FACULDADE DE MEDICINA
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Mitochondrial respiratory chain complexes and antioxidant enzymes analysis in diabetes and chronic periodontitis-derived human blood mononuclear cells

Olga Fokt

Orientadora: Professora Doutora Ana Cristina Carvalho Rego
Co-orientadora: Professora Doutora Isabel Poiares Baptista
Supervisor de laboratório: Doutora Ildete Luísa Ferreira

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Fokt O[1], Ferreira IL[2,3], Baptista IP[1,4], Rego AC[1,2]

[1] Área de Medicina Dentária, Faculdade de Medicina da Universidade de Coimbra
[2] CNC-Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
[3] IIIUC-Institute for Interdisciplinary Research, University of Coimbra, Coimbra, Portugal
[4] Instituto de Periodontologia, Faculdade de Medicina da Universidade de Coimbra, Portugal

Área de Medicina Dentária da Faculdade de Medicina da Universidade de Coimbra
Av. Bissaya Barreto, Bloco de Celas
3000-075 Coimbra
Portugal
Tel: +351 239 484 183
Fax: +351 239 402 910
E-mail: olga.fokt@gmail.com

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Abbreviations

BOP – Bleeding on probing

CAL – Clinical attachment level

CP – Chronic periodontitis

CS – Citrate synthase

Cx I – Complex I

Cx III – Complex III

DM – Diabetes mellitus

GPx – Glutathione peroxidase

GRed – Glutathione reductase

GSH – Reduced glutathione

GSSG – Oxidized glutathione

IL-1β – Interleukin 1β

IL-6 – Interleukin - 6

MRC – Mitochondrial respiratory chain

PB – Probing depth

PBMC - Peripheral blood mononuclear cells

PBS – Potassium buffer saline

PPB – Potassium phosphate buffer

ROS – Reactive oxygen species

SOD – Superoxide dismutase

Abstract

Periodontitis is a chronic inflammatory disease, initiated by the presence of a bacterial biofilm affecting the tissues that support the teeth and culminating in bone resorption. Diabetes *mellitus* (DM) is a group of metabolic diseases characterised by hyperglycaemia that induces an excessive proinflammatory state. Current evidence points to a bidirectional interrelationship between diabetes and periodontitis, which has become known as the sixth complication of DM and could be a risk factor for diabetic decompensation. These pathologies associate with oxidative stress due to increased production of reactive oxygen species (ROS). Thus, in this work we aimed to define dysfunctional activity of mitochondrial complex I and III, two major producers of mitochondrial ROS, and the activities and protein levels of cellular antioxidants using peripheral blood mononuclear cells (PBMCs) from patients with chronic periodontitis (CP) and DM plus CP (DM-CP), when compared to control individuals. Patient and control individuals were selected from dentistry appointments at the Hospital Centre of Coimbra University (CHUC) where clinical parameters for the diagnosis of periodontal health were determined. We analysed the enzyme activities of complex I and III from mitochondrial electron transport chain, and related citrate synthase, an enzyme of the citric acid/Krebs cycle, as well as the activity of glutathione redox cycle, Mn-superoxide dismutase (SOD)/SOD2 and catalase levels in isolated PBMCs. Our results show decreased activity of complex III in DM-CP group, and reduced activity of glutathione cycle, namely decreased levels of reduced and oxidized glutathione (GSH and GSSG) and diminished activities of glutathione peroxidase (GPx) and glutathione reductase (GRed) in DM-CP group. Furthermore, we found a tendency for increased levels of acetyl(K68) SOD2 and reduced protein levels of catalase in PBMCs from DM-CP patients. These data indicate potential increased mitochondrial generation of ROS and reduced antioxidant profile in blood cells from DM-CP, thus explaining exacerbated ROS levels in DM-CP group observed in our previous study.

Keywords: Chronic Periodontitis; Diabetes Mellitus Type 2; Oxidative Stress; Mitochondria; Antioxidants

Introduction

Periodontitis is a chronic multifactorial and inflammatory disease initiated and sustained by plaque biofilm accumulation apically to the gingival margin, containing gram-negative anaerobic bacteria affecting the supporting tissues of the teeth (gingiva, periodontal ligament, cementum and bone) (Fig. 1) [(1)(2)(3)(4)(5)]. It is a major public health problem due to its high prevalence, as well as because it may lead to tooth loss and disability[(4)]. This disorder result of an aberrant inflammatory host response to the biofilm characterized by secretion of inflammatory mediators such as interleukin (IL-1β, IL-6), prostaglandin E2, tumour necrosis factor α, matrix metalloproteinases, and regulatory T-cells that produce cytokines and chemokines, being the gingival inflammatory infiltrate mainly composed by neutrophils[(1)(3)(6)]. The diagnosis of periodontitis is made by clinical evaluation (bleeding on probing (BOP), probing depth (PB) and clinical attachment level (CAL)) and radiological, and biological signs and symptoms[(4)(5)].

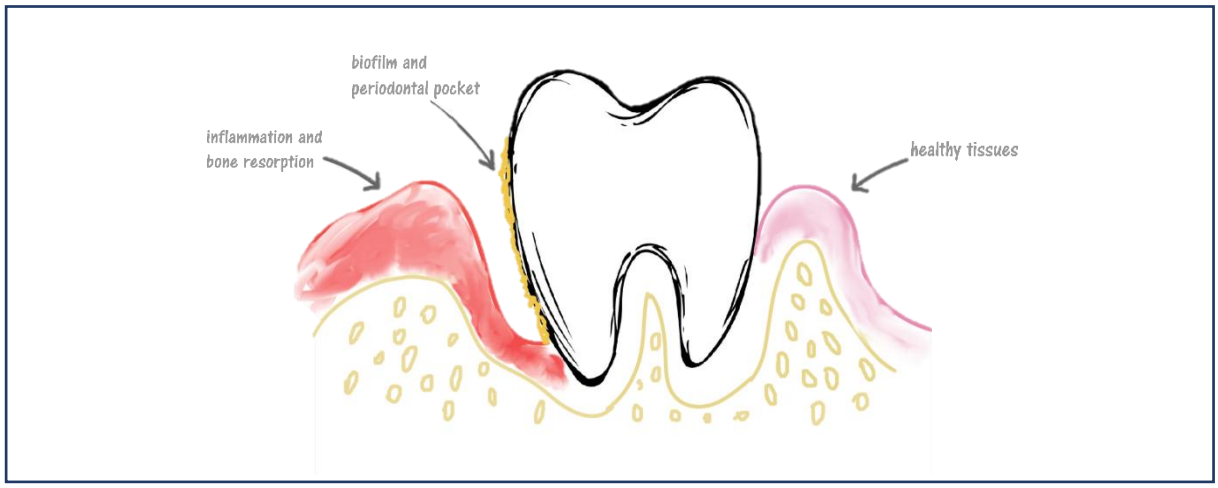

Figure 1. Periodontal condition vs healthy condition. Periodontal state (left) is characterized by the presence of biofilm, gingival inflammation, increase of probing depth along with bone loss. Healthy condition (right) is represented by the presence of healthy tissues. Adapted from: https://www.vectorstock.com/royalty-free-vector/hand-drawn-tooth-icon-vector-18759105

The determinants of the rate of periodontal disease development, its severity and extent are in accordance with both local risk factors, known as predisposing factors, and systemic risk factors, referred to as modifying factors[(5)]. Diabetes mellitus (DM) is one of the systemic risk factors of periodontal disease, considered the sixth complication of DM[(1)(2)].

Considered as a group of metabolic diseases, DM is characterized by an increase in blood glucose concentration caused by inherited and/or acquired deficiency in insulin production and/or action[(1)(7)]. This disorder can be classified into the following general categories: 1) type 1 DM, also known as insulin-dependent diabetes, characterized by an

autoimmune pancreatic β-cell destruction, normally leading to complete insulin deficiency, 2) type 2 DM, largely associated to insulin resistance, also resulting in a progressive loss of β-cell insulin secretion, 3) gestational diabetes mellitus, that is diagnosed in the second or third trimester of pregnancy and 4) diabetes associated with monogenic diabetes syndromes, diseases of exocrine pancreas and drug- or chemical-induced diabetes[8].

Several studies have pointed out that chronic periodontitis (CP) and diabetes have a bi-directional association[1][2][9][10]. In fact, periodontitis is considered a complication of diabetes and diabetes a modifier factor of CP. In that way, the progress of periodontitis can be enhanced by diabetes, especially when there are reduced control of glycaemic levels and the presence of periodontitis in patients with DM, impairs their glycaemic control [3][9][10].

Mitochondrial dysfunction is known to play a significant role in several pathological conditions such as DM, where inflammation is perpetuated by immune cells[11]. Several studies have characterized mitochondrial function in peripheral blood mononuclear cells (PBMC) and platelets in different conditions such as diabetes showing increased production of mitochondrial reactive oxygen species (ROS), resulting in oxidative stress, and altered mitochondrial morphology and dynamics[12][13]. ROS production has been identified as an early step in the activation of the inflammatory response, stimulating pro-inflammatory cytokines production. Mitochondria are the main intracellular source of ROS during electron transport chain redox reactions. Electrons from NADH and $FADH_2$ formed in the Krebs cycle flow in the mitochondrial respiratory chain (MRC) through a series of membrane proteins (Complexes I, III, IV) and generate a proton gradient across the membrane. These protons then flow through ATP synthase (Cx V) to generate ATP from ADP and inorganic phosphate. Oxygen is the final electron acceptor of the electron-transport chain. Under physiological conditions, about 1-5% of oxygen consumed by mitochondria in tissue cells is converted to ROS. In this way, leakage of electrons from MRC can combine with O_2 forming a reactive oxygen specie named superoxide anion ($O_2^{\bullet -}$) (Fig.2).

Defects in the MRC in affected tissues may contribute to increased production of $O_2^{\bullet -}$ being the antioxidants defence (non-enzymatic and enzymatic systems) work against the harmful effects of ROS[10][14][15][16][17][18][19]. Among non-enzymatic mechanisms, molecules such as ascorbic acid, α-tocopherol, β-carotene, reduced glutathione (GSH), thioredoxin, albumin, transferrin and caeruloplasmin act in ROS scavenging and by chelating transition metal ions. Superoxide dismutase (SOD) catalyses the dismutation of $O_2^{\bullet -}$ to H_2O_2, and catalase, glutathione peroxidase (GPx) and peroxiredoxins accelerate H_2O_2 reduction to water[17][18][19]. Cu/Zn-SOD (SOD1) acts in the cytosol, whereas Mn-SOD (SOD2) is an important enzyme located in the mitochondrial matrix. Although these enzymes can dispose

most of the ROS and free radicals, sometimes ROS formation surpasses the cellular defence system and oxidative stress is imposed, leading to critical damage of cellular macromolecules, including nucleic acids, proteins and lipids[17][19].

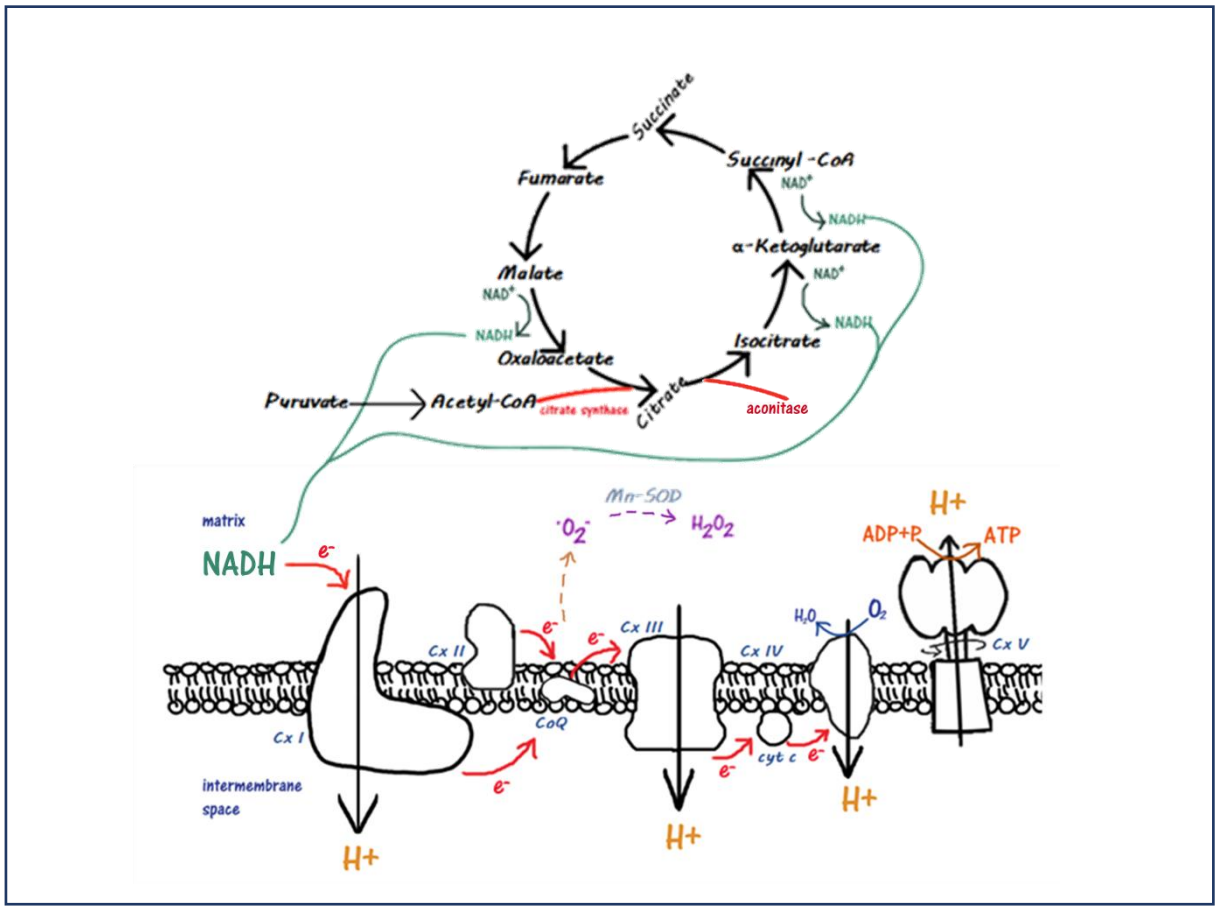

Figure 2. Simplified mitochondria respiratory chain and Krebs cycle. The citric acid cycle includes a series of oxidation-reduction reactions. The pyruvate generated from glucose is decarboxylated to form Acetyl-CoA and CS catalyse a condensation reaction between acetyl-CoA and oxaloacetate to form citrate. The electrons removed from acetyl-CoA are used to form NADH and $FADH_2$. Electrons released from these molecules flow through a series of membrane proteins (Complex I, II, III, IV) to generate a proton gradient across the membrane. These protons then flow through ATP synthase (Cx V) to generate ATP from ADP and inorganic phosphate. O_2 is the final electron acceptor of the electron-transport chain.

Recently, induced DM and CP in Wistar rats caused increased ROS, impaired mitochondrial function and compromised bioenergetics contributing to aggravated pathogenesis of periodontitis in diabetic gingival samples obtained from mice[10]. In accordance are our previous study showing that patients with both pathologies, DM and CP, show exacerbated H^+ leak along with decreased bioenergetic health index (BHI) values impacting on increased ROS levels production[20]. These observations raised the question whether ROS would be produced as a result of reduced antioxidant activity and/or alteration in the activity of MRC complexes in PBMCs obtained from these patients.

Thus, the aim of this study was to analyse the activity of MRC complexes, namely complexes I and III (Cx I and Cx III) that have been largely implicated in mitochondrial ROS production, and the activity of citrate synthase (CS), an enzyme of the Krebs cycle (Fig. 2). In addition, we aim to determine the activity of cellular antioxidants, namely glutathione cycle enzymes, reduced and oxidized glutathione levels, acetyl-SOD2, and catalase levels in order to explain the high amounts of ROS production and their relationship with selective modifications of the MRC in patients with DM and CP observed in our previous work[20].

Material and methods

Reagents

- 2,6-Dichlorophenolindophenol sodium salt hydrate (DCPIP; Sigma, cat. no. 33125)
- 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB; Sigma, cat. no. D218200)
- Acetyl CoA lithium salt (Ac CoA; Sigma, cat. no. A2181)
- Antimycin A (Sigma, cat. no. A8674)
- β-Nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH; Sigma, cat. no. N4505)
- Bovine serum albumin, essentially fatty acid free (Sigma, cat. no. A6003)
- Cytochrome c from equine heart (Sigma, cat. no. C2506)
- Decylubiquinone (DUB; Sigma, cat. no. D7911)
- EDTA (Sigma, cat. no. ED2P)
- Oxaloacetic acid
- Potassium cyanide (KCN)
- Potassium phosphate dibasic
- Potassium phosphate monobasic (Sigma, cat. no. ED2p)
- Sodium phosphate monobasic dihydrate (Sigma cat. no. 71505)
- Rotenone (Sigma, cat. no. R8875)
- Tris(hydroxymethyl)aminomethane (Fisher, cat. no. BP152-1)
- Triton X-100 (Sigma, cat. no. X100)
- Tween-20 (Sigma cat. no. P5927)
- Ubiquinone1 (Sigma, cat. no. C7956)
- L-Glutathione reduced (GSH; Sigma cat. no. G4251)
- Glutathione reductase (GRd; Sigma cat. no. G3664)
- L-Glutathione oxidized (GSSG; Sigma cat. no. G4501)
- tert-Butyl Hydroperoxide Solution (t-BHP; Aldrich cat. no. 458139)
- Phosphoric acid (H3PO4;
- O-phthaldehyde (OPT; Sigma cat. no. P1378)
- N-ethylmaleimide (NEM; Sigma cat. no. E3876)
- Manganese chloride (MnCl2;
- DL-Isocitric acid trisodium (Sigma cat. no. I1252)
- Sodium dodecyl sulfate (SDS; Fisher BP1311-1)
- DL-Dithiothreitol (DTT; Nzytech MB033101)
- Bromephenol Blue sodium salt (Sigma B8026)
- NaCl (Merk, cat. no. 106404)
- Polyvinylidene difluoride (PVDF) membranes
- ECF substracte (GE healthcare, 1067873)
- Anti-SOD2/MnSOD (acetyl K68) antibody (Abcam ab 137037)
- Anti-SOD2/MnSOD antibody (Abcam ab13533)
- Anti-Catalase antibody - Peroxisome Marker (Abcam ab15834)
- Actin Antibody **(**Sigma A5316)

Equipment

- Microplate reader Spectra Max Plus 384 (Molecular Devices, USA)
- Perkin–Elmer Luminescence Spectrometer LS 50B
- BioRad ChemiDoc Touch Imaging System (BioRad, Hercules, USA)
- Image Lab analysis software (BioRad, Hercules, USA)

1.1 Subject selection

This study was carried out from January 2018 to June 2019. Samples containing PBMC pellets were obtained in 2017 during the master thesis of *Costa A. S. 2017*[20] and were stored at -80°C .The patient's selection was screened from dentistry appointments in Hospital Centre of Coimbra's University (CHUC). Informed written consent was obtained from all subjects that agreed to participate voluntarily (see attachment 1). The individuals were categorized into 3 diseased study groups (CP group: 10 subjects with history of periodontal disease, with probing depths > 3mm; DM group: 10 subjects with no history of periodontal disease, but with clinical diagnosis for diabetes mellitus type 2; DM-CP group: 10 subjects with history of periodontal disease, with probing depths >3mm and clinical diagnosis of diabetes mellitus type 2) and a control group (Control group: 10 subjects with no history of either periodontal disease or diabetes mellitus type 2[20].

Isolation of peripheral blood mononuclear cells from buffy coat

To optimize the experimental design for a reliable assessment of the respiratory chain enzymatic activities, a buffy coat sample from a human blood sample was used to isolate PBMCs. An aliquot (1 mL) was diluted with 9 mL phosphate buffered saline (PBS) [containing (in mM): 137 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 $Na_2HPO_4 \cdot 2H_2O$, pH 7.4] and carefully layered onto 8 mL of Ficoll-Paque™ solution in 50 mL Falcon tubes and then centrifuged at 2500 rpm for 20 minutes at 18ºC in a swing-out rotor without brake. After centrifugation, the lymphocyte-containing ring was removed with a Pasteur pipette, collected in another 50 mL Falcon tube and further diluted with PBS to a final volume of 45 mL. The tubes were then centrifuged at 1500 rpm for 10 min at 18°C with maximum acceleration and breaking, and pelleted cells stored at -80°C until use.

1.2 Preparation of total extracts and protein quantification

The PBMCs pellets obtained from all samples (10 controls, 10 CP: 10 DM and 10 DM-CP) were resuspended in hypotonic potassium phosphate buffer (PPB) containing: 0.5 M potassium phosphate dibasic (K_2HPO_4) and 0.5 M potassium phosphate monobasic (KH_2PO_4), pH 7.5, homogenised by pipetting up and down several times, snap-freezed in liquid nitrogen 3 times and stored at -80°C

Total protein concentration measurements were performed using the Bio-Rad protein assay (Bio-Rad 500-0006) by Bradford method.

1.3 Measurement of complex I activity

To optimize the amount of protein required to perform the assay, the PBMCs samples obtained from the buffy coat were used to evaluate Cx I activity. The Cx I activity was evaluated using 20 µg, 30 µg and 50 µg protein by following the decrease in absorbance due to oxidation of NADH and by the reduction of ubiquinone at 340 nm in 96 multiwell UV plates at 30°C using a microplate reader Spectra Max Plus 384 (Molecular Devices, USA), according to protocol described by *Spinazzi et al., 2012*[21]. For that purpose, and to the desired amount of protein, 10 µL PPB (0.5 M, pH 7.5), 6 µL BSA (Stock concentration (SC) 50 mg/mL), 3 µL KCN (SC 10 mM) and 1 µL NADH (SC 10 mM) were added, and the volume adjusted to 100 µL with water. In parallel experiments the same quantity of reagents and sample were prepared, but with the addition of 1 µL of rotenone, a Cx I inhibitor (SC 1 mM). The baseline was read for 2 min being the reaction started by adding 0.6 µL of ubiquinone (SC 10 mM) for additional 2 min. Unfortunately, we were only able to obtain consistent results using 50 µg of protein, so we decided to proceed with other trials due to the low protein content in our samples, as also described in Results section.

1.4 Measurement of complex III activity

This assay measures the activity of Cx III by following the increase in absorbance due to cytochrome c reduction. The analysis was performed in 96 multiwell plates at 550 nm at 30°C by using a microplate reader Spectra Max Plus 384 (Molecular Devices, USA), according to protocol described by *Spinazzi et al., 2012*[21]. For that purpose, samples containing 3 µg of protein plus 5 µL PPB (0.5 M, pH 7.5), 7.5 µL of oxidized cytochrome c (SC 1mM), 5 µL of KCN (SC 10 mM), 2 µL of EDTA (SC 5 mM, pH 7.5) and 1 µL of Tween-20 (SC 2.5% (v/v)), were mixed and the final volume adjusted to 100 µL with water. The baseline absorbance values were recorded for 2 min and the reaction started by adding 1 µL of decylubiquinol solution (SC 10 mM) followed by 2 min readings.

1.5 Evaluation of citrate synthase activity

Citrate synthase is a Krebs cycle matrix enzyme, used as a marker of the abundance of mitochondria within a tissue/cell. Thus, CS activity measurements are widely used to normalize other MRC enzyme activities[21]. The assay measures the activity of CS by following 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) reduction to 2-nitro-5-thiobenzoic acid (TNB) which has a yellowish colour. The analysis was performed in 96 multiwell plates at 412 nm at 30°C by using a microplate reader Spectra Max Plus 384 (Molecular Devices, USA), according to

protocol described by *Spinazzi et al., 2012*[21]. Briefly, 6 µg of protein sample was added plus 50 µL of Tris (SC 200 mM, pH 8.0) with Triton X-100 (SC 0.2% (vol/vol)), 10 µL of DTNB (SC 1mM) and 3 µL of Acetyl CoA (SC 10 mM) and the final volume adjusted to 100 µL with water. The baseline was read for 2 min and the reaction started by adding 5 µL of oxaloacetic acid (SC 10 mM) and absorbance followed for additional 3 min.

1.6 Measurement of glutathione levels by fluorimetry

The intracellular total levels of reduced and oxidized glutathione (GSH and GSSG, respectively) (Fig.3) were determined using a fluorometric assay, according to method described by *Resende et al.,2008 and Ribeiro et al., 2012*[22][23] with some modifications, in 96 multiwell UV plates. GSH levels were measured in 20 µg sample protein after the addition of 5 µL o-Phthalaldehyde (stock concentration (SC) of 1 mg OPT/mL methanol) and phosphate buffer (100mM NaH_2PO_4, 5mM EDTA, pH 8.0) for 100µL of final volume. After 15 min incubation, the end point fluorescence was measured using an excitation wavelength of 350 nm and an emission wavelength of 420 nm.

For GSSG total levels determination, 25 µg protein was incubated for 30 min with 5 µL N-ethylmaleimide (SC 5 mg NEM/mL methanol). Then, the mixture was incubated for 15 min in 5 µL OPT (1 mg OPT/ml methanol) and adjusted with NaOH (SC 100 mM) for 100 µL of final volume. The fluorescence was measured with excitation at 350 nm and emission at 420 nm in a Perkin–Elmer Luminescence Spectrometer LS 50B.

1.7 Quantification of GPx and GRed activities

Measurement of glutathione reductase (GRed) and glutathione peroxidase (GPx) activities (Fig.3) was performed spectrophotometrically at 340 nm in 96 multiwell UV plates, through the analysis of NADPH oxidation, as described previously[22][23] with some minor modifications. Briefly, for the activity of GPx, 15 µg of protein samples was incubated for 5 min in the dark at 30°C with phosphate buffer (0.25 M KH_2PO_4, 0.25 M K_2HPO_4, and 0.5 mM EDTA, pH 7.0), 10 µL of GSH (SC 10mM) and 3,9 µL of GRed (SC 100-300 units/mg protein). The reaction occurred after the addition of 15 µL of NADPH (SC 2.5 mM) and 15 µL of tert-butyl hydroperoxide (t-BHP, SC 12 mM) for 150 µL final volume, and absorbance read for 5 min. For the activity of GRed, 15 µg of sample protein was incubated for 30 sec in the dark at 30°C, in phosphate buffer (0.2 M K_2HPO_4 and 2 mM EDTA, pH 7.0) and 10 µL of NADPH (SC 2mM). The measurements were initiated with the addition of 15 µL of GSSG (SC 20 mM). GRed and

GPx activities were determined using a *SpectraMax Plus384* microplate spectrophotometer (Molecular Devices, USA).

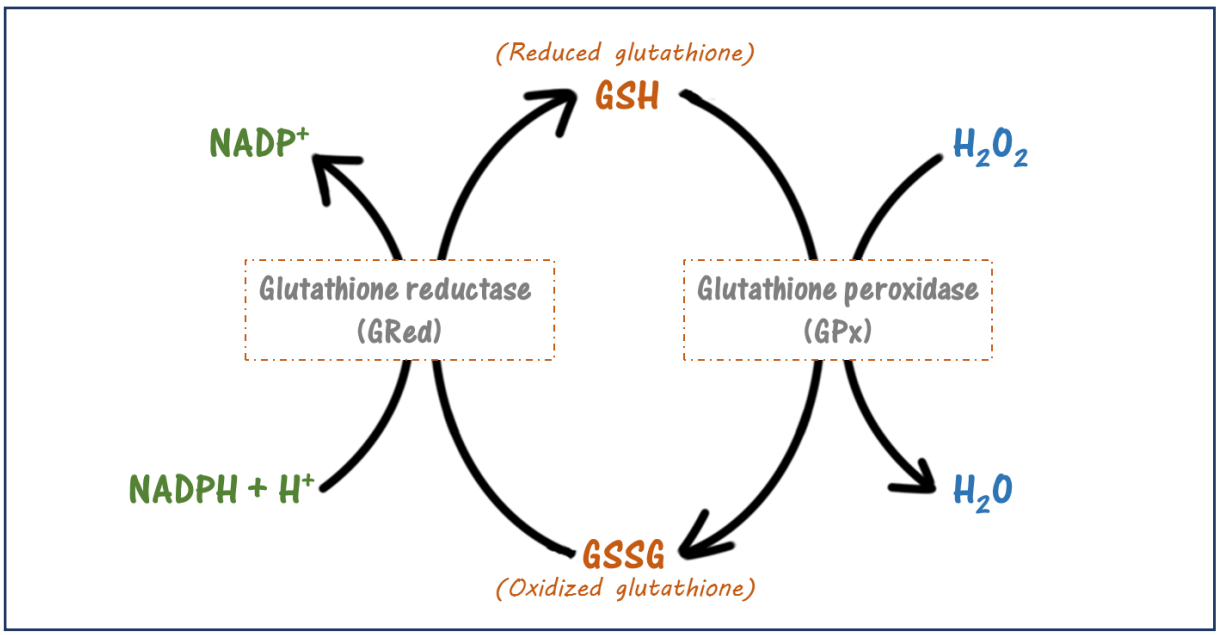

Figure 3. Glutathione cycle. GSH undertakes a redox reaction using GPx to detoxify ROS like hydrogen peroxide (H_2O_2). GSH is converted to an oxidized form (GSSG) and is recycled back to GSH by the enzymatic reaction of GRed which requires the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) to form a redox cycle.

1.8 Sample preparation and Western blotting

Total extracts obtained for lysed PBMCs as described before (section 1.2 of Material and Methods) were denatured with 6x concentrated loading buffer (containing 300 mM Tris-HCl pH 6.8, 12% SDS, 30% glycerol, 600 mM DTT, 0.06% bromophenol blue) at 75°C for 10 min. Equivalent amounts of protein (20 µg) were separated in 7.5% SDS-PAGE gel electrophoresis (accordingly to the molecular weight of proteins of interest) and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were further blocked with 5% BSA in TBS-Tween: 25 mM Tris-HCl, 150 mM NaCl, pH 7.6,)/0.1% Tween-20 (v/v), during 1 h at room temperature and further incubated overnight at 4°C with gentle agitation with primary antibodies, Anti-acetyl-SOD2 (K68), Anti-SOD2 and Anti-Catalase-peroxisome marker, prepared in 1% BSA-containing TBS-Tween. Membranes were incubated with anti-mouse or anti-rabbit IgG secondary antibodies prepared in 1% BSA-containing TBS-Tween for 1 h, at room temperature. In order to normalize the amount of protein per lane, anti-actin was used as loading control for catalase labelling. Immunoreactive bands were visualized by alkaline phosphatase activity after incubation with ECF reagent on BioRad ChemiDoc Touch Imaging System (BioRad, Hercules, USA) and quantified using Image Lab analysis software (BioRad, Hercules, USA).

Statistical analysis

Data were analysed by using Excel and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) software and results expressed as the mean ± SEM. Complex I, III, GPx and GRed activities as well as the measurement of GH and GSSG levels were performed in duplicates. The measurement of CS activity was performed in triplicates, in 10 individuals per group (Control, CP and DM-CP). Comparison among groups was performed by one-way ANOVA followed by Tukey's post-hoc test. Significance was defined as $p<0.05$.

Results

Mitochondria are defined as the largest producers of ROS in eukaryotic cells. In a previous work we observed a significant increase in ROS production by PBMCs obtained from individuals with CP and DM-CP, when compared with control individuals, as shown by Costa A.S. 2017[20]. Thus, in the first part of the present work we aimed to evaluate the activities of Cx I and Cx III of mitochondrial respiratory chain since they are known as the main producers of $O_2^{\bullet -}$ by this organelle[24]. Measurement of Cx I activity in PBMCs obtained from the buffy coat was only observed for high concentrations of protein. Unfortunately, and due to the low protein content in our samples, we were not able to perform these studies.

Our results demonstrated a significant decrease in Cx III activity (about 37%) in PBMCs mitochondria from individuals with DM-CP compared to the control subjects ($p<0.05$) (Fig.4A). A trend for decreased, although not statistically significant, CX III activity in CP patients was also observed (Fig.4A).

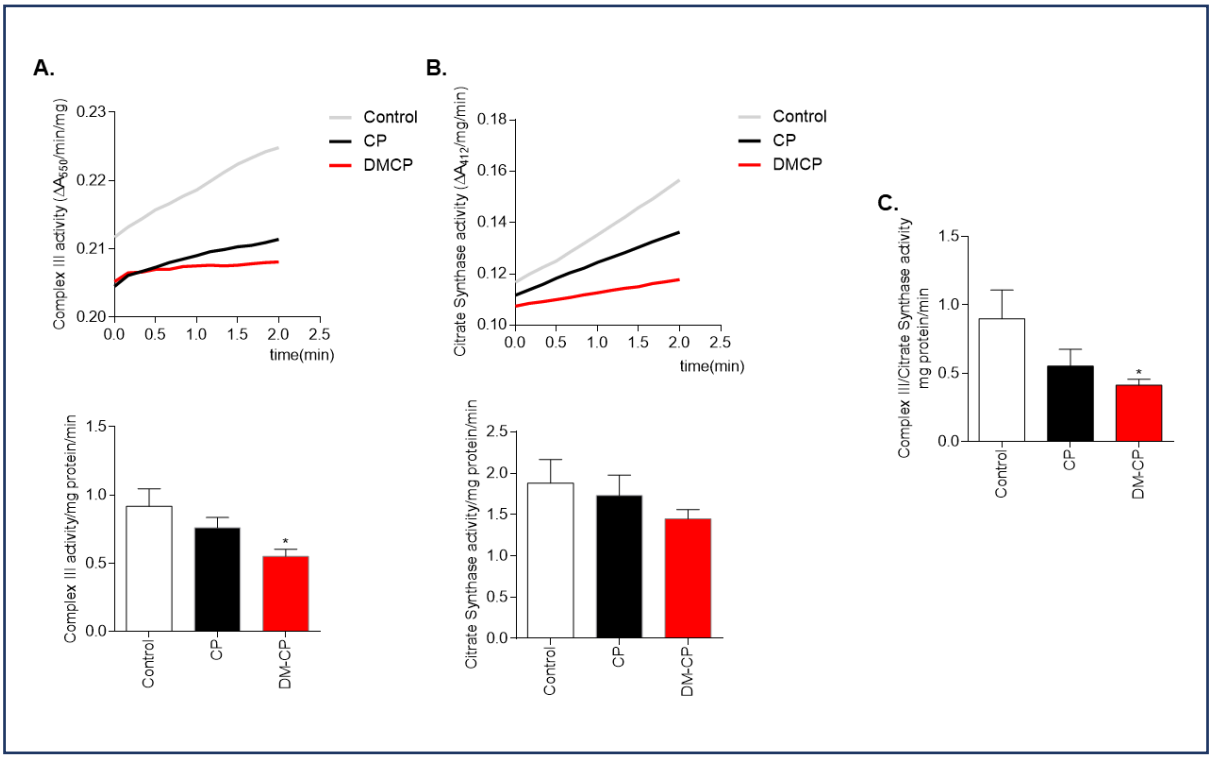

Figure 4. Complex III and citrate synthase activity. (A) Complex III activity was evaluated in PBMCs obtained from control individuals, chronic periodontitis (CP) and diabetes mellitus type 2 and chronic periodontitis (DM-CP) patients. **(B)** CS activity was evaluated in the same individuals. **(C)** Ratio between Cx III and CS activities. Data are presented as mean ± SEM performed in PBMCs isolated from 10 individuals per group, run in duplicates or triplicates. Statistical analysis: *$p<0.05$ by One-way ANOVA followed by Tukey's Multiple Comparison test.

The activity of CS was also measured in order to normalize the Cx III activity (Fig.4B). Despite a slight decrease in CS activity in DM-CP group, no significant differences were observed when compared to control individuals (Fig.5B). However, when the ratio between the Cx III and CS activities was calculated, a decrease of about 50% in DM-CP group was observed in relation to the control ($p<0.05$) (Fig. 4C).

In order to investigate if a disturbance in the prooxidant-antioxidant balance could contribute for the increased production of ROS in DM-CP group, the levels of glutathione (GSH and GSSG), and the activities of GPx and GRed, SOD2 and CAT levels were also determined (Figs 5, 6 and 7).

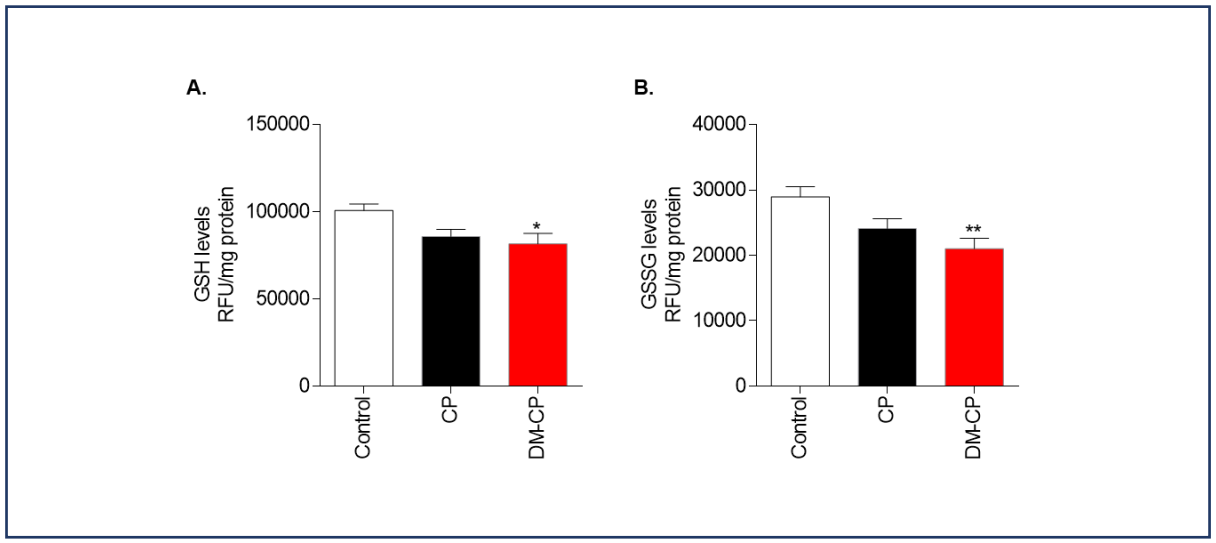

Figure 5. GSH and GSSG levels. (A) Reduced glutathione levels and **(B)** oxidized glutathione levels presented in RFU/mg of protein. Data are presented as the mean ± SEM, performed in PBMCs isolated from 10 individuals per group, run in duplicates. Statistical analysis: *p<0.05 and **p<0.01 by One-way ANOVA followed by Tukey's Multiple Comparison test.

GSH is used in antioxidant defence by GPx, which catalyses the reduction of H_2O_2 into H_2O and GSSG. The oxidized form of glutathione (GSSG) is potentially toxic to the cells; however, high levels of GRed activity maintain most of the GSH in the reduced form. Our results evidence a significant decrease in both GSH and GSSG levels in DM-CP group when compared with the control group (Fig. 5A, 5B), and a significant decrease of about 40% and 48%, in GPx and GRed activities, respectively (Fig.6A, 6B), suggesting a compromised glutathione cycle.

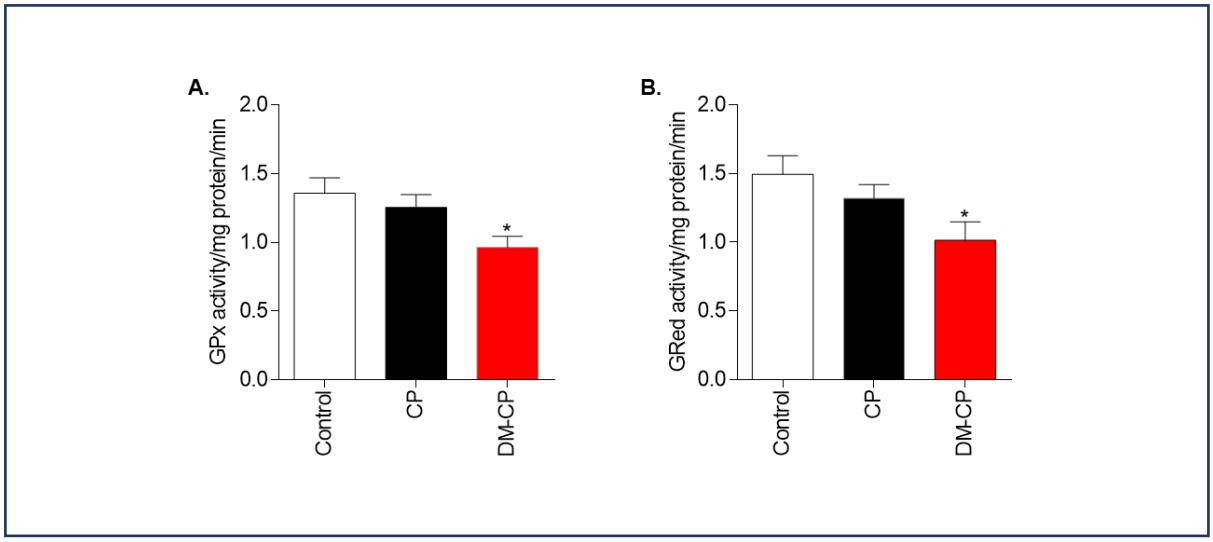

Figure 6. Glutathione peroxidase and glutathione reductase activities. (A) GPx activity and **(B)** GRed activity presented in mg of protein per minute. Data are presented as the mean ± SEM, performed in PBMCs isolated from 10 individuals per group, run in duplicates. Statistical analysis: *p<0.05 and by One-way ANOVA followed by Tukey's Multiple Comparison test.

Mitochondrial SOD2 or Mn-SOD catalyses dismutation of $O_2^{\bullet -}$ to H_2O_2, while CAT accelerate H_2O_2 reduction to water. Previously, increased acetylation of SOD2 at lysine 68 (K68) was shown to be linked to decreased enzymatic activity and excessive production of ROS in mouse diabetic oocytes[25]. Our results demonstrate a slight increase, although not significant, in protein levels of acetyl(K68)-SOD2 in DM-CP group, suggesting a tendency for a decrease in SOD2 activity. We also observed a tendency for decreased catalase protein levels in CP and DM-CP groups, when compared to control group (Fig. 7), suggesting reduced antioxidant defences in PBMCs derived from DM-CP patients.

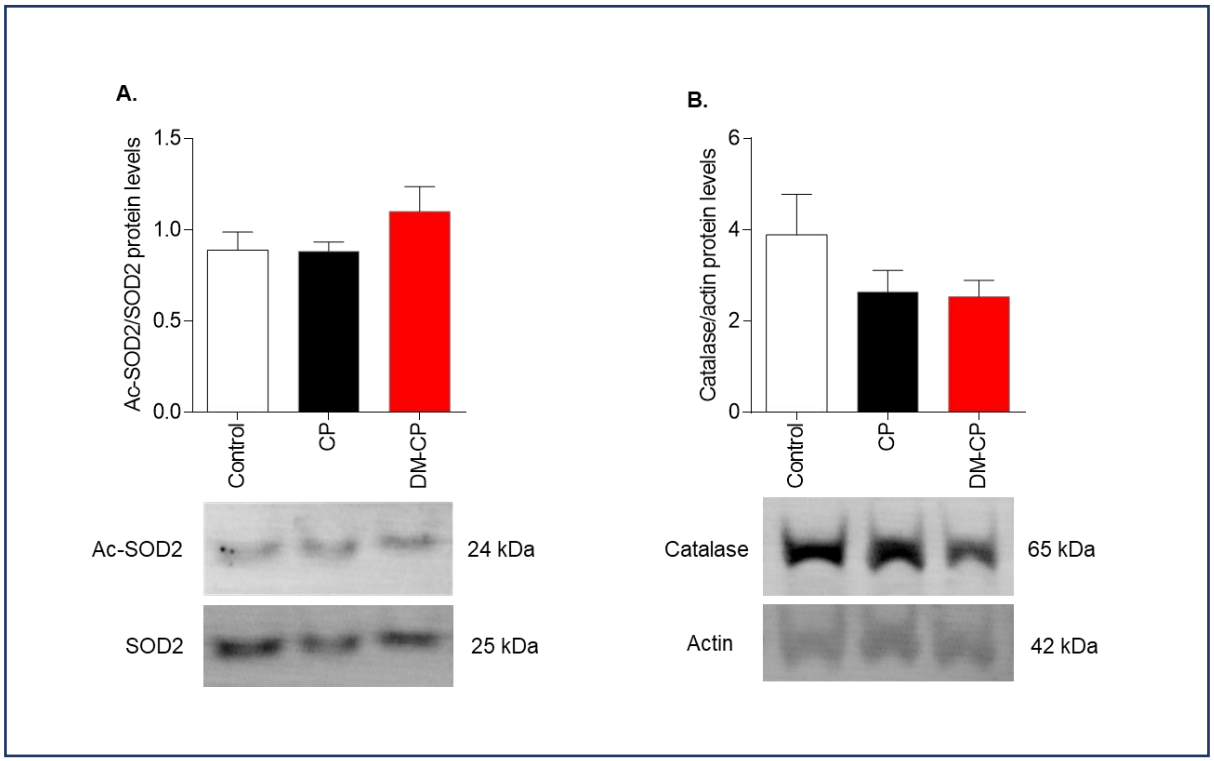

Figure 7. Ac-SOD2/SOD2 and catalase protein levels. (A) Ac-SOD2/SOD2 protein levels and **(B)** catalase/actin protein levels in PBMCs total lysates. Preliminary data are presented as the mean ± SEM, performed in PBMCs, (Control: n=8, CP: n=9, DM-CP: n=8).

Discussion and Conclusion

In this study we evaluated the influence of diabetic conditions on periodontitis by analysing mitochondrial function, more specifically the activity of Cx III of MRC and glutathione cycle enzymes, acetyl-SOD2 and catalase levels in PBMCs isolated from patients with chronic periodontitis (CP) and chronic periodontitis plus diabetes mellitus type 2 (DM-CP) *versus* healthy controls, in order to understand the increased production of ROS observed in a previous study in the same group of patients.

A decrease in Cx III activity in DM-CP patients is in accordance with data presented by Costa A. S., 2017, where an increased H^+ leak in DM-CP group and increased ROS production was shown[20]. The present data largely suggest that increased superoxide radical is generated at the level of Cx III in cells from DM-CP patients, potentially perpetuating a cycle of oxygen radical–induced damage. In the same work, an increase in ROS was observed also in the CP group, however in our study, we did not observe a statistically significant decrease in the activity of Cx III in CP patients that could explain the increase in ROS production.

The decrease of GSH and GSSG levels and GPx and GRed activities in DM-CP group observed in our study, may also explain the high levels of ROS, namely H_2O_2. Reduced GPx activity evidences decreased capacity to reduce H_2O_2 into H_2O and thus decreased conversion of GSH into GSSG, while reduced GRed indicates decreased regeneration of GSSG into GSH. *Arana C., et al., 2017,* showed that GPx and GRed activities, measured in saliva, were decreased in patients with poor metabolic control compared with diabetic patients with good metabolic control levels[26]. *Monea A. et al., 2014*, also reported that GSH was one of the antioxidants found to be decreased in periodontal tissues of patients with DM and periodontitis[27]. Although decreased levels of GSH and GSSG may be accounted for by reduced activities of GPx e GRed, one cannot exclude the possibility that enzymes involved in GSH synthesis, such as glutamate-cysteine ligase or glutathione synthase, are also affected in DM-CP patients.

According to *Patil V.S. et al., 2016*[3] study in red blood cells, SOD activity and catalase levels are significantly decreased in periodontitis patients with type 2 DM. Our results also show a slight decrease, although not significant, in SOD2 levels for CP and possibly DM-CP group and a tendency for decreased activity of SOD2 in DM-CP, as well as reduced catalase levels in PBMCs from CP and DM-CP patients. Opposing data was found by *Trivedi S. et al., 2013*[18], showing an increase in SOD and catalase activities in red blood cells from patients with diabetes and periodontitis, suggesting that increased ROS production in periodontitis may enhance the antioxidant defence system, counterbalancing the pro-oxidant environment. Another study performed in the gingival tissue from patients with periodontitis evidenced an

increase in expression of SOD2 genes when this condition was associated with DM with poor glycaemic control; however, catalase gene expression was not significantly influenced by any of these inflammatory disorders[19]. These apparent discrepancies may due to the differences in the biological fluid/tissue (PBMCs, erythrocytes, gingival tissue). In periodontitis there is a predominance of local ROS activity; therefore, evaluation of antioxidant status in gingival tissues could better clarify the role of antioxidant enzymes in the pathogenesis of periodontal diseases, although it would not be so appropriate when considering the study of patients with diabetes.

Excessive ROS production associated with decreased antioxidant defence mechanisms results in tissue destruction in periodontal diseases. It has been shown that superoxide and H_2O_2 activate and promote osteoclast formation, thus facilitating bone resorption and increasing the progression of periodontitis[28]. More severe destruction of periodontal tissues, related to excessive ROS, occurs when periodontal disease is associated with type 2 DM[3]. In this way it is important to lower ROS levels, for example, by increasing antioxidant defences. Alternatively, and according to *Masi S. et al., 2018,* an intense periodontal treatment was able to diminish mitochondrial ROS production of immune-inflammatory cells, being associated with an improved endothelial function in patients with DM and CP, and a significant improvement in the metabolic control in the same study population[29].

One of the limitations of our study was the non-inclusion of the diabetic group in our experiments. Following this study, it will be essential to perform the analysis in DM group, to serve as a control group in relation to the DM-CP group, since, for example, the reduction of GSH levels verified in this study group, could be present in the group of DM and thus be related only to diabetes mellitus and not be influenced by periodontitis. According to *Chang et al., 2003*, there is a decrease in GSH levels in rats with diabetes[30].

The data presented in this study corroborate the hypothesis that the increase in ROS levels may be related to a decrease in the Cx III activity of the MRC and to the reduction of antioxidant enzymes, such as SOD2 and CAT (although these analyses need to be completed) and glutathione redox cycle enzymes; nevertheless, it is important to evaluate the activity of the other complexes of the MRC, namely Cx I. We believe that there may be some Cx I dysfunction, since Cx I is another main source in ROS production; in this way it would be necessary to study the activity of Cx I in other patient samples to test this hypothesis.

The knowledge about the mechanisms that lead to the increase in oxidative stress in patients with DM and CP is expected to contribute to determine new therapeutic targets in order to better control the progression of chronic periodontitis in these patients and thus improve the metabolic control of patients with type 2 diabetes mellitus.

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Attachments

Attachment 1. Informed Consent

FORMULÁRIO DE INFORMAÇÃO E CONSENTIMENTO INFORMADO

TÍTULO DO PROJECTO DE INVESTIGAÇÃO: Caracterização sanguínea em doentes com periodontite e diabetes mellitus.

PROTOCOLO Nº não aplicável

PROMOTOR (Entidade ou pessoa(s) que propõe(m) o estudo): Mestrado Integrado em Medicina Dentária

INVESTIGADOR COORDENADOR Prof.ª Ana Cristina Rego, Prof.ª Dr.ª Isabel Poiares Baptista

CENTRO DE ESTUDO

INVESTIGADOR PRINCIPAL Ana Solange Gomes Costa

MORADA Av. Bissaya Barreto e Praceta Prof. Mota Pinto
3000-075 Coimbra

CONTACTO TELEFÓNICO B. central/ Bloco de Celas: Tel: 239 400 400

NOME DO DOENTE _____

(LETRA DE IMPRENSA) _____

É convidado(a) a participar voluntariamente neste estudo no âmbito da identificação de um marcador em situações de inflamação relacionadas com a diabetes mellitus e doença periodontal.

Este procedimento é chamado consentimento informado e descreve a finalidade do estudo, os procedimentos, os possíveis benefícios e riscos. A sua participação poderá

contribuir para melhorar o conhecimento sobre a identificação de marcadores no controlo da hiperglicémia e influência na doença periodontal e relação com a diabetes.

Receberá uma cópia deste Consentimento Informado para rever e solicitar aconselhamento de familiares e amigos. O Investigador ou outro membro da sua equipa irá esclarecer qualquer dúvida

que tenha sobre o termo de consentimento e também alguma palavra ou informação que não possa entender.

Depois de compreender o estudo e de não ter qualquer dúvida acerca do mesmo, deverá tomar a decisão de participar ou não. Caso queira participar, ser-lhe-á solicitado que assine e date este formulário. Após a sua assinatura e a do Investigador, ser-lhe-á entregue uma cópia. Caso não queira participar, não haverá qualquer penalização nos cuidados que irá receber.

1. INFORMAÇÃO GERAL E OBJECTIVOS DO ESTUDO

Desde há alguns anos, têm sido desenvolvidos estudos que relacionam a diabetes mellitus e a doença periodontal. Entende-se que a doença periodontal é uma patologia que implica a perda de osso alveolar e toda a afetação dos tecidos de suporte dentários por ação de diferentes microorganismos.

A diabetes mellitus pode ser subdividida em dois tipo: diabetes tipo I e diabetes tipo II. Os diabéticos tipo I são normalmente doentes mais jovens em que a função do pâncreas para produção de insulina foi perdida na totalidade, não estando associados especificamente a casos de obesidade, mas sim com a genética. Os diabéticos do tipo II são normalmente mais velhos e com obesidade ou com tendência para tal. Sendo uma doença que cada vez mais é frequente na população mundial, reveste-se de grande interesse a investigação sobre biomarcadores que possam relacionar a diabetes com a periodontite. Neste estudo, em específico, será abordada a diabetes mellitus tipo II.

A nível do plasma, componente sanguíneo, a resistina é um polipéptido de sinalização derivado dos adipócitos (células que constituem o tecido adiposo/ gordura) e que, tal como o nome indica, está relacionado com a resistência à insulina.

Através da colheita de sangue, é possível, com recurso a testes bioquímicos, detetar os níveis desta proteína. Pretende-se então, com este projeto de investigação, verificar se os níveis da proteína estão alterados nesta amostra de doentes e se a presença ou ausência de doença periodontal é um fator que influencia os mesmos.

Este estudo irá decorrer na área de Medicina Dentária em colaboração com o serviço de Bioquímica da Faculdade de Medicina da Universidade de Coimbra, com o objetivo de explorar a correlação entre os níveis plasmáticos de proteínas (como a resistina) em doentes com doença periodontal e diabetes.

Trata-se de um estudo observacional, pelo que não será feita nenhuma alteração na sua medicação ou tratamentos habituais.

Este estudo foi aprovado pela Comissão de Ética da Faculdade Medicina da Universidade de Coimbra (FMUC) de modo a garantir a proteção dos direitos, segurança e bem-estar de todos os doentes ou outros participantes incluídos e garantir prova pública dessa proteção.

Como participante neste estudo, beneficiará da vigilância e apoio do seu médico, garantindo assim a sua segurança.

Serão incluídos 20 doentes e 10 participantes saudáveis.

2. PROCEDIMENTOS E CONDUÇÃO DO ESTUDO

2.1. Procedimentos

O doente será sujeito a uma atualização da história clínica médica geral e oral. Em todos os doentes intervenientes no estudo será identificada a medicação e patologias. Especialmente dedicada à caracterização da doença periodontal, será preenchido um periodontograma.

Será feita uma recolha analítica e não invasiva de sangue em jejum.

(Colheita de sangue)

As colheitas de sangue serão feitas de acordo com os processos habituais para este tipo de análises, sendo as mesmas realizadas pela Enfermeira destacada para o serviço no dia em que o doente comparecer à consulta.

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2.2. Calendário das visitas/ Duração (exemplo)

Os doentes necessitarão de comparecer a duas consultas, em dias diferentes, sendo que na primeira necessitarão jejuar, efetuando-se a colheita de sangue e atualização da história clínica médica geral. Nesta primeira fase, está prevista uma duração máxima de 30 minutos; na segunda consulta será feita uma análise clínica mais detalhada que demorará no máximo 2 horas. No âmbito da medicina dentária, existe um interesse em relacionar algumas patologias sistémicas com patologias específicas da cavidade oral. Como já descrito na literatura, a doença periodontal ocupa a sexta complicação da diabetes mellitus.

Descrição dos Procedimento (exemplo):

Serão realizados os seguintes procedimentos/exames:

1ª consulta:

- Atualização da história clínica do paciente
- Recolha de sangue

2ª consulta:

- Preenchimento de um periodontograma
- No laboratório, efetuar-se-á o isolamento de células sanguíneas e análise
- bioquímica, de acordo com os métodos convencionais determinados.

Após obtenção de todos os dados:

- Tratamento dos resultados e análise estatística

2.3. Tratamento de dados/ Randomização

Foi selecionada uma amostra de 5 doentes para cada grupo. Todos os parâmetros clínicos e bioquímicos serão calculados de acordo com os testes estatísticos mais indicados para o estudo.

3. RISCOS E POTENCIAIS INCONVENIENTES PARA O DOENTE

O preenchimento de um periodontograma consiste na medição da profundidade de sondagem, hemorragia à sondagem, recessão gengival, mobilidade e verificação do envolvimento das furcas de todos os dentes da cavidade oral do doente, a fim de se fazer um diagnóstico. Este procedimento não implica quaisquer riscos nem inconvenientes para o doente.

A recolha de sangue será efetuada por enfermeiras que respeitarão os manuais de boas práticas para a recolha de fluidos.

4. POTENCIAIS BENEFÍCIOS

Este estudo permitirá confirmar a relação entre as duas patologias, doença periodontal e diabetes, através da identificação de vários marcadores sanguíneos. Assim, permitirar-se-á alargar conhecimentos acerca destas patologias, tratando-se de uma temática recente na bibliografia.

5. NOVAS INFORMAÇÕES

Ser-lhe-á dado conhecimento de qualquer nova informação que possa ser relevante para a sua condição ou que possa influenciar a sua vontade de continuar a participar no estudo.

6. TRATAMENTOS ALTERNATIVOS NÃO APLICÁVEL

7. SEGURANÇA Não aplicável

8. PARTICIPAÇÃO/ ABANDONO VOLUNTÁRIO

O participante é inteiramente livre de aceitar ou recusar participar neste estudo. Pode retirar o seu consentimento em qualquer altura sem que haja qualquer consequência, não necessitando de explicar razões. Assim, o doente não será sujeito a nenhum tipo penalidade ou perda de benefícios, não comprometendo a relação com o Investigador que lhe propõe a participação neste estudo. Ser-lhe-á pedido para informar o Investigador se decidir retirar o seu consentimento.

O Investigador do estudo pode decidir terminar a sua participação neste estudo se entender que não é do melhor interesse para a sua saúde continuar nele. A sua participação pode ser também terminada se não estiver a seguir o plano do estudo, por decisão administrativa ou decisão da Comissão de Ética. O médico do estudo notificá-lo-á se surgir uma dessas circunstâncias, e falará consigo a respeito da mesma.

9. CONFIDENCIALIDADE

De acordo com as leis e regulamentos aplicáveis e, não violando normas de confidencialidade, será atribuído o acesso aos registos médicos a auditores e autoridades reguladoras para verificação dos procedimentos realizados e informação obtida no estudo. De acordo com os regulamentos e leis aplicáveis, todos os registos manter-se-ão confidenciais e anonimizados. Se os resultados deste estudo forem publicados a sua identidade manter-se-á confidencial.

Ao assinar este Consentimento Informado autoriza este acesso condicionado e restrito.

Pode ainda em qualquer altura exercer o seu direito de acesso à informação. Pode ter também acesso à sua informação médica diretamente ou através do seu médico neste estudo. Tem também o direito de se opor à transmissão de dados que sejam cobertos pela confidencialidade profissional.

Os registos médicos que o identificarem e o formulário de consentimento informado que assinar serão verificados para fins do estudo pelo promotor e/ou por representantes do promotor, e para fins regulamentares pelo promotor e/ou pelos representantes do promotor e agências reguladoras noutros países. A Comissão de Ética responsável pelo estudo pode solicitar o acesso aos seus registos médicos para assegurar-se que o estudo está a ser realizado de acordo com o protocolo. Não pode ser garantida confidencialidade absoluta devido à necessidade de passar a informação a essas partes.

Ao assinar este termo de consentimento informado, permite que as suas informações médicas neste estudo sejam verificadas, processadas e relatadas conforme for necessário para finalidades científicas legítimas.

Confidencialidade e tratamento de dados pessoais

Os dados pessoais dos participantes no estudo, incluindo a informação médica ou de saúde recolhida ou criada como parte do estudo (tais como registos médicos ou resultados de testes), serão utilizados para condução do estudo, designadamente para fins de investigação científica relacionados com a patologia em estudo.

Ao dar o seu consentimento à participação no estudo, a informação a si respeitante, designadamente a informação clínica, será utilizada da seguinte forma:

1. O promotor, os investigadores e as outras pessoas envolvidas no estudo recolherão e utilizarão os seus dados pessoais para as finalidades acima descritas.
2. Os dados do estudo, associados às suas iniciais ou a outro código que não o (a) identifica diretamente (e não ao seu nome) serão comunicados pelos investigadores e outras pessoas envolvidas no estudo ao promotor do estudo, que os utilizará para as finalidades acima descritas.
3. Os dados do estudo, associados às suas iniciais ou a outro código que não permita identificá-lo(a) diretamente, poderão ser comunicados a autoridades de saúde nacionais e internacionais.
4. A sua identidade não será revelada em quaisquer relatórios ou publicações resultantes deste estudo.

5. Todas as pessoas ou entidades com acesso aos seus dados pessoais estão sujeitas a sigilo profissional.
6. Ao dar o seu consentimento para participar no estudo autoriza o promotor ou empresas de monitorização de estudos/estudos especificamente contratadas para o efeito e seus colaboradores e/ou autoridades de saúde, a aceder aos dados constantes do seu processo clínico, para conferir a informação recolhida e registada pelos investigadores,

designadamente para assegurar o rigor dos dados que lhe dizem respeito e para garantir que o estudo se encontra a ser desenvolvido corretamente e que os dados obtidos são fiáveis.

7. Nos termos da lei, tem o direito de, através de um dos médicos envolvidos no estudo/estudo, solicitar o acesso aos dados que lhe digam respeito, bem como de solicitar a rectificação dos seus dados de identificação.
8. Tem ainda o direito de retirar este consentimento em qualquer altura através da notificação ao investigador, o que implicará que deixe de participar no estudo/estudo. No entanto, os dados recolhidos ou criados como parte do estudo até essa altura que não o(a) identifiquem poderão continuar a ser utilizados para o propósito de estudo/estudo, de forma a manter a integridade científica do estudo, a sua informação médica não será removida do arquivo do estudo.
9. Se não der o seu consentimento, assinando este documento, não poderá participar neste estudo. Se o consentimento agora prestado não for retirado e até que o faça, este será válido e manter-se-á em vigor.

10. COMPENSAÇÃO

Este estudo é da iniciativa do investigador e, por isso, se solicita a sua participação sem uma compensação financeira para a sua execução, tal como também acontece com os investigadores e o Centro de Estudo. O Centro de Estudo suportará todos os custos inerentes aos procedimentos laboratoriais. Não haverá portanto qualquer custo para o participante pela sua participação neste estudo, nomeadamente, não lhe será cobrada nenhuma taxa sobre as análises efetuadas. Como compensação, ser-lhe-á efetuado um tratamento periodontal.

11. CONTACTOS

Se tiver perguntas relativas aos seus direitos como participante deste estudo, deve contactar:

Presidente da Comissão de Ética da FMUC,

Azinhaga de Santa Comba, Celas – 3000-548 Coimbra

Telefone: 239 857 707

e-mail: comissaoetica@fmed.uc.pt

Se tiver questões sobre este estudo deve contactar:

(Nome, morada e contactos do Investigador Principal)

NÃO ASSINE ESTE FORMULÁRIO DE CONSENTIMENTO INFORMADO A MENOS QUE TENHA TIDO A OPORTUNIDADE DE PERGUNTAR E TER RECEBIDO

RESPOSTAS SATISFATÓRIAS A TODAS AS SUAS PERGUNTAS.

CONSENTIMENTO INFORMADO

De acordo com a Declaração de Helsínquia da Associação Médica Mundial e suas actualizações:

1. Declaro ter lido este formulário e aceito de forma voluntária participar neste estudo.

2. Fui devidamente informado(a) da natureza, objectivos, riscos, duração provável do estudo, bem como do que é esperado da minha parte.

3. Tive a oportunidade de fazer perguntas sobre o estudo e percebi as respostas e as informações que me foram dadas. A qualquer momento posso fazer mais perguntas ao médico responsável do estudo. Durante o estudo e sempre que quiser, posso receber informação sobre o seu desenvolvimento. O médico responsável dará toda a informação importante que surja durante o estudo que possa alterar a minha vontade de continuar a participar.

4. Aceito que utilizem a informação relativa à minha história clínica e os meus tratamentos no estrito respeito do segredo médico e anonimato. Os meus dados serão mantidos estritamente confidenciais. Autorizo a consulta dos meus dados apenas por pessoas designadas pelo promotor e por representantes das autoridades reguladoras.

5. Aceito seguir todas as instruções que me forem dadas durante o estudo. Aceito em colaborar com o médico e informá-lo(a) imediatamente das alterações do meu estado de saúde e bem-estar e de todos os sintomas inesperados e não usuais que ocorram.

6. Autorizo o uso dos resultados do estudo para fins exclusivamente científicos e, em particular, aceito que esses resultados sejam divulgados às autoridades sanitárias competentes.

7. Aceito que os dados gerados durante o estudo sejam informatizados pelo promotor ou outrem por si designado.

   Eu posso exercer o meu direito de rectificação e/ ou oposição.

8. Tenho conhecimento que sou livre de desistir do estudo a qualquer momento, sem ter de justificar a minha decisão e sem comprometer a qualidade dos meus cuidados médicos. Eu tenho conhecimento que o médico tem o direito de decidir sobre a minha saída prematura do estudo e que me informará da causa da mesma.

9. Fui informado que o estudo pode ser interrompido por decisão do investigador, do promotor ou das autoridades reguladoras.

Nome do Participante_____

Assinatura : _____

Data:_____/_____/____

Nome de Testemunha / Representante Legal:_____

Assinatura: _____

Data:_____/_____/____

Confirmo que expliquei ao participante acima mencionado a natureza, os objectivos e os potenciais riscos do Estudo acima mencionado.

Nome do Investigador:_____

Assinatura: _____

Data:_____/_____/____