Molecular tools for preventing and improving diagnosis of peri-implant diseases

FRANCESCO CARINCI[1], GEORGIOS ROMANOS[2], LUCA SCAPOLI[3]

[1]*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy*

[2]*Department of Periodontology, School of Dental Medicine, Stony Brook University, Stony Brook, NY, USA*

[3]*Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy*

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Corresponding Author:
Francesco Carinci
Department of Morphology, Surgery and Experimental Medicine
University of Ferrara
Via Luigi Borsari, 46
Ferrara
Italy
phone/fax: +39.0532.455874
e-mail: crc@unife.it
Web: www.carinci.org

Abstract

Peri-implantitis is an inflammatory process of tissues surrounding osseointegrated dental implants. Inflammation affecting soft and hard tissues causes alveolar bone resorption and subsequent implant loss. Clinical surveillance and early diagnosis are of paramount importance to reduce clinical failures and improve implant survival.

Today diagnosis is based on clinical and radiological signs. Molecular tests are emerging tools, which can potentially help clinicians to prevent and early detect peri-implantitis, as well as monitoring efficacy of therapy.

Scientific research has proposed a plethora of potential markers to support clinical diagnosis of peri-implantitis. However, conflicting evidences were common, mainly due to weak statistical results due to limited sample size or disease ascertainment heterogeneity.

The present paper reviewed candidate diagnostic markers of peri-implantitis, including infective agents, genetic susceptibility factors, and key proteins related to inflammation and tissue remodeling.

Key words: *bacteria, diagnostic tests, genetic susceptibility, implants, peri-implantitis*

Introduction

Peri-implant mucositis (PM) and peri-implantitis (PI) are inflammatory processes around implants, where bacteria and immunologic response are considered the natural target of scientific investigation.

Today diagnosis is based on clinical and radiological evidences (Figs. 1, 2). Ideally, a molecular test consists in a reliable tool for preventing diseases, make early diagnosis, tailoring therapy, check treatment, customize clinical recalls and improve patient compliance and therapeutic alliance. Molecular test integrate (with additional information) a clinical and radiological diagnosis.[1,2]

In regards to timing, molecular test should be done in conjunction with radiological evaluation. Usually, when patient is clinically evaluated for the first time by the dentist, a radiograph is performed to check the status of hard tissues. In a similar way it is important to evaluate type and load of different bacteria resident in the oral cavity. Samples are collected by means of a paper tip, shipped to lab and a report is emailed to doctor after few days (Fig. 3). Then the treatment planning is implemented starting from oral hygiene to more complex treatment approaches (Fig. 4). If surgery is scheduled, a second microbiological test should be performed in order to verify the efficacy of pre-surgical hygiene condition before operation. Finally, after the end of treatment, patient should be evaluated with a test to detect if his home care is adequate or not. A tailored recall program is therefore planned.

To become a routine tool of daily practice, molecular tests should be easy and rapid to perform, gave a result in a short time, easy to be understood (possible also by patient), and cheap. Generally speaking there are two types of tests: screening test, such as pregnancy test (that is performed by patient at home and investigate one marker) and lab test (that is performed by dentists or dental hygienist with a precise sampling protocol and investigate several markers). Lab test usually follows screening test. In medicine and specifically in gynecology, a woman

performs a screening test at home and then she goes to the specialist, who will ask for a panel of markers. In a similar way, microbiological tests should be performed by the dentists.

Since PM and PI became a burning topic, molecular tests are seen as a potential tool to ameliorate diagnosis and treatment of both diseases. Although it certainly is, dentist should keep in mind that molecular tests should be routinely used to plan therapy and to build a prevention program rather than for rescue operations, exactly similarly to radiographic evaluation.[1,2]

Infective factors

It is commonly accepted that bacteria are major player in PM and PI as well as in periodontal diseases. It is also well known that periodontally compromised patients have a higher risk of PM and PI than edentulous subjects.[3,4] However, it is evident that there are substantially different in terms of anatomy and physiology between periodontium and the interface implant-mucosa/bone, which reflected in difference of bacterial profile[5,6] and immune response between the two disease groups.[7]

Initially, the aim of researches was to detect specific bacteria differentially associated (in terms of quality and quantity) to PM and PI, and to periodontal diseases. For this purpose a limited panel of periodontal pathogenic bacteria was investigated first[8-10] and then high quality technologies were used.[6,11-14] The result was more complex than expected one and it obligates researchers to change their starting hypothesis from few specific pathogens to a dynamic condition, where a relative amount of selected bacteria growth and coordinate other species and the host immune defense.[15]

Cortelli et al.[8] investigated *Porphyromonas gingivalis*, *Tannerella forsythia*, *Campylobacter rectus*, *Prevotella intermedia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans* to test the hypothesis that there is a higher bacterial frequency in

PI/PM, followed by mucositis/gingivitis and peri-implant/periodontal health and also similar bacterial frequency between comparable peri-implant and periodontal clinical status. They concluded that there was a trend towards higher bacterial frequency around teeth than around implants.

Topcuoglu et al.[10] analyzed 10 periodontal bacteria in 84 patients with generalized aggressive periodontitis, generalized chronic periodontitis, PI, localized aggressive periodontitis and refractory chronic periodontitis. The red complex bacteria were the most prevalent with very high levels in all groups.

Persson et al.[9] identified that a cluster of seven bacterial species is associated with PI. The microbiological data also identified that the total bacterial load in PI for these seven species (*T. forsythia*, *P. gingivalis, Treponema socranskii*, *Staphylococcus aureus*, *Staphylococcus anaerobius*, *Streptococcus intermedius*, and *Streptococcus mitis*) is approximately four times higher than at healthy peri-implant sites. Thus, the bacterial burden as such may be an important factor in PI.

Subsequently, more extensive investigations were performed, by using high quality technologies. Dabdoub et al.[11] performed a deep-sequencing approach to identify the degree of congruence between adjacent peri-implant and periodontal bacteria in healthy and diseased conditions by analyzing partially edentulous subjects. About 80% of patients shared less than 10% of microbes between teeth and implants. Additionally, the periodontal microbiome demonstrated significantly higher diversity than the implant, and distinct bacterial lineages were associated with health and disease in each ecosystem. Authors concluded that simple geographic proximity is not sufficient for the colonization of topographically distinct niches, and that the periodontal and peri-implant microbiomes represent microbiologically distinct ecosystems.

Tsigarida et al.[13] studied the influence of smoking on microbiome composition either in healthy and inflammatory peri-implant conditions. In smokers, the transition from healthy to mucositis is characterized by the loss of bacterial species important for oral health and a consequent reduction of microbiome diversity. In non-smoking subjects, the transition from healthy to mucositis is characterized by the acquisition of different species without replacement of pioneers' microorganisms, creating a significant increase in diversity. PI is not very different from mucositis in terms of number and type of microbial species, both in smokers than in non-smokers subject. Smoke encourages the development of pathogenic species in the peri-implant microbiome, even in states of clinic health. However, the PM appears to be a key event in the progression of the disease to periodontitis, favoring the creation of high-risk pathogenic communities. Periodontal pathogens can play an important role in the transition from health system to peri-implant disease.

Zheng et al.[14] studying the subgingival microbiome in patients with healthy and compromised dental implants showed that microbial diversity increase in compromised implants. The pathogens usually associated with PI, *P. gingivalis*, *T. forsythia*, and *P. intermedia*, are present although in smaller quantities, also in the peri-implant mucositis, suggesting that mucositis is an important transitional phase in the development of PI. The 75% of the oral bacterial flora consists of not more than four bacterial species, which differ from individual to individual, defining periodontitis as a heterogeneous disease. Periodontitis-associated communities demonstrate greater diversity and richness when compared to healthy teeth.

By contrast PI is a simple infection. Its microbiome shows less diversity and fewer species than the periodontitis.[5]

Shiba et al.[12] characterized the taxonomic profiles of microbial species in PI and periodontitis samples by quantifying 16S ribosomal ribonucleic acid (rRNA). They found similarities in metabolic pathways and virulence factors, whereas taxonomic profiles were dissimilar between

the two diseases. They detected high abundances of *P. gingivalis*, *T. denticola*, *T. forsythia* and *Eubacterium nodatum* in both diseases. In periodontitis *E. nodatum* is in the core taxa, whereas in PI, *P. gingivalis* and *Prevotella nigrescens* were found more abundant.

From the above mentioned and additional studies[16,17] is emerging that "red complex" bacteria (i.e. *T. denticola*, *T. forsythia* and *P. gingivalis*) and "orange complex" (i.e. *Fusobacterium nucleatum*, *P. intermedia*, *Peptostreptococcus micros*, *E. nodatum*, *Campylobacter rectus*) increasing the risk of PM and PI. However, the role of periodonto-pathogens should see under the light of "keystone pathogen" hypothesis.[15] This hypothesis indicates that similar bacteria are presented in both healthy and disease[15] conditions, while the change in bacterial proportion represent the key that broken the healthy/disease ratio. Therefore, periodonto-pathogens provide permissive condition for the overgrowth of opportunistic bacteria, which shift in pathologic ones. In this regard, an evaluation of opportunistic bacteria is the key focus of recent studies. Among them are *Staphylococcus aureus* and *warneri*,[9,18] *Parvimonas micra*,[19,20] *E. nodatum*,[21] and *Streptococcus mutans*,[5] which seem to be promising candidates to be used of sentinel markers of PI.

A role of viruses in periodontal diseases have been suggested in the last 20 years, mainly focused on human herpesviruses (HHVs). Eight human HHVs with distinct biological and clinical characteristic have been described: Human Herpesvirus 1 (Herpes simplex virus type 1), Human Herpesvirus 2 (Herpes simplex virus type 2), Human Herpesvirus 3 (Varicella-Zoster virus), Human Herpesvirus 4 (Epstein-Barr Virus), Human Herpesvirus 5 (Cytomegalovirus), Human Herpesvirus 6, Human Herpesvirus 7, Human Herpesvirus 8 (Kaposi sarcoma-associated Herpesvirus 8).

The role of HHVs in periodontal diseases was extensively studied by Slots' group which demonstrated and reviewed that coinfection of periodontal HHVs and bacterial pathogens is implicated in the onset of aggressive periodontitis.[22-28]

Real Time PCR has become the benchmark methodology for the detection of HHVs nucleic acids in oral fluids.[27] Consequently, curative treatments that target both HHVs and pathologic bacteria, rather bacteria alone, could be more effective in arresting periodontitis.

A specific treatment protocol to shot both bacteria and virus was reported.[28] It is based on a couple of inward treatments associated with patients self-care at home. The first clinical therapy uses 10% povidone-iodine to reduce the virus and bacterial spread during mechanical maneuver, gross scaling with ultrasonic instrument and valacyclovir twice daily for 10 days to get a systemic anti-viral effect. Then oral rinse with 0.1% sodium hypochlorite for 30 seconds twice weekly have to be performed by patient at home to remove and preventing dental biofilm. After 10 days, a second clinical treatment is delivered by using 10% povidone-iodine during definitive scaling and root planing and subsequent prescription of antibiotics for 8 days (generally amoxicillin plus metronidazole, both 250 mg, 3 time daily).

A potential role of HHVs in PI is arising in the recent literature. Two recent papers critically analyzed the available literature.[29,30] An association between HHVs and PI was reported, but authors concluded that further investigations are needed to firmly establish this association. A not invasive diagnostic protocol detecting both relevant bacteria and HHVs nucleic acids by real time PCR could be routinely used to perform a tailored therapy in PI, likely mimicking the periodontitis therapy protocol.

Genetic susceptibility

The human genome includes a large number of sequence polymorphisms and structural variations. The 1,000 genome-project estimated that a typical genome differs from the reference human genome at 4.1 million to 5.0 million sites.[31] Inherited sequence variations, together with postnatal somatic mutations and epigenetics regulation account for the human genetic diversity.

Most of sequence variations is likely to have no phenotypic effect, while rare mutations or common polymorphisms affecting coding and regulatory sequences could have an impact on individual characteristics, including disease susceptibility. Indeed, gene sequence variation affecting regulatory elements could influence gene expression levels, while coding sequence variation could impair protein function or alter mRNA stability.[32]

It was observed that implant failure risk was higher if the patient had already had one implant removed. This suggests the existence of systemic/genetic factors affecting implant survival. Among other known or potential patient-related systemic risk factors, such as systemic diseases, genetic traits, chronic drug or alcohol consumption, and smoking status, genetic inherited susceptibility factors have been taken in account by scientific research. The case-control association analysis of candidate genes has been the main research strategy adopted for the discovery of genetic risk factors of PI. Candidate genes have been selected based on the current knowledge of pathogenesis of PI. Bacterial biofilm and particularly lipopolysaccharides of the cell walls of gram-negative bacteria can induce monocytes and macrophages to release pro-inflammatory mediators, such as interleukin-1α (IL-1α), interleukin-1β (IL-1β), and tumor necrosis factor-alpha (TNF-α). These cytokines stimulate residential cells, such as fibroblasts to produce prostaglandins and metalloproteinases (MMPs) promoting the destruction of extracellular matrix and further loss of alveolar bone (Fig. 1).[33]

Genes IL1A and IL1B codes the pro-inflammatory proteins IL-1α and IL-1β, respectively, while the gene IL1-RN controls the synthesis of the interleukin-1 receptor antagonist, an anti-inflammatory non-signaling molecule, which inhibits the effect of both IL-1α and IL-1β. These three genes map in a gene cluster within a ~360-kb region.[34] Several studies investigated polymorphisms of these loci for association with PI. Discordant results were obtained, with some investigations supporting association between interleukin-1 and PI, other only when additional factors were considered, while other negating any association.[35,36] It is worth noting

that many studies lacked of statistical power to reach relevant conclusion, because the number of patients involved was too small to detect medium or low effect sizes. Liao and colleagues (2014) performed a meta-analysis of the association between interleukin-1 polymorphisms and dental implant failure.[37] The pooled data indicated that no interleukin-1 variant by itself was associated, while the composite genotype of IL1A (-889) and IL1B (+3954) was associated with an increased risk of implant failure/loss (OR 1.76, 95% CI 1.21–2.57) and PI (OR 2.34, 95% CI 1.03–5.33). The analysis revealed also population heterogeneity with lower risks among Europeans. Based on the current knowledge, it can be assumed a correlation between interleukin-1 polymorphism and PI; however, the level of association and the risk rise for variants carriers appear weak for fruitful diagnostic applications.

The association between TNF-α polymorphisms and dental implant disease risk was investigated in another meta-analysis.[38] In this case, no support for a role of TNF-α was found; however, authors concluded that further studies on different ethnicities with large sample sizes should be conducted to support this conclusion.[38]

A recent investigation reported remarkably association with additional candidate genes. Coelho and colleagues investigated association between PI and bone morphogenetic proteins (BMPs) or fibroblast growth factors (FGFs) members because of their involvement in bone remodeling.[39] This study, involving 215 patients, found elements supporting a possible role for BMP6 and FGF10 in PI.

Matrix metalloproteinases (MMPs) represent the major class of degrading extracellular proteins, including type I collagen, contributing to degradation and tissue remodeling.[40] Interestingly, evidence of association between polymorphisms of the MMP8 promoter and early implant failure was reported.[41]

The membrane protein CD14 is considered the major endotoxin receptor, it is able to recognize lipopolysaccharides of Gram-negative outer membrane and initiate the innate immune response

to bacterial invasion. The CD14 modulates the subsequent production of pro-inflammatory cytokines and appears to have a central role in bone adsorption triggered by lipopolysaccharides.[42] Interestingly, a functional SNP in the CD14 gene promoter was found associated with PI.[43] Considering the high level of association observed (OR > 5), CD14 represent promising markers for PI diagnosis.

Altogether, the research effort spent so far to identify genetic factors influencing implant failure did not produce sufficient data to develop diagnostic applications now. However, several promising data need to be rigorously confirmed by independent studies. An elevated number of patients to accurately calculate risk levels, as well as diagnostic standards for case and control selection are determinant factors needed to transfer research data to clinical application.

A great advantage of genetic-based diagnostic tool is that it is either applicable before or after treatment or disease onset. Furthermore, polymorphism genotyping of genomic DNA is more robust and easier to perform with respect to evaluation of biomarkers in peri-implant fluids, which is subject to several technical and operator-based variables. An accurate patient tailored risk evaluation based on DNA analysis, may be by a combination of different susceptibility factors, could help clinical practice for treatment planning, selection of appropriate treatment solution, as well as for prognosis of dental implants.

Molecular markers

The saliva, a multi-constituent oral fluid, has been demonstrated to be an optimal diagnostic source with high potential for disease detection and the health surveillance.[44] Saliva has been proposed as a diagnostic fluid not only for oral diseases, such as caries, periodontitis and oral cancer, but also for systemic diseases, including diabetes, non-oral cancer, autoimmune, viral, bacterial and cardiovascular diseases.[45,46] Although saliva-based diagnostics has many advantages, because the collection of saliva samples is noninvasive, safe, and inexpensive, the

peri-implant fluids appear a more suitable source of biomarkers to obtain more precise diagnostic information from a specific sampling site.[47]

A broad research effort has been spent to identify biochemical markers associated with peri-implant disease. Ideally, a diagnostic test able to evaluate molecular markers in peri-implant sulcus fluids could flank clinical and radiographic examination to monitor the health level of peri-implant tissues. To date, different cytokines, enzymes, and proteases have been investigated because of their known roles in inflammation or in tissues damage.[48] The pro-inflammatory mediators IL-1β and TNF-α, were the most investigated and promising biomarkers to assist in the early diagnosis of PI.[48,49]

Most studies reported increased levels of IL-1β in PM and PI,[50-53] while other authors did not find significant differences between healthy implant sites and implants with PI[54,55] as reviewed before.[49,56] Significant heterogeneity between published investigations - in terms of study design, disease definition, assessed parameters, as well as measured outcomes - make difficult study comparisons. For instance, biomarker levels were commonly reported either as total amount, or as concentration, thus preventing the quantitative synthesis of the results by meta-analysis.[49] A meta-analysis that combined a small number of homogeneous investigations concluded that IL-1β, as well as TNF-α, can be used as additional criteria for diagnosis of peri-implant infection, but cannot help to discriminate between PM and PI.[56] The scientific community should make a serious attempt to promote some levels of standardization in both clinical and technical procedure in order to make studies more comparable and speed up translation from scientific evidences to clinical applications.[47] Another issue that prevents making strong conclusion is the limited number of patients considered in each investigation. Remarkably, Ramseier and colleagues[57] reported biomarkers evaluation at teeth and implants of hundreds of patients 10 years after implant placement. In regards to IL-1β, they observed no concentration differences between samples from implants and adjacent teeth, but significant

differences were detected when different periodontal and peri-implant conditions were compared. Indeed, IL-1β was elevated in inflamed peri-implant tissues and correlated with increased probing depths.[57]

In the same investigation the Matrix Metalloproteinase-8 (MMP-8), displayed a trend similar to IL1-β: elevated in inflamed implants and correlation with clinical parameters, such as bleeding on probing and increased probing depth.[57] This confirms earlier reports about elevated MMP-8 levels in inflamed tissues around implants.[58,59] MMPs are responsible of the irreversible connective-tissue degradation and loss of attachment in both periodontitis and PI. Among different MMPs and tissue inhibitor of metalloproteases (TIMPs), the MMP-8 (also called neutrophil collagenase or collagenase 2) has been selected as more promising for diagnostic tool.[60,61] Indeed, MMP-8 is the most competent proteinase to initiate type I collagen and extracellular matrix degradation and it is present in periodontitis and PI affected tissue, gingival crevicular fluid, peri-implant sulcular fluid, saliva and mounthrinse samples.

Recently, Sorsa and Coll[62] outlined the effectiveness of MMP-8 as a periodontal and peri-implant disease biomarkers to monitor the active process of destruction around tooth and implant. Diagnosis of periodontal and peri-implant disease is currently based on the measurement of attachment loss, pocket depth and bleeding on probing together with radiological evaluation. The limit of these investigations it that they ascertain the past history of tissue destruction rather than disease activity. The chance to testing MMP-8 in real time and at point-of-care is of paramount importance (1) to define starting illness with minimal clinical signs, (2) to intercept disease activity, (3) to tailor therapy, and (4) to monitor the effectiveness of treatment and home-care maintenance.

Molecular tests on the market

Routine monitoring of dental implants is essential to prevent biological complications or failures. Clinical and radiological assessments are usually sufficient to determine implant health or inflammatory complications. Although there are no approved diagnostic guidelines for molecular testing, a number of scientific studies suggest that such tests could be useful to identify risk factors associated with developing peri-implant diseases thus favoring early diagnosis. On these bases, the free market offers molecular analysis service for evaluation of bacterial, genetic and inflammatory factors that may be relevant for both periodontitis and PI. Some examples of companies offering analysis service of saliva or peri-implant fluids specimens include: LAB SRL (http://www.labsrl.com/en/), GEN-TREND (www.gentrend.cz), Advanced Dental Diagnostic (www.addinternational.nl), OralDNA® Labs (www.oraldna.com), IMD labor Berlin-Potsdam (www.imd-berlin.de), Carpegen (www.carpegen.de), OralVital (www.oravitalpro.com), PerioPrevention (www.periopreventionnetwork.com).

Conclusions

Molecular tests integrate clinical diagnosis and radiographic evaluation. Differently from radiographs, molecular tests objectively identify the dynamic interplay between bacteria and human defense and thus it is relevant not only to verify the status of bacteria and cytokines but also how they change over time.

As regard bacteria, periodonto-pathogens provide permissive condition for the overgrowth of opportunistic bacteria, which shift in pathologic ones. High level of red complex pathogens (i.e. *T. denticola*, *T. forsythia* and *P. gingivalis*) increase risks of PM and PI, whereas a list of opportunistic bacteria are now investigated as promising candidates to be used as sentinel markers.

Genetic susceptibility studies did not produce any clinically applicable markers until now. Among pro-inflammatory cytokines, MMP-8 and IL1-β were elevated around inflamed

implants and correlation with clinical parameters was found, such as bleeding on probing and probing pocket depth. Therefore, molecular analysis may be relevant to diagnose, predict disease progression and manage peri-implant diseases.

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Figure legends

Fig. 1: Immediate post-operative radiograph performed in December 2012

Fig. 2: Radiograph performed in July 2017 showing bone resorption around the middle implant

Fig. 3: Peri-implant sampling for molecular based test

Fig. 4: Surgical field showing bone resorption around implant

Table 1 Potential targets for PI diagnostic tests. Table includes the most investigated and the most promising targets

Diagnostic type	Targets	References
Infective factors	*Treponema denticola*	{14,16,17}
	Tannerella forsythia	{10,14,16,17}
	Porphyromonas gingivalis	{10,16,17}
	Staphylococcus aureus	{10,18}
	Parvimonas micra	{19,20}
	Eubacterium nodatum	{14,16,17,21}
	Streptococcus mutans	{5}
	Human Herpesviruses	{28-30}
Genetic factors	Interleukin-1 (IL-1A and IL-1β)	{35-37}
	Interleukin-1 Receptor Antagonist (IL1RN)	{35,36}
	Matrix Metalloproteinase-8 (MMP-8)	{39}
	Fibroblast Growth Factors (FGF10)	{37}
	CD-14 Molecule	{40}
Molecular markers	Interleukin-1 beta (IL-1β)	{48-57}
	Tumor Necrosis Factor-alpha (TNF-α)	{41,51}
	Matrix Metalloproteinase-8 (MMP-8)	{56-59}