Gut-derived low-grade endotoxaemia, atherothrombosis and cardiovascular disease

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Abstract | Systemic inflammation has been suggested to have a pivotal role in atherothrombosis, but the factors that trigger systemic inflammation have not been fully elucidated. Lipopolysaccharide (LPS) is a component of the membrane of Gram-negative bacteria present in the gut that can translocate into the systemic circulation, causing non-septic, low-grade endotoxaemia. Gut dysbiosis is a major determinant of low-grade endotoxaemia via dysfunction of the intestinal barrier scaffold, which is a prerequisite for LPS translocation into the systemic circulation. Experimental studies have demonstrated that LPS is present in atherosclerotic arteries but not in normal arteries. In atherosclerotic plaques, LPS promotes a pro-inflammatory status that can lead to plaque instability and thrombus formation. Low-grade endotoxaemia affects several cell types, including leukocytes, platelets and endothelial cells, leading to inflammation and clot formation. Low-grade endotoxaemia has been described in patients at risk of or with overt cardiovascular disease, in whom low-grade endotoxaemia was associated with atherosclerotic burden and its clinical sequelae. In this Review, we describe the mechanisms favouring the development of low-grade endotoxaemia, focusing on gut dysbiosis and changes in gut permeability; the plausible biological mechanisms linking low-grade endotoxaemia and atherothrombosis; the clinical studies suggesting that low-grade endotoxaemia is a risk factor for cardiovascular events; and the potential therapeutic tools to improve gut permeability and eventually eliminate low-grade endotoxaemia.
**Key points**

* Gut permeability can be altered by gut microbiota dysbiosis, favouring lipopolysaccharide (LPS) translocation into the systemic circulation, with ensuing development of low-grade endotoxaemia.

* Low-grade endotoxaemia induces an inflammatory state in the arterial wall that ultimately leads to initiation and progression of atherosclerosis.

* Low-grade endotoxaemia has effects on several cell types, such as leukocytes, platelets and endothelial cells, shifting them to a procoagulant phenotype that contributes to thrombosis.

* Gut permeability-derived low-grade endotoxaemia might contribute to atherosclerosis and be associated with cardiovascular events in patients at risk of or with overt cardiovascular disease.

* Modulation of gut permeability-derived low-grade endotoxaemia is a potential tool to counteract inflammation-related atherothrombosis and its clinical sequelae.

LPS is a pathogen-associated molecular pattern, and is recognized by innate immune system receptors, such as Toll-like receptors (TLRs), a family of highly conserved, membrane pattern-recognition receptors that are implicated in atherosclerotic and thrombotic processes\(^{11-15}\). For example, TLR2, TLR4 and TLR9 are involved in platelet activation and thrombus formation\(^{16,17}\).

Together, these data indicate the existence of a gut-systemic circulation axis, which might contribute to atherothrombosis via a sequence of events including increased LPS translocation from the gut to the portal circulation and impaired degradation of LPS in the liver. The LPS can then reach the systemic circulation to induce arterial inflammation and ultimately thrombus growth via binding to TLR4 (Fig. 1). In this Review, we describe the mechanisms underlying LPS translocation from the gut to the systemic circulation and the ensuing low-grade endotoxaemia, the effect of low-grade endotoxaemia on the atherosclerotic and thrombotic processes, the clinical settings associated with low-grade endotoxaemia and cardiovascular complications, and the potential therapeutic tools to improve gut permeability and eventually eliminate low-grade endotoxaemia.

**Gut barrier and permeability**

The human intestine has several lines of defence that protect against the translocation of microorganisms or microbial products into the bloodstream. This multilayer barrier constitutes the largest interface between the external environment and the host. The first line of defence consists of mucus that separates the gut microbiota from a single layer of different types of epithelial cells that forms the intestinal epithelial barrier\(^{18-20}\). The continuous intercellular barrier of intestinal epithelial cells tightly regulates the absorption, secretion and transport of water, ions and organic molecules across the epithelium\(^{21}\). The intestinal epithelial barrier consists of a physical barrier formed by the apical plasma membrane of enterocytes, which are held together by tight junction proteins, adherens junction proteins (cadherins and catenins), gap junction proteins (connexins) and desmosomes (desmoglein and desmocollins)\(^{22}\) (Fig. 2). Tight junction proteins are the most apical junctional complex regulating gut permeability and paracellular diffusion and, therefore, are crucial for maintaining cell-to-cell adhesion and gut barrier health\(^{23}\). The tight junction complex includes transmembrane proteins, such as claudins, occludin, tricellulin and junctional adhesion molecules, and intracellular scaffold proteins such as the zonula occludens proteins ZO1, ZO2 and ZO3 that bind the transmembrane structure to the cytoskeleton\(^{24,25}\) (Fig. 2). The gut–vascular barrier, an additional cellular barrier, is situated below the epithelial barrier and is also involved in controlling the translocation of microorganisms into the portal vein\(^{26,27}\).

Gut barrier permeability is regulated by intrinsic and extrinsic mechanisms in the intestinal epithelial cells, and can be altered by exogenous factors, such as excessive alcohol intake and non-steroidal anti-inflammatory drugs, or endogenous factors, such as inflammation linked to systemic disease\(^{28}\). The gut microbiota has a protective role in the digestive mucosa by participating...
Intestinal adhesion proteins
The scaffold of the epithelial cell barrier that protects from the translocation of microorganisms or microbial products into the systemic circulation.

in the maintenance of the physiological integrity of tight junction proteins\(^1\). The gut microbiota generates a variety of metabolites from dietary products that have important effects on gut barrier function and immune responses\(^2\). Microbiota metabolites with a protective role for the gut barrier include short-chain fatty acids (SCFA), indole and indole derivatives (which are generated from tryptophan microbial digestion\(^4\)), bile acid metabolites, polyamines and polyphenols (FIG. 2). Moreover, gut dysbiosis is a prerequisite for the modification of gut barrier functionality and the translocation of microorganisms or microbial products into the systemic circulation\(^4\). For example, in animal models of diabetes and obesity, treatment with a large spectrum of antibiotics reduced the levels of LPS in the systemic circulation coincidentally with a reduction in gut permeability via upregulation of tight junction proteins such as ZO1 and occludin\(^5\).

Diet is an important factor affecting gut permeability. In rats fed a high-fat diet, changes in gut microbiota are characterized by an imbalance in the ratio of Gram-negative to Gram-positive bacteria that might predispose to alterations in gut barrier defence\(^6\). The relationship between a high-fat diet, metabolic disease and blood LPS levels was first outlined by Cani and colleagues\(^7\). They demonstrated in mouse models that a 4-week high-fat diet chronically increased the plasma LPS concentration two to five times, corresponding to one to two orders of magnitude lower than the levels attained with infections\(^8\). The high-fat diet also affected gut barrier integrity by reducing the number of Bifidobacteria\(^9\), which have been shown to protect intestinal barrier integrity via upregulation of tight junction proteins such as ZO1 and occludin\(^10\). Finally, Cani and colleagues showed that chronic, experimental metabolic endotoxaemia induced by LPS infusion triggered the development of obesity, diabetes and liver insulin resistance, mimicking the negative effects elicited by a high-fat diet alone\(^11\).

Ageing is another important factor in gut barrier dysfunction, which in turn can promote low-grade systemic inflammation, a characteristic observed in older individuals\(^12\). A reduction in gut microbiota diversity and an imbalance between opportunistic and commensal bacteria (with increased numbers of opportunistic bacteria such as Enterobacteriaceae, Clostridium perfringens and Clostridium difficile, and decreased numbers of commensal bacteria such as Bacteroides, Bifidobacteria and Lactobacilli) have been reported in older individuals\(^13\). Age-related gut dysbiosis negatively influences gut barrier permeability and is responsible for translocation of microbial products into the systemic circulation, with ensuing systemic inflammation and ultimately impaired bacteria-killing immune responses\(^12\).

Together, these data indicate that diet-induced gut microbiota alterations are the primus movens of a sequence of events including dysbiosis-mediated increase in gut permeability, in which LPS has a key role in the disassembly of intestinal adhesion proteins (FIG. 2). LPS binding to TLR4 in intestinal cells begets an inflammatory process that ultimately downregulates the levels of tight junction proteins and favours the translocation of LPS into the systemic circulation. Several studies in experimental models using TLR4 inhibitors or Tlr4-knockout animals have demonstrated a crucial role for the LPS–TLR4 pathway in modulating gut barrier integrity\(^14\). Nevertheless, the intestinal barrier includes factors that can protect from LPS-induced damage. For example, intestinal alkaline phosphatase (IAP) removes one of the two phosphate groups of the LPS lipid A moiety, resulting in LPS degradation to monophosphoryl LPS, which can still bind to TLR4 but acts as an antagonist\(^15\). In animals fed a high-fat diet, IAP over-expression improved intestinal function by maintaining the integrity of intestinal mucosa, thereby reducing LPS translocation into the systemic circulation and lipid accumulation in the liver, resulting in the attenuation of atherosclerotic plaque burden\(^16\). Biosynthesis of HDL...
Increased gut permeability | CARDiOLOGY

Gut permeability and cardiovascular risk. Emerging evidence indicates that gut dysbiosis is a risk factor for cardiovascular disease in mice with type 1 diabetes mellitus9, obesity90 or hypertension91. Studies to assess gut permeability in patients at risk or with coronary heart disease have mostly been conducted by measuring serum levels of zonulin, which is an indirect marker of gut permeability. Zonulin is a 47-kDa protein released by epithelial cells of the small intestine after stimulation by gliadin or gut dysbiosis92. In interstitial epithelial cells, the signalling pathway downstream of zonulin leads to protein kinase C phosphorylation, which triggers the disassembly of tight junction proteins such as ZO1 [REF[92]]. Increased serum levels of zonulin together with elevated levels of LPS have been detected in individuals with type 2 diabetes, obesity93–96 or acute or chronic cardiovascular disease97 compared with the levels in healthy individuals. Increased gut permeability and low-grade LPS endotoxaemia related to gut dysbiosis have also been detected in patients with non-septic pneumonia98,99 or in the acute phase of myocardial infarction, as well as in experimental models of intestinal anoxia100 and systemic inflammation-associated overproduction of pro-inflammatory cytokines, such as interferon-γ and tumour necrosis factor (TNF)101 [FIG. 2].

Of note, given that analysis of serum zonulin levels is an indirect measure of gut permeability, other assays should be used to assess changes in gut permeability in humans. In this regard, analysis of d-lactate might be an alternative approach because an increase in d-lactate levels in the blood is considered to be a marker of increased intestinal permeability secondary to bacterial infection or experimentally induced gut injury102,103. However, measurement of urinary excretion of dextrose and mannitol after oral ingestion is likely to be a better approach for analysing gut barrier dysfunction because dextrose and mannitol are absorbed through the paracellular and transcellular pathways, respectively104, but such an analysis is cumbersome and requires specific expertise105. Nevertheless, the relationship between gut permeability and low-grade endotoxaemia in patients at risk of or with cardiovascular disease should be further investigated.

LPS and atherosclerosis

Atherogenesis is a complex process that is thought to be initiated by LDL crossing the arterial wall, where LDL is trapped in the subendothelial layer and undergoes oxidative changes that lead to the formation of oxidized LDL (oxLDL).106 OxLDL elicits recruitment of inflammatory cells, production of inflammatory cytokines and a shift in endothelial cells to a pro-atherogenic phenotype, all of which perpetuate the inflammation in the arterial intima107. The relevance of oxidative stress in atherogenesis has been supported by evidence of impaired macrophage uptake of oxLDL in patients undergoing carotid endarterectomy who received vitamin E (which has antioxidant properties) and injected with native radiolabelled LDL108.

Experimental studies have suggested that gut dysbiosis is involved in the aforementioned mechanisms of atherosclerosis by promoting the development of metabolic diseases that favour arterial inflammation, such as dyslipidaemia, obesity and hypertension109. Pathogenic gut microbiota are more frequent in patients with symptomatic atherosclerosis than in those with asymptomatic atherosclerosis, and are predictive of the risk of coronary heart disease110–112. Further support for the connection between gut microbiota and cardiovascular disease has been provided by studies in germ-free mouse models that have shown a causal link between gut dysbiosis, hypertension, vascular dysfunction, systemic inflammation and atherothrombosis113,114.

In this context, data on the role of LPS in atherosclerosis might provide insights into the mechanisms...
NADPH oxidase

A ubiquitous, multisubunit cellular enzyme that is a major producer of reactive oxygen species.

linking gut microbiota to atherosclerosis and its complications. LPS is a pro-atherogenic molecule through its pro-oxidant properties, which are mediated by the activation of NOX2 (REF 80), the catalytic core of NADPH oxidase, among the most important cellular producers of reactive oxygen species (ROS). LPS concentrations similar to those found in the plasma of individuals without sepsis amplified platelet responses in vitro to common agonists via TLR4-mediated, NOX2-derived ROS formation, which is a mechanism favouring LDL oxidation81–88. A significant correlation between the presence of low-grade endotoxaemia and elevated circulating levels of oxLDL has been found in patients with impaired fasting glucose84, which provides indirect evidence for the pro-oxidant properties of LPS.

Support for the putative role of LPS in atherosclerosis has been provided by immunohistochemistry analysis of carotid atherosclerotic plaques from patients undergoing endarterectomy, which revealed the presence of LPS adjacent to plaque macrophages with high TLR4 levels. By contrast, LPS was not detected in atherosclerosis-free thyroid arteries from the same patients85. In humans, LPS in circulation is mostly bound to lipoproteins (80–97%)80,87, with the highest concentration in LDL (35.7%) and the lowest in VLDL (13.9%)80. However, VLDL particles carry a higher number of LPS molecules81. The observation that circulating LPS is transported by pro-atherogenic lipoproteins, such as VLDL and LDL, might be relevant in the pathogenesis of atherosclerosis. LPS could enter the arterial wall bound to these pro-atherogenic lipoproteins, which would favour LDL oxidation80 and thereby contribute to propagation of arterial inflammation. A further contributor to the pro-atherogenic process is LPS-binding protein-mediated LPS transfer from HDL to LDL82 (FIG. 3).

Experiments in animal models have been conducted to substantiate the role of LPS as a trigger of atherosclerosis. One study found that a single LPS infusion induced a profound systemic inflammatory response without changes to the atherosclerotic plaque86. However, other experiments have consistently shown an association between LPS infusion and arterial injury81,82. Daily intravenous or intraperitoneal LPS infusions in animals accelerated atherosclerosis in the aorta, together with increased production of pro-inflammatory cytokines, such as IL-8 and TNF, and autoantibodies against oxLDL, and increased accumulation of activated lymphocytes and deposition of IgG and IgM in the arterial intima81,82. In isolated human saphenous vein samples, LPS at concentrations as low as 0.1 ng/ml increased the production of ROS and chemotactic cytokines, such as IL-8 and CCL2, via interaction with TLR4 (REF 83). These changes were mitigated by administration of statins, which are known to have antioxidant properties beyond their LDL-lowering effect84. The relevance of the LPS–TLR4 axis in atherosclerosis is further supported by studies in mice. In atherosclerosis-prone hypercholesterolaemic Apoe−/− mice, LPS-induced TLR4 activation increased neointima formation, whereas lack of either the adaptor molecule MyD88 or its upstream receptor TLR4 reduced atherosclerotic burden85,86. Tlr4−/−Ldlr−/− mice fed a carbohydrate-rich diet or a control diet had markedly reduced aortic atherosclerotic lesion areas compared with Ldlr−/− mice85.

In accordance with these experiments, the analysis of human atherosclerotic plaques revealed TLR4 overexpression in several cell types, including macrophages, vascular smooth muscle cells and dendritic cells87,88. However, it is unclear whether TLR4 overexpression is mediated by LPS or other TLR4 ligands, such as oxLDL, cleaved fibrinogen or heparin sulfate proteoglycan. Furthermore, studies in patients with the Asp299Gly variant in the TLR4 region on chromosome 9, which is associated with impaired TLR4 signalling, have provided inconclusive results89.

LPS has also been shown to destabilize atherosclerotic lesions, which renders the atherosclerotic plaque more vulnerable to rupture or erosion. Intratracheal injection of LPS in Apoe−/− mice fed a Western diet90 induced progression from stable to unstable phenotypes in aortic arch atherosclerotic plaques; histological features of plaque vulnerability included reduced cap thickness, leukocyte infiltration, increased necrotic core and thrombus formation at the edges of atherosclerotic plaque91. Consistent with these findings, a marked increase in atherosclerotic plaque size was detected in hypercholesterolaemic mice after a single LPS infusion mimicking the acute

Fig. 2 | Mechanisms of gut permeability-mediated low-grade endotoxaemia. The gut epithelial barrier consists of the apical plasma membrane of enterocytes, held together by tight junction proteins (claudin and occludin) and adherens junction proteins (E-cadherin and catenin), as well as the zonula occludens proteins ZO1 and ZO2, which are adaptor proteins necessary for the structural and regulatory functions of tight junctions. a | Upregulation of junctional proteins can be induced by microbiota metabolites including polyphenols, indole and indole derivatives, short-chain fatty acids (SCFAs) and polyamines. b | Downregulation of junctional proteins is mediated by: lipopolysaccharides (LPS) through binding to Toll-like receptor 4 (TLR4); by zonulin, a protein that activates the EGF receptor (EGFR) through transactivation of the proteinase-activated receptor 2 (PAR2), thereby inducing protein kinase C (PKC) phosphorylation; and by pro-inflammatory cytokines, including IL-1β, interferon-γ (IFNγ) and tumour necrosis factor (TNF). JAM, junctional adhesion molecules.
macrophages, inducing foam cell formation and inflammatory cytokine leading to the formation of oxidized LDL (oxLDL). OxLDL is taken up by receptor domain-containing adaptor protein (TIRAP) and recruitment of the

**Mechanisms of LPS-mediated atherosclerosis.** a | LDL can cross into the arterial wall and undergo oxidation in the subendothelial space, leading to the formation of oxidized LDL (oxLDL). OxLDL is taken up by macrophages, inducing foam cell formation and inflammatory cytokine production. b | Lipopolysaccharides (LPS) can cross into the arterial wall, either together with LPS binding protein (LBP) or by LBP-mediated LPS transfer from HDL to LDL particles. LPS binds to Toll-like receptor 4 (TLR4) in several cell types, leading to phosphorylation of Toll–interleukin-1 receptor domain-containing adaptor protein (TIRAP) and recruitment of the myeloid differentiation primary response protein 88 (MyD88) to the cytoplasmic domain of TLR4. Downstream signalling induces the activation of the transcription factor nuclear factor-xB (NF-xB), which increases the production of pro-inflammatory cytokines, such as IL-8 and tumour necrosis factor (TNF); oxidative stress via upregulation of NADPH oxidase 2 (NOX2)-derived reactive oxygen species, which further promotes LDL oxidation; and destabilization of the atherosclerotic plaque via activation of the arachidonic acid pathway and biosynthesis of leukotriene B4 (LTB4), which attract leukocytes to the atherosclerotic lesion. O$_2^-$, superoxide.

**LPS and thrombosis**

Studies have shown that gut microbiota might be implicated in the atherothrombotic process and suggested production of TMAO as a potential mechanism. LPS translocation into the systemic circulation might be another pathway.

The relationship between low-grade endotoxaemia and thrombosis was first hypothesized based on observations in patients with advanced liver disease, who have an increased risk of bleeding and thrombotic complications in the portal and peripheral circulation. Low-grade endotoxaemia was detected in both vascular trees in these patients and was associated with increased biosynthesis and activity of tissue factor in monocytes, a glycoprotein that converts factor X to factor Xa after binding to factor VIIa. Stimulation of human monocytes with LPS in vitro resulted in increased thrombin generation, an effect that was blunted by treatment with the antioxidant vitamin E. In endothelial cells in vitro, LPS addition caused a shift to a prothrombotic phenotype characterized by overexpression of tissue factor and overproduction of thrombin-activatable fibrinolysis inhibitor (also known as carboxypeptidase B2) and plasminogen activator inhibitor 1. Overexpression of tissue factor by LPS-treated mouse endothelial cells in vitro was related to LPS binding to TLR4 because tissue factor activity and mRNA were significantly higher in wild-type endothelial cells than in Tlr4$^{-/-}$ endothelial cells. Under the same experimental conditions, LPS-induced tissue factor activation was inhibited in wild-type endothelial cells by treatment with an anti-TLR4 antibody. Further evidence of the prothrombotic effects mediated by LPS comes from an in vitro study showing that stimulation of human endothelial cells with LPS induced the release of prothrombotic molecules, such as von Willebrand factor and factor VIII, via formation and secretion of Weibel–Palade bodies. This phenomenon was blunted by pretreating the cells with a TLR4 inhibitor.

Further support for a role of the LPS–TLR4 axis in thrombosis has been provided by immunohistochemistry analysis of coronary thrombi from patients with myocardial infarction. The analysis revealed the presence of LPS within the thrombus alongside the overexpression in leukocytes of TLR4 and cathepsin G, a protein favouring leukocyte–platelet binding and activation. The interplay between LPS and leukocytes might also contribute to thrombosis via release of NETs, which
comprise DNA and histones and are released upon neutrophil stimulation by pattern-recognition receptor activation or chemokines. The release of NETs requires ROS formation and calcium mobilization into the neutrophil, which in turn activate protein–arginine deaminase type 4 (PAD4) to deaminate arginine residues on histones. LPS stimulates NET formation in vitro in a dose-dependent manner and requires binding to TLR4 and increased NOX-generated ROS, as indicated by the observation that the TLR4 inhibitor TAK242 and the NOX inhibitor diphenyleneiodonium suppress LPS-mediated NETosis.

To explore whether the prothrombotic effect of LPS could be recapitulated in vivo, we developed a mouse model of low-grade endotoxaemia that involved intraperitoneal LPS injection (0.5 mg/kg) at a concentration that corresponded with the LPS level detected in human thrombus (40 pg/ml). In LPS-treated animals, thrombus growth was accelerated and was associated with increased levels of systemic biomarkers of platelet activation. Both changes were inhibited by co-administration of a TLR4 inhibitor, reinforcing the hypothesis that TLR4 has a pivotal role in the prothrombotic effect of LPS. This finding is consistent with the significant correlation between platelet TLR4 upregulation and low-grade endotoxaemia detected in patients with coronary thrombosis.

Other components of the TLR family are also implicated in the thrombotic process mediated by gut microbiota. For instance, TLR2 recognizes and is activated by lipoprotein components of Gram-positive or Gram-negative bacteria and has direct pro-thrombotic effects. Germ-free or TLR2-deficient mice had reduced thrombus growth after carotid artery injury compared with controls, and this effect was counteracted by intestinal colonization with microbiota. However, the interplay, if any, between LPS and TLR2 in this context remains to be established.

**LPS and cardiovascular events**

Circulating LPS levels have been assessed in the general population and in patients at risk of cardiovascular events and in patients with metabolic diseases, clinically overt cardiovascular disease (such as atrial fibrillation or myocardial infarction) or acute infections. The association between circulating LPS levels and atherosclerotic burden and the clinical sequelae of atherosclerosis have

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**Fig. 4 | Mechanisms of LPS-mediated thrombosis.** Low-grade endotoxaemia caused by lipopolysaccharide (LPS) promotes thrombus formation at the site of atherosclerotic plaque rupture or erosion by binding to Toll-like receptor 4 (TLR4) in different cell types, including endothelial cells, monocytes, neutrophils and platelets. a | In endothelial cells, TLR4 activation induces the release of von Willebrand factor (vWF) and factor VIII (FVIII) via formation and secretion of Weibel–Palade bodies (WPb) and the upregulation of tissue factor (TF) secretion, which converts factor X (FX) to activated factor X (FXa) to generate thrombin. b | In monocytes, TLR4 induces upregulation of TF release. c | In neutrophils, TLR4 signalling triggers the formation of neutrophil extracellular traps (NETs) and cathepsin G-mediated platelet activation. d | In platelets, LPS-mediated TLR4 signalling leads to platelet activation via NADPH oxidase 2 (NOX2)-mediated oxidative stress. FVIIa, activated factor VII; FIX, factor IX; FIXa, activated FIX; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor-κB; PAR4, proteinase-activated receptor 4; O2−, superoxide; TIRAP, Toll–interleukin-1 receptor domain-containing adaptor protein.
Table 1 | Low-grade endotoxaemia in various clinical settings

| Study (year)       | Study design       | Study cohort (n)                                      | Follow-up | Endotoxaemia evaluation | Main results                                                                                                                                     | Ref. |
|-------------------|--------------------|------------------------------------------------------|-----------|-------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|------|
| Carpino et al. (2020) | Case–control study | NAFLD (n = 211); no NAFLD (n = 65)                  | NR        | LPS                     | Increased serum LPS level and higher LPS localization in hepatocytes in patients with NAFLD versus patients without NAFLD; LPS was associated with liver inflammation | 31   |
| Simonsen et al. (2020) | Prospective study  | Type 1 diabetes (n = 3,781)                          | 13.7 years | LAL                     | LPS activity increased in patients with type 1 diabetes and incident CHD                                                                         | 125  |
| Wiedermann et al. (1999) | Prospective study  | General population, cohort from the Bruneck study (n = 516) | 5 years   | LAL                     | LPS >50 pg/ml in the plasma increased the risk of carotid artery atherosclerosis                                                              | 18   |
| Asada et al. (2019) | Prospective study  | General population, cohort from Japan (n = 2,568)    | 10 years  | LBP                     | Serum LBP level was associated with cardiovascular events                                                                                     | 121  |
| Pussinen et al. (2007) | Prospective study  | General population, cohort from FINRISK study (n = 7,927) | 10 years  | LAL                     | The LPS to HDL-cholesterol ratio was associated with systemic inflammation and cardiovascular events                                                | 121  |
| Leskelä et al. (2021) | Genome-wide association study | Three cohorts from Finnish studies (total n = 11,296); FinnDiane, type 1 diabetes (n = 4,242); FINRISK, general population (n = 6,323); Finnish T1w Cohort study, general population (n = 731) | NR        | LAL                     | Five genetic loci were associated with serum endotoxin activity; the genetic risk score of endotoxaemia was associated with venous thromboembolism | 122  |
| Zhou et al. (2018) | Case–control, prospective study | STEMI (total n = 100); stable angina (n = 50); control group (n = 50) | 3 years   | LAL                     | Serum LPS was increased in patients with STEMI and was associated with cardiovascular events                                                   | 68   |
| Carnevale et al. (2020) | Case–control study | STEMI (total n = 50); stable angina (n = 50); control group (n = 50) | NR        | LPS                     | Serum LPS level was increased in patients with STEMI and correlated with serum zonulin levels; LPS localized in coronary thrombi from patients with STEMI | 15   |
| Pastori et al. (2017) | Prospective study  | Atrial fibrillation (n = 912)                        | 3 years   | LPS                     | Serum LPS > 100 pg/ml was associated with increased risk of MACE                                                                               | 129  |
| Zhang et al. (2021) | Cross-sectional study | Atrial fibrillation (total n = 1,152); young participants (aged 18–44 years; n = 694); middle-aged participants (aged 45–64 years, n = 357); older participants (65–75 years, n = 101) | NR        | LAL                     | Serum LPS levels were increased in older patients with atrial fibrillation                                                                    | 127  |
| Cangemi et al. (2016) | Prospective, observational study | CAP (n = 278); control group (n = 50)          | NR        | LPS                     | Serum LPS levels increased in the acute phase of CAP and correlated with serum zonulin levels                                                   | 66   |
| Oliva et al. (2021) | Case–control, prospective study | COVID-19 (n = 81); control group (n = 81)          | 18 days   | LPS                     | Serum LPS level was associated with thrombotic events in patients with COVID-19 and correlated with serum zonulin level                   | 19   |

CAP; community-acquired pneumonia; CHD, coronary heart disease; COVID-19, coronavirus disease 2019; LAL, limulus amoebocyte lysate; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; MACE, major adverse cardiovascular events; NAFLD, non-alcoholic fatty liver disease; NR, not reported; STEMI, ST-segment elevation myocardial infarction.

been assessed in cross-sectional studies and prospective analyses (Table 1).

Wiedermann and colleagues were among the first to investigate the effect of low-grade endotoxaemia on the risk of atherosclerosis in a study that included 516 individuals with risk factors for atherosclerotic disease. After 5 years of follow-up, individuals with LPS >50 pg/ml at baseline had a threefold increased risk of incident carotid artery atherosclerosis compared with individuals with LPS levels <50 pg/ml. These findings were supported and extended by a study that included 2,568 individuals without previous cardiovascular disease. After 10 years of follow-up, baseline serum levels of LPS-binding protein were significantly associated with incident cardiovascular disease, after adjusting for conventional risk factors for cardiovascular disease. This finding was corroborated by a study that analysed circulating LPS levels in 7,927 individuals from a population-based chronic disease risk factor survey. The study found a significant association between the LPS to HDL cholesterol ratio and cardiovascular events after a follow-up of 10 years. Evidence for a relationship between endotoxaemia and thrombosis was also provided by an analysis of the genetic profile linked to endotoxaemia and its association with thrombosis in participants of three Finnish cohorts. The study...
endotoxaemia was also associated with large VLDL particle diameters and small in the liver. The increased clearance of amino acids from plasma in endotoxaemia is tissue lipolysis, together with increased lipid biosynthesis and decreased lipid oxidation with endotoxaemia might be explained by lipopolysaccharide (LPS)-induced adipose amino acids and glucose. The available data on plasma metabolite changes during indicated by changes in the plasma levels of major metabolites, including lipoproteins, levels, including fatty acid levels in arterial tissue and plasma levels of monounsaturated fatty acids were inversely associated with endotoxaemia. In studies in healthy volunteers, an endotoxin bolus injection induced progressive hyperglycaemia within 2–6 h after the endotoxin challenge, as well as a significant increase in lipid levels, including fatty acid levels in arterial tissue and plasma levels of monounsaturated fatty acids, but decreased amino acid levels in the plasma.

The observed changes in lipoprotein composition and plasma lipid levels associated with endotoxaemia might be explained by lipopolysaccharide (LPS)-induced adipose tissue lipolysis, together with increased lipid biosynthesis and decreased lipid oxidation in the liver. The increased clearance of amino acids from plasma in endotoxaemia is likely to be due to increased LPS-induced synthesis of acute-phase proteins in the liver and the use of amino acids in energy production.

Identified single-nucleotide polymorphisms at five genetic loci that were associated with serum endotoxin activity; several of the single-nucleotide polymorphisms were associated with expression of nearby genes affecting the clotting system. Interestingly, a significant association between the genetic risk score of endotoxaemia and venous thromboembolism was also identified. Low-grade endotoxaemia has also been investigated in patients with stable or unstable cardiovascular disease in cross-sectional and prospective studies. Elevated circulating levels of LPS and D-lactate, a marker of gut permeability, have been reported in patients in the early phase of acute myocardial infarction, suggesting that coronary ischaemia is a trigger for gut barrier dysfunction. This hypothesis was corroborated by a study in an animal model of coronary ischaemia, in which coronary ischaemia was associated with increased gut permeability and downregulation of tight junction proteins. Furthermore, in patients with myocardial infarction, a significant association was observed between circulating LPS levels detected during the acute phase of the disease and major adverse cardiovascular events after 3 years of follow-up. Furthermore, elevated circulating LPS levels in the peripheral and coronary circulation have been observed in patients with myocardial infarction compared with the levels in patients with stable cardiovascular disease and healthy individuals. Of note, circulating LPS levels correlated with the serum levels of zonulin and several markers of inflammation, such as IL-1β and TNF, reinforcing the hypothesis that coronary ischaemia is associated with intestinal barrier dysfunction.

A growing body of evidence indicates that low-grade endotoxaemia occurs in patients with metabolic disease, such as type 2 diabetes, obesity, NAFLD and NASH. A systematic review of studies in patients with diabetes and obesity found a significant association between circulating LPS levels and triglyceride and total cholesterol levels, fasting glycaemia, insulinemia, HbA1c levels and C-reactive protein levels. The elevation in circulating LPS levels was more marked in patients with macroalbuminuria and was reduced in patients taking hypoglycaemic drugs, and insulin therapy exacerbated endotoxaemia compared with other antidiabetic drugs. In a prospective study involving 3,781 patients with type 1 diabetes, the number of antibiotic purchases, a surrogate of bacterial infection, as well as high LPS activity were independently associated with incident cardiovascular events during a follow-up of 15 years.

Metabolic endotoxaemia can also have a negative effect on liver cells, in which LPS can localize and elicit liver damage. Higher serum LPS levels and LPS hepatocyte localization have been detected in patients with NAFLD and in mouse models of these conditions compared with the levels in controls. Immunohistochemistry analysis revealed a potential causal interplay between the liver steatosis and fibrosis and the TLR4 overexpression in macrophages and platelets. Of note, a significant association was observed between platelet TLR4 levels and circulating LPS levels, suggesting a role for LPS in increasing platelet activation. This finding might provide insights on the role of platelets in liver inflammation as well as in cardiovascular events in patients with liver disease.

Atrial fibrillation is another clinical setting in which the role of LPS as a trigger of arrhythmia or cardiovascular events has been explored. In patients with atrial fibrillation, a progressive increase in circulating LPS levels has been observed with increasing age, with high LPS levels found in middle-aged and older patients with atrial fibrillation. A potential cause–effect relationship between endotoxaemia and atrial fibrillation was investigated in experimental models of atrial fibrillation, which demonstrated a role for gut dysbiosis in promoting atrial fibrillation in old rats and a reduction in atrial fibrillation susceptibility in old rats that received faecal transplantation from young animals. Overexpression of TLR4 and activation of the NLRP3 inflammasome in the atria were key factors driving atrial fibrosis and increased susceptibility to atrial fibrillation. The pro-inflammatory activity of LPS might also be relevant for the clinical outcomes of atrial fibrillation, as evidenced by a higher incidence of major adverse cardiovascular events during a follow-up of 3 years in patients with blood LPS levels >100 pg/ml than in patients with LPS <100 pg/ml.

Low-grade endotoxaemia has also been detected in the acute phase of infection in patients with community-acquired pneumonia or with coronavirus disease 2019 (COVID-19), even in the absence of overt sepsis. Of note, both community-acquired pneumonia and COVID-19 can be complicated by venous and/or arterial thrombosis during the hospital stay, with patients with COVID-19 having an incidence double than that seen in patients with community-acquired pneumonia. In patients with COVID-19, circulating LPS levels correlated with serum zonulin levels and...
Prebiotics  
Non-digestible food ingredients (fibres) that promote a shift to a healthier gut microbiota profile.

Probiotics  
Live microorganisms that confer a health benefit in the host when administered in adequate amounts.

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in adequate amounts. The Mediterranean diet, which is rich in fibre, is likely to be one of the best approaches to improving gut permeability and eventually eliminating low-grade endotoxaemia. Indeed, high adherence to the Mediterranean diet has been associated with beneficial microbiome-related metabolic profiles, an increase in plasma SCFA levels and a reduction in circulating LPS levels compared with low adherence to the Mediterranean diet. Administration of extra virgin olive oil, a key component of the Mediterranean diet, reduced endotoxaemia and serum zonulin levels and improved the metabolic profile of healthy individuals and patients with impaired fasting glucose. These findings have been corroborated by an interventional study that found decreased circulating levels of LPS in patients with metabolic syndrome who received a fibre-rich diet for 75 days.

Prebiotics, which are plant-derived fibres with beneficial effects on gut microbiota, gut permeability and low-grade endotoxaemia, is another potential strategy to reduce low-grade endotoxaemia. Indeed, mice fed a diet supplemented with a fermentable fibre (oligo-fructo) showed improved gut barrier function and reduced circulating LPS levels compared with control mice. Specific mechanisms that might account for the improved gut permeability included upregulation of ZO1 and occludin. An increase in glucagon-like peptide 1 (GLP1) and GLP2 levels is another intriguing and potential beneficial effect mediated by prebiotics. Indeed, mice fed a prebiotic-rich diet had an upregulation of GLP1 and GLP2 in the portal circulation compared with control mice, with a significant correlation between high GLP2 levels in the portal blood and improved gut barrier function. This finding was recapitulated in another study in mice showing that GLP2 administration improved gut permeability, upregulated ZO1 and occludin levels in epithelial cells, lowered circulating LPS levels and reduced liver steatosis. All these changes were associated with a reduction in systemic inflammation and oxidative stress. However, the effect of these molecules on atherothrombosis remains to be established. Of note, administration of inulin, a prebiotic polysaccharide, in Apoe mice slowed atherosclerosis progression and reduced liver steatosis.

Another potential nutrition-related strategy to improve gut barrier permeability is the high intake of fish oils to increase the ratio of n-3 polyunsaturated fatty acids (n-3 PUFAs) to n-6 PUFAs. However, the role of n-3 PUFAs in the modulation of gut permeability needs to be further clarified.

Other approaches to improving gut permeability include the use of probiotics, which are live bacteria, such as Lactobacillus plantarum or Akkermansia muciniphila, or microbial metabolites such as urolithin A or oxyberberine. For example, urolithin A administration in mice improved gut barrier function via upregulation of claudin 4, occludin and ZO1 levels in intestinal epithelial cells, whereas oxyberberine administration downregulated the expression of Tlr4 and the production of pro-inflammatory cytokines such as Il-1β, IL-6 and TNF in colonic tissue. Similar protective effects on barrier function have been achieved in vitro by incubation of epithelial cells with SCFAs, such as propionate, butyrate and acetate, that stem from dietary fibre and indigestible carbohydrate fermentation by commensal microbiota such as Bifidobacterium, Bacteroides, Enterobacter, Fusobacterium and Roseburia. In addition, administration of a diet rich in SCFAs to patients with metabolic syndrome resulted in upregulation of epithelial adhesion proteins. However, the implementation of these strategies is so far limited to patients with inflammatory bowel disease. Therefore, further studies are needed to assess the effect on gut permeability and low-grade endotoxaemia in patients at risk of or with cardiovascular disease.

Statins are lipid-lowering drugs that effectively reduce atherosclerosis and its complications. Evidence indicates that statins have beneficial effects on gut barrier function by modulating gut dysbiosis or upregulating the levels of intestinal adhesion proteins. Furthermore, in vitro experiments have shown that atorvastatin inhibits the pro-inflammatory effects caused by low-grade endotoxaemia.

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Fig. 5 | Potential therapeutic strategies to reduce low-grade endotoxaemia. Gut dysbiosis downregulates junction proteins, which leads to increased gut permeability and increased translocation of lipopolysaccharide (LPS) into the systemic circulation. Dietary and pharmacological interventions might improve gut barrier dysfunction by upregulating the levels of tight junction proteins and the zonula occludens protein ZO1 and thereby lower circulating LPS levels. Potential interventions include a Mediterranean diet, probiotics (live bacteria or microbial metabolites such as urolithin A), prebiotics (fermentable fibres), interventions that increase glucagon-like peptide 1 (GLP1) and GLP2 levels, and statins.

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**Box 3 | Methods of LPS detection**

**Rabbit pyrogen test**

The rabbit pyrogen test is the first method to be developed for detecting lipopolysaccharide (LPS) and was approved by the FDA in 1942. This test is based on measuring the increase in body temperature in rabbits after intravenous injection of endotoxin. The detection limit is greater than 0.1 ng of LPS.

**Limulus amoebocyte lyase assay**

The limulus amoebocyte lyase (LAL) assay is the gold-standard method for the detection of lipid A in LPS. The LAL assay is based on the discovery by Bang in 1956 that blood cells (amoebocytes) from horseshoe crabs (Limulus polyphemus) agglomerase after reacting with endotoxin. The test is based on the coagulation cascade of horseshoe crab blood triggered by endotoxins, which involves several serine proteases and is initiated by the combination of LPS with zymogen factor C. The detection limit is 0.06 EU/mL. However, improved methods, such as the chromogenic and turbidimetric methodologies, increase the LAL test sensitivity to 0.015 EU/mL and 0.005 EU/mL, respectively.

**ELISA**

The enzyme-linked immunosorbent assay (ELISA), a technique developed in 1971, can be used to detect either the LPS antigen or LPS antibody titres. The ELISA detects LPS by primary capture antibody. To overcome the problems related to cross-reactivity or false positive results due to the heterogeneity of LPS, a microplate assay EndoLISA was developed in 2011, which directly detects endotoxin by using the target analyte (LPS) selectively bound to the surface of the precoated plate followed by detection with factor C. However, the assay cannot differentiate between bacterial serotypes. To date, specific immunological assays have been developed by using LPS preparations purified from different serotypes, which primarily differ in the chemical nature of the LPS O-antigen, or antibodies targeted against the hypervariable O-antigen region to ensure discriminatory detection of the assays.

**Mass spectrometry-based methods**

Gas chromatography (GC) mass spectrometry (MS) provides a direct measure of LPS mass by enabling the quantification of 3-hydroxytetradecanoic acid, the most abundant hydroxylated fatty acid of the lipid A moiety of most LPS molecules. However, only small amounts of lipid-rich samples can be loaded onto GC columns. High-performance liquid chromatography (HPLC) tandem MS (MS/MS) methods overcome the technical issues of GC–MS or GC–MS/MS, providing a sensitive, accurate and direct quantification of total amounts of LPS in the plasma. The HPLC–MS/MS method has high specificity and a low limit of detection and quantification, allowing the detection of the low concentrations of LPS found in the context of low-grade endotoxaemia.

**Biosensors**

In the past 10 years, biosensing techniques with high specificity, low cost and easy-to-operate and fast methods have been developed. Biosensors detect the presence of a specific analyte by using a biological recognition element on their surface, and can be monitored by the optical, electronic, mass or magnetic signal variation induced by the interaction with the analyte of interest. Different types of biosensors to detect LPS have been developed, including biosensors based on proteins, peptides, aptamers and antibodies. In particular, aptamers, single-stranded oligonucleotides that bind to specific targets with high affinity and specificity, are emerging molecular recognition tools for biosensing LPS.

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F.V. developed the concept and design of the review and wrote the manuscript. All the authors contributed to the discussion of content and reviewed and edited the manuscript before submission.

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