

A contributory role of gut microbiota in lipid metabolism

The gut microbiota as novel player in the development of atherosclerosis?

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Abstract - Atherosclerosis is the main underlying cause of a lot of cardiovascular diseases. Several risk factors contribute to the development of atherosclerosis, including dyslipidaemia. Recent studies identified a contributory role of the gut microbiota in the development of atherosclerosis by affecting lipid metabolism. The aim of this thesis is to investigate the mechanisms by which gut bacteria influence lipid metabolism of the host and to determine if this contributes to the development of atherosclerosis. Two main pathways will be discussed in which gut bacteria are involved: the bile acid metabolism pathway and the so-called trimethylamine pathway. Gut bacteria produce secondary bile acids which act on a receptor in cells of the liver and intestine leading to increased reverse cholesterol transport (RCT) and decreased intestinal cholesterol absorption. Next to that, gut bacteria contribute to the production of the metabolite trimethylamine-*N*-oxide out of products such as meat and eggs. This gut-microbial-derived metabolite promotes foam cell formation and suppresses RCT and bile acid secretion. Taken together, both pathways might be potential therapeutic targets to prevent atherosclerosis. However, a lot of research is needed to identify the exact mechanisms and bacterial species involved in both pathways.

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1. Introduction

Cardiovascular diseases (CVD) is one of the leading causes of death in the world (1,2). The main underlying cause of a lot of cardiovascular diseases is the process of atherosclerosis, in which a plaque made of cholesterol, fatty substances and other cellular waste products builds up inside the arteries (3,4). This plaque can partially or totally block the blood's flow, leading to diseases as coronary artery disease (CAD) or peripheral artery disease (PAD) (2,4). Despite the widespread use of statin-based lipid lowering therapy, several clinical trials revealed that at least a 50% residual cardiovascular risk remains (5). To improve the prevention and treatment of atherosclerosis, innovative progress in the identification of novel pathways that underlie CVD pathology is needed (4).

Both genetic and environmental risk factors contribute to the pathogenesis of atherosclerosis (2,5). A well established environmental risk factor for the development of atherosclerosis is a diet rich in lipids. The relationship between blood cholesterol and triglyceride levels and cardiovascular risk is well identified (2). Diet is also an important determinant of the composition of the gastrointestinal microbiota (6). The gut microbiota has been recently established to play an important role in human health. The bacterial community itself or metabolites they produce have been associated with increased susceptibility to a variety of diseases, such as obesity and related metabolic abnormalities, but also neurobehavioral conditions, such as autism (7-10).

In addition, recent studies demonstrated that gut bacteria have a contributory role in the development of cardiovascular diseases, such as atherosclerosis (2,11). Gregory *et al.* showed that atherosclerosis susceptibility can be transmitted with gut microbial transplantation in germ-free mice (12). The mechanisms by which gut bacteria affect the development of atherosclerosis seem to be very diverse. Bacteria have long been known to activate inflammatory pathways. Recent data demonstrated that the gut microbiota also affect lipid metabolism (13). A study by Fu *et al.* provides some of the first evidence in humans that variation in gut bacteria are associated with blood lipid levels. They investigated which gut bacteria were associated with different blood lipids and how much of the variation in blood lipids could be explained by the gut microbiome (3).

So, the gut microbiota may contribute to the development of atherosclerosis by affecting lipid metabolism. However, the mechanisms involved in this process are still unclear. The main question in this thesis is: by which mechanisms do gut bacteria affect lipid metabolism of their host and does this influence the development of atherosclerosis? In the next sections, two main pathways will be discussed in which gut bacteria, lipid metabolism and atherosclerosis risk are involved: bile acid metabolism and the so-called trimethylamine pathway. Recent studies showed that gut bacteria convert bile acids, which are involved in the maintenance of cholesterol balance in the body, suggesting a role for gut microbiota in lipid metabolism (14). The other pathway which will be discussed, involves the conversion of certain components in meat and other dairy products by the gut microbiota. These bacterial-derived metabolites are also suggested to play a role in atherosclerosis by affecting cholesterol balance (1,2).

2. Cardiovascular diseases: the process of atherosclerosis

As described in the introduction, CVD is a main cause of death in the world (1,2). A lot of factors contribute to the pathogenesis of CVD, including genetic and environmental factors (2,5). Despite extensive research into the genetic factors contributing to CVD, less than one-fifth of attributable cardiovascular risk has been accounted for from genetic causes (5). This finding suggest that environmental factors are also an important player in the pathogenesis of CVD. A diet rich in lipids is one of those environmental risk factors for the development of CVD. A relationship between blood cholesterol and triglyceride levels and cardiovascular risk is well established (2). The main underlying cause of cardiovascular diseases such as stroke, carotid artery disease (CAD) and peripheral artery

disease (PAD) is the process of atherosclerosis (2,4). Atherosclerosis is considered as a disorder of lipid accumulation in which also chronic inflammation is involved (4). In the process of atherosclerosis, a plaque made of cholesterol, fatty substances, cellular waste products, calcium and fibrin builds up inside the arteries. When the plaque partially or totally blocks the blood's flow through an artery in for example the heart, legs or arms diseases as CAD or PAD may develop (2,4).

The first step in the process of atherosclerosis is endothelial dysfunction and low-density lipoprotein (LDL)-cholesterol deposition in the arterial wall. This process is initiated when cardiovascular risk factors, such as dyslipidaemia, hypertension, diabetes mellitus and smoking activate and/or injury the endothelium, leading to dysfunction and fragmentation (4, 15). Lipid-containing lipoproteins accumulate in the intima of the artery wall, where the accumulated LDL is oxidized resulting in the formation of oxidized LDL (OxLDL). The oxidation of LDL leads to an inflammatory response, which is characterised by the recruitment of circulating leukocytes and the production of growth factors which encourage cell migration and proliferation (Figure 1).

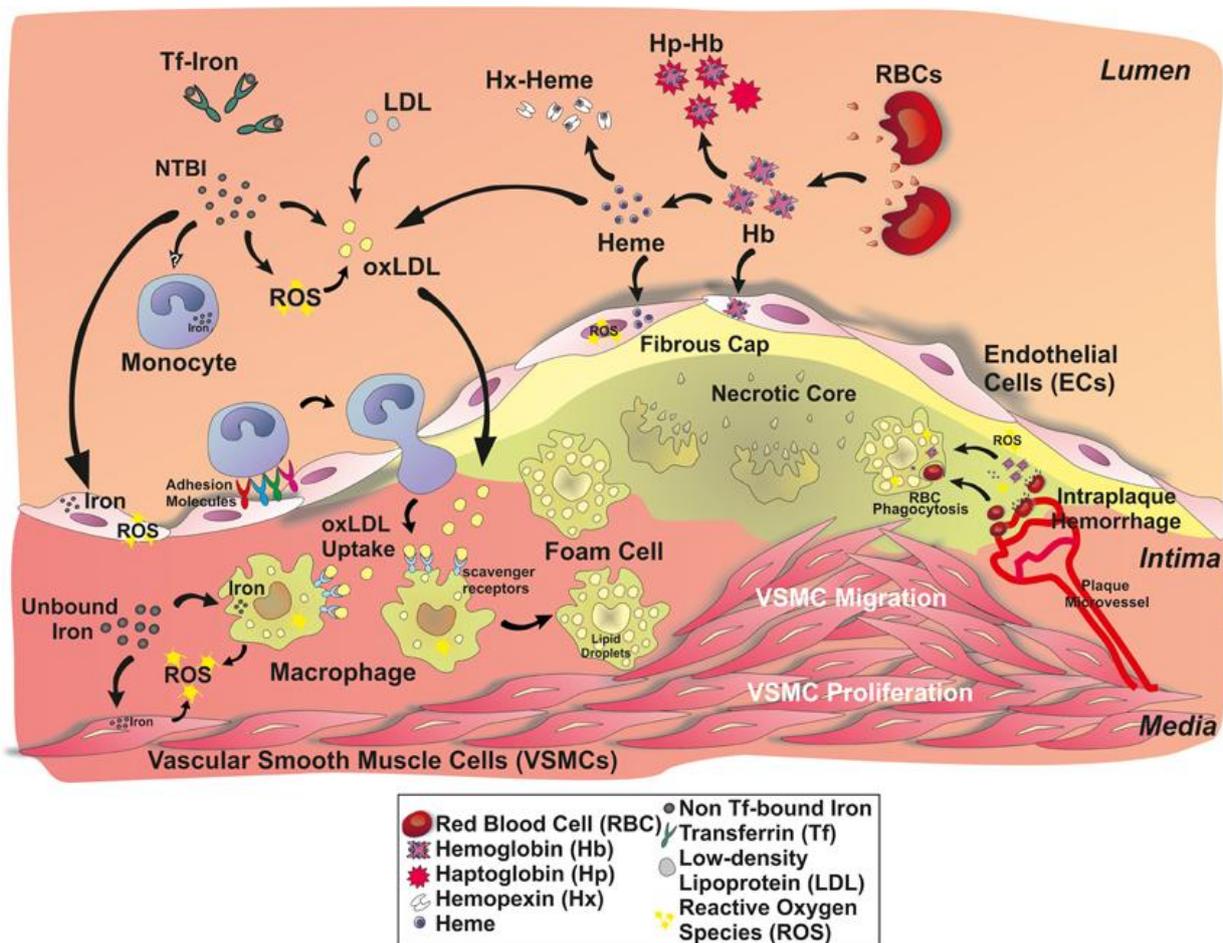


Figure 1. The process of atherosclerosis, in which accumulated LDL is oxidized to OxLDL. The formation of OxLDL leads to the recruitment of inflammatory cells, which pass into the sub endothelial space and take up OxLDL. As the process progress, the lesion becomes more complex and vascular smooth muscle cells proliferate and migrate into the lesion, forming an complex atherosclerotic plaque (18).

Monocytes and various other types of leukocytes adhere to the activated endothelium at sites of injury or inflammation. Upregulation of adhesion molecules leads to the migration of monocytes into the arterial wall. The monocytes pass into the sub endothelial space, where they differentiate into macrophages. Subsequently, these macrophages take up OxLDL via scavenger receptors, leading to

the formation of lipid-laden foam cells. Now the initial fatty streaks are developed, which consist of lipids and numerous immune cells such as macrophages, dendritic cells and T lymphocytes (16).

As the atherosclerotic process progresses, the lesion becomes more complex. Next to macrophages, smooth muscle cells may also migrate into the sub endothelial space and transition into foam cells. Foam cells aggregate within the developing arterial plaque. In the core of the plaque some foam cells undergo necrotic death, resulting in the release of harmful cellular contents. These cellular contents can promote plaque rupture and development of blood clots. Together, these processes result in the formation of a complex lesion, consisting of migrated smooth muscle cells (SMCs), apoptotic cells and extracellular matrix such as collagen and proteoglycans. When such complex atherosclerotic plaques suddenly rupture, they can induce life-threatening coronary thrombosis presenting as an acute coronary syndrome. The notable features of unstable rupture-prone plaque include infiltration of many inflammatory cells, large lipid core, and thin fibrous cap (4,17).

3. Cholesterol balance in the human body

As described in the previous section, lipid accumulation in macrophages is a critical event in atherosclerosis (4). Influx of lipids into the arterial wall leads to an increased influx of macrophages, which take up oxidized LDL. The major lipid component in atherosclerotic plaques is cholesterol, either in its free form or esterified to a fatty acid (4). An imbalance of cholesterol uptake, synthesis, and export by macrophages underlies the development of foam cells.

Cholesterol is synthesized in extrahepatic tissues or imported from plasma lipoproteins. Both dietary cholesterol and that synthesized *de novo* are transported through the circulation in lipoprotein particles. To prevent accumulation or abnormal deposition of cholesterol in the body, which we see in for example atherosclerosis, the synthesis and utilization of cholesterol must be tightly regulated. Cells in the body have to export excess cholesterol to achieve a neutral cholesterol balance (19). The pathways involved in the export of excess cholesterol from foam cells and its removal from the body could provide new targets for anti-atherosclerotic therapies.

Excess cholesterol from cells in peripheral tissues and organs, including macrophages in vessel walls, is transported to the liver by a process called reverse cholesterol transport (RCT). High-density lipoprotein (HDL) is generally believed to protect against atherosclerosis by acting as the specific cholesterol carrier in the RCT pathway. HDL delivers cholesterol from the artery wall to the liver, which removes cholesterol from the body through excretion as neutral sterols or bile acids via the bile into the feces (20-22). Recently, several researchers have demonstrated that RCT can also proceed through a non-biliary pathway known as trans intestinal cholesterol excretion (TICE) (23-28). However, under normal physiologic conditions the hepatobiliary route predominates and TICE only contributes to ~30% of the total cholesterol loss through the feces in mice. TICE is a minor pathway which persists in both the surgical or genetic absence of biliary cholesterol secretion (19,23).

4. The enterohepatic circulation

The enterohepatic circulation refers to the circulation of biliary acids, bilirubin, drugs or other substances from the liver to the bile, followed by entry into the small intestine, absorption by the enterocyte and transport back to the liver (29). As described above, the production of bile and bile acids is an important component in the removal of cholesterol from the body. Bile is a complex fluid containing water, electrolytes and organic molecules such as bile salts, cholesterol, phospholipids and bilirubin (29). Bile acids are synthesized in hepatocytes from cholesterol by a process that requires the actions of at least 14 liver enzymes (30,31). In humans, the primary bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA) (14). Bile salts are formed when primary bile acids are conjugated with glycine or taurine (29). They are then excreted through the canaliculi to the biliary system (14). Next to the elimination of excess cholesterol and waste products, bilirubin, drugs and toxic

compounds bile serves another main function. They aid digestion of fats by facilitating the absorption of triglycerides, cholesterol, and lipid-soluble vitamins.

After synthesis of bile acids, they are stored in the gallbladder and released into the duodenum upon ingestion of a meal (32,33). In the duodenum bile acids, which have detergent properties, emulsify fat globules into smaller micelles. The emulsifying of fat globules into smaller micelles leads to an increase in surface accessible to lipid-hydrolyzing enzymes. Bile acids also keep fat-soluble compounds in solution for uptake (vitamins) or excretion (cholesterol, lipophilic toxins/drugs) (29). Mixed micelles of bile salts and phospholipids carry cholesterol and other lipophilic compounds through the intestine. In the terminal ileum, 90-95% of the bile salts are efficiently reabsorbed and returned to the liver. Reabsorption of bile salts mainly occurs by active transport mediated by the ileal bile acid transporter (IBAT). In the upper small intestine and colon also passive diffusion of bile salts occurs (30). The remaining 5-10% of the bile salts are transported to the colon. In the colon, primary bile salts are transformed into secondary bile salts by bacterial metabolism. A part of the secondary bile salts produced by bacteria are reabsorbed from the colon. The remaining is excreted with the feces. All the reabsorbed primary and secondary bile salts return to the liver via the portal circulation. When bile salts reach the liver, they are taken up into hepatocytes, thereby completing the enterohepatic cycle (29). The process of enterohepatic circulation occurs four to twelve cycles each day (14).

5. Composition of the gut microbiota

As mentioned in the introduction, the gut microbiota are suggested to contribute to the development of atherosclerosis by interfering with cholesterol and bile acid metabolism. The intestinal microbiota consist of at least 1000 distinct species of bacteria. Colonization of the gastrointestinal tract begins after birth, and continues throughout life. The composition of the microbiota in the gut changes during childhood and youth. By adulthood, the composition is almost stabilized (34).

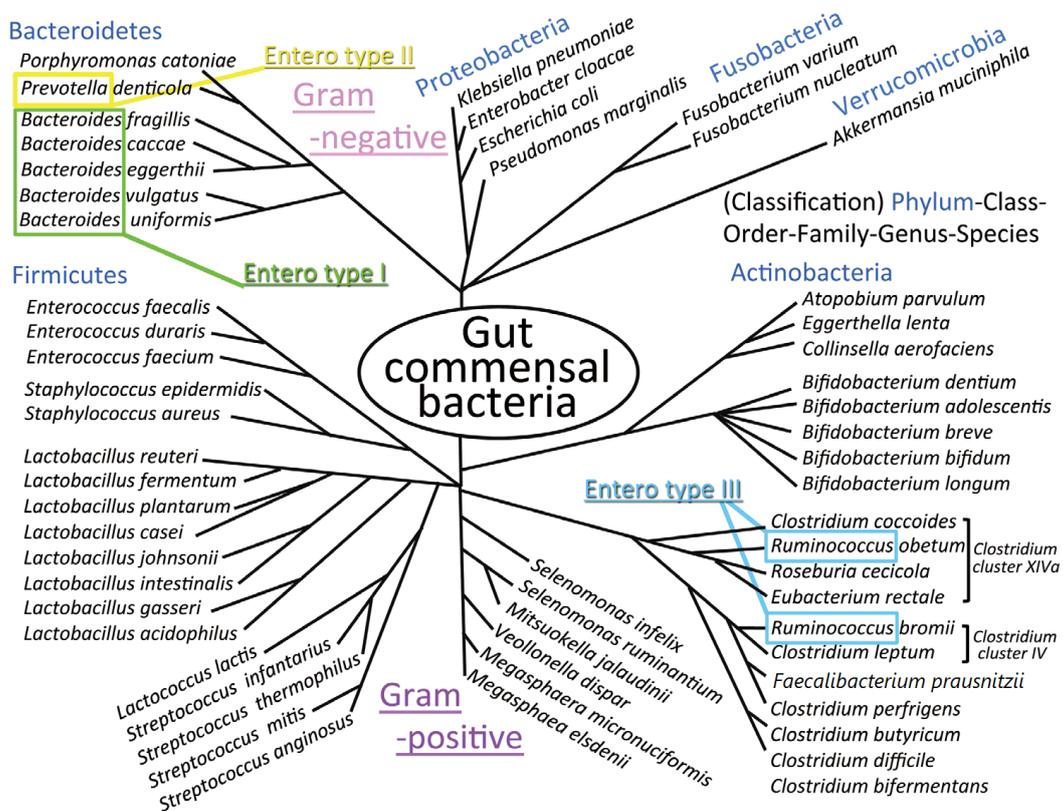


Figure 2. Human gut commensal microbiota and their classification. The 4 major phyla of Firmicutes, Bacteroides, Actinobacteria, and Proteobacteria account for more than 98% of all human gut microbiota. The total number of bacteria in the human intestine is more than one hundred trillion. Those bacteria are classified into several hundreds of species. (Adapted from Yamashita et al (4)).

The predominant phyla in the gut are the phyla *Firmicutes* and *Bacteroides*. They colonize the gastrointestinal tract together with less abundant bacterial phyla such as *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* (Figure 2) (4). *Firmicutes*, *Bacteroides*, *Actinobacteria* and *Proteobacteria* are the 4 major phyla, occupying more than 98% of all human gut microbiota. The microbial community is diverse and dynamic, because exposure to different diets affects the composition of the gut microbiota. However, its composition over time seems to be remarkably stable within individuals and their family members (7).

Using metagenomics, 3 major clusters of gut bacteria, called enterotypes, are identified in humans. These enterotypes are based on the predominant bacterial general in fecal samples. The first enterotype is characterized by high levels of *Bacteroides*. Type II had few *Bacteroides*, but *Prevotalla* are common. The third enterotype is marked by high levels of *Ruminococcus* (35).

6. The relation between gut microbial taxa and lipid levels

In 2010, Velagapudi *et al.* proposed that gut microbes are involved in modulating lipid levels and host energy metabolism, using germ-free and conventionally raised mice (36). The design of this study however, did not permit the identification of candidate bacteria responsible for the observed effects (37). A recent study by Fu *et al.* now provides the first evidence in humans that variation in gut bacteria are associated with blood lipid levels, as well as clues toward discovering the bacterial species involved (3). They performed a systematic analysis of host genome, gut microbiome, body mass index (BMI) and blood lipids in 893 human subjects from the Dutch LifeLines-Deep cohort (38). In this study, Fu *et al.* tested for association between individual bacterial profiles, BMI and blood lipid levels.

They found that, although most of the associated bacterial taxonomies were shared between lipid metabolites and BMI, several microbes were predominantly linked to lipids rather than BMI. The family *Lachnospiraceae* for example, was especially associated with decreased LDL levels, but not with BMI or other lipids (3). Further, the family *Pasteurellaceae* (Proteobacteria), genus *Coproccoccus* (Firmicutes) and genus *Collinsella stercoris* (Actinobacteria) were strongly associated with decreased triglyceride (TG) levels. They had nominal significance to other lipids and no association with BMI (3).

Previously, several bacterial associations to obesity were described. Fu *et al.* confirmed some of them. For example, an increased abundance of genus *Akkermansia* was associated with a decrease in BMI. Next to that, they confirmed the association of both the family *Christensenellaceae* (phylum Firmicutes) and the phylum Tenericutes with low BMI. These particular bacteria were also strongly associated with lower levels of TG and higher levels of HDL, which was a novel finding (3).

Also several other new associations between bacteria and BMI, TG and HDL levels were observed. *Eggerthella* was associated with increased TG and decreased HDL. Family *Pasteurellaceae* however, was associated with decreased TG. The study of Fu *et al.* also showed that the genus *Butyricimonas* is strongly associated with decreased TG and nominally associated with BMI and HDL (3).

Fu *et al.* corrected their observations for age, gender and genetics. They demonstrated that the microbiota described above still contribute significantly to lipid variation, thus independently of age, gender and genetics (3). *Bacteroidales* (phylum Bacteroidetes) and family *Clostridiaceae* (phylum Firmicutes) are both negatively correlated with BMI and TG. Next to that, they are also known to be involved in bile acid metabolism. Fu *et al.* found support for the role of bacterial bile acids in lipid metabolism. Bile acid activity of commensal bacteria are involved in digestion and absorption of dietary lipids. The mechanisms by which gut bacteria are involved in bile acid metabolism will be discussed in the next section.

7. Gut-microbiota-driven bile acid metabolism pathway

One mechanism by which gut bacteria may affect cholesterol balance in the body and thereby the development of atherosclerosis is through interference with bile acid metabolism. (37,39). Bile acids play a role in the excretion of cholesterol from the body. Next to that, recent studies showed that bile acids also function as signalling molecules (39). They do not only regulate their own biosynthesis, but also affect metabolic pathways involved in lipoprotein, glucose drug and energy metabolism by activation of nuclear receptors such as farnesoid X receptor (FXR) and the G protein-coupled receptor TGR5 (39-41).

7.1. Conversion of bile acids by several bacterial genera

Gram-positive gut bacteria including *Lactobacillus* and *Bifidobacterium* convert the two primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) that reach the colon to over 20 different secondary bile acids (14). The main bile acid conversions in the human gut include deconjugation, oxidation and epimerization, 7-dehydroxylation, esterification and desulfatation, which are all catalysed by enzymes of different bacterial genera (Table 1) (14,42). Enzymes of the microbiota regulate the conversion of bile acids, but intestinal bacteria growth is in turn regulated by bile acids, as biliary obstruction causes bacterial overgrowth that can be reversed by administration of bile acids (43,44).

Dehydroxylation of primary bile acids is the most physiologically significant conversion of bile acids in humans (14,45). However, dihydroxylation can only occur after deconjugation of primary bile acids. Deconjugation is mediated by the enzyme bile salt hydrolase (BSH), which is expressed by gut microbes (46). BSH genes have been detected in the main bacterial genera of the gut microbiota (14,47). BSH-enzymes have been purified from *Bacteroides fragilis*, *B. vulgatus*, *Clostridium perfringens*, *Listeria monocytogenes* and several species of *Lactobacillus* and *Bifidobacterium*. (14). Probiotics with high BSH activity promote the deconjugation of bile acids in the gut to secondary amino acid conjugates. When these secondary conjugates are excreted, cholesterol is broken down to replace the processed bile acids. This process promotes the catabolism of cholesterol, which leads to reduced serum levels of cholesterol (48,49).

After deconjugation of primary bile acids, dehydroxylation can occur. The 7 α -dehydroxylation of CA and CDCA leads to the formation of the most abundant secondary bile acids in humans, lithocholic acid (LCA) and deoxycholic acid (DCA) (50,51). In mice, CDCA is converted into muricholic acid (MCA) instead of DCA (43). DCA may account for up to 25% of the total bile acid pool (14). The main bacterial species known to possess 7 α -dehydroxylation activity are *Clostridium* and *Eubacterium*, which are members of the *Firmicutes* phylum (14).

Table 1. Bacterial genera of the gut microbiota involved in bile acid metabolism (14).

Reactions	Bacterial genera
Deconjugation	<i>Bacteroides</i> , <i>Bifidobacterium</i> , <i>Clostridium</i> , <i>Lactobacillus</i> , <i>Listeria</i>
Oxidation and epimerization	<i>Bacteroides</i> , <i>Clostridium</i> , <i>Escherichia</i> , <i>Egghertella</i> , <i>Eubacterium</i> , <i>Peptostreptococcus</i> , <i>Rumminococcus</i>
7-dehydroxylation	<i>Clostridium</i> , <i>Eubacterium</i>
Esterification	<i>Bacteroides</i> , <i>Eubacterium</i> , <i>Lactobacillus</i>
Desulfatation	<i>Clostridium</i> , <i>Fusobacterium</i> , <i>Peptococcus</i> , <i>Pseudomonas</i>

7.2. The FXR receptor as main bile acid receptor

Recent studies revealed that bile acids play a role in a wide range of biological pathways (52). The regulatory functions of bile acids are mainly mediated by signalling through different receptors (43). The nuclear receptor FXR and the membrane receptor TGR5 are the best studied bile acid receptors (43). Bile acids can also activate other nuclear receptors which are implicated in lipid and glucose metabolism, such as the human steroid and xenobiotic receptor (SXR) and its rodent homolog pregnancy X receptor (PXR), vitamin D receptor (VDR), and constitutive androstane receptor (CAR) (53, 54). FXR and TGR5 are differentially activated by CDCA>DCA>LCA>>CA and LCA>DCA>CDCA>CA (affinity from high to low), respectively. The receptors VDR and PXR are activated by LCA (46, 55), however activation of these receptors seems to occur at non-physiological bile acid concentrations (43). At this moment, the FXR receptor seems to be most involved in the development of atherosclerosis, so we will discuss the role of this receptor in the next section.

The FXR receptor is the main bile acid sensor in the liver and intestine (56, 57, 58). Activation of the nuclear receptor FXR by a ligand, leads to translocation of FXR to the cell nucleus, where it binds to FXR response elements (FXRE) in the promoter of its target genes, regulating their transcription (43). Next to that, FXR can also indirectly regulate gene expression (43). This occurs for example in the liver, where FXR-activated small heterodimer partner (SHP) inhibits different factors that are necessary for optimal transcription of CYP7A1 (43). CYP7A1 is a key enzyme in the synthesis of bile acids out of cholesterol. (43). By activating FXR receptors in the intestine, bile acids also induce an intestinal-hepatic signalling pathway, which leads to the release of FGF-19 in humans and FGF-15 in mice. FGF-19/15 reaches the circulation, which also leads to the repression of CYP7A1 by binding to the FGF-4 receptor in hepatocytes (59, 60).

As described above, the regulation of bile acid synthesis is mediated by the FXR receptor. The synthesis and circulation of bile acids represents a major pathway of cholesterol catabolism (43). This suggests that the FXR receptor might be involved in lipid metabolism and the development of atherosclerosis. Several studies investigated the effect of FXR deficiency on lipid levels in mice (61, 62, 63). A study performed by Lambert *et al.* (61) evaluated the major pathways of cholesterol metabolism in FXR-deficient mice. They showed that FXR-deficient mice had increased plasma HDL, TG, LDL and VLDL levels and increased intestinal cholesterol absorption. Concomitantly, FXR-deficient mice had reduced expression of hepatic genes involved in reverse cholesterol transport (61). However, biliary cholesterol elimination was increased, despite decreased expression of hepatic genes thought to be involved in this process. These findings suggest that FXR is a positive regulator of HDL-mediated reverse cholesterol transport and a negative regulator of intestinal absorption of cholesterol (61). As mentioned above, FXR-deficient mice showed increased plasma TG levels, which implies that the FXR receptor plays a role in TG metabolism. A recent study showed that the activation of FXR improves plasma clearance of TG in wild-type mice, but not in FXR-deficient mice. (64). In addition, VLDL receptor expression, which is known to contribute to plasma TG clearance, increased due to FXR activation. (64). Taken together, several studies demonstrate that FXR plays a regulatory role in normal cholesterol metabolism and that changes in FXR function could influence the development of atherosclerosis.

Loss of FXR function in mouse models of atherosclerosis showed contradictory results, depending on the animal model and gender (43). On the one hand, FXR deficiency in LDL-receptor deficient mice is atheroprotective (65, 66). However, on the other hand, more severe atherosclerosis was observed in FXR-deficient mice who were also deficient in apoE, an apolipoprotein essential for normal catabolism of triglyceride-rich lipoprotein constituents (67). Next to expression of FXR in liver and intestine, FXR is also expressed in cell types of the blood vessel, including endothelial cells and vascular smooth muscle cells (VSMC) (68, 69). In endothelial cells, FXR activation leads to endothelium-dependent vasodilation through increased production of nitrogen oxide (NO). However, chronic stimulation of FXR leads to impaired relaxation in rabbit arteries, due to decreased sensitivity

of VSMC to NO (70). In VSMCs, FXR activation inhibits inflammation and migration of VSMCs in rats, effects that may attenuate vascular remodelling and atherosclerosis development (71). Taken together, activation of the FXR receptor might play a protective role against atherosclerotic plaque formation (43).

Sayin *et al.* investigated the difference in bile acid metabolism and FXR receptor activation between germ-free (GF) and conventionally raised (CONV-R) mice. They found out that, in the presence of a gut microbiota, mice had a smaller bile acid pool, with specific reductions in muricholic acid (MCA) rather than cholic acid (CA) (39). Bile acid levels were reduced in the gallbladder and small intestine of CONV-R mice compared with GF mice (39, 72, 73). In contrast, CONV-R mice had higher levels of bile acids in the cecum, colon, and feces compared with GF mice. Also the diversity of bile acids in the cecum, colon, and feces of CONV-R animals was greater than the diversity in GF mice. Next to that, no secondary bile acids were observed in GF mice (39). Taken together, this suggests that the reduced bile acid pool size in CONV-R mice may also reflect reduced bile acid reabsorption from the distal ileum and increased bile acid excretion in the feces (39). Earlier studies support a role for increased reabsorption of bile acids that may further contribute to the larger bile acid pool in GF mice. Gustafsson *et al.* and Riottot *et al.* demonstrated that TCA uptake is increased in ileal epithelium isolated from GF rats and that the half life of ¹⁴C-labeled CA is four to five times longer in GF. From these findings we can conclude that without gut microbiota, bile acids are reabsorbed in the distal ileum and are less excreted in the feces (74, 75). This results in higher levels of bile acids in the circulation and also a higher cholesterol content in the body, which is a risk factor for the development of atherosclerosis.

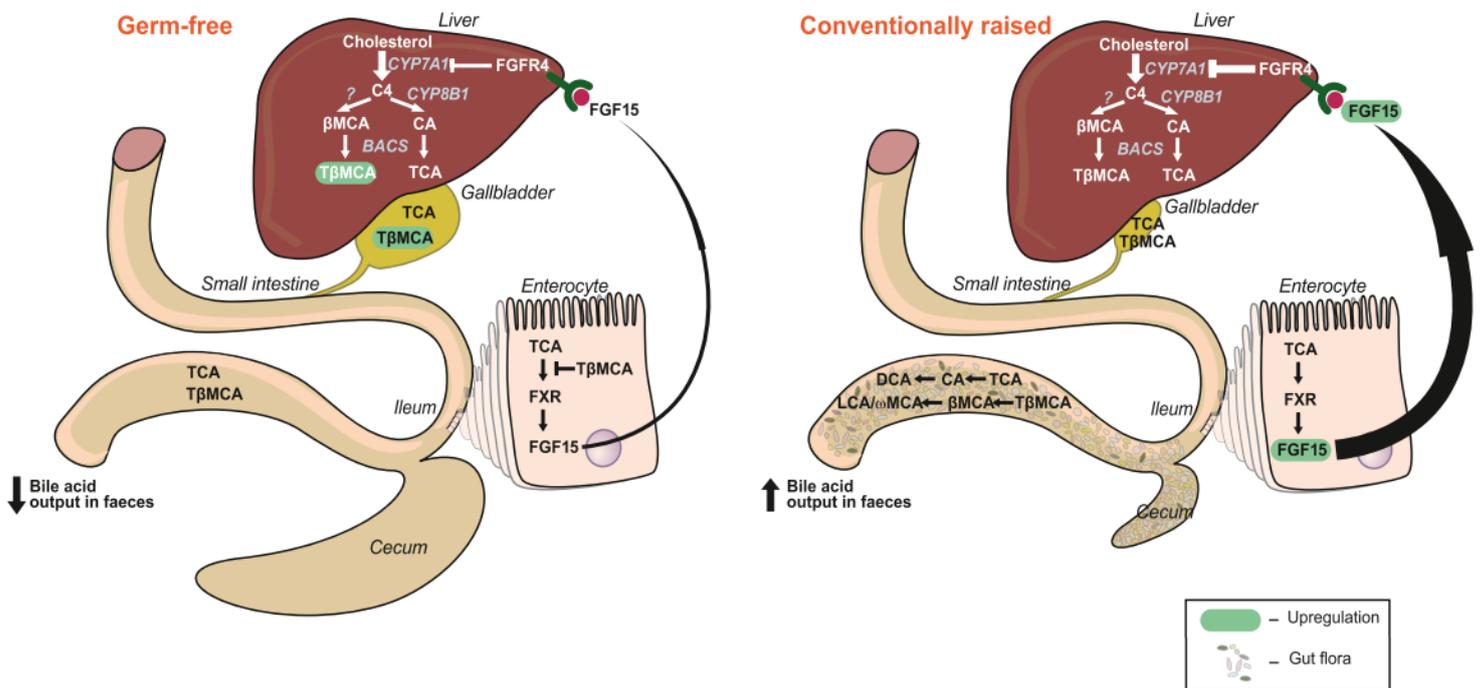


Figure 3. Model for microbial regulation of bile acid metabolism. GF mice have a larger total bile acid pool size compared with CONV-R mice. The activity and expression of CYP7A1 and levels of TβMCA are increased in GF mice. TβMCA functions as a natural antagonist of FXR, which may result in reduced inhibition of CYP7A1 in GF mice. In CONV-R mice, the reduction in TβMCA leads to increased activation of FXR in enterocytes, which upregulates FGF15, a potential suppressor of CYP7A1 in the liver. Furthermore, the presence of gut microbiota in the intestine of CONV-R mice causes variation in the bile acid profile due to the presence of secondary bile acids, as well as increased excretion of bile acids. Enzymes are indicated in gray (39).

Sayin *et al.* also found out that the presence of a gut microbiota increases the hydrophobicity of the bile acid pool and thus generates a bile acid profile that is a more potent activator of FXR (39). As described above, activation of the FXR receptor leads to more RCT and less reabsorption of bile acids in the intestine. This suggests that activation of the FXR receptor might be atheroprotective by reducing the levels of cholesterol in the body. Furthermore, FXR activation leads to the release of FGF-15 in mice, which leads to the suppression of CYP7A1 and thus bile acid synthesis. This finding sounds contradictory with the other atheroprotective effects of FXR, while bile acid synthesis contributes to cholesterol excretion. However, Sayin *et al.* found out that CYP7A1 is required for CDCA formation, which is rapidly converted to MCAs in mice (39). The study of Sayin *et al.* identified two taurine-conjugated forms of MCA, α MCA and β MCA, that seem to act as antagonists of the FXR receptor. CONV-R mice showed a lower T-MCA/CA ratio than GF mice, which results in increased activity of the FXR receptor in CONV-R mice. Furthermore, they showed that the antagonist β MCA significantly reduced the taurine-conjugated cholic acid (TCA) induced expression of FGF15 in the ileum *ex vivo* as well as *in vivo* (Figure 3) (39). Taken these findings together, the release of FGF15 upon FXR receptor activation leads to the inhibition of CYP7A1, an key enzyme in the synthesis of FXR receptor antagonists. The gut microbiota promotes FXR-dependent FGF15 expression in the ileum. Next to that, the gut microbiota promotes RCT and inhibits reabsorption of bile acids by activating the FXR receptor.

8. Gut-microbiota-driven trimethylamine pathway

Recent studies have identified that the metabolite trimethylamine-*N*-oxide (TMAO) in plasma is associated with CVD risks (2). TMAO, an oxidation product of trimethylamine (TMA), is a relatively common metabolite of choline and L-carnitine in animals (2, 76, 77). Choline and L-carnitine contain a TMA structure and can be metabolized to TMAO. The main dietary source for L-carnitine is red meat, whereas eggs, milk, liver, poultry, shell fish and fish are believed to be the major sources for choline and hence TMAO production (78).

8.1. The relation between increased TMAO levels and atherosclerosis

Recently, Hazen *et al.* have reported that the metabolites TMA and TMAO are pro-atherogenic in both mice and humans (2, 4, 79). They found out that oral feeding of choline, rather than parental delivery was necessary to generate the metabolite TMAO. This suggests that a necessary phase in the conversion of choline to TMAO was performed within the intestine (79). Dietary choline and L-carnitine were shown to be metabolized by intestinal bacteria to TMA (4, 80). Subsequently, the generated TMA is absorbed into the host and rapidly metabolized to TMAO in the liver by at least one member of the hepatic flavin monooxygenase (FMO) family of enzymes, FMO3 (Figure 4) (2, 4).

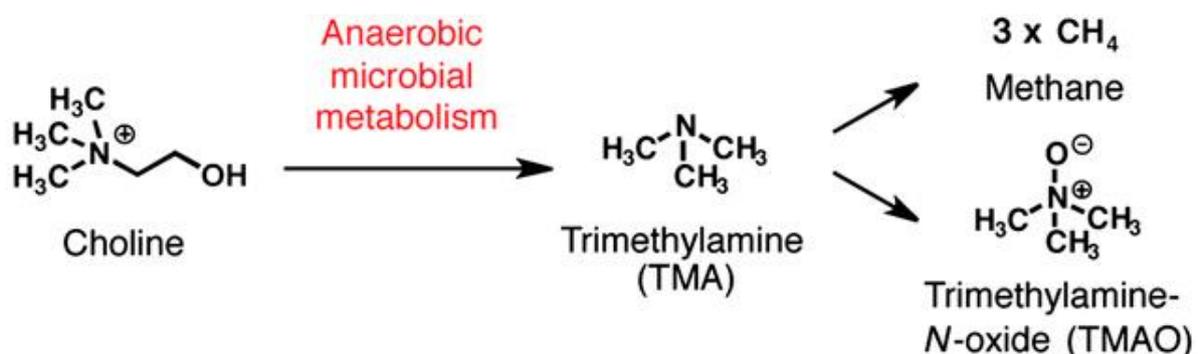


Figure 4. Choline metabolism to TMAO. Generation of trimethylamine (TMA) from choline by bacterial metabolism. Subsequently, TMA can be metabolized to TMAO by a member of the hepatic flavin monooxygenase (FMO) family of

The gut-microbiota-driven trimethylamine pathway has been well studied (80). Gut microbes take in choline or L-carnitine containing compounds, and release TMA, which is metabolized in the liver to TMAO (2, 4). Several studies have linked this pathway to the development of cardiovascular diseases, such as atherosclerosis in mice and humans. Wang *et al.* observed that aortic root atheroma sizes in mice and clinical plaque burdens increase in parallel with TMAO (2, 80). With preserved intestinal microflora, choline supplementation augmented atherosclerosis in mice nearly threefold. In contrast, suppression of intestinal microbiota by antibiotics completely inhibited dietary choline-mediated enhancement in atherosclerosis (2). A study by Koeth *et al.* also showed a gut-microbiota dependent increase in atherosclerosis in mice upon L-carnitine supplementation. However, this occurred in the absence of pro-atherogenic changes in lipid, lipoprotein and glucose levels (81). Also in a cohort study, researchers observed a relationship between high plasma TMAO levels and cardiovascular events. This correlation remained robust even following adjustment for traditional risk factors (79, 80). Because plasma TMAO levels are associated with atherosclerosis (2, 81), it is interesting to find out if intake of compounds that contain a TMA-structure, such as choline or L-carnitine, is linked with increased TMAO levels (80). In several studies, when humans or mice were fed with isotope-labelled choline or L-carnitine, indeed tracer-labelled TMAO was subsequently noted in both the plasma and urine (2, 81).

So as described before, the intake of foods that contain choline or L-carnitine leads to the production of the gut-dependent metabolites TMA and TMAO. High TMAO levels lead to an increase risk of cardiovascular diseases, such as atherosclerosis (Figure 5). These findings raise the question whether we need choline and L-carnitine containing compounds or not. Several studies showed that choline and L-carnitine do not only have negative effects on the human body. Trimethylamine-containing nutrients such as choline and L-carnitine play a central role in human metabolism (80). Choline, for example, is a component of the cell membrane and plays a role in lipid metabolism, anti-platelet aggregation and immune modulation (82). Choline can also be oxidized to betaine, which is involved in both membrane structure and lipid metabolism. Choline and betaine have also been linked to reduced inflammation and plasma homocysteine levels (83, 84). Events such as dyslipidaemia, inflammation and platelet aggregation are linked to cardiovascular disease. Given that choline has some protective effects by preventing inflammation and dyslipidaemia, choline supplementation could even be cardio protective (82, 84). Taken together, our body needs choline supplementation to a certain degree because of its cardio protective effects, however a lot of choline leads to the production of high levels of TMAO, which is pro-atherogenic in contrast. So we need TMA-containing nutrients, however the safety of supplementation of compounds as choline and L-carnitine with respect to the management of TMAO levels and cardiovascular disease has to be determined on large-scale (80).

As described above the intake of TMA-containing nutrients may be cardio protective, but can also be a risk for development of atherosclerosis, through metabolites as TMAO. To protect people from the negative effects of metabolites as TMAO, it is important to know something about the underlying mechanism of the relation between TMAO, atherosclerosis and the gut microbiota. The formation of lipid-rich macrophages within the arterial wall is the fundamental pathological change in atherosclerosis (80). Macrophages within the arterial wall take up oxidized LDL, which is mediated by macrophage 'scavenger' receptors. As mentioned earlier in this thesis, HDL is believed to protect from atherosclerosis by the transport of lipids out of the arterial wall, a process called 'reverse cholesterol transport'. The process of atherosclerosis can be accelerated by compounds that affect lipid influx, lipid synthesis or lipid clean up (80). Several researchers investigated if TMAO accelerates atherosclerosis by influencing one of those processes.

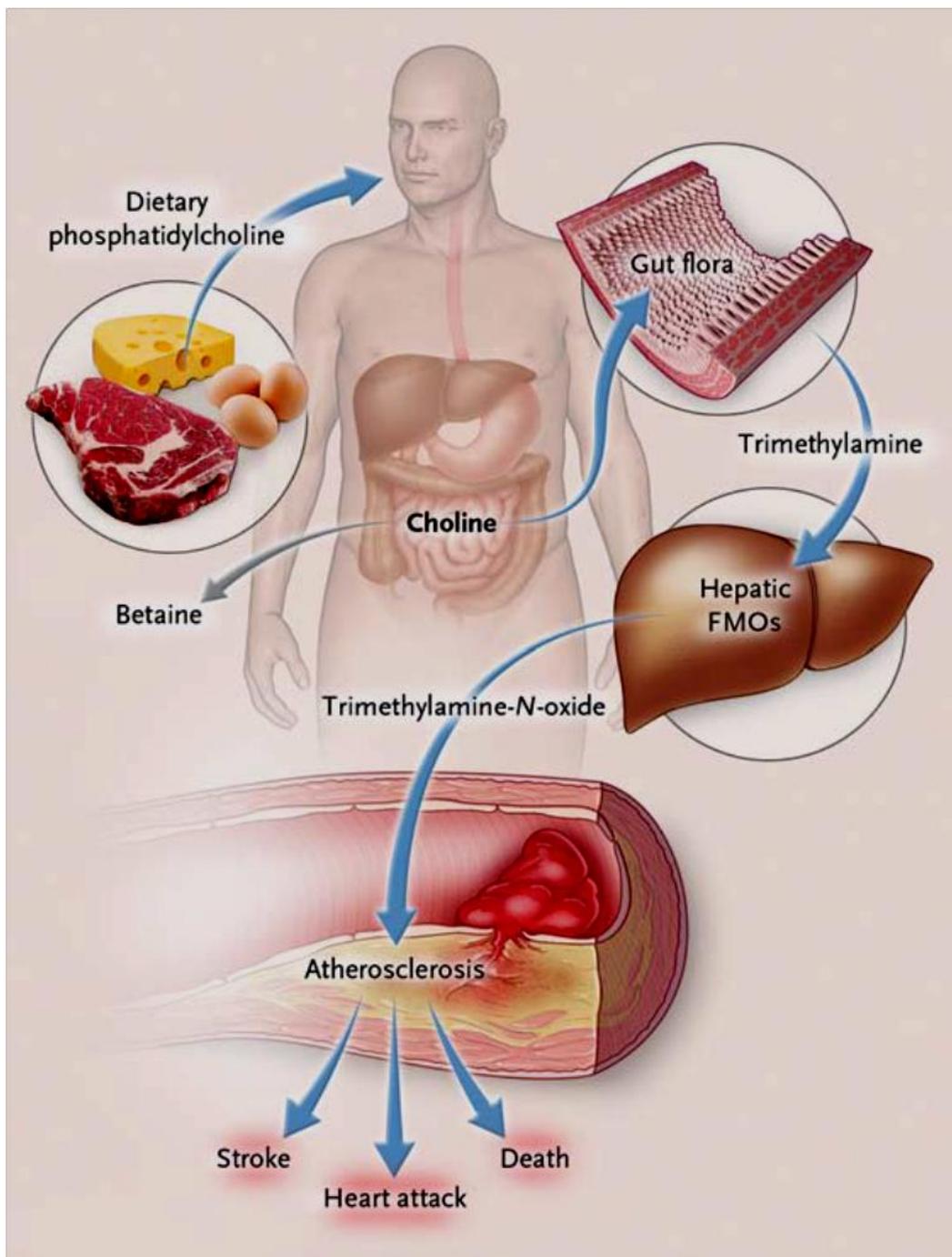


Figure 5. Pathways linking dietary choline, intestinal microbiota and atherosclerosis. Choline-containing nutrients that reach the cecum and large bowel may serve as fuel for intestinal microbiota, producing TMA. TMA is rapidly further metabolized to TMAO, which is associated with increased atherosclerosis risk. TMAO enhances the accumulation of cholesterol in macrophages. Choline can also be oxidized to betaine, which also serves as a substrate for bacteria to form TMA and presumably TMAO (79).

8.2. Potential mechanisms underlying the involvement of TMAO in atherosclerosis

Koeth *et al.* investigated whether TMAO affects cholesterol synthesis by regulation of cholesterol biosynthesis genes. They cultured macrophages with cholesterol and observed that in the presence or absence of physiologically relevant TMAO concentrations, TMAO failed to alter mRNA levels of the LDL receptor or cholesterol synthesis genes. (81). Parallel studies observed that also inflammatory gene expression in macrophages didn't change in the presence of TMAO. (85).

In contrast to cholesterol synthesis, TMAO does affect foam cell formation. Wang *et al.* supplied mice with choline, betaine or TMAO and observed an increase in the levels of two atherosclerosis-related macrophage scavenger receptors, cluster determinant 36 (CD36) and SR-A1. They also examined the impact of dietary choline and gut microbiota on endogenous formation of foam cells. Mice on the choline supplemented diet showed markedly enhanced lipid-laden macrophage development compared to mice on normal diet. In contrast, suppression of the intestinal bacteria using antibiotics significantly inhibited dietary-choline-induced foam cell formation. Plasma TMAO levels were only significant higher in mice on the high-choline diet in the absence of antibiotics, suggesting TMAO-dependent foam cell formation (2).

TMAO also affects the removal of cholesterol from the body by suppressing RCT as well as by blocking bile acid secretion (81). Koeth *et al.* tested whether dietary sources of TMAO (choline or L-carnitine) inhibit RCT in mice. Mice on choline or L-carnitine supplemented diets showed significantly less RCT compared to normal chow-fed controls (81). When they suppressed the intestinal microbiota (and thus plasma TMAO concentrations) with antibiotics, the suppression of RCT was reversed, suggesting that a microbiota-generated product, such as TMAO, is responsible for the inhibition of RCT. Indeed a TMAO-containing diet led to a 35% decrease in RCT compared to mice on a normal diet (81). So far, how TMAO exactly alters RCT remains unclear (80). The study by Koeth *et al.* also showed that the gut microbiota-dependent metabolite TMAO affects a major pathway for cholesterol elimination from the body, the bile acid synthetic pathway, at multiple levels. For example mRNA levels in the liver of the key bile acid synthetic enzymes CYP7A1 and CYP27A1 were significantly lower in mice upon TMAO supplementation. Also a dietary-TMAO induced decrease in expression of multiple bile acid transporters (Oatp1, Oatp4, Mrp2, and Ntcp) in the liver was observed. Next to the effects of TMAO on bile acid transporter gene expression, supplementation of TMAO also affects total bile acid pool size. Mice on a TMAO-diet had a smaller bile acid pool size compared to normal fed mice. (81.) As the bile acid pathway plays a major role in cholesterol elimination, blocking this pathway may have accelerated atherosclerosis (80).

Taken together, the findings described above show that TMAO accelerates the process of atherosclerosis by facilitating foam cell formation and suppressing RCT and bile acid secretion.

The studies of Wang *et al.* and Koeth *et al.* also showed that gut bacteria are essential for the generation of TMAO. When gut microbiota were completely suppressed by antibiotics, TMA and TMAO levels were also decreased in mice (2, 81). Trials in humans also showed decreased TMAO levels following the administration of antibiotics (79). Furthermore, plaque burdens and numbers of macrophages in the plaques were decreased in mice upon antibiotic administration (2, 81).

These findings support the relation between gut microbiota, TMAO and atherosclerosis.

8.3. Identification of bacteria responsible for TMA-generation

The next step in understanding the relationship between gut microbiota, TMAO and atherosclerosis is the identification of the bacteria responsible for TMA generation. Koeth *et al.* compared the TMAO production upon L-carnitine supplementation between vegans/vegetarians and omnivores, to test whether L-carnitine modulates gut flora taxa and thereby influencing the ability of microbes to generate TMAO (80, 81). Significant lower baseline TMAO levels in plasma and urine were observed in vegans and vegetarians compared with omnivores. Even following L-carnitine intake, vegans and vegetarians showed a markedly reduced capacity to synthesize TMAO (81). By sequencing bacterial 16S rRNA in fecal samples, several bacterial taxa were linked with both dietary habits (vegetarians vs omnivores) and TMAO levels (81). Individuals with an enterotype characterized by enriched proportions of the genus *Prevotella* had higher plasma TMAO concentrations than subjects with an enterotype notable for enrichment in the *Bacteroides* genus (81). So the component L-carnitine in meat may modulate the gut microbiota and thereby affect the ability to generate TMAO.

The identification of bacterial enzymes responsible for TMA production may provide more information about specific bacterial taxa responsible for TMA and thus TMAO generation. Zhu *et al.* identified an enzyme, composed of both an oxygenase (CntA) and a reductase (CntB) and associated gene cluster proposed to be involved in L-carnitine metabolism in representative genomes of the human microbiota (86). They identified genetic and biochemical mechanisms for TMA production from L-carnitine in representative human microbiota: *Gammaproteobacteria*, *Betaproteobacteria* and *Firmicutes*. Using *Acinetobacter baumannii* as model, it was demonstrated that cntAB is essential in the metabolism of L-carnitine to TMA (86). Another study by Craciun *et al.* identified enzymes responsible for the generation of TMA out of choline. The co-expression of choline utilization C (CutC) and choline utilization D (CutD) is associated with the conversion of choline to TMA. Bacteria in which gene clusters for the enzymes CutC and CutD are found, include some strains of *D. askensis*, *D. desulfuricans*, *C. hathewayi* and *C. sporogenes*.(87).

Romano *et al.* revealed eight bacterial species representing two different phyla (*Firmicutes* and *Proteobacteria*) and six genera that showed choline consumption and TMA accumulation in vitro: *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Escherichia fergusonii*, *Proteus penneri*, *Providencia rettgeri*, and *Edwardsiella tarda* (88). Seven of the eight identified species encode CutC and CutD, components involved in the conversion of choline to TMA. Only *E. tarda* did not contain these genes, suggesting another pathway of choline metabolism. Subsequently the eight bacterial species were colonized in germ-free mice. Addition of the TMA-producing species *C. sporogenes*, which represented only 0,15% of the cecal community, to a core community of five non-TMA producing bacteria, resulted in the significant reduction of fecal choline, accumulation of TMA in the cecum, and appearance of TMAO in serum. However surprisingly, addition of the other 7 TMA-producing bacteria to the core + *C. sporogenes* did not significantly change the levels of choline, TMA and TMAO compared to the core and *C. sporogenes* alone (88). Taken together, the results describe above demonstrate a link between gut microbial TMA-production and TMAO accumulation in vivo. However, phylogeny is a poor predictor of microbial TMA formation from choline. Genes encoding for enzymes involved in TMA production may be transferred to non-TMA producing bacteria (80). Next to that, additional TMA-producing enzymes have to be identified. Beside the abundance of TMA-producing bacteria other factors, such as host genotype, may also account for differences in TMA accumulation. Romano *et al.* for example shows gender differences in FMO3 expression, leading to differences in TMAO levels. Gender differences in TMAO accumulation can be derived from differences in FMO3 expression, instead of higher microbiota-generated TMA levels (88).

9. The gut microbiota as therapeutic target in the treatment and prevention of atherosclerosis

Although many questions regarding the bile acid metabolism pathway and the trimethylamine pathway have yet to be elucidated, the previous sections suggest a contributory role of the gut microbiota in the development of atherosclerosis by interacting with these two pathways. This knowledge might provide new strategies to treat and prevent atherosclerosis, by for example selective manipulation of the gut microbiome. Nowadays, four different interventions are known to manipulate the composition of bacteria in the gut: antibiotics, diet, fecal microbiota transplant (FMT) and probiotics (Figure 6) (37).

9.1. Antibiotics

Modern medicine has relied heavily on the prescription of antibiotics to eliminate bacterial species or their products (37, 48). Before the development of other therapeutic interventions, gut infections with bacteria such as *Helicobacter pylori* and *Clostridium difficile* were controlled with the use of antibiotics (48, 89). The use of antibiotics may also be beneficial in controlling the development of atherosclerosis by interfering with the trimethylamine pathway. Elimination of the bacteria

responsible for generation of the TMAO-metabolite might be favourable, as this bacterial product is associated with increased atherosclerosis risk by mechanisms described in the sections above. Indeed, Wang *et al.* and Koeth *et al.* showed that administration of antibiotics in mice led to a decrease in TMAO levels and atherosclerosis risk, as mentioned above (2, 81). However antibiotics are relatively non-discriminating between pathogenic and non-pathogenic bacteria (48) and thus long term administration may be complicated by other problems such as diarrhea but also organ injury and drug tolerance (48, 80).

9.2. Diet/Prebiotics

Diet might be one of the most important interventions to avoid high TMAO levels, which can contribute to accelerated atherosclerosis. Consuming foods rich in L-carnitine (predominantly red meat) and choline can increase the levels of TMAO in plasma. Meats and dairy products, which contain L-carnitine and choline, are abundant components of the Western diet (81). In a recent dietary study, 30% reduction in cardiovascular events was observed in subjects consuming a Mediterranean diet, with specific avoidance of red meat, compared with individuals consuming a control diet (90). Next to that, Koeth *et al.* recently suggested that reduced intake of L-carnitine and choline by vegans and vegetarians leads to decreased TMAO levels (81). This may contribute to their observed cardiovascular health benefits. However, if intake of L-carnitine and choline containing nutrients should be limited to reduce atherosclerosis risk has to be further investigated.

Nutritional intervention such as the promotion of beneficial bacteria growth by prebiotic supplementation may also be a possible intervention in the bile acid metabolism pathway. Prebiotics are substrates that are used by bacteria as energy sources and promote the growth of beneficial bacteria (91). If certain prebiotics could promote the growth of gut bacteria responsible for generating secondary bile acids, which leads to FXR activation and thereby reduced cholesterol levels in the body, prebiotics could be a therapeutic intervention in individuals with increased atherosclerosis risk. Cani *et al.* demonstrated that the abundance of *Bifidobacteria* and *Lactobacilli*, which are involved in bile acid metabolism, increased upon supplementation with a prebiotic containing oligo-fructans type fibres (92). Next to that, dietary supplementations with prebiotics in mouse models was also associated with modification of lipid metabolism, such as downregulation of proteins involved in VLDL synthesis (93, 94).

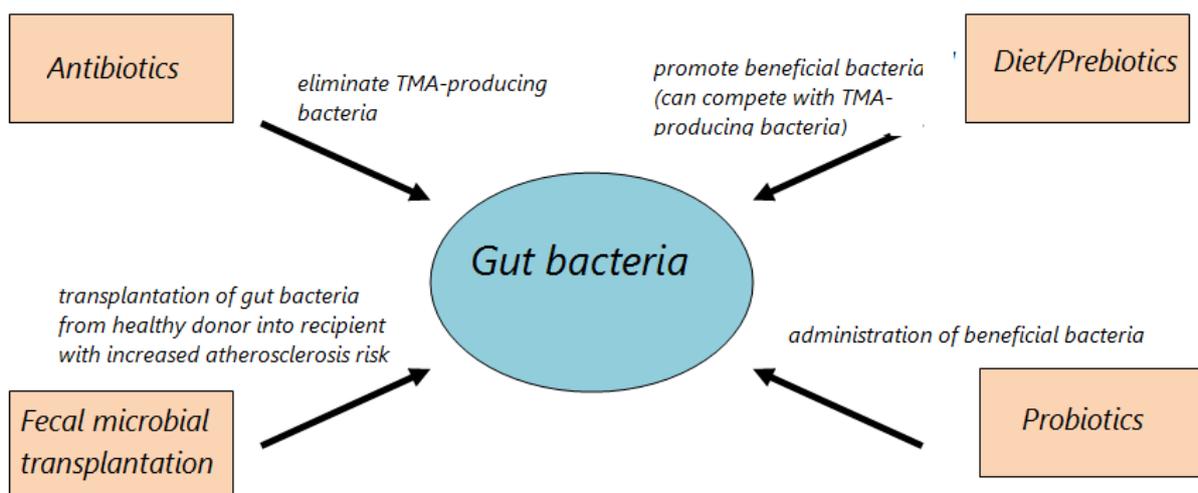


Figure 6. Overview of the gut microbiota as therapeutic target in the development of atherosclerosis. The use of antibiotics can lead to decreased levels of TMA-producing bacteria, which can be beneficial for atherosclerosis. Next to that, pre- and probiotics might increase the level of beneficial bacteria. Another therapeutic target is fecal microbial transplantation.

9.3. Probiotics

In addition to antibiotics and diet/prebiotics, administration of probiotics may also be useful in the manipulation of gut microbiota. Probiotics are defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host (95). One of the most investigated applications for probiotic therapy in atherosclerosis is the reduction of serum cholesterol (48). The ability of bacteria to lower cholesterol is strain and species specific and involves several mechanisms of action (48). The mechanism we discussed in this thesis, the processing of bile acids in the gut by Gram-positive bacteria including *Lactobacillus* and *Bifidobacterium*, is one of the most accepted mechanisms. Gut microbes expressing the enzyme bile salt hydrolase (BSH) mediate the metabolism of cholesterol to several bile acids (96, 97). Different probiotic strains contain different BSH phenotypes and many probiotic strains express more than one BSH homolog, which potentially helps the bacteria to survive in the gut when exposed to different types of bile acids (49, 98). A recent study by Degirolamo *et al.* showed that intake of an 8 strain probiotic VSL#3 increased fecal bile acid deconjugation and excretion in mice (99). VSL#3 consists of *Lactobacillus acidophilus*, *Lactobacillus paracasei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus plantarum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, and *Streptococcus thermophilus* (99, 100). If such a probiotic could be used in the prevention or treatment of atherosclerosis has to be further investigated.

Intervention with probiotics may also play a role in the trimethylamine pathway.

Recently, Brugère *et al.* identified an archaea strain known as *Methanomassiliicoccus luminyensis* B10, which was able to catabolize TMA (101). Administration of this strain might be beneficial for an individual's health by reducing TMAO levels due to catabolism of TMA. However, further research is needed to determine if this strain will be able to effectively deplete TMA in the gut and to determine the best way to deliver such oxygen-sensitive microorganism into the gut. Further research has to determine whether other bacterial strains are able to catabolize TMA and if administration of TMA-catabolizing bacteria can protect against atherosclerosis. Probiotics might not only contribute to an increase in TMA-catabolizing bacteria, but could also lead to a decrease in TMA-generating bacteria. Administration of bacteria that are beneficial for human health may lead to increased competition between beneficial bacteria and TMA-generating bacteria, leading to reduced numbers of TMA-generating bacteria.

9.4. Fecal microbiota transplant

The fourth option to manipulate the composition of bacteria in the gut is that of fecal microbiota transplant (FMT). In this process, fecal bacteria are transplanted from a healthy human into a recipient. Previous studies have demonstrated FMT as an effective therapy in patients infected by *Clostridium difficile* (102). Next to that another study showed that transplantation of microbiota from healthy individuals into recipients with metabolic syndrome led to an improvement in insulin sensitivity (103). If we take that into account, FMT might be a new therapeutic strategy for patients with increased atherosclerosis risk, caused by the presence or absence of gut bacteria associated with for example bile acid metabolism or TMAO production. Indeed, Gregory *et al.* demonstrated that atherosclerosis susceptibility can be transmitted via transplantation of the gut microbiota in mice (12). However, the application of FMT in humans and the influence of FMT on the bile acid pathway and the trimethylamine pathway has to be further investigated.

10. Discussion

The aim of this thesis was to gain some insight in the mechanisms by which gut bacteria affect lipid metabolism of their host and if this could influence the development of atherosclerosis in the host. Taken the findings in several studies together, gut bacteria seem to affect lipid metabolism of their host by acting on two main pathways, the bile acid metabolism pathway and the trimethylamine pathway. Both pathways indeed seem to be associated with the development of atherosclerosis.

Bile acids play an important role in the excretion of excess cholesterol from the body and function also as signalling molecules. Several bacterial species are involved in the metabolism of primary bile acids into secondary bile acids. By acting on the FXR receptor, these gut-microbial-derived bile acids are important players in normal cholesterol metabolism. FXR receptor activation leads to increased HDL-mediated RCT and decreased intestinal cholesterol absorption, which results in decreased serum cholesterol levels and thus reduced atherosclerosis risk. Sayin *et al.* showed that the presence of gut microbiota is essential in these processes, given that, in the absence of gut microbiota, mice displayed less excretion of bile acids in the feces and thus higher serum cholesterol levels (39).

Next to that, gut bacteria are involved in the trimethylamine pathway. Choline or L-carnitine containing nutrients, such as milk, eggs and red meat, are metabolized by gut bacteria to TMA, which is subsequently converted to the pro-atherogenic metabolite TMAO in the liver. Several studies mentioned earlier in this thesis proved that choline or L-carnitine supplementation resulted in increased atherosclerosis risk due to high levels of TMAO only in the presence of preserved gut microbiota. TMAO accelerates atherosclerosis by facilitating foam cell formation and suppressing of RCT and bile acid secretion (2, 81).

The findings described above seem to explain a clear relationship between gut microbiota, lipid metabolism and atherosclerosis risk. However, some contradictions in literature are observed about the role of microbiota in the bile acid metabolism pathway and the link with atherosclerosis. As described earlier in this thesis, Sayin *et al.* (39) described that gut microbiota promotes FXR-dependent FGF15 expression in the ileum, which leads to inhibition of CYP7A1, an key enzyme in the synthesis of FXR receptor antagonists, two taurine-conjugated forms of MCA. This suggests that gut bacteria promote FXR receptor activation and thereby increase RCT and decrease intestinal cholesterol absorption by reducing the levels of FXR antagonists, which might protect against atherosclerosis. Another study by Degirolamo *et al.* (99) showed that colonization of gut flora with VSL#3 promoted increased BA deconjugation and fecal excretion and these events were associated with increased hepatic synthesis and biliary output and repression of the enterohepatic FXR/FGF15 axis. These events could also protect against atherosclerosis, because increased biliary output leads to decreased cholesterol levels in the body. However, the mechanisms described by Sayin *et al.* include FXR/FGF15 axis activation and those described by Degirolamo *et al.* include FXR/FGF15 axis repression, which is contradictory (39, 99).

Degirolamo *et al.* suggests that VSL#3 administration limits the availability of conjugated bile acids, which are FXR ligands. The bacterial species in VSL#3 contain BSH-activity, which leads to deconjugation of bile acids. This results in decreased levels of conjugated bile acids, which leads to repression of the FXR/FGF15 axis and thus upregulation of the CYP7A1 gene. However, a functional FXR transcriptional activity is required for probiotic bacteria to promote fecal bile acid loss and induce hepatic bile acid synthesis, as FXR and FGF-15 deficient mice showed no alterations in bile acid metabolism upon VSL#3 administration (99). Taken these findings together, the FXR/FGF15 axis seems to play an important role in bile acid metabolism and thus in cholesterol balance in the body. This suggests that the FXR/FGF15 axis and the gut bacteria that metabolize bile acids affecting this axis might play a role in the development of atherosclerosis. The differences observed between the studies of Sayin *et al.* and Degirolamo *et al.* might be the result of the existence of different bile acids that arise from metabolic actions of different bacterial species. Some bile acids might have activating actions on the FXR/FGF15 axis, while other bile acids have inhibiting actions on the same axis, which results in various effects on bile acid metabolism and thus cholesterol balance in the body. **It has to be investigated if there is a certain balance between activation and repression of the FXR/FGF15 axis, which leads to most favourable conditions with regard to cholesterol balance in the body and thus atherosclerosis risk.**

The findings mentioned in this thesis suggest a contributory role of the gut microbiota in the development of atherosclerosis by interacting with the bile acid metabolism pathway and the trimethylamine pathway. However, a lot of research is needed to reveal which bacteria affect lipid metabolism and in which manner. Further research should be directed towards revealing these questions. As mentioned earlier, further research considering bile acid metabolism should focus on specific bacterial species and the chemical conversions of bile acid metabolism caused by these bacteria. In the next step the actions of bacterial-derived bile acids on the FXR-receptor and the consequences of activation or inhibiting of this receptor has to be better established. The outcome of such studies might in the future lead to the development of therapeutic interventions, such as probiotics, which can reduce the risk of atherosclerosis in for example people with high cholesterol levels. In addition, the trimethylamine pathway gives some starting points for further research about the relation between gut microbiota and the development of atherosclerosis. Manipulation of intestinal microbiota may lead to decreased TMAO levels. Therefore, it is necessary to determine which types of bacteria are associated with elevated TMAO levels and if certain bacteria are atherogenic. Next to that, it is important to determine if intake of choline or L-carnitine containing compounds should be limited to prevent the development of atherosclerosis due to high TMAO levels. **In conclusion, a lot of further research is needed to determine if the gut microbiota might be a therapeutic target to prevent or even treat the process of atherosclerosis, but the studies performed so far suggest at least a contributory role of gut microbiota in lipid metabolism. Gut microbiota might be a good target to prevent from atherosclerosis and thereby one of the leading causes of death in the world in the future.**

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