The gut microbiota in cardiovascular disease
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CHAPTER 1

Introduction
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Cardiovascular disease (CVD) is a major burden for the society worldwide leading to 17.7 million deaths per year and causing 31% of all global deaths. The majority of cardiovascular deaths is caused by heart attacks and strokes with atherosclerosis as the main underlying cause (World Health Organization). Atherosclerosis starts by the formation of fatty streaks, which are formed by the deposition of lipids and cholesterol and the infiltration of blood leukocytes into the intima of the vessel wall. Consequently, the infiltrated monocytes differentiate into macrophages, which take up lipids via scavenger receptors to form foam cells. Smooth muscle cells (SMC) infiltrate from the media into the intima to form the fibrous cap. Cell death of SMC and foam cells leads to extracellular lipid accumulation and formation of the necrotic core (Libby et al., 2011). Rupture of the fibrous cap can cause thrombus formation, which can block the blood flow of coronary or carotid arteries leading to myocardial infarction or a stroke respectively. Therefore, prevention and adequate treatment of atherosclerosis is essential to reduce the number of cardiovascular deaths worldwide.

Treatment of cardiovascular disease
In 1913, Anitschkov discovered the importance of dietary cholesterol in the atherogenic process (Anitschkov et al., 2011) and over the last century the importance of hyperlipidemia as a driving force for atherosclerosis has become well-established (Dawber et al., 1951; Ference et al., 2017; Gofman et al., 2007). The discovery of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, better known as statins, by Akio Endo in 1976 was a major step in cardiovascular disease management (Endo et al., 1976a; Endo et al., 1976b). Inhibition of HMG-CoA reductase by statins affects cholesterol metabolism by preventing endogenous cholesterol synthesis, promoting uptake and degradation of plasma LDL-cholesterol and inhibiting scavenger receptors (Stancu and Sima, 2001) (Figure 1). This leads to a successful reduction of plasma LDL-cholesterol levels and a reduction in cardiovascular death (Group, 1994). The use of genetic linkage analysis has led to the discovery of new target genes for treatment of cardiovascular disease, including proprotein convertase subtilisin/kexin type 9 (PCSK9) (Abifadel et al., 2003). PCSK9 is an important modulator of the LDL receptor, increased
levels of PCSK9 in blood lead to downregulation of the LDL receptor in the liver, hence LDL-cholesterol levels rise in the circulation (Bjorklund et al., 2014). Recent studies have shown a 60% decrease in LDL-cholesterol on top of statin treatment following treatment with the PCSK9-inhibitor Evolocumab (Sabatine et al., 2017). Combined treatment by statins and PCSK9-inhibitors leads to a reduction of LDL-cholesterol levels to 0.78 mmol/L, which is well within the normolipidemic range (Sabatine et al.,
Nevertheless, these strategies do not lead to complete protection against atherosclerosis. Combining statins and PCSK9-inhibitors, which successfully protect against hyperlipidemia, reduces the number of cardiovascular events by 50% (Sabatine et al., 2017), indicating that factors independent of hyperlipidemia contribute substantially to atherosclerosis. Next to hyperlipidemia, associative studies in human and mechanistic studies in mice support an important role for inflammation in atherosclerosis (Libby et al., 2011; Moss and Ramji, 2016; Ramji and Davies, 2015). This is supported by the CANTOS-trial, which investigated the efficacy of Canakinumab, an anti-inflammatory drug that targets the pro-inflammatory cytokine IL-1β. In the CANTOS-trial patients with previous myocardial infarction and increased systemic inflammation were treated with Canakinumab (Ridker et al., 2017). The study showed a significant reduction of 15% in the number of cardiovascular events following treatment with Canakinumab on top of statin treatment, thereby showing that targeting inflammation in atherosclerosis is therapeutically effective in the protection against cardiovascular disease.

**Inflammation in atherosclerosis**

Leukocytosis is associated with atherosclerosis in humans and specifically increased numbers of circulating monocytes and neutrophils are well-known to contribute to atherogenesis in Ldlr-/- and ApoE-/- mice (Swirski and Nahrendorf, 2013). Accumulation of cholesterol into hematopoietic and progenitor cells (HSPCs) leads to the production of GM-CSF and increased expression of the beta chain of the IL-3 receptor (IL-3Rβ) thereby promoting increased hematopoiesis leading to leukocytosis (Murphy et al., 2011; Yvan-Charvet et al., 2010). Circulating monocytes and neutrophils can consequently migrate into the intima of the vessel wall by binding to the adhesion molecules ICAM-1 and VCAM (Radi et al., 2001). In healthy conditions arterial epithelial cells resist binding of leukocytes, however dyslipidemia, hypertension or pro-inflammatory stimuli can upregulate the expression of ICAM-1 and VCAM thereby facilitating the uptake of leukocytes into the intima of the vessel wall (Libby et al., 2011). Following migration into atherosclerotic lesion areas neutrophils promote oxidative stress which leads to endothelial cell dysfunction, lesion growth and plaque instability (Swirski and Nahrendorf, 2013). In addition, neutrophils also stimulate further migration of monocytes into the lesion
area (Drechsler et al., 2010). Following migration of monocytes into the intima of the vessel wall monocytes can differentiate into macrophages (Libby et al., 2011). Intracellular lipid accumulation into macrophages via uptake of lipids transforms these macrophages into foam cells (Libby et al., 2011). Production of pro-inflammatory cytokines including IL-1β and TNF-α by macrophages progresses atherogenesis (Chi et al., 2004; Elhage et al., 1998; Kirii et al., 2003) and facilitates the infiltration of other leukocytes, including T-cells. Although the number of T-cells in atherosclerotic plaques is low, specific subsets do seem to be important in atherogenesis (Swirski and Nahrendorf, 2013). T-helper 1 and T-helper 17 cells are important producers of IFN-γ and IL-17 respectively and promote atherogenesis, whereas regulatory T-cells protect against atherosclerosis development by producing the anti-inflammatory cytokines IL-10 and TGF-β. Deficiency of the Th1 cytokine IFN-γ or the Th17 cytokine IL-17 reduces atherogenesis whereas administration of the anti-inflammatory cytokine IL-10 protects against atherosclerosis (Ramji and Davies, 2015).

To summarize, atherosclerosis is associated with increased circulating numbers of leukocytes and specific leukocyte subsets, including monocytes, neutrophils and T-helper cells infiltrate into the atherosclerotic lesion area and produce pro-inflammatory cytokines and reactive oxygen species, altogether this further exacerbates atherogenesis. Hyperlipidemia is one of the driving forces of the inflammatory process, nevertheless the anti-inflammatory drug Canakinumab effectively reduced the number of cardiovascular events by 15% during normolipidemic conditions (Ridker et al., 2017). This indicates that factors independent of hyperlipidemia contribute to the inflammatory process in the etiology of atherosclerosis. Although the CANTOS-trial effectively reduced cardiovascular events, an important side-effect was the increased incidence of fatal infections or sepsis. Increased susceptibility for infections is an intrinsic risk factor of drugs targeting inflammation, therefore it is important to understand which factors contribute to systemic inflammation in the atherogenic process. A better understanding of the mechanisms leading to systemic inflammation in the context of atherosclerosis development could lead to new therapeutic targets, where factors causing systemic inflammation could be directly targeted instead of the inflammatory process itself.
The microbiota in cardiovascular disease

Recently, the gut microbiota was identified as an additional player affecting atherosclerosis development (Koeth et al., 2013; Li et al., 2016; Tang and Hazen, 2014). Atherosclerosis is associated with changes in microbiota composition (Emoto et al., 2016; Jie et al., 2017; Karlsson et al., 2012). The presence of Collinsella in the gut microbiota of patients with symptomatic atherosclerosis is increased, whereas a decrease is observed for Eubacterium and Roseburia (Karlsson et al., 2012). In addition, a large case control study confirmed the reduction of Roseburia intestinalis and identified novel associations between gut microbiota composition and atherosclerosis, including a reduction in Faecalibacterium Praunitzii and increased abundance of Ruminococcus Gnavus, Escherichia Coli, Klebsiella and Enterobacter aerogenes in CVD patients (Jie et al., 2017). Increased presence of Ruminococcus Gnavus was previously associated with inflammatory bowel disease and intestinal barrier function (Matsuoka and Kanai, 2015). A decrease in intestinal barrier function is associated with leakage of bacteria or endotoxins (e.g lipopolysaccharide, LPS) from the gut into the systemic circulation (Brandsma et al., 2015). In addition, bacteria or endotoxins can translocate from the gut into systemic circulation via leakage between intestinal epithelial cells or via active cellular transport, to be discussed in depth in Chapter 2 and Chapter 4. Interestingly, taxonomies of the gut microbiota have been detected in atherosclerotic lesions (Koren et al., 2011) and systemic infection with Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis has been shown to promote systemic inflammation and atherogenesis (Hayashi et al., 2011; Zhang et al., 2010). Moreover, translocation of LPS from the gut lumen into the systemic circulation has been shown to promote systemic inflammation and atherogenesis (Li et al., 2016). Thus, translocation of bacteria or bacterial components (e.g LPS) from the gut into the systemic circulation may contribute to systemic inflammation and atherogenesis. In addition, the microbiota may also affect development of cardiovascular disease through systemic immune responses. Mono-colonizing germfree mice with 52 different bacteria increased or decreased the percentage of regulatory T-cells and macrophages in the colon as well as in systemic lymphoid organs (e.g spleen)(Geva-Zatorsky et al., 2017). A correlation of the frequencies of these immune cell subsets in the colon and systemic lymphoid organs suggests that gut bacteria may locally affect expansion
of immune cells in the colon, which consequently migrate to the systemic
circulation (Geva-Zatorsky et al., 2017). Furthermore, the gut microbiome
is associated with an altered ex-vivo cytokine production by peripheral
blood mononuclear cells, indicating a relationship between the gut
microbiome and systemic cytokine responses (Schirmer et al., 2016). A
role for the gut microbiome in the regulation of the systemic immune
system is further supported by the capacity of the gut microbiome to
control hematopoiesis in primary immune sites, including the bone
marrow (Khosravi et al., 2014). Altogether, the gut microbiome affects
systemic immune cell populations, systemic cytokine responses and
hematopoiesis, which have all been implicated in the development of
cardiovascular disease.

**Gut microbiota metabolites in inflammation and CVD**

Next to translocation of bacteria or endotoxins, production of metabolites
by the gut microbiome may also affect cardiovascular disease. The gut
microbiota is important for the production of short-chain fatty acids
(SCFA). SCFA are produced by the fermentation of dietary fibers by
the gut microbiota, and acetate, butyrate and propionate are the most
prominent SCFA (Besten et al., 2013). Interestingly, CVD mortality is
negatively correlated with consumption of dietary fibers (Sahyoun et
al., 2006) and a reduction in SCFA producing bacteria (e.g Roseburia
intestinalis) has been observed in CVD patients (Karlsson et al., 2012).
Furthermore, administration of butyrate reduces atherogenesis in
ApoE-/- mice (Aguilar et al., 2014). Altogether this indicates a protective
effect for the microbiota derived SCFA in atherogenesis. SCFA can be
taken up from the intestinal lumen by enterocytes and can consequently
be transported basolaterally to reach the systemic circulation where they
can affect metabolic function (discussed in **Chapter 2** and inflammation.
The SCFA receptor GPR43 is highly expressed on leukocytes and
acetate, butyrate and propionate all suppress NF-κB activity (Cox et al.,
2009; Tedelind et al., 2007). Consequently this leads to a reduction in
multiple pro-inflammatory cytokines, including several cytokines affecting
atherogenesis, such as IFN-γ, IL-1β and IL-2 (Aoyama et al., 2010;
Cavaglieri et al., 2003; Cox et al., 2009). Thus, changes in the capacity
of the microbiota to produce the anti-inflammatory SCFA may influence
systemic inflammation and atherogenesis.
Thesis aim and outline
Recent studies have linked microbiota composition to cardiovascular disease and inflammation. It is however unknown whether alterations in microbiota composition can contribute to cardiovascular disease development and if interactions between the gut immune barrier, diet and gut microbiota can affect systemic inflammation and atherogenesis. Therefore, we aimed to understand how the interaction between the diet, gut microbiota and intestinal immune barrier contributes to systemic inflammation and atherosclerosis.

Chapter 2 describes in depth how the gut microbiota is controlled by the mucosal immune system and how aberrancies in the control of the gut microbiota affects development of non-alcoholic fatty liver disease, type 2 diabetes and cardiovascular disease. In Chapter 3 we investigated the effect of a pro-inflammatory gut microbiota on the development of systemic inflammation and atherosclerosis. Furthermore, we explored multiple mechanisms to understand how the pro-inflammatory microbiome of Caspase1\(^{-/-}\) mice may affect atherosclerosis development. These questions were studied by transplanting the gut microbiota of Caspase1\(^{-/-}\) mice into Ldlr\(^{-/-}\) mice. Chapter 4 explores the role of the antimicrobial peptide REG3y in the development of atherosclerosis. We investigated the development of atherosclerosis in Reg3y\(^{-/-}\) mice to understand whether infiltration of the intestinal epithelial barrier by bacteria from the gut microbiota naturally residing in the lumen is involved in atherosclerosis development. Chapter 5 discusses the role of western type diets in the development of type 2 diabetes. Here we aimed to provide better insight into the effect of high fat diet (HFD) feeding and dietary cholesterol on the intestinal epithelial barrier and how this relates to the development of type 2 diabetes. In Chapter 6 we will discuss the major findings of this thesis and put them into the context of the field. Furthermore, we will discuss the current status and future perspectives for microbiota research in the field of cardiovascular diseases.
References


