REVIEW ARTICLE

ENDOTHELIAL ACTIVATION: THE BASIS FOR THE INITIAL ASSOCIATION BETWEEN CHRONIC PERIODONTITIS AND CARDIOVASCULAR DISEASE

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ABSTRACT

A significant association between chronic periodontitis and cardiovascular disease has been described in various meta-analysis studies. Various potential molecular mechanisms linking chronic periodontitis and subclinical atherosclerosis has been studied, and although no direct causal relationship has been proven, a possible associated increased risk of cardiovascular disease in periodontally affected patients has been described. Bacteraemias, endotoxemias, systemic inflammatory mediators, reactive oxygen species and acute phase reactants generated by chronic periodontitis has been shown to be statistically associated with endothelial activation. Interactions between these various pathognomonic elements of chronic periodontitis and vascular endothelial cells may cause a shift from normal endothelial function to that of activation, including a proinflammatory and prothrombotic state of the endothelium. An updated summary and illustration of these potential interactions is given whereby an overall and improved understanding by both the dental and medical practitioner of these mechanisms will positively influence the comprehensive clinical care of especially periodontally diseased patients who may also be experiencing an increased risk of cardiovascular disease.

Key Words: *endothelial activation, cardiovascular disease, periodontal disease, risk factors, biological plausibility.*

INTRODUCTION

There is much heterogeneity in various studies regarding the association between chronic periodontitis (CP) and cardiovascular disease (CVD), however, epidemiological meta-analysis studies have suggested a modest but significant association, that is independent on the effects of confounders.[1,2,3] These studies however, do not support a causative relationship.[4] CP is a multifactorial disease, comprising elements such as the genetic background of patients, the presence of pathogenic bacteria, the activation of both innate and acquired immunity, including autoimmunity, as well as an increase in locally produced and systemic oxidative stress.[5,6] The resultant systemic inflammation generated by CP which includes increased circulating levels of inflammatory mediators and reactive oxygen species (ROS), has also been shown to be statistically associated with endothelial dysfunction (ED).[4,6,7] Experiments in animal models, as well as *in vitro* and *in vivo* studies have described the plausibility of potential molecular mechanisms linking CP and subclinical atherosclerosis[8,9], thereby also indicating a possible associated increased risk of CVD.[10] Normal physiological vascular endothelial function includes the regulation of various processes, such as the response to infection and sepsis, coagulation and fibrinolysis, control of vessel lumen and alteration of blood flow, wound healing, neutrophil recruitment, inflammatory cell adhesion, the generation of cytokines and ROS.[11,12,13] ED, which may be present long before the occurrence of atherosclerotic CVD, predisposes the vascular endothelium to various pathologies[14], whereby ED has been described to include a functional shift from normal endothelial function, towards a proinflammatory and prothrombotic state of the endothelium, i.e. endothelial activation[13], this then being considered as the initial step in the process of atherosclerosis[12]. It is therefore the purpose of this narrative review to illustrate, and to give an updated general summary of the potential various interactions between the pathognomonic elements of CP and vascular endothelial cells (EC); these interactions thus comprising the initial manifestations of ED. By doing so, a supplementary and overall understanding of these potential interactions is envisaged for both the general dental and medical practitioner, thereby facilitating the comprehensive clinical care of especially periodontally diseased patients who may also be experiencing an increased risk of CVD.

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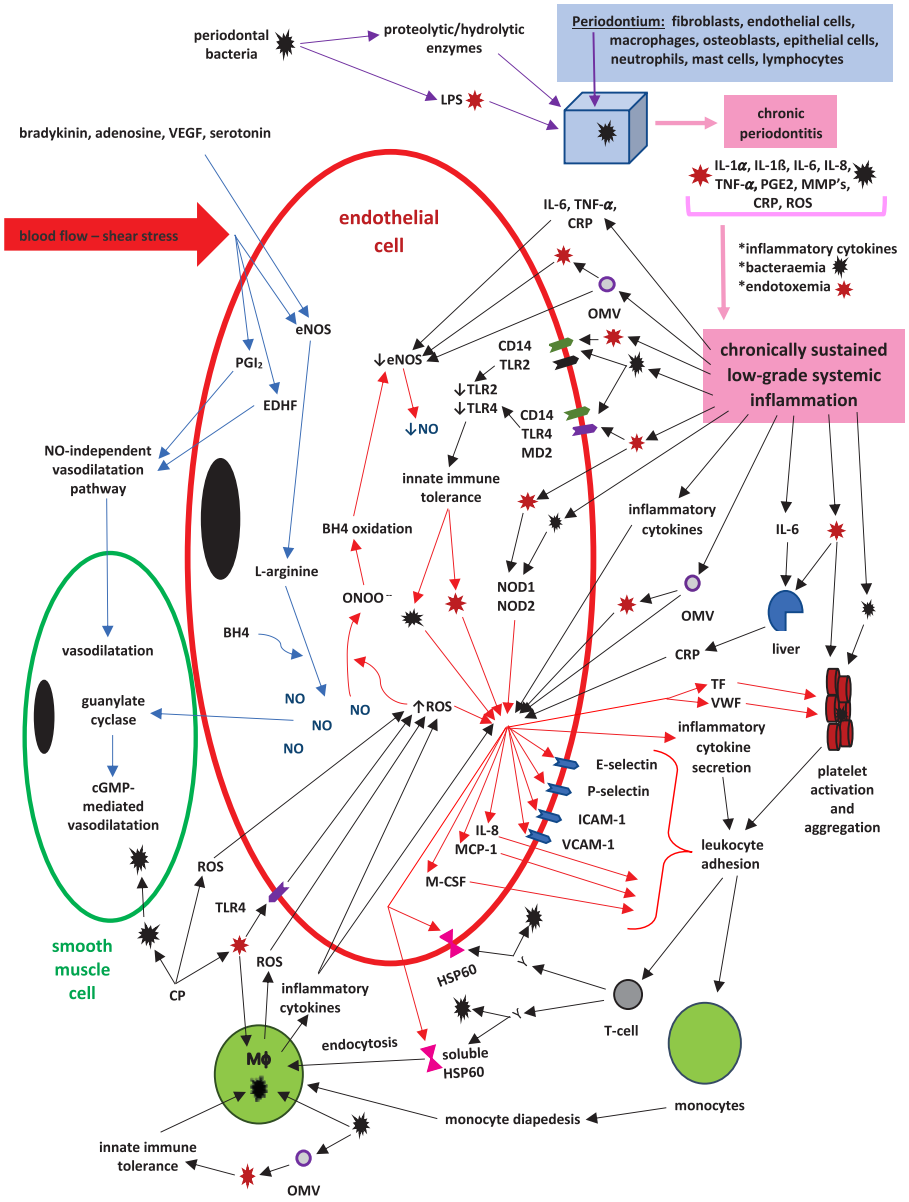
Endothelial function: normal vascular homeostasis: *(See Fig 1)*

Endothelium is a key regulator of vascular homeostasis and responds to physical and chemical stimuli by producing factors that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell (SMC) proliferation, and vessel wall inflammation.[15] Long-term organ perfusion necessitates tissue metabolic and oxygen supply, this being affected by endothelium-derived regulation of vascular tone and vasomotion.[16] ECs modulate vasomotion by means of releasing vasodilator and vasoconstrictor agents. Laminar shear stress induced by blood flow activates



= periodontal bacteria, = lipopolysaccharides **(LPS)**, = outer membrane vesicles **(OMV)**, **BH4** = tetrahydrobiopterin, **CD14** = CD14 receptor, **CP** = chronic periodontitis, **CRP** = C-reactive protein, **EDHF** = endothelium-derived hyperpolarizing factor, **eNOS** = endothelial nitric oxide synthase, **HSP60** = heat-shock protein 60, **ICAM-1**, intercellular adhesion molecule -1, **Mϕ** = macrophage, **MCP-1** = monocyte chemoattractant protein-1, **M-CSF** = macrophage colony stimulating factor, **MD2** = accessory protein, **NO** = nitric oxide, **NOD** = nucleotide-binding oligomerization domain, **$ONOO^-$** = peroxynitrite, **PGI_2** = prostacyclin, **ROS** = reactive oxygen species, **TF** = tissue factor, **TLR** = Toll-like receptor, **VCAM-1** = vascular cell adhesion molecule-1, **VEGF** = vascular endothelial growth factor, **VWF** = von Willebrand factor

Fig 1: Interaction of chronic periodontal disease with endothelial cells

endothelial nitric oxide synthase (eNOS) in the presence of cofactors, such as tetrahydrobiopterin (BH4) which acts upon L-arginine, to produce nitric oxide (NO).[17] eNOS is also activated by bradykinin, adenosine, vascular endothelial growth factor (VEGF) and serotonin.[18] NO is the principal regulator of vasodilatation.[19] NO diffuses to the vascular SMCs in the medial layer of the vascular wall and activates guanylate cyclase, which leads to cGMP-mediated vasodilatation.[20] Hyperpolarization of vascular SMCs is also mediated by NO-independent pathways, namely by means of the release of endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2).[15] Important functions of NO include the inhibition of SMC proliferation and platelet activation[15,21], and inhibiting the expression of adhesion molecules that mediate leucocyte attachment (anti-inflammatory).[21] NO also has a direct effect on leucocytes by preventing their activation to motile forms, thus inhibiting the diapedesis of these cells into the tissues.[19] Locally acting vasoconstrictor agents involved in the endothelium modulation of vasomotion include endothelin-1, prostanoids and the conversion of angiotensin I to angiotensin II.[21,22] Normal vascular homeostasis (endothelial function) is thus regarded as the vascular wall being in a state of quiescence, involving the predominant NO-mediated silencing of cellular processes including the inhibition of inflammation, cellular proliferation and thrombosis.[15,23]

Chronic periodontitis and chronic low-grade systemic inflammation: *(See Fig 1)*

CP is characterized by the infection and invasion of bacteria into the periodontal tissues, with the accompanying release of proteolytic enzymes and lipopolysaccharides (LPS) into the periodontal tissues. Resident cells in the periodontium such as fibroblasts, endothelial cells, osteoclasts, epithelial cells, neutrophils, macrophages, lymphocytes and mast cells consequently react to the bacterial invasion and their products, by releasing various pro-inflammatory products[24]. This host-induced expression of pro-inflammatory factors, such as interleukin-1-alpha (IL-1α) and interleukin-1-beta (IL-1β), IL-6, IL-8, tumor necrosis factor-alpha (TNF-α), prostanoids [prostaglandin E2 (PGE2)], and matrix metalloproteinases (MMPs), orchestrates host-mediated bone resorption and periodontal tissue destruction.[25] Some individuals may harbor a hyper-inflammatory monocyte phenotype, resulting in the release of an abnormally high amount of pro-inflammatory mediators, especially when stimulated by bacterial LPS.[26]

TABLE 1: CHRONIC PERIODONTITIS AND ASSOCIATED BACTEREMIA AND ENDOTOXEMIA

- Tissue destruction causes interruption of the oral sulcular epithelium in the periodontal pocket, resulting in contact between invading periodontal pathogens and the adjacent microvessels.[30]
- The possibility therefore arises that periodontal microbes and inflammatory cytokines from within the infected tissues disseminate into the systemic circulation, causing a bacteremia, causing the induction and maintenance of inflammation at sites distant from the periodontium.[27,31]
- The gingival sulcus and the progressively deepening periodontal pocket is thus considered to be the major source and portal for entry of periodontal bacteria to the circulation.[32]
- The extent of the bacteremia depends on the magnitude of the tissue trauma, the bacterial density and the severity of local inflammation[33], as well as inflammatory cytokines having sufficient concentrations together with their preservation of bioactivity within the circulation.[34]
- Inflammatory mediators are present in higher concentrations in the systemic circulation of patients with CP than in those who are periodontally healthy.[34] Also, certain clinical identifiers of CP, such as increased probing depth, bleeding on probing, and clinical attachment loss, have been shown to be linked with ED.[35]
- The ensuing bacteremia and endotoxemia can elicit a state of chronic low-grade systemic inflammation whereby bacteria, endotoxins and accompanying inflammatory mediators can reach distant organs[21], thereby leading to bacterial attachment and invasion of various cells, including ECs and SMCs.[36] Studies have reported *P. gingivalis*-specific DNA to be present in inflammatory atherosclerotic plaques.[37]
- *P. gingivalis* can also reach distant sites by entering immune cells, such as monocytes/macrophages or dendritic cells in the diseased periodontium. *P. gingivalis* 40-kDa outer membrane proteins (OMP) are expressed on the surface of bacteria and are responsible for cell invasion and the survival of engulfed bacteria in macrophages.[38]
- *P. gingivalis* can bind to dendritic cell-specific intercellular adhesion molecules by means of their fimbriae proteins, to then become internalized and routed in large numbers to intracellular vesicles within dendritic cells.[39] These cells may then leave the inflamed tissues, enter the circulation, localize, and diapedese into the vascular intima at sites of activated vascular endothelium.[40]

TABLE 2: CHRONIC PERIODONTITIS AND OXIDATIVE STRESS

- The pathophysiological progression of CP is associated with an increased production of ROS contributing to oxidative stress.[28,41] Under physiological conditions, low concentrations of ROS production stimulate the growth of fibroblasts and epithelial cells, however, at higher concentrations it results in tissue injury.[42]
- Oxidative stress plays a central role in tissue damage caused during CP, either as a direct result of excess ROS activity/antioxidant deficiency, or indirectly as a result of the activation of redox-sensitive transcription factors, thereby creating a proinflammatory state.[42] This tissue destruction leads to over-production of lipid peroxides, inflammatory mediators, as well as oxidized proteins. These products further activate macrophages, neutrophils, and fibroblasts to generate more ROS, thus forming a vicious circle.[42]
- Studies have suggested that comparatively higher oxidative stress levels may be correlated with the presence of specific types of bacteria, such as *P. gingivalis, Aggregatibacter actinomycetemcomitans, Tannerella forsythia* and *Treponema denticola.*[43,44]
- LPS and DNA from periodontopathogens, via CD14 receptor and Toll-like receptor-4 (TLR-4), cause activation of both activating protein-1(AP-1) and nuclear factor kappa-β (NF-kβ) pathways in gingival fibroblasts, and the production of inflammatory cytokines. The activation of NF-kβ and AP-1 also causes the activation of osteoclasts, further increasing the concentration of MMPs, which ultimately results in periodontal tissue damage.[42]
- Bacterial cells and inflammatory cytokines in gingival tissues cause the recruitment and activation of hyper-responsive PMNs, and in response to TNF-α, primed PMNs undergo a respiratory burst, releasing superoxide anion ($O2^{-}$)[45], thereby speeding up the production of ROS.[42,46] Other studies have indicated an increased production of $O2^{-}$ in gingival crevicular fluid[47] and enhanced $O2^{-}$ production by PMNs in CP.[48]
- NOX4 [which is a nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase enzyme] has been detected in endothelial cells, gingival and periodontal ligament (PDL) fibroblasts, keratinocytes, and osteoclasts.[49,50,51] Physiological levels of NOX4-generated ROS in PDL cells are responsible for alveolar bone remodeling, maintenance and repair of the extracellular matrix, but are however upregulated in response to endoplasmic reticulum stress[52], shear stress[53] and hypoxia, or ischemia.[54] Cytokines, like transforming growth factor-β1 (TGF-β1), TNF-α and insulin-like growth factor-1 (IGF-1), also induce the upregulation of NOX4 expression.[48,49]
- Inflammation and inflammatory cytokines, cause tissue hypoxia, due to an increase in oxygen consumption by invading immune cells.[55] Hypoxic gingival sulci and diseased periodontal tissues favor proliferation of anaerobic *P. gingivalis,* as well as expose these tissues to *P. gingivalis* LPS.[56] The hypoxia and LPS stimulate upregulated NOX4 production in PDL fibroblasts, with subsequent production and accumulation of proinflammatory cytokines, as well as local ROS, especially $O2^{-}$ and hydrogen peroxide (H_2O_2), leading to the activation of macrophages, which secrete MMPs, thus causing tissue and alveolar bone destruction.[55,56]
- Regarding *P. gingivalis* invasiveness and virulence, the bacterium possesses its own protective antioxidants, for example, rubrerythrin, providing defense against the oxidative burst of the host.[57] Bacteria in the oral cavity and periodontal pockets may consume local tissue antioxidants and suppress ROS detoxification[58], including a significant and progressive reduction in catalase and superoxide dismutase.[59] This then enables the entry of ROS from the periodontal tissues into the bloodstream, inducing circulating oxidative stress.[58]
- Chronic low-grade systemic inflammation can also be associated with an attenuated total plasma antioxidant capacity in patients with severe CP[60,61], thereby attributing to the promotion of inflammation in the endothelial vascular wall.[21]

CP is a chronic pathologic inflammatory disorder, which induces chronic low-grade systemic inflammation.[7,21] This includes an associated bacteremia and endotoxemia[27], the production of reactive oxygen species[28] and acute phase reactants.[29] Many studies have been done on these aspects, and Tables 1, 2 and 3 summarize the findings of various authors on these abovementioned aspects, respectively.

Endothelial activation: *(See Figure 1)*

Endothelial activation represents a fundamental switch from a quiescent endothelial phenotype, which involves a NO-mediated silencing of cellular processes[15], towards one that involves a loss of NO bioactivity in

TABLE 3: PERIODONTAL DISEASE AND ACUTE PHASE REACTANTS

- C-reactive protein (CRP), which is an acute-phase protein, is primarily synthesized by hepatocytes[62,63], while the extrahepatic synthesis of CRP has also been reported in peripheral blood lymphocytes[64], most cell types in gingival connective tissues, including gingival epithelial cells, where it is constitutively expressed.[65]
- With active inflammation in periodontal tissues, the production of CRP in gingival tissues is associated with increased IL-6 activity, whereby the gingiva may constitute a local source of CRP, and thus partially contribute to CRP levels in gingival crevicular fluids, saliva and serum.[65] In the oral cavity, CRP has been detected in saliva[66] and gingival crevicular fluid.[67]
- The *in vivo* activities of CRP are both anti-inflammatory and pro-inflammatory, and CRP activates the classical complement cascade, thereby contributing to the clearance of bacteria and damaged cells in inflamed tissues and the bloodstream.[68] C5a generated by complement activation, together with bacterial LPS, acts in concert with inflammatory cytokines, such as IL-6 and/or IL-1ß, to promote the up-regulation of CRP gene expression.[69]
- The plasma levels of IL-6 is associated with the extent of periodontitis[70], and as a result of CP, the dissemination into the systemic circulation of elevated numbers of neutrophils, as well as LPS and IL-6 may occur[71], thereby inducing hepatic inflammation, also resulting in the production and release of CRP.[72] In hepatocytes, CRP is also induced principally at the transcriptional level by IL-6.[62,63]
- Studies have described an association between CP and CRP[73,74,75], whereby periodontal therapy decreases serum CRP levels.[76] Some studies have described that elevated CRP levels may indicate a pathological link between CP and atherosclerosis[77,78,79], whereby ED has been shown to be restored after periodontal therapy reduced initially increased levels of CRP.[78,79]
- Other studies have however not observed such an association[80] and have suggested that elevated CRP levels are merely markers of systemic inflammation in periodontitis patients.[34]
- The role of CRP in the development or progression of atherosclerosis is also however controversial.[81] CRP and IL-6 have been shown to be predictors of CVD development and can be actively involved in the progression of atherosclerotic diseases.[82,83] Clinical studies have shown that high levels of CRP directly impair endothelial function by causing a reduced capacity to activate eNOS mRNA, leading to a reduced production of NO.[77,84,85] Other studies have shown that a reduction in plasma levels of CRP can reduce the risk of CVD.[86]
- However, other studies have shown a lack of meeting some of the criteria to prove causality between CRP and CVD, whereby CRP has thus been described to be merely a bystander of CVD.[81]
- Another acute-phase reactant, namely fibrinogen, has been shown to be abnormally elevated during CP, which reduced to normal levels after periodontal therapy, suggesting that PD may increase the risk of CVD.[87] However, this still remains controversial, as other studies have shown no association between CP and increased levels of fibrinogen.[88]

the endothelial vessel wall, thus leading to an impairment of endothelium-dependent vasodilation, altered anticoagulant and anti-inflammatory properties of the endothelium, the impaired modulation of vascular growth, and the dysregulation of vascular remodeling.[89] This includes a host defense response, which includes the expression of chemokines, cytokines, and adhesion molecules for the purposes of interacting with leukocytes and platelets, as well as directing inflammation to specific tissues to remove bacterial pathogens.[90] Endothelial function is modulated by a balance of endothelium-derived vasodilators, especially NO, and ROS, whereas endothelial function can become impaired by an imbalance of the reduced production of NO and the increased production of ROS during oxidative stress.[91] Table 4 summarizes the studies by various authors regarding endothelial activation, including the decline in NO bioavailability, the normal functions of ROS in ECs, the effects of excessive ROS production, and the effects of ROS on endothelial barrier function.

In a bacteremia, periodontal pathogens evade clearance by immune cells through the mechanism of invasion of ECs. The vascular endothelium responds to infection by *P. gingivalis* and antigens by the production of cytokines, chemokines, and surface molecules that serve as a stimulus to drive immune cell localization and activation.[127] In addition to endothelial activation induced by chronic low-grade systemic inflammation,

TABLE 4: ENDOTHELIAL ACTIVATION

Normal functions of reactive oxygen species (ROS):

- ROS are produced in all aerobic cells, including vascular SMCs, endothelial cells and mononuclear cells[91], and are imperative for healthy cell function, acting as signaling molecules to regulate cell physiology.[92]
- ROS are produced by oxidase enzymes, including NADPH oxidase, xanthine oxidase, uncoupled eNOS, cyclooxygenase, glucose oxidase, lipooxygenase, and mitochondrial electron transport.[91]
- ROS include free radicals with potent oxidation ability, such as $O2^-$, hydroxyl radical (OH) and NO. ROS which are non-free radicals, but also have oxidation ability, are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$).[92]
- The primary ROS, $O2^-$, is mainly produced by NADPH-oxidase (NOX) proteins, at complexes I and III of the electron transport chain.[93] They catalyze the reduction of oxygen to O2– using NADPH as an electron donor93. Five NOX enzymes (NOX 1-5) have been identified.[94]
- There is a tonal level of production of ROS, and the subcytotoxic release of ROS is utilized as a method of communication between mitochondrial function and other cellular processes, so as to maintain homeostasis and to promote adaptation to stress.[92] ROS generate various cellular messengers and cofactors that regulate further downstream cellular activities, including protein kinases and phosphatases, and in addition, directly modulate the activity of downstream molecules.[95]
- Cellular processes regulated by ROS are cellular adaptation to hypoxia, the regulation of autophagy, as well as the regulation of innate as well as adaptive immune function, including early T-cell activation, antiviral, antibacterial, and antiparasitic responses.[96]
- ROS are essential for multiple TLR-initiated pathways, including inflammatory cytokine signaling through ROS pathways.[97,98] Intracellular inflammasomes which recognize microbial pathogen-associated molecular patterns (PAMP)s and endogenous damage-associated molecular patterns (DAMPs) require ROS for the induction of proteolytic processing and activation of pro-inflammatory cytokines IL-1β and IL-18.[99]
- ROS play a significant role in the differentiation of embryonic stem cells[92,100], as well as in the regulation of aging, whereby low levels of ROS activate stress responses that are beneficial and can extend an individual's lifespan.[92]

Decrease in NO bioavailability and endothelial activation:

- A decrease in NO bioavailability, either by means of a decrease in NO production and/or an increase in NO inactivation, can induce endothelial dysfunction.[91]
- A decline in NO bioavailability may be caused by the decreased expression of eNOS, a lack of substrate or cofactors for eNOS, as well as alterations of cellular signaling thereby causing disruption in eNOS activation.[101]
- eNOS can switch to generate ROS during endothelial activation, by means of eNOS uncoupling.[102] eNOS uncoupling occurs when the key cofactor BH4 is not present, resulting in $O2^-$ formation, or the generation of H_2O_2 if the substrate L-arginine is deficient.[102]
- Pro-inflammatory mediators such as TNF-α and IL-6, expressed in CP, reduce the endothelial production of eNOS.[21] TNF-α decreases the half-life of eNOS mRNA in ECs.[103]
- LPS and LPS-induced TNF-α production causes suppression of eNOS expression, by means of mitogen-activated protein kinases which decrease the half-life of eNOS mRNA, leading to the reduction of NO levels in ECs.[104,105]
- $O2^-$ can also react with NO to form ONOO–, a potent oxidant, which causes the oxidation of cofactor BH4, thereby resulting in a decrease in eNOS levels in ECs.[106]
- Increased levels of CRP also cause reduced eNOS mRNA in human aortic ECs.[77,107]

Reactive oxygen species and endothelial activation:

- The susceptibility of vascular cells to ROS is a function of the overall balance between the degree of oxidative stress and the antioxidant defense capability. Protective antioxidant mechanisms are complex and multifactorial, which scavenge ROS in the vasculature, resulting in the inhibition of NO degradation.[91]
- The excessive production of ROS, known as oxidative stress, can overwhelm endogenous antioxidant

defense mechanisms, which leads to the oxidization of biological macromolecules, DNA, protein, carbohydrates, and lipids, as well as accelerated NO degradation.[15,101,108]

- Endothelial ROS signaling may be initiated by the exposure to inflammatory cytokines and growth factors, and the interaction of the endothelium with leukocytes.[15]
- LPS binding to endothelial TLR4 promotes the expression and secretion of TNF-α through NF-κB activation, incorporating the atypical protein kinase C pathway[109,110], whereby TNF-α activates NADPH oxidase proteins, thus generating ROS in ECs[109].
- A study has shown a novel mechanism of early LPS-induced ROS generation in human umbilical ECs, this being independent of pro-inflammatory TNF-α synthesis and is accomplished by TLR4 and the direct activation of NADPH oxidase proteins, incorporating the phosphatidylinositol-3-kinase (PI3-K) pathway.[110]
- Another study has shown a direct interaction of TLR4 with NADPH oxidase proteins for LPS-mediated generation of ROS, via NF-κB activation.[111]
- ROS, such as H_2O_2, leads to the up-regulation of adhesion molecules such as P-selectin[112], intercellular adhesion molecules (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and chemotactic molecules like macrophage chemoattractant peptide-1 (MCP-1), thereby promoting leukocyte adhesion and extravasation.[21,113]
- EC oxidant stress also stimulates the production and extracellular release of platelet-activating factor (PAF) in ECs, which then binds to the EC surface, mediating PMN adhesion.[114]
- The adherence of primed PMNs to endothelium, together in response to TNF-α, causes the release of $O2^-$, whereby $O2^-$ can interact with endothelial NO, forming $ONOO^-$.[113,115]
- The recruitment of macrophages into the arterial wall, together with their activation and subsequent secretion of various ROS, furthermore leads to increased generation of localized vascular oxidative stress.[116]
- Activated leukocytes which are adherent to the endothelial cell surface are a major source of ROS. Therefore, at sites of vascular inflammation, the endothelium is exposed to high levels of ROS, as well as cytokines and chemokines, for prolonged periods of time.[113] Inflammatory cytokines, such as IL-1 and IFN-Y, and vasoactive peptides such as bradykinin, also cause $O2^-$ release by endothelial cells.[117]

Endothelial activation, ROS and endothelial barrier dysfunction:

- ROS are also implicated in endothelial barrier dysfunction, leading to an increase in vascular transendothelial permeability, for example, to albumin.[118,119]
- ROS cause vascular EC intercellular gap formation, cell shape change, and actin filament reorganization, leading to impaired cell-cell adhesion, adherens junctions and intercellular junctions.[119,120]
- ROS in ECs causes a rapid fall in cellular ATP levels[121,122], resulting from the inactivation of the glycolytic and mitochondrial pathways of ADP phosphorylation[119,122], thereby leading to actin microfilament disruption[119], and thus increased endothelial permeability.[121]
- The impairment in expression and organization of adherens and tight junctional proteins in response to ROS, is furthermore exacerbated by inflammatory mediators, such as histamine, TNF-α and IFN-Y.[123]
- Another study has indicated that systemic LPS and ROS, such as H_2O_2, increases intracellular oxidative stress in ECs, which then facilitates the expression and secretion of TGF-β1 and TGF-β2 in ECs, thereby inducing the conversion of ECs into myofibroblasts, together with an overexpression of extracellular matrix proteins and collagen type III. This conversion of ECs into myofibroblasts causes ECs to lose their cell-to-cell connection, thereby losing their capacity to function as a selectively permeable barrier, thus promoting increased filtration from the intravascular lumen.[124] H_2O_2 has also been shown to furthermore facilitate leukocyte transmigration.[125]
- Thus, an imbalance between the production of ROS and their effective removal by non-enzymatic and enzymatic antioxidant systems induces ED with alterations of vascular tone, increases in cell adhesion properties (leukocytes and platelet adhesion), an increase in vascular wall permeability as well as a pro-coagulant state.[126]

TABLE 5: BACTERIAL OUTER MEMBRANE VESICLES AND CP:

- *P. gingivalis* releases OMVs which are secreted portions of the bacterial outer membrane, containing constituents of the periplasm and cytoplasm.[128] As a naturally occurring process, bacteria have the capacity to modulate vesiculation, for example, the upregulation thereof by conditions that activate a stress response in bacteria, such as harsh antimicrobial environments.[129]
- OMVs facilitate interbacterial interactions such as promoting the growth of cocolonizing pathogens, biofilm formation and colonization, the promotion of quorum sensing, the elimination of competing bacterial strains, and the securing of the survival of mixed bacterial infective populations by actively destroying host defenses, as well as producing an environment that is resistant to antibiotics and antibacterials.[130,131,132]
- This may be accomplished by OMVs being involved in DNA transfer, cellular communication, and the delivery of virulence constituents including proteins, toxins, enzymes, LPS, muramic acid, fimbriae as well as other PAMPs.[129,133]
- OMVs are produced and situated proximally to host cells in the bacterial biofilm and can be present at sites disseminated from direct sites of bacterial colonization[128], and by this means deliver active toxins and proteases to degrade host cells.[134,135,136]
- OMVs from one species can contribute indirectly to the pathogenicity of another species, this being accomplished by the binding and depleting of complement in the adjacent environment.[137]
- *P. gingivalis* can influence bacterial cell-host cell associations, by enhancing the attachment and invasion of *T. forsythia* in periodontal epithelial cells.[138]
- Released OMVs cause increased inflammation, leading to exposure of host ECM proteins, and together with the upregulation of epithelial cell surface receptors on other bacteria, this may then become beneficial to the colonization by other strains.[137]
- Attachment to, and entry of OMVs into host cells can occur via a membrane fusion event, or via adhesin-receptor-mediation, whereby the receptors can be identical to those used by the bacteria themselves.[139]
- Subsequent to host cell internalization of OMVs via endocytoses, OMVs can mediate OM surface remodeling within phagosomes of the host cell. This contributes to the virulence of OMVs, whereby bacterial surface remodeling may inhibit fusion with lysosomes, as well as promote the remodeling of the bacterial surface to an intracellular replicative form.[140]
- OMVs play a major role in the export and activity of bacterial toxins. Active toxins can become enriched in vesicles, be associated with the exterior surface of vesicles, and be more active than the toxins alone.[141]
- For example, vesicle-associated external enterotoxin (LT), which is an adhesin responsible for vesicle interactions with host cells, can bind LPS, thus forming a bridge between vesicle-associated LPS and the host cell.[142] LT is thus not only toxic, but also causes the internalization of other bacterial components, including membrane proteins, periplasmic proteins, and endotoxin, into the host cell.[143]
- Furthermore, the host cell response to the delivery of vesicle-associated toxins can differ from that of soluble toxins, if the uptake of vesicle-associated toxins occurs during the uptake with OMVs.[144]
- Macrophages and dendritic cells which are activated by *P. gingivalis* OMVs, can increase the levels of surface major histocompatibility complex class II (MHC-II) receptor expression on their membranes, thereby activating CD4+ T-cells, including the increase of production and expression of proinflammatory mediators, such as TNF-α and IL-12.[145]
- The direct recognition, adherence and internalization of OMVs by epithelial cells, macrophages, endothelial and dendritic cells, followed by the processing of toxin components of vesicles, triggers an immediate innate and acquired host immune response, subsequently leading to the induction and modulation of inflammatory pathways and receptor expression.[129,145,146]

many studies have described the various pathognomonic elements of CP which includes the induction of chronic low-grade systemic inflammation, which interact with ECs, further inducing the activation of ECs. This includes periodontopathic bacteria and the activity of their outer membrane vesicles (OMV) (See

TABLE 6: BACTERIAL OUTER MEMBRANE VESICLES AND ENDOTHELIAL ACTIVATION:

- *P. gingivalis* OMVs induce and regulate an acute inflammatory cellular response, characterized by the accumulation of neutrophils in connective tissue. This cellular response is accomplished by the biosynthesis and expression of E-selectin and ICAM-1 by vascular ECs, together with the inhibition of IFN-γ.[147]
- Furthermore, OMV-induced suppression of eNOS expression, at both mRNA and protein levels has also been described, whereby virulence factors within OMVs, such as gingipains, fimbriae and LPS, can lead to reduced eNOS production.[146]
- LPS in OMVs are the most potent immune-stimulating component of OMVs[128] and have a higher biological activity than whole-bacterial cell LPS.[148]
- LPS is a main vesicular component involved in OMV-induced EC activation. LPS activates inflammatory factors such TNF-α, which then utilizes the NF-κB pathway, leading to a reduction of eNOS expression.[142] NF-κB is considered to be an integral regulator of the immune system. The activation of NF-κB leads to increased transcription of genes related to innate immunity and inflammatory responses.[149]
- LPS-mediated NF-κB translocation also leads to cytokine and adhesion protein synthesis, namely TNF-α, IL-8 and significantly increased levels of IL-6, as well as an increase in ICAM-1 and E-selectin expression.[129,146]
- IL-6 and TNF-α are responsible for the activation of inflammatory and ECs and facilitate the recruitment of leukocytes to activated ECs. TNF-α has been shown to activate the endothelium and can cause changes in endothelial permeability, as well as cause apoptosis.[150] IL-6 also initiates an effective endothelial response against an infection.[146]
- ICAM-1 is constitutively expressed at low levels by the endothelium, however following infection, it becomes up-regulated.[151] Inflammation in ECs can thus also be directly initiated by OMVs, independently of leucocytes, including both adhesion protein and cytokine expression, through the NF-κB pathway.[146]

TABLE 7: BACTERIAL FIMBRIAE AND INTERACTION WITH ENDOTHELIAL CELLS:

- The attachment to, as well as endothelial cell invasion by *P. gingivalis* is accomplished by attachment pilli, namely fimbriae, of which fimbrillin is a structural component of fimbriae.[127]
- Fimbriae interact with pattern recognition receptors (PRP)s namely Toll-like receptors (TLRs) TLR2 and TLR4 on ECs.[152] TLR2 and TLR4 monitor the extracellular environment and recognize PAMP of *P. gingivalis* (such as fimbriae), as well as LPS.[153] The innate immune signaling pathways used by *P. gingivalis* during ligation and activation of PRR Toll-like receptors depends on the host cell type and the bacterial PAMP.[154]
- Regarding the interaction of fimbriae of *P. gingivalis* with TLRs, both TLR2 and TLR4 do not bind fimbriae[155], however, a novel function for TLR2 has been suggested involving an inside-out signaling for integrin activation.[156]
- The fimbriae proteins of *P. gingivalis* initially bind to CD14 receptors, whereby CD14 functions as a co-receptor for TLR2.[157] This leads to the activation of TLR2, followed by signaling events through activated TLR2 and PI3K, leading to affinity up-regulation and activation of the ligand-binding capacity of β_2 integrins, namely CD11b/CD18, which are needed for the effective ligand binding of fimbriae.[156,157]
- The CD11b/CD18 receptor is the most prevalent integrin expressed by monocytes, neutrophils and endothelial cells[158], and the clustering of CD14, TLR2 and CD11b/CD18 innate immune receptors has been suggested to have a cooperative role in constituting central signal-transducing elements for the triggering of innate immunity[159], including pathogen recognition and cellular activation, this specifically by the fimbriae of *P. gingivalis.*[152]
- The pro-inflammatory effect of *P. gingivalis* fimbriae activating CD11b/CD18 has been suggested to occur by the fimbriae either in a bacterial-cell-associated form, or as free molecules shed from the bacterial cell surface, or as components of released OMVs.[160]

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- *P. gingivalis* and fimbriae as well as LPS have also been demonstrated to stimulate TLR2 surface expression on monocytes/macrophages[161], resulting in a strongly associated increase in TNF-α secretion.[162]
- Regarding the interaction with TLR4, *P. gingivalis* fimbriae activate ECs in a TLR4-dependent manner through the presence of MD2, which is an accessory protein.[163] Therefore, *P. gingivalis* fimbriae are involved in TLR2- and TLR4-dependent activation of ECs, as well as in the upregulation of adhesion molecules such as ICAM-1, VCAM-1, E-selectin, and P-selectin.[164]
- EC activation then induces the release of pro-inflammatory cytokines[152], including the recruitment of neutrophils and monocytes due to the upregulation of adhesion molecules.[165] CD11b/CD18 is a key mediator of leukocyte migration and interacts with ICAM-1[166], resulting in monocyte adhesion to arterial endothelium and transendothelial migration to atherosclerotic plaques.[166,167]
- *P. gingivalis* fimbriae activation of CD11b/CD18 may be further exploited by *P. gingivalis* to evade host immune defenses, by mediating CD11b-CD18-dependent interactions with β_1 integrins, thus enabling *P. gingivalis* to invade gingival epithelial cells and to replicate themselves[168], as well as by mediating CD11b/CD18-dependent down-regulatory signals that inhibit IL-12 production in monocytes/macrophages.[169]

TABLE 8: BACTERIAL INTERACTION WITH CYTOSOLIC PRR IN ECS AND LEUKOCYTES:

- Another class of PRRs are soluble cytosolic nucleotide-binding oligomerization domain (NOD1 and NOD2) proteins, which complement the host defense by providing an intracellular layer of surveillance. NOD1 and NOD2 are important for microbial recognition and host defense after stimulation of TLRs.[170]
- There is controversy in various studies whether NOD proteins directly bind to bacteria or their products.[153] However, NOD2 receptors have been shown to recognize structural patterns of bacteria, such as muramyl dipeptide.[171] It has further been speculated that the source of these structural proteins may be from the degradation of phagocytosed bacteria within phagocytic vacuole in macrophages, containing intracellular hydrolases that break down bacterial peptidoglycans.[172]
- Upon invasion of ECs by *P. gingivalis,* host genes may become regulated whereby an IL-1β response is induced, causing the expression of monocyte chemoattractant protein-1 (MCP-1) receptors, and the secretion of IL-8 chemokine, thereby increasing the adhesion of mononuclear leukocytes.[173]
- *P. gingivalis*-mediated stimulation of monocyte TLR expression sensitizes monocytes to microbial ligands, or other endogenous TLR ligands, resulting in an enhanced inflammatory response, thereby inducing the secretion of soluble inflammatory cytokines.[154]
- The ligation and activation of Toll-like as well as NOD1/NOD2 receptors initiates the activation of several transcription factors including NF-κB, resulting in the expression of inflammatory genes[174], with the subsequent induction of intracellular signaling in both ECs and macrophages. This triggers a proinflammatory response, including the secretion of proinflammatory cytokines.[169,175]
- This response in activated ECs also includes the upregulation of adhesion molecules, namely ICAM-1, VCAM-1, MCP-1, E-selectin, and P-selectin, as well as macrophage colony stimulating factor (M-CSF), thereby initiating the recruitment, attachment and diapedesis of monocytes.[155]

TABLE 9: PERIODONTAL BACTERIA, LPS AND INNATE IMMUNE TOLERANCE:

- Cell-mediated immunity, involving Th1 cells, is needed for the removal of intracellular pathogens, however, *P. gingivalis* LPS predominantly induces a Th2-mediated humoral response, thereby facilitating immune-deviation towards a non-clearing response, leading to pathogen persistence.[176]
- Studies have indicated that *P. gingivalis* can be found within autophagosomes and may use components of the autophagocytic pathway as a means to survive.[177]
- Studies have described a response by endothelial cells whereby a constant or repetitive low-level exposure to *P. gingivalis* bacteraemias causes a subsequent decrease in TLR expression levels, resulting in a muting of Toll-like receptor signaling.[153] Other studies have shown that low-level stimulation with LPS followed by the subsequent stimulation by LPS of the same PRR, namely TLR2, causes a reduction in TNF-α levels, thus inducing a state of innate immune tolerance, also described as endotoxin tolerance.[162]

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- Studies have also demonstrated that an initial exposure of LPS followed by a subsequent challenge causes a reduction of TLR2 and TLR4 mRNA levels. It was suggested that this reduction may be due to alterations in the transcription factors bound to the Toll-like receptor promoters, or due to epigenetical alterations in the Toll-like receptor gene, as well as the silencing of Toll-like receptor mRNA.[178] Thus, by implication the muting of the innate immune response by *P. gingivalis,* enabling it to evade the host immune response and to thrive in infected cells.[153]
- Bacterial persistence in dendritic cells, this being accomplished by an immunosuppressive mechanism entailing the muting of the T-helper type 1 inflammatory response, has been suggested to be by means of the minor fimbriae of *P. gingivalis* uncoupling dendritic cell maturation from the cytokine response within these cells.[179]
- Intracellular survival of *P. gingivalis* in macrophages has been shown to be by means of the manipulation of complement protein C3 and TLRs in macrophages.[180] *P. gingivalis* LPS has also been described to attenuate macrophage cytokine responses by means of complement protein C5a modulation of TLR4 signaling.[181]
- Also, *P. gingivalis* OMV components other than LPS, such as gingipains, have been shown to modulate the sensing of LPS by host cells[129,182], this being accomplished by decreasing the level of membrane-bound expression of CD14 on macrophage surfaces, as well as by binding and actively degrading soluble CD14, leading to the suppression of inflammatory phenotype macrophages, thus causing a decreased ability of inflammatory macrophages to trigger LPS-stimulated cytokine production.[129,182] A loss of CD14 has been shown to be prevalent in cases of CP.[129]
- Additionally, *P. gingivalis* OMV vesicles have been shown to degrade IgG, IgM, and complement factor C3, thus attenuating the host immune response.[129,183]

TABLE 10: ENDOTHELIAL CELLS AND PLATELET ACTIVATION:

Endothelial activation and platelet aggregation:

- Pro-inflammatory molecules, such as LPS, TNF-α, IL-1β, thromboxane A2, vascular endothelial growth factor, as well as vasoactive histamine, bradykinin, and thrombin, have been shown to activate ECs[184], leading to the subsequent expression of, among other factors, tissue factor (TF), von Willebrand factor (VWF) and P-selectin by endothelial cells. This leads to clot formation, platelet adhesion and aggregation, as well as the recruitment of leukocytes, respectively.[184]
- Platelet adhesion then initiates the expression of platelet adhesive receptors, such as αIIβ3, P-selectin and CD40 ligand; thereby initiating platelet binding to other platelets, to ECs, and to immune cells (monocytes, neutrophils, lymphocytes); and furthermore, the exocytosis of proinflammatory mediators, such as chemokines, cytokines, growth factors, and soluble CD40 (sCD40); and the expression of TF.[185]
- Activated platelets therefore have a major role in the activation and proliferation of the endothelium, by altering the chemotactic and adhesive properties of ECs, contributing also to a pro-coagulant state in CP.[186]

***P. gingivalis,* LPS and platelet aggregation:**

- Due to CP-induced bacteraemia, *Streptococcus mutans,* A. *actinomycetemcomitans, S. sanguinis, P. gingivalis,* and *T. denticola* have been shown to activate platelets[187] and may act synergistically to stimulate platelet adhesion at sites of endothelial activation or damage, thereby stimulating the migration of inflammatory cells, as well as thrombus formation.[184,188,189]
- A study has described *P. gingivalis* gingipains to process the expression of Hgp44 adhesins on the bacterial cell surface, which is also considered to be essential for platelet aggregation.[190]
- *P. gingivalis* vesicles containing gingipains have been shown to activate protease-activated receptors (PAR) in endothelial cells, causing the initiation of EC signals, inducing the expression of TF and VWF, thereby activating platelet aggregation.[185,191]

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- Furthermore, studies have shown that *P. gingivalis* gingipains are able to cleave PARs on the platelet surface.[192] PAR ligation on platelets leads to internal phospholipase C-β signaling, leading to the subsequent increase of intracellular free calcium, leading to shape change of platelets and thus platelet aggregation.[193]
- The secretion of RANTES (a chemokine which interacts with endothelial cells to allow for monocyte and T-cell adhesion), as well as macrophage migration inhibitory factor (MIF) and plasminogen activator inhibitor-1 (PAI-1) by platelets are however cleaved and/or modulated by proteolytic gingipains, thus allowing *P. gingivalis* to remain undetected by inflammatory cells during platelet activation.[193]
- Both *P. gingivalis* and LPS have been shown to engage TLR2 and TLR4 receptors on platelets.[184,194] Platelet TLR2 interaction with *P. gingivalis* is responsible for the formation of platelet–neutrophil aggregates in whole blood and subsequent proinflammatory reactions.[195] Platelet TLR4 interaction with LPS stimulates TNF-α release[196] and IL-1 synthesis[197], whereby TNF-α and IL-1 upregulate the production of TF and VWF in endothelial cells[184], thus suggesting a role for platelets in the innate response to bacteraemias.[185]
- *P. gingivalis* has also been shown to be localized not only on the surface between adherent platelets, but also to be present in engulfment vacuoles of aggregated platelets[190,198], thereby enabling them to replicate within platelets, as well as to sustain inflammation.[193]

TABLE 11. HEAT-SHOCK PROTEINS AND AUTOIMMUNITY:

- Heat-shock proteins (HSPs) are found in several intracellular compartments, including the nucleus, cytoplasm, endoplasmic reticulum, and mitochondria of endothelial cells.[199] HSPs function as mediators of protective pathways in stressful conditions affecting the arterial wall. This includes the principal function of protein folding and unfolding. Also, by modulating misfolded proteins, HSPs prevent their aggregation within the cell.[200] In addition to protein folding, HSP60 also has a role in the assembly of polypeptides, the transportation and chaperoning of proteins to various cellular locations, as well as protein translocation across membranes.[200,201]
- Under normal physiological conditions, HSP60 is not expressed on vascular EC surfaces. However, with the induction of stress to the EC surface by traditional risk factors for atherosclerosis, as well as infections, mechanical stress and changes in temperature, mitochondrial expressed HSP60 can become biochemically modified autologous HSP60, which is then translocated to the cytoplasm, to then be expressed on the cell surface of damaged or dying ECs.[202]
- These various stressors furthermore also induce the expression of adhesion molecules on the EC surface, including VCAM-1, ELAM-1 (endothelial-leukocyte adhesion molecule 1), and ICAM-1.[203] Autologous HSP60 itself, furthermore also induces E-selectin, VCAM-1, ICAM-1, and IL-6 production within the EC.[204]
- Thus, under conditions of stress, autologous HSP60 in ECs is transported into the cytosol and is finally expressed on the cell surface, and together with the upregulated expression of the abovementioned adhesion molecules, antigen recognition by HSP60-reactive T-cells is induced.[205]
- The immune system consequently mounts a physiological T-cell-mediated and humoral autoimmune response against the biochemically modified autologous HSP60[201], whereby it thus has been suggested that HSP60-reactive T-cells induce the initial EC activation in atherosclerosis, and that antibodies to HSP60 further accelerate and perpetuate the disease.[201] Therefore, the cell surface expression of autologous HSP60 may act as a danger signal for pre-existing anti-HSP60 immunity, thus establishing a HSP60-directed autoimmune pathogenesis in the initial events of atherosclerosis.[201,206]
- Besides being expressed on the EC surface, HSP60 can also be shed into the circulation in a soluble form[207], whereby they can function as potent activators of the innate immune system.[208] HSP60 has been shown to activate monocytes and macrophages via the TLR2/IL-1-receptor signaling pathway, inducing endocytosis by means of the LPS receptor CD14 and the p38 mitogen-activated protein kinase pathway.[209,210]
- The host with CP also acquires a cellular and humoral immunological response against bacterial HSP60 as a protective defense against invading periodontopathogens. However, the risk of cross-reactivity with autologous HSP60 expressed by stressed ECs can be increased[201], as bacterial HSP60 are homologous with host HSP60, and also have a strong immunogenic nature.[12] This homology is unrecognizable by the host T-cells, resulting in antibodies which are directed against the bacterial HSP60 to cross-react with HSP60 on ECs, thereby inducing autoimmune responses leading to ED with an ensuing inflammatory cascade.[12,201]

Table 5), the interaction of these OMV with ECs (See Table 6), the interaction of bacterial fimbriae with ECs (See Table 7), the bacterial interaction with cytosolic pathogen recognition receptors (PRR) in ECs as well as leukocytes[175] (See Table 8). *P. gingivalis* is an intracellular pathogen which evades recognition and uptake by neutrophils, infecting oral epithelial cells, fibroblasts, dendritic cells, macrophages and endothelial cells, where it survives and replicates, thereby inducing innate immune tolerance (See Table 9), leading to the persistence of the bacterium in these cells, as well as a proinflammatory response within these cells.[182] Furthermore, various studies on platelet activation and the development of autoimmunity associated with bacterial heat-shock proteins (HSP) which are integral to EC activation, are discussed in Tables 10 and 11 respectively.

CONCLUSIONS

The pathophysiological progression of CP is complex and multifactorial, whereby the dissemination into the systemic circulation of bacteria, endotoxins and inflammatory mediators can induce chronic, low-grade systemic inflammation which may induce and maintain inflammation at sites distant from the periodontium, such as the cardiovascular endothelium. The normal functioning of ECs may then become disrupted, whereby the inflammatory activation of ECs can further develop into the initial lesion of atherosclerosis. Although no causal relationship has been proven, many *in vivo* and *in vitro* studies have shown statistical associations between CP and EC activation, including a proinflammatory and prothrombotic state, thereby increasing the risk of CVD in periodontally diseased patients. Further studies in future may however investigate and clarify the scientific basis of a more descriptive causal relationship. Practitioners who have a thorough knowledge and understanding of the various potential interactions between CP and EC activation will be in an advantageous position to better serve their periodontally-affected patients, specifically from a CVD prophylactic point of view.

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