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The gut microbiome and thromboembolism

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Abstract

The gut microbiome plays a critical role in various inflammatory conditions, and its modulation is a potential treatment option for these conditions. The role of the gut microbiome in the pathogenesis of thromboembolism has not been fully elucidated. In this review, we summarize the evidence linking the gut microbiome to the pathogenesis of arterial and venous thrombosis. In a human host, potentially pathogenic bacteria are normal residents of the human gut microbiome, but significantly outnumbered by commensal anaerobic bacteria. Several disease states with an increased risk of venous thromboembolism (VTE) are associated with an imbalance in the gut microbiome characterized by a decrease in commensal anaerobic bacteria and an increase in the abundance of pathogenic bacteria of which the most common is the gram-negative Enterobacteriaceae (ENTERO) family. Bacterial lipopolysaccharides (LPS), the glycolipids found on the outer membrane of gram-negative bacteria, is one of the links between the microbiome and hypercoagulability. LPS binds to toll-like receptors to activate endothelial cells and platelets, leading to activation of the coagulation cascade. Bacteria in the microbiome can also metabolite compounds in the diet to produce important metabolites like trimethylamine-N-oxide (TMAO). TMAO causes platelet hyperreactivity, promotes thrombus formation and is associated with cardiovascular disease. Modulating the gut microbiome to target LPS and TMAO levels may be an innovative approach for decreasing the risk of thrombosis.

Keywords

Thrombosis; Microbiota; Lipopolysaccharides; Dysbiosis

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Author contributions

R. Hasan drafted the manuscript. A. Koh and A. Zia provided supervision and critically edited the manuscript.

Conflicts of interest

None

Introduction

Venous and arterial thromboembolic events are rapidly growing clinical problems. The rates of venous thromboembolism (VTE) in pediatric patients have increased by almost 70% based on a retrospective cohort from 2001 to 2007[1, 2]. The incidence of VTE continues to rise rapidly in adulthood after the fourth decade of life to 5–6 per 1000 people annually [3]. The morbidity associated with VTE – such as bleeding events from anticoagulation, recurrence, lack of thrombus resolution and post-thrombotic syndrome (PTS) -- is substantial. Similarly, cardiovascular disease (CVD) leading to arterial thromboembolic events is an emerging problem in both children and adults[4]. The pathogenesis of thrombosis is complex and occurs from the additive effects of genetic and environmental risk factors[5]. Hence, there is a strong need to identify modifiable risk factors to prevent thrombotic events and by extension, adverse long-term outcomes.

The role of the gut microbiome in various aspects of human health (ranging from obesity and CVD to autoimmune phenomena) is becoming increasingly recognized as having a significant impact on host physiology [6–10]. The gut microbiome is a modifiable host factor for several chronic diseases and is the focus of novel therapies such as fecal microbial transplant (FMT), probiotics, dietary changes, selective antibiotic use, and targeted inhibition of microbial enzymes [6, 11–15]. Perturbations of the gut microbiome from various environmental or genetic factors can lead to activation of inflammatory pathways in vascular endothelial cells, platelets, and innate immune cells resulting in release of various coagulation proteins leading to a prothrombotic state [16–21]. This review discusses current data on the association of gut microbiome perturbations and thrombosis and its potential as a preventive treatment option for VTE.

Overview of the gut microbiome

The human body is colonized by trillions of resident microorganisms that inhabit numerous host body niches with the highest density residing in the gastrointestinal tract[22]. The gut *microbiota* refers to all the microorganisms in the intestinal tract, and the *microbiome* is a collective term that includes all the genes of the microbiota and their by-products. Colonization of the gastrointestinal tract begins at birth and rapidly gains diversity during the first few years of life as diet, genetics, and environmental insults, such as antibiotics, come into play[23–25]. The microbiome composition can vary significantly from person to person and within the same person as they age, but in general, family members tend to have similar gut microbiome signatures, which is likely a combination of both genetic and environmental (e.g., diet) factors[6]. The microbiome maintains a complex balance with environmental and host genetic factors, the disruption of which leads to the development of various disease states[6, 7]. The gut epithelial barrier typically restricts the microbiomes to the intestinal lumen, but its function can be impaired by various factors, including inflammation, nutrition, and antibiotic use[26]. This “leaky” epithelial barrier allows for the translocation of gut microbial products and metabolites into the portal and then systemic circulation leading to multiple pathologies including potentially thrombosis [26].

Microbiome composition

Microbial composition and density vary along the gastrointestinal tract with a limited number and lower burden of bacterial species in the small intestine (< 10^4 cells per gram feces) as compared to the colon with ~ 10^{11} to 10^{12} cells per gram feces [6, 27, 28]. Usually, the gut microbiota is diverse with an assortment of gram-positive, gram-negative, obligate and facultative anaerobic bacteria [25]. In a healthy human adult, the gut microbiota is comprised of an abundance (>95%) of commensal obligate anaerobic bacteria, of which the majority is the phyla Firmicutes and Bacteroidetes[25]. Potentially pathogenic bacteria also referred to as pathobionts (e.g., facultative gram-negative bacteria, such as *Escherichia coli*), are normal residents of the human gut microbiome, but are greatly outnumbered (<1% relative abundance) by commensal anaerobic bacteria[29]. Dysbiosis is defined as a microbial imbalance that typically manifests as a decrease in microbial diversity: for example, a decrease in commensal anaerobic gut bacteria and an overgrowth of pathobionts such as the bacterial family Enterobacteriaceae (ENTERO)[30, 31]. ENTERO are gram-negative facultative anaerobes able to thrive with or without oxygen and are recognized as harmful, pathogenic, and proinflammatory bacteria[32]. The microbiome promotes immune system development. On the contrary, dysbiosis and ENTERO expansion is associated with obesity, diabetes, inflammatory diseases, and other chronic disease states [6].

Metabolite production

Another mechanism by which the gut microbiome induces host response is via gut microbiota derived metabolites. Western diets are rich in trimethylamine (TMA) containing nutrients such as choline, carnitine, and phosphatidylcholine. The gut microbiota metabolizes these nutrients from the host's diet into metabolites such as trimethylamine-N-oxide (TMAO)[33]. Western diets promote enrichment of a gut microbiome that is associated with increased TMAO production (figure 1a)[19]. Higher plasma TMAO levels are associated with adverse outcomes of CVD and thrombotic events and TMAO is currently being studied as a therapeutic option for CVD prevention[19, 34, 35].

Profiling the microbiome

In the past, culture-based approaches have not accurately characterized the composition of the gut microbiome, given that many obligate anaerobic species are difficult or impossible to culture[36]. Quantitative polymerase chain reaction (qPCR) allows for determining absolute levels of specific species or groups of gut microbiota but cannot provide community microbiome taxonomic composition data (Table 1). With the advent of next-generation sequencing approaches, investigators focused on the bacterial 16S ribosomal RNA (16S rRNA) gene, which contains genetic sequences common to all bacteria but also variable regions that are unique for specific groups or species of bacteria[37]. Hence, by taking genomic DNA recovered from a complex bacterial community (e.g. human fecal sample), the variable regions of all the bacterial 16S rRNA genes could be simultaneously amplified, sequenced, and then analyzed (by interrogation of a 16S rRNA sequence database) to produce a readout of the relative abundances of various gut microbiota in that community[25, 36, 38]. More recently, metagenomic shotgun sequencing (MSS), which directly sequences all the genomic DNA and forgoes amplicon libraries, has led to higher

taxonomic resolution and an unbiased approach to profiling the gut microbiome[36]. Thus, there is great interest in identifying a distinct microbiome signature associated with CVD or increased TMAO production. For example, 16s RNA sequencing of atherosclerotic plaques shows an increased density of certain bacterial species also present in the oral cavity and gut of these patients and correlated with higher levels of cholesterol[39].

Yet both 16S rRNA and MSS are time-consuming, laborious, and not suited for real-time gut microbiota monitoring in patients (Table 1). A major limitation of these methods is they only allow for determining the relative abundance of microorganisms, not the absolute levels, which can be misleading as an increase in relative abundance may not always correlate with an increase in absolute abundance . As such, novel methods are needed to provide point of care gut microbiota profiling in the future.

Inflammation and dysbiosis associated VTE

Inflammation is an important risk factor for VTE. Inflammation activates endothelial cells, platelets, and leukocytes to initiate coagulation [40]. It also leads to a consumptive coagulopathy and increase in pro-inflammatory cytokines, chemokines and various leukocyte subtypes [41, 42]. Several inflammatory states such as obesity, sepsis/infection, inflammatory bowel disease (IBD) and intestinal failure (IF) are associated with both dysbiosis and higher incidence of VTE (Table 2)[19, 30, 31, 43–55].

Dysbiosis, LPS, and hypercoagulability

One of the links between dysbiosis and thrombosis is lipopolysaccharides (LPS), the glycolipid found on the outer membrane of gram-negative bacteria [56]. LPS is a heat-stable endotoxin that can enter the blood either by local or systemic infection [57]. In sepsis, patients have LPS levels a hundred-fold higher than healthy controls, which leads to overproduction of inflammatory cytokines and the clinical findings of gram-negative septic shock (organ failure, hypotension, respiratory distress, and disseminated intravascular coagulation (DIC) from dysregulation of coagulation cascade) [58]. Metabolic endotoxemia is a condition with chronically elevated plasma levels of LPS that are 10–50 times lower than sepsis resulting in a chronic inflammatory state [57]. Metabolic endotoxemia results from bacteria and their endotoxins, such as LPS, entering systemic circulation by translocating across an impaired intestinal lumen due to inflammation and injury [59]. LPS can translocate across different regions of GI tract from the lymphatic system in the oral cavity to the defective mucosal barrier in the intestinal mucosa (figure 1b)[49, 50, 60, 61].

LPS and metabolic endotoxemia

There has been difficulty finding a reliable LPS assay to measure these low levels of LPS in metabolic endotoxemia. The horseshoe crab (*Limulus Polyphemus* or *Tachypleus species*) has blood that is very sensitive to and will clot in the presence of LPS. One of the first assays used for LPS detections was a limulus amebocyte lysate (LAL) assays that would determine the presence of LPS in the patient's plasma by measuring activation of the coagulation cascade in a horshecrab's blood[62]. Since then, further assays have been developed including ELISA and Endotoxin activity assay (EAA), however, their reliability

and interpretation are inconsistent and levels can fluctuance quickly within the bloodstream [62]. Another major limitation, is that not all LPS is the same as it can have differing structures and immunogenecity depending on the species to induce a host response. For example, *Bacteriodes* are commensal gram negative obligate anaerobes which also produce LPS but their LPS is structurally distinct and is beneficial as it inihibits innate immune signaling compared to LPS from *E. Coli* which is a potent immune activator[63, 64].

Despite these limitations, metabolic endotoxemia has been shown to be present in a variety of disease states such as atherosclerosis, autoimmune diseases, metabolic syndrome, cirrhosis, depression, type 2 diabetes, obesity, traumatic brain injury, multi-organ failure, depression and HIV [62]. To further highlight how dysbiosis plays a critical role in metabolic endotoxemia, in IBD, abundance of ENTERO in the microbiome of patients is associated with increased serum LPS levels compared to healthy controls[50]. Patients with cirrhosis similarly have higher levels of circulating systemic LPS secondary to translocation of bacteria and their by-products from the intestinal lumen into the portal and systemic circulation[65, 66]. Much higher levels of LPS are found in portal circulation than systemic circulation, suggesting a mechanistic process of spillover from the gut lumen into the portal and then systemic circulation[66–68]. These findings have also been recapitualated in a mouse model as high-fat diets in mice (as model of obesity) induce a change in gut microbiota populations towards an increase in abundance of gram-negative bacteria and increased intestinal permeability, which leads to LPS translocating into the systemic circulation with resultant metabolic endotoxemia[69].

Evidence for LPS-induced hypercoagulability

How increased LPS may lead to hypercoagulability has been evidenced in studies across various disease states and in mouse models(Table 3). In cirrhosis patients, elevated serum LPS levels positively correlate with factor (F) VIII levels, von Willebrand factor (VWF), and thrombin generation (as measured by prothrombin fragments F1+2 and D-dimer levels) [67, 68, 70]. Administration of enteral non-absorbable antibiotics led to a decrease in LPS levels and a concomitant decrease in thrombin generation [70]. Similarly, in IBD patients, LPS levels correlated with D-dimer and prothrombin fragment levels, and this association was stronger in patients with colonic disease where there is a higher bacterial burden than the rest of the GI tract [50].

Wang et al. have studied the effect of LPS on both arterial and venous thrombosis in a murine model [71]. In this model, high levels of ferric chloride (FeCl3) are used to induce vessel injury either to the carotid artery or vena cava[71]. Dose-dependent thrombosis; time to thrombosis and thrombus size are used as metrics of hypercoagulability[71]. Wang et al. discovered that administration of LPS potentiated thrombus formation even at lower ferric chloride concentrations where vessel injury was not induced[71].

Toll-like receptor pathways in hypercoagulability

LPS is a key activator of the innate immune system via toll-like receptors (TLRs) expressed on both immune and non-immune cells, including macrophages, lymphocytes, endothelial cells, dendritic cells and platelets[72]. TLRs are key activators of the innate immune system

that recognize pathogen-associated molecular patterns (PAMPs) found in bacteria, viruses, and fungi and play a key role in the activation of the innate immune system[72, 73]. Over 10 different TLRs have been identified of which the best studied are TLR2 and TLR4, which are expressed on nucleated endothelial cells, white blood cells, and platelets[50, 74, 75]. Platelet TLRs are the bridge in “thromboinflammation” with their role in inflammation, platelet adhesion and aggregation and the formation of neutrophil extracellular traps (NETs) [73]. There have also been studies to demonstrate that higher levels of TLR transcript expression can be associated with cardiovascular risk and inflammatory biomarkers to further highlight the association with thrombosis[76].

TLR4

TLR4 is the primary receptor for LPS on nucleated cells and platelets[77]. In clinical studies of IBD patients, LPS levels correlate positively with TLR4 concentrations to highlight the role of LPS-TLR4 pathway in triggering coagulation in these patients [50]. *Ex vivo* studies of platelets from cirrhosis patients with elevated LPS levels show platelet aggregation is increased in response to subthreshold concentrations of common agonists (such as Adenine di-Phosphate or collagen) compared to healthy controls and regardless of platelet count [78]. This response is blunted in the presence of TLR4 inhibition to further highlight the importance of this pathway [78].

TLR4 is found on endothelial cells which contain Weibel-Palade bodies (WPBs) that store and release modulators of vascular inflammation, including VWF, factor VIII and P-selectin [68, 79]. I*n vitro* studies of human umbilical vein endothelial cells (HUVEC) incubation with LPS at concentrations observed in cirrhosis patients show that TLR4 activation induces VWF and factor VIII expression from endothelial cells (figure 1c)[68]. When these cells were incubated with a TLR4 inhibitor, this effect was mitigated suggesting that TLR4 pathways mediates exocytosis of Weibel-Palade bodies. Binding of LPS to TLR4 leads to activation of a MyD88-dependent pathway which causes the release of proinflammatory cytokines[80].

On platelets, TLR4 mediates platelet-neutrophil interactions by increasing binding to neutrophils, formation of NETs, and histone-mediated platelet responses that increase thrombin generation[73].

LPS has also been shown to activate platelets directly through aggregation, dense/alpha granule release and fibrinogen binding (figure 1d)[17]. However this role is controversial in other studies that show LPS may inhibit platelets or not induce any response either directly or through potentiation [81]. This discordant data may be due to a variety of factors including platelet preparation, insufficient amounts of soluble CD14 (needed for LPS mediated platelet response), the strain and concentration of LPS, stimulation time and biphasic response to cGMP signaling[73].

TLR2

TLR2 is also found on endothelial cells and platelets. TLR2 normally forms a heterodimer with either TLR1 or TLR6 and recognizes the PAMPs on a wide range of peptidoglycans on mainly gram-positive and some gram-negative bacteria[73, 77].

TLR2 pathway is vital for endothelial cells as TLR2 receptors are found on the hepatic endothelial cells and their activation will lead to an increase in VWF and an increase in arterial thrombus growth [21]. Germ-free mice (mice without microbiota) and TLR2 deficient mice have reduced thrombus formation highlighting the importance of the microbiota for TLR2 activation. Furthermore, treatment of *in vitro* HUVEC with TLR2 agonists leads to procoagulant effects with increased production of tissue factor, plasminogen activator inhibitor-1 (PAI-1), and decreased production of tissue plasminogen activator and tissue factor pathway inhibitor (TFPI) [82].

In platelets, activation of the TLR2 pathway leads to platelet activation, aggregation with leukocytes, and release of alpha and dense granules [83–85]. There are some conflicting studies suggest TLR2/6 may inhibit platelet activation but further studies are needed to understand the true pathophysiology [86].

Understanding immunothrombosis

"Immunothrombosis" is a term coined by Englemann and Massberg on the role of thrombosis in preventing intravascular antimicrobial spread through the formation of thrombi in microvessels by the recruitment of innate immune cells and platelets [87]. Neutrophils, monocytes, and dendritic cells work together to inhibit pathogen dissemination in the vasculature by initiating thrombosis through fibrin formation and platelet activation [87]. "Immunothrombosis" is distinct from the process of primary and secondary hemostasis as it occurs mostly in the intravascular compartment and could potentially be a novel treatment option for preventing pathological thrombosis [87]. Hemostasis and immunothrombosis work as a continuum for preventing blood loss and intravascular antimicrobial dissemination, but aberrant activation can lead to pathologic thrombosis seen in CVD and DVTs [87].

Neutrophils

Neutrophils are the major effectors of innate immunity and plan a dual function in host defense and thrombosis (Table 4). They aggregate rapidly at the site of vessel injury with platelets and can contribute to the formation of large-vessel thrombosis in cardiovascular diseases [88]. Upon activation, neutrophils will release NETs that are a matrix of DNA and histones projected from the neutrophils. NETs are protective against infections as they work as scaffolds to enhance neutrophil-mediated trapping and killing of pathogens[87, 89]. However, they can also induce a strong procoagulation response and be injurious to tissues by leading to pathologic consequences such as inflammatory diseases and transfusion-related acute lung injury (TRALI)[90].

NET formation in "immunothrombosis" leads to platelet-neutrophil interactions that activate the contact factor and contribute to aggregation (figure 1e). Histone components of the extracellular nucleosomes in NETs can activate TLR2 and TLR4[91]. Activated neutrophils will deposit serine proteases that inactivate important anticoagulants like TFPI and thrombomodulin, which leads to resultant hypercoagulability[92, 93]. NETs also activate the contact factor pathway by activating factor XII and bind to VWF to facilitate platelet recruitment and activation[94]. TLR4 pathway also induces NET formation by increasing

endothelial expression of important adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) [20, 95, 96]. Furthermore, blocking NET formation in preclinical models by treatment of mice with deoxyribonuclease (DNase) that breakdown NETs, will prevent thrombus formation [97].

Platelets

Platelets play a critical supportive role in immunothrombosis through pathogen recognition and augmentation of prothrombotic pathways involved in innate immune cells. Platelet will bind to LPS on circulating bacteria and present these pathogens to neutrophils and other innate immune cells[98]. In arterial thrombus, they are the first cells recruited to the sites of endothelial disruption and lead to the expression of adhesion molecules and release of chemokines to recruit innate immune cells[99]. In DVTs, platelets accumulate at the inflammatory sites and participate in receptor-dependent binding to neutrophils and monocytes and direct platelet-endothelial cell interactions that foster additional immune cell recruitment [94].

LPS and the coagulation cascade

Contact activation (intrinsic) pathway

Elevated LPS levels in endotoxemia can directly activate kallikrein-kinin system (KKS) [100]. Generated kallikrein reciprocally activates factor XII which leads to activation of the intrinsic coagulation system and cleaves high molecular weight kininogen (HK) to lead to the liberation of the proinflammatory mediator, bradykinin[100]. HK is also a critical LPS carrier as mice lacking HK are resistant to LPS-induced mortality, and replenishing HK results in restored susceptibility of animals [12]. In mononuclear cell cultures, FXII-induced release of interleukin (IL)-1 is augmented in the presence of LPS[101]. Furthermore, FXII-treated splenic dendritic cells exposed to LPS, produce higher amounts of IL-6 and IL-23 further corroborating that FXII and the KKS are instrumental in mediating host immune responses[102].

6.2 Tissue factor (extrinsic) pathway

LPS can also affect the extrinsic coagualation cascade. Phosphatidylserines enhance activity of tissue factor (TF) by increasing TF binding to FVII and formation of cofactor-protease complexes in the coagulation cascade[103]. Phosphatidylserines are normally found in the inner leaflet of the plasma membrane and will externalize in the presence of LPS to the outer leaflet of the plasma membrane leading to TF activation[103, 104]. The mechanism of LPS induced phosphatidylserine expression is through LPS activation of an intracellular cytosolic LPS receptor, Caspase-11 (Casp11) which triggers gastrodermin D (GSDMD) pore formation leads to calcium influx and activation of transmebrane proteins that mediate phosphatidylserine expression[105]. Deletion of Casp11 will lead to decrease LPS-induced thrombin generation and platelet aggregation in mice[105]. Prior studies have also suggested an important link between extrinsic pathway and endotoxemia as inhibition of TF prevents endotoxemia-induced DIC[106]. This combination of platelet hyperreactivity, release of coagulation factors, cytokine release and NET formation can lead to hypercoagulablity and possibly thrombus formation (figure 1f). This supports

an important role of immunothrombosis in the pathogenesis of VTE and as a possible therapeutic target for future studies. Further details on mechanisms of LPS-induced hypercoagulability are extensively reviewed elsewhere[85, 107–110].

Microbiome metabolites and thrombosis risk

The gut microbiome processes the undigested dietary polysaccharides from the host's diet and can alter metabolite production in the host [24]. A western diet, high in fat and sugar, has increased content of phosphatidylcholine, choline and carnitine[24]. TMA lyases in the gut microbiome metabolize choline from the diet into TMA, which then goes through the portal circulation to the liver where it is converted into TMAO[24, 34, 111]. Microorganisms associated with increase TMAO production were difficult and inconsistently identified with 16s rRNA sequencing as production of a specific metabolite like TMAO in the abudance a certain bacterial taxa does not necessarily imply causality[112–115]. Reference genomic studies have been more helpful in identify TMAO producing species in the phylum *Actinobacteria*, *Firmicutes* and *Proteobacteria* through sceening for species with pontential to be involved in metabolic pathway of TMA production[115, 116]. TMAO has gained importance because it enhances cardiovascular and thrombosis risk by atherosclerosis progression through vascular inflammation, endothelial cell dysfunction, foam cell formation, and platelet hyperreactivity[19, 117–120].

Other microbiome metabolites that correlate with the risk of CVD are bile acids (BA) and short-chain fatty acids (SCFA)[121]. BA are synthesized from cholesterol in the liver and are vital for cholesterol excretion in the feces. Changes in the microbiome can decrease BA synthesis, leading to decreased excretion of cholesterol and increased risk for CVD[122]. SCFA result from fermentation of indigestible fibers by the microbiota, and while elevated levels are thought to generally have an anti-inflammatory effect, uncontrolled levels can be a risk factor for CVD[123–125]. While there have been advances in our ability to detect these metabolites and study correlations, our understanding of the exact mechanisms remains unclear.

Mechanisms of TMAO

Atherosclerotic diseases, such as myocardial infarction and stroke, are multifactorial in origin however, there is increasing evidence that changes in the microbiome may influence the progression of atherosclerosis[125]. Atherosclerotic disease has been shown to be associated with increased metabolism of dietary lipid phosphatidylcholine to TMAO [24]. Plasma TMAO levels are thought to correlate with coronary atherosclerotic plaque burden and are an independent predictor of CVD disease in humans (Table 5) [19, 24, 114]. TMAO increases the deposition of cholesterol-laden macrophage foam cells onto vessels, which is one of the earliest steps in the development of atherosclerotic disease (figure 1g) [24]. The microbiome plays an important role in this as a choline-rich diet will augment atherosclerosis while the depletion of gut flora with antibiotics, even in the presence of a choline-rich diet, will suppress this response[24].

In mouse models of thrombosis, higher levels of TMAO in choline-supplemented mice are associated with a shorter time to cessation of blood flow after FeCl3 injury[19].

Furthermore, when germ-free mice with no gut colonization are supplemented with choline alone, there is no effect on the time to cessation of blood flow, underscoring the critical role of the gut microbiome in the prothrombotic phenotype with TMAO[19]. A choline-rich diet induces dysbiosis by an increase in the phyla of *Firmicutes* and *Proteobacteria* and transplantation of this microbiome composition to germ free mice results in increased platelet responsiveness (as measured by *ex vivo* platelet aggregometry) and *in vivo* thrombosis potential (as measured by the FeCl3 carotid artery injury model)[19, 126]. TMAO does not stimulate platelets directly but enhances the release of Ca^{2+} and activation to platelet agonists to show a TMAO-dependent enhancement [19].

Unfortunately, these findings are not consistent as other studies have shown that TMAO correlated inversely with atherosclerotic lesion size, and further studies are needed to fully understand its role in thrombosis[127]. Jonsson et al. determined that while choline supplementation did increase TMAO levels, the size of aortic lesions was not affected by choline supplementation and did not correlate with TMAO levels[113]. However these studies differed significantly in methods as Jonsson et al. used germ free mice as microbiota depleted controls and supplemented choline at an older age of 8 weeks (when atherosclerotic would have already developed in mice) as compared to Wang et al. who used antibiotic to deplete the gut microbiota and started choline supplementation at only 4 weeks[24]. TMAO is also not as clear of a predictor for VTE as a cross-sectional study of patients with acute VTE, higher levels of TMAO correlated with mortality but not with VTE recurrence[128].

Treatment options

Dysbiosis

Many treatment options to modulate dysbiosis in the gut microbiome are currently being explored (Table 6). Prebiotics, foods, or dietary supplements that “induce” the growth or activity of beneficial gut commensal anaerobes , and probiotics, live organisms that are ingested, have been studied as treatment options in IBD and *Clostridium difficile* (*C. difficile*) infection with mixed reports on efficacy[11, 129, 130]. The use of certain broad-spectrum antibiotics, such as vancomycin and cephalexin, can lead to long term changes in the composition of the microbiome with the expansion of pathobionts, such as ENTERO, which can potentially lead to opportunistic infections[13, 131]. The use of clindamycin in the hematopoietic stem cell transplantation (HSCT) population leads to depletion of commensal anaerobes (Firmicutes), which is associated with adverse outcomes of graft-versus-host disease (GVHD)[7]. How modulating the gut microbiome with antibiotics can lead to ENTERO expansion, and increased thrombotic risk has not been studied.

FMT is the transplantation of a donor’s fecal samples by either oral or rectal delivery to restore gut microbiota homeostasis. FMT is a safe and effective treatment option for recurrent *C. difficile* infection with remission rates of over 80% compared to antibiotics alone[14, 132]. The role of FMT in metabolic syndrome has been studied in animal models and humans with favorable results[133, 134]. Autologous FMT also has promising potential as a therapeutic option in HSCT, where the loss of microbial diversity during transplant is associated with systemic infections, *C. difficile* infections and GVHD[135]. The expansion of commensal anaerobes, specifically phyla *Firmicutes*, in successfully transplanted patients

is thought to have an important role in restoring the microbiome and reducing *C. difficile* infections[14, 136]. Healthy donor FMT can also affect hypercoagulabilty in metabolic syndrome patients by leading to a prolonged lag time on thrombin generation and downregulation of coagulation related proteins[137].

FMT, however, is not without risks. Recently in 2019, there was an FDA warning about the use of FMT after two immunocompromised patients developed invasive extended spectrum-beta lactamase *Escherichia coli* infections after transplants from the same donor (https://www.fda.gov/vaccines-blood-biologics/safety-availability-biologics/important-safety-alert-regarding-use-fecal-microbiota-transplantation-and-risk-serious-adverse). The donor had not been tested for multi-drug resistant organisms, which is typically performed by many groups administering FMT.

LPS:

Treatment options to target LPS have also long been explored. One option is to block LPS with therapies such as a HMW Kininogen antibody (C11C1), a monoclonal antibody directed to the LPS binding site on domain 5 of HK [138]. C11C1 has been shown to reduce LPS-induced systemic inflammation and circulating LPS levels in mice[138]. Several TLR4 antagonists have been developed and investigated in clinical trials but with little efficacy[138, 139].

Another option is to increase the clearance of LPS. CD14 is the LPS sensing receptors on monocytes that binds to LPS and transfer it to the TLR4 complex[140]. Anti-CD 14 antibodies can lead to TLR4 internalization, decrease LPS response and may be a novel therapeutic option[141]. LPS is also cleared through the cholesterol pathway, including the low density lipoprotein receptor (LDLR)[142]. Statins increase expression of LDLR and have general anti-inflammatory effect but in clinical trials of sepsis do not shown a survival benefit against sepsis. The use of proprotein convertase subtilisin/ kexin type 9 (PCSK9) inhibitors to block degradation of LDL receptors and lead to increase LPS clearance can also be considered[143, 144]

Targeting the downstream effectors of the TLR-4 signaling pathway are yet another therapeutic possibility. Activator protein 1,signal transducers and activators of transcription family of transcription factors and interferon regulatory factors are currently being explored as potential options for modulating inflammation and sepsis[145]. The Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial has shown that IL-1B inhibitors, downstream of the TLR4 pathway, lead to a lower incidence of cardiovascular events[146]. Another option that has yet to be explored is using DNase, which inhibits NET formation as a possible therapeutic drug for thrombolysis[89]. Whether these therapies will be effective in decreasing thrombosis risk in dysbiosis has not been explored.

TMAO

The discovery of TMAO as a risk factor for arterial thrombosis has led to research targeting this pathway as a treatment modality to decrease the risk of CVD. Flavin monooxygenases (FMOs), mainly FMO3, convert TMA into TMAO [34]. There is a genetic disorder of FMO3 deficiency known as fish odor syndrome, but recreating this in humans is clinically

limited as it has the unfortunate side effect of a strong door from the increase in TMA secretion through the sweat glands, urine, and breath[147]. A structural analog of choline, 3,3-dimethyl-1-butanol (DMB), is found naturally in food products and decreases TMAO production by inhibiting microbial TMA lyases to lower TMA levels [15]. CutC/D inhibitors that target microbial proteins in choline metabolism are another treatment option. These inhibitors would accumulate only in the intestinal microbes and block TMA and TMAO production to reduce thrombosis potential [35].

Conclusion

The role of the gut microbiome in various aspects of human health is becoming increasingly recognized. The etiology of thrombosis is a complex interplay between the coagulation system, innate immune system, and inflammation. Inflammation is further associated with dysbiosis, increase in intestinal permeability and production of specific metabolites. Identification of important metabolites related to the gut microbiome and cardiovascular risk, such as TMAO, has led to research in the development of targeted therapies [19, 35]. The role of gut microbiota derived LPS in hypercoagulability and VTE continues to be studied and treatment options are yet to be explored. The added inflammatory effect of dysbiosis that activates the innate immune system is yet another pathway that could be targeted. The authors are currently leading an ongoing single institutional prospective pilot study on new pediatric VTE with the primary objectives of determining if patients who develop VTE have a distinct gut microbiome signature with expansion of ENTERO. Furthermore, they are determining whether these patients have elevated plasma LPS levels and if these levels correlate with hypercoagulability as determined by thrombin generation assay. The primary goal is to establish a gut microbiome signature for VTE and elevated LPS levels as a possible biomarker of VTE in high-risk patients. Causality could then be tested by utilizing a murine model of thrombosis to gain mechanistic insight into how dysbiosis leads to hypercoagulability. Our ability to understand the microbiome and its role in thrombosis is critical in optimizing treatment and developing new preventative and therapeutic strategies with limited bleeding risk.

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Abbreviations:

VTE	Venous thromboembolism

ENTERO	Enterobacteriaceae
LPS	Lipopolysaccharides
TMAO	Trimethylamine-N-oxide
PTS	Post-thrombotic syndrome
CVD	Cardiovascular Disease
FMT	Fecal microbial transplant
TMA	Trimethylamine
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal RNA
MSS	Metagenomic shotgun sequencing
IBD	Inflammatory bowel disease
IF	Intestinal failure
DIC	Disseminated intravascular coagulation
LAL	Limulus amebocyte lysate
EAA	Endotoxin activity assay
F	Factor
VWF	von Willebrand factor
FeCl3	Ferric chloride
TLRs	Toll-like receptors
PAMPs	Pathogen-associated molecular patterns
NETs	Neutrophil extracellular traps
WBP	Weibel-Palade bodies
HUVEC	Human umbilical vein endothelial cells
PAI-1	Plasminogen activator inhibitor-1
TFPI	Tissue factor pathway inhibitor
TRALI	Transfusion-related acute lung injury
ICAM-1	Intercellular adhesion molecule-1
DNase	Deoxyribonuclease
KKS	Kallikrein-kinin system

HK	High molecular weight kininogen
IL	Interleukin
TF	Tissue factor
Casp11	Caspase-11
GSDMD	Gastrodermin D
BA	Bile acids
SCFA	Short-chain fatty acids
HSCT	Hematopoietic stem cell transplantation
GVHD	Graft-versus-host disease
C11C1	HMW Kininogen antibody
LDLR	Low density lipoprotein receptor
PCSK9	Proprotein convertase subtilisin/ kexin type 9
CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcome Study
FMOs	Flavin monooxygenases
DMB	3,3-dimethyl-1-butanol

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Highlights:

- Several disease states with high risk of thrombosis are associated with dysbiosis
- Bacterial LPS from the gut can bind to TLRs and activates the coagulation cascade
- The microbiome can affect the innate immune system and lead to “immunothrombosis”
- Gut metabolites like TMAO can promote cardiovascular disease and thrombosis

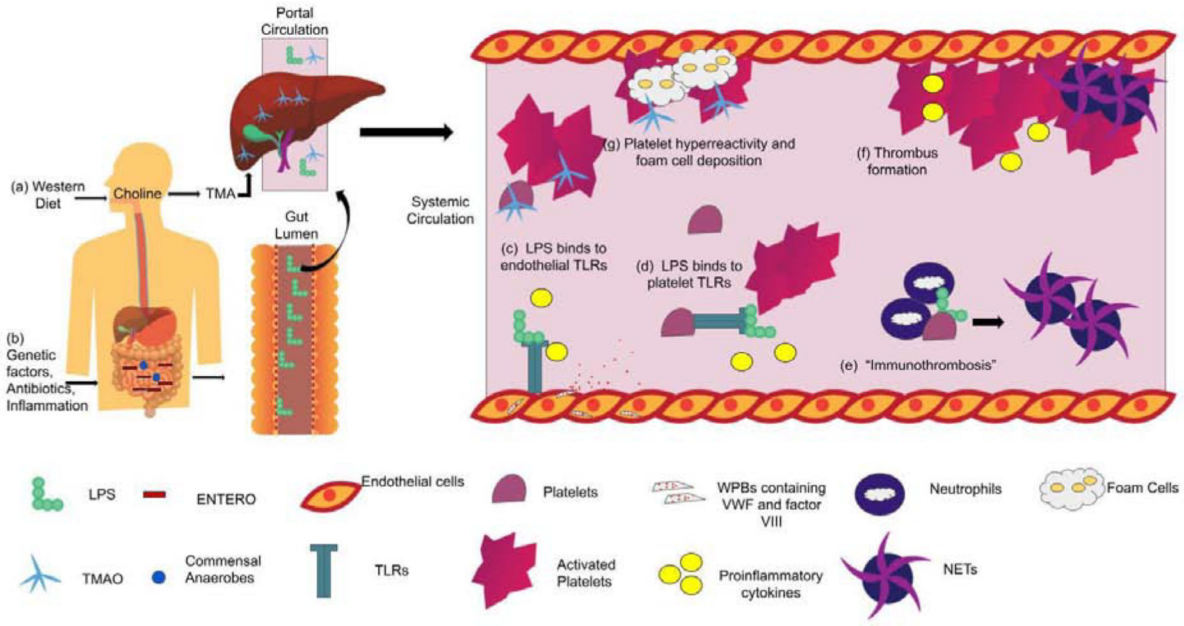

Figure 1:

Proposed mechanisms of how the microbiome induces thrombosis

(a) Western diet are choline-rich which is metabolized to TMA by TMA lyases in the gut. TMA enter portal circulation in the liver where it is converted to TMAO and released into circulation.

(b) Presence of genetic and environmental factors lead to dysbiosis with expansion of ENTERO and decrease in microbial diversity. Increase in ENTERO leads to increase in LPS production in the gut lumen. LPS translocates through an impaired gut epithelial barrier into portal and then systemic circulation.

(c) LPS leads to activation of TLR4 on endothelial cells which leads to release of proinflammatory cytokines and exocytosis of Weibel palade bodies to release factor VIII and vWF.

(d) LPS binds to platelet TLRs leading to activation of platelets, release of alpha and dense granules and release of cytokines.

(e) TLRs also mediate platelet-neutrophil interactions and “immunothrombosis” by binding to neutrophils to increase formation of NETs, which can activate the contact factor pathway and contribute to aggregation.

(f) Platelet hyperreactivity, release of coagulation factors, NET formation and proinflammatory cytokines lead to thrombus formation.

(g) Upon entering systemic circulation, TMAO leads to platelet hyperresponsiveness and increases deposition of foam cells in the early steps in atherosclerosis.

Table 1:

Comparison of various gut microbiome profiling approaches

Technique	Population	Method	Pros	Cons
Quantitative polymerase chain reaction (qPCR)	Bacteria, viruses, fungi	Group specific primers are used to clone segment of 16S rRNA gene	Low cost and labor	Offers no metagenomic insight
			Only method that determines absolute concentration	Can only detect one bacterial group per experiment (i.e. 16S rRNA for *E. coli* to determine ENTERO)
			Can be performed within a day	
16S ribosomal RNA gene sequencing (16S)	Bacteria only	Amplifies the V1-V9 region of 16S rRNA gene and clusters sequences into operational taxonomic units (OTUs)	More affordable	Can have taxonomic bias (primer design, amplification conditions)
			Less complex informatics than MSS	
		OTUs are references against database of 16S rRNA sequences to determine taxa	Determines relative abundance	
			Can simultaneously detect thousands of different taxa	
Metagenomic Shotgun Sequencing (MSS)	Bacteria, viruses, fungi	Unrestricted sequencing genome of all microorganisms in a sample	Can detect low abundance organisms	High cost
			Can pair with metabolomic profiling to detect metabolites such as TMAO	Complex bioinformatics analysis
		Uses marker genes for taxonomic identification	Can simultaneously detect thousands of different taxa	Takes weeks to months to perform

rRNA, ribosomal RNA; ENTERO, Enterobacteriaceae, V, variable; ENTERO, Enterobacteriaceae; TMAO, trimethylamine-N-oxide;

Table 2:

Disease states associated with high risk of VTE and dysbiosis

Disease State:	VTE Risk:	Study:	Microbiome Changes:	Study:
ICU patients	Sepsis patients have 1.7 to 3.9-fold higher risk of developing VTE	Kaplan, 2015 [43]	Microbiome changes within 48 hours to day 10 ICU admission Decrease in commensal Firmicutes and Bacteroidetes Increase in abundance of ENTERO Did not exclude use of antibiotics in patients	McDonald, 2016 [31]
IBD	1.5 to 3.6-fold higher risk of VTE independent predictor of VTE	Bernstein 2001 [44] Kappelman 2011 [45] Miehsler 2004 [46]	Less biodiversity, decrease in commensal anaerobes and increase in proinflammatory ENTERO Dysbiosis correlated with disease status Defective intestinal mucosal barrier allow translocation of bacterial components	Manichanh, 2006 [47] Frank, 2011 [48] Pastorelli, 2013 [49] Pastorelli, 2015 [50]
Obesity	2.2 to 2.7-fold higher risk of VTE	Ageno 2008 [51]	Less bacterial diversity, increase in pathogenic microorganisms Changes in proportion of Bacteroidetes and firmicutes Increase TMAO production	Zhu, 2016 [19] Karlsson, 2012 [52] Ley, 2005 [53]
Intestinal Failure	57% with central lines with develop line associated VTE 40% will have recurrence	Journeycake 2015[54] Gonzalez-Hernandez 2016 [55]	ENTERO expansion and paucity of commensal anaerobes	Piper, 2017 [30]

ENTERO, Enterobacteriaceae; TMAO, trimethylamine-N-oxide;

Table 3:

LPS Mediated hypercoagulability

Pathways	Finding:	References:
General	Administration of LPS potentiated thrombus formation in murine models	Wang, 2008 [71]
	LPS levels correlated with elevated levels of vWF and factor VIII	Carnavale, 2017 [68]
Platelet activation	Platelets exposed to LPS have increase aggregation	Raparelli, 2017 [78]
TLR4 mediated platelet activation	Degranulation of alpha and dense granules	Andonegui, 2005 [17]
	Release of inflammatory cytokines	Zhang, 2009 [80]
	Release vWF and potentiates thrombin	Rivadeneyra, 2014 [85]
	Mediate platelet-neutrophil interactions and NET formation	D'Atri and Schattner, 2017 [73]
TLR4 mediated activation of endothelial cells	Release of vWF, Factor VIII and P-selectin from Weibel Palade bodies	Carnevale, 2017 [68] Pan, 2016 [79]
TLR 2 mediated platelet activation	Platelet activation, aggregation with leukocytes, release of alpha and dense granules	Blair, 2009 [83] Rex, 2009 [84] Rivadeneyra, 2014 [85]
TLR 2 mediated activation of endothelial cells	Increase vWF, adhesion and arterial thrombus growth	Jäckel, 2017 [21]
	Increase tissue factor, PAI-1 and decrease TPA and TFPI	Shin, 2011 [82]

LPS, lipopolysaccharides; VWF, von Willebrand factor; TL4, toll-like receptor 4 ; NET, neutrophil endothelial traps ; PAI-1, plasminogen activator inhibitor-1; TPA, tissue plasminogen activator; TFPI, tissue factor pathway inhibitor

Table 4:

Immunothrombosis in hypercoagulability

Pathway/ Cell:	Key Finding:	Reference
Neutrophils	NETs illicit a strong procoagulant response	Fuchs, 2012 [89]
	LPS induces expression of adhesion VCAM-1 and ICAM1	Sawa, 2008 [95]
	Breakdown of NETs with DNAse will lead prevent thrombus formation	Brill, 2012 [97]
Platelets	Bind to LPS and present it to neutrophils and immune cells	Semple, 2007 [98]
	Bind to neutrophils and endothelial cells to foster additional immune cell recruitment	von Brühl, 2012[94]
Contact activation (intrinsic) pathway	Elevated LPS levels activate KKS	Wu, 2015 [100]
	LPS increased FXII release of Interleukins	Toossi, 1992 [101] Gobel, 2016 [102]
Tissue factor (extrinsic) pathway	LPS leads to externalization of phosphatidylserines that lead to increase TF activation	Yang, 2019 [105]
	Inhibition of TF prevents DIC findings in endotoxemia	Pawlinski, 2010 [106]

NETS, neutrophil endothelial traps; VCAM, vascular cell adhesion molecule; ICAM, intracellular adhesion molecules; LPS, lipopolysaccharides; DNAase, deoxyribonuclease; KKS, Kallikrein-Kinin system

Table 5:

TMAO mediated hypercoagulability

Finding:	References:
TMAO levels are independent predictors of CVD	Wang, 2011 [24]
Choline dependent atherosclerosis is suppressed in microbiota depleted mice	
TMAO increases cholesterol laden foam cell formation	
Intestinal microbiota is needed to produce TMAO and accelerate atherosclerosis	Koeth, 2013 [114]
Dietary choline and elevated TMAO has a direct prothrombotic effect in humans	Zhu, 2016 [19]
Use of DMB to lower TMAO levels leads to reduced size of atherosclerotic lesions	Wang, 2015 [15]

CVD, cardiovascular disease; TMAO, trimethylamine-N-oxide; DMB, 3,3-dimethyl-1-butanol

Table 6:

Treatment Options

	Options:	Mechanism:
Dysbiosis	Prebiotics, probiotics	Ingestion of live organisms or supplements to induce growth of certain commensal anaerobes
	Antibiotics	Depletion of pathogenic microbiota leading to overgrowth of healthy gut flora
	Fecal microbial transplant	Transplantation of donor fecal samples
LPS	C11C1	Monoclonal antibody that blocks LPS binding site
	CD14 antibodies	Lead to TLR4 internalization and decrease LPS response
	Statins	Lead to increase expression of LDL-R and increase clearance of LPS
	PCSK9	Inhibit degradation of LDL-R leading to increase LPS clearance
	IL-1B inhibitors	Block downstream TLR4 pathway leading to decrease in CVD
TMAO	FMO inhibitors	Block conversion of TMA into TMAO
	DMB	Inhibit microbial TMA lyases to inhibit production of TMA
	CutC/D	Target microbial proteins

C11C1, HMW Kininogen antibody; LPS, lipopolysaccharides; PCSK9, proprotein convertase subtilisin/ kexin type 9; LDL-R, low density lipoprotein receptor; IL, interleukin; TLR4,toll-like receptor 4; CVD, cardiovascular disease; FMO, Flavin monooxygenases; DMB, 3,3-dimethyl-1-butanol;