

**Low-grade endotoxemia and platelets: a deadly aggregation**

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Acute thrombotic complications of atherosclerosis such as myocardial infarction and ischemic stroke are major causes of morbidity and mortality worldwide despite considerable therapeutic advances.<sup>1</sup> The formation of an arterial thrombus is a complex pathological process originating from endothelial erosion or plaque rupture and involving platelet activation, adhesion and aggregation.<sup>2</sup> The labile platelet clot is then stabilized via deposition of insoluble fibrin as a result of tissue factor-mediated activation of the coagulation cascade.<sup>3</sup> Lastly, fibrinolysis is triggered to further remodel the arterial thrombus eventually leading to complete degradation of fibrin polymers, clot dissolution and restoration of the anti-thrombotic homeostasis. As such, thrombus stability has recently emerged as an important mediator of the clinical sequelae of thrombosis, determining the extent of downstream tissue damage and irreversible organ dysfunction.<sup>4</sup>

Thrombotic events generally punctuate the chronic course of atherosclerosis. Beside its role as marker of disease activity, chronic low-grade inflammation has been shown to significantly contribute to the pathophysiology of cardiovascular (CV) disease up to the point of defining a “residual inflammatory risk”, distinct from the previously defined “residual cholesterol risk” and assessed by circulating levels of high sensitivity C-reactive protein (CRP) as opposed to lipoprotein particles.<sup>5, 6</sup> Non-resolving inflammation and atherothrombosis share common risk factors such as chronic infection, obesity and ageing (i.e. inflamm-aging), which are characterized by endotoxemia, namely increased levels of circulating endotoxin lipopolysaccharide (LPS).<sup>7, 8</sup> LPS is a constituent of the outer cellular membrane of most gram negative bacteria which can enter the systemic circulation when mucosal barriers, predominantly in the gut or airways, become injured or dysfunctional. In the blood

stream, LPS binds to carrier molecules such as LPS-binding protein (LBP) or serum lipoproteins and triggers toll-like receptor 4 (TLR4) on different cell membranes through its co-receptor CD14.<sup>9</sup> TLR4 activation leads to recruitment of different adaptor proteins (such as MyD88 and TIRAP), ultimately triggering an inflammatory response through NF- $\kappa$ B- and IRF3-dependent transcription of pro-inflammatory cytokines and induction of procoagulant proteins, including tissue factor (TF), factor VIII, urokinase-type plasminogen activator (uPA), and plasminogen activator inhibitor-1 (PAI-1). Experimental and clinical evidence indicates that low-grade endotoxemia is associated with chronic inflammatory disease such as atherosclerosis,<sup>9</sup> and has been linked to arterial thrombosis through induction of pro-coagulants by macrophages and endothelial cells or platelet activation.<sup>10, 11</sup> Although a link between chronic inflammation and atherosclerosis is well established, whether low-grade endotoxemia plays a causative role in thrombus formation at the site of an unstable coronary plaque, is unclear.

This hypothesis has been explored by Carnevale *et al.* in their translational work entitled “Low-grade endotoxemia enhances artery thrombus growth via toll-like receptor 4. Implication for Myocardial Infarction” in this issue of the *European Heart Journal*, in which the authors explore the complex interplay between chronic inflammation and thrombosis in the context of CV disease. The current study builds on previous work from the same group showing that LPS from *E. Coli* localizes in human atherosclerotic plaque but not in normal arteries and may trigger inflammation via interaction with TLR4.<sup>12</sup> Here the authors assessed circulating levels of inflammation, pro-coagulants, LPS, soluble platelet-selectin (sP-selectin) and zonulin -a marker of gut permeability- in a cohort of patients with ST-elevation myocardial

infarction (STEMI), stable angina (SA) and in controls without overt CV disease. Specifically, higher levels of white blood cells, hs-CRP, oxLDL, TF, proinflammatory cytokines [tumor necrosis factor (TNF) $\alpha$  and interleukin (IL)-1 $\beta$ ], LPS, sP-selectin and zonulin were found in patients with STEMI compared to patients with SA and controls. Furthermore, LPS was found to positively correlate with these markers in the whole cohort suggesting common regulatory mechanisms. Analysis of coronary thrombi from STEMI and intracoronary blood from SA patients yielded similar results. Again, samples from STEMI patients showed higher levels of LPS, sP-selectin, TF and pro-inflammatory cytokines compared to those from SA patients. Of note, STEMI patients with raised zonulin level, indicating a highly permeable intestinal mucosa, showed higher levels of endotoxemia and greater platelet activation. With immunohistochemistry, fresh thrombi from STEMI patients showed positivity for monocytes, TLR4 and cathepsin G, a serine protease released by neutrophils upon TLR4 stimulation, which plays a role in platelet aggregation. On the other hand, only 25% of thrombi were positive for *E.coli*-LPS. To gain further mechanistic insight, supporting *in vitro* studies exposed human cells to LPS at the concentration detected in coronary thrombi and showed that this stimulated polymorphonuclear leukocyte/platelet aggregation in a dose-dependent manner, via TLR4 and cathepsin G. To further investigate a causative role of LPS in thrombosis, in an *in vivo* mouse model of arterial thrombosis (i.e. wire injury), administration of LPS to achieve circulating levels similar to those observed in STEMI patients resulted in enhanced thrombus formation and platelet activation, and these effects were blunted by treatment with a TLR4 inhibitor. The authors conclude that low-grade endotoxemia, resulting from increased gut permeability, contributes to thrombus formation via

increased platelet activation through TLR4 and cathepsin G at the site of arterial damage.

Although the results point towards enhanced platelet aggregation as a mechanistic explanation for endotoxemia-related increased thrombogenicity, platelet-independent mechanisms of LPS-mediated thrombus formation cannot be excluded. Together with platelet activation, coagulation and thrombolytic cascades critically regulate the formation of an arterial thrombus.<sup>2</sup> As acknowledged by the authors, LPS may directly induce endothelial expression of TF through TLR4-mediated NF- $\kappa$ B nuclear translocation thus enhancing the extrinsic pathway of the coagulation cascade.<sup>13</sup> Furthermore, LPS may exert a leukocyte-mediated prothrombotic response, through NF- $\kappa$ B activation, resulting in the induction of adhesion molecules potentiating leukocyte transmigration and thrombotic potential, with expression of tissue factor and the formation of neutrophil extracellular traps (NETs), which in addition to antibacterial functions, also induce a strong coagulant response and enhance thrombus stability.<sup>14, 15</sup> Impacting on thrombus stability, the fibrinolytic pathway is increasingly recognized as an important mediator of arterial thrombus formation and therapeutic target in the context of CV disease. In this sense, LPS have been shown to induce the expression of the fibrinolysis inhibitor PAI-1 in endothelial cells directly in an IKK $\beta$ /NF- $\kappa$ B -dependent manner or indirectly through cathepsin G.<sup>16-18</sup> Whether the observed effect of LPS on thrombus formation might be partially due to its effect on fibrinolysis deserves further investigation.<sup>19</sup>

The study by Carnevale and co-authors adds to the growing body of evidence that intestinal microbiota may be an important modulator of CV disease. The myriad of

microbial cells hosted in the human intestine produce different physiological and pathogenic mediators which may directly impact on CV function. Amongst these, trimethylamine N-oxide (TMAO) is one of the best characterized and has been linked to atherosclerosis and acute CV events through clinical association and experimental evidence.<sup>20</sup> The paper in this issue of the *European Heart Journal* supports the link between gut microbes and CV events, with TLR4 and cathepsin G mediating the enhanced thrombotic status during low-grade endotoxemia. Further studies are needed to confirm the applicability and effectiveness of therapeutic approaches targeting TLR4 signalling to reduce CV disease burden. In this sense, TLR4 genetic or pharmacological inhibition has shown preclinical efficacy in ameliorating several inflammatory-driven models of disease.<sup>21</sup> Furthermore, some inhibitors have shown good safety profiles in man and are now in phase 2 and 3 clinical investigation. Specifically, eritoran -a TLR4 antagonist- after showing no additional effect on sepsis-related mortality,<sup>22</sup> is being tested in diabetic and obese individuals to reduce chronic low-grade inflammation and improve glucose metabolism [NCT02267317]. Additionally, NI-0101, an anti-TLR4 antibody, is currently being tested in patients with rheumatoid arthritis as an adjuvant to methotrexate [NCT03241108]. Of note, TLR4 is a promiscuous receptor mediating the effect of several damage-associated molecular patterns (DAMPs) beside LPS, most of those with proven pathogenic role in the development of CV disease (e.g. heat shock proteins, oxLDL and fibrinogen).<sup>21</sup> In this sense, the paper published in this issue of the *European Heart Journal* further highlights the need for clinical studies to specifically investigate the effect of TLR4 inhibition in patients with residual inflammatory risk to reduce acute CV events.

The study by Carnevale *et al.* reinforces the link between intestinal microbiota and CV disease and points toward modulation of microbiota, the regulation of gut permeability or TLR4 inhibition as potential strategies for CV disease prevention. Further insights into the mechanisms linking low-grade endotoxemia with arterial thrombosis may help identify novel targets to prevent and treat cardiovascular and cerebrovascular disease.

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## Figure legend

**Figure 1: Novel insights into the relationship between endotoxemia and thrombosis.** Mucosal barrier impairment in the intestine allows *E.coli*-derived LPS to leak into the bloodstream (i.e. endotoxemia). LBP-carried LPS reaches the coronary circulation where it triggers TLR4-mediated cathepsin G release by neutrophils. Then, cathepsin G enhances platelet aggregation and aggravates atherothrombosis. LBP: lipopolysaccharide-binding protein; LPS: lipopolysaccharide; TLR: toll-like receptor.