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REVIEW ARTICLE

Contribution of the commensal microbiota to atherosclerosis and arterial thrombosis

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The commensal gut microbiota is an environmental factor that has been implicated in the development of cardiovascular disease. The development of atherosclerotic lesions is largely influenced not only by the microbial-associated molecular patterns of the gut microbiota but also by the meta-organismal trimethylamine N-oxide pathway. Recent studies have described a role for the gut microbiota in platelet activation and arterial thrombosis. This review summarizes the results from gnotobiotic mouse models and clinical data that linked microbiota-induced pattern recognition receptor signalling with atherogenesis. Based on recent insights, we here provide an overview of how the gut microbiota could affect endothelial cell function and platelet activation, to promote arterial thrombosis.

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Abbreviations

ApoE, apolipoprotein E; CVD, cardiovascular disease; DMB, 3,3-dimethyl-1-butanol; MAMPs, microbial-associated molecular patterns; NLR, NOD-like receptor; Pam3CSK4, N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine; PG, peptidoglycan; PRR, pattern recognition receptor; TLR, toll-like receptor; TMA, trimethylamine; TMAO, trimethylamine N-oxide; VLDL, very low density lipoprotein; VWF, von Willebrand factor

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Introduction

The intestinal microbiota is the sum of trillions of microorganisms that reside in the gastrointestinal tract (Bäckhed *et al.*, 2015; Xiao *et al.*, 2015). The microbiome is largely influenced by host genetics, body site, diet, antibiotics and lifestyle factors (Gilbert *et al.*, 2018). This complex microbial ecosystem regulates, among others, the vascularization and architecture of the small intestine (Reinhardt *et al.*, 2012; Khandagale and Reinhardt, 2018), the maintenance of the structural integrity of the gut mucosal barrier (Cani *et al.*, 2008; Muccioli *et al.*, 2010), inflammatory tone (Bain *et al.*, 2014; Balmer *et al.*, 2014a; Zhang *et al.*, 2015a) and host energy metabolism (Bäckhed *et al.*, 2007; Heiss and Olofsson, 2017).

Nutrition and related changes in the gut microbiota influence the intestinal barrier function, increasing gut permeability (Cani *et al.*, 2008; Thaiss *et al.*, 2018). Bacterial products, such as **peptidoglycan**s and **LPS** constantly leak into the portal circulation (Clarke *et al.*, 2010; Balmer *et al.*, 2014a,b), promoting the development of metabolic inflammation (Cani *et al.*, 2008; Caesar *et al.*, 2012). The remote signalling of microbiota-derived metabolites and microbial-associated molecular patterns (MAMPs) can promote disease progression, for instance, of liver fibrosis (Lelouvier *et al.*, 2016), non-alcoholic fatty liver disease (Bashiardes *et al.*, 2016; Janssen *et al.*, 2017) and hepatocellular carcinoma (Roderburg and Luedde, 2014; Li *et al.*, 2016a).

Interestingly, the microbiota is not only associated with liver pathologies. During the past decade, a number of clinical and animal studies have provided a substantial amount of association-based evidence, linking the commensal microbiota with the development of cardiovascular disease (CVD) (Ott *et al.*, 2006; Koren *et al.*, 2011; Fåk *et al.*, 2015; Emoto *et al.*, 2017) and cerebrovascular diseases (Koren *et al.*, 2011; Karlsson *et al.*, 2012; Fåk *et al.*, 2015; Benakis *et al.*, 2016), which include arterial thrombosis (Table 1). However, the underlying mechanisms that are triggered by signalling-active molecules derived from gut microbial communities, contributing to the progression of cardiovascular disease and promoting the development of arterial thrombosis, are largely unresolved (Komaroff, 2018).

There is increasing evidence for the contributory role of the gut microbiota in the development of CVD and in arterial thrombosis. Initial mechanistic studies have revealed that **trimethylamine** (TMA), a choline-derived metabolite produced by the TMA-lyases (cutC) and the carnitine oxygenase (cntA) of gut microbes that is absorbed into the portal circulation and converted to **trimethylamine N-oxide** (TMAO) by flavin monooxygenases in the liver, is associated with CVD. TMAO is associated with increased atherogenesis in mice and humans (Wang *et al.*, 2011; Koeth *et al.*, 2013). Furthermore, this metabolite was demonstrated to facilitate platelet activation, thus promoting arterial thrombus formation (Zhu *et al.*, 2016).

In addition, innate immune pathways contribute to the development of atherosclerosis (Björkbacka *et al.*, 2004) and foster arterial thrombosis (Ren *et al.*, 2014). A role of gut microbiota-induced **toll-like receptor-2** (TLR2) signalling in promoting arterial thrombus growth has recently been demonstrated with germ-free mouse models (Jäckel *et al.*, 2017) (Figure 1). Furthermore, clinical studies have provided strong evidence for the involvement of innate immune pathways in CVD, showing that the blockade of IL-1β signalling reduced cardiovascular mortality (Ridker *et al.*, 2017). Hence, it will be intriguing to reveal the molecular mechanisms that link the gut microbiota to arterial thrombosis.

In this review, we provide an overview of the role of the commensal microbiota as a modulating factor of atherogenesis and its involvement in augmenting prothrombotic platelet function. We specifically discuss the functional role of the intestinal microbiota as a novel risk factor in arterial thrombosis.

Microbial metabolites and microbe-associated molecular patterns from the gut microbiota float in the circulation

Under physiological conditions, the barrier function of the epithelial lining (Marchiando *et al.*, 2010) and the gut vascular barrier (Spadoni *et al.*, 2015) restrict colonizing gut microbes to the intestinal lumen. However, small amounts of bacteria are constantly taken up by dendritic cells and **CX3CR1^{hi}** mononuclear phagocytes, reaching the mesenteric lymph nodes (Huang *et al.*, 2000; Diehl *et al.*, 2013) to preserve peripheral tolerance (Probst *et al.*, 2014). In contrast to live bacteria, bacterial breakdown products and bacterial metabolites leak into the portal circulation, thus reaching remote body sites (Balmer *et al.*, 2014b) and could be detected in the plasma of mice and humans (Amar *et al.*, 2008; Clarke *et al.*, 2010). Factors that affect gut microbial ecology (e.g. nutrition, antibiotics and oxidative stress) can perturb the intestinal barrier function. High-fat diet increases the leakage of gut microbial products, such as LPS, into the circulation (Cani *et al.*, 2008). This 2.5-fold rise in plasma LPS was termed metabolic endotoxemia and results in low-grade inflammation. MAMPs (e.g. LPS, peptidoglycans, **muramyl dipeptide**, lipoteichoic acid and **flagellin**) are recognized by **pattern-recognition receptors** (PRRs) such as TLRs, **NOD-like receptors** (NLRs) and **retinoic acid inducible gene 1 protein** receptors. Interestingly, antibiotic treatment abolished the effect of high-fat diet, indicating the involvement of the gut microbiota in increasing gut permeability, which was associated with a reduced expression of epithelial tight junction proteins (Cani *et al.*, 2008). Under conditions of acute intestinal inflammation, the intestinal barrier function is severely perturbed. Then, the liver acts as a firewall to clear blood-borne gut bacteria from the mesenteric and systemic vasculature and to prevent systemic spreading (Balmer *et al.*, 2014b).

In addition to innate immune receptor agonists, gut microbial metabolites, such as TMA, are also translocated from the gut into the circulation (al-Waiz *et al.*, 1992). A detailed understanding of the link between diet, microbiota profile and intestinal barrier is crucial, as both PRR signalling and the meta-organismal TMAO pathway promote CVD (Björkbacka *et al.*, 2004; Wang *et al.*, 2011; Koeth *et al.*, 2013) and arterial thrombosis (Ren *et al.*, 2014; Zhu *et al.*, 2016).

Table 1

Gut microbes associated with atherosclerosis

Link to atherosclerosis	Bacterial species	Habitat	Reference
Bacteria found in the blood that were associated with cardiovascular disease risk	*Helicobacter pylori*	GI tract	Patel *et al.* (1995)
	Prevotella nigrescens[a]	Oral flora	Yakob *et al.* (2011)
	Porphyromonas gingivalis[a]	Oral flora, upper GI tract, respiratory tract and colon	Yakob *et al.* (2011) and Haraszthy *et al.* (2000)
Microbiota isolated from carotid plaque	*Bacteroides forsythus*	Oral cavity	Haraszthy *et al.* (2000)
Bacteria found in atherosclerotic plaque	*Staphylococcus* sp.[a] *(epidermidis, aureus, haemolyticus and hominis)*	Skin and respiratory tract	Ott *et al.* (2006) and Koren *et al.* (2011)
	Proteus vulgaris	Intestinal tract	Ott *et al.* (2006)
	Klebsiella pneumonia	Oral flora, skin and intestine	
	Enterobacteriaceae bacterium	Gut flora	
	Enterobacter dissolvens	Gut flora	
	Pantoea agglomerans	Gut flora	
	Citrobacter freundii	GI tract	
	Enterobacter cloacae	Gut flora	
	Streptococcus[a]	Skin	
	Nocardia sp.	Oral cavity	
	Propionibacterineae[a]	Skin	Koren *et al.* (2011)
	Granulicatella[a]	GI tract	
	Streptococcus[a]	Gut flora	
	Subdoligranulum[a]	Gut flora	
	Ruminococcus[a]	Gut flora	
	Veillonella[a]	Oral cavity and gut flora	
	Chryseomonas	Oral cavity	
	Lachnospiraceae[a]	Gut flora	
Microbiota from faecal samples associated with atherosclerosis	*Collinsela*	Gut flora	Karlsson *et al.* (2012)
	Ruminococcus[a]	Gut flora	Jie *et al.* (2017) and Emoto *et al.* (2017)
	Clostridium[a]	Gut flora	
	Escherichia	Gut flora	
	Subdoligranulum	Gut flora	
	Streptococcus[a]	Gut flora	
	Coprobacillus	Gut flora	
	Enterococcus	Gut flora	
	Bifidobacterium	GI tract	Emoto *et al.* (2017)
	Prevotella	Oral flora	
	Lactobacillales	Disperse	
	Bacteroides	GI tract	
	Lachnospiraceae[a]	Gut flora	Koren *et al.* (2011)
	Veillonella[a]	Oral cavity and gut flora	
	Enterobacter cloacae	Gut flora	
	Subdoligranulum[a]	Gut flora	
Microbiota, isolated from oral cavity, associated with atherosclerosis	*Streptococcus*[a]	Gut flora	Koren *et al.* (2011)
	Propionibacterineae[a]	Skin	
	Rothia	Oral cavity	
	Corynebacterium	Gut flora	
	Staphylococcus[a]	Skin and respiratory tract	

continues

Table 1

(Continued)

Link to atherosclerosis	Bacterial species	Habitat	Reference
	Veillonella[a]	Oral cavity and gut flora	Koren *et al.* (2011) and Fåk *et al.* (2015)
	Anaeroglobus	Oral flora	Fåk *et al.* (2015)
	Odoribacter	Oral flora	
	Porphyromonas[a]	Oral flora, upper GI tract, respiratory tract and colon	
	Prevotella[a]	Oral flora	
	Coprobacillus	Gut flora	

GI, gastrointestinal.
[a]Bacteria found in different samples.

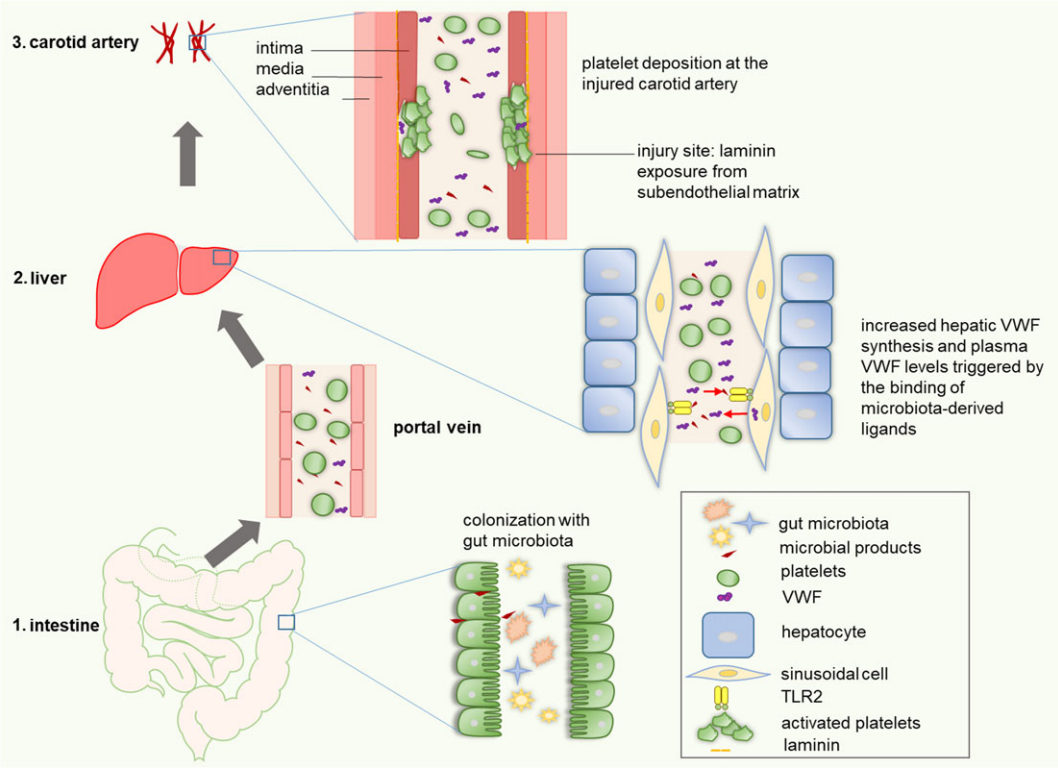

Figure 1

Remote signalling by the microbiota stimulates VWF synthesis in the hepatic endothelium of mice, promoting thrombus growth in the ligation-injured carotid artery. (1) Microbial products from the gut lumen translocate from the gut lumen to the liver, where they (2) stimulate TLR2, leading to increased hepatic VWF synthesis and release into the circulation. (3) Elevated VWF plasma levels promote platelet deposition and thrombus growth in the injured carotid artery.

Microbial-associated molecular patterns derived from the gut microbiota affect the development of atherosclerotic lesions

Rupture (Badimon and Vilahur, 2014) and erosion (Quillard *et al.*, 2017) of atherosclerotic plaques are considered the primary cause of arterial thrombosis. Pattern recognition receptors (PRRs), such as **TLR4**, are functionally expressed by myeloid cells (Xu *et al.*, 2001), platelets (D'Atri and Schattner, 2017) and the vascular endothelium (Dunzendorfer *et al.*, 2004). In atherosclerotic plaques from patients undergoing endarterectomy and in biopsies of the internal mammary artery from patients undergoing bypass surgery, the expression of **TLR1**, TLR2 and TLR4 was increased and localized

to endothelial cells and macrophages (Edfeldt *et al.*, 2002). Epidemiological studies on carotid artery atherosclerosis (Kiechl *et al.*, 2002) and various animal models (Björkbacka *et al.*, 2004) established that innate immune receptor signalling is an important determinant of atherogenesis. Furthermore, in hypertensive endarterectomy patients, blood LPS levels were significantly elevated, and macrophages in atherosclerotic plaque specimens and carotid arteries stained positive for LPS (Carnevale *et al.*, 2018). Demonstrating the functional involvement of LPS, the intravenously administered TLR4 agonist LPS from *Escherichia coli* accelerated the formation of atherosclerotic lesions, in a hypercholesterolemic rabbit model, as evaluated by increased lesion size, lesion thickness and lesion volume (Lehr *et al.*, 2001). Collectively, these studies demonstrate a role for MAMPs in the development of atherosclerosis.

Strong evidence for the role of TLR signalling in atherogenesis comes from genetic mouse studies. In $TLR4^{-/-} \times ApoE^{-/-}$ atherosclerotic mice that were fed for 6 weeks with 0.15% cholesterol-rich diet, the aortic plaque area and macrophage infiltration of the aortic sinus plaques were reduced (Michelsen *et al.*, 2004). Importantly, the effect of MAMPs on atherogenesis is not restricted to LPS and TLR4 signalling of myeloid cells (Coenen *et al.*, 2009), as TLR2-deficiency led to a reduction in atherosclerotic lesion size in the LDL receptor (LDLR)-deficient and the apolipoprotein E (ApoE)-deficient atherosclerosis model (Mullick *et al.*, 2005; Liu *et al.*, 2008). In contrast to the atherogenic role of TLR4 under low-fat diet conditions, in the LDLR-deficient atherosclerosis mouse model (Coenen *et al.*, 2009), bone marrow transplantation experiments showed that the lack of TLR2 in myeloid cells did not reduce the development of atherosclerotic lesions in hypercholesterolemic LDLR-deficient mice, indicating that TLR2 signalling in the vascular endothelium promotes the development of atherosclerotic lesions (Mullick *et al.*, 2005, 2008). The role of endothelial TLR2 in atherogenesis was further corroborated, as stimulation of TLR2/1-mediated signalling with the synthetic ligand **Pam3CSK4** increased atherosclerotic burden in hypercholesterolemic LDLR-deficient mice, but not in mice with total TLR2-deficiency or with TLR2-deficiency in myeloid cells (Mullick *et al.*, 2005). Taken together, there is strong evidence for the contribution of TLR signalling of macrophages and endothelial cells in atherogenesis.

The commensal gut microbiota is a tonic-activating factor of TLRs and other PRRs not only in the intestine (Hörmann *et al.*, 2014; Nigro *et al.*, 2014) but also in remote organs, such as the bone marrow (Khosravi *et al.*, 2014; Balmer *et al.*, 2014a). In a clinical study, the functional characterization of the gut metagenomes of patients with symptomatic atherosclerosis showed an enrichment in genes encoding for the peptidoglycan biosynthesis pathway (Karlsson *et al.*, 2012). Therefore, MAMPs derived from the gut microbiota have to be considered as drivers of atherosclerotic lesion formation.

Causal evidence for the role of the gut microbiota in atherosclerosis comes from antibiotic-treated and germ-free mouse atherosclerosis models (Table 2). When the commensal gut microbiota of ApoE-deficient mice was extensively reduced by treatment with a cocktail of the antibiotics vancomycin, neomycin, metronidazole and ampicillin, no difference in atherosclerotic lesion size was found on a standard chow diet, whereas the antibiotic cocktail reduced the proatherogenic effect of a 1% choline-enriched diet (Wang *et al.*, 2011). Similarly, when atherosclerosis-prone ApoE-deficient mice on an atherogenic diet were treated with the broad-spectrum antibiotic ampicillin, the reduction of the gut microbiota had beneficial effects on cardiovascular risk factors, such as improved glucose tolerance, reduced plasma LDL and very LDL (VLDL) levels and decreased atherosclerotic lesion size (Rune *et al.*, 2016). In contrast, germ-free ApoE-deficient mice that were kept for 20 weeks on a chow diet were reported to have increased total plasma cholesterol levels, increased LDL and VLDL levels, but decreased triglyceride levels (Kasahara *et al.*, 2017). In this study, germ-free ApoE-deficient mice on a chow diet showed reduced lesion size and a reduced quantity of macrophages in the aortic sinus plaques (Kasahara *et al.*, 2017). However, a previous study has reported that germ-free ApoE-deficient mice that were fed a diet that lacked cholesterol had a significantly reduced vessel lumen and an increased volume of the atherosclerotic plaque in the thoracic aorta, compared with conventionally raised ApoE-deficient mice on the same diet (Stepankova *et al.*, 2010). When 8 week old germ-free ApoE-deficient mice and conventionally raised ApoE-deficient mice were kept on a diet containing 2% cholesterol for 3–4 months, no significant difference was found in the area fraction of the free vessel lumen or in the volume of the atherosclerotic plaque. In agreement with the study of Kasahara *et al.*, serum cholesterol levels were also increased in germ-free ApoE-deficient mice in this study (Stepankova *et al.*, 2010). In a recent study with germ-free ApoE-deficient mice, it was confirmed that 12 weeks of Western diet feeding did not result in changed atherosclerotic lesion size in the aortic root, excluding a proatherogenic effect of dietary choline supplementation (Lindskog Jonsson *et al.*, 2018). Interestingly, the colonization of conventionally raised ApoE-deficient mice that were on an atherogenic Western diet for 8 weeks with *Akkermansia muciniphila*, an abundant colonizer of the Verrucomicrobia phylum that counteracts metabolic endotoxemia and is decreased in obese leptin-deficient and in diet-induced obese mice (Everard *et al.*, 2013), resulted in reduced atherosclerotic lesion size (Li *et al.*, 2016b). The authors of this study suggested that the association of hyperlipidaemic ApoE-deficient mice with *Akkermansia muciniphila* ameliorated vascular inflammation in the aorta. Furthermore, in this study, gavage of ApoE-deficient mice on a Western diet with *Akkermansia muciniphila* reduced intestinal permeability, a critical determinant of metabolic endotoxemia (Cani *et al.*, 2008). However, due to the low numbers of mice used in these studies, the different types of diets used, the partly controversial results and the severity of the ApoE atherosclerosis model (Table 2), additional germ-free mouse studies, addressing also early atherosclerosis, are indispensable to clarify the role of gut commensals in atherogenesis.

Pattern recognition receptor signalling induced by the gut microbiota and microbiota-dependent choline metabolism promote arterial thrombus growth

PRR signalling of platelets (Zhang *et al.*, 2015b; Biswas *et al.*, 2017) and endothelial cells (Ren *et al.*, 2014; Jäckel *et al.*,

Table 2

The outcome of different diets in antibiotic-treated and germ-free ApoE mouse models

Diet	Microbiota-dependent influence on atherogenesis	Reference
ApoE$^{-/-}$ mice were raised on chow diet until 4 weeks of age and then transferred on a control diet (0.08% choline) or a normal chow with high choline content (1% choline) for 16 weeks.	When C57BL/6J ApoE$^{-/-}$ mice were treated with a cocktail of vancomycin (0.5 g·L^{-1}), neomycin sulfate (1 g·L^{-1}), metronidazole (1 g·L^{-1}) and ampicillin (1 g·L^{-1}) at weaning *via* the drinking water, the choline-diet dependent increase in foam cell formation and macrophage total cholesterol content were suppressed. Also, the choline-diet induced increase in atherosclerotic lesion size and macrophage content in the aorta was reduced by the antibiotics.	Wang *et al.* (2011)
ApoE$^{-/-}$ mice were fed gluten-free Western diet (21% fat, 50% carbohydrate, 20% protein, 2% choline bitartrate and 1.5% cholesterol) from weaning until 16 weeks of age.	When B6.129P2-ApoE^{tm1Unc} N11 mice received tap water with ampicillin (1 g·L^{-1}), the treated mice gained more body weight, transiently improved glucose tolerance, and lowered total plasma cholesterol, LDL and VLDL. The antibiotic-treated group on Western diet was protected from the development of atherosclerotic lesions (*en face* plaque assessment).	Rune *et al.* (2016)
ApoE$^{-/-}$ mice were fed a chow diet composed of 20% calories from fat, 50% calories from carbohydrates and 30% calories from protein.	Germ-free C57BL/6 ApoE$^{-/-}$ mice showed increased plasma cholesterol and LDL cholesterol levels and a decrease in triglyceride levels compared to conventionally raised controls. Also, liver total cholesterol was increased in the germ-free state. In contrast to conventionally raised ApoE$^{-/-}$ mice, the germ-free ApoE$^{-/-}$ mice had conjugated bile acids. Germ-free ApoE$^{-/-}$ mice were resistant to the development of atherosclerosis, as shown by reduced plaque area in the aortic root and reduced macrophage-positive area per plaque area.	Kasahara *et al.* (2017)
ApoE$^{-/-}$ mice were fed a standard diet, containing 0% cholesterol and 3% fat. These mice were compared to a hypercholesteric diet containing 2% cholesterol, 5% tallow fat and 3% fish fat fed at the age of 8 weeks for 3–4 months.	Germ-free B6.129P2-ApoE^{tm1Unc}/J mice on the standard diet had significantly reduced vessel lumen and an increased atherosclerotic plaque volume in the thoragic aorta. This difference was abolished when mice were kept on the hypercholesteric diet. This study also reported increased plasma cholesterol levels in the germ-free ApoE$^{-/-}$ mice.	Stepankova *et al.* (2010)
8-week-old conventionally raised ApoE$^{-/-}$ mice on a normal chow or a Western diet for 8 weeks. Mice were gavaged daily for a period of 8 weeks with 5×10^9 cfu *Akkermansia muciniphila*.	*Akkermansia muciniphila* was reduced in the faeces of the Western diet-fed ApoE$^{-/-}$ mice. Treatment with live *Akkermansia muciniphila* substantially reduced the lesion area and size in ApoE$^{-/-}$ mice fed a Western diet but did not alter lipid metabolism in this model. *Akkermansia muciniphila* ameliorated aortic and systemic inflammation in Western diet-fed ApoE$^{-/-}$ mice, as shown by MOMA, CCL2 and ICAM-1 staining in the aortic arch. This was associated with decreased permeability and reduced serum LPS.	Li *et al.* (2016b)

2017) promotes arterial thrombosis in the mouse ferric chloride and the ligation injury model of the carotid artery. Arterial thrombus formation is supported by **NOD2**, TLR2 and **TLR9** in platelets (Panigrahi *et al.*, 2013; Zhang *et al.*, 2015b; Biswas *et al.*, 2017) as well as TLR2 and TLR4 in endothelial cells (Ren *et al.*, 2014; Jäckel *et al.*, 2017). As the gut microbiota is a source of physiologically active PRR agonists in the plasma (Clarke *et al.*, 2010; Balmer *et al.*, 2014a), it is crucial to explore the role of this microbial ecosystem in arterial thrombosis.

There is emerging evidence from germ-free mouse models that colonization with a gut microbiota promotes thrombus formation in the carotid artery, as demonstrated in the ligation injury model on normal chow diet (Jäckel *et al.*, 2017) and in the ferric chloride injury model by feeding a choline-rich diet (Zhu *et al.*, 2016). As endothelial cells in the liver encounter blood from the intestine *via* the portal vein, it is interesting to note that hepatic **von Willebrand factor** (VWF) expression, an integral component of the acute phase response, is increased by the presence of a gut microbiota

(Jäckel *et al.*, 2017). Consistent with these results, plasma levels of VWF and **coagulation factor VIII** levels were decreased in germ-free mice compared with those in their conventionally raised counterparts. Similar to germ-free mice, hepatic endothelial VWF expression and VWF plasma levels were reduced in TLR2-deficient mice compared with wild type control mice, but this difference was abolished when comparing germ-free TLR2-deficient mice with germ-free wild type littermate controls (Jäckel *et al.*, 2017). Demonstrating the involvement of the gut microbiota, recolonization of germ-free TLR2-deficient mice and their wild type littermate controls re-established the difference in hepatic VWF expression. In this study, we could show that MAMPs taken up *via* the enteric route are clearly important for the function of the liver endothelium, as hepatic VWF expression could also be up-regulated by administration of the TLR2/6 agonist lipoteichoic acid to germ-free mice *via* the drinking water. In the carotid artery ligation model, platelet deposition to the injury site was dependent on microbiota-triggered TLR2 signalling and the TLR2-dependent increase in VWF plasma levels. Importantly, platelet depletion experiments virtually excluded a role of platelet TLR2 in platelet deposition to the arterial ligation injury site. In static adhesion experiments on laminin coatings, the **arginine-glycine-asparaginic** acid-motif in the C4 domain of VWF, which primarily interacts with the platelet **integrin $\alpha_{IIb}\beta_3$**, was identified to mediate this platelet deposition defect observed in TLR2-deficient mice. Taken together, these data from gnotobiotic mouse models support a functional role of microbiota-stimulated TLR2 signalling in arterial thrombosis (Figure 1).

In addition to the pattern recognition of endothelial cells, the TMAO meta-organismal pathway has been uncovered as a microbiota-dependent factor that is predictive for thrombotic event risk and augments thrombus formation in the carotid artery of laboratory mice (Zhu *et al.*, 2016). **Choline** is catabolized by the intestinal microbiota, leading to the formation of TMA by bacterial TMA-lyase enzymes. Meanwhile, a number of TMA-producing bacterial strains have been identified and characterized (Table 3). TMA is in turn metabolized to TMAO in the liver by the hepatic flavin monooxygenase enzyme family (Wang *et al.*, 2011; Koeth *et al.*, 2013). TMAO was proposed to promote microbiota-dependent atherosclerosis, but there are experimental and clinical studies that could not confirm this link (Meyer *et al.*, 2016; Lindskog Jonsson *et al.*, 2018). Although, in mice, the choline-derived metabolite TMAO induced vascular inflammation in the aorta and evoked phosphorylation of the **p38 mitogen-activated protein kinase/extracellular signal-related kinase/**

Table 3

Overview on identified TMA-producing bacteria

Bacterial species	Study description	Reference
Shigella alkalescens	The choline reaction was suggested to separate *S. alkalescens* from *S. paradysenteriae*.	Wood and Keeping (1944)
Vibrio cholinicus	*Vibrio cholinicus N* sp., a choline-fermenting organism, was isolated from black mud from a small stagnant creek.	Hayward and Stadtman (1959) and Hayward and Stadtman (1960)
Clostridium sp.	Identification of 26 betaine, choline, creatine and ethanoamine degrading *Clostridium* species.	Möller *et al.* (1986)
Proteus mirabilis	The cleavage of choline to trimethylamine and acetaldehyde by extracts of *Proteus mirabilis* was described.	Sandhu and Chase Jr (1986) and Jameson *et al.* (2016)
Streptococcus sanguis I	Mixed bacterial flora was cultured from dental plaque and saliva and the only TMA-forming bacterium was identified as *Streptococcus sanguis I*.	Chao and Zeisel (1990)
Desulfovibrio desulfuricans G20	By position-specific iterative (PSI)-BLAST, genes encoding homologues of EutG, EutE and microcompartment protein EutM from *Salmonella enterica* were found in the genome of *Desulfovibrio desulfuricans* (ATTC 27774) and the choline utilization (cut) gene cluster was demonstrated to be responsible for choline metabolism and TMA production.	Craciun and Balskus (2012)
Klebsiella pneumoniae	The structure and function of the cutC choline lyase from the human microbiota bacterium *Klebsiella pneumoniae* was characterized.	Kalnins *et al.* (2015)
cutC: *Clostridium XIVa* strains and *Eubacterium* sp. strain AB3007 cntA: Gammaproteobacteria, in particular *Escherichia*/*Shigella*	Databases for genes of the main TMA-synthesis pathways (cutC, cntA) were established and gene-targeted assays were designed for quantitative PCR coupled to sequencing of PCR products. The TMA-producing communities in the faecal samples of 50 individuals were characterized.	Rath *et al.* (2017)

NF-κB pathway in human aortic endothelial cells and vascular smooth muscle cells, the prothrombotic phenotype caused by TMAO was due to platelet hyperreactivity (Zhu *et al.*, 2016, 2017). Intraperitoneal injections of TMAO, yielding plasma TMAO concentrations of approximately 100 μM, 0.12% v/v TMAO in drinking water, or dietary 1% choline supplementation of the diet, reduced the time to occlusion in the ferric chloride injury model of the carotid artery. In this study, the effects of dietary TMAO and choline were further confirmed in a photochemical mouse thrombosis model (Zhu *et al.*, 2016). The prothrombotic phenotype of dietary choline supplementation in the ferric chloride carotid artery model was absent in germ-free mice, but importantly, it could be re-established in conventionalized mice. Furthermore, Zhu and co-workers demonstrated that the TMAO-dependent augmentation in platelet aggregation and ferric chloride-induced carotid artery thrombosis could be transplanted into germ-free recipient mice depending whether the transplanted cecal microbiota was derived from a high TMA-producing donor mouse strain (C57BL/6J) or from a low TMA-producing donor mouse strain (NZW/LacJ). Independent of the agonist used, TMAO increased the sensitivity of the aggregation response of human platelets in PRP and washed platelets. Moreover, TMAO increased platelet deposition to a collagen-coated surface in whole blood under flow conditions. Mechanistically, TMAO treatment of platelets resulted in an enhanced stimulus-dependent release of Ca^{2+} from intracellular stores, which in washed human platelets correlated with the augmentation of the second messenger **inositol-1,4,5-trisphosphate**. Of note, the effect of the choline diet, increasing platelet aggregation, was mediated by a factor contained in platelet-poor plasma. An interventional clinical study has recently demonstrated that a choline-rich diet for 2 months augments the extent of ADP-induced platelet aggregation in vegetarians and omnivores (Zhu *et al.*, 2017). Interestingly, in this study, administration of aspirin prior to the choline-supplementation attenuated the plasma TMAO-dependent increase in ADP-induced platelet aggregation (Zhu *et al.*, 2017). To date, the exact prothrombotic action of microbiota-derived TMAO is still not completely resolved, in so far as no 'chemical sensor' for TMAO has been identified and characterized in platelets.

The gut microbiota as a novel therapeutic target in CVD and arterial thrombosis

As plasma levels of TMAO correlated with CVD progression and with the incidence of arterial thrombosis (Wang *et al.*, 2011; Zhu *et al.*, 2016, 2017), and as the bacterial strains that exert TMA-lyase enzyme activity and the wide distribution of the choline utilization (cut) gene cluster in the human intestinal tract are increasingly recognized (Craciun and Balskus, 2012; Koeth *et al.*, 2013; Martínez-del Campo *et al.*, 2015; Rath *et al.*, 2017) (Table 3), microbiome analyses of faecal samples from patients with diagnosed early atherosclerosis could become an interesting diagnostic option in the future. This may especially be useful to predict disease progression of CVD patients and to decide on nutritional or probiotic interventions. However, it is essential that future clinical studies clarify the conditions under which the TMAO metabolite is associated with increased CVD and arterial thrombosis risk, particularly as others have not found this association (Meyer *et al.*, 2016).

Targeting the enzymes of gut microbes with the aim of reducing the synthesis of microbial metabolites that promote cardiovascular disease and arterial thrombosis in the human host or to enhance beneficial microbial synthesis pathways is currently developing as a promising therapeutic option (Brown and Hazen, 2017). The specific non-lethal inhibition of gut microbial TMA-lyase enzymes could be of therapeutic value to reduce the progression of atherosclerotic lesion development, and TMA-lyases could be a useful target for antithrombotic prophylaxis (Wang *et al.*, 2015). Indeed, in mice fed a high choline or **carnitine** diet, the inhibition of TMA production by **3,3-dimethyl-1-butanol** (DMB), a non-lethal inhibitor of the TMA-lyases of gut microbes, lowered plasma TMAO levels. In the ApoE-deficient mouse atherosclerosis model, fed a chemically defined chow diet containing 1% choline, the administration of 1% (v/v) DMB in the drinking water reduced foam cell formation in the atherosclerotic plaques and reduced atherosclerotic lesion size in the aortic root (Wang *et al.*, 2015). Because of the risk of toxic side effects, further studies need to address whether pharmacological targeting of gut bacterial TMA-lyases can be considered a possible and safe approach.

LPS-induced activation of TLR4 has been demonstrated in murine and human platelets (see Vallance *et al.*, 2017), and in addition to TLR2, TLR4 expressed by the vascular endothelium promoted arterial thrombosis in mice (Ren *et al.*, 2014). However, it appears challenging if not impossible to target pharmacologically, the microbiota-derived microbial patterns or the innate immune receptors that drive atherogenesis and arterial thrombosis, as their balanced function is vital for host defence and tissue homeostasis (Rakoff-Nahoum *et al.*, 2004). Nevertheless, the CANTOS trial, including patients with a history of myocardial infarction and elevated **C-reactive protein**, has shown that therapeutic inhibition of the inflammatory pathways downstream of pattern recognition, that is, the blockade of IL-1β with canacinumab, reduced **IL-6** plasma levels and lowered the incidence of recurrent cardiovascular events (Ridker *et al.*, 2017).

In summary, distinct metabolic pathways of the human gut microbiota are currently being identified as promising druggable targets to combat the progression of cardiometabolic diseases.

Concluding remarks

Meanwhile, there is compelling evidence from metagenomics analyses of patient stool samples, clinical studies with patient specimens and gnotobiotic mouse models that implicate the commensal gut microbiota in the development of cardiovascular and cardiometabolic disease. Only recently, the gut microbiota has been identified as a factor affecting arterial thrombosis, but clearly, further investigations with

gnotobiotic mouse models are needed to pinpoint the microbiota-dependent mechanisms that can modulate arterial thrombus growth. In the future, well-designed prospective clinical studies are required to analyse the translational significance of the identified microbiota-regulated factors. This should lead to interventional studies with selective inhibitors that target the meta-organismal pathways promoting CVD and arterial thrombosis.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c).

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Author contributions

K.K. and C.R. wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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