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Relationship between invasion of the *periodontium* by periodontal pathogens and periodontal disease: a systematic review

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ABSTRACT

Background: Bacterial invasion of the periodontal tissues has been suggested as a relevant step in the etiopathogenesis of periodontal disease. However, its exact importance remains to be defined. The present systematic review assessed the scientific evidence concerning the relationship between the quality or quantity of periodontal microbiota in periodontal tissues and development of periodontal disease.

Methods: The databases Medline-PubMed, Cochrane-CENTRAL, ISI Web of Knowledge and SCOPUS were searched, up to January. Studies that reported evaluation of periodontal pathogens invasion on human tissues were selected.

Results: The screening of 440 title/abstracts elected 26 papers for full-text reading. Twenty three papers were subsequently excluded because of insufficient data or a study protocol not related to the objectives of this systematic review. All included studies were case-control studies that evaluated intracellular or adherent bacteria to epithelial cells from periodontal pockets *versus* healthy sulci. Study protocols presented heterogeneity regarding case and control definitions and methodological approaches for microbial identification. No consistent significant differences were found related to the presence/absence or proportion of specific periopathogens across the studies, as only one study found statistically significant differences regarding the presence of *A. actinomicetemcomitans* (p=0.043), *T. forsythia* (p<0.001), *P. intermedia* (p<0.001), *C. ochracea* (p<0.001) and *C. rectus* (p=0.003) in epithelial cells from periodontal pockets *versus* healthy sulci. All studies reported a larger unspecific bacterial load in or on the epithelial cells taken from a diseased site compared to a healthy sulcus.

Conclusion: The current available data is of low to moderate quality and inconsistent mainly due to study design, poor reporting and methodological diversity. As so, there is insufficient evidence to support or exclude the invasion of periodontal pathogens as a key step in the etiopathogenesis of periodontal disease. Further research is needed.

58   **INTRODUCTION**

59   Over the past 50 years the role of invasion of bacteria in the complex pathogenic process of

60   periodontal disease has undergone cycles of acceptance and rejection. Since Listgarten [1], and its

61   first electron microscopy images of spirochetal infiltration in gingival tissues from patients with

62   necrotizing ulcerative gingivitis, the invasiveness of oral bacteria emerged as a potentially

63   important mechanism that mediates initiation and progression of periodontal disease.

64   It is not difficult to conjecture that the presence of microorganisms within gingival tissues would

65   not augur good things to the *periodontium*. Tissue invasion allows direct discharge of destructive

66   bacterial products[2-4] and promotes the release of lysosomal contents from neutrophils in the

67   periodontal tissues.[5,6] Besides that, invasive bacteria seem to have mechanisms to evade host

68   defenses. To be sheltered from the humoral immune surveillance invasive bacteria penetrate

69   and remain within the epithelial cells, in a nutritious environment, where they can replicate and

70   spread to neighboring cells.[7] Additionally, *in vitro* studies have shown that *Porphyromonas*

71   *gingivalis* impedes transepithelial migration of neutrophils and prevents epithelial cells from

72   secreting IL-8 in response to bacterial challenge.[8,9] Several studies have also suggested that

73   periodontal bacteria actively suppress cell-mediated immunity and this, presumably,

74   contributes to periodontal lesion development.[10] Standard periodontal treatment is also

75   undermined as intracellular bacteria are less likely to be physically removed by scaling and root

76   planning [11] and are more resistant to antibiotics. [12]

77   Although there is a theoretical basis for a role of invasion in the etiopathogenesis of periodontal

78   disease and *in vitro* studies that support that bacteria possess the molecular machinery to

79   perform invasion and deceive host defenses[7], few *in vivo* studies were conducted[13-16], letting the

80   idea in abeyance throughout the years.

81   Findings of putative periodontal pathogens in healthy sulci[17], the impossibility to discriminate

82   periodontal disease by microbiologic analysis[18], the inability to categorically refute the

83   possibility that bacteria were not artificially introduced into the tissues during collection or

84 processing, in some studies[19], and evidence of internalized putative periodontal pathogens in

85 epithelial buccal cells of healthy individuals[20,21] helped to sustain this doubt.

86 As the comprehensive knowledge of the whole dynamic of the periodontal interface is crucial

87 for improving diagnostics and setting effective and rational treatments and the exact

88 importance of bacteria invasiveness in the etiopathogenesis of periodontal disease remains

89 unclear there is a need for a systematic assessment of the literature on this topic. The aim of the

90 present systematic review was to assess the existing scientific literature to ascertain the

91 relationship between the quality or quantity of periodontal microbiota in periodontal tissues

92 and periodontal disease.

93

94 **RESULTS**

95

96 **Search and selection results**

97 The search resulted in 440 unique papers, in which titles and abstracts were screened (for details

98 see Fig. 1). Of these, 26 met the eligibility criteria[14,22-46] and were selected for full text reading.

99 One study was in Japanese and was excluded.[36] Twenty two studies were subsequently excluded

100 for specific reasons: 3 studies[34,35,37] had a study protocol not related to the objectives of this

101 systematic review, 8 studies[14,25-27,30,39,40,42] had absence of control group (non-diseased samples),

102 one study[38] only presented disease samples from a single patient, 7 studies[28, 29, 31-33,41,44] hadn't

103 clearly defined inclusion and/or exclusion criteria, and finally three studies[43, 45, 46] were excluded

104 because they only addressed opportunistic pathogens. Additional hand searching of the

105 reference lists of the selected papers didn't retrieve any additional studies. As such, only three

106 papers were included in the present systematic review.

107

108 **General trial characteristics and heterogeneity**

109   The three included studies were case-control studies with considerable heterogeneity. In all

110   studies subjects with systemic diseases and antibiotic intake in the previous 6 months were

111   excluded. Two studies (#2, #3) also excluded pregnant women. In two studies (#2, #3) diseased

112   sites were defined as having probing pocket depth (PPD) and clinical attachment lost (CAL) ≥ 4

113   mm and healthy sites as having PPD ≤3 mm and CAL<4 mm while in the other study (#1) diseased

114   sites were defined as having PPD>5, bleeding on probing (BOP) and suppuration and healthy

115   sites as having PPD<5 with no BOP or suppuration. No information about CAL thresholds was

116   provided in this last study. One study (#2) provided smoking habits information of the

117   participants while in the other two (#1, #3) this information was not reported. Two studies (#1,

118   #3) had separate case and control groups of participants. In one study (#2) each participant

119   provided samples from healthy sites and diseased sites.

120   Two studies used DNA-DNA checkerboard (#1, #2) to assess the presence of intracellular or

121   adherent bacteria contrasting with study #3 where fluorescence *in situ* hybridization (FISH) was

122   used. All studies applied probes for the detection of *Porphyromonas gingivalis, Aggregatibacter*

123   *actinomycetemcomitans, Tannerella forsythia and Treponema denticola.* Study #1 additionally

124   aimed to detect *Prevotella intermedia, Prevotella nigrescens, Capnocytophaga ochracea,*

125   *Fusobacterium nucleatum subsp vicentii, Campylobacter rectus, Veillonella parvula,*

126   *Streptococcus sanguis, Streptococcus oralis, Streptococcus intermedius* and *Peptostreptococcus*

127   *micros.* In addition to all these, study #2 also aimed to detect *Actinomyces naeslundii,*

128   *Actinomyces viscosus, Actinomyces odontolyticus, Actinomyces israelii, Actinomyces*

129   *gerencseriae, Capnocytophaga sputigena, Capnocytophaga gingivalis, Campylobacter showae,*

130   *Eubacterium nodatum, Eikenella corrodens, Fusobacterium periodonticum, Fusobacterium*

131   *nucleatum* subsp. *polymorphum, Fusobacterium nucleatum* subsp. *nucleatum, Gemella*

132   *morbillorum, Leptotrichia buccalis, Neisseria mucosa, Propionibacterium acnes, Streptococcus*

133   *anginosus, Streptococcus constellatus, Streptococcus gordonii, Streptococcus mitis* and

134   *Selenomonas noxia.* For global bacteria counts study #3 used DNA probe EUB338. This probe is

135   universal for eubacteria. The studies #1 and #3 inferred global bacteria counts by the available

136   data but didn´t use any specific method. Detailed information regarding the studies

137   characteristics is presented in table 1.

138

139   **Quality analysis**

140   Methodological quality scores were given according to predetermined criteria (table 2). The

141   highest level of evidence, with low risk of confounding or bias and a moderate probability that

142   the relationship is causal, was attributed to Study #3.

143

144   **Outcome measurements**

145   *Primary outcome* - Study #1 found statistically significant differences (Fisher´s exact test)

146   regarding the presence of *A. actinomycetemcomitans* (16.7% vs. 0%, p=0.043), *T. forsythia* (75%

147   vs. 11,1%, p<0.001), *P. intermedia* (54.2% vs. 7.4%, p<0.001), *C. ochracea* (37.5% vs. 0%,

148   p<0.001) and *C. rectus* (29.2% vs. 0%, p=0.003) in epithelial cells from periodontal pockets *versus*

149   healthy sulci. Studies #2 and #3 did not found statistically significant differences regarding

150   specific bacteria.

151

152   *Secondary outcome* - All three studies reported that there were quantitatively more unspecific

153   bacteria in or on the epithelial cells taken from a diseased site when compared with a healthy

154   sulcus, however objective data is only shown in study #3. This study presented a statistically

155   significant higher percentage (chi-square test) of epithelial cells with more than 100 bacteria

156   (>100) in periodontal pockets of subjects with periodontitis compared with epithelial cells from

157   healthy sulci of periodontally healthy subjects (35.8% vs. 0%, p<0.05). No statistically significant

158   differences were found for cells harboring 1-20 and 21-100 bacteria. Interestingly, there were

159   also no differences in the percentage of epithelial cells with 1-20, 21-100 or >100 bacteria from

160   periodontal pockets and healthy sulci of periodontitis subjects.

161

Evidence profile

For quality rating of evidence a several aspects were taken into consideration. The case-control

design (observational) of all included studies, the low number of studies, the imprecise or sparse

data, the uncertainty about directness and the high risk of bias suggest that results are subject

to limitations and therefore must be interpreted with caution (very low quality rating).

167

DISCUSSION

This systematic review attempted to gather the available evidence on the *in vivo* presence of

periodontal pathogens in periodontal tissues and its relationship with periodontal disease.

171

Summary of main results

All selected studies describe the presence of intracellular and/or adherent bacteria to gingival

epithelial cells, *in vivo*, either in periodontal pockets or in healthy sulci. These observations not

only corroborate the *in vitro* findings of this bacterial ability[47-52], but also sustain that invasion of

periodontal tissues occurs even in the presence of a hostile and apparently competent (potential

cases with systemic diseases were excluded) immune system. They also substantiate that

bacterial invasion, *per se*, is a common event both in health and in disease as suggested by

several authors.[16,20,21,53]

Study #1 found significantly more *A. actinomicetemcomitans*, *T. forsythia*, *P. intermedia*, *C.*

ochracea and *C. rectus* intracellularly or adhered to epithelial cells from periodontal pockets

when compared to healthy sulci. However, this observation was not supported by the other two

case-control studies. In fact, none of these two latter studies found a statistically significant

difference in any of the putative periodontal pathogens when comparing health and disease.

The lack of consistency in the findings could result from different methodological approaches

regarding microbial identification and case and control definitions. Sudies #1 and #2 used the

187   DNA-DNA checkerboard technique. In this technique a positive reaction was recorded only when

188   a signal was greater or equal to (10^4) bacterial cells of the searched specie. This means that only

189   substantial differences in the bacterial load were detected. Study #1 hypothetically potentiated

190   differences by defining cases as having suppuration besides PPD >5 and BOP. A much higher

191   polymorphonuclear neutrophil (PMN) and microbial load in suppurative pockets when

192   compared with healthy sulci[54] could be a possible explanation for the significant differences

193   found, with such a small sample. Study #2 hypothetically attenuated possible existing

194   differences by comparing periodontal pockets and healthy sulci from the same subjects with

195   periodontitis. It has been recognized that healthy sulcus from subjects with periodontal disease

196   harbor greater number of pathogens when compared with a subject with a healthy

197   *periodontium.*[55,56] Study #3 used the FISH technique. In this technique a sample was considered

198   positive when at least one epithelial cell in each microscopic field observed presented

199   fluorescent bacteria from the hybridized species-specific probe. Bacterial numbers were

200   estimated by direct counting. Unlike DNA-DNA checkerboard, FISH allows smaller differences to

201   be detected, however as clusters of bacteria are not dispersed the real number of bacteria could

202   be underestimated. Despite all this, all studies seemed to be unanimous in considering that

203   there were quantitatively more bacteria in or on the epithelial cells taken from a diseased site

204   than from a healthy sulcus.

205   The absence of a significantly higher amount of the 'traditional' periopathogens and the

206   apparent increase of the 'commensal' flora in disease could also be interpreted with reference

207   to the concept of keystone periopathogen described by Hajishengallis.[57] Accordingly to

208   Hajishengallis, keystone pathogens, specifically *P. gingivalis*, seemed to be able to remodel a

209   symbiotic community into a dysbiotic state by imparing innate immunity in ways that enhanced

210   uncontrolled growth of other species, while being a quantitatively minor constituent of the

211   periodontal microbiota.

212

Limitations

213

214 The studies included in this review are all non-randomized case-control studies. Although having

215 inherent limitations and less level of significance, systematic reviews of observational studies of

216 etiology are especially important. This type of studies are often limited in size and so, only by

217 the simultaneous examination of data from similar studies we can achieve insight into real and

218 spurious associations.[58]

219 Concerning, however, the scope of this review, the reduced number and the limitations of the

220 included studies preclude a high or even moderate evidence of association between invasion of

221 the *periodontium* by periodontal pathogens and periodontal disease. The current available data

222 is of low to moderate quality and inconsistent mainly due to study design, poor reporting and

223 methodological diversity.

224

CONCLUSION

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226 There is insufficient evidence to support or exclude the invasion by periodontal pathogens as a

227 key step in the etiopathogenesis of periodontal disease. This review highlights the need of

228 further studies on this topic. Future research should rely on large sample studies, with clear case

229 and control definitions ensuring representativeness of cases and comparability of cases and

230 controls. It´s also important to ascertain exposure to invasive periodontal pathogens with

231 maximum detail (e.g. strains, intracellular/intercellular, synergisms) using a blinded operator.

232

METHODS

233

234 This systematic review was conducted in accordance with the guidelines of Cochrane

235 Collaboration[59] and Transparent Reporting of Systematic Reviews and Meta-Analyses [PRISMA

236 statement].[60] The focused question was given in the following: What is the relationship between

237 the quality or quantity of putative periodontal pathogens in periodontal tissues and periodontal

238 disease?

239

Eligibility criteria

All studies that reported evaluation of periodontal pathogens invasion on human tissues were considered eligible. To be as inclusive as possible, no restrictions were applied with regard to the year of publication or to language. Exclusion decision of non-English papers was delayed until the next step. Reviews and case reports were excluded.

Search Strategy

Four Internet sources of evidence were used in search of appropriate papers that fulfill the purpose of the study: the National Library of Medicine, Washington D.C. (MEDLINE-PubMed), the Cochrane Central Register of Controlled Trials (CENTRAL), ISI Web of Knowledge (Thomson Reuters) and SCOPUS (Elsevier). The databases were searched up to 22nd of January, 2014. A structured search strategy was developed to include any published paper that investigated the invasion of periodontal pathogens in patients with periodontal disease. The following strategy was used in the search: (((periodontitis OR "periodontal disease" OR "periodontal pocket") AND (identification OR detection OR localization) AND bacteria*) AND invas* OR intracellular OR tissue* OR "epithelial cells")). Key words were combined with Boolean operators and the asterisk symbol (*) was used as truncation. The search strategy was customized according to the database being used.

Screening and selection

Firstly, two independent reviewers (LM and MP) screened titles and abstracts for eligible papers. Secondly, the full texts of those papers were obtained and screened in relation to the study purpose, protocol and reported data. An additional hand search across the reference list of the selected studies was made as an attempt to find additional papers that could meet the eligibility criteria.

265

Data extraction and analysis

Data from the papers that met the selection criteria were processed for analysis. Information

regarding year of publication, study design, population, inclusion and exclusion criteria, case and

control definitions, characteristics of participants, type of intervention, ascertainment of

exposure, cell sample, microorganisms found and its location and statistical analysis were

collected by LM e MP using data extraction sheets.

Heterogeneity of trials

The heterogeneity across the trials was detailed according to the following factors: population

characteristics, study design, case and control definition, used methodology and type pathogens

found.

Quality assessment

The methodological quality of the selected studies was independently scored by two reviewers

(LM and MP). Any disagreement was resolved after additional discussion. As all eligible papers

were non-randomized studies (case-control studies) methodological quality was assessed

combining several proposed criteria of the STROBE statement[61], the Newcastle – Ottawa quality

assessment scale[62], the Downs and Black checklist for non-randomized studies[63] and SIGN50

guidelines[64] as suggested by the Quality Assessment Tools Project Report elaborated by the

Canadian Agency for Drugs and Technologies in Health.[65] In short, a study was classified as high

quality with a very low risk of confounding or bias and a high probability that the relationship is

causal (++) when all of the following criteria were adequately addressed: appropriate and clearly

focused question, defined exclusion criteria, case selection, control selection,

representativeness of cases, comparability of cases and controls, ascertainment of exposure,

blindness, funding and statistical analysis. When some of the criteria have not been fulfilled or

291 not adequately described but this fact is thought unlikely to alter the conclusions the study was

292 classified as well conducted study with low risk of confounding or bias and a moderate

293 probability that the relationship is causal (+). When some of the criteria have not been fulfilled

294 or not adequately described and this fact is thought likely to alter the conclusions (-) the study

295 was classified as a study with a high risk of confounding or bias and a significant risk that the

296 relationship is not causal (-).

297

298 **Outcome variables**

299 Primary outcome measures of interest were intracellular or adherent (to cell /periodontal

300 tissues) mean value or percentage of known pathogens. Secondary outcomes of interest were

301 global counts of intracellular or adherent (to cell /periodontal tissues) unspecific bacteria.

302

303 **Evidence profile**

304 The Grading of Recommendations Assessment, Development and Evaluation (GRADE) system[66]

305 as proposed by the GRADE working group was used for grading the collective evidence emerging

306 from this review. Two reviewers (LM and MP) rated the quality of the evidence for outcomes

307 across studies. Any disagreement was resolved after additional discussion.

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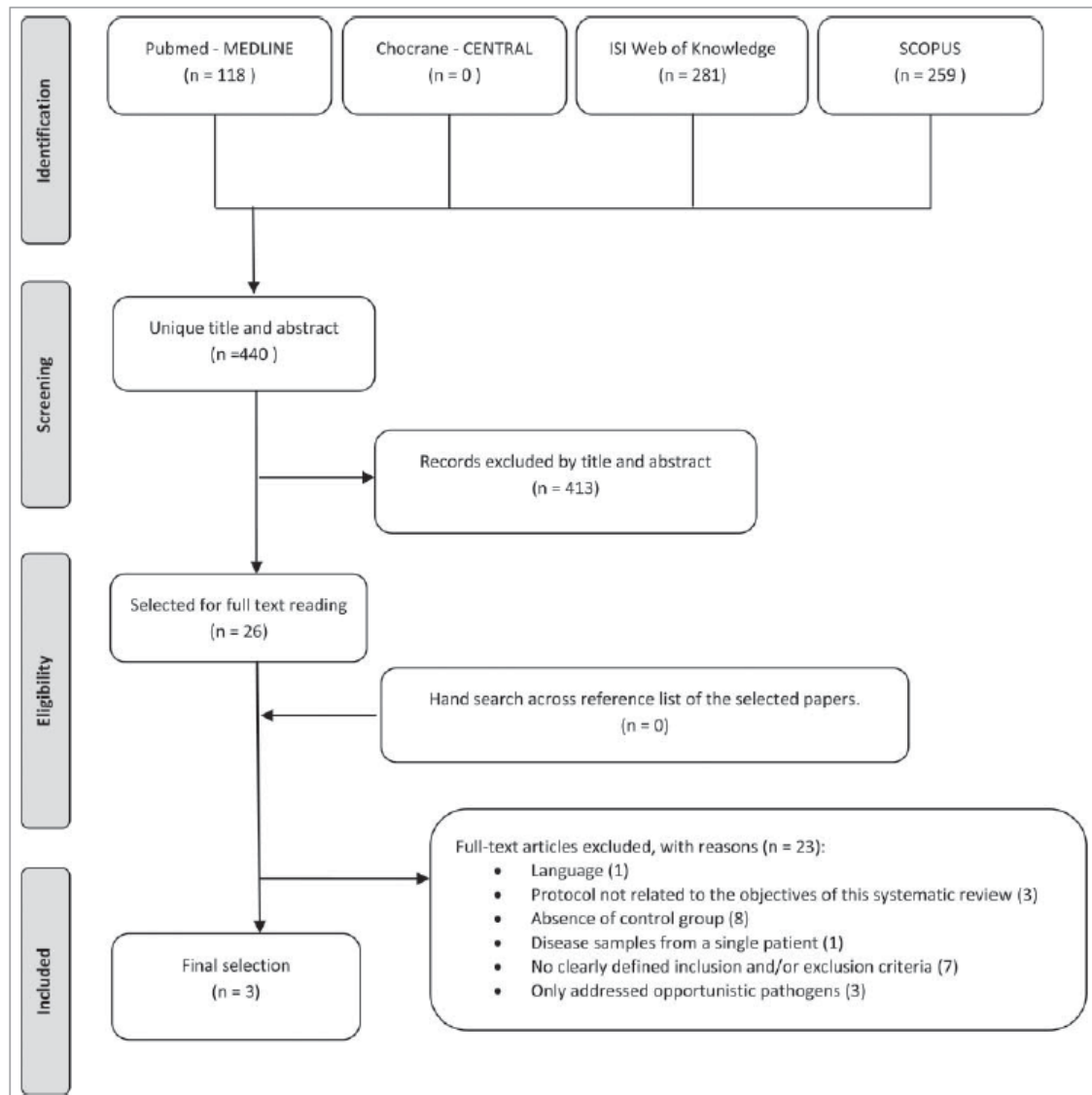559   *BMJ* 2004; 328(7454):1490.
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562   **FIGURES**
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566   *Fig. 1*. Flowchart of literature search and study selection

Table 1. Characteristics of included studies

#	Study design		n	Population	Male/Female%(age±SD)	Smokers (%)
1	Dibart et al. (1998)	Case-control	**Cases** 24	Patients from Center for Clinical Research in Periodontal Disease	*Unknown*	*Unknown*
			Control 27	Periodontal healthy dental students		
2	Colombo et al. (2006)	Case-control	**Cases** 120	Patients with moderate to severe CP from Federal University of Rio de Janeiro	43/57% (46.3±1.4)	Non-smokers (51%) Past smokers (27%) Smokers (22%)
			Controls 92			
3	Colombo et al. (2007)	Case-Control	**Cases** 175	Patients with CP from Federal University of Rio de Janeiro	57/43% (44.2 ±1.4)	*Unknown*
			Controls 68	Periodontal healthy subjects	21/79 % (29.9 ± 2.3)	

A. Subject characteristics

PPD = Probing pocket depth (mm); CAL = Clinical attachment loss (mm); PI= Plaque index (%); BOP = Bleeding on probing

Table 1. Characteristics of included studies

| | | | B. | Intervention characteristics | | |
#	Sample	Sample site characteristics		Intervention	Microorganisms	Microbiological differences *Bacteria (disease vs. health, p)*
1	Epithelial cells from periodontal pockets	Cases	PPD= 6.5	DNA-DNA checkerboard	*P. gingivalis, A. actinomycetemcomitans, T. forsythia,T. denticola, P. intermedia, P. nigrescens, C. ochracea, F. nucleatum subsp vicentii, C. retus, V. parvula, S. sanguis, S. oralis, S. intermedius* and *P. micros*	Aa. (16.7% vs. 0%, p=0.043) T f. (75% vs. 11,1%, p<0.001) P i. (54.2% vs. 7.4%, p<0.001) Co. (37.5% vs. 0%, p<0.001) Cr. (29.2% vs. 0%, p=0.003)
		Controls	PPD = 2.5			
2	Epithelial cells from periodontal pockets	Cases	PPD = 5.8 ± 0.3 CAL = 6.8 ± 0.3	DNA-DNA checkerboard	*P. gingivalis, A. actinomycetemcomitans, T. forsythia,T. denticola, P. intermedia, P. nigrescens, C. ochracea, F. nucleatum subsp vicentii, C. retus, V. parvula, S. sanguis, S. oralis, S. intermedius, P. micros A. naeslundii, A. viscosus, A. odontolyticus, A. israelii, A. gerencseriae, C. sputigena, C. gingivalis, C. showae, E. nodatum, E. corrodens, F. periodonticum, F. nucleatum* subsp. *polymorphum, F. nucleatum* subsp. *nucleatum, G. morbillorum, L. buccalis, N. mucosa, P. acnes, S. anginosus, S. constellatus, S. gordonii, S. mitis* and *S. noxia,*	No statistically significant differences regarding specific bacteria .
		Controls	PPD = 1.8 ± 0.1 CAL = 2.3 ± 0.1			
3	Epithelial cells from periodontal pockets, gingival crevice and buccal mucosa	Cases	PPD = 5.8 ± 0.3 CAL = 6.8 ± 0.3 PI = 86 ± 11 BOP = 70 ± 18	Fluorescence in situ hybridization laser-scanning confocal microscopy	*P. gingivalis, A. actinomycetemcomitans, T. forsythia* and *T. denticola.*	No statistically significant differences regarding specific bacteria GBC (35.8% vs. 0%, p<0.05).
		Controls	PPD = 1.8 ± 0.1 CAL = 2.3 ± 0.1 PI = 27 ± 15 no BOP			

PPD = Probing pocket depth (mm); CAL = Clinical attachment loss (mm); PI= Plaque index (%); BOP = Bleeding on probing; Aa.=A. actinomycetemcomitans; Tf.=T.

Forsythia; Pi.=P. intermedia; Co.= *C. ochracea; Cr.= C. retus; GBC= global bacterial counts in or on* epithelial cells (>100).

Table 2. Quality assessment of included studies

#		Appropriate and clearly focused question	Defined Exclusion Criteria	Case Selection	Control Selection	Representativeness of cases	Comparability of cases and controls	Ascertainment of Exposure	Blindness	Statistical Analysis	Funding	Overall assessment
1	Dibart et al. (1998)	2	2	1	1	1	0	1	0	1	2	-
2	Colombo et al. (2006)	2	2	2	1	1	2	1	0	1	2	-
3	Colombo et al. (2007)	2	2	2	1	1	2	2	0	1	2	+

(0 = not addressed, 1 = poorly addressed, 2 = adequately addressed)

(++ All or most of the criteria have been fulfilled. Where they have not been fulfilled the conclusions of the study or review are thought very unlikely to alter; + some of the criteria have been fulfilled. Those criteria that have not been fulfilled or not adequately described are thought unlikely to alter the conclusions; - some of the criteria have been fulfilled. Those criteria that have not been fulfilled or not adequately described are thought likely to alter the conclusions.)