

REVIEW ARTICLE

Invasion of *Porphyromonas gingivalis* strains into vascular cells and tissue

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Porphyromonas gingivalis is considered a major pathogen in adult periodontitis and is also associated with multiple systemic diseases, for example, cardiovascular diseases. One of its most important virulence factors is invasion of host cells. The invasion process includes attachment, entry/internalization, trafficking, persistence, and exit. The present review discusses these processes related to *P. gingivalis* in cardiovascular cells and tissue. Although most *P. gingivalis* strains invade, the invasion capacity of strains and the mechanisms of invasion including intracellular trafficking among them differ. This is consistent with the fact that there are significant differences in the pathogenicity of *P. gingivalis* strains. *P. gingivalis* invasion mechanisms are also dependent on types of host cells. Although much is known about the invasion process of *P. gingivalis*, we still have little knowledge of its exit mechanisms. Nevertheless, it is intriguing that *P. gingivalis* can remain viable in human cardiovascular cells and atherosclerotic plaque and later exit and re-enter previously uninfected host cells.

Keywords: *P. gingivalis*; invasion; vascular cells; vascular tissue

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The invasion of cells and tissue (Fig. 1) (1) is an important virulence property for many bacteria including *Porphyromonas gingivalis* (2). *P. gingivalis* is considered to be a key pathogen in adult periodontitis and has been associated with a number of systemic diseases, such as cardiovascular diseases (e.g. 2, 3). Both periodontal diseases and cardiovascular diseases are common and constitute an enormous burden to the populations and health budgets of the western civilization. *P. gingivalis* can invade various cell types, some of which include epithelial, endothelial, and smooth muscle cells (4). Although oral epithelia probably are the primary colonization sites for *P. gingivalis*, multiple daily bacteremias such as through tooth brushing and chewing, especially in those with periodontitis, can facilitate the entry of this bacterium into the circulation and thus subsequent direct contact with distant tissues, for example, the large vessels. In fact, it has been estimated that individuals may have the equivalent of 3 h of bacteremia per day from oral bacteria (5). Evidence of the existence of *P. gingivalis* in atheromas includes not only the presence of specific bacterial DNA but also the demonstration of live *P. gingivalis* in these tissues (6). It has been suggested that invasion provides a

privileged niche where the bacterium has access to host proteins and iron. This enables persistence of *P. gingivalis* in the infected tissue sequestered from the surveillance of both the humoral and cellular immune response (7). Intracellular life can also provide bacterial protection against antibiotics.

Five stages of bacterial invasion have been identified: 1) attachment, 2) entry/internalization, 3) trafficking, 4) persistence, and 5) exit (2). Persistence is particularly important for pathogens causing chronic disease such as periodontitis. A number of studies have reported cell and tissue invasion by oral bacteria (2), but this concept was quite controversial and against the prevailing dogma when first reported (8).

Efforts to demonstrate that *P. gingivalis* can invade cardiovascular cells/tissue culminated with the report that human atherosclerotic plaque contains viable *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* cells (6). Additional support for their viability was the *in situ* presence of these organisms on the DNA level and by the prerequisite that they must be viable to be able to invade (8, 9).

It should be noted that other bacteria in the plaque microbiota such as *Fusobacterium nucleatum* and *Filifactor*

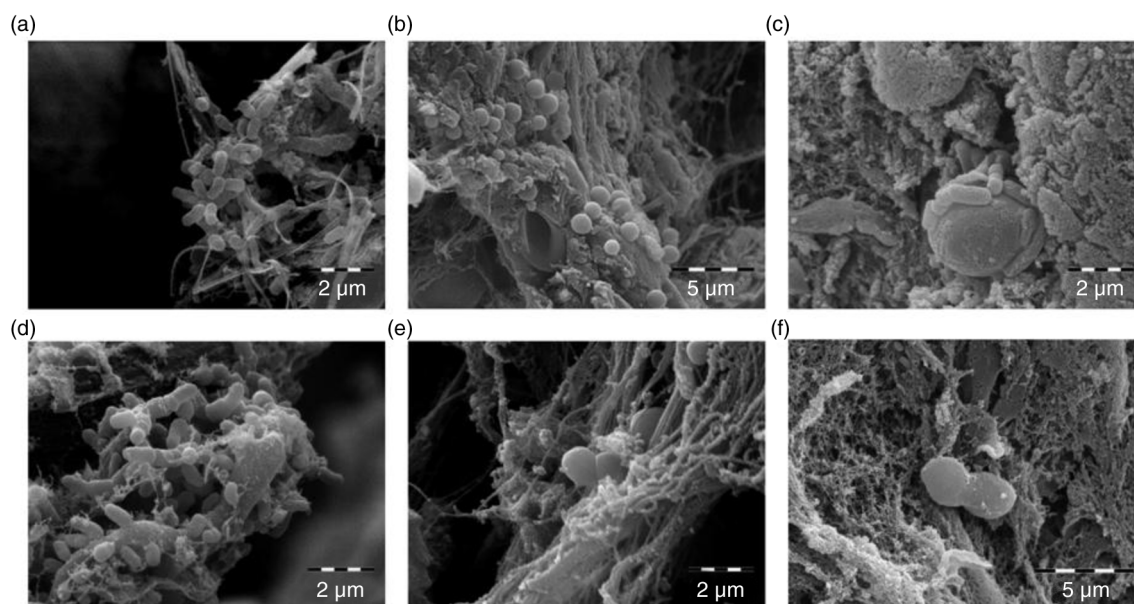


Fig. 1. Scanning electron micrographs of bacteria in vascular biopsies from patients with periodontitis. (a) From aneurysmal wall with bacteria in a meshwork of fibers. (b) Aneurysmal wall with bacteria in fibers and remnants of intravascular plaque. (c) Rods with remnants of intravascular plaque on aneurysmal wall. (d) Aneurysmal wall with bacteria in fibers and remnants of intravascular plaque. (e, f) Aneurysmal wall with coccus-shaped bacteria in fibers and remnants of intravascular plaque. From Armingohar et al. (1).

alocis can stimulate invasion of endothelial cells by *P. gingivalis* (10, 11). In addition, other oral bacteria can also invade vascular cells and tissue, for example, *Streptococcus mutans* invading human coronary artery endothelial cells (HCAEC) (12) and multiple oral and intestinal bacteria have been reported to invade aneurysmal walls (1, 13). *P. gingivalis*–epithelial cell interactions have previously been reviewed (14) as well as invasion of oral bacteria in general into vascular cells (e.g. 2, 4, 7). Consequently this review will be focused on the topic of mechanisms of *P. gingivalis* invasion of cardiovascular cell types and tissues and the significance of strain differences in relation to cardiovascular disease.

Adhesion

The process of bacterial invasion begins with adherence to the host cell surface which is a prerequisite for bacteria to enter the cells. A number of adhesins have been reported for *P. gingivalis* such as fimbriae and hemagglutinating adhesins that have been proposed as ligands for initial attachment to and subsequent invasion by *P. gingivalis* of host cells (15, 16). The importance of FimA in this regard was first established by the fact that strain FDC 381 was highly invasive in several cell types, but its mutant strain, DPG3, lacking FimA was unable to adhere to and invade these cell types (16, 17). In addition, it has been reported that non-fimbriated strains have a reduced capacity to invade host cells (15, 18, 19). The fimbrial genotypes Pg-II and to a lesser extent, Pg-I, are the most potent genotypes with regard to adhesive and cell-invasive capacities of

fimbriae (20–22). Gingipains also have a significant role in *P. gingivalis* adhesion (14) as do other adhesins including hemagglutinins (23) and glycolipid sugar chains (24). An association between an increased number of repeat domains in hemagglutinin A (HagA) and the invasive ability of *P. gingivalis* has also been reported (25).

Specific host cell receptors for *P. gingivalis* adhesins include β integrins (26), CD14, and β 2 integrins CD11b/CD182 (27, 28). However ICAM-1 appears to be the most significant receptor for *P. gingivalis* for some endothelial cells (Progulske-Fox, personal communication).

Entry/internalization

Once attached, *P. gingivalis* entry into endothelial cells is an active process that requires actin polymerization and protein synthesis in metabolically active host cells (29, 30). Once adhered, *P. gingivalis* internalizes via lipid rafts in the host cell's membrane (31). As mentioned previously, entry of *P. gingivalis* in oral cells is facilitated by interaction between fimbriae and β 1 integrin receptors (32), although the type of fimbriae cannot fully explain the relative and absolute values of adherence and invasion of cardiovascular endothelial cells (33). Cytoskeletal rearrangements are also required for entry of *P. gingivalis* into host cells (9, 26).

Rodrigues et al. (34) reported that W83 was highly invasive in HCAEC, despite the presence of a capsule but they also reported that another strain, A7463, had a capsule that could reduce invasiveness. For comparison, the capsule of *P. gingivalis* was thought to make it less

efficient in attaching to and therefore, invading gingival fibroblasts (35). Thus the presence or absence of a capsule does not appear to be an overriding determinate of invasiveness and the extent to which a capsule influences invasion of cardiovascular cells needs further investigation.

Trafficking

P. gingivalis trafficks through the autophagic pathway in cardiovascular cells, Fig. 2 (36) but it controls and usurps this pathway to its own advantage. *P. gingivalis* actually ‘sets up’ or signals the host cell to turn on autophagy prior to its entry, a process that is not yet well understood (37). Once *P. gingivalis* is localized within autophagosomes of HCAEC, 4 to 6 h post entry (Fig. 3), it prevents the final step of the pathway, fusion of a lysosome with the late autophagosome in which it resides. This fusion creates an autolysosome which results in the degradation of the contents of the original autophagosome but because *P. gingivalis* prevents this fusion, instead of being degraded, it resides in a novel replicating or persistence vesicle (7). It is interesting to speculate that this vesicle is ideal for persistence of *P. gingivalis* intracellularly because the vesicle is still tagged as an autophagosome and the cell would continue to deliver cellular proteins to the vesicle. The *P. gingivalis* proteases could then degrade the cellular proteins, providing the peptides and amino acids necessary for its survival.

This trafficking of *P. gingivalis* may depend to an extent on the concentration of invading bacteria or multiplicity of infection (MOI). For example, when *P. gingivalis* was cocultured with host cells at an MOI of 100, the bacteria

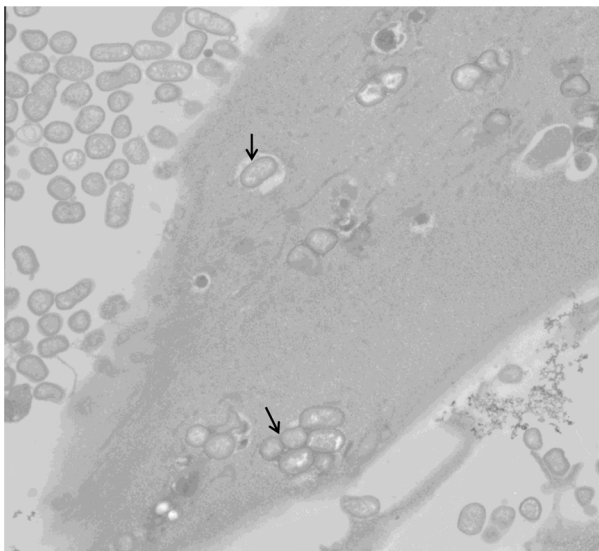


Fig. 2. Transmission electron micrograph of *P. gingivalis* strain W83 entering and inside HCAEC after 2.5 h of coinubation. Arrows indicate *P. gingivalis* inside autophagic vesicles. Adherent and bacteria in the midst of entry are also visible.

entered and trafficked through the autophagic pathway, whereas at an MOI of 1,000, the majority of *P. gingivalis* trafficked through the phagocytic pathway (31). However, this shifting of *P. gingivalis* to the phagocytic pathway could be explained as the result of the autophagic pathway being saturated at this especially high MOI.

Persistence

The intracellular survival of *P. gingivalis* in vascular cells can extend in time, again depending on strain (4). For example, strains W83 and A7436 remained viable up to 48 h, whereas strain 381 was cleared by 48 h and ATCC 33277^T by 24 h in HCAEC (35). These differences in persistence could be related to differences in intracellular trafficking. For example, although strains W83 and 381 trafficked via the autophagic pathway (36) in HCAEC and cardiovascular smooth muscle cells (SMC), strains A7436 and 33277 did not (35). Because most internalized 33277 were found within lysosome-associated membrane glycoprotein positive vacuoles, it was suggested that the internalized 33277 trafficked from the early endosome to the endosome/lysosome compartment (35). In contrast, internalized A7436 probably does not sort into either the late endosome/lysosomes or autophagosomes but is instead sorted out into the endocytic recycling pathway. Consequently, there is significant variation in mechanisms of trafficking and persistence among *P. gingivalis* strains and this likely determines their intracellular fate.

When internalized, *P. gingivalis* survived *in vitro* antibiotic treatment with metronidazole or amoxicillin alone or in combination (34). It was suggested that this ability to survive was promoted by activation of a variety of apoptotic pathways (38) and by regulating distinctive *P. gingivalis* proteins and genes (39).

When *P. gingivalis* was exposed directly to 100 µg/mL cotinine – an important tobacco substance – invasion of epithelial cells was significantly increased (40). However nicotine had no such effect. This is interesting considering that smoking significantly affects onset, progression, and outcome of periodontal disease but the mechanisms involved in this are unclear. The effects of cotinine on *P. gingivalis* invasion of cardiovascular cells have not yet been studied.

Exit

P. gingivalis exits epithelial cells through the endocytic recycling pathway (41) which likely allows the bacteria to exit from infected cells by a mechanism that allows them to invade neighboring cells via cell-to-cell spreading. The exit step itself remains largely undescribed except that inhibition experiments revealed that bacterial exit from human gingival epithelial cells is dependent upon actin polymerization, lipid rafts, and microtubule assembly (42). The involvement of the endocytic recycling pathway in *P. gingivalis* interactions with endothelial cells is

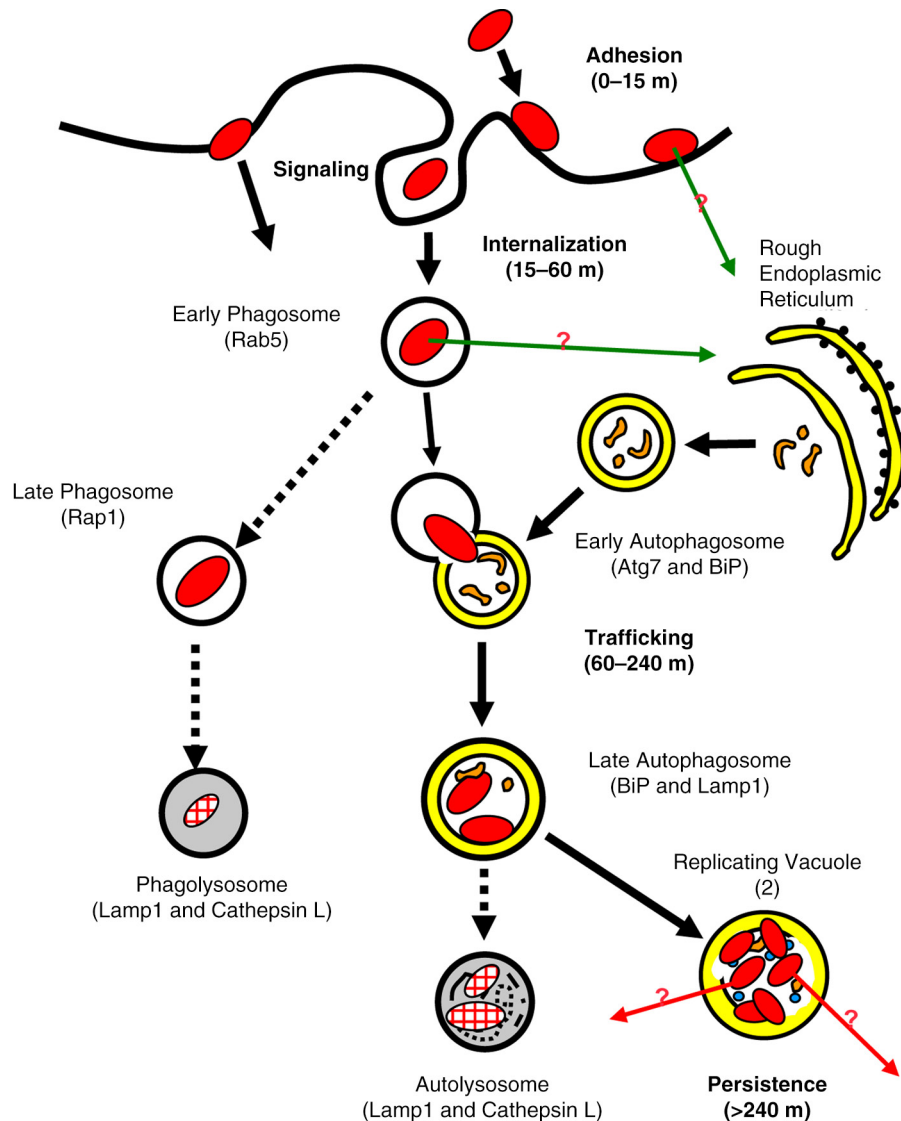


Fig. 3. Diagram of proposed entry, trafficking, and persistence of strains of *P. gingivalis* that utilize the autophagic pathway in HCAEC.

unknown (4), but recent data suggest that *P. gingivalis* rapidly exits oral endothelial cells via this pathway (Progulsk-Fox, personal communication). In addition, it has been reported that *P. gingivalis* that exited from endothelial cells could then infect SMC (4).

Transmission

P. gingivalis can directly transit from one cell to another *in vivo* (4, 43). Furthermore, it can transit between the same and even different cell types (4). Cell-to-cell contact between infected cells and host cells promotes the rate of transmission but is not required for transmission (4). This means that cell-to-cell transmission can transfer intracellular bacteria from dormancy to a cultivable state (4), but *P. gingivalis* can also exit into the milieu and then reinfect from the outside.

It is also possible that *P. gingivalis* can be reactivated from dormancy after internalization by phagocytes (44). Bacteria internalized in monocytes/macrophages or in dendritic cells can thus use a ‘Trojan horse’ approach to disseminate to endothelia where they can transit further to the lamina and tunica media due to extravasation of the carrier (7). It has been reported that *P. gingivalis* interferes with normal dendritic cell function, promoting a migratory immunosuppressive cell phenotype that supports spread of bacteria from focal periodontal lesions to gain access to the systemic circulation (45). It is also interesting that differentially regulated genes of *P. gingivalis* such as genes involved in protein synthesis, transcription, and energy metabolism were found to be down-regulated during the earlier stages of invasion (4). The hypothesis of reactivation of *P. gingivalis* cells was also supported

by the finding that *P. gingivalis* could be recovered from atherosclerotic tissue when cultured with fresh cells (6).

Interestingly, during a period of 48 h, the number and presence of intracellular *P. gingivalis* in vascular cells as viewed by microscopy did not change, although the recovery of viable bacteria from these cells on blood agar plates diminished significantly (4). However, *P. gingivalis* resident in vascular cells at the end of the 48-h period could be recovered as viable bacteria if exposed to fresh cells. It was thus suggested that *P. gingivalis* may persist intracellularly for an extended period of time in an uncultivable stage of dormancy. These dormant cells can then become activated, especially in phagocytic cells, which allow them to escape and infect new host cells. This could be a mechanism of chronicity and persistence in host tissues. Dormancy could also explain why antibiotics have failed to treat atherosclerosis (46).

Not all *P. gingivalis* that have entered host cells survive intracellularly and reinfect other cells. Survival time can be limited and depends on the number of internalized bacteria, available nutrients, and iron. Control of intracellular *P. gingivalis* may be achieved by the host cells by trafficking the bacteria to the lysosomal compartments for degradation. However, *P. gingivalis* controls, at least to some degree its own survival by escaping from the host and by infecting fresh host cells.

Strain differences

Strain differences in the bacterial world can influence virulence and *P. gingivalis* is no exception. This can involve both phenotypic and genetic characteristics. For example, as mentioned above, only certain strains of *P. gingivalis* usurp autophagy in cardiovascular endothelial cells and strains of this species vary in the number of direct repeats in the HagA adhesion (25).

Baek et al. (47) also reported interstrain variability when the invasion ability of *P. gingivalis* was assessed by flow cytometry. The invasion ability, but not the cytokine proteolytic activities, showed a strong positive correlation with clinical parameters of periodontitis among subjects harboring the isolates. Furthermore, when 20 different strains of *P. gingivalis* were examined for their capacity to invade cardiovascular cells, they could be divided into categories including high-, moderate-, and non-invaders (33).

When intergenic spacer region sequence analysis was used to type strains in clinical samples of *P. gingivalis*, 22 heteroduplex types were detected (48). The relatedness corresponded to that found on the basis of genome content (microarray analysis). This supported studies done previously (49). It was considered likely that the genomic differences identified accounted in part for the interstrain variation in disease-associated phenotype and virulence of *P. gingivalis*.

The invasive genotype of *P. gingivalis* was examined by Dolgilevich et al. (50) using comparative genomics. Their results indicated that more than 100 genes are absent from the genome of non-invading strains. The true degree of clonal genetic diversity in *P. gingivalis* was significant. The authors also detected putative invasion-associated genes in 9 of 11 tested isolates from patients, and *PG185* was identified as an invasion-related gene.

Suwannakul et al. (51) presented evidence that both highly invasive and poorly invasive subpopulations (bistable phenotypes) existed in cultures of two strains of *P. gingivalis* (NCTC11834 and W50) exhibiting different abilities to invade and/or to survive within an epithelial cell line and in normal keratinocytes. Not surprisingly, there was a difference in gene expression between the two subtypes. For example, the surface-associated protease enzyme complex Arg-gingipain was a key invasion factor preferentially expressed by the bistable phenotype. It is reasonable to expect that similar subpopulations exist with respect to invasion of cardiovascular cells as well.

Of clinical importance is that *P. gingivalis* fresh isolates demonstrated increased invasive capabilities with increasing probing depth and that these capabilities varied in strains from healthy and diseased periodontal sites (52). In support of this, the laboratory strain 381 showed a 1,000-fold greater capacity than the non-invasive strain AJW4 to invade epidermoid carcinoma cells (33). There were no reports on endothelial cells.

Genes and invasion of cardiovascular cells

Using microarray and RT-PCR analysis, Rodrigues et al. (53) identified 11 genes of W83 that were up-regulated and 52 that were down-regulated during invasion of HCAEC compared to W83 growing in culture. Among the up-regulated genes identified were several likely involved in intracellular trafficking and/or interaction with autophagosomal vesicles or other virulence functions. Down-regulated genes (21 of 52) included those whose functions were predicted to be involved in protein synthesis, transcription, and energy metabolism, indicating a limited but more specific metabolic activity in those *P. gingivalis* in the process of entering or trafficking within host cells. This study also reported that gene expression can differ between different phases of cell invasion and between different host cell types invaded.

In addition to *PG185* and others discussed above, *ClpB*, a component of the stress response, is significantly involved in cellular invasion by *P. gingivalis* (54) because a *clpB* mutant was found to be defective in trafficking and was more readily killed intracellularly in HCAEC.

The IVET system is a powerful tool in discovering virulence genes expressed *in vivo* and the function *ivi* genes have in the pathogenesis of *P. gingivalis* infection (55). Among the *in vivo* expressed genes identified, the *ivi10*

gene was found to have a significant role in adherence and invasion of *P. gingivalis*.

Invasion by outer membrane vesicles

Outer membrane vesicles (OMV) produced by Gram-negative bacteria are present both on the cell surface and in the surrounding milieu of the bacteria (56). Vesicles from *P. gingivalis*, because of their small size (50 to 250 nm in diameter), spread more readily in tissues than their larger parent cells (57). The vesicles, carrying a number of virulence factors, have been described as bacterial bombs, virulence bullets, and even decoys (58). They have also been suggested to act as long-range virulence factors that can protect their luminal cargo from extracellular proteases during transport to the receptor or target. OMV of *P. gingivalis* affect HCAEC and may even be involved in control of the host cell signaling involved in its autophagic pathway. For comparison, the invasive capacity of OMV from *P. gingivalis* W83, 33277 and their mutants was recently examined in human oral keratocytes (HOK) and human gingival fibroblasts (HGF) (59). Significantly more vesicles derived from strain 33277 than from W83 or mutant strains were internalized into HOK and HGF. In this case, FimA was not essential for the invasive capabilities of *P. gingivalis* but minor components of long fimbriae were. Therefore, pathogenicity of *P. gingivalis* vesicles may largely depend on expression of the *mfa* locus. OMV may thus be the/a mechanism by which *P. gingivalis* strain W83 signals the target host cell to turn on autophagy even prior to the adherence/entry of *P. gingivalis* into the cell (37).

Possible systemic effects post invasion

It is likely that different *P. gingivalis* strains induce different degrees of endothelial dysfunction. *P. gingivalis* 381 (fimbriae type I) induced fimbriae-dependent phenomena such as gene expression of GroA, GroE, IL-6, IL-8, the sequestering of vascular cell adhesion molecule (VCAM-1), and endothelial leukocyte adhesion molecule (ELAM-1) in HCAEC (60). The expression of these molecules is mediated through Toll-like receptors (TLR) (61). W83, which has a capsule but no fimbriae, activates TLR2 moderately and causes an attenuated inflammation response in HCAEC (35). The capsule-positive A7436 with type IV fimbriae also induced a moderate inflammatory response in HCAEC (2). Fimbriae are known to promote chemokine production in human aortic endothelial cells (HAEC) through actin cytoskeletal rearrangements as well as through the proinflammatory cytokines, IL-1 β , IL-8 and MCP-1 (62). Furthermore, expression of ICAM-1, VCAM-1, and P- and E-selectins were induced in human endothelial cells by *P. gingivalis* strains 381 and A7436, but not by a non-invasive non-fimbriated mutant (63). *P. gingivalis* strain 381 also induced apoptosis in

HAEC which is another mechanism of tissue damage in vasculopathies (64). Dying cells in atherosclerotic plaque tend to undergo necrosis which can contribute to plaque instability (65) and *P. gingivalis* induced apoptosis in the host's endothelium (64). Interestingly, the ability of the arterial cells to induce or inhibit autophagy is reported to be important in the progression of plaque (66, 67) so the fact that some strains of *P. gingivalis* usurp the host cell's autophagic pathway is likely significant in this regard. In addition to endothelial cells, *P. gingivalis* invasion also affects SMC in a prothrombotic (68) and a proliferative manner (69).

It is likely that additional mechanisms of *P. gingivalis* interactions with cardiovascular tissues can promote atherosclerosis as well. For example, W83 and A7436 accelerated atherosclerosis in ApoE null mice by inducing arterial plaque progression (70) and by causing a shift in the ACD lipid profile (71).

Although there are common mechanisms used by *P. gingivalis* to invade both oral and cardiovascular cells and tissues, it is clear that *P. gingivalis* as a species has a repertoire of invasive and intracellular trafficking mechanisms that are adaptable and specific to the host cell and tissue type. Thus the mechanism(s) of invasion of cardiovascular cells is quite different from the invasion of oral cells, even given the same cell type. Further complicating our understanding is the fact that strains of *P. gingivalis* differ widely with respect to mechanism and outcome of invasion of host cells. It is thus reasonable to suggest that certain strains of *P. gingivalis* are virulent or not with regard to a particular pathology and/or disease, depending on the bacterium's ability to invade and adapt to the particular intracellular environment.

Finally, we do not propose that all cases of atherosclerosis are mediated or related to *P. gingivalis*. There are many other bacterial species and infectious agents that can also be involved, and there are also non-infectious mechanisms of atherosclerosis development. However, given the incidence of atherosclerosis, if *P. gingivalis* is related to even a small percentage of the cases, the number of total cases would nevertheless be quite significant with regard to public health. As provided in this paper, we presently have some fundamental knowledge of the mechanisms and role of *P. gingivalis* invasion of cardiovascular cells and arterial-cardiovascular disease, primarily from *in vitro* studies, but there remain many questions to be answered. Furthermore, based on present knowledge, one cannot assume that what occurs or is a virulence factor in periodontal disease applies to *P. gingivalis* as a cardiovascular pathogen and *vice versa*.

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Conflict of interest and funding

There is no conflict of interest.

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