CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

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"Gum Bug, Leave My Heart Alone!"—Epidemiologic and Mechanistic Evidence Linking Periodontal Infections and Atherosclerosis

ABSTRACT

Evidence from epidemiologic studies suggests that periodontal infections are independently associated with subclinical and clinical atherosclerotic vascular disease. Although the strength of the reported associations is modest, the consistency of the data across diverse populations and a variety of exposure and outcome variables suggests that the findings are not spurious or attributable only to the effects of confounders. Analysis of limited data from interventional studies suggests that periodontal treatment generally results in favorable effects on subclinical markers of atherosclerosis, although such analysis also indicates considerable heterogeneity in responses. Experimental mechanistic *in vitro* and *in vivo* studies have established the plausibility of a link between periodontal infections and atherogenesis, and have identified biological pathways by which these effects may be mediated. However, the utilized models are mostly mono-infections of host cells by a limited number of 'model' periodontal pathogens, and therefore may not adequately portray human periodontitis as a polymicrobial, biofilm-mediated disease. Future research must identify *in vivo* pathways in humans that may (i) lead to periodontitis-induced atherogenesis, or (ii) result in treatment-induced reduction of atherosclerosis risk. Data from these studies will be essential for determining whether periodontal interventions have a role in the primary or secondary prevention of atherosclerosis.

KEY WORDS: periodontal, infection, atherosclerosis, epidemiology, mechanisms.

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INTRODUCTION

In 2005, Robin Warren and Barry Marshall won the Nobel Prize in Physiology or Medicine "for their discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease" (Pincock, 2005). Although the award was granted fairly recently, their original research dates back to the early 1980s (Warren and Marshall, 1983) and was becoming widely accepted by the mid-1990s (Thagard, 1998). On the heels of this novel hypothesis, research studies began to explore the possible causal role of infections in the pathophysiology of other chronic diseases. In 1989, two studies were published, almost simultaneously, that posited oral infection to have an etiologic role in cardiovascular disease (Mattila *et al.,* 1989; Syrjanen *et al.,* 1989). Since that time, a substantial body of literature has developed, the majority of which supports the oral infection hypothesis. However, several authors have appropriately highlighted the fact that while this research discipline has generated a large number of publications, the majority of these tend to be either original studies with low levels of evidence or reviews (Dietrich and Garcia, 2005). Our purpose with this review is to summarize the state of the science with special emphasis on recent findings from epidemiologic and basic science articles, to highlight areas where corroborating findings from both disciplines strengthen various mechanistic hypotheses, and to identify areas that need to be substantiated further by new research data. In so doing, we will primarily focus on studies related to atherosclerotic vascular disease (AVD) and particularly on coronary heart disease (CHD) and ischemic stroke, as well as on subclinical assessments of atherosclerosis and endothelial function.

OBSERVATIONAL STUDIES

Several earlier reviews have discussed the findings from the major epidemiologic studies (Meurman *et al*., 2004; Behle and Papapanou, 2006; Demmer and Desvarieux, 2006), and at least three meta-analyses have been published summarizing the association between periodontal disease and clinical cardiovascular outcomes (Janket *et al.,* 2003; Mustapha *et al*., 2007; Humphrey *et al*., 2008), consistently concluding that the available evidence suggests a moderate, positive association between periodontal diseases and AVD. Several recent publications support these conclusions and have extended previous research in important ways. Key findings from selected observational studies published between 2006 and 2009 exploring associations between periodontal disease and CHD or stroke are summarized in Table 1. Among these, two

Table 1. Summary of Selected Epidemiologic Observational Studies Exploring Associations between Periodontal Disease and Clinical CHD or Stroke between 2006 and 2009

Study	N	Country	Age Range	Design	Exposure	Outcome	Adjustments	Measure of Association (95% Confidence Interval)
Geismar *et al.*, 2006	250	Denmark	NA	Case-control	Radiographic	CHD	1,2,5,6	OR 2.0 (0.77, 5.08)
Briggs *et al.*, 2006	171	Ireland	40+	Case-control	PD	CHD	1–6,10	OR 3.06 (1.02–9.17)
Holmlund *et al.*, 2006	4254	Sweden	20–70	Cross-sectional	Tooth #	Self-reported	1,3,5	OR 0.80 (0.64, 0.96)
					Radiographic	CHD		2.69 (1.12, 6.46)
Rech *et al.*, 2007	114	Brazil	NR	Case-control	Periodontitis (clinical)	ACS	1,3,6	OR 4.5 (1.3, 15.6)
Rubenfire *et al.*, 2007	440	USA	NR	Case-control	Positive BANA test	ACS	1,3,5	OR In BANA+ participants 3.95 (1.61, 9.71)
Andriankaja *et al.*, 2007	1461	USA	35–69	Case-control	CAL	Non-fatal MI	1,3,5–8	OR 1.46 (1.36, 1.69)
Gotsman *et al.*, 2007	201	Israel	NR	Cross-sectional	CAL (% of teeth with CAL ≥ 5)	ACS	1,5,7,8	OR 1.03 (1.01, 1.04)
Nonnenmacher *et al.*, 2007	90	Germany	40–80	Case-control	CAL	CHD	1–3,5,9	OR 3.2 (1.2, 9.0)
Dietrich *et al.*, 2008*	1203	USA (NAS)	21–59	Cohort	Radiographic	CHD	1–10	HR 2.12 (1.26, 3.60)
		USA (NAS)	60–84	Cohort	Radiographic	CHD	1–10	HR 1.81 (NA)
Senba *et al.*, 2008	6816	Japan	< 66	Cross-sectional	Self-report periodontitis or tooth loss	CHD men	1–3,5,6,8,9	OR 1.51 (0.90, 2.52) 1.54 (0.90, 2.62)
Senba *et al.*, 2008	23,088	Japan	< 66	Cross-sectional	Self-report periodontitis	CHD women	1–3,6,8,9	OR 1.48 (0.95, 2.32) 1.68 (1.08, 2.61)
Lund Håheim *et al.*, 2008	1173	Norway	48–77	Case-control	IgG (*Aa*, *Pg*, *Td*, or *Tf*)	CHD	1–3,5–9	OR 1.31 (1.01, 1.69)
Pussinen *et al.*, 2007b	505	Finland	25–64	Nested case-control	IgA *Aa*	CHD & stroke	1–9	HR
					IgG *Aa*	combined		1.43 (0.88, 2.31)
					IgA *Pg*			1.64 (1.00, 2.69)
					IgG *Pg*			1.53 (0.95, 2.44)
								1.53 (0.93, 2.50)
Tu *et al.*, 2007	12,223	Scotland	≤ 30	Cohort	Tooth loss	Fatal CVD	1,3–5,8,9	HR
						Fatal CHD		1.35 (1.03, 1.77)
						Fatal stroke		1.19 (0.84, 1.69)
								1.64 (0.96, 2.80)
Syrjala *et al.*, 2009	392	Finland	75+	Cross-sectional	Tooth loss	Stroke & CHD combined	1–10	CPR (dendate *vs.* edentulous)*** 0.9 (0.5,1.8)
Pussinen *et al.*, 2007a	893	Finland	30–59	Nested case-control	IgA *Aa*	Stroke	1,2,4–10	OR 0.83 (0.62–1.10)
					IgG *Aa*			0.93 (0.66–1.32)
					IgA *Pg*			1.22 (0.91–1.65)
					IgG *Pg*			1.31 (0.97–1.76)

(continued)

Table 1. (continued)

Study	N	Country	Age Range	Design	Exposure	Outcome	Adjustments	Measure of Association (95% Confidence Interval)
Lee *et al.*, 2006	5123	USA (NHANES)	60+	Cross-sectional	Periodontal Health Status (PHS; a composite index of periodontitis and tooth loss)	Self-reported stroke	1,5,6,8,10,14	OR for PHS Class 2-5 *vs.* 1 1.34 (0.75, 2.38) 1.97 (0.85, 4.56) 1.99 (1.06, 3.71) 1.56 (0.95, 2.57)
Sim *et al.*, 2008	479	Korea	40–79	Case-control	CAL	Stroke**	1–6,8–10	OR 4.30 (2.27, 8.16)
Jimenez *et al.*, 2009	1137	USA (NAS)	27–84	Cohort	Radiographic	Stroke	1–10	HR 3.52 (1.59, 7.81)
Pradeep *et al.*, 2010	200	India	33–68	Case-control	PD	Stroke	1–3	OR 8.5 (1.1, 68.2)
You *et al.*, 2009	22,862	USA	45+	Cross-sectional	Tooth loss	Stroke**	1–8	OR 1.27 (1.09, 1.49)
Choe *et al.*, 2009	867,256	Korea	30+	Cohort	Tooth loss	Stroke**	1–3,5–10	HR 1.3 (1.2, 1.4)

Adjustments: 1, age; 2, race; 3, sex; 4, SES (income and/or education); 5, smoking status; 6, diabetes; 7, hyperlipidemia (or continuous LDL-cholesterol and/or HDL-cholesterol); 8, hypertension (or diastolic and/or systolic blood pressure); 9, obesity; 10, alcohol consumption. *Abbreviations:* PD, probing depth; CAL, clinical attachment loss; CHD, coronary heart disease; ACS, acute coronary syndrome; OR, odds ratio; HR, hazard ratio; CPR, cumulative prevalence ratio; NA, not available; NAS, Normative Aging Study, Boston, MA; IgG, immunoglobulin G; IgA, immunoglobulin A; Pg, *Porphyromonas gingivalis;* Aa, *Aggregatibacter actinomycetemcomitans.**Dietrich *et al.* (2008) reported results only in age subgroups, so the hazard ratio for the association between radiographic periodontal disease and incident stroke among the full cohort is not available.**Hemorrhagic strokes included.***The study by Syrjala *et al.* (2009) computed a cumulative prevalence ratio by comparing risk of prevalent stroke or CHD among dentate participants in the numerator with that of edentulous participants in the denominator; therefore, it can be inferred that there was a modest, non-significant increased prevalence of stroke or CHD among edentulous participants relative to dentate participants.

publications from the Normative Aging Study (NAS) cohort report positive associations between periodontal disease and both incident CHD (Dietrich *et al*., 2008) and stroke (Jimenez *et al*., 2010). Importantly, these studies also had the ability to test two previously described trends as *a priori* hypotheses. Specifically, these studies reported stronger associations among younger *vs*. older participants as well as for stroke *vs.* CHD outcomes (see discussion below). In addition, recently reported data from Korea (Sim *et al*., 2008; Choe *et al.,* 2009) and India (Pradeep *et al*., 2010) are, to our knowledge, among the first publications reporting on associations between periodontal disease and clinical AVD events that arise from Asian populations. Therefore, these data enhance the consistency of previous findings that predominantly stem from European and North American cohorts. The growing diversity of study populations from which results have been reported helps assuage concerns about spurious findings related to health behaviors (smoking, dietary patterns, physical activity), health care systems (access to care, clinical guidelines, availability of pharmaceuticals), or environmental risk factors (environmental tobacco smoke, pollution, diet).

Another important development is the publication of at least three studies using direct assessments of periodontal bacterial colonization (Renvert *et al.,* 2006; Spahr *et al.,* 2006; Nonnenmacher *et al*., 2007), all of which report positive associations between specific oral bacterial colonization levels and CVD outcomes. These findings corroborate and extend the earlier reported observations from the Oral Infections and Vascular Disease Epidemiology Study (INVEST): that high colonization by a cluster of species assumed to be either causative for periodontal disease or strong correlates of underlying causative species (*Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia,* and *Treponema denticola*) was associated with significantly increased intima-media thickness in the carotid artery (Desvarieux *et al*., 2005).

In summary, at least 13 observational studies have been published in the last four years, all of which report at least marginal evidence of a positive association between periodontal disease and clinical CVD. These recent findings support and extend earlier observations, and have directly addressed several key themes relevant to the hypothesized role of periodontal infection/inflammation as an independent contributor to the pathogenesis of atherosclerotic vascular disease, which are discussed below in greater detail.

The Role of Confounding

A prominent and persistent concern regarding the positive associations between periodontitis and AVD is the potential for spurious findings induced by known correlations between established risk factors for AVD and poor periodontal health, or, in epidemiologic terms, *confounding,* which occurs when a variable is both associated with the exposure (*i.e*., periodontitis) and is an independent cause of outcome (*i.e.,* AVD) (Hernan *et al.,* 2002). For example, smoking is an established risk factor for both periodontal disease and AVD, which raises the possibility that the increased risk of AVD commonly observed among groups with periodontal disease is actually due to a higher rate of smoking in these groups. Strong arguments have been made to demonstrate the importance of smoking-related confounding, particularly in the context of periodontal infection and cancer (Hujoel *et al.,* 2003), but the conclusions apply to AVD research as well (Hujoel *et al.,* 2002). In response, epidemiologic approaches have been sharpened by many investigators. For example, it is now common practice to carry out multivariable adjustments that include not only smoking status (current, former, never) but also pack-years of smoking and years since cessation. Alternatively, stratified analyses conducted among never-smokers are also common; this ensures that the association exists among participants in whom smoking-related confounding is theoretically impossible (barring second-hand smoking and information bias). In addition to initial key studies that performed these stratified analyses, several recent studies have consistently reported positive associations between periodontal infections and AVD. For example, a Korean case-control study reported an odds ratio (OR) of 3.3 (95%CI: 1.7,6.7) for non-fatal stroke among never-smokers (Sim *et al*., 2008). In Finland, a nested case-control study observed an OR for incident stroke among male never-smokers of 3.31 (95%CI: 1.31-8.40), while the OR among female never-smokers was 2.36 (95%CI: 1.44-3.88) (Pussinen *et al.,* 2007a). In another publication from Finland (Ylöstalo *et al.,* 2006), an OR of 1.62 (95%CI: 0.70, 3.74) was reported for prevalent angina pectoris associated with gingivitis. Analysis of US data obtained from the Behavioral Risk Factor Surveillance Survey, including 41,891 participants from 22 states, showed that among never-smokers, the respective ORs for CHD among participants missing 1-5 or 6-31 teeth were 1.39 (95%CI: 1.05-1.85) and 1.76 (95%CI: 1.26-2.45) (Okoro *et al.,* 2005).

Despite these advances, smoking remains a critical threat to the validity of findings concerning periodontal infection and AVD. Nevertheless, it is clear that the preponderance of evidence to date in support of a positive association between periodontal infection and CVD cannot be completely explained by smoking, and therefore exists independent of smoking behaviors. With an example from the cancer epidemiology literature, it has been convincingly demonstrated that it is unrealistic to conclude that smoking alone, particularly residual confounding by smoking or environmental tobacco smoke, can completely explain the reported associations in observational epidemiological studies (Michaud *et al*., 2007; Taguchi, 2007). Nevertheless, future research can benefit from careful methodological work with specific focus on confounding using modern epidemiological tools such as Directed Acyclic Graphs (DAGs) (Merchant and Pitiphat, 2002), which provide a quick visual method for the selection of potential confounders and minimization of bias in the design and analysis of epidemiological studies.

Of equal importance to the problems posed by known confounders is the potential for *unknown* confounders to underlie the consistent findings in the literature. For example, a recent candidate-gene association study identified a common genetic susceptibility locus, shared by both coronary heart disease and aggressive periodontitis, which may partly account for the observed associations (Schaefer *et al*., 2009). Unfortunately,

very little, if anything, can be done in observational studies to account for this possibility. The remedy for this problem lies in randomization, a key feature required of intervention studies testing the oral infection-AVD hypothesis.

The Role of Effect Modification

Effect modification (also referred to as interaction) is a phenomenon that occurs when "two or more risk factors modify the effect of each other with regard to the occurrence or level of a given outcome" (Szklo *et al*., 2000). In the context of this review, we are referring to a situation where the effects of periodontal infections on AVD are either stronger or weaker across levels of an additional factor (*e.g*., age, smoking status, or a polymorphism conferring genetic susceptibility). Two earlier publications have indeed discussed the concept of effect modification in the context of periodontal infection-AVD association (Hyman, 2006; Ylöstalo and Knuuttila, 2006).

From a statistical standpoint, assessing the evidence for effect modification is difficult because it generally requires much larger sample sizes than traditional analyses. Nevertheless, many publications often include subgroup results to inform the potential for effect modification, despite the fact that there is usually poor statistical power for meaningful null hypothesis testing in regard to interaction. This practice is still quite helpful, since it provides alternative views of the data to help assuage concerns regarding confounding—which is a phenomenon distinct from effect modification—and also allows for qualitative assessments regarding effect modification. However, it is important that these subgroup analyses not be over-interpreted in the absence of formal statistical testing for interaction with statistically significant p-values, as well as a clear rationale and *a priori* hypotheses.

A good example of potential effect modification in the context of periodontal infections and CVD is in regard to the influence of age. A pattern emerged in earlier studies in which the analyzed periodontal disease-AVD associations were consistently stronger among younger individuals (DeStefano *et al*., 1993; Morrison *et al*., 1999; Hujoel *et al.,* 2000; Joshipura *et al.,* 2003; Desvarieux *et al*., 2004; Demmer and Desvarieux, 2006). In terms of clinical CVD, the meta-analysis conducted by Janket and colleagues (Janket *et al*., 2003) reported that the overall CVD risk associated with periodontal disease was 1.19 (95%CI: 1.08, 1.32), while the CVD risk among individuals under 65 years of age was 1.44 (95%CI: 1.2, 1.7).

Recent reports confirm these trends (Table 2). Accordingly, Dietrich *et al.* (2008) reported a two-fold increase in the risk of incident CHD among male participants with *vs*. those with little or no evidence of clinically and radiographically defined periodontal disease at baseline. These positive findings from the Veterans Affairs Normative Aging Study (NAS) were limited to participants under age 60, while the association was not evident among older individuals. Similar findings for stroke were recently reported from the same study (Jimenez *et al*., 2009). These results are important and convincing, since: (i) they stem from a well-conducted population-based study that was initiated in the 1960s (*i.e*., 20 years prior to the publication of the first reports of a periodontal-CVD association), which minimizes the potential for bias; (ii) they represent both fatal and non-fatal CVD events occurring during 35 years of longitudinal follow-up; and (iii) the periodontal disease/age interaction hypothesis was specified *a priori* based on the previously reported observations of increased risk among younger participants mentioned above (DeStefano *et al*., 1993; Mattila *et al*., 2000; Joshipura *et al*., 2003; Grau *et al.,* 2004) and is likely not a spurious *post hoc* finding.

In addition, analysis of case-control data from a Danish population showed an odds ratio of 6.6 (95%CI: 1.69, 25.6) for the association between periodontal disease and CHD among participants < 60 years of age, while there was no association observed among participants 60+ years (Geismar *et al.,* 2006). Even more dramatic were the reported case-control data from Korea, with a 25-fold increase in the odds of ischemic stroke among participants with *vs*. those without periodontitis in ages 40-59 years, as compared with a 2.5-fold increase among participants in ages 60-79 years (Sim *et al*., 2008).

These age-associated trends were not corroborated by data from Finland (Pussinen *et al*., 2007a,b) that reported moderate or no associations between serum antibody levels to specific periodontal pathogens and both CHD and stroke among participants under the age of 65 years. However, it must be realized that the exposure definition in this study was limited to serum antibody titers to two periodontal pathogens (*A. actinomycetemcomitans* and *P. gingivalis*). In addition, the prevalence of edentulism in the study population was not accounted for, which is problematic given its impact on antibody levels discussed above. Therefore, while these findings add counter-balance to the otherwise convincing trends of stronger risk among younger individuals, they should be viewed in the context of their epidemiological limitations.

The age trends reported in studies with clinical AVD outcomes have been extended to studies of subclinical AVD among young participants in at least two separate studies. Analysis of data from the population-based Study of Health in Pomerania (SHIP) demonstrated that, when comparing participants with high *vs*. those with low levels of periodontal disease, the prevalence of carotid artery plaque increased by approximately 15% among men under the age 59 years, while among participants aged 59 years and older, the increase was only approximately 5% (Desvarieux *et al*., 2004). Likewise, the odds of having increased carotid artery intima-media thickness (IMT) among periodontitis patients under 40 years of age was increased over eight-fold when compared with odds in periodontally healthy control individuals matched on age, gender, obesity, and smoking behaviors (Cairo *et al.,* 2008).

Analyses of some data have also reported smoking (Hyman *et al.,* 2002) and gender (Desvarieux *et al.,* 2004; Grau *et al*., 2004; Volzke *et al.,* 2006; Desvarieux *et al.,* 2010) to be potential effect modifiers of the periodontal infection/AVD association. However, we are unaware of any studies that have reported statistically significant interactions between periodontal infections and either smoking status or gender for the prediction of incident clinical cardiovascular disease. As stated above, conducting the appropriate analyses for these tests generally requires

Table 2. A Subset of the Observational Studies Reported in Table 1 that Provides Information on Age and CVD Outcome Trends (Data are presented separately for individuals younger than 68 yrs or over 65 yrs of age.)

Study	N	Country	Age Range	Design	Exposure	Outcome	Adjustments	Measure of Association (95% Confidence Interval)
Geismar *et al.*, 2006	250*	Denmark	< 60	Case-control	Radiographic	CHD	1,2,5,6	OR 6.6 (1.69, 25.6)
Tu *et al.*, 2007	12,223	Scotland	≤ 30	Cohort	Tooth loss	Fatal CHD	1,3–5,8,9	HR 1.19 (0.84, 1.69)
Dietrich *et al.*, 2008	1203*	USA (NAS)	21–59	Cohort	Radiographic	CHD	1–10	HR 2.12 (1.26, 3.60)
Pussinen *et al.*, 2007b	505	Finland	25–64	Nested case-control	IgA *Aa*	CHD & stroke combined	1–9	HR 1.43 (0.88, 2.31)
					IgG *Aa*			1.64 (1.00, 2.69)
					IgA *Pg*			1.53 (0.95, 2.44)
					IgG *Pg*			1.53 (0.93, 2.50)
Pussinen *et al.*, 2007a	893	Finland	30–59	Nested case-control	IgA *Aa*	Stroke	1,2,4–10	OR 0.83 (0.62–1.10)
					IgG *Aa*			0.93 (0.66–1.32)
					IgA *Pg*			1.22 (0.91–1.65)
					IgG *Pg*			1.31 (0.97–1.76)
Tu *et al.*, 2007	12,223	Scotland	≤ 30	Cohort	Tooth loss	Fatal stroke	1,3–5,8,9	HR 1.64 (0.96, 2.80)
Sim *et al.*, 2008	160	Korea	40–59	Case-control	CAL	Stroke	1–6,8–10	OR 25.9 (5.77, 117)
Jimenez *et al.*, 2009	NA	USA (NAS)	27–64	Cohort	Radiographic	Stroke	1–10	HR 5.81 (1.63,20.68)
Pradeep *et al.*, 2010	200	India	33–68	Case-control	PD > 4.5 mm	Stroke	1–3	OR 8.5 (1.1, 68.2)
Geismar *et al.*, 2006	250*	Denmark	65+	Case-control	Radiographic	CHD	1,2,5,6	OR 0.8(0.26, 2.69)
Dietrich *et al.*, 2008	1203*	USA (NAS)	60–84	Cohort	Radiographic	CHD	1–10	HR 1.81 (NA)
Sim *et al.*, 2008	197	Korea	60–79	Case-control	CAL	Stroke	1–6,8–10	OR 2.45 (1.06, 5.67)
Jimenez *et al.*, 2009	NA	USA (NAS)	65–84	Cohort	Radiographic	Stroke	1–10	HR 2.39 (0.91, 6.25)

Adjustments: 1, age; 2, race; 3, sex; 4, SES (income and/or education); 5, smoking status; 6, diabetes; 7, hyperlipidemia (or continuous LDL-cholesterol and/or HDL-cholesterol); 8, hypertension (or diastolic and/or systolic blood pressure); 9, obesity; 10, alcohol consumption. *Abbreviations:* PD, probing depth; CAL, clinical attachment level; CHD, coronary heart disease; OR, odds ratio; HR, hazard ratio; NA, not available; NAS, Normative Aging Study, Boston, MA; IgG, immunoglobulin G; IgA, immunoglobulin A; Pg, *Porphyromonas gingivalis;* Aa, *Aggregatibacter actinomycetemcomitans*. *Represents total cohort—sample size in age subgroups not specified.

large sample sizes and reasonable AVD event rates. Thus, additional research is required to determine whether smoking and gender are true effect modifiers.

The Appropriate Exposure Measure

Relative to the large body of literature that has accumulated on the topic of periodontitis-AVD associations, there has been very little methodological work to help inform and optimize the appropriate case definition of periodontitis that best reflects the infectious etiological agents that are hypothesized as the primary exposure of interest in this context (Fong, 2002).

The currently accepted paradigm on the pathogenesis of periodontal disease is based on the notion that bacterial colonization elicits an immune response by the host that, under additional conditions, may result in the manifestation of clinical disease. From an epidemiologic standpoint, there is a need for strong methodological research in this area so that bias related to misclassification of exposure status can be better understood and minimized. Bias of this nature is insidious but critically important. As has been demonstrated in simulation studies, misclassification of periodontal infection exposure status can yield substantially biased parameter estimates of periodontal infection-AVD associations (Dietrich and Garcia, 2005).

A majority of studies reporting on this field have used either clinical or radiographic measures of periodontal disease as a surrogate for periodontal infection. Clinical measures have included assessments of attachment loss, pocket depth, bleeding on probing, and even tooth loss, while radiographic measures have included linear or proportional assessments of alveolar bone loss. However, the definition of a "periodontitis case" has varied greatly across studies. Moreover, when oral infection is considered as a risk factor for AVD, restricting the exposure to a distinct clinical phenotype may be inappropriate. For example, attachment loss is not always the result of an infectious process, since, in certain instances, it may be due to trauma. Therefore, careful consideration of appropriate exposure definitions based on clinical periodontal measures is required.

Beck and Offenbacher (2002) were the first to publish data regarding appropriate exposure definitions in this context. Their approach helped to introduce two important concepts: (i) periodontal measures that are relevant in the context of exposure for AVD are possibly different from definitions used to classify the various clinical periodontal disease entities and periodontitis in particular; and (ii) when oral infection models are used to study infectious etiologies for AVD, clinical exposure definitions should be "predicated upon those clinical signs that best represent the underlying mechanisms and temporal sequence that may affect that systemic outcome." Illustrating this point, they reported that attachment loss showed a weaker association with acute-phase systemic biomarkers of AVD risk than probing depth or bleeding on probing. Subsequent research has extended these initial findings by performing robust sensitivity analyses allowing for a side-by-side comparison of the

association between systemic AVD risk markers and periodontal exposure definitions (Demmer *et al*., 2008a), and demonstrated that optimal definitions varied according to whether the particular marker represented an acute or chronic condition. For example, pocket depth was more strongly correlated with the acute-phase marker fibrinogen, while attachment and tooth loss tended to be more strongly correlated with the chronic marker hemoglobin A1c or carotid artery atherosclerosis.

Going beyond clinical definitions, several studies described above have used assessments of periodontal bacterial colonization as exposures associated with subclinical AVD (Desvarieux *et al*., 2005) or coronary heart disease outcomes (Renvert *et al*., 2006; Spahr *et al*., 2006; Nonnenmacher *et al*., 2007). No data are so far available addressing the association between colonization levels and stroke outcomes. Alternatively, at least 11 studies have now been published using serum antibody responses to periodontal bacteria as the main exposure of interest (Pussinen *et al*., 2003, 2004a,b, 2005, 2007a,b; Taniguchi *et al.,* 2003; Beck *et al.,* 2005a,b; Johansson *et al.,* 2005; Lund Håheim *et al.*, 2008). A meta-analysis including several of these studies (Mustapha *et al.,* 2007) reported the overall trend to suggest a 36% increased risk for CVD outcomes associated with elevated systemic antibody responses, although the summary measure was not statistically significant ($p = 0.09$). When only CHD outcomes were considered, the findings were stronger and statistically significant: odds ratio 1.75 (95% CI, 1.32 to 2.34; $p < 0.001$).

While the aforementioned studies have attempted to relate CVD outcomes to bacterial exposures and systemic immune responses, there are persisting limitations in the exposure assessments used. For example, despite a substantial body of literature regarding the microbial etiology of periodontal diseases and the several hundreds of bacterial species that colonize the periodontal niche (Paster *et al.,* 2001; Socransky and Haffajee, 2005), only a handful have been recognized as etiologic agents (Proceedings of the 1996 World Workshop in Periodontics), while the subset of microbiota investigated in most studies to date is largely biased toward cultivable species. Another important limitation in regard to bacterial exposure assessments is the high resource burden it puts on research studies. The development of high-throughput molecular techniques such as bacterial microarray platforms (Colombo *et al*., 2009) is likely to facilitate large-scale "periodontal microbiome" assessments in the future, but these data will be subject to analytical and statistical limitations, including issues related to multiple comparisons. Techniques that utilize knowledge about bacterial clusters (Socransky *et al*., 1998), as well as analytical techniques to combine key species across clusters, are likely to be part of the solution (Desvarieux *et al*., 2005; Demmer *et al.,* 2008b), but more research in this area is necessary.

Likewise, the utilization of systemic antibody titers for measuring the degree of infectious exposure is also subject to limitations. Recent studies have demonstrated complex, species-specific associations between serum antibody to periodontal bacteria and clinical periodontal conditions, with certain high titers likely suggesting the presence of a protective adaptive response, with others being positively correlated with the severity of periodontitis (Dye *et al*., 2009). In turn, these observations complicate the interpretation of findings from studies using systemic antibody levels to define periodontitis-associated infectious exposure for AVD outcomes. Thus, it is unclear whether the few studies reporting inverse associations between antibody titer levels and AVD (Mustapha *et al*., 2007) are due to bias, chance findings, or true associations, the latter possibility suggesting that a robust antibody response to periodontal pathogens is protective against AVD. This hypothesis is intriguing when one considers the fact that among studies included in recent meta-analyses (Janket *et al.,* 2003; Humphrey *et al*., 2008) utilizing mainly clinical measures of periodontal disease and clinical CVD outcomes, only one study has reported an inverse association (Tuominen *et al.,* 2003). Furthermore, results from studies using exposure definitions based on antibody titers also need to consider the influence of partial or complete edentulism, since edentulous participants, despite a likely history of periodontal infection, tend to display lower antibody titers than their dentate counterparts (Vlachojannis *et al.,* 2010). Beyond these issues, additional problems include the potential cross-reactivity among antibodies and the differential correlation of different immunoglobulin subclasses with chronic, cumulative bacterial stimuli (typically IgG responses) or more recent, transient exposures (better represented by IgA responses).

To date, no large-scale methodological studies have systematically assessed inter-relationships among clinical, microbial, and antibody population-based data to examine whether combinations of these measures may enhance the precision of exposure definitions and enhance the prediction of AVD-related outcomes.

Significance of AVD Outcomes

Evidence suggests that periodontal infections may be a stronger risk factor for ischemic stroke outcomes as compared with coronary outcomes. The most compelling data supporting this notion originate from three separate studies that have reported associations between periodontal disease and both coronary and stroke outcomes in the same populations, using the same exposure definitions. Specifically, Wu *et al*. (2000) reported strong associations between periodontal disease and stroke in NHANES I and its follow-up study, while Hujoel *et al*. (2000) reported weak/null associations for CHD. Accordingly, analysis of data published by Joshipura *et al*. from the Health Professionals Follow-Up Study reported positive associations for stroke (Joshipura *et al.,* 2003), but weak/null findings for CHD (Joshipura *et al.,* 1996). More recently, two publications mentioned above from the Normative Aging Study (NAS; Table 1) also corroborate that clinical periodontal disease may be more strongly associated with stroke (Jimenez *et al.,* 2009) than CHD (Dietrich *et al*., 2008; Jimenez *et al.,* 2009). Interestingly, analysis of the NAS data regarding periodontal disease and CHD did demonstrate a statistically significant association [HR(95%CI) = 2.12(1.26, 3.60)] among younger individuals (aged 21-59 years, Table 1). Accordingly, the NHANES I results reported by Hujoel *et al.* (2000) also indicated a positive statistically significant association between periodontal index and incident CHD [HR(95%CI) = 1.80 (1.04-3.10)] among the

subgroup of participants aged 35-44 years. While these subgroup findings were not particularly relevant at the time of publication in 2000, they have become noteworthy in light of more recent trends suggesting age interactions as discussed above.

However, these trends were not confirmed by a recent meta-analysis (Mustapha *et al*., 2007) that included only studies that used exposure definitions based on systemic antibody responses to two periodontal bacteria (*P. gingivalis* and *A. actinomycetemcomitans*) and reported stronger associations between periodontal infection and CHD as opposed to stroke outcomes. In addition to the limitation of serological exposure definitions discussed above, this meta-analysis included only two studies reporting stroke outcomes, both of which also included hemorrhagic stroke (Pussinen *et al.*, 2004a; Johansson *et al*., 2005). This is important, since the currently considered plausible association between periodontal infection and cerebrovascular disease is limited to atherogenic stroke. Future studies that can analyze results for stroke and CHD outcomes using multiple exposure definitions in the same population sample will be informative in this context.

INTERVENTION STUDIES

Treatment studies of patients with periodontitis have contributed with additional insights into the potential link between periodontal infections and AVD, and may serve as an intermediate, translational link between the observational studies referenced above and the mechanistic studies reviewed below. Typically, these studies have examined whether periodontal therapy may induce favorable changes in markers of systemic inflammation or on surrogate markers of subclinical AVD, and point to potential biological pathways that can be modulated *in vivo* by periodontal therapy and may contribute to the promotion of an anti-atherogenic phenotype.

It has been well-established that multiple cytokines and inflammatory markers, including IL-1, IL-6, IL-8 and TNF, are abundantly produced locally in pathological periodontal tissues and can be recovered in gingival crevicular fluid (GCF) samples obtained from periodontally involved tooth sites (Ebersole, 2003; Lamster and Ahlo, 2007). It has been postulated that these locally produced inflammatory mediators are introduced into the bloodstream, although periodontitis has not been shown to induce a sustained elevation of plasma IL-1 beta (Mengel *et al*., 2002) or TNF-alpha (Meyle, 1993). Nevertheless, parallel to the induction of short-lived bacteremias, described under the review of potential mechanisms below, chronic periodontal infection has been shown to contribute to a state of systemic inflammation characterized by plasma elevation of acute-phase proteins such as CRP (Loos *et al*., 2000; Noack *et al.,* 2001; Ebersole *et al*., 2002; Loos, 2005), inflammatory cytokines such as IL-6 (Loos *et al*., 2000), and coagulation factors such as fibrinogen (Loos, 2005).

A review of the intervention studies investigating the effect of periodontal therapy on plasma levels of inflammatory mediators reveals somewhat inconsistent findings. Patients treated by non-surgical periodontal therapy were shown to display a significant increase in plasma TNF-alpha, CRP, and IL-6 levels immediately after intervention, suggesting a systemic acute-phase response, possibly due to a massive bacterial inoculation in conjunction with the mechanical instrumentation of the periodontal tissues (D'Aiuto *et al*., 2004a; Ide *et al*., 2004; D'Aiuto *et al.,* 2005b; Tonetti *et al*., 2007). Two small-scale studies (Ide *et al.,* 2003; Yamazaki *et al*., 2005), the latter also involving surgical periodontal therapy followed by a short course of systemic antibiotics, reported no significant changes of serum levels of CRP, IL-6, or TNF-alpha 3 months after the completion of therapy. In contrast, a 6-month post-treatment follow-up of individuals enrolled in a single-arm intervention study (D'Aiuto *et al*., 2004b) involving conventional periodontal therapy demonstrated significant reductions in serum IL-6 (median decrease 0.2 ng/L, 95% CI 0.1-0.4 ng/L) and CRP (median decrease 0.5 mg/L, 95% CI 0.4-0.7). A subsequent pilot randomized control trial that compared mechanical periodontal therapy alone *vs*. identical therapy supplemented by local adjunctive antibiotics (D'Aiuto *et al*., 2005a) reported significant reductions in serum CRP and IL-6 in both treatment arms, and a significant reduction in total and low-density lipoprotein (LDL) cholesterol in the group that received adjunctive antibiotics. Yet, a larger randomized controlled trial by the same research group (Tonetti *et al.,* 2007) reported no significant differences in post-treatment plasma levels of CRP, IL-6, and plasminogen activator inhibitor-1 (PAI-1) levels between the treatment and control groups at 6 months, although the treatment group experienced lower levels of serum-soluble E-selectin and lower neutrophil counts. The most recent available systematic review of six treatment studies investigating the effects of periodontal therapy (scaling and root planing, with or without adjunctive local or systemic antibiotics) on serum CRP levels (Paraskevas *et al*., 2008) concluded that there is modest evidence for a treatment-induced reduction (weighted mean difference of reductions of 0.50 mg/L (95% CI 0.08-0.93). Exploring further the apparent heterogeneity in the short-term post-treatment responses in serological inflammatory marker levels, Behle *et al.* (2009) used a composite score (Summary Inflammatory Score) to represent the aggregate post-treatment response to a panel of 19 individual biomarkers. These investigators demonstrated that approximately one-third and one-fourth, respectively, of the treated patients showed a marked reduction or a pronounced increase in systemic inflammation, while the remainder remained seemingly unchanged. Interestingly, periodontal therapy resulted in significant differential regulation of multiple genes expressed in peripheral blood monocytes, especially in genes related to innate immunity, apoptosis, and cell signaling, in a manner compatible with the promotion of an anti-atherogenic phenotype (Papapanou *et al*., 2007). Thus, although it appears that the above studies indicate a general trend toward a treatment-induced suppression of systemic inflammation, the effects of therapy on specific markers are not entirely consistent across studies, and their sustainability over time has not been convincingly established.

Another group of studies has addressed the effect of periodontal therapy on endothelial dysfunction, which is characterized by reduced vasodilator capability of peripheral blood vessels (Verma *et al*., 2003) and is assessed by measurement of the difference in diameter of a peripheral artery prior to and after reactive hyperemia induced through occlusion of blood flow

(Celermajer *et al.,* 1992). Endothelial dysfunction has been shown to be associated with adverse long-term outcomes of coronary artery disease in at-risk patients or patients with early stages of AVD (Schachinger *et al.,* 2000; Suwaidi *et al*., 2000; Perticone *et al*., 2001). Association studies have established that endothelial dysfunction is more pronounced in patients with periodontitis when compared with periodontally healthy control individuals (Amar *et al*., 2003; Mercanoglu *et al.,* 2004). This is thought to occur due to "endothelial stunning", *i.e*., a transient reduction in endothelium-dependent dilatation (EDD) when endothelial cells are challenged by bacterial products such as endotoxins (Bhagat *et al*., 1996). Numerous periodontal treatment modalities have been shown to improve EDD in small-sized (N range, 22-30) single-arm intervention studies involving mechanical (non-surgical and surgical) periodontal therapy alone (Mercanoglu *et al*., 2004; Elter *et al*., 2006) or supplemented by systemic antibiotics (Seinost *et al*., 2005). Recently, a randomized controlled trial involving a total of 120 patients with severe periodontitis, 61 of whom received full-mouth subgingival debridement completed within a single session and accompanied by extensive application of local antibiotics in all deep periodontal pockets (Tonetti *et al*., 2007), demonstrated a significant improvement in EDD in the treatment group at a 6-month follow-up examination. Notably, this intense intervention resulted in a transient deterioration of EDD and a significant increase in multiple inflammatory mediators in the plasma immediately after the intervention.

Extending the observations of several association studies demonstrating that severe periodontitis (Beck *et al*., 2001), high subgingival colonization levels by specific periodontal pathogens (Desvarieux *et al.,* 2005), and high serum IgG titers against individual periodontal bacteria (Beck *et al*., 2005b) are significantly related with increased carotid artery IMT in adjusted analyses, a recent pilot study suggested that mechanical treatment of mild to moderate periodontitis in otherwise healthy individuals may result in a significant reduction of carotid artery IMT 12 months after completion of treatment (Piconi *et al*., 2009).

To date, only a single, multi-center pilot study has examined the effects of periodontal therapy on the secondary prevention of cardiac events. The Periodontitis and Vascular Events (PAVE) study (Beck *et al.,* 2008; Offenbacher *et al.,* 2009) randomized patients with periodontitis and a history of severe CVD to either community care or a study protocol that consisted of oral hygiene instruction and mechanical periodontal therapy. Over a 25-month follow-up period, cardiovascular adverse events occurred with similar frequency in the community control and the periodontal treatment groups, but the periodontal therapy administered resulted in a rather limited improvement of periodontal status at 6 months after the intervention, and these positive effects were not sustainable at the one-year follow-up. What further complicates the interpretation of the findings of this study is the fact that a substantial proportion of the individuals randomized in the community care group did receive some form of preventive or periodontal care outside the study. Last, obesity appeared to nullify the effects of periodontal treatment on a reduction in serum CRP levels. Important lessons were thus learned by this pilot trial that will inform the design of future randomized controlled trials with respect to: (i) the required intensity of the protocol-provided periodontal intervention to result in clinically and biologically meaningful and sustainable positive effects on the periodontal status; (ii) the role of co-existing risk factors for AVD that may negate the treatment-induced positive modulation of systemic inflammation; and (iii) the overall feasibility of the study design.

POTENTIAL MECHANISMS LINKING PERIODONTAL INFECTIONS AND ATHEROSCLEROSIS

In this section, we will review recent publications on the potential pathogenic mechanisms mediating the direct or indirect effects of periodontal infections on the initiation and perpetuation of AVD, as well as the plausible biological pathways that may account for the observed systemic effects of periodontal therapy. Delineation of the mechanisms and pathways that link oral infections to atherogenesis is critical for establishing causality, for the identification of patient populations that may benefit from periodontal intervention in the context of prevention/arrest of AVD, and for the design of relevant treatment strategies.

To facilitate an understanding of the various potential mechanisms involved in different stages of atherogenesis, we have summarized the most relevant pathways in three schematic figures that depict critical events in the development of AVD: Fig. 1 illustrates the potential role of periodontal pathogens and their products in the development of endothelial dysfunction; Fig. 2 summarizes their potential contributions to the formation of fatty streaks and atherosclerotic plaques; and finally, Fig. 3 illustrates potential pathways that modulate the maturation of atheromatic plaques and facilitate their rupture and vascular thrombosis. For a detailed review of the pathogenesis of AVD, the reader is referred to the article by Libby (2002).

The Role of Bacteremias

Entry of oral bacteria and/or bacterial products into the bloodstream [recently reviewed by Iwai (2009)] is thought to be one of the key initiators of biological events that link oral infections to AVD. Transient bacteremias are common after dental procedures, regardless of periodontal status (Olsen, 2008), occurring frequently after mastication or after personal oral hygiene (Lockhart *et al.,* 2008; Crasta *et al*., 2009). The incidence and intensity of these bacteremias correlate positively with the extent and severity of periodontitis (Kinane *et al*., 2005; Forner *et al.,* 2006) and are in line with histopathologic observations demonstrating disruption of the epithelial integrity of the periodontal pocket, a sizeable ulcerated surface amounting to up to 8 to 20 cm^2 (Hujoel *et al.,* 2001), and the proximity of highly vascularized tissue to the subgingival biofilm (Nanci and Bosshardt, 2006).

Oral and periodontal bacteria have been occasionally incriminated as causative for infections at distant organs, including the lung (De Soyza *et al.,* 2000), the central nervous system (Ewald *et al.,* 2006; Mueller *et al.,* 2009), or endovascular protheses (Grace *et al*., 1988), suggesting that they are able to establish

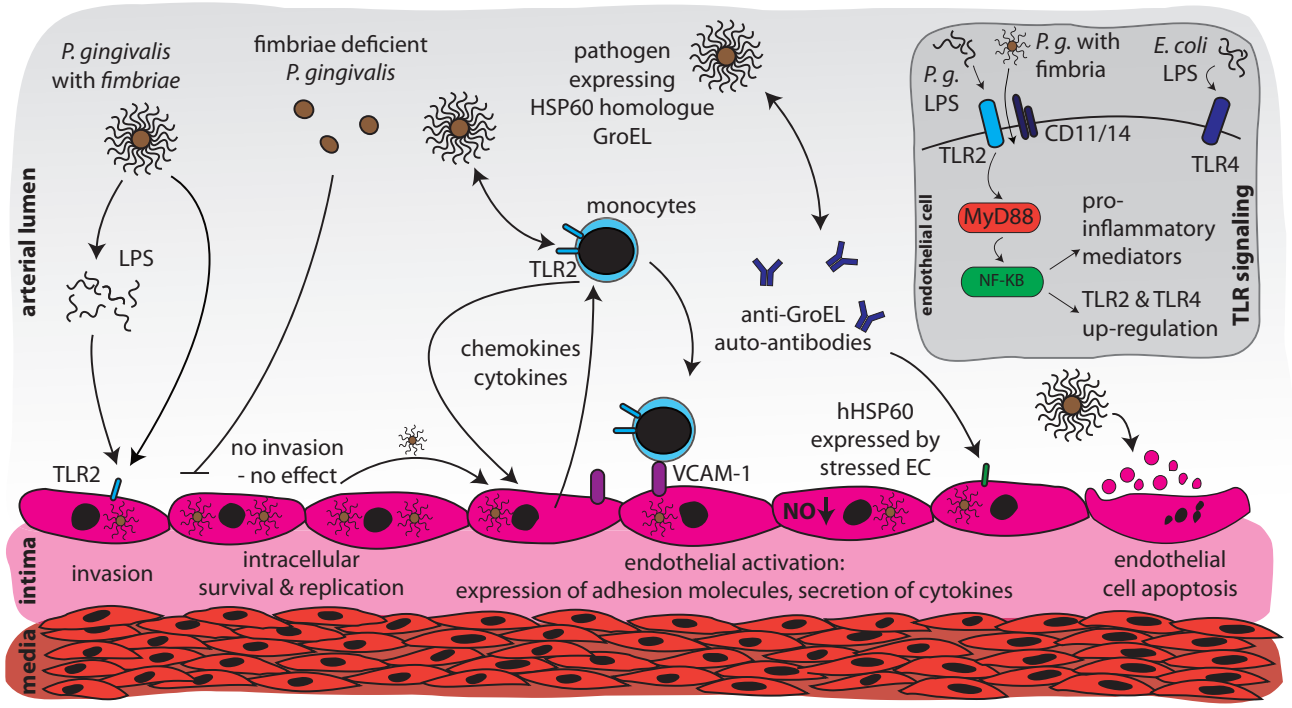

Figure 1. Schematic overview of potential mechanisms linking periodontal infections and endothelial dysfunction/incipient atherosclerosis. Vascular endothelial cells are invaded by fimbriated pathogens, *e.g., P. gingivalis*. These pathogens can persist and multiply intracellularly. Activation of Toll-like receptor 2 (TLR2) by fimbriated bacteria or LPS results in release of pro-inflammatory mediators and up-regulation of cell adhesion molecules. Monocytes are recruited by a gradient of chemotactic cytokines, such as MCP-1. Antibodies against bacterial heat-shock proteins, such as HSP60-related GroEL, auto-react with mammalian HSP60 expressed by activated endothelium, resulting in cell destruction. Further, *P. gingivalis* induces apoptosis of endothelial cells.

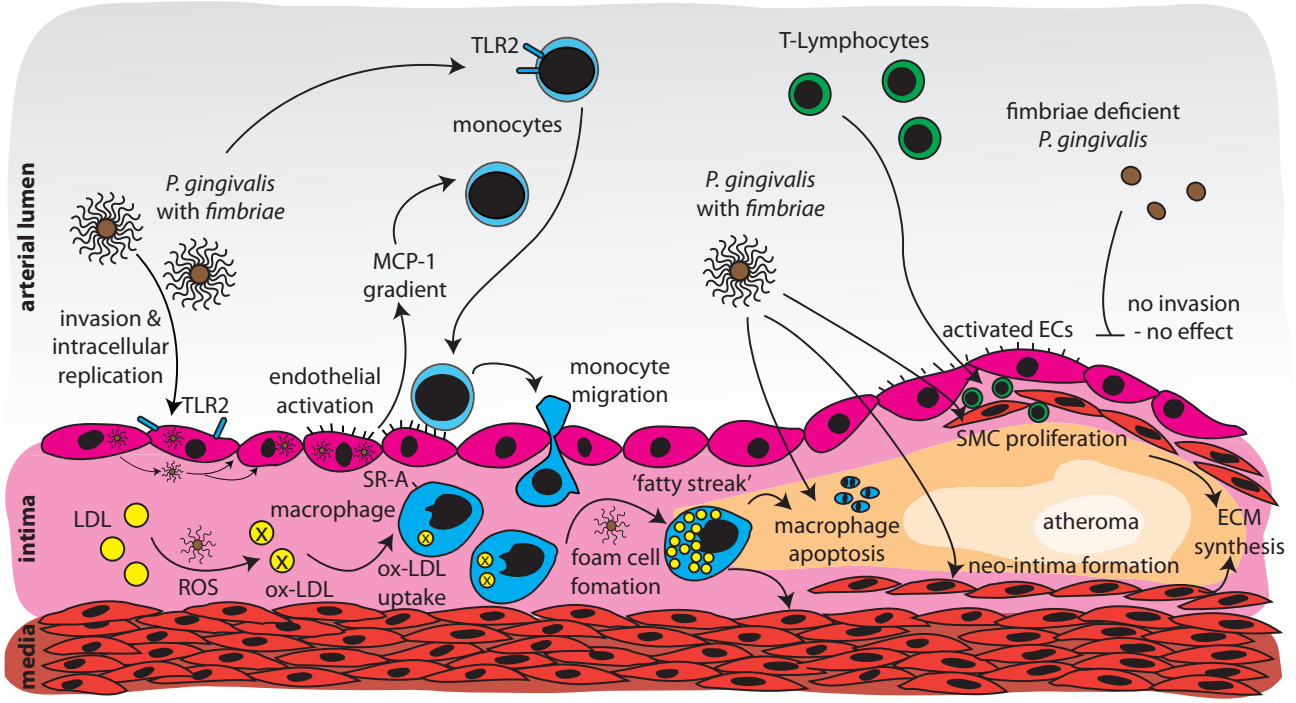

Figure 2. Potential mechanisms linking periodontal infections and fatty-streak formation/plaque maturation. Monocytes activated by periodontal pathogens chemotactically migrate into the sub-endothelial space, and transform into macrophages and, subsequently, into foam cells after uptake of oxidized LDL. Apoptosis of LDL-laden macrophages results in accumulation of lipids in the sub-endothelial space. Furthermore, periodontal pathogens induce smooth-muscle-cell proliferation in the intima and neo-intima formation. Extracellular matrix build-up and extravasation of T-cells consummate the formation of a fibrous cap covering the plaque.

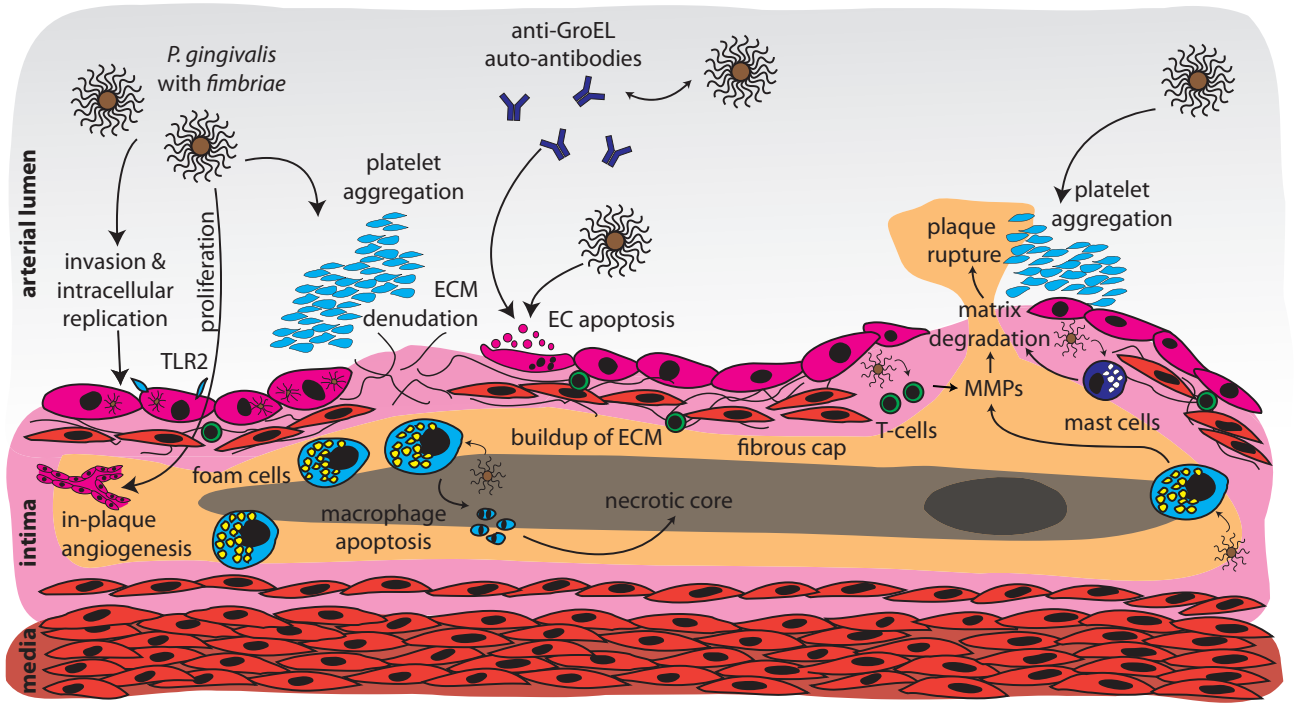

Figure 3. Potential mechanisms linking periodontal infections to mature atherosclerotic plaques and plaque rupture. Pathogen-mediated in-plaque angiogenesis is a hallmark of plaque organization. Denudation of the fibrous cap and its pro-thrombotic components occurs after endothelial cell apoptosis mediated by whole periodontal pathogens, or anti-endothelial auto-antibodies. Plaque rupture is induced by pathogen-mediated extracellular matrix degradation by endothelial cells, plaque macrophages, T-cells, and plasma cells, leading to exposure of pro-thrombotic plaque components, and subsequent vessel occlusion.

themselves at extra-oral locations. A great many studies have thus evaluated whether bacteria of oral or periodontal origin are detectable, retrievable, and cultivable from atherothrombotic plaques or vascular biopsies. Bacterial DNA from several periodontal pathogens has been detected in human endarterectomy specimens by PCR (Haraszthy *et al.,* 2000; Stelzel *et al*., 2002; Fiehn *et al.,* 2005; Ford *et al.,* 2005; Kozarov *et al.,* 2006; Padilla *et al.,* 2006; Nakano *et al.,* 2007; Pucar *et al*., 2007; Nakano *et al.,* 2008; Gaetti-Jardim *et al.,* 2009), by a combination of anaerobic culture and subsequent PCR identification (Padilla *et al.,* 2006), by checkerboard DNA-DNA hybridizations (Zaremba *et al.,* 2007), or by fluorescence *in situ* hybridizations (FISH) (Cavrini *et al*., 2005). Furthermore, viable *A. actinomycetemcomitans* and *P. gingivalis* were recovered and cultured from human atheromatous plaques originating from a patient with periodontal disease (Kozarov *et al.,* 2005). Two studies have so far failed to detect periodontal pathogen DNA in atheromatous plaques by PCR (Cairo *et al.,* 2004; Romano *et al*., 2007), but given the majority of positive studies, it appears that the assumption that periodontal pathogens may disseminate through the circulation and localize within atheromatic lesions is likely correct. It must be recognized, however, that this is likely not an exclusive property of periodontal pathogens. For example, Nakano *et al.* (2006) used PCR and subsequent sequencing, and identified *Streptococcus mutans* DNA in 74% of the investigated atheromatous plaque samples, while DNA from other species, including periodontal pathogens, was detected at far lower frequencies and levels. Thus, it appears that the disruption of the pocket epithelial integrity that occurs in periodontitis may also provide a point of entry for non-periodontal pathogens, such as the highly prevalent *S. mutans* in caries-affected dentitions. A recent study utilizing 16S rRNA sequencing identified 98 different bacterial species in the peripheral blood from 151 individuals with bacteremia; of these, 19 species were novel (Bahrani-Mougeot *et al.,* 2008). Given that the oral microbiome is comprised of approximately 700 species (Parahitiyawa *et al*., 2009), it is highly unlikely that the effects of periodontal infections on AVD are mediated by the limited number of periodontal pathogens studied so far.

Bacteria/Bacterial Products and Key Atherogenesis-promoting Processes

In the following text, we briefly summarize recent findings describing interactions of oral bacteria and bacterial products with specific host cells involved in the atherosclerotic process. The majority of the reviewed studies are primarily *in vitro* investigations of the effects of specific mono-infections on cultured host cells. Experimental animal studies and relevant data from human studies are presented whenever available. A critical appraisal of the limitations of these studies is offered at the end of this section.

Vascular Endothelial Activation

Upon entering the bloodstream, bacteria are rapidly cleared by host immune cells. To survive and elicit effects at distant sites, they have evolved several host-evasion strategies that have been investigated in multiple *in vitro* studies with *P. gingivalis* as a model periodontal pathogen. One such strategy is the ability of this micro-organism to invade vascular endothelial cells (Deshpande *et al*., 1998; Dorn *et al*., 1999; Progulske-Fox *et al.,* 1999). Within the endothelial cell, the survival of *P. gingivalis* depends on the concurrent activation of autophagy and suppression of apoptosis, which provides an intracellular niche where the pathogen can replicate unobstructed by host immune responses. In fact, repression of autophagy by chemical inhibitors, such as wortmannin, results in transition of the bacteria to the phagolysosome and subsequent degradation (Bélanger *et al*., 2006).

P. gingivalis' invasion of endothelial cells is dependent on fimbriae and a specific hemagglutinin (Takahashi *et al.,* 2006). Infection of human aortic endothelial cells by the invasive *P. gingivalis* strain 381 results in up-regulation of the chemokine IL-8, adhesion molecules such as ICAM-1, VCAM-1, and E-selectin, and cyclo-oxygenase-2 (COX-2). In contrast, corresponding expression profiles in cells infected with a non-invasive *fimA* mutant remain largely unchanged (Chou *et al*., 2005). Thus, fimbriae appear to be critical for both the invasive and pro-atherogenic properties of *P. gingivalis.* Induction of IL-6 in vascular endothelial cells has also been shown to be a process dependent on fimbriae, nuclear factor kappa B (NF-κB), and meiosis-specific kinase 1, which is regulated by the autocrine IL-6 signal transducer gp130 (Ho *et al.,* 2009). Again, it should be noted that *in vitro* invasion of endothelial cells is not an exclusive feature of specific periodontal pathogens, since *S. mutans* has also been shown to invade human aortic endothelial cells and to persist intracellularly over prolonged periods of time (Abranches *et al.,* 2009).

Nevertheless, the infection-induced effects on endothelial cells also show species-specific variation. For example, expression of the chemokine monocyte chemoattractant protein-1 (MCP-1), an important regulator of monocyte migration from the vessel lumen to the sub-endothelial space, was strongly induced in human umbilical vein endothelial cells after *P. gingivalis* infection, while it was minimally up-regulated after infection with *T. forsythia* and virtually unaffected after infection by *T. denticola* (Niu and Kolattukudy, 2009). Induction of MCP-1 expression was attenuated by inhibition of several pathways, including the mitogen-activated (MAP) kinase, NF-κB, c-Jun N-terminal-kinase (JNK), and activator-protein 1 (AP-1) pathways (Choi *et al.,* 2005). *P. gingivalis* infection resulted in activation of NF-κB and AP-1.

In addition to whole bacteria-endothelial cell interactions, studies have examined the effects of specific bacterial products such as microbial proteases and lipopolysaccharide (LPS). Arginine-specific gingipain, a *P. gingivalis*-specific protease (Fitzpatrick *et al.,* 2009), increased the responsiveness of endothelial cells to live *P. gingivalis* and *P. gingivalis* LPS, by inducing Weibel-Palade body exocytosis through activation of protease-activated receptors (PARs). Weibel-Palade bodies are vesicles in endothelial cells that store vaso-active substances, such as angiopoietin-2, which may enhance IL-8 production by LPS-stimulated cells (Inomata *et al.,* 2007). Another class of biologically active bacterial products investigated in this context is outer membrane vesicles (OMVs), *i.e.,* vesicles 'budding off' from growing bacterial cells and comprising a protein fraction and LPS. *P. gingivalis* OMVs were found to impair growth and tube formation in human umbilical vein endothelial cells, an effect mediated by the protein fraction of OMVs, since it was effectively inhibited by heat inactivation (Bartruff *et al*., 2005). In addition, a free-soluble surface material, released by *A. actinomycetemcomitans* grown either in a biofilm or in a planktonic form, was found to induce production of several pro-inflammatory cytokines in human whole blood. Interestingly, the effect was only partially LPS-dependent, as shown in LPS blocking experiments with polymyxin B, and did not depend on the presence of *A. actinomycetemcomitans* toxins, including the cytolethal distending toxin, leukotoxin, or peptidoglycan-associated lipoprotein (Oscarsson *et al*., 2008). Since both OMVs and free-soluble surface material are abundantly produced locally in the plaque biofilm, their potential entry into the circulation may constitute a significant source of inflammatory stimulants along with the planktonic bacteria in the bloodstream.

Induction of apoptosis in vascular endothelial cells, a hallmark of developing endothelial dysfunction (Hotchkiss *et al.,* 2009; Pober *et al*., 2009), is another bacterial strategy of critical importance in atherogenesis. *P. gingivalis* gingipains were shown to induce cell adhesion molecule cleavage, detachment, and apoptotic cell death in bovine coronary artery endothelial cells (Sheets *et al*., 2005, 2006). Given that the effector enzymes in apoptosis are caspases, a family of cysteine proteases (Pop and Salvesen, 2009), these effects could efficiently be blocked by pre-incubation with cysteine-protease inhibitors. Interestingly, both gingipains (Sheets *et al*., 2005, 2006) and whole *P. gingivalis* (Desta and Graves, 2007) were also able to induce caspase-independent programmed cell death, as shown with the irreversible caspase inhibitor z-VAD-fmk. However, apoptosis in endothelial cells was strongly dependent on the relative proportion of planktonic pathogens to cultured cells, *i.e*., the multiplicity-of-infection (MOI), with invasive *P. gingivalis* consistently inducing apoptosis at MOI ranging between 500 and 1000, but not at MOI between 50 and 100 (Roth *et al.,* 2007a).

Conversely, the potential ability of periodontal bacteria or their products to induce vascular cell proliferation is also relevant in the context of atherogenesis, since smooth-muscle-cell proliferation results in thickening of the vessel media, and endothelial proliferation is needed for local angiogenesis within atheromatous plaques. Interestingly, only cell-free products of *P. gingivalis,* but not whole bacteria, induced proliferation of aortic smooth-muscle cells *in vitro* after pre-incubation with human plasma (Inaba *et al.,* 2009). Proliferation could not be induced with live *P. gingivalis* in the absence of plasma, and was shown to be dependent on the up-regulation of S100 calcium-binding protein A9, a hitherto-unrecognized proliferation factor. These findings are important, since they indicate that the presence of live bacteria is seemingly not required for the induction of these proliferative effects that may be mediated by OMVs or free-soluble surface material. *P. gingivalis*-mediated proliferation in human

endothelial cells, including tube formation, and angiogenesis in matrigel plugs was found to be dependent on activation of the mitogen-activated extracellular signal-regulated kinase-1 and -2 (ERK1/2) (Koo *et al*., 2007). Similarly, *E. corrodens* was found to possess angiogenic, proliferative, and pro-inflammatory effects on endothelial cells utilizing a MAPK-dependent mechanism (Yumoto *et al*., 2007).

Interactions with Monocytes/Tissue Macrophages

Interactions of periodontal bacteria with other host cells that participate in atherogenesis have included studies involving monocytes, which are central to the formation of fatty streaks (see Fig. 2) (Webb and Moore, 2007). Roth *et al*. (2007b) observed an increased adhesion of monocytes to human aortic endothelial cells infected with invasive *P. gingivalis* when compared with adhesion to non-infected controls, or to cells infected with a fimbriae-deficient *P. gingivalis* mutant, mediated by elevated expression of adhesion molecules and chemotactic cytokines in the endothelial cells. Complementing these observations, infection of monocytes with invasive strains of *P. gingivalis* enhanced migration and elicited the expression of the pro-inflammatory cytokines TNF-alpha and IL-6, whereas infection by the fimbriae-deficient mutant had virtually no effect (Pollreisz *et al*., 2010). Likewise, monocyte infection with invasive *P. gingivalis* strains promoted enhanced LDL-uptake and foam cell formation to a greater extent than infection with a non-invasive fimbriae-deficient mutant (Giacona *et al*., 2004). Interestingly, similar experiments with *A. actinomycetemcomitans* LPS suggest that the presence of whole bacterial cells is not necessary for these effects. Indeed, LPS-challenged monocyte-derived macrophages showed enhanced secretion of TNF-alpha and interleukin-1beta and induction of foam cell formation and accumulation of LDL. LPS stimulation also decreased mRNA levels of scavenger receptor B, and ATP-binding cassette transporter-1, *i.e*., of two receptors that mediate the efflux of cholesterol from macrophages (Lakio *et al.*, 2006). These results are in agreement with findings from a recent *in vivo* study that demonstrated that sub-acute endotoxinemia resulted in a significantly impaired reverse cholesterol transport independent of plasma HDL levels (McGillicuddy *et al.,* 2009).

Pro-thrombotic and Pro-coagulant Effects

Platelets can be activated either by direct interaction of pathogens or their products, or indirectly *via* the vascular endothelium (Jennings, 2009). Several recent *in vitro* studies addressed direct and indirect effects of the model organism *P. gingivalis* on platelet aggregation.

P. gingivalis induces platelet aggregation *via* a TLR2-dependent mechanism, since its pro-coagulant properties were effectively blocked by pre-treatment with a TLR2-blocking antibody, or by inhibition of the downstream phosphoinositide 3-kinase (PI3-K)/Akt signaling pathway activated by TLR2 (Blair *et al*., 2009). Platelet aggregation in plasma was shown to depend on the adhesion molecule Hgp44 and the *P. gingivalis* protease Lys-gingipain (Kgp), but not on active Arg-gingipain (Rgp) (Naito *et al*., 2006). Importantly, in experiments with an MOI below the required threshold to activate platelet aggregation, *P. gingivalis* had a sensitizing effect on human platelets, enhancing epinephrine-induced aggregation. This effect was attributed to a limited activation of protease-activated receptors (PARs) on the platelet surface by gingipains, with subsequent mobilization of Ca^{2+}, leading to a marked coagulant response to epinephrine binding to the alpha(2) adrenergic receptor (Nylander *et al.,* 2008).

Evidence of indirect pathogen-induced promotion of a pro-thrombotic state has been provided in studies of the interactions of *P. gingivalis* and vascular endothelial cells and smooth-muscle cells. *P. gingivalis* gingipains induced hydrolysis of platelet endothelial cell adhesion molecule 1 (PECAM-1/CD31), suggesting ability to enhance vascular permeability (Yun *et al*., 2005). Infection of endothelial cells with invasive *P. gingivalis* strains resulted in enhanced tissue factor expression and activity, suppressed levels of tissue factor inhibitor, decreased levels and activity of tissue plasminogen activator, and increased plasminogen activator inhibitor-1 antigen levels (Roth *et al.,* 2006). Interestingly, these effects were most prominent at later time-points, suggesting that they are due to downstream intracellular pathways triggering pro-coagulant mechanisms. In aortic smooth-muscle cells, whole *P. gingivalis,* but not *P. gingivalis* LPS, induced a pro-thrombotic phenotype by down-regulation of tissue factor pathway inhibitor (Roth *et al.,* 2009). *P. gingivalis* arginine- and lysine-specific gingipains induced degradation of vascular endothelial cell thrombomodulin *in vitro,* an observation corroborated by the reduced expression of thrombomodulin in the gingival microvascular endothelia of patients with periodontitis (Inomata *et al*., 2009).

Recent human *in vivo* studies support and extend these observations. A comparison of platelet-activating factor (PAF) levels in sera and GCF from patients with periodontitis, patients with coronary heart disease (CHD) without periodontitis, patients with periodontitis and CHD, and healthy control individuals showed significantly higher serum and GCF levels in all patient groups when compared with levels in control individuals (Chen *et al.,* 2009). In another case-control study (Papapanagiotou *et al.,* 2009), significantly elevated soluble P-selectin, a marker of platelet activation, was documented in the plasma of patients when compared with periodontitis-free control individuals. Furthermore, platelets from periodontitis patients showed an increased binding of the glycoprotein IIb-IIIa complex, a direct measure of platelet activation, which correlated positively with the extent and severity of periodontitis of the donor. The same research group (Nicu *et al*., 2009) stimulated platelets and leukocytes from periodontitis patients and periodontally healthy control individuals with four oral bacteria (*A. actinomycetemcomitans, P. gingivalis, Tannerella forsythia,* and *Streptococcus sanguis*) and reported higher platelet expression of P-selectin, and increased formation of platelet-monocyte complexes in periodontitis donors. Furthermore, platelet/monocyte complexes displayed a better ability to bind and phagocytose *A. actinomycetemcomitans,* suggesting that increased atherothrombosis was paralleled by enhanced bacterial clearance.

It thus appears that: (i) there is cross-sectional evidence of increased platelet activation in periodontitis patients; (ii) this

activation can be attributed to either direct effects of a periodontal "model" organisms on platelets, or indirect, pro-thrombotic effects on vascular endothelial and smooth-muscle cells; and (iii) the pro-thrombotic/pro-coagulant state also appears to serve as an antimicrobial defense.

Finally, a conceivable alternative pathway by which periodontal infections may exert pro-thrombotic effects is by means of pathogen-mediated apoptosis of vascular endothelial cells, a key event in the development of endothelial dysfunction, which exposes the basement membrane and allows for interaction of platelets with collagen, resulting in local thrombosis (Bombeli *et al.,* 1997). Activated endothelium by either direct interaction with periodontal pathogens, or *via* systemic inflammatory molecules, is known to express tissue factor (thrombokinase), an important mediator of thrombin formation (Pober *et al.,* 2009). However, tissue factor expressed on vascular endothelium is often 'encrypted', *i.e.,* is rendered ineffective by post-translational modification and, thus, is unable to exert its pro-coagulant function (Bach, 2006). Apoptosis of endothelial cells can 'decrypt' tissue factor by increasing calcium concentrations and proteolytic cleavage and thereby can trigger thrombosis, even when the basement membrane is not uncovered by endothelial desquamation (Greeno *et al.,* 1996).

It is important to note that thrombosis induction is not an exclusive feature of periodontal pathogens. In fact, it has been well-established that other oral bacteria, including *Streptococcus* species (Herzberg *et al.,* 2005), can also induce platelet aggregation.

Oral Bacteria and Atheromatic Plaque Disruption

A role of bacteria and bacterial products is also conceivable in plaque disruption, one of the final and critically important events in atherosclerosis that is caused either by rupture of the fibrous cap of an unstable plaque, leading to exposure of the pro-thrombotic contents of the plaque, or through plaque erosion by apoptosis, triggering local thrombosis (Virmani *et al.,* 2006; Libby, 2009; Ward *et al*., 2009) (see Fig. 3.). These events result in the clinical presentation of atherosclerotic vascular disease in the form of a myocardial or cerebrovascular infarction. Degradation of fibrous caps is mediated by matrix metalloproteinases (MMPs) produced within the plaques by macrophages (Galis *et al.*, 1994; Sukhova *et al.,* 1999). *P. gingivalis* and other periodontal bacteria (Ding *et al.,* 1995), including *P. intermedia* (Guan *et al*., 2009), have been reported to induce production of several MMPs in different cell types, including macrophages and endothelial cells, while at the same time reducing the expression of the MMP antagonist tissue inhibitor of MMPs (TIMPs) (Sato *et al.,* 2009). *In vitro, P. gingivalis* was shown to degrade fibrous cap material isolated from human autopsy plaque samples (Kuramitsu *et al.*, 2001). The pro-apoptotic effects of periodontal pathogens and their products were discussed above and can also contribute to plaque erosion.

Last, the reported pro-inflammatory effects of *A. actinomycetemcomitans* in human mast cells (Oksaharju *et al*., 2009) may also be relevant in this context. Although mast cells are uncommon in vascular tissues, they do localize in atherosclerotic plaques and particularly in shoulder regions of rupture-prone plaques (Lindstedt *et al.,* 2007). Activation of this cell population and subsequent production of levels of proteases capable of destabilization of atheromatic plaques correlate with intra-plaque hemorrhage, endothelial cell and macrophage apoptosis, and vascular leakage (Bot *et al.,* 2007). *In vivo,* mast-cell-deficient mice showed increased collagen content and fibrous cap development, and decreased atherosclerosis (Sun *et al.,* 2007). Activation of mast cells may thus be a possible route of bacterially triggered plaque rupture, although the available data are sparse.

Activation of Innate Immune Signaling Associated with Atherosclerosis by Periodontal Bacteria

Accumulating evidence suggests that periodontal pathogens and their bacterial products may exert pro-atherogenic effects in vascular endothelial cells *via* Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs). These primary receptors of the innate immune system recognize highly conserved pathogen-associated molecular patterns (PAMPs). Activation of TLRs and their downstream signaling pathways leads to cellular activation and a specific response to microbial infection. Expression of TLRs is strongly induced in endothelial cells and macrophages in atherosclerotic lesions (Xu *et al*., 2001; Edfeldt *et al*., 2002), but exposure of *in vitro* cultured endothelial cells to laminar flow down-regulated TLR2 (Dunzendorfer *et al*., 2004). Patients suffering from chronic inflammatory diseases show higher B-cell expression of TLR2 and TLR4 (Jagannathan *et al*., 2009), while these receptors are also induced on monocytes in diabetes (Devaraj *et al.,* 2008). Deficiency in MyD88, a central downstream signaling molecule for most TLRs, was shown to result in decreased atherosclerosis *in vivo* (Bjorkbacka *et al.,* 2004). Similarly, deletion of TLR2 (Mullick *et al.,* 2005) and TLR4 (Michelsen *et al.,* 2004) in mice had an atheroprotective effect, suggesting that agonists of these receptors play a role in advancing atherosclerosis (Erridge, 2008). However, a recent study utilizing an *in vitro* model of atherosclerosis found that only blockade of TLR2, but not of TLR4, resulted in significantly attenuated levels of inflammatory mediators and tissue-degrading MMPs (Monaco *et al.,* 2009).

Interestingly, *P. gingivalis* LPS appears to interact with different TLRs in a cell-type-dependent manner (Kocgozlu *et al.,* 2009). For example, activation of human vascular endothelial cells, monocytes, and mouse macrophages by *P. gingivalis* was not mediated by TLR4, the typical receptor interacting with LPS from Gram-negative bacteria, but by TLR2 (Triantafilou *et al.,* 2007; Hajishengallis *et al.,* 2008). As discussed above, *P. gingivalis* mediates its own uptake as a host defense evasion strategy, by interacting with TLR2 and the integrins CD11b/CD18 (Harokopakis and Hajishengallis, 2005). In fact, *P. gingivalis* fimbriae interacting with human monocytes and mouse macrophages induce CXCR4/TLR2 co-association in lipid rafts, *i.e*., cholesterol-enriched micro-domains involved membrane fluidity and cellular trafficking, which in turn inhibit TLR2-mediated pro-inflammatory and antimicrobial responses. Cholesterol depletion inhibits pathogen uptake and attenuates pathogen-induced signaling, suggesting that hypercholesterolemia may result in accelerated TLR2-mediated atherosclerosis (Wang and

Hajishengallis, 2008). TLR2 deletion in a mouse model of periodontitis resulted in a significant attenuation of periodontitis-aggravated atherosclerosis (Liu *et al.,* 2008), supporting a predominant role for this receptor in atherogenesis.

Stimulation of human aortic endothelial cells by *P. gingivalis* fimbriae resulted in increased expression of both TLR2 and TLR4, resulting in sensitization of these cells to enterococcal LPS that acts *via* TLR4 (Yumoto *et al.,* 2005). These observations suggest the synergistic potential of multiple bacterial stimuli in eliciting enhanced pro-atherogenic responses in vascular endothelial cells. *P. gingivalis* LPS stimulation of human coronary-artery-derived endothelial cells, but not of venous endothelial cells, resulted in increased expression of TLR2, and an induction of IL-8 secretion, E-selectin expression, and increased monocyte adhesion (Erridge *et al.,* 2007; Kocgozlu *et al.,* 2009). Thus, the selective pathogen-induced expression of TLR2 in arterial endothelial cells supports its involvement in atherogenesis.

Autoimmune Responses to Periodontal Bacteria

Molecular mimicry is a term used to describe the possibility that antibody responses targeted against bacterial antigens can essentially function as autoimmune responses, due to the high degree of homology between specific bacterial antigenic peptides and mammalian proteins, and has been considered as a biologically plausible mechanism linking infection and atherosclerotic vascular disease (Wick *et al.,* 1999; Epstein *et al.,* 2000; Lamb *et al*., 2003; Rajaiah and Moudgil, 2009). Central to this notion is a family of highly conserved heat-shock proteins (HSPs) (Fink, 1999), which is expressed on certain bacterial membranes, as well as by eukaryotic cells when exposed to stress (Polla, 1988). Bacterial HSPs are considered major antigenic determinants (Kaufmann, 1990) that elicit antibodies and specific reactive T-cells (Young and Elliott, 1989) that can cross-react with host cells expressing homologous molecules, resulting in auto-aggressive destruction (Mayr *et al*., 1999).

The mammalian host-protective heat-shock protein 60 (hHSP60) can be induced in vascular endothelial cells, macrophages, and smooth-muscle cells by several pro-atherogenic stimuli, including bacterial endotoxin, oxidated lipids, inflammatory mediators, hypertension, or mechanical shear stress (Seymour *et al*., 2007; Galkina and Ley, 2009). Although hHSP60 is not a trans-membrane protein and is thus seemingly unreachable by circulating antibodies, it synergizes with mitochondrial HSP70 (mtHSP70), which is expressed on the surfaces of stressed cells. hHSP60 and mtHSP70 associate within lipid rafts, resulting in endothelial activation with up-regulation of adhesion molecules and pro-inflammatory mediators, or in programmed cell death in endothelial cells (Alard *et al*., 2009), and in cytokine production by macrophages (Van Eden *et al*., 2007).

A high degree of homology between HSP from *C. pneumoniae,* a pathogen implicated in AVD, and that of human HSPs has been demonstrated (Kol *et al.,* 1998; Huittinen *et al.,* 2002). Likewise, high homology was found between the *P. gingivalis* HSP60—termed GroEL—and mammalian HSP60 family members (Maeda *et al*., 1994). *P. gingivalis* GroEL was shown to be highly immunogenic, and was recognized by serum antibodies isolated from patients suffering from periodontal disease (Ford *et al.,* 2005, 2006).

In comparisons of antibody titers to hHSP60 and GroEL in patients with atherosclerosis and periodontal disease, systemically healthy periodontitis patients, and healthy control individuals, the highest titers for both human and bacterial HSP60 were found in patients with both conditions, followed by the systemically healthy group with periodontitis, while the control group showed the lowest antibody levels. In addition, clonal analysis of the T-cells found both hHSP60- and GroEL-reactive populations in the circulation of atherosclerosis patients. These T-cell populations were also found in atherosclerotic plaques in several patients (Yamazaki *et al*., 2004).

The effects of auto-antibodies to pathogens were evaluated in a series of *in vivo* studies in mice. In ApoE-deficient mice immunized with intra-peritoneal injections of live *P. gingivalis* and/or C. *pneumoniae,* only *P. gingivalis* inoculations had a pro-atherogenic effect. A positive correlation of atherosclerotic lesion development and anti-GroEL titers was found, as well as hHSP60 in the lesions themselves (Ford *et al*., 2007). The presence of circulating anti-HSP60 auto-antibodies resulted in an increased expression of P-selectin and von-Willebrand factor (vWF), with an altered morphology of endothelial cells in uninjured carotid arteries. In a model of injured carotid arteries, auto-antibodies promoted thrombosis and recruitment of inflammatory cells (Dieude *et al*., 2009).

Interestingly, *H. pylori,* a gastrointestinal tract pathogen that expresses HpHSP60, a HSP60 family member similar to GroEL, was recently found to induce atherosclerosis in mice. Subcutaneous immunization with HpHSP60, or eradication of *H. pylori* infection by the use of antibiotics, resulted in significantly reduced atherosclerosis, decline of Th1-immune response, and reduction of T-cell chemotaxis beyond the endothelium (Ayada *et al*., 2009).

Induction of Oxidative Stress by Periodontal Pathogens—Role of ox-LDL

A potential pathway through which periodontitis may contribute to atherogenesis is through induction of oxidative stress. Oxidation of LDL *via* reactive oxygen species (ROS) is a prerequisite for cholesterol uptake by macrophages and the formation of foam cells (Stoll and Bendszus, 2006; Itabe, 2009), but also results in several additional pro-atherogenic effects (Verhoye and Langlois, 2009). Thus, apart from its involvement in the formation of fatty streaks, ox-LDL also affects the vascular endothelium both directly and indirectly. Direct effects include the induction of cellular activation and apoptosis by interaction with lectin-like oxidized low-density lipoprotein receptor (LOX-1) (D Li *et al*., 2002; Ma *et al*., 2006). Indirect effects are exerted through down-regulation of the expression of endothelial nitric oxide synthase (eNOS), which results in increased production of ROS, ongoing LDL oxidation, and endothelial dysfunction (Victor *et al.,* 2009). Ox-LDL also inhibits differentiation and induces apoptosis (D Li *et al.,* 2002; Ma *et al.,* 2006) of endothelial progenitor cells (EPCs), a subpopulation of bone-marrow-derived stem cells that participates

in vascular repair (Friedrich *et al*., 2006; Wassmann *et al.,* 2006; Zenovich and Taylor, 2008). Low levels of EPCs in the peripheral blood were found in states of poor vascular health (Werner *et al.,* 2007), and were predictive of adverse cardiovascular outcomes (Werner *et al*., 2005). Last, it has been established that ox-LDL up-regulates pro-atherogenic chemokines and adhesion molecules *via* the CD40/CD40L pathway (Li *et al*., 2003) and triggers IL-6, TNF-alpha, and CRP secretion (Hulthe and Fagerberg, 2002).

In *in vitro* studies with *P. gingivalis* as a model organism, incubation of whole bacteria with blood resulted in higher levels of oxidized LDL, increased proportions of apolipoprotein M, and cleavage of apolipoprotein B-100, a part of the LDL core, by arginine-specific gingipains (Bengtsson *et al.,* 2008). In addition, *P. gingivalis*-modified ox-LDL induced vascular smooth-muscle-cell proliferation *in vitro,* suggesting a potential role in intima-media thickening. Anti-oxidant treatment of endothelial cells infected with *P. gingivalis* resulted in an attenuated production of MCP-1 (Choi *et al*., 2005), suggesting an inhibition of monocyte migration into subendothelial spaces, the site where oxidation of LDL primarily takes place, likely due to the limited activity of anti-oxidants outside the vessel lumen (Verhoye and Langlois, 2009).

In an *in vivo* setting, higher levels of ROS and oxidative stress-related genes were found in the aortas of rats with experimental periodontitis (Ekuni *et al.,* 2009a). An intervention with vitamin C, a potent anti-oxidative agent, resulted in attenuated oxidative stress, and decreased lipid deposition in the aorta (Ekuni *et al.,* 2009b).

Periodontal Pathogens in Animal Models of Atherogenesis

Several studies conducted in ApoE-deficient mice, a mouse model prone to accelerated atherosclerosis, evaluated the effect of *P. gingivalis* infection on atherogenesis. Thus, intravenous injection of *P. gingivalis* (L Li *et al.,* 2002), *P. gingivalis* LPS (Gitlin and Loftin, 2009), or repeated oral/anal bacterial applications (Lalla *et al.,* 2003) resulted in enhanced atherosclerosis in infected animals when compared with uninfected controls. Periodontal tissue destruction correlated positively with atherosclerotic lesion formation, serum levels of IL-6 expression, and VCAM-1 expression in the aorta (Lalla *et al.,* 2003). Consistent with the *in vitro* data discussed above, only invasive *P. gingivalis,* as compared with a fimbriae-deficient mutant, was able to induce periodontitis, increase atherogenesis, and trigger up-regulation of Toll-like receptors TLR2 and TLR4 (Gibson *et al*., 2004).

P. gingivalis-mediated atherosclerosis was prevented by prior immunization with *P. gingivalis* (Miyamoto *et al.,* 2006; Koizumi *et al*., 2008, 2009). Interestingly, it appeared that pro-atherogenic changes, *i.e.,* development of atheromatous plaques in the aortic sinus, were dependent on innate immune responses occurring early during the course of the infection, rather than on the establishment of chronic systemic inflammation (Miyamoto *et al.,* 2006). It must be realized that the above studies tested the preventive effects of immunization prior to the induction of periodontitis, and can thus not be extrapolated to represent also the effects of periodontal therapy on atherogenesis-related events. In an experimental study that modeled the effects of periodontal therapy on systemic inflammation and atherosclerosis—bearing in mind that mechanical periodontal therapy is not feasible in mice—systemic doxycycline was found to reduce atherosclerotic lesion size and pro-atherogenic cytokine production in ApoE heterozygotic mice (Madan *et al.,* 2007). In this context, it is important to note that human periodontal disease is a chronic infection that is mediated by a biofilm rather than by planktonic bacteria. Pathogens in a biofilm are largely protected from antibiotics (del Pozo and Patel, 2007), and disruption of the dental plaque by mechanical means is considered essential for the successful management of periodontal disease, and for any downstream systemic effects (Schaudinn *et al*., 2009). Therefore, the reported lack of efficacy of systemic antibiotic therapy in the secondary prevention of cardiac events (O'Connor *et al*., 2003; Cannon *et al*., 2005; Grayston *et al*., 2005; Stassen *et al.,* 2008) should not be interpreted to demonstrate the inability of periodontal therapy to reduce the incidence of atherosclerosis-related events, since concomitant mechanical periodontal therapy was not administered in these studies. In contrast, the pro-atherogenic changes induced in the mouse model studies above (Madan *et al.,* 2007) were mediated by repeated injections of planktonic *P. gingivalis,* without formation of a dental plaque biofilm. Therefore, the observed anti-atherogenic effects of antibiotic monotherapy cannot be readily extrapolated to the setting of human periodontitis. In addition, the study did not control for the inherent anti-inflammatory and MMP-inhibiting (Gu *et al.,* 2010) effects of doxycycline.

Interestingly, treatment of *P. gingivalis* LPS induced atherosclerosis in $ApoE^{-/-}$ mice with a COX-2 inhibitor increased the extent of atherosclerotic lesions. This effect could be mediated by an inhibition of COX-2-dependent prostaglandin E2 expression by the drug, leading to increased TNF-alpha production and subsequent increased atherosclerosis (Gitlin and Loftin, 2009). Similarly unanticipated was the observation that deletion of IL-6, a major pro-atherogenic cytokine (Schuett *et al*., 2009), resulted in a significant increase in lesion size and alterations in atheromatic plaque composition toward an unstable phenotype in mice (Madan *et al*., 2008). These findings underscore the complexity of the process of atherogenesis and the apparent redundancy of the atherosclerosis-promoting pathways that complicate the interpretation of experimental mechanistic studies.

It should be emphasized that the above studies modeling the periodontitis/atherosclerosis associations used genetically modified ApoE-deficient mice on a high-fat, high-cholesterol diet ('Western diet'), and the obtained findings may have limited relevance to the human situation. In contrast, wild-type mice were shown to be relatively resistant to a plethora of atherogenic stimuli, and did not develop atherosclerosis within reasonable time periods (Graves *et al*., 2008). However, in other animal models involving pigs, *P. gingivalis* was shown to induce rapid atherogenesis, even in normo-cholesterolemic situations (Brodala *et al*., 2005). Nevertheless, cost-related issues and,

more importantly, the possibility to generate knock-out or knock-in animals make mouse models extremely useful in the study of specific molecular targets of atherogenesis.

The effects of periodontal pathogens were further studied in the liver, an organ of central importance for lipid metabolism, and for the production of acute-phase proteins, such as CRP. Repeated intravenous injections of live *A. actinomycetemcomitans* in mice resulted in detection of the pathogen in liver tissue, along with pronounced inflammatory cell infiltration, elevated mRNA levels for pro-inflammatory genes, and elevated serum amyloid A and LPS (Hyvarinen *et al*., 2009). Thus, induction of hepatic inflammation is conceivably another route by which periodontal pathogens may contribute to atherogenesis by interfering with lipid metabolism and promoting systemic inflammation.

Limitations of Available Mechanistic Studies

The majority of the mechanistic studies reviewed above used a 'model' periodontal pathogen, in most instances *P. gingivalis,* to dissect pathways relevant to the course of a poly-microbial infection such as periodontitis. Studies that utilized multiple bacterial stimuli simultaneously, *e.g*., in a co-infection model of human aortic endothelial cells with both *P. gingivalis* and *F. nucleatum* (Metzger *et al.,* 2009; Polak *et al*., 2009), demonstrated a two- to 20-fold increased invasive capacity of *P. gingivalis* (Saito *et al.,* 2008), suggesting that inferences based on mono-infections may not adequately portray the *in vivo* capabilities of the investigated pathogens. Furthermore, the selection of the particular strain of the model organism to be used in experimentation is of importance. For example, in the case of *P. gingivalis,* its virulence and potency to induce a pro-inflammatory host response depend on the types of fimbriae expressed, with Type II strains such as those frequently isolated from clinical lesions eliciting significantly stronger responses than Type I strains, which are most often used as model organisms (Wang *et al.,* 2009). In future studies, the use of artificial biofilms (Thurnheer *et al.,* 2004) incorporating multiple species rather than of planktonic bacterial cells will provide the possibility to account for properties ascribed to biofilm-derived bacterial products such as outer membrane vesicles, and may further enhance the ability of *in vitro* systems to model the *in vivo* situation. Similar issues arise from the selection of specific cell lines to be used as the effector targets, the properties of which may vary widely (Bouis *et al*., 2001; Staton *et al*., 2009). Last, the MOIs used in *in vitro* studies are likely much higher than a reasonable pathogen/host cell ratio that may plausibly be encountered in an *in vivo* setting. For example, the capacity of low *P. gingivalis* inocula to induce pro-inflammatory responses in human endothelial cells was rather weak in comparison with the effects of TNF-alpha or interleukin-1 beta at concentrations commonly measured in serum from periodontal patients (Honda *et al*., 2005). Likewise, Roth *et al.* (2007a) reported a lack of pro-apoptotic capability of invasive *P. gingivalis* on vascular endothelial cells in MOIs lower than 500. However, intracellular replication has recently been considered as a mechanism through which a pathogen may reach sufficiently high MOIs *in vivo* to exert biologic effects. In fact, *P. gingivalis* was recently shown to exploit its ability for intercellular transmission and conversion from a latent state of dormancy to a viable state, and thereby to be able to multiply and persist in vascular tissues (Li *et al.,* 2008).

CONCLUDING REMARKS

Evidence from observational epidemiologic studies that has accumulated over the past few years has extended earlier observations and suggests that periodontal infections are independently associated with cardiovascular and cerebrovascular clinical outcomes. Although the strength of the reported associations is generally modest, the consistency of the data that have emerged from geographically and ethnically diverse populations across a variety of exposure and outcome variables suggests that the findings cannot be ascribed solely to the effects of confounders. Analysis of limited data from interventional epidemiologic studies suggests that treatment of periodontal infections results in lower levels of systemic inflammation and favorable effects on subclinical markers of atherosclerosis, although analysis of the data suggests substantial heterogeneity in responses. However, there are no data available to date suggesting that the prevention or amelioration of periodontal infections will result in reduced incidence of cardiovascular or cerebrovascular clinical events. A great many experimental mechanistic *in vitro* and *in vivo* studies have established the plausibility of a link between periodontal infections and atherogenesis, and have identified biological pathways by which these effects are mediated. Future research must expand into the identification of *in vivo* pathways in humans that lead to periodontitis-mediated atherogenesis or result in treatment-induced reduction in atherosclerosis risk. Ultimately, these findings will pave the way for the conduction of appropriately designed clinical trials that will determine if periodontal interventions have a role in the primary or secondary prevention of AVD.

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