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Mitochondrial and redox-based transcriptional changes in type-2 diabetes mellitus and periodontitis

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1. Abbreviations

3-NT - 3-Nitrotyrosine

4-HNE - 4-Hydroxy-2-nonenal

8-OH-dG - 8-Hydroxy-deoxyguanosine

AGE - Advanced glycation end product

ATP - Adenosine-5′-triphosphate

CAT- Catalase

CRP - C-reactive protein

DM - Diabetes Mellitus

ETC - Electron transport chain

GCF - Gingival crevicular fluid

GPx - Glutathione peroxidase

GR - Glutathione reductase

H_2O_2 - Hydrogen peroxide

HIF - Hypoxia-inducible factor

$HO^{\bullet}$ - Hydroxyl radical

IL – Interleukin

INF - Interpheron

Keap1 - ECH-associated Kelch-like protein 1

MMP - Matrix metalloproteinases

miR - MicroRNA

NF-κB - Nuclear factor k B

NO - Nitric oxide

Nrf2 - Nuclear factor erythroid 2–related factor 2

$O_2^{\bullet-}$ - Superoxide anion

OPG - Osteoprotegerin (OPG)

OS - Oxidative stress

PBMC - Peripheral blood mononuclear cells

PGE2 - Prostaglandin E2

PMN - Polymorphonuclear cells

PDL - Periodontal ligament

PD – Periodontitis

RAGE - Advanced glycation end product receptor

RANK - receptor activator for nuclear factor-κB ligand

ROS - Reactive oxygen species

SOD - Superoxide dismutase

T2DM - Type 2 Diabetes Mellitus

T2DM-PD – Type 2 Diabetes Mellitus and Periodontitis

TNF-alpha – Tumor necrosis factor alpha

TLR - Toll-like receptors

2. Abstract

Introduction: Periodontitis (PD) and Type 2 Diabetes Mellitus (T2DM) are inflammatory diseases with a high prevalence in the world population and which may be related to severe complications, including death. The bi-directional relationship between PD and T2DM has been suggested. Mitochondria play a very relevant role in the pathogenesis of these diseases. Mitochondrial dysfunction has been pointed out as a relevant contributor for oxidative stress (OS), which is implicated in protein, lipid and genetic modifications, reflected in the decrease of the antioxidant activity and in transcriptional alterations.

Materials and Methods: A PubMed search was conducted using the terms MesH and keywords "Periodontitis", "Type 2 Diabetes Mellitus", "Oxidative Stress", "Mitochondria", "Oxidation-Reduction", "Antioxidants" with the bullet connectors "AND" and "OR". All systematic reviews with and without meta-analysis were excluded. In addition to study type, filtering was performed based on language, including English and Portuguese, and articles published between January 2011 and 2021 May. All publications were screened for relevance after reading the abstracts.

Discussion: In individuals with simultaneous Pedriodontitis and Type 2 Diabetes Mellitus (T2DM-PD), poor glycaemic control was associated with accumulation of advanced glycation end products (AGEs), increased numbers of red complex pathogens in the subgingival biofilm and proinflammatory mediators. They showed higher levels of pro-inflammatory mediators such as tumour necrosis factor alpha (TNF-alpha), interleukin 6, 17 and decreased anti-inflammatory mediators such as interleukin 10 and interleukin 8. In both animal models and humans, DM and PD caused mitochondrial dysfunction, manifested through decreased ATP production, mitochondrial DNA copy number, expression of complex I subunits and increased reactive oxygen species (ROS). Moreover, OS was associated with the stimulation of pro-inflammatory cytokines such as TNF-alpha and interferon gamma (IFN-gamma) and decreased antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR). Reduced antioxidant activity may be associated with changes in transcription factors, such as nuclear factor erythroid 2–related factor 2 (Nrf2), hypoxia-inducible factor-1 (HIF-1) and nuclear factor kB (NF-κB). In both T2DM and PD, HIF-1alpha and NF-κB levels were increased. The transcription of antioxidant enzymes can be alsomodulated by some microRNA (miRNA, miR), such as miR-223, associated with T2DM, which are involved in the expression of antioxidants such as HO-1, SOD1 and SOD2.

Conclusion: PD exacerbates the T2DM immune-inflammatory response and the opposite is also true, clinically reflected through increased periodontal destruction and insulin resistance. Confirmed mitochondrial dysfunction in these two pathologies was associated with increased ROS and decreased antioxidant activity, highly pronounced in T2DM-PD subjects. There is an influence of ROS on the regulation of the transcription of antioxidant enzymes, through the downregulation of transcription factors such as Nrf2, HIF-1alpha and NF-κB. The processes involved in the downregulation of antioxidant enzyme transcription are not fully understood. miRNAs may be involved in this deregulation through modulation of transcription, constituting an important subject of study in understanding the transcriptional changes associated with inflammatory pathologies such as PD and T2DM and important for future studies.

Keywords: Diabetes Mellitus type 2, Periodontitis, Mitochondria, Oxidative Stress, Reactive oxygen species, Antioxidants

3. Resumo

Introdução: A Periodontite (PD) e a Diabetes Mellitus tipo 2 (T2DM) são patologias inflamatórias com uma prevalência elevada na população mundial e que podem estar relacionadas com complicações graves, nomeadamente a morte. A relação bi-direcional entre a Periodontite e a Diabetes Mellitus Tipo 2 tem sido amplamente estudada. A mitocôndria tem um papel de grande relevância na patogénese destas patologias, mais concretamente na resposta imuno-inflamatória. A disfunção mitocondrial tem sido apontada como um contribuinte importante para o stress oxidativo (OS), implicado em modificações proteicas, lipídicas e genéticas que se refletem na diminuição da atividade antioxidante e em alterações transcripcionais.

Materiais e Métodos: Foi realizada uma pesquisa PubMed usando os termos MesH e palavras-chave "Periodontitis", "Diabetes Mellitus Tipo 2", "Stress Oxidativo", "Mitochondria", "Oxidation-Reduction", "Antioxidants" com os conectores de bala "AND" e "OR". Todas as revisões sistemáticas com e sem meta-análise foram excluídas. Para além do tipo de estudo, a filtragem foi realizada com base na língua, incluindo inglês e português, e artigos publicados entre Janeiro de 2011 e 2021 de Maio. Todas as publicações foram rastreadas pela sua relevância após a leitura dos resumos.

Discussão: Em indivíduos com Periodontite e a Diabetes Mellitus tipo 2 (T2DM-PD) em simultâneo, o deficiente controlo da glicémia foi associado à acumulação de produtos finais de glicação avançada (AGEs), ao aumento do número de patogénios complexos vermelhos no biofilme subgengival e mediadores pró inflamatórios. Estes mostraram níveis mais elevados de mediadores pró-inflamatórios como o factor de necrose tumoral alfa (TNF-alfa), a interleucina 6, 17 e a diminuição de mediadores anti-inflamatórios como a interleucina 10 e a interleucina 8. Tanto em modelos animais como em humanos, a DM e PD causaram disfunção mitocondrial, manifestada através da diminuição da produção de ATP, do número de cópias de ADN mitocondrial, da expressão de subunidades do complexo I e no aumento de espécies reativas de oxigénio (ROS). Além disso, o OS foi associado à estimulação de citocinas pró-inflamatórias como o TNF-alfa e o interferon gama (IFN-gamma) e à diminuição da capacidade antioxidante de enzimas como superóxido dismutase (SOD), catalase (CAT), glutatião redutase (GR). Verificou-se que a redução da atividade antioxidante poderá estar associada a alterações transcripcionais nomeadamente em fatores de transcrição como Fator nuclear eritróide 2 relacionado com o factor 2 (Nrf2), o factor-1 induzido pela hipóxia (HIF-1) e o factor nuclear kB (NF-κB). Tanto na T2DM como na PD, os níveis de HIF-1 alfa e NF-κB estavam aumentados. A transcrição de enzimas antioxidantes é modulada por alguns MicroRNA (miRNA, miR), como o miR-223, associado à T2DM, que promoveu a expressão de antioxidantes como HO-1, SOD1 e SOD2.

Conclusão: A DP exacerba a resposta imuno-inflamatória T2DM e o oposto também é verdadeiro, reflectido-se, clinicamente, através do aumento da destruição periodontal e da resistência à insulina. A disfunção mitocondrial confirmada nestas duas patologias foi associada ao aumento da ROS e à

diminuição da atividade antioxidante, altamente pronunciada em sujeitos T2DM-PD. Existe influência da ROS na regulação da transcrição de enzimas antioxidantes, através da desregulação de fatores de transcrição como o Nrf2, HIF-1 e NF-κB. Os processos envolvidos na desregulamentação da transcrição de enzimas antioxidantes não estão totalmente compreendidos. Os miRNAs podem estar envolvidos nessa desregulação através da modulação da transcrição, constituindo um importante objeto de estudo na compreensão das alterações transcricionais associadas a patologias inflamatórias como a DP e o T2DM e importantes para estudos futuros.

Palavras-chave: Diabetes Mellitus tipo 2, Periodontite, Mitocôndria, Stress Oxidativo, Espécies Reativas de Oxigénio, Antioxidantes

4. Introduction

Periodontitis (PD) is the most prevalent pathology worldwide, affecting 50% of the world population. It is characterised by the destruction of the periodontal tissues that support the teeth. When untreated, it can lead to severe tooth loss with several functional consequences, not only locally, but also systemically, with great impact on the quality of life of individuals (1).

PD is considered the sixth complication of Type 2 Diabetes Mellitus (T2DM), which according to the World Health Organization, affects 6% of the world population (2014 data) and 30% of the elderly over 60 years old (according to the Report "Diabetes: Facts and Figures 2020"). T2DM is described as a state of chronic hyperglycemia and associated with various comorbidities and, in more extreme situations, cause the death of the patients (2). According to the World Health Order T2DM was among the top ten causes of death in 2019.

The bidirectional relationship between these two pathologies has been widely studied (3). In addition to sharing risk factors such as higher age, male gender, low socioeconomic status, genetic predisposition (mainly for poor immune/inflammatory responses), socioeconomic status, smoking, obesity, sedentary lifestyle and unhealthy diet (4), PD and T2DM are both chronic inflammatory-based pathologies (5). Previous studies indicate that PD impacts on T2DM control, which in turn is linked to an increased risk of T2DM, both contributing to a systemic inflammatory burden (2)

A chronic inflammatory process has been presented as a key issue behind the complications associated with these pathologies and is related to an altered/changed immune-inflammatory response (5). The increased inflammatory burden is related to the increased number and activity of immune cells such as peripheral blood mononuclear cells (PBMC), inflammatory mediators and reactive oxygen species (ROS) (6). Severe periodontal tissue destruction has been positively correlated with excessive ROS generation and decreased antioxidant capacity in subjects with T2DM. Studies have linked oxidative stress (OS) caused by increased ROS with mitochondrial dysfunction (7). Indeed, mitochondria are essential for producing adenosine-5′-triphosphate (ATP) to meet the energy needs of cells, namely of PBMCs (8). That said, and given the importance of these cells in the immune-inflammatory response, PBMCs are an excellent model to study mitochondrial dysfunction in T2DM and PD (4).

OS associated with mitochondrial dysfunction, is at the origin of several alterations in lipid proteins and genetic material, having implications for the degree of severity and progression of complications associated with PD and T2DM (9,10). The balance between ROS and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), is crucial for systemic health. Of relevance, both PD and T2DM have been shown to be associated with decreased antioxidant capacity (11).

Based on these evidences, the aim of this review is to describe mitochondrial dysfunction and oxidative stress and their link to inflammatory burden and transcriptional alterations occurring in PD and T2DM, which may help to identify potential therapeutic targets for these pathological conditions.

5. Methods

A PubMed search was performed using the terms MesH and keywords "Periodontitis", "Type 2 Diabetes Mellitus", "Oxidative Stress", "Mitochondria", "Oxidation-Reduction", "Antioxidants" with the bullet connectors "AND" and "OR". All systematic reviews with and without meta-analysis were excluded. In addition to the type of study, filtering was performed based on language, including English and Portuguese, and articles published between january 2011 and may 2021. All publications were screened for their relevance after reading the abstracts.

6. Discussion

Periodontitis (PD) is a chronic multifactorial immune-inflammatory disease triggered by a dysbiotic oral microbiome associated with persistent plaque biofilm and characterized by progressive destruction of the teeth supportive tissues, eventually leading to tooth loss (1,12,13). Epidemiological evidence showed that PD affects more than 50% of adults worldwide, being currently the sixth most common chronic disease in the world. Diagnosis is made radiographically (alveolar bone loss) and clinically (presence of periodontal pocketing and gingival bleeding, clinical attachment loss (CAL)). Since 2017, the new classification of periodontitis introduced a multi-dimensional staging and grading system, in which ”staging” is based upon disease severity and case management complexity, and “grading” addresses biological features such as rate of disease progression, assessment of the risk for further advancement and potential threats to general health. Until now, grading encompasses grade modifiers, smoking and level of metabolic control in diabetes, as risk factors (1).

DM is a progressive systemic disease characterised by chronic hyperglycemia, associated with a failure of blood glucose regulation associated with various complications and, in more severe cases, death (2,14). There are two main types of DM: 1) type 1 DM, a form of autoimmune disease in which the pancreatic beta cells that produce insulin are destroyed by the human immune system itself or after a virus infection, among other factors; and 2) type 2 DM (T2DM), the most common form, characterised by insulin resistance. Being the most prevalent disease in adults, the review will focus on this type. According to the World Health Organization, the 2014 prevalence of T2DM was 8% of the world population, corresponding to over 422 million people. By 2030, it is estimated to increase to 570 million people. Hence, DM is presently a huge public health problem and the principal systemic disease affecting periodontitis (15).

PD is highly associated with T2DM, being considered as its sixth complication. Indeed, when DM is uncontrolled or poorly controlled, the hyperglycemic chronic state increases the prevalence and severity of PD, which can even compromise periodontal treatment (13,14,16). Although this evidence focuses essentially on T2DM, the effect appears to be similar in type 1 DM, though less studied. Additionally, the effects of comorbidities often seen in subjects with metabolic syndrome, including obesity and hypertension, can act as confounders. The pathogenic mechanisms responsible for the effects of hyperglycemia on periodontitis comprise, among others, a hyperinflammatory response to a bacterial challenge including neutrophil defects, increased release of proinflammatory cytokines, oxidative stress and impaired healing responses. On the other hand, PD is associated with difficulties in controlling serum glucose levels. The association between these two conditions was observed in a clinical study by Purnamasari and co-authors (14), where the inflammatory response was compared in a group consisting of individuals with T2DM and PD, another with only diabetic subjects and a control group. The results confirmed that PD severity was higher in the group with T2DM plus periodontitis, when compared to periodontitis without T2DM (14). Liu and co-authors (17) further demonstrated that periodontal disease can aggravate pancreatic beta-cell failure and insulin resistance in diabetic rats. Similar results were obtained by Blasco-Baque and co-workers (18) who, by inoculating specific

periodontal pathogens, namelys Porphyromonas gingivalis, Fusobacterium nucleatum and Prevotella intermedia, and consequently inducing PD in female C57Bl/6 mice subjected to diabetogenic/non-obesogenic fat-enriched diet for 3 months, found that periodontal microbiota-induced periodontal dysbiosis affected regional and systemic immune response, impaired glucose metabolism, increasing insulin resistance (18).

6.1 Immune-inflammatory response associated with Diabetes and Periodontitis

6.1.1 The impact of Periodontitis on the immune-inflammatory response

Both PD and DM are chronic inflammatory diseases. Thus, the immune-inflammatory response plays a crucial role in the pathogenesis of PD and DM, bridging the gap between these two pathologies (3). What has been found to be associated with these pathologies is increased inflammation and an impaired immune response.

The dysbiosis (Fig.1) that is at the origin of PD is due to the presence, in the subgingival biofilm, of specific pathogenic bacteria such as Treponema denticola, Tannerella forsythia, Porphyromonas gingivalis, associated with the progression of the pathology when it is not treated (15,19). These red complex pathogens, strongly associated with PD pictures, through the release of endotoxins and exotoxins into the bloodstream, trigger a systemic inflammatory process with stimulation of the production of pro-inflammatory cytokines, namely some interleukins (IL-1beta, IL-6), interferon gamma (IFN-gamma), tumour necrosis factor alpha (TNF-alpha), matrix metalloproteinases (MMP), prostaglandin E2 (PGE2), chemokines and adhesion molecules (Fig.1) (15)

IL-6 is associated with the adaptive function activity through stimulation of C-reactive protein (CRP), an acute phase protein, important in phagocytosis. Chemokines, such as IL-8, increase permeability of gingival capillaries, whereas adhesion molecules are responsible for the recruitment of immune cells (peripheral blood mononuclear cells (PBMC)) from the bloodstream to the site of inflammation (20). Neutrophils are the first to be recruited, constituting the inflammatory infiltrate, the first defence barrier. By analysing the plasma of PD patients, it has been found that cytokines can participate in the induction of hyper-active (IL-8, GM-CSF, IFN-alpha) and hyper-reactive (IFN-alpha) phenotypes in neutrophils (21). Hyper-reactive neutrophils are associated with increased production of ROS, collagenase and elastase, as well as pro-inflammatory cytokines and chemokines that perpetuate the chronicity of inflammation (5).

CD4+ helper T-cells, specifically Th-1, Th-2, Th-17 (Fig. 1) and T-regulatory cells are an important part in controlling inflammation. Th-1 secrete IL-2 and INF-gamma (more involved in cellular immunity), while Th-2 secrete IL-4, IL-6, IL-9, IL-10 and IL-13 and are more involved in humoral immunity through B-cell activation, mast cells, and production of immunoglobulin E (20).

Significant increases in the levels of IL-1beta, IL-6, TNF-alpha, IL-4 have been found in the blood serum of PD patients, when compared to healthy individuals (22) and an increase in CCL28, IL-8, IL-1beta and TNF-alpha in gingival crevicular fluid with the most aggressive clinical presentations of

PD, being associated with higher levels of these mediators (Fig. 1) (23). Moreover, inflammation inhibitors and immunosuppressants such as IL-10 recorded lower levels in blood serum samples from PD patients (22). As an inflammation inhibitor and immunosuppressant, IL-10 and IL-4, their inhibition entails consequences on the ability to resolve inflammation (22). Increased IL-10 levels have been shown to be associated with a decrease in clinical signs such as probing depth (15). In PD, IL-10 participates in the inhibition of pro-inflammatory cytokine production by macrophages and Th1 cells. Shi and co-authors (24) have recently reported that, in animal models, IL-10 can modulate local host immune responses and prevent inflammatory damage to alveolar bone by reducing pro-inflammatory cytokine expression and decreasing the local proliferation of Th17 cells that play a crucial role in mediating osteoclast activity (24).

6.1.2 The immune-inflammatory response as a bridge between Periodontitis and Type 2 Diabetes Mellitus

The interconnection between periodontitis and diabetes is primarily established based on direct and indirect changes in the inflammatory status of the periodontal tissue. Hyperglycemic conditions promote pro-inflammatory host response in the periodontal environment in a direct path and via advanced glycation end product (AGE) and its receptor, the RAGE (Fig. 1). Additionally, an indirect link is suggested, as the promotion of periodontal dysbiotic microbiome in patients with diabetes with poor glycemic control (3,25). With a view to a better understanding of this relationship, T2DM appears to influence the immune-inflammatory response, namely related with periodontal pathogens, which (as mentioned) are responsible for triggering the inflammatory process associated with PD. Indeed, in cross-sectional study, significant differences were found in the subgingival microbiota between subjects with T2DM-PD and PD, with the former showing higher levels of Aggregatibacter actinomycetemcomitans, Neisseria, Gemella, Eikenella, Selenomonas, Actinomyces, Capnocytophaga, Fusobacterium, Veillonella and Streptococcus, and lower percentages of Porphyromonas gingivalis, Filifactor, Eubacterium, Synergistetes, Tannerella forsythia and Treponema denticola, using ribosomal RNA amplicon sequencing (25). Additionally, other studies focused on red complex pathogens, Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia, associated with higher virulence, in greater numbers in subjects with uncontrolled T2DM-PD than in subjects with PD alone (26). Porphyromonas gingivalis was detected more frequently in individuals with increased HbA_{1c} values (26). Of note, Castrillon and co-authors (27) associated Porphyromonas gingivalis to patients with PD only and Aggregatibacter actinomycetemcomitans to patients with simultaneous T2DM and PD. The results, regarding this issue, were not linear and therefore the differences in periodontal pathogens in subjects with T2DM compared to subjects without T2DM are still inconclusive. However, poor glycaemic control was found to be associated with increased number of red complex pathogens in the subgingival biofilm in subjects with T2DM-PD (26). The observed findings may be related to the hyperglycemia

associated with T2DM, and subsequently in the gingival crevicular fluid, which may stimulate selective growth of specific pathogens, which is then reflected in the severity of PD (25).

Besides the impact of poor glycemic control on periodontal microbiota, hyperglycemia also forces the irreversible formation of AGEs and the expression of its receptors as well as toll-like receptors, already identified at gingival tissues and saliva from periodontitis and diabetic patients. When AGEs bind to RAGE, they have direct pro-inflammatory and pro-oxidant effects (3). RAGE from endothelial cells can act as a receptor for integrins present on the surface of inflammatory cells, thus playing an important role in transendothelial migration of inflammatory cells. The interaction of AGEs with their surface receptor induces oxidative stress through the generation of ROS (Fig.1) and also activation of protein kinase C (28). The presence of periodontal infection further potentiates this vicious cycle in the susceptible diabetic host, leading to accelerated and augmented severity of periodontal destruction (3).

In subjects with T2DM, of increased levels of AGEs interact with RAGEs that are present in macrophages, stimulating the production of cytokines such as IL-1 beta (29). In this context, IL-1beta, IL-6 and TNF-alpha have been the subject of several studies. IL-1 beta is considered one of the major cytokines in the inflammatory destruction of periodontal tissue (20). In gingival crevicular fluid (GCF), an inflammatory exudate derived from the periodontal tissues, of Sprague-Dawley rats, a significant increase of this interleukin was found in the T2DM-PD group of rats compared to the PD-only or T2DM groups (29). In gingival biopsies from humans, Duarte and coworkers (30), recorded that the levels of IL-1beta did not differ between subjects with T2DM-PD and only PD. Thus, it was concluded that, regardless of whether T2DM exists or not, IL-1beta production was associated with PD (20,30). TNF-alpha, a promoter of osteoclast activation, has also been the subject of study. In both animal (29) and human models (30) there was a more pronounced increase in this mediator in the groups that had T2DM-PD, which clinically translated into greater alveolar bone loss. In the latter study, there was also an increase in IL-6 levels in subjects with both pathologies (30). In subjects with T2DM-PD, a trend towards a higher concentration of IL-8 (chemotactic agent) and lower concentrations of IL-10 were observed at the sites, compared to subjects with PD (30). As mentioned previously, in addition to Th1 and Th2 cells, Th17 cells have been shown to play a key role in PD with the release of the pro-inflammatory cytokine IL-17 (Fig.1) (30). Th1 and Th17 cells were associated with a higher nuclear factor-κB ligand (RANKL)/osteoprotegerin (OPG) ratio, whereas Th2 cells correlated with a lower RANKL/OPG ratio RANK is responsible for bone resorption and OPG for bone remodelling. Thus, the balance between RANK and OPG allow us to assess the degree of bone resorption associated with the pathological process of PD (31).

Considering the in vivo and in vitro human and animal data, the identification of inflammatory markers at the periodontium (GCF, saliva and gingival tissues) demonstrated that diabetes with poor glycemic control affects periodontal inflammatory and noninflammatory cells, mainly through higher expression of pro-inflammatory mediators, but also by the activation of the Th17 pathway and immunomodulation by local fibroblasts.

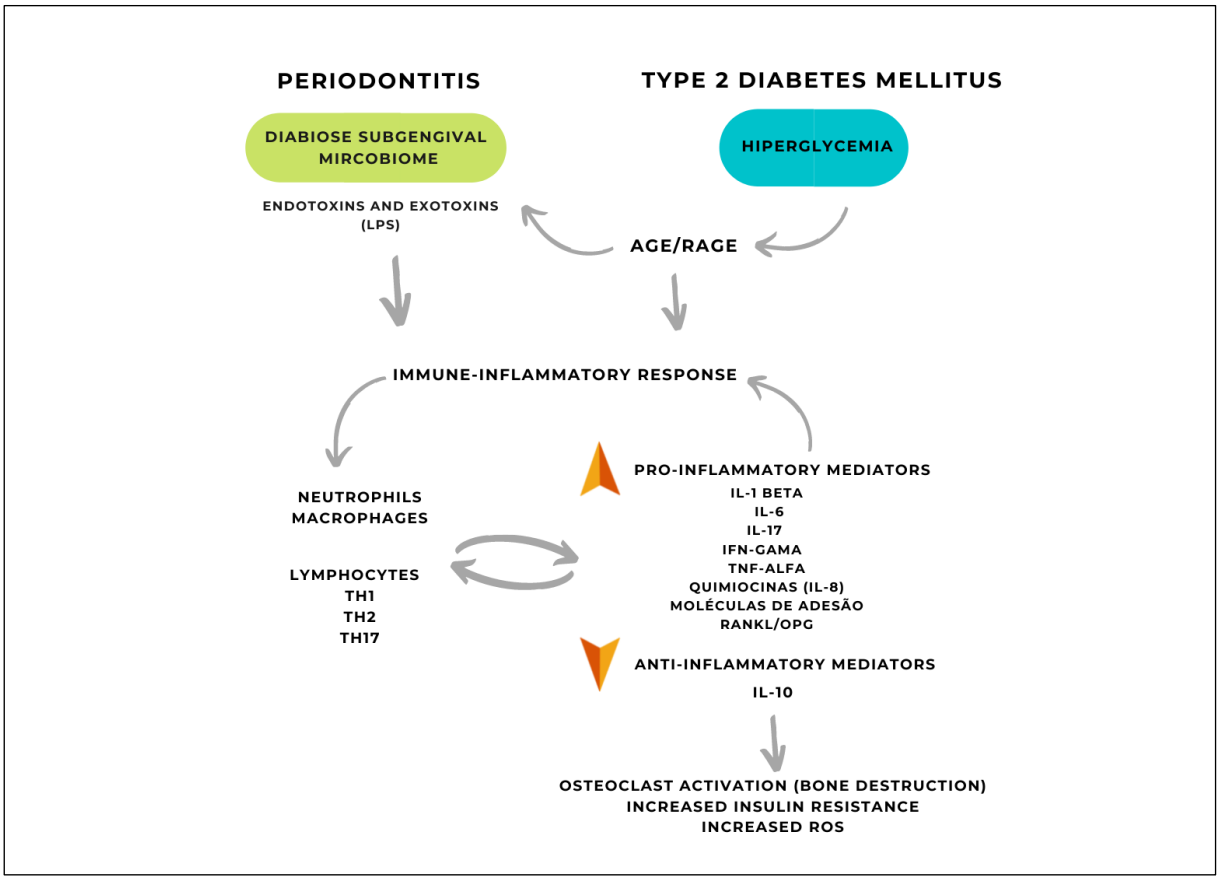

Figure 1. The immune-inflammatory response as a link between Periodontitis and Type 2 Diabetes Mellitus. Periodontitis is associated with subgingival microbial dysbiosis and Type 2 Diabetes Mellitus is characterised by a state of hyperglycaemia that leads to the formation of Advanced glycation end product (AGEs). AGEs besides favouring microbial dysbiosis, their binding to their receptors triggers an immune-inflammatory response. In turn, the immune-inflammatory response is also stimulated by endotoxins and exotoxins, such as lipopolysaccharide, from periodontal bacteria. The immune-inflammatory response is characterised by the activation of innate immunity, in which neutrophils and macrophages are involved, and by acquired immunity with the activation of Th1, Th2 and Th17 lymphocytes. The immune-inflammatory response is characterised by an increase of pro-inflammatory mediators, produced by immune cells, such as interleukin 6 (IL-6), interleukin 17 (IL-17), interferon gamma (INF-gamma), chemokines such as interleukin 8 (IL-8) and adhesion molecules. It is also characterised by a decrease in anti-inflammatory mediators such as interleukin 10 (IL-10). The production of certain inflammatory mediators activates osteoclasts, leading to increased bone destruction and has an influence on increased insulin resistance. The immune-inflammatory response is also associated with an increase in reactive oxygen species (ROS).

Regarding the expression of immuno-inflammatory markers, elevated mRNA levels of IL-17, RANKL, toll-like receptors (TLR), and RAGEs were detected in gingival biopsies from all groups with PD, regardless of the presence of DM, when compared with healthy subjects (30).

Comparative results suggest that hyperglycaemia, more specifically, the accumulation of AGEs at the gum level in individuals with T2DM-PD, increases the the immune-inflammatory response to periodontal pathogens by decreasing anti-inflammatory markers and increasing relevant pro-inflammatory markers such as TNF-alpha and C-reactive protein (CRP) (Fig.1) (20). The imbalance between pro-inflammatory

and anti-inflammatory mediators associated with the hyperinflammatory state stimulated by hyperglycemia leads to increased ROS. In turn, increased expression of RAGE in infected periodontal tissues, increases the sensitivity of periodontal tissues to ROS damage (10). On the other hand, the increase in inflammatory mediators associated with PD may have implications on glucose metabolism, as found in a study with human participants in which intravenous administration of TNF-alpha was accompanied by increased IL-6 and insulin resistance in healthy patients.

7. The role of mitochondria on Type 2 Diabetes Mellitus and Periodontitis

7.1 Mitochondrial Dysfunction and Oxidative Stress

In light of some of the most recent advances in the link between PD and T2DM, a new field of interest is emerging that links mitochondrial dysfunction and oxidative stress to both diseases. Mitochondria play a crucial role in fundamental cellular functions including cellular metabolism, regularly producing ROS with a role in intracellular redox signalling, in the regulation of intracellular calcium levels, and in intrinsic apoptotic pathways, in cell proliferation and differentiation, and inflammation (8,32). This organelle is responsible for much of the production of adenosine-5′-triphosphate (ATP) (Fig. 2) and a determinant of the effectiveness of the immune response, as it is directly related to the proper functioning of cells participating in the immune-inflammatory response, such as PBMCs (33). Increased oxygen consumption in stimulated PBMCs, specifically CD4+ T cells, has been confirmed by analysing blood samples collected from individuals with T2DM (33). Indeed, T2DM and PD are two chronic inflammatory diseases. As such, the inflammatory process, characteristic of these pathologies, will stimulate an immune response in order to control inflammation. Since immune cells are metabolically active and dependent on mitochondria for energy production, mitochondrial function is closely related to the quality and efficiency of the immune response. That said, mitochondrial dysfunction has emerged as an important key process in understanding the mechanisms underlying these pathologies (33).

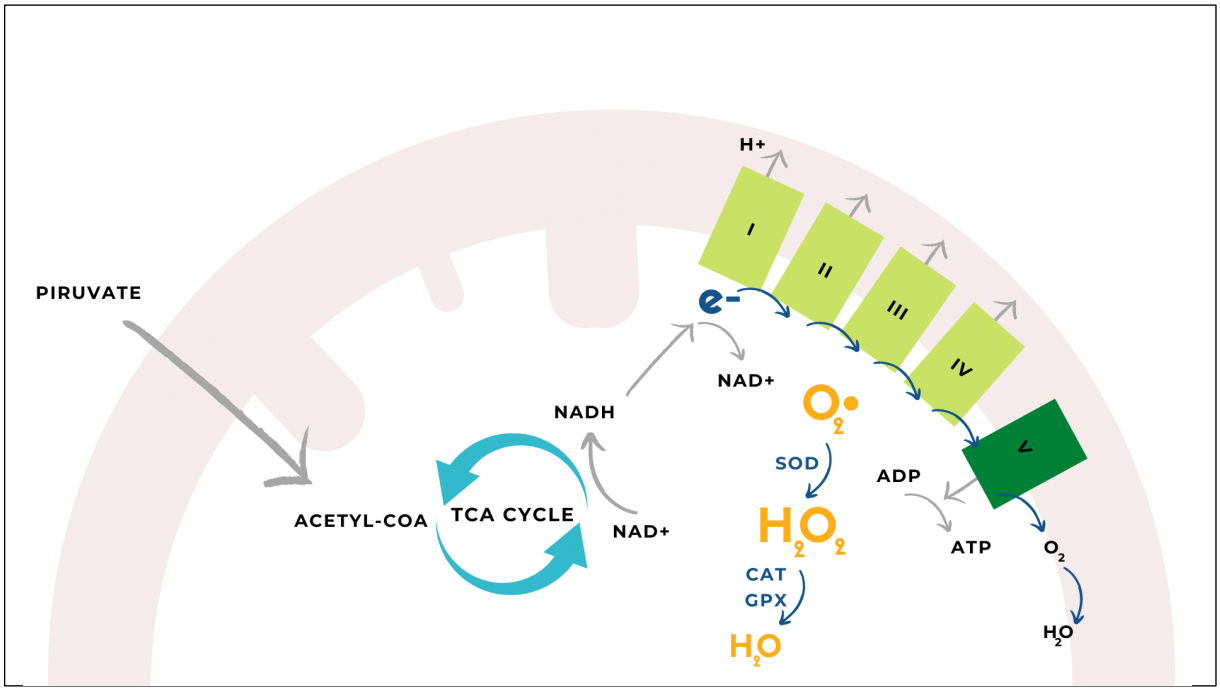

Figure 2. Simplified mitochondria respiratory chain, Krebs cycle and antioxidant activity. Mitochondrial respiration and ROS production show a rhythmic activity. Mitochondrial respiration is the result of electron transfer with Oxygen as the final acceptor of the electron transport chain (ETC). The pyruvate generated from glucose, forming Acetyl-CoA enters the Krebs cycle. From this cycle electrons are transported by NADH to the ETC, where energy (ATP) formation occurs. The ETC is also the main source of reactive oxygen species (ROS) as superoxide anion ($O_2^{\bullet-}$). Superoxide dismutase (SOD), an antioxidant enzyme, reduces $O_2^{\bullet-}$ into hydrogen peroxide (H_2O_2) and catalase (CAT), glutathione peroxidase (GPx) accelerate the reduction of H_2O_2 into water.

Mitochondria are a major source of ROS, including superoxide anion ($O_2^{\bullet-}$), which can then generate hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) and reactive nitrogen species like nitric oxide (NO). The primary source of $O_2^{\bullet-}$ in mitochondria is the electron transport chain (ETC), in particular complexes I and III (Fig. 2) (7). Increased production of ROS is an essential step in the activation of lymphocytes and is a potent trigger of pro-inflammatory cytokine production (33). The excessively generated ROS, related to persistent inflammation, induce oxidative damage to mitochondrial proteins, lipids, cell membranes and genetic material (e.g. mitochondrial DNA, which is free of histones), which subsequently leads to the structural alterations and altered mitochondrial function (7,34) This dysfunction manifests through decreased mitochondrial membrane potential, reduced electron flow along the electron transport chain and decreased ATP levels, with consequences linked to impaired cellular function, potentially leading to intrinsic apoptotic pathway (35). Induction of DM and PD in Wistar rats caused mitochondrial dysfunction, impaired ATP production, decreased mitochondrial DNA copy number and reduced expression of complex I subunits and increased oxidative stress in gingival samples. Clinically, mitochondrial dysfunction resulted in a worsening of PD, with greater alveolar bone loss (7). In a study using PBMC from PD patients, an increase in ROS production was determined in PD subjects compared to non-PD subjects (35).

Enhanced ROS levels in T2DM has been attributed to the regulation of glucose metabolism by mitochondria. When the available glucose does not meet the energy needs of cells, there can be a decrease in the rate of electron flow, which extends the lifetime of reactive intermediates in complexes I and III. In turn, this increase in ROS impacts on the increase in insulin-resistance, potentially establishing a vicious cycle (32).

In PD, the activation of immune defence mechanisms leads to increased ROS release by PMBCs, due to the increased number and activity of these cells (11). The increase in ROS stimulates the production of pro-inflammatory cytokines, such as TNF-alpha or IFN-gamma that induce the expression of endothelial cell adhesion molecules, necessary for leukocyte recruitment (32). Stimulation of these cytokines impacts on insulin resistance and systemic endothelial dysfunction, reflected in the perpetuation of systemic inflammation (36). Stimulation of osteoclastogenesis and apoptosis of osteoblasts also occurs with consequences on periodontal attachment loss (35) Chen et al. 2019;; Yu et al. 2012). (11).

Oxidative stress (OS) arises when antioxidant mechanisms fail to cope with the exacerbated increase in ROS. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) prevent tissue damage caused by excessive ROS production (22). SOD is a key antioxidant enzyme that dismutates $O_2^{\bullet-}$ into H_2O_2 and O_2. In turn, CAT and GPx accelerate the reduction of H_2O_2 into water (11). In a study by Trivedi and co-authors (11). there was a decrease in the activity of SOD, CAT and GR in the saliva of PD patients. Interestingly, in the group with T2DM and PD, with the exception of GR that was increased, the activity of SOD and CAT suffered a greater decrease when compared with PD. In erythrocytes, SOD, CAT and GR activities were higher in T2DM and PD group, when compared to the T2DM group. This increase can be related to the fact that PD triggers compensatory antioxidant mechanisms. However, it should be noted that since PD is a chronic inflammatory disease, antioxidant enzymes are ultimately unable to counterbalance oxidative damage (11).

Decreased activities of antioxidant enzyme systems in diabetes is linked to progressive glycation of several proteins (11). ROS play a role in T2DM and PD associated complications through the impairment of antioxidant gene expression responsible for ROS degradation and maintenance of vascular health (9,35).

7.2 Changes in transcription: Factor erythroid 2–related factor 2 and Hypoxia-inducible factor

The regulation of antioxidant activity plays an important role in progression of PD and T2DM. The decrease in antioxidant activity may be accounted for by reduced transcription of antioxidant enzymes. There are several transcription factors regulated by OS, such as nuclear factor erythroid 2–related factor 2 (Nrf2), nuclear factor kappa B (NF-κB) and hypoxia-inducible factor (HIF-1) (37). The Nrf2 signalling pathway is essential for regulating the antioxidant defence system in response to oxidative stress (9). Under basal conditions Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1) in the cytosol, however, under OS conditions, oxidation of the cysteines responsible for

Nrf2/Keap 1 binding occurs, resulting in their dissociation. The complex separates and Nrf2 binds to p21, a protein that prevents Nrf2-Keap-1 binding from re-establishing. The new complex migrates to the nucleus and binds to antioxidant response element (ARE, 5′-TGACXXXGC-3′) in the promoter region of Nrf2 target genes, which include NADPH quinone dehydrogenase 1 (Nqo1) and glutathione S-transferase (Gst), catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase, heme oxygenase-1 (HO-1), glutathione peroxidase (GPx), glutamate cysteine ligase (GCL), and peroxiredoxin 1 (Prx-1) (34). The partial deletion of the Keap-1 gene in mice with DM and the consequent accumulation of Nrf2, led to decreased blood glucose levels through improved insulin secretion, as well as reduced insulin resistance (38).

Under pathological conditions and deregulation of this pathway, antioxidant production is compromised and may be associated with worsening and progression of inflammatory pathologies such as T2DM and PD. In comparison with healthy subjects, the Nrf2 pathway was found to be downregulated in polymorphonuclear cells (PMN) from subjects with PD, leading to inhibition of antioxidant production. Given that PMN chemotaxis is a ROS-dependent process, the inability to neutralise ROS led to a continuous recruitment of these cells, making it impossible to resolve inflammation and clinically translating into a worsening of periodontal destruction (34). Of note, dysregulation of the Nrf2 pathway can promote osteoclast formation and increase bone resorption (39). It has been shown that beta cells have a high sensitivity to oxidative damage, so induction of the Nrf2 signalling pathway may reduce the risk of developing DM by reducing the level of oxidative stress (9). In studies with Wistar rats, there was a downregulation of Nrf2 in the DM-PD group when compared with the PD and DM groups, which, associated with increased local and systemic oxidative damage contribute to the development and progression of PD in subjects with T2DM (7,12). Of note, compared to the control group, in the group with only PD, the increase in oxidative damage occurred at the local level, whereas in the group with DM occurred at the systemic level. As expected, in the group with DM-PD, oxidative damage was more severe at both local and systemic levels. In this group there was a clear correlation between loss of alveolar bone, increased apoptosis of periodontal cells, increased protein damage (assessed by 3-nitrotyrosine (3-NT)) in DNA (assessed by 8-hydroxy-deoxyguanosine (8-OHdG)), increased lipid peroxidation (assessed by 4-hydroxy-2-nonenal (4-HNE)), decreased SOD activity and Nrf2 expression (12,40).

HIF-1 is composed of HIF-1alpha and HIF-1 beta, the former being degraded in the presence of oxygen, through the hydroxylation of HIF-1alpha, and activated under hypoxic conditions. The progression of PD is associated with the appearance of deeper and deeper periodontal pockets, characterised by an increasingly oxygen-poor and acidic microenvironment that favours the proliferation of anaerobic bacteria such as Porphyromonas gingivalis. Hypoxia increases the formation of ROS that lead to OS in periodontal ligament cells (41). HIF-1 regulates the transcription of genes associated with cellular adaptation to hypoxic conditions through upregulation of glycolysis, angiogenesis through vascular endothelial growth factor (VEGF) expression, recruitment of immune cells and inhibition of apoptotic factors (37,42,43). It is also important for the activation of macrophages and neutrophils, as well as for the promotion of phagocytosis (44). Analysis of human tissue samples from healthy and

inflamed gingiva and periodontal ligament under hypoxic conditions revealed a significantly more pronounced (30-fold) increase in HIF-1alpha expression in pathological samples (Gingivitis and PD). In PD tissue samples, a dramatic and widespread immunoreactivity for HIF-1alpha was observed in the cytosol and nuclei of almost all cell types, namely of fibroblasts and immune cells of the infiltrate (41). The same was found in another in vivo study with human gingival fibroblasts (12) and in both saliva and GCF (42) increased levels of HIF-1alpha, VEGF and TNF-alpha were observed in periodontitis subjects. In T2DM, exposure to high glucose levels has been shown to induce hypoxia and hypoxia-induced pathways in beta cell lines, namely the HIF pathway (45).

The Nrf2 pathway is a key factor in the HIF-1 pathway. A study in pancreatic and lung tumour cells found that in the absence of Nrf2, under hypoxic conditions, the HIF-1alpha subunit was also absent. The absence of both factors (Nrf2 and HIF-1alpha) under hypoxic conditions led to the questioning of the existence of a stabilising protein interaction of HIF-1alpha. This interaction was subsequently confirmed (43). As there is a dependence relationship of HIF-1alpha on Nrf2, downregulation of the latter may lead to downregulation of the former confirmed (43).

NF-κB is an important transcription factor in the immune response, activated by inflammation. It is involved in processes such as cell proliferation (T, B and dendritic cells), differentiation, apoptosis and immune response, through the expression of pro-inflammatory cytokines such as IL-1β, chemokines, adhesion molecules, and enzymes such as MMPs. Analysis of periodontal tissue samples (healthy gingiva and periodontal ligament (PDL) and in conditions of gingivitis and periodontitis), revealed a strong and generalised immunoreactivity of NF-κB in PD cases, compared to the other samples. The increased activity of this factor may lead to an overproduction of these mediators, which, as already mentioned, promote inflammation and aggravate the local reaction (46).

Dysregulation of the NF-κB pathway is associated with an impaired or uncontrolled response that, in turn, translates into a lack of immune response or an over-response. The interaction between HIF-1alpha and NF-κB is known and important in the inflammatory response, but it is non-linear, since, depending on the cell type, these factors can cooperate or antagonise each other. NF-κB and HIF share some functions when activating the immune response to infection, promoting the expression of pro-inflammatory cytokines and increasing bacterial anti-phagocytosis activity. In neutrophils, HIF increases NF-κB activity stimulating, as mentioned, the release of pro-inflammatory cytokines and reducing apoptosis (44). In turn, NF-κB plays an important role in basal and stimulated HIF-1α mRNA expression. In tissues with periodontal pathology and under hypoxic conditions, it was hypothesised that NF-κB activation may have induced HIF-1alpha mRNA expression (46).

The synergy between the afore mentioned signalling pathways is critical for homeostasis, which is compromised in chronic inflammatory pathologies such as PD and T2DM. Downregulation of Nrf2 at the expense of exacerbated ROS increase has direct consequences on the inefficiency of antioxidant activity and is indirectly related to changes in the inflammatory immune response through HIF-1alpha.

The mechanisms behind the dysregulation of transcription caused by OS are not fully explained. MicroRNA (miRNA, miR), non-coding RNA sequences that modulate gene expression by suppressing

or stimulating it, play important biological functions, notably in the immune response and in the response to SO (47).

miRNAs can modulate the Nrf2 signalling pathway through changes in Keap1, Nrf2 expression, Nrf2 migration to the nucleus and through modulation of mediators upstream of the Nrf2 signalling pathway. miRNAs such as miR-34, miR-223 and miR-27a stimulate the Nrf2 pathway. miR-223 is associated with T2DM, and is characterised by promotion of antioxidant activity through enhanced expression of HO-1, SOD1 and SOD2 (9). In T2DM, another miRNA, miR21, was increased, which promotes Nrf2 migration into the nucleus and triggers the antioxidant response.

One study, found an overregulation of miR-21 in subjects with PD and in mice with induced PD. A decrease in the production of pro-inflammatory cytokines such as IL-6 and TNFalpha by macrophages was observed, leading to the conclusion that this miRNA has an anti-inflammatory activity. Being IL-6 and TNFalpha pro- osteoclastogenic factors, the decrease of this miRNA, would lead to gingival and alveolar bone loss (48).

Another study identified miR-146a, miR-146b, miR-155 as promising in PD and T2DM. These microRNAs were analysed from saliva samples of T2DM, PD and T2DM-PD patients and healthy patients. Higher levels were found in patients with both pathologies compared to the other groups. miR155 is implicated in the regulation of NF-kB activation. The activity of the NF-kB-miR-155 axis in combination with the NF-kB-miR146a axis regulates the intensity and duration of inflammation (47).

MiRNAs play important roles in the transcriptional regulation of antioxidant enzymes, so their downregulation is expected to have implications in the clinical manifestations of inflammatory pathologies such as PD and T2DM.

8. Conclusion

The elevated levels of key inflammatory mediators support the hypothesis that PD exacerbates the T2DM immune-inflammatory response and the opposite is also true, clinically reflected through increased periodontal destruction and insulin resistance.

Mitochondrial dysfunction was confirmed in these two pathologies and associated with the increased ROS that together with reduced antioxidants underlie OS. As such, decreased activity of SOD and CAT was shown to be highly pronounced in T2DM-PD subjects.

The influence of ROS on the regulation of the transcription of antioxidant enzymes, through downregulation of transcription factors such as Nrf2, has been also reported. Due to the synergy between transcription factors, namely between Nrf2, HIF-1 and NF-κB, downregulation of one of them can lead to downregulation of the others. In both PD and T2DM alone, there was an increase in HIF-1alpha transcription. In PD, HIF-1 increased NF-κB activity, which in turn increased HIF-1alpha expression, contributing to the exacerbation of the immune-inflammatory response. The processes involved in the downregulation of antioxidant enzyme transcription are not fully understood. Additionally, miRNAs have shown an increasing importance in modulating transcription, constituting an important object of study in understanding the transcriptional alterations associated with inflammatory pathologies such as PD and T2DM.

Future studies on the molecular and cellular mechanisms involved in T2DM-PD may help to develop more efficient therapeutic strategies to alleviate the clinical consequences and progression of this pathological condition.

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