New tendencies in non-surgical periodontal therapy

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Abstract: The aim of this review was to update the evidence of new approaches to non-surgical therapy (NSPT) in the treatment of periodontitis. Preclinical and clinical studies addressing the benefits of adjunctive antimicrobial photodynamic therapy, probiotics, prebiotics/synbiotics, statins, pro-resolving mediators, omega-6 and -3, ozone, and epigenetic therapy were scrutinized and discussed. Currently, the outcomes of these nine new approaches, when compared with subgingival debridement alone, did not demonstrate a significant added clinical benefit. However, some of these new alternative interventions may have the potential to improve the outcomes of NSPT alone. Future evidence based on randomized controlled clinical trials would help clinicians and patients in the selection of different adjunctive therapies.

Keywords: Fatty Acids, Omega-3; Periodontal Debridement; Probiotics; Ozone; Epigenomics.

Introduction

Periodontal therapy comprises a broad range of interventions applied in a stepwise approach with the aim of controlling the infection and arresting the inflammation.[1] The first step of periodontal therapy includes the control of supragingival biofilm, both by the patient and the professional, as well as the control of those proven risk factors in the etiopathogenesis of periodontal diseases.[2,3] For patients diagnosed with gingivitis, this step of therapy should be enough to arrest gingival inflammation once biofilm accumulation has been removed. For patients diagnosed with periodontitis, the first step is a prerequisite before implementing the second step, based on the removal of subgingival biofilm and calculus, which is the basic mode of periodontal therapy. Subgingival instrumentation may include adjunctive local/systemic antimicrobial or anti-inflammatory medications.

A variety of endpoints have been evaluated in periodontal literature to assess the efficacy of these two steps of treatment, most frequently average reductions in probing pocket depth (PPD) and gains in clinical attachment level (CAL). More recently, there has been a concern to define more appropriate endpoints that should be achieved after periodontal therapy.[4] For instance, in the clinical practice guideline from the European Federation of Periodontology (EFP),[1] a clear definition of the desired outcome after treatment of Stage I-II periodontitis has been established.[5] After the completion of periodontal therapy, a stable periodontitis patient is

defined by gingival health on a reduced periodontium (bleeding on probing in < 10% of the sites; shallow probing depths of 4 mm or less and no 4 mm sites with bleeding on probing). When these criteria are met after the completion of periodontal treatment, but bleeding on probing is present at > 10% of the sites, then the patient is diagnosed as a stable periodontitis patient with gingival inflammation.

If the aims of periodontal therapy have not been achieved with this sequence of steps 1 and 2, the step 3 of periodontal therapy needs to be implemented. It consists of either repeated subgingival instrumentation or other periodontal surgical interventions. Finally, the results of step 3 should be reevaluated for the accomplishment of the therapy endpoints and, if these endpoints are achieved, a strict maintenance program should be implemented.[6,7,8]

Non-surgical periodontal therapy (NSPT) is the term used to generally describe subgingival mechanical instrumentation performed during the second step of treatment. Standard NSPT, mainly performed by means of scaling and root planing (SRP), is the gold-standard treatment for Stage I-III periodontitis.[1] Nevertheless, some sites and/or patients may present poor response to standard NSPT. This may be related to microbial factors, when this mode of therapy is not capable of converting the dysbiotic infectious process to a homeostatic/commensal balance, probably due to residual subgingival biofilm in the periodontal pocket after SRP[9] and/or tissue invasion by periodontopathic bateria,[10] or the maintenance of a non-resolving chronic inflammatory response in spite of the subgingival debridement. Therefore, there is a continuous search for adjunctive therapies that can improve the outcomes of subgingival instrumentation alone. Thus, the purpose of this narrative review was to evaluate the evidence from clinical or preclinical studies of new therapeutic approaches used as adjuncts to NSPT.

Antimicrobial photodynamic therapy (aPDT)

The major aim of aPDT is to eradicate or reduce microorganisms. This is achieved when a non-toxic light-sensitive "photosensitizer", excited by a visible light or near infrared with the appropriate wavelength, stimulates the formation of free radicals of singlet oxygen that will act as toxic agents to the bacteria. Toluidine blue O and methylene blue are the most used photosensitizers in Dentistry, although others have been studied. Both have shown to be effective for periodontal pathogen control in planktonic bacteria and in biofilms.[11,12,13] The gold-standard light for applying aPDT in the treatment of periodontitis is the low level laser (LLLT), which has shown favorable healing conditions.[14] LLLT has a potency of 30–100 MW, wavelength of 630–904 nm, and minimum thermal heat. Its efficacy depends on the amount of absorbed light. When appropriate, low intensity laser can help restore the cell balance, especially because of its influence in the inflammatory process *per se*.[15]

One of the major advantages of aPDT is that in the clinical treatment of local infections it might be less injurious to indigenous/commensal biofilms than systemic antibiotic therapy.[16] The use of aPDT was first evaluated in a split-mouth randomized controlled trial (RCT) as a sole therapy, compared with SRP in the treatment of aggressive periodontitis patients.[17] Although the clinical outcomes and biomarker levels in gingival crevicular fluid (TNF-α and RANK-L)[18] were similar 3 months after therapy, the microbiological outcomes (reduction of red complex pathogens) were more favorable in the SRP group.[19] These findings indicated that aPDT should not be considered as a single method to eliminate subgingival biofilm, but should be implemented as adjunctive to SRP.

Several preclinical and clinical studies have evaluated aPDT as an adjunctive method to SRP[17-25] with the goal of penetrating the soft tissues, thus reaching the periodontal pathogens that might have infiltrated into the periodontium.[22] Moreover, the low intensity laser is capable of favoring tissue repair by improving cell behavior and collagen synthesis.[26]

A recent systematic review with meta-analysis has summarized the findings of RCTs assessing the clinical outcomes achieved with aPDT.[27] A total of 17 trials were included and analyzed separately for participants with chronic (n = 13) and aggressive (n = 4) periodontitis. Six additional studies tested aPDT in residual sites after maintenance therapy. In general,

the achieved additional benefits did not lead to a significant effect compared to standard NSPT. In agreement with the conclusions of this systematic review, the EFP Clinical Practice Guidelines for the treatment of Stage I-III periodontitis do not recommend aPDT in a single application (wavelength 660–670 nm or 800–900 nm) in association with SRP for patients with periodontitis based on this available evidence.[1]

The frequency of application of aPDT also deserves some discussion, since there are indications that it may lead to different clinical outcomes. It has been demonstrated that multiple sessions of aPDT bring additional benefits. The first study evaluating the multiple episodes of aPDT in adjunctive to concomitant NSPT[25] found greater probing depth (PD) reduction and more clinical attachment gain 6 months after NSPT alone. Another split-mouth RCT[28] demonstrated that repeated aPDT applications (baseline, 2, 7 and 14 days) adjunctive to SRP were more effective to treat initially deep pockets (≥ 7 mm) in aggressive periodontitis than SRP alone 90 days after the therapies.

Other aspects still need investigation, for example, the specific patient populations that may benefit more from aPDT. It has been hypothesized that patients with systemic conditions with impaired healing or patients with uncontrolled diabetes may benefit from this adjunctive therapy. One study found greater reduction in HbA1c after 3 months with the association of aPDT with SRP, and IL1-β levels were also lower for the association.[29] Smokers would also represent a target population to be treated with aPDT; however, the adjunctive effect of aPDT has failed to improve clinical parameters in such patients. Also, aPDT associated with SRP did not alter periodontal biofilm composition in a positive way, either when applied in single[30] or multiple sessions.[31] Nevertheless, this is only the beginning of studies in this area of aPDT.

In summary, there is evidence that aPDT, as an adjunct to SRP and when applied in multiple applications, can improve microbiological and immunological outcomes. This application could be of interest in patients with systemic comorbidities. The advent of new LED lights and/or nano-photosensitizers may better target the pathogenic biofilm, possibly leading to more significant improvements and, consequently, further encouraging new studies to assess the use of these technologies.

Probiotics

Current concepts of etiopathogenesis of periodontal diseases consider the existence not only of specific periodontopathogens, but of a synergistic and dysbiotic microbial community.[32] Microbial dysbiosis leads to non-resolving inflammations due to alterations in the host's immunoinflammatory response. Therefore, new therapies seeking to regain the microbiome ecological balance are currently being investigated and appear to be more closely aligned with current concepts aiming to restore a healthy oral microbiome and periodontal health.[33]

In a proof of concept study, Teughels et al.[34,5] introduced *Streptococcus* species within periodontal pockets after SRP in dogs. This adjunctive therapy reduced the periodontal tissue inflammation and modified the microbial composition of the treated sites, reducing and delaying their recolonization by periodontopathogens. The study introduced the concept of guided recolonization of periodontal pockets by using oral health-compatible bacteria as a treatment strategy for periodontitis. Therefore, host microbiota modulation and direct interaction with the immune system are the basic mechanisms that can explain the beneficial effects of probiotics on periodontal health.

The term "probiotics" was introduced by Lilly and Stillwell in 1965.[35] The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have defined probiotics as "living microorganisms that, when administered in appropriate amounts, confer health benefits to the host".[36] Most of currently used probiotics are lactic acid bacteria of the *Lactobacillus* and *Bifidobacterium* genera, although fungi, species of *Bacillus, Clostridium, Propionibacterium* and Gram-negative bacteria like *Escherichia coli* have also been used as probiotics.[37] It is well documented that lactic acid-producing bacteria have antimicrobial effects on several periodontopathogens.[38-43] A recent *in vitro* study has shown that probiotics alter biofilm

formation and the transcription of *Porphyromonas gingivalis* virulence-associated genes.[44] Probiotic microorganisms provide nutrients, help the host to digest food, and compete for space and nutrients with potential pathogens. Also, they participate in the host's immunoinflammatory response, since they can induce the secretion of antimicrobial peptides or anti-inflammatory molecules through interaction with various cell populations.[45-48] Some clinical and preclinical studies analyzing saliva, gingival crevicular fluid, and gingival biopsies have shown that the ingestion of probiotics can interfere with the levels of several pro- and anti-inflammatory markers.[48-55]

Although the scope of this article is NSPT for periodontitis, probiotics have been mainly evaluated for the treatment of gingivitis, either in experimental gingivitis models[56-59] or in patients with established gingivitis.[60-62] A recent systematic review with meta-analysis[63] including 10 RCTs has summarized the results of using *L. reuteri* as a probiotic. It has shown that, in half of the included studies,[61,63-66] clinical parameters of inflammation significantly improved when compared with the control group, whereas in five studies comparable results in clinical inflammatory parameters were reported between test and control groups.[58,62,67-69] The meta-analysis, however, did not demonstrate a statistically significant difference between probiotic and placebo groups in clinical periodontal inflammatory parameters, although the results are very heterogeneous and prevent a fair evaluation of the results.

In regard to the adjunctive application of probiotics for the treatment of periodontitis, a recent systematic review assessed the impact of probiotics on clinical, microbial, and immunological outcomes when used as adjuncts to NSPT.[70] Ten RCTs were included, with all patients receiving NSPT with probiotics or placebo administration or SRP alone. Both 3- and 12-month data have shown significant benefits in the use of probiotics, with greater magnitude at 12 months in pooled estimates of PPD reductions. The results have shown a significant benefit in probiotics in both PPD and CAL when baseline mean PPD values were ≥ 5 mm. There was no significant difference in periodontal pathogen levels between groups at 3 months. Immunological data were not sufficient for quantitative analysis.

Significant inter-individual human microbiome variability mediated by factors such as age, diet, antibiotic usage, food supplements, underlying medical conditions, and patterns of circadian activity can impact the effects of probiotics.[71,72] In humans, marked person-, strain- and gut region-specific mucosal probiotic colonization patterns clustered individuals into those "permissive" or "resistant" to mucosal probiotic colonization. Importantly, these distinct colonization states had different impacts on probiotics-associated changes in the gut microbial community structure and host transcriptome.[71] Regarding the effects of probiotics on periodontal diseases, it is also mandatory to analyze the influence of the vehicle by which the probiotic strain is administered in its therapeutic potential and oral colonization, as well as its survival in the oral cavity. Additionally, more studies are required to evaluate modes of application, different therapeutic regimens, and the persistence of probiotic microorganisms in the oral cavity after discontinuation of probiotic therapy. This will provide further evidence to support the use of probiotics for NSPT. Meanwhile, this therapeutic alternative is not yet indicated in daily clinical practice.

Prebiotics and synbiotics

Prebiotics are substrates, either naturally present in certain foods or synthetically produced, which are selectively used by beneficial microorganisms colonizing the host. These substrates are intended to multiply and/or become activated metabolically to beneficially alter the composition of the host microbiota in the intestine or elsewhere in the organism.[73,74,75]

The concept of using prebiotics involves three assumptions: a substance (substrate), a mechanism, and a beneficial effect.[73] Oligosaccharide carbohydrates inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), and polydextrose are among the most studied prebiotics.[76] Several health benefits have been attributed to their application: relief of poor digestion of lactose, increased resistance to bacterial infection, and improved immune response.

The main mechanism of action of prebiotics is through the selective stimulation of the growth of beneficial bacteria in the host. Some of these microorganisms have specific enzymes that can hydrolyze prebiotic oligosaccharides, which results in the proliferation of these beneficial bacteria.[77] This selective action prevents changes in the microbial composition associated with diseases, suppressing the growth of pathogenic species.[75,78] Furthermore, prebiotics can offer protection against pathogens through direct interactions.[79] Oligosaccharide derivatives contain sugars that are specific receptors for epithelial cells. Therefore, these receptors become unavailable for the adhesion of pathogenic bacteria, which will lose their ability to colonize and will be eliminated.[80,81]

The fermentation of prebiotics in the large intestine leads to the production of short-chain fatty acids.[82] These molecules increase the intestinal absorption of calcium, reducing intestinal pH, and promote the development of intestinal villi, leading to changes in the intestinal microbiota.[83] In addition, there is evidence that some prebiotics have direct effects on the host immune system, regardless of their effects on resident bacterial populations.[80,84] These effects include stimulation of IL-10 and interferon-γ expression, increased IgA secretion, modulation of inflammatory responses to pathogens, and stabilization of the intestinal mucosal barrier.[80]

The ability of certain prebiotics to increase the growth of resident commensal intestinal bacteria, particularly bifidobacteria and lactobacilli, is well documented.[80,85] It is expected that prebiotics can cause changes in any host microbial ecosystem.[79] There is evidence in the medical literature that prebiotics may be useful in preventing several diseases, like atherosclerosis, osteopenia and/or osteoporosis, and other bone pathologies,[83,86] but only recently has the oral cavity been suggested as a relevant target for the application of this approach.[87] In Periodontics, the first studies identified potential prebiotics that could stimulate beneficial bacteria and suppress the growth of pathogenic species *in vitro*.[75,87] Two compounds, named beta-methyl-D-galactoside and N-acetyl-D-mannosamine, were able to modify two-species biofilm communities to a predominantly beneficial composition.[75] In another experiment, using multispecies biofilms, N-acetyl-D-mannosamine led to a biofilm composition with more than 97% beneficial microorganisms.[87]

The pioneering study to evaluate the effects of a prebiotic on periodontitis was carried out with β-glucans fiber from *Saccharomyces cerevisiae*, administered to rats that underwent diabetes and periodontitis inductions.[81] It demonstrated that prebiotic therapy led to reduction of alveolar bone loss and serum levels of TNF-α, and improved pancreatic β-cell function and intestinal morphology.[81] Another preclinical study, which evaluated the effects of the mannanoligosaccharide prebiotic in animals with experimental periodontitis, demonstrated that the therapy led to a reduction in periodontal destruction and levels of TNF-α and IL-1β, and an increase in TGF-β. Also, it is important to highlight that animals with experimental periodontitis have been shown to present changes in intestinal morphology, pointing to a possible interrelation between oral and intestinal ecosystems.[53,88,89] In the preclinical study with the mannanoligosaccharide prebiotic, the animals treated have shown intestinal morphology more similar to that of animals without disease, which demonstrated the protective role of prebiotics in the intestinal environment under conditions of oral dysbiosis.[88] When the results of this study are compared to other preclinical studies with probiotics, similar beneficial effects are found. However, to date, there are no clinical studies evaluating the effects of prebiotics in the management of periodontitis.

Since oligosaccharides that act as prebiotics can also improve the ability of probiotic strains to adhere to intestinal cells and mucin, it has been suggested that the use of prebiotics with probiotics, that is, synbiotic compounds, may be a potential tool to increase the numbers of probiotic bacteria and the time they remain in the body. This could even reduce the period of administration of the probiotic therapy in some cases.[90,91] A randomized placebo-controlled clinical study evaluated the effects of a synbiotic containing multispecies probiotics and FOS prebiotic as an adjunct to SRP in patients with periodontitis and type 2 diabetes mellitus.[92] The group that received

the synbiotic presented better results than patients in the control group in relation to CAL gain, plaque, bleeding on probing, total antioxidant capacity, glutathione peroxidase, and also in the reduction of IL-1β and malondialdehyde.

In this context, prebiotic or synbiotic therapies can be considered potential adjunct strategies for the prevention and/or treatment of periodontal diseases. The concept of prebiotic was only recently introduced in oral health, and knowledge about its possible effects is still in its "childhood stage" in Dentistry.[87] It is necessary to perform studies to assess the effects of prebiotics and synbiotics on periodontal diseases, as well as on the modulation of oral and intestinal microbiomes, in order to help maintain and enhance the benefits provided by the beneficial microbiota to the host.

Statins

Statins are inhibitors of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-CoA reductase) and act directly in the inhibition of cholesterol biosynthesis.[93] The mechanism of action of statins is through their anti-inflammatory, anticoagulant, and antioxidant effects.[94] In addition, statins inhibit the hepatic synthesis of apolipoprotein B100 and decrease the synthesis and secretion of triglyceride-rich lipoproteins.[95,96] Furthermore, it has been proposed that statins have pleiotropic properties, which leads to improved endothelial cell function and modulation of the inflammatory response.[97] Statins constitute an important group of drugs used in the treatment of several conditions, such as hypercholesterolemia, and are aimed to decrease the risk of developing cardiovascular diseases.[98] There are several types of statins, such as atorvastatin, ezetimibe (usually administered in combination with another drug), fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin.[99]

The application of statins in the treatment of periodontitis has been evaluated by local application in the periodontal pockets. Preclinical studies have evaluated the effects of statins (simvastatin, rosuvastatin, atorvastatin) in animals subjected to periodontitis induction. In general, it was observed that the treatment led to reduction in bone resorption, reductions in pro-inflammatory markers and inflammatory infiltrate, as well as to an increase in anti-inflammatory mediators and antioxidant substances.[100-104]

Clinical investigations have assessed the association of several types of statins with SRP in the treatment of periodontitis.[8,105,106] A recent systematic review with meta-analysis included 12 studies that applied 1.2% atorvastatin, simvastatin, or rosuvastatin gel subgingivally as an adjunct to non-surgical periodontal therapy. Considering intrabony defects, there was an overall mean difference of 2.25 mm in PPD reduction and 2.19 mm in CAL gain at 9 months between the use of statin gels and placebo. Moreover, statin gels provided greater reductions in the mean intrabony/furcation defect depth than placebo at 6 months.[107] However, very high heterogeneity was found, putting into question the efficacy and predictability of statins as adjuncts to SRP. Other systematic reviews with meta-analyses corroborate these findings.[108,109]

Considering the data available so far, it is not possible to conclude which type of statin is the most effective.[107,109] Two studies concluded that rosuvastatin gel is more effective than atorvastatin gel in the reduction of PD in both intrabony and class II furcation defects.[110,111] One study did not find any difference in PD improvements when locally applied atorvastatin and simvastatin gel were compared.[113] Nevertheless, a systematic review with meta-analysis and meta-regression concluded that simvastatin was the only statin to provide significant improvements in all parameters analyzed (PPD reduction, CAL gain, resolution of intrabony defects), when compared with placebo.[108]

The use of statins in the management of periodontitis can be considered a potential adjunctive approach. In addition, statins have low cost, are widely accessible and do not appear to pose risks of complications or allergic/adverse reactions. Nevertheless, it is important to consider that the same research group performed all the clinical trials using locally applied statins until the present moment. Therefore, additional controlled and randomized clinical studies performed by other research centers, including greater numbers of

patients and longer follow-up times, are needed to corroborate the current findings and determine the best protocol (dose, duration, and type of statin) to be used in periodontal treatment.

Pro-resolving mediators

Specialized pro-resolving mediators (SMP) are endogenously biosynthesized chemical mediators released during acute inflammation that are both pro-resolving and anti-inflammatory. Omega-3 polyunsaturated fatty acids (PUFA)—eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in particular—are the precursors of these molecules, namely lipoxins, resolvins, protectins, and maresins.[113,14,115] The mechanisms underlying the mode of action of such mediators comprise a complex cascade of events during the acute phases of inflammation, and these mediators are potent agonists that control their duration and magnitude. In general, they act as receptor agonists, controlling the resolution of the inflammation and promoting healing.[116]

When compared to conventional anti-inflammatory drugs, these synthetically developed molecules may have the advantage of controlling inflammation through positive agonist/receptor mediated cellular signals without eliciting any side-effects. Their application, therefore, aims to enhance the off-signal rather than inhibit the on-signal.[113,114,116]

Evidence for the application of pro-resolving mediators in the treatment of periodontitis is still in preclinical phases,[117] and two SMPs have been investigated in animal models: resolvin E1 (RvE1) and benzo-lipoxin A4 (bLXA4). Their activity on the periodontal tissues in these experimental *in vivo* investigations up to 2017 has been recently summarized in a systematic review.[118] A total of 6 studies[119-124] from a single research group were reported to have applied pro-resolving mediators locally in periodontal defects. Major findings revealed that these mediators lead to bone gain and consequently bone regeneration by reducing the inflammatory cell infiltration and osteoclastic activity. Although these mediators do not have a direct effect on the microbiota, the resulting regulation of the inflammation shifted the composition of

the microbiota, probably through changes in the local environment secondary to the inflammation resolution.[118] Between 2017 and 2020, other preclinical studies confirmed previous findings and provided new insights on their biological mechanisms[126-128] In summary, these preclinical studies have shown promising results that need to be confirmed with clinical studies.

Omega-3 and -6

Fatty acids (FA) mainly act as an essential source of energy and as precursors of inflammatory and anti-inflammatory signaling molecules.[128] Long-chain FAs (LCFAs) comprise those with more than 12 carbons and can be sub-grouped into saturated, mono-saturated, or polyunsaturated (PUFAs). The first carbon double bond position denominates n-6 or n-3 PUFAs, or Omega-6 and Omega-3 respectively.[129]

Different chemical routes of Omega-6 and Omega-3 may drive the cellular inflammatory response. Omega-6 (linoleic acid) cascade originates arachidonic acid (ARA).[128] After being incorporated in the phospholipid membrane of mammalians' cells, ARA acts as a substrate to cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 enzymes that originate the so-called eicosanoids. Eicosanoids [prostaglandins (PGs), thromboxanes (Tr), and leukotrienes (LTs)] are responsible for a pro-inflammatory condition and linked to several systemic chronic conditions, such as rheumatoid arthritis, atherosclerotic plaque rupture, critical illness, sepsis,[128,130,131,132] and periodontal diseases. On the other hand, Omega-3 (α-linolenic acid) originates eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids that, in turn, produce resolvins, which possess anti-inflammatory effects, as described above.

The effect of Omega-3 supplements on the treatment of periodontal diseases was first investigated using the experimental gingivitis model, and a tendency to improve the inflammatory condition was reported.[133,134] In regard to periodontitis, cross-sectional data have shown an association between dietary intake of Omega-3 and Omega-6 and periodontal health.[135] Additionally, there is evidence that Omega-3 intake may improve clinical signs of periodontitis

in interventional studies. A systematic review of the use of Omega-3 supplements has concluded that, depending on the duration and dosage of the supplementation, periodontal disease progression might be reduced. Higher plasma concentrations of EPA and DHA correlated with CAL gain, less severe periodontitis, and a higher number of teeth.[136] More recently, one RCT[138] and a meta-analysis[138] concluded that the intake of Omega-3 as an adjunct to periodontal therapy reduced pocket depth and increased CAL gain.

Altogether, these results show that Omega-3 has the potential benefit of reducing periodontal disease progression and improving NSPT outcomes. However, clinical studies are still needed and should evaluate the ideal dosage of Omega-3, duration and origin of supplementation (diet or capsules), side effects, and a better description of periodontal outcomes.

Ozone therapy

Ozone, also known as triatomic oxygen and trioxygen, is a highly oxidative gas found in the atmosphere. Based on its alleged healing and antimicrobial properties, it has been used in Dentistry for various indications, such as treating early caries, ulcerations, herpetic lesions, and disinfecting root canals. Additionally, the application of ozone as an adjunctive treatment has been suggested as a new strategy in the management of periodontitis.[139] Its *in vitro* mechanism of action is based on the production of free radicals leading to an acute and controlled oxidative stress in human cells, resulting in the modulation of antioxidant response, oxygen metabolism, and cellular energy, which will foster positive biological responses.[140] Moreover, ozone's high solubility and instability warrant its entire absorption and prevent toxicity in the tissues.[141] Its effects would not lead do antimicrobial resistance. In addition to the ozone action in bacteria, viruses, and fungi, there is *in vitro* evidence of action against biofilms.[142,143]

Ozone therapy has been administrated in different ways, but applications in gas and aqueous formulations have been the most common. There are studies that tested ozone as ozonized/ozonated water,[139,144] gaseous ozone,[145,146] and ozonized oil.[147] Despite its promising *in vitro* evidence, the clinical application of ozone in the treatment of periodontal diseases has not achieved a minimum level of efficacy. In general, most clinical trials did not show statistically significant differences in plaque index, gingival index, PPD, and CAL when any kind of ozone therapy was used as an adjunct to NSPT as compared to NSPT alone. In addition, the impact of ozone application on microbiologic and biochemical outcomes was limited.[144,145,148] These limited outcomes may be due to its high volatility and instability, resulting in low substantivity and activity when applied clinically. Additionally, the low quality of studies may have also impacted the clinical outcomes. These findings were summarized in a recent systematic review with meta-analysis of randomized clinical trials, which concluded that there is no scientific support for the use of ozone therapy for NSPT.[149]

Epigenetic therapy

Epigenetics has been defined as the change in gene expression that is not encoded in the DNA sequence, and includes chemical changes in the DNA and its related proteins (called histones), which leads to the remodeling of chromatin and the resulting activation/inactivation of the targeted gene.[150] Epigenetic mechanisms dynamically regulate gene functions (activation or inactivation) and can change in response to different stimuli.[151] For example, systemic (smoking and diabetes mellitus) and local influences (bacteria and their virulence factors) can lead to changes in the epigenetic status of cells. These events may induce changes in immunological responses and thus contribute to the pathogenesis of chronic inflammatory diseases, such as periodontitis.[152] In spite of the early stage of studies about epigenetics in periodontitis, the recent accumulation of evidence has suggested the possible etiopathogenic role of epigenetic changes in the initiation and progression of periodontitis. It has been reported that the levels of multiple cytokine-encoding genes and inflammatory response-related genes may be regulated epigenetically.[153]

Although several epigenetic changes have been described in periodontitis, there is little information on the resolution of these changes after periodontal therapy. In addition, the possible effect of adjunctive treatments using epigenetic drugs (known as Epidrugs) is currently unknown. Only *in vitro* and preclinical *in vivo* studies are available assessing the effect of these molecules on gingival tissues.[154,155,156]

DNA methylation

DNA methylation is a regular epigenetic modification in nuclear cells, characterized by the addition of methyl groups in cytosines within cytosine-guanosine (CpG) dinucleotides, regulated by different DNA methyltransferases. DNA methylation related to disease can occur by hypermethylation or hypomethylation as a result of elevating or suppressing the expression of specific genes.[157] Several studies have investigated DNA methylation of inflammatory cytokines such as IL-6, -8 and TNF-α in different forms of periodontitis.[156,158-161] In addition, other inflammation-related genes were analyzed in periodontitis, such as Toll-like receptors (TLRs)[163] and Interferon gamma (IFN-γ).[163]

It is noteworthy that studies have not tested interventions aiming to change DNA methylation; however, it has been demonstrated that periodontal treatment may alter DNA methylation. For instance, Asa'ad et al.[156] demonstrated that periodontal therapy was able to redefine the DNA methylation status of the inflammatory gene for COX-2 in patients with periodontitis, even though DNA methylation levels for TNF-α and IFN-γ remained similar in the experimental groups. Andia et al.[164] found no change in methylation levels between groups after three months of periodontal therapy. However, variations in methylation status between groups were not assessed at the beginning of the study. As a whole, these findings suggest that periodontal therapy can influence epigenetic changes.

Histone acetylation

Histones can be acetylated or deacetylated on the tails of amino acids in chromatin. While histone acetylation (addition of acetyl groups) is regulated by histone acetyltransferases (HATs), histone deacetylation (removal of acetyl groups) is regulated by histone deacetylases (HDACs). Unlike DNA methylation, which is associated with gene expression, histone acetylation is associated with gene transcription (activation or repression).[165]

Different histone deacetylase inhibitors have been investigated as a potential treatment for bone-related diseases, since they epigenetically regulate the expression of genes associated with osteoclast differentiation, maturation, and activity. In this sense, some histone deacetylase inhibitors have already been approved by the US Food and Drug Administration (FDA), and evidence across therapeutic modalities has shown good results in the treatment of different cancers.[112]

In Periodontics, some promising results in preclinical research have been demonstrated. The early results seem to point to an increase in histone acetylation in induced-periodontitis models. Cantley et al.[166] reported that 1179.4b, a histone deacetylase inhibitor, suppresses bone loss in the induced-periodontitis model by oral inoculation with *Porphyromonas gingivalis*. However, the analysis revealed that 1179.4b reduced bone loss despite having no effect on gingival inflammation. The authors suggested HDACi as a potential therapeutic option for periodontitis in the future. Moreover, HDACi MS-275 has been shown to have an effect on osteoclastogenesis *in vitro* and *in vivo*.[167] MS-275 inhibited osteoclast differentiation of bone marrow-derived macrophages by suppressing RANKL-induced expression, suggesting a potential therapeutic value of HDACi for bone disorders associated with increased bone resorption. Bromodomain and extra-terminal protein (BET) inhibitors have also been studied in alveolar bone loss. BET proteins are regulatory molecules of chromatin that bind to acetylated histones. JQ1, a BET inhibitor, was studied by Meng et al.[168] in an experimental periodontitis model, which demonstrated that JQ1 suppressed the transcription of inflammatory cytokines activated by LPS and osteoclast markers promoted by RANKL.

Although the results with histone acetylation inhibitors are promising, there are numerous types of cells present at the site of bone destruction, including inflammatory cells. Thus, inhibition of histone

deacetylases might have the opposite effect on those cells, resulting in increased bone resorption or raised inflammatory reaction. Therefore, further research is required to prove the combined effect of histone deacetylase inhibitors in the treatment of localized bone destruction.

MicroRNAs (miRNAs)

miRNAs are also epigenetic mechanisms that regulate gene expression through post-transcriptional modifications. These molecules describe a group of small non-coding RNAs.[169] MicroRNAs play critical roles in inflammatory responses by modulating cellular processes, such as cell growth, apoptosis, and differentiation. Given this fact, miRNAs are linked to the development of diseases such as rheumatoid arthritis and cancer.[170] In the last years, miRNAs have been investigated in bone-related diseases and bone-remodeling processes due to their importance in osteoclastogenesis, osteogenesis, and osteoclast/ osteoblast differentiation. In addition to that, miRNAs have been shown to play a relevant function in the differentiation of periodontal ligament stem cells. An *in vitro* study demonstrated that one type of miRNA stimulated osteogenic differentiation of periodontal ligament stem cells.[171]

miRNAs have also been involved in the control of TLR reaction to bacteria. A recent study[172] reported that infection by three important periodontal pathogens (*Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*) increased the expression levels of miR-146a in mice with experimental periodontitis. The authors also reported that cultured cells simultaneously stimulated with all three periodontal pathogens showed that TNF-α

imbalance may be signal for this expression of miRNA.

In summary, our current understanding of the role of epigenetic factors in periodontitis development is still insufficient. However, emerging studies highlight that DNA methylation, histone modifications, and miRNAs are modified during the oral mucosa response to bacteria, environmental factors, and inflammatory processes. Future translational studies are needed to further explore and understand these discoveries and enable the development of highly desirable therapies for periodontal disease based on the regulation of the host immune response.

Concluding remarks

This review addressed the available literature for nine therapeutic approaches with potential benefits over standard NSPT. At the moment, there is a lack of solid and sound evidence to recommend most of these new tendencies in NSPT in daily practice. SRP alone reduces bleeding in up to 63% of the sites and results in 74% of closed pockets (PPD ≤ 4mm and no BOP).[173] Clinical outcomes observed with new therapies approached in this review have not demonstrated minimally acceptable consistency and benefits over those achieved with SRP alone. Consequently, SRP should still be considered the gold-standard treatment for periodontitis. Importantly, evidence is growing, and some of these new tendencies have great potential to improve NSPT outcomes. Future RCTs applying comparable estimates of clinical outcomes are encouraged in order to assist clinicians and patients in their clinical decision about alternative treatments.

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