedrichs S, Kerksiek A, Lovgren-Sandblom A, Geilenkeuser WJ, et al. First international descriptive and interventional survey for cholesterol and non-cholesterol sterol determination by gas- and liquid-chromatography-Urgent need for harmonisation of analytical methods. J Steroid Biochem Mol Biol. 2019;190:115-25.
36. Silbernagel G, Fauler G, Genser B, Drechsler C, Krane V, Scharnagl H, et al. Intestinal cholesterol absorption, treatment with atorvastatin, and cardiovascular risk in hemodialysis patients. J Am Coll Cardiol. 2015;65(21):2291-8.
37. Berge KE, von Bergmann K, Lutjohann D, Guerra R, Grundy SM, Hobbs HH, et al. Heritability of plasma noncholesterol sterols and relationship to DNA sequence polymorphism in ABCG5 and ABCG8. J Lipid Res. 2002;43(3):486-94.
38. Calpe-Berdiel L, Escola-Gil JC, Blanco-Vaca F. New insights into the molecular actions of plant sterols and stanols in cholesterol metabolism. Atherosclerosis. 2009;203(1):18-31.
39. Mel'nikov SM, Seijen ten Hoorn JW, Eijkelenboom AP. Effect of phytosterols and phytostanols on the solubilization of cholesterol by dietary mixed micelles: an in vitro study. Chem Phys Lipids. 2004;127(2):121-41.
40. Demonty I, Ras RT, van der Knaap HC, Duchateau GS, Meijer L, Zock PL, et al. Continuous dose-response relationship of the LDL-cholesterol-lowering effect of phytosterol intake. J Nutr. 2009;139(2):271-84.
41. Katan MB, Grundy SM, Jones P, Law M, Miettinen T, Paoletti R, et al. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. Mayo Clin Proc. 2003;78(8):965-78.
42. Musa-Veloso K, Poon TH, Elliot JA, Chung C. A comparison of the LDL-cholesterol lowering efficacy of plant stanols and plant sterols over a continuous dose range: results of a meta-analysis of randomized, placebo-controlled trials. Prostaglandins Leukot Essent Fatty Acids. 2011;85(1):9-28.

43. Ras RT, Geleijnse JM, Trautwein EA. LDL-cholesterol-lowering effect of plant sterols and stanols across different dose ranges: a meta-analysis of randomised controlled studies. Br J Nutr. 2014;112(2):214-9.
44. Altmann SW, Davis HR, Jr., Zhu LJ, Yao X, Hoos LM, Tetzloff G, et al. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. Science. 2004;303(5661):1201-4.
45. Davies JP, Scott C, Oishi K, Liapis A, Ioannou YA. Inactivation of NPC1L1 causes multiple lipid transport defects and protects against diet-induced hypercholesterolemia. J Biol Chem. 2005;280(13):12710-20.
46. Dujovne CA, Ettinger MP, McNeer JF, Lipka LJ, LeBeaut AP, Suresh R, et al. Efficacy and safety of a potent new selective cholesterol absorption inhibitor, ezetimibe, in patients with primary hypercholesterolemia. Am J Cardiol. 2002;90(10):1092-7.
47. Kalogirou M, Tsimihodimos V, Gazi I, Filippatos T, Saougos V, Tselepis AD, et al. Effect of ezetimibe monotherapy on the concentration of lipoprotein subfractions in patients with primary dyslipidaemia. Curr Med Res Opin. 2007;23(5):1169-76.
48. Knopp RH, Gitter H, Truitt T, Bays H, Manion CV, Lipka LJ, et al. Effects of ezetimibe, a new cholesterol absorption inhibitor, on plasma lipids in patients with primary hypercholesterolemia. Eur Heart J. 2003;24(8):729-41.
49. Ose L, Shah A, Davies MJ, Rotonda J, Maccubbin D, Tribble D, et al. Consistency of lipid-altering effects of ezetimibe/simvastatin across gender, race, age, baseline low density lipoprotein cholesterol levels, and coronary heart disease status: results of a pooled retrospective analysis. Curr Med Res Opin. 2006;22(5):823-35.
50. Jeu L, Cheng JW. Pharmacology and therapeutics of ezetimibe (SCH 58235), a cholesterol-absorption inhibitor. Clin Ther. 2003;25(9):2352-87.
51. Lin X, Racette SB, Lefevre M, Ma L, Spearie CA, Steger-May K, et al. Combined effects of ezetimibe and phytosterols on cholesterol metabolism: a randomized, controlled feeding study in humans. Circulation. 2011;124(5):596-601.
52. Miettinen TA, Gylling H, Lindbohm N, Miettinen TE, Rajaratnam RA, Relas H, et al. Serum noncholesterol sterols during inhibition of cholesterol synthesis by statins. J Lab Clin Med. 2003;141(2):131-7.
53. Smith JL, Roach PD, Wittenberg LN, Riottot M, Pillay SP, Nestel PJ, et al. Effects of simvastatin on hepatic cholesterol metabolism, bile lithogenicity and bile acid hydrophobicity in patients with gallstones. J Gastroenterol Hepatol. 2000;15(8):871-9.
54. Gylling H. Cholesterol metabolism and its implications for therapeutic interventions in patients with hypercholesterolaemia. Int J Clin Pract. 2004;58(9):859-66.
55. Santosa S, Varady KA, AbuMweis S, Jones PJ. Physiological and therapeutic factors affecting cholesterol metabolism: does a reciprocal relationship between cholesterol absorption and synthesis really exist? Life Sci. 2007;80(6):505-14.
56. de Jong A, Plat J, Lutjohann D, Mensink RP. Effects of long-term plant sterol or stanol ester consumption on lipid and lipoprotein metabolism in subjects on statin treatment. Br J Nutr. 2008;100(5):937-41.

57. Blair SN, Capuzzi DM, Gottlieb SO, Nguyen T, Morgan JM, Cater NB. Incremental reduction of serum total cholesterol and low-density lipoprotein cholesterol with the addition of plant stanol ester-containing spread to statin therapy. Am J Cardiol. 2000;86(1):46-52.
58. Neil HA, Meijer GW, Roe LS. Randomised controlled trial of use by hypercholesterolaemic patients of a vegetable oil sterol-enriched fat spread. Atherosclerosis. 2001;156(2):329-37.
59. O'Neill FH, Brynes A, Mandeno R, Rendell N, Taylor G, Seed M, et al. Comparison of the effects of dietary plant sterol and stanol esters on lipid metabolism. Nutr Metab Cardiovasc Dis. 2004;14(3):133-42.
60. Simons LA. Additive effect of plant sterol-ester margarine and cerivastatin in lowering low-density lipoprotein cholesterol in primary hypercholesterolemia. Am J Cardiol. 2002;90(7):737-40.
61. Baumgartner S, Bruckert E, Gallo A, Plat J. The position of functional foods and supplements with a serum LDL-C lowering effect in the spectrum ranging from universal to care-related CVD risk management. Atherosclerosis. 2020;311:116-23.
62. Rubino J, MacDougall DE, Sterling LR, Kelly SE, McKenney JM, Lalwani ND. Lipid lowering with bempedoic acid added to a proprotein convertase subtilisin/kexin type 9 inhibitor therapy: A randomized, controlled trial. J Clin Lipidol. 2021;15(4):593-601.
63. Ballantyne CM, Banach M, Mancini GBJ, Lepor NE, Hanselman JC, Zhao X, et al. Efficacy and safety of bempedoic acid added to ezetimibe in statin-intolerant patients with hypercholesterolemia: A randomized, placebo-controlled study. Atherosclerosis. 2018;277:195-203.
64. Ballantyne CM, Laufs U, Ray KK, Leiter LA, Bays HE, Goldberg AC, et al. Bempedoic acid plus ezetimibe fixed-dose combination in patients with hypercholesterolemia and high CVD risk treated with maximally tolerated statin therapy. Eur J Prev Cardiol. 2020;27(6):593-603.
65. Goldberg AC, Leiter LA, Stroes ESG, Baum SJ, Hanselman JC, Bloedon LT, et al. Effect of Bempedoic Acid vs Placebo Added to Maximally Tolerated Statins on Low-Density Lipoprotein Cholesterol in Patients at High Risk for Cardiovascular Disease: The CLEAR Wisdom Randomized Clinical Trial. JAMA. 2019;322(18):1780-8.
66. Latimer J, Batty JA, Neely RD, Kunadian V. PCSK9 inhibitors in the prevention of cardiovascular disease. J Thromb Thrombolysis. 2016;42(3):405-19.
67. Laufs U, Banach M, Mancini GBJ, Gaudet D, Bloedon LT, Sterling LR, et al. Efficacy and Safety of Bempedoic Acid in Patients With Hypercholesterolemia and Statin Intolerance. J Am Heart Assoc. 2019;8(7):e011662.
68. Penson P, McGowan M, Banach M. Evaluating bempedoic acid for the treatment of hyperlipidaemia. Expert Opin Investig Drugs. 2017;26(2):251-9.
69. Peach M, Xu R, Fitzpatrick D, Hamilton L, Somaratne R, Scott R, et al. Effect of evolocumab on cholesterol synthesis and absorption. J Lipid Res. 2016;57(12):2217-24.
70. Brandt EJ, Benes LB, Lee L, Dayspring TD, Sorrentino M, Davidson M. The Effect of Proprotein Convertase Subtilisin/Kexin Type 9 Inhibition on Sterol Absorption Markers in a Cohort of Real-World Patients. J Cardiovasc Pharmacol Ther. 2019;24(1):54-61.

71. Mora S, Cook N, Buring JE, Ridker PM, Lee IM. Physical activity and reduced risk of cardiovascular events: potential mediating mechanisms. Circulation. 2007;116(19):2110-8.
72. Varady KA, Houweling AH, Jones PJ. Effect of plant sterols and exercise training on cholesterol absorption and synthesis in previously sedentary hypercholesterolemic subjects. Transl Res. 2007;149(1):22-30.
73. Wilund KR, Feeney LA, Tomayko EJ, Weiss EP, Hagberg JM. Effects of endurance exercise training on markers of cholesterol absorption and synthesis. Physiological research. 2009;58(4):545-52.
74. Cho AR, Moon JY, Kim S, An KY, Oh M, Jeon JY, et al. Effects of alternate day fasting and exercise on cholesterol metabolism in overweight or obese adults: A pilot randomized controlled trial. Metabolism. 2019;93:52-60.
75. Lee JJ, Pedley A, Hoffmann U, Massaro JM, Levy D, Long MT. Visceral and Intrahepatic Fat Are Associated with Cardiometabolic Risk Factors Above Other Ectopic Fat Depots: The Framingham Heart Study. Am J Med. 2018;131(6):684-92 e12.
76. Liu J, Fox CS, Hickson D, Bidulescu A, Carr JJ, Taylor HA. Fatty liver, abdominal visceral fat, and cardiometabolic risk factors: the Jackson Heart Study. Arterioscler Thromb Vasc Biol. 2011;31(11):2715-22.
77. Beath SV, Kelly DA. Total Parenteral Nutrition-Induced Cholestasis: Prevention and Management. Clin Liver Dis. 2016;20(1):159-76.
78. Clayton PT, Whitfield P, Iyer K. The role of phytosterols in the pathogenesis of liver complications of pediatric parenteral nutrition. Nutrition. 1998;14(1):158-64.
79. Gabe SM. Lipids and liver dysfunction in patients receiving parenteral nutrition. Curr Opin Clin Nutr Metab Care. 2013;16(2):150-5.
80. Calder PC. Omega-3 fatty acids and inflammatory processes: from molecules to man. Biochem Soc Trans. 2017;45(5):1105-15.
81. Zhong VW, Van Horn L, Cornelis MC, Wilkins JT, Ning H, Carnethon MR, et al. Associations of Dietary Cholesterol or Egg Consumption With Incident Cardiovascular Disease and Mortality. JAMA. 2019;321(11):1081-95.
82. Zhuang P, Wu F, Mao L, Zhu F, Zhang Y, Chen X, et al. Egg and cholesterol consumption and mortality from cardiovascular and different causes in the United States: A population-based cohort study. PLoS Med. 2021;18(2):e1003508.
83. Farnier M, Averna M, Missault L, Vaverkova H, Viigimaa M, Massaad R, et al. Lipid-altering efficacy of ezetimibe/simvastatin 10/20 mg compared with rosuvastatin 10 mg in high-risk hypercholesterolaemic patients inadequately controlled with prior statin monotherapy - The IN-CROSS study. Int J Clin Pract. 2009;63(4):547-59.
84. Teoh H, Mendelsohn AA, Goodman SG, Jaffer S, Chen RY, Tjia S, et al. Usefulness of statin-ezetimibe combination to reduce the care gap in dyslipidemia management in patients with a high risk of atherosclerotic disease. Am J Cardiol. 2009;104(6):798-804.
85. Fellstrom BC, Jardine AG, Schmieder RE, Holdaas H, Bannister K, Beutler J, et al. Rosuvastatin and cardiovascular events in patients undergoing hemodialysis. N Engl J Med. 2009;360(14):1395-407.

86. Hou W, Lv J, Perkovic V, Yang L, Zhao N, Jardine MJ, et al. Effect of statin therapy on cardiovascular and renal outcomes in patients with chronic kidney disease: a systematic review and meta-analysis. Eur Heart J. 2013;34(24):1807-17.
87. Upadhyay A, Earley A, Lamont JL, Haynes S, Wanner C, Balk EM. Lipid-lowering therapy in persons with chronic kidney disease: a systematic review and meta-analysis. Ann Intern Med. 2012;157(4):251-62.
88. Wanner C, Krane V, Marz W, Olschewski M, Mann JF, Ruf G, et al. Atorvastatin in patients with type 2 diabetes mellitus undergoing hemodialysis. N Engl J Med. 2005;353(3):238-48.
89. Ferguson JF, Patel PN, Shah RY, Mulvey CK, Gadi R, Nijjar PS, et al. Race and gender variation in response to evoked inflammation. J Transl Med. 2013;11:63.
90. Plat J, Hendrikx T, Bieghs V, Jeurissen ML, Walenbergh SM, van Gorp PJ, et al. Protective role of plant sterol and stanol esters in liver inflammation: insights from mice and humans. PLoS One. 2014;9(10):e110758.
91. Spann NJ, Garmire LX, McDonald JG, Myers DS, Milne SB, Shibata N, et al. Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. Cell. 2012;151(1):138-52.
92. Zhang X, McDonald JG, Aryal B, Canfran-Duque A, Goldberg EL, Araldi E, et al. Desmosterol suppresses macrophage inflammasome activation and protects against vascular inflammation and atherosclerosis. Proc Natl Acad Sci U S A. 2021;118(47).
93. Draisma A, Pickkers P, Bouw MP, van der Hoeven JG. Development of endotoxin tolerance in humans in vivo. Crit Care Med. 2009;37(4):1261-7.

APPENDICES

Impact
Summary
Abbreviations
Acknowledgments
About the author
List of publications
Summary and acknowledgments in Arabic

Impact

Societal and economic relevance

Cardiovascular disease (CVD) remains the major cause of deaths worldwide. It causes 3.9 millions of deaths in Europe and represents 32% for all global deaths, every year. In 2019, approximately 113 million habitants in Europe had CVD. It is estimated that the costs of CVD eventually will reach 210 billion euros a year [1, 2]. Due to the high numbers and costs for CVD, it has become a major economic burden. Therefore, understanding the underling metabolic processes related to CVD development is of utmost importance to design strategies to prevent or decrease CVD risk. Atherosclerosis is the process underlying the development of CVD [3]. Several factors are known to increase risk for atherosclerosis development such as cigarette smoking, hypertension, physical inactivity, dyslipidemia, obesity and type 2 diabetes [4]. For dyslipidemia, the causal role of increased serum cholesterol concentrations, especially in the low-density lipoprotein (LDL) fraction, in the atherogenic process is well recognized [5, 6]. However, not only lipids, but inflammation is also involved in initiation and progression of atherosclerosis development [7].

Physiological processes including intestinal cholesterol absorption, endogenous cholesterol synthesis, and bile acid synthesis are important for the maintenance of cholesterol homeostasis [8-10]. Therefore, it is essential to understand in more detail 1) the association between characteristics of cholesterol metabolism with different diseases, and 2) the possibilities to modulate these processes to improve cardiovascular health. Interestingly, a relatively high intestinal cholesterol absorption was associated with higher risk of developing CVD [11]. In addition, there is accumulating evidence that increased intestinal cholesterol absorption and decreased cholesterol synthesis are associated with CVD risk [12]. It has been suggested in recent studies that a high intestinal cholesterol absorption is atherogenic [13, 14]. In addition, it is estimated that approximately 30% of the population is characterized by a high intestinal cholesterol absorption, which relates to a 2-fold CVD risk [15]. This might suggest that interventions focussing on lowering cholesterol absorption may be a possible strategy in CVD risk management. European guidelines recommended lifestyle and nutrition interventions for CVD risk reduction include amongst others, increased physical activity and weight loss. Thus, it is of interest to investigate how these interventions could affect and/or improve cholesterol metabolism. In this thesis, we aimed to understand the roles of physical activity, weight loss and inflammation on cholesterol metabolism characteristics in relation to CVD risk management. For aerobic exercise, we observed a trend to a reduction in cholesterol absorption. For diet induced weight loss we found an increased cholesterol absorption and a deceased cholesterol synthesis, and finally for inflammation induced by a pro-inflammatory trigger (lipopolysaccharides [LPS]) we found a decrease in cholesterol synthesis and bile acid synthesis.

Measurement relevance

The studies described in this thesis are essential to better understand how cholesterol metabolism characteristics relate to disease and how these characteristics can be changed by (lifestyle) interventions. Young and older adults were included in the studies presented in this thesis. The prevalence of overweight and obesity in adult population is high, representing over 1.9 billions worldwide [16], which increases risk of CVD development in these people. Physical inactivity and sedentary lifestyle are common in people with overweight and obesity. In this regard, measuring the effects of aerobic exercise on cholesterol metabolism characteristics is of interest. Furthermore, the effects of weight loss on cholesterol metabolism characteristics are also interesting in terms of CVD risk. In addition, inflammation plays important role in almost all stages of anthogenesis and obese subjects at risk to develop atherosclerosis are characterized by a pro-inflammatory state.

Translation into practice

The results presented in this thesis provide knowledge for scientists and researchers with an interest in cholesterol metabolism, highlighting changes in cholesterol metabolism characteristics underlying the effects of aerobic exercise and diet-induced weight loss, as well as the pro-inflammatory effects of LPS on cholesterol metabolism. Most studies described in this thesis have been published in international journals and presented at several international congresses. Before these findings can be translated to the clinic, more studies are needed in other population groups to confirm or refute our findings.

References

1. Timmis A, Vardas P, Townsend N, Torbica A, Katus H, De Smedt D, et al. European Society of Cardiology: cardiovascular disease statistics 2021. Eur Heart J. 2022;43(8):716-99.
2. World Health Organization (WHO). Cardiovascular diseases (CVDs) fact sheet. June 2021. Available from: https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).
3. Frostegard J. Immunity, atherosclerosis and cardiovascular disease. BMC Med. 2013;11:117.
4. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med. 2011;17(11):1410-22.
5. Zarate A, Manuel-Apolinar L, Saucedo R, Hernandez-Valencia M, Basurto L. Hypercholesterolemia As a Risk Factor for Cardiovascular Disease: Current Controversial Therapeutic Management. Arch Med Res. 2016;47(7):491-5.
6. Gidding SS, Allen NB. Cholesterol and Atherosclerotic Cardiovascular Disease: A Lifelong Problem. J Am Heart Assoc. 2019;8(11):e012924.
7. Ridker PM, Luscher TF. Anti-inflammatory therapies for cardiovascular disease. Eur Heart J. 2014;35(27):1782-91.
8. Yeagle PL. Cholesterol and the cell membrane. Biochim Biophys Acta. 1985;822(3-4):267-87.
9. Trapani L, Segatto M, Pallottini V. Regulation and deregulation of cholesterol homeostasis: The liver as a metabolic “power station”. World J Hepatol. 2012;4(6):184-90.
10. Cohen DE. Balancing cholesterol synthesis and absorption in the gastrointestinal tract. J Clin Lipidol. 2008;2(2):S1-3.
11. Silbernagel G, Fauler G, Genser B, Drechsler C, Krane V, Scharnagl H, et al. Intestinal cholesterol absorption, treatment with atorvastatin, and cardiovascular risk in hemodialysis patients. J Am Coll Cardiol. 2015;65(21):2291-8.
12. Weingartner O, Lutjohann D, Bohm M, Laufs U. Relationship between cholesterol synthesis and intestinal absorption is associated with cardiovascular risk. Atherosclerosis. 2010;210(2):362-5.
13. Zhuang P, Wu F, Mao L, Zhu F, Zhang Y, Chen X, et al. Egg and cholesterol consumption and mortality from cardiovascular and different causes in the United States: A population-based cohort study. PLoS Med. 2021;18(2):e1003508.
14. Zhong VW, Van Horn L, Cornelis MC, Wilkins JT, Ning H, Carnethon MR, et al. Associations of Dietary Cholesterol or Egg Consumption With Incident Cardiovascular Disease and Mortality. JAMA. 2019;321(11):1081-95.
15. Helgadottir A, Thorleifsson G, Alexandersson KF, Tragante V, Thorsteinsdottir M, Eiriksson FF, et al. Genetic variability in the absorption of dietary sterols affects the risk of coronary artery disease. Eur Heart J. 2020;41(28):2618-28.
16. World Health Organization (WHO). Obesity and overweight fact sheet. June 2021. Available from https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight.

Summary

For decades, cardiovascular disease (CVD) has been the major cause of morbidities and mortalities worldwide. It is known that atherosclerosis is the process underlying the development of CVD. Elevated blood cholesterol concentrations, especially low-density lipoprotein cholesterol (LDL-C), is a well-defined causal risk factor for the atherogenic process. Therefore, investigating processes regulating cholesterol homeostasis will provide important information to further improve our understanding of cholesterol metabolism and consequently cardiovascular health. Cholesterol homeostasis is maintained by the interplay between intestinal cholesterol absorption, endogenous cholesterol synthesis, and bile acid synthesis and excretion. A high intestinal cholesterol absorption is associated with a higher risk for CVD. Furthermore, inflammation plays an important role in almost all stages of atherosclerosis. Lifestyles and dietary changes have been recommended for CVD prevention i.e. increasing physical activity and promotion of weight loss. However, effects of physical activity, diet-induced weight loss and inflammation on cholesterol metabolism characteristics are largely unknown.

The aim of the studies described in this thesis was to investigate the effects of aerobic exercise and diet-induced weight loss as well as of a pro-inflammatory trigger (lipopolysaccharides [LPS]) on cholesterol metabolism characteristics. In addition, effects of plant sterol content in three different lipid emulsions used for home parenteral nutrition (HPN) on liver function and inflammatory markers were studied.

In **chapter 2**, characteristics of cholesterol metabolism i.e., intestinal cholesterol absorption and endogenous cholesterol synthesis in various metabolic disturbances were systematically reviewed. This chapter also described the validity of non-cholesterol sterol concentrations as markers for intestinal cholesterol absorption and endogenous cholesterol synthesis. Overall, there was an indication of distinctive patters for cholesterol absorption and cholesterol synthesis, suggesting that individuals with very different metabolic conditions can be classified as cholesterol absorber or cholesterol synthesizers. **Chapter 3** describes the effects of an 8-week aerobic exercise program training on markers of cholesterol absorption and cholesterol synthesis. In this study, 17 apparently healthy overweight and obese older men participated in a randomized, cross-over study. Compared with the control period, total cholesterol (TC)-standardized level of the cholesterol absorption marker campesterol tended to decrease with no change in the cholesterol synthesis marker lathosterol after 8 weeks. In **chapter 4**, we investigated the effects of diet-induced weigh loss on markers for cholesterol absorption and synthesis in abdominally obese men. In this chapter, we also examined cross-sectionally baseline differences between abdominally obese and normal weight men. For this, 54 apparently healthy abdominally obese and 26 normal weight men were recruited. Abdominal obese

men were randomized either into a weight loss group or a non-weight loss control group. Subjects in the weight loss group consumed a caloric restricted diet for 6 weeks followed immediately by a 2-week weight maintenance period to reach a waist circumference below 102 cm. In non-weight loss control group, subjects were instructed to maintain their habitual dietary intakes and physical activity levels. After weight loss, the TC-standardized levels of the cholesterol absorption marker cholestanol increased and the cholesterol synthesis marker lathosterol decreased. Cholesterol metabolism characteristics between previously abdominal obese and normal weight men became comparable. Changes in TC-standardized levels of cholestanol were not only negatively related to weight loss, but also negatively to changes in visceral fat volume. Cross-sectionally, mediation analyses revealed roles of visceral fat and intrahepatic fat in mediating the relationships between body mass index and markers for cholesterol absorption and synthesis. **Chapter 5** describes the effects of the acute proinflammatory trigger LPS on lipid and lipoprotein concentrations, high-density lipoprotein (HDL) functionality as well as markers of cholesterol metabolism. From a randomized study including 32 healthy young male subjects, we selected the eight subjects from the placebo arm which means they were infused with LPS only. LPS infusion decreased LDL-C concentrations, HDL functionality, markers of endogenous cholesterol synthesis as well as bile acid formation, but increased triglycerides concentrations. No effect on cholesterol absorption markers was observed. This study also demonstrated that desmosterol (endogenous cholesterol synthesis marker) and 7α-hydroxycholesterol (bile acid formation marker) were positively correlated with various markers for inflammatory responses, while there were negative correlations between changes in desmosterol and 7α-hydroxycholesterol and inflammatory response markers. The aim of a pilot study with 58 stable adult patients with intestinal failure receiving HPN was to investigate the effect of the plant sterol content in three different lipid emulsions on markers of inflammation and liver function (**Chapter 6**). It was concluded that patients receiving Intralipid had higher plasma plant sterol concentrations compared to those receiving ClinOleic or SMOFLipid emulsions. There were significant positive correlations between plasma plant sterol sterols and markers of liver function. Furthermore, patients receiving SMOFLipid had concentration of triglycerides and liver function markers apparently within normal values compared to those receiving ClinOleic or Intralipid emulsions.

Overall, in this thesis we focused on human cholesterol metabolism; more specifically the effects of aerobic exercise training, nutrition and inflammation on markers for intestinal cholesterol absorption and endogenous cholesterol synthesis. it was demonstrated that increased aerobic exercise, diet-induced weight loss and infusion of proinflammatory trigger (LPS) are related to changes in cholesterol metabolism characteristics. Future studies are needed to assess whether these changes have beneficial effects on the risk of CVD.

List of abbreviations

ABCG5/G8: ATP-binding cassette sub-family G member 5 and 8
ACAT: Acyl-CoA cholesterol acyltransferase-2 enzyme
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
BMI: Body mass index
BW: Body weight
CVD: Cardiovascular disease
DHA: Docosahexaenoic acid
EPA: Eicosapentaenoic acid
GC-FID: Gas chromatography with a flame ionization detector
GC-MS: Gas-liquid chromatography-mass spectroscopy
GGT: Gamma-glutamyltranspeptidase
HDL-C: High-density lipoprotein cholesterol
HMG-CoA: Hydroxymethyl-glutaryl-CoA
HPN: Home parenteral nutrition
IFN: Interferon
IHL: Intrahepatic lipid content
IL: Interleukin
IPAQ: International Physical Activity Questionnaire
LDL-C: Low-density lipoprotein cholesterol
LEs: Lipid emulsions
LPS: Lipopolysaccharide
LXR: Liver X receptors
NPC1L1: Niemann-Pick C1-Like 1
PCSK9: Proprotein convertase subtilisin/kexin type 9
Pmax: Maximal power
PN: Parenteral nutrition
ST: Subcutaneous fat
TC: Total cholesterol
TG: Triglyceride
TNF: Tumour necrosis factor
VLCD: Very low caloric diet
VO_2 peak: Peak oxygen consumption
VT: Visceral fat

Acknowledgements

The journey of obtaining the PhD has finally come to end. First and foremost, I am thankful to **Almighty Allah** for providing me with the motivation, faith and patience to accomplish this achievement.

Throughout this journey, I also have been fortunate to have the great support and constant advice of supervisors, colleagues, friends and family. Therefore, there are numerous people I would like to thank and dedicate this section to them.

I would like to start extending my appreciation and gratitude towards my supervising team and this thesis would not have been possible without their guidance. **Jogchum**, thank you for the endless efforts and great feedback and your insightful contributions to my PhD work. Thank you for welcoming me to your office after a short notice and even sometimes without an appointment. I am overwhelmed with your great support especially during the corona pandemic when I experienced and spent the lockdown period lonely. I had really good times with you while we had outings for our group and department. Thank you Jogchum for all of this and I am looking forward to our collaboration in the future. **Ronald**, thank you for all your critical feedback and constructive comments on the content of this thesis. I did get worried before receiving your feedback, however, with time I have noticed how the critical eye of you contributes into the quality of my thesis. Our meetings have taught me to critic my work and organize my thoughts. Thank you very much for being patient with me and explaining to me if there is something unclear to me. Sorry, I still forget to say properly your name in Dutch, by the way, I usually do it in Arabic too!. **Sabine**, thank you for the smile every time I came by your office, your door was always open to the discussions and my endless questions. I learnt a lot from you during our discussions and meetings and I wish you a great success in the new job.

I would like to thank the members of the assessment committee: Prof. dr. M.K.C Hesselink, Prof. dr. M. Poeze, Prof. dr. J.M. Geleijnse, Prof. dr. E.A. Trautwein and Dr. T.C.M. Adam for taking time to review my thesis.

Maurice, thank you for the willingness to give your time so generously to answer my questions and helping me a lot, especially in the part of GC peaks integration. You were being so patient with me when I made mistakes in the lab work. I had fun during our talks in the office and coffee breaks. I am also thankful to my pervious officemate, **Maud**, for the small chats and many things we shared.

I am grateful to my paranymphs, **Kevin and Micah**, for organizing the details of my defence ceremony and assuring everything would run smoothly. Your efforts are highly appreciated.

Jordi and Peter, thanks for your contributions that led to the creation of two published articles presented in this thesis and for taking time to answer my questions about the study details. I appreciate your kindness, cooperation and help.

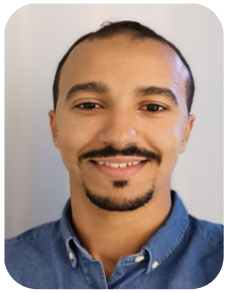
To all my former and current colleagues in the PHuN group: **Cara, Dena, Ellen, Eva, Fatma, Herman, Jehad, Jordi, José, Kevin, Kim, Kyle, Lea, Lieve, Maite, Marco, Mathijs, Merel, Micah, Nathalie, Peter, Sanne, Tania and Willem**, thank you all for the nice times during quizzes via Kahoot, puzzles solving, coffee breaks and our group outings. Dear secretaries of our department, Yolanda and Desiree, scheduling my meetings with supervisors and arranging all the administrative paperwork would not have been done without your help, I am so grateful to you both.

I would also like to thank the **Saudi government** and **AlBaha University** for providing me with the scholarship to pursue and accomplish my PhD and for the financial support of my researches. I also do not forget to thank my academic advisor in **Saudi Arabia Cultural Bureau** in The Hauge, **Lieke Gieles** for her kindness and help throughout the PhD trajectory.

My sincere thanks go to my former and current PhD students. **Adriyan and Harry**, thank you both for our chats during lunch breaks and phone calls. **Manuel**, thank you for all good times spend together while walking and for many invitations to your place for Board games. **Nader and Turki**, thank you for the nice outings, dinners and watching movies. **Uthman**, thank you for all the awesome times while having coffee and dinner also for taking care of me, especially when I was feeling unwell, your friendship means a lot to me. I am also fortunate for being surrounded by **Saudi friends** in Maastricht.

Last but not least, to **my father (Qassem), mother (Saleha), sisters (Amani, Fatma, Zahra, Khulood), brothers (Majed, Jaber, Mohammed), grandparents, relatives and friends** in Saudi, many thanks for your support, encouragement, prayers and phone calls while I am abroad.

About the author



Sultan Mashnafi was born on 4th of October 1985 in Makkah, Saudi Arabia. After completion of secondary school in 2003, he started his bachelor studies in laboratory medicine at Umm Al-Qura university. His thesis for Bachler degree was about Levels of Blood Glucose, HbA1c, Cholesterol, Creatinine and Urea in Umm Al-Qura University Male Medical Students. After receiving his bachelor in 2008, he was appointed at the same year as a demonstrator in the faculty of applied medical sciences at AlBaha university, AlBaha city, Saudi Arabia. He then received a scholarship from AlBaha University to continue his postgraduate studies with a major in Nutrition. In 2012, he was awarded a postgraduate diploma in Human Nutrition from University of Glasgow, Scotland. In 2014, he moved to Aberdeen to study Human Nutrition with a specialization in metabolic nutrition at Aberdeen University. His master research was conducted in the human Nutrition Unit at the Rowett institute. He undertook research into nitrated fatty acids formation during pickling and the digestion processes under the supervision of Dr. Frank thies. By this research he developed a personal interest regarding the effect of dietary components on cardiovascular function, cardiovascular risk and inflammatory processes. In 2015, he graduated from the University of Aberdeen with an MSc in Human Nutrition. After obtaining his master degree, he worked as a lecturer in the faculty of applied medical sciences at AlBaha university. In January 2018, he started his PhD in the Department of Nutrition and Movement sciences at Maastricht University under the supervision of Prof. dr. Jogchum plat, Prof. dr. ir. Ronald P. Mensink and Dr. Sabine Baumgartner. During his PhD research, he investigated the effects of aerobic exercise training, nutrition and inflammation on human cholesterol metabolism. He has presented his research results at several national symposiums and international congresses. After his PhD, Sultan will continue his career in Saudi. He is looking forward to continuing his research and teaching activities in the field of Human Nutrition.

List of publications

Published manuscripts

Mashnafi S, Plat J, Mensink RP, Baumgartner S. Non-Cholesterol Sterol Concentrations as Biomarkers for Cholesterol Absorption and Synthesis in Different Metabolic Disorders: A Systematic Review. Nutrients. 2019 Jan 9;11(1):124. doi: 10.3390/nu11010124.

Mashnafi S, Plat J, Mensink RP, Joris PJ, Kleinloog JPD, Baumgartner S. Effects of an 8-week aerobic exercise program on plasma markers for cholesterol absorption and synthesis in older overweight and obese men. Lipids Health Dis. 2021 Sep 21;20(1):112. doi: 10.1186/s12944-021-01537-2

Mashnafi S, Plat J, Mensink RP, Joris PJ, Kusters YHAM, Houben AJHM, Stehouwer CDA, Schalkwijk CG, Baumgartner S. Effects of Diet-Induced Weight Loss on Plasma Markers for Cholesterol Absorption and Synthesis: Secondary Analysis of a Randomized Trial in Abdominally Obese Men. Nutrients. 2022 Apr 8;14(8):1546. doi: 10.3390/nu14081546.

Osowska S, Kunecki M, Sobocki J, Tokarczyk J, Majewska K, Burkacka M, Radkowski M, Fisk HL, **Mashnafi S**, Baumgartner S, Plat J, Calder PC. Do omega-3 fatty acids protect against the adverse effect of phytosterols? A pilot study comparing three different lipid emulsions in adult patients on home parenteral nutrition.

Submitted manuscript

Mashnafi S, Plat J, Mensink RP, Perlee D, van Vught LA, Lutjohann D, Baumgartner S. Effects of a transient systemic inflammatory response via lipopolysaccharide (LPS) infusion on markers of cholesterol metabolism in healthy normocholesterolemic young men

Presentations

Mashnafi S, Plat J, Mensink RP, Baumgartner S. Non-Cholesterol Sterol Concentrations as Biomarkers for Cholesterol Absorption and Synthesis in Different Metabolic Disorders: A Systematic Review. Presented at the European Atherosclerosis Society (EAS) congress 2019 in Maastricht, The Netherlands. Poster presentation

Mashnafi S, Plat J, Mensink RP, Joris PJ, Kleinloog JPD, Baumgartner S. Effects of an 8-week aerobic exercise program on plasma markers for cholesterol absorption and synthesis in older overweight and obese men. Presented at The Dutch Nutritional Science Days (NSD) 2019 in Heeze, The Netherlands. Oral presentation

Mashnafi S, Plat J, Mensink RP, Joris PJ, Kusters YHAM, Houben AJHM, Stehouwer CDA, Schalkwijk CG, Baumgartner S. Effects of Diet-Induced Weight Loss on Plasma Markers for Cholesterol Absorption and Synthesis: Secondary Analysis of a Randomized Trial in Abdominally Obese Men. Presented at Joint European Congress on Obesity of the European Association of the Study of Obesity and the International Federation for the Surgery of Obesity and Metabolic Disorders-European Chapter 2022 in Maastricht, The Netherlands. Poster presentation

يصف الفصل الخامس تأثيرات LPS على مستويات الدهون والبروتينات الدهنية، ووظيفة الكوليسترول الصحي (HDL) وكذلك علامات خصائص أيض الكوليسترول. من دراسة عشوائية مضبوطة شارك فيها إثنان وثلاثون رجلا شاباً يتمتعون بصحة جيدة، كان ثمانية رجال منهم في مجموعة الدواء الوهمي وقد تم حقنهم بمركب LPS فقط . أدى حقن LPS لهؤلاء الرجال إلى خفض تركيزات LDL-C ووظيفة HDL وعلامات تصنيع الكوليسترول بالإضافة إلى زيادة تكوين حمض الصفراء ومستويات الدهون الثلاثية. وقد لاحظنا غياب التأثير على علامات امتصاص الكوليسترول. أظهرت هذه الدراسة أيضًا بأن كلا مستويات ديسموسترول (يستخدم أيضا كعلامة لتصنيع الكوليسترول في داخل الجسم) وعلامة تكوين حمض الصفراء (7α-hydroxycholesterol) مرتبطة ارتباطًا إيجابيًا بعلامات مختلفة للاستجابات الالتهابية، بينما كان الارتباط سلبياً بين تغييراتهم وتغييرات علامات الاستجابات الالتهابية.

يصف الفصل السادس من هذه الأطروحة دراسة تجريبية تضمنت خمسة وثمانين مريضًا بالغًا مستقرًا يعانون من فشل معوي ويتلقون HPN وكان الهدف من هذه الدراسة هو التحقق من تأثير محتوى مركبات الستيرول النباتية في ثلاث مستحلبات دهنية مختلفة على علامات الالتهاب ووظيفة الكبد. تم الاستنتاج من هذه الدراسة أن المرضى الذين يتلقون مستحلب Intralipid لديهم مستويات أعلى من مركبات الستيرول في البلازما مقارنة مع أولئك الذين يتلقون مستحلبات ClinOleic أو SMOFLipid. وكانت هناك علاقة ارتباط موجبة ظهرت بين هذه المركبات وعلامات وظائف الكبد. علاوة على ذلك، كان لدى المرضى الذين يتلقون مستحلب SMOFLipid مستويات دهون ثلاثية وعلامات وظائف الكبد على ما يبدو ضمن المستويات الطبيعية في البلازما مقارنة مع أولئك الذين يتلقون مستحلبات ClinOleic أو Intralipid.

بشكل عام، ركزنا في هذه الأطروحة على أيض الكوليسترول البشري. وبشكل أكثر تحديدًا، آثار التمارين الهوائية والتغذية والالتهابات على علامات امتصاص الكوليسترول المعوي وتصنيع الكوليسترول داخل الجسم. تم الإثبات من هذه الأطروحة أن عوامل مثل ممارسة التمارين الهوائية، وفقدان الوزن الناجم عن النظام الغذائي، وحقن بمركب LPS قد ارتبطت بتغيرات في خصائص أيض الكوليسترول. ولازال هناك حاجة لدراسات مستقبلية لتقييم ما إذا كانت هذه التغيرات لها آثار مفيدة على تقليل خطر الإصابة بأمراض القلب والشرايين.

نبذه مختصرة عن الأطروحة

لعقود من الزمن، عُدَت أمراض القلب والشرايين من أحد الأسباب الرئيسية للوفيات في جميع أنحاء العالم. ومن المعروف أن تصلب الشرايين هو السمة المعروفة وراء تطور هذه الأمراض. أشارت الأبحاث العلمية إلى أن مستويات الكوليسترول المرتفعة في الدم، وخاصة الكوليسترول الضار (LDL-C)، هو عامل يسهم في خطر الإصابة بتصلب الشرايين. ولذلك، فإن التحقيق في العمليات الحيوية التي تنظم اتزان الكوليسترول في داخل جسم الإنسان قد يزودنا بمعلومات مهمة لغرض زيادة تحسين فهمنا لعملية أيض الكوليسترول وبالتالي صحة القلب والشرايين. يتم الحفاظ على توازن الكوليسترول في الجسم من خلال التوازن بين العمليات الحيوية والتي تتضمن امتصاص الكوليسترول، تصنيع الكوليسترول، وتصنيع وإفراز حمض الصفراء. وقد أشارت الدراسات إلى أن ارتفاع امتصاص الكوليسترول المعوي مرتبط بزيادة خطر الإصابة بأمراض القلب والشرايين. علاوة على ذلك، يلعب الالتهاب دورًا مهمًا في جميع مراحل تصلب الشرايين تقريبًا. وقد تمت التوصية بالتغيير في أنماط الحياة والغذاء للوقاية من أمراض القلب والشرايين، على سبيل المثال زيادة النشاط البدني والتحفيز على فقدان الوزن. ومع ذلك، فإن تأثيرات النشاط البدني، فقدان الوزن الناجم عن النظام الغذائي والالتهابات على خصائص أيض الكوليسترول غير معروفة إلى حد كبير. ولهذا كان الهدف من الدراسات المذكورة في هذه الأطروحة هو التحقيق في آثار التمارين الهوائية وفقدان الوزن الناجم عن النظام الغذائي بالإضافة إلى المحفزات المسببة للالتهاب (متعدد سكريد الدهني [LPS]) على خصائص أيض الكوليسترول. بالإضافة إلى ذلك، تمت دراسة تأثير محتوى مركبات الستيرول النباتية المستخدمة في ثلاث مستحلبات دهنية مختلفة للتغذية الوريدية في المنزل (HPN) على وظائف الكبد وعلامات الالتهاب.

تضمن الفصل الثاني من هذه الأطروحة مراجعة منهجية لكل ما نشر من بحوث علمية عن خصائص أيض الكوليسترول، أي امتصاص الكوليسترول وتصنيعه داخل الجسم، في العديد من الاضطرابات الأيضية المختلفة. وقد وصف هذا الفصل أيضًا صلاحية مستويات non-cholesterol sterols كعلامات لامتصاص الكوليسترول المعوي وتصنيع الكوليسترول داخل الجسم. بشكل عام، كان هناك مؤشر يدل على وجود أنماط مميزة لامتصاص الكوليسترول وتصنيع الكوليسترول، مما قد يشير إلى أن الأفراد الذين يعانون من اضطرابات أيضية يمكن تصنيفهم إلى ممتصين للكوليسترول أو صانعين للكوليسترول.

وصف الفصل الثالث من هذه الأطروحة تأثير برنامج التمارين الرياضية الهوائية لمدة ثمانية أسابيع على علامات امتصاص وتصنيع الكوليسترول. في هذه الدراسة، شارك ما إجماليه سبعة عشر رجُلاً من كبار السن يعانون من زيادة الوزن وبدانة في دراسة عشوائية مضبوطة. بالمقارنة مع فترة الانضباط، فإن مستويات علامة امتصاص الكوليسترول (الكامبيسترول) قد مال إلى الانخفاض مع عدم وجود أي تغيير في علامة تصنيع الكوليسترول (اللاثوستيرول) بعد فترة ثمانية أسابيع من ممارسة هذه التمارين.

في الفصل الرابع من هذه الأطروحة، قمنا بالتحقيق في تأثير فقدان الوزن الناجم عن النظام الغذائي على علامات امتصاص الكوليسترول وتصنيعه في رجال يعانون من بدانة عند منطقة البطن. في هذا الفصل، قمنا أيضًا بفحص بشكل مقطعي الاختلافات الأساسية بين هؤلاء الرجال وأولئك ذوي الوزن الطبيعي. لهذا الغرض، تم إشراك ما إجماليه أربعةً وخمسون رجلاً لديهم بدانة عند منطقة البطن وستة وعشرون رجلاً يتمتعون بوزن طبيعي. تم توزيع الرجال الذين يعانون من بدانة عند منطقة البطن بشكل عشوائي إما في مجموعة فقدان الوزن أو مجموعة المنضبطة. استهلك الأشخاص في مجموعة فقدان الوزن نظامًا غذائيًا مقيدًا بالسعرات الحرارية لمدة ستة أسابيع متبوعًا مباشرة بفترة حفاظ على الوزن لمدة أسبوعين للوصول إلى محيط خصر أقل من 102 سنتيمتر. تم إرشاد الأشخاص المشاركين في المجموعة المنضبطة إلى الحفاظ على النمط الغذائي المعتاد ومستوى النشاط البدني. ، زاد مستوى علامة امتصاص الكوليسترول (كوليستانول) بينما انخفض علامة صنع الكوليسترول (اللاثوستيرول) بعد فقدان الوزن. وجدنا أيضا تشابه في خصائص أيض الكوليسترول بين الرجال الذين يعانون من بدانة عند منطقة البطن سابقًا والرجال ذوي الوزن الطبيعي. بالإضافة لذلك، لم تكن التغيرات في مستويات الكوليستانول مرتبطة بشكل سلبي بفقدان الوزن فحسب، بل كانت أيضًا مرتبطة بشكل سلبي بالتغيرات في حجم الدهون الحشوية. وكشفت تحليلات الدراسة المقطعية عن دور الدهون الحشوية والدهون داخل الكبد في التوسط في العلاقات بين مؤشر كتلة الجسم وعلامات امتصاص الكوليسترول وتصنيعه.

شكر وتقدير

لك ربي كُل الحمد والشكر والثناء والفضل على عونك وإعانتك وتيسير أموري والتوفيق لإتمام هذه الأطروحة، وأسألك ان تجعلها خالصة لوجهك الكريم.

إلى من هم سبب وجودي في الحياة بعد ربي سبحانه، أبي وأمي، لا أجد الكلمات الكافية التي توفي دعمكم وحبكم الدائم، شكراً لكما من أعماق قلبي على دعائكما المستمر لي بالنجاح والتوفيق.

إلى أخواني وأخوتي، شكراً على تحفيزكم وإدخال السرور لقلبي وتواصلكم معي خلال مسيرتي الدراسية، سأبقى دوماً ممتن لكم وفخور بكم.

إلى الأقارب والأصدقاء، شكراً لكم على سؤالكم عني والاطمئنان عليَ ودعائكم لي بالخير والتسيير والتوفيق وتحقيق هدفي، أنتم تستحقون الشكر والثناء.

إلى وطني الغالي وحكومتي الرشيدة، شكرا لكم على دعمكم السخي طيلة فترة دراستي.

إلى جامعة الباحة وكلية العلوم الطبية التطبيقية، شكرا لكم على الدعم والتشجيع.

سلطان قاسم مشنفي