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Mitochondrial function analysis in diabetes and chronic periodontitis-derived human blood cells: role of resistin

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Mestrado Integrado em Medicina Dentária
Coimbra, 2017

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Abbreviations

AAP - American academy of periodontology
ATP - Adenosine triphosphate
BHI - Bioenergetic health index
BMI - Body mass index
BOP - Bleeding on probing
CAL - Clinical attachment level
DM - Diabetes *mellitus* type II
DMEM - Dulbecco's modified Eagle's medium
EDTA - Ethylenediamine tetraacetic acid
ELISA - Enzyme-linked immunosorbent assay
FBS - Fetal bovine serum
FCCP - Trifluoromethoxy carbonylcyanide phenylhydrazone
HbA_{1c} - Glycated hemoglobin
IL-6 - Interleukin 6
OCR - Oxygen consumption rate
OXPHOS - Oxidative phosphorylation
PBMCs - Peripheral blood mononuclear cells
PBS - Phosphate buffered saline
PD - Probing depth
PI - Plaque index
PGE2 - Prostaglandin E_2
ROS - Reactive oxygen species
RPMI 1640 - Roswell park memorial institute 1640 medium
TNF-α - Tumor necrosis factor alfa

Resumo

A doença periodontal (DP) é considerada uma doença inflamatória da cavidade oral onde, por ação de microrganismos patogénicos presentes no biofilme subgengival, o periodonto é afetado, culminando na reabsorção óssea. A diabetes *mellitus* tipo II (DM) é uma doença metabólica inflamatória classificada pelo aumento dos níveis de glicose sanguínea, existindo uma relação bi-direcional com a DP. Assume-se que a diabetes pode conduzir ao desenvolvimento de periodontite e que esta última pode influenciar a ocorrência ou agravamento da diabetes. A resistina, proteína secretada pelo tecido adiposo, é associada com a resistência à insulina, permitindo relacionar a obesidade com a DM. Estas doenças inflamatórias têm sido referidas como estando associadas ao *stress* oxidativo (*e.g.* devido ao aumento da produção de espécies reativas de oxigénio) e à disfunção mitocondrial, dando informação acerca do estado energético celular. As células mononucleares do sangue periférico (PBMCs, do inglês 'peripheral blood mononuclear cells') constituem modelos úteis para o estudo da função mitocondrial e do *stress* oxidativo associado a doenças inflamatórias como a DM e periodontite crónica (PC). Neste trabalho, procedeu-se à seleção de doentes inseridos na base de dados da consulta de medicina dentária do Centro Hospitalar da Universidade de Coimbra, onde foram avaliados parâmetros clínicos para o diagnóstico da saúde periodontal e determinado o índice de massa corporal. Procedeu-se à recolha de sangue venoso periférico de indivíduos com DM, PC, DM-PC e em indivíduos controlo, por forma a avaliar os níveis de hemoglobina glicada níveis plasmáticos de resistina e a produção de peróxido de hidrogénio (H_2O_2) bem como a bioenergética mitocondrial no plasma e PBMCs dos diferentes grupos de doentes e controlos. Os nossos resultados mostram um aumento dos parâmetros clínicos para o diagnóstico de DP nos doentes com PC e DM-PC e um concomitante aumento da produção de H_2O_2. O aumento dos níveis plasmáticos de resisina em indivíduos com DM e DM-PC foram correlacionados com o índice de massa corporal, ocorrendo um aumento dos níveis de hemoglobina glicada nos doentes em que se verificou um aumento dos níveis de resistina (DM-PC). A nível da atividade mitocondrial, verificou-se uma diminuição da respiração máxima nas PBMCs isoladas dos doentes com PC. Em contraste, observou-se um aumento da respiração basal, respiração máxima e da produção de ATP mitocondrial nas PBMCs dos doentes com DM-PC. Este incremento na capacidade respiratória, associado a um aumento do H^+ *leak* nos doentes com DM-PC poderá explicar a diminuição do BHI, um índice de avaliação da capacidade bioenergética,nestes doentes.

Palavras-chave: periodontite crónica; diabetes *mellitus* type II; PBMCs; mitocôndria; *stress* oxidativo; taxa de consumo de oxigénio

Abstract

Periodontal disease is an oral inflammatory disease due to pathogenic microorganisms present on subgingival biofilm that affect the periodontium, culminating with bone resorption. Diabetes *mellitus* type II (DM) is an inflammatory and metabolic disorder classified by increased blood glucose levels, fot which a bi-directional relationship with periodontal disease has been described. It is assumed that diabetes can lead to the development of periodontitis, and that the latter may influence the occurrence or worse diabetes condition. Resistin is released by the adipose tissue and related with insulin resistance, linking obesity and DM. These inflammatory diseases associate with oxidative stress (*e.g.* due to increased production of reactive oxygen species) and mitochondrial dysfunction, giving information about cellular energy status. Peripheral blood mononuclear cells (PBMCs) are useful models for studying mitochondrial function and oxidative stress associated with inflammatory diseases such as DM and chronic periodontitis (CP). The patient's selection was screened from dentistry consultations at the Hospital Center of Coimbra University (CHUC) where clinical parameters for the diagnosis of periodontal health and body mass index were determined. Venous peripheral blood was collected from DM, CP, DM-CP and control individuals in order to evaluate glycated hemoglobin levels and resistin plasma levels and H_2O_2 production and mitochondrial bioenergetics in isolated PBMCs. Our results show increased clinical parameters for periodontal disease diagnosis in patients with CP and DM-CP, along with increased production of H_2O_2. Increased plasma resistin levels in DM and DM-CP patients correlated with body mass index. Moreover, the increase in glycated hemoglobin levels also occurred in DM-PC patients. When studying mitochondrial function, we observed decreased maximal mitochondrial respiration in PBMC isolated from CP patients. In contrast, an increase in basal and maximal respiration and enhanced mitochondrial ATP production was detected in PBMCs from DM-CP patients. Such exacerbated parameters associated with and an increase in H^+ leak in DM-CP may explain the decrease in BHI, an index of bioenergetic health, in these patients.

Key-words: chronic periodontitis; diabetes *mellitus* type II; oxidative *stress*; mitochondria; oxygen consumption rate

Introduction

Traditionally, periodontitis has been classified as a localized oral infection that affects periodontium[1][2]. Nowadays, it is considered as a chronic infection in which periodontal tissue inflammation occurs due to the chronic presence of specific pathogenic microorganisms of subgengival biofilm, requiring a susceptible host and culminating with bone resorption. Periodontitis is caused by an increase of local immune-inflammatory factors like TNF-α (tumor necrosis factor alfa), IL-6 (Interleukin 6), and PGE2 (prostaglandin E_2)[3] being the inflammatory infiltrate mainly composed by neutrophils[1][4][5][6][7][2][8]. Periodontal disease include disorders ranging from gingivitis to chronic periodontitis (CP) affecting more than 90% of world's population (Fig. 1)[1][4]. The classification of this oral disease is complex taking into account the clinical evolution, the rate of disease progression, systemic factors and rate of local risk factors [1]. Clinical diagnosis of periodontal disease is currently made by specific symptoms related by the patients and also by signals in periodontal tissues, such as gingival bleeding [9].

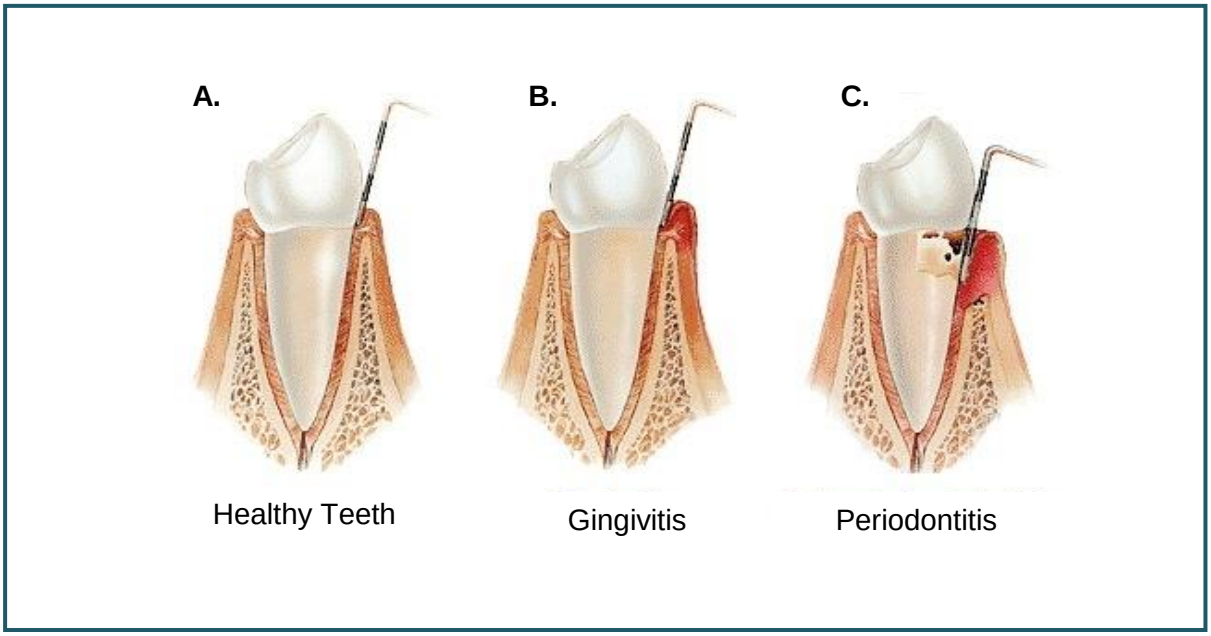

Figure 1. Teeth probing depth in healthy teeth (A), in gingivitis condition (B) and in periodontitis disease (C). *Adapted from:* http://www.dentalelements.com/wp-content/uploads/dental-gum-disease.jpg

The activity or inactivity of the disease can be evaluated accordingly to inflammation signals as bleeding on probing (BOP) that change accordingly to patient's plaque control levels that are present in all disease's states[9][10][11]. There are three major risk factors that influence periodontitis, namely smoking, diabetes and patients medications [2][5].

Characterized by hyperglycemia, diabetes *mellitus* (DM) is defined as a group of metabolic diseases that result from a defect in insulin secretion, insulin defect action, or both[2][12][13][14]. DM can be sub-divided in two major classifications: 1) diabetes *mellitus* type I, classified as an autoimmune disease where pancreatic β-cells do not secrete insulin - this type of diabetes is also known as insulin-dependent diabetes, affecting mostly young people; and 2) diabetes *mellitus* type II, in which pancreatic β-cells secrete low insulin levels along with resistance to insulin action. It is characterized by being associated with obesity and aging. The prevalence of this type of diabetes, when compared to the other types, varies between 90-95%. Although not so frequent, diabetes is also associated with pregnancy, diseases of exocrine pancreas and endocrinopathies[8][12].

Scientific evidence that associate DM with periodontitis had been found. Two-way relationship between these diseases has been demonstrated, being diabetic individuals two or three times more susceptible to periodontitis[1][2][12][15][16][17]. Some authors refer that a poor glycemic control can influence the periodontitis state[2]. Thus, periodontal disease is an important factor to increase the risk of diabetic complications, being considered the 6th complication of diabetes[7][16][18].

Obesity is considered a risk factor for diabetes *mellitus* type II, caused by a high caloric intake in combination with a sedentary lifestyle[5]. The body mass index (BMI), an indicator of obesity, corresponds to the body fat percentage accumulated using a formula that relates individuals' weight and height, allowing us to classify if someone is within the interval of normal weight (18.5 – 24.9 kg/m^2)[19]. Glycated hemoglobin (HbA_{1c}) occurs after binding of the NH_2 terminal of hemoglobin β-chain to excess and continuous glucose, diffused from plasma into the erythrocytes, along their life cycle. In this way HbA_{1c} is considered a reliable marker of glucose concentration, indicating hyperglycemia severity in individuals with diabetes[15][20].

Adipose tissue abundance, associated to insulin resistance, is responsible for altered secretion of adipokines that play an important role in pathological and physiological inflammatory response[6][21]. Currently, there are many different types of adipokines described on literature and some of them are associated with diabetes *mellitus*. Apokines, like resistin, are immune-inflammatory factors secreted by adipose tissue[22]. Described as being associated with insulin resistance and as markers of weight regulation, these adipokines are involved in conditions such as cardiovascular diseases, diabetes *mellitus*, and other inflammatory diseases[6][7]. Resistin, a cysteine-enriched protein, was firstly found in mice white adipose tissue; however, low resistin levels are produced by adipocytes in humans being produced by peripheral blood mononuclear cells (PBMCs), like monocytes and lymphocytes, and also by macrophages[7][22][23].

Recent findings concerning immunometabolism have focused on the importance of immune cells in inflammation associated with obesity and diabetes *mellitus*[24]. Derived from the lymphoid lineage, the uni-nucleated lymphocytes play a very important role in adaptive immunity, using mitochondria to meet the energetic demands[25]. The heterogeneity of these cells make them ideal to investigate the relationship between bioenergetics and disease processes related to inflammation[25]. Mitochondria are cellular organelles that play a major role in generating cellular energy in the form of adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS); apart from that they are also involved in the regulation of the intracellular calcium concentration, intracellular redox signaling, heme synthesisand apoptosis. Cellular energy requirements are dependent on environment and redox balance[26][27][28]. About 80-90% of energy (ATP) required for the process of inflammation is produced by mitochondria through oxidative processes. It is recognized that alterations in mitochondrial function can contribute to aging process and to several diseases, like neurodegenerative diseases, atherosclerosis and cancer[26]. In addition, mitochondrial dysfunction can be a crucial process that can explain the metabolic alterations like insulin resistance or the etiopathogenic process of other systemic diseases[27][29]. Lymphocytes, the main population in isolated PBMCs, are a heterogeneous cell population that largely dependents on mitochondria to meet energetic demands[30]. In this way, PBMCs obtained from human blood have been widely used for mitochondrial function evaluation. In this work, we assume that PBMCs reproduce systemic changes occurring in inflammatory diseases like periodontitis. Bioenergetic analysis in blood cells, particularly PBMCs, has suggested that they may be used to measure oxygen consumption rates (OCR), indicating potential mitochondrial dysfunction[25][30].

Reactive oxygen species (ROS), including both oxygen radicals and also non-radical species, are continuously generated in organisms during mitochondrial oxidative metabolism[8]. However, recent studies suggested that ROS, like hydrogen peroxide (H_2O_2), play a role on diabetic and periodontal complications contributing to impairment of the antioxidant gene expression responsible for ROS degradation and maintenance of vascular health. Deregulation of these defense mechanisms or high ROS production can lead to “oxidative stress" state causing tissue damage in individuals with chronic diseases like diabetes or chronic periodontitis. Moreover, it was shown that increased HbA_{1c} levels lead to an increase of ROS production[31]. Within periodontal tissues, an increase in ROS production causes deregulation in cellular homeostasis, further inducing tissue damage. In diabetes *mellitus* there are several evidences suggesting that oxidative stress can be associated to pre-diabetic and diabetic states [8], which can also be responsible for diabetic complications like chronic periodontitis[32]. In this way, oxidative stress can act as a link between metabolic

syndrome (associated to the combination of obesity, hypertension, hyperinsulinemia and dyslipidemia associated with diabetes *mellitus*) and periodontitis[3][27].

Research on mitochondrial dysfunction and oxidative stress in inflammatory diseases is a current topic, eliciting special interest in the study of these mechanisms related to diabetes *mellitus* and periodontitis, as well as in finding a specific and sensitive biomarker that can be used for risk and screening assessment of these diseases[6][26][22]. Thus, the aim of this study is to investigate the relationship between mitochondrial bioenergetics with the disease processes associated with individuals with type-II diabetes and periodontitis and also to evaluate the levels of resistin in serum of the same patients.

Material and methods

Material

Resistin kit was from RayBio®. Amplex® Red was obtained from Molecular Probes, Life Technologies (Eugene, OR, USA). Ficoll-Paque solution was from GE Healthcare (GE Healthcare Bio-Sciences, PA, USA). RPMI 1640, DMEM, poly-*D*-lysine, oligomycin, trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP), antimycin A and rotenone, were from Sigma Chemical Co. (St Louis, MO, USA). All other reagents were of analytical grade.

1.1. Subject selection

This study was carried out from September 2016 to May 2017. The patient's selection was screened from dentistry consultations in Hospital Centre of Coimbra's University (CHUC). The study population consisted in patients with 45 to 75 years of age, and balanced individuals (20 men and 20 women) presenting a minimum of 15 teeth. Informed written consent was obtained from all subjects that agreed to participate voluntarily (see attachment 1). All the patients were evaluated for medications, weight, height and HbA_{1c} levels.

The individuals satisfying the above criteria were categorized into 3 diseased study groups (DM group: 10 subjects with no history of periodontal disease, but with clinical diagnosis for diabetes *mellitus* type II; CP group: 10 subjects with history of periodontal disease, with probing depths > 3mm; DM-CP group: 10 subjects with history of periodontal disease, with probing depths >3mm and clinical diagnosis of diabetes *mellitus* type II) and a control group (CONT group: 10 subjects with no history of either periodontal disease or diabetes *mellitus* type II). The clinical parameters plaque index (PI), probing depth (PD), bleeding on probing (BOP) and clinical attachment level (CAL) were clinical parameters assessed for all the subjects, using a periodontal probe. Periodontitis were classified as: slight, when PD values are between 3 and 5 mm; moderate, when PD values are between more or equal to 5 mm and less than 7 mm and severe, when PD values are more or equal than 7mm; an healthy probing depth is considered between 1-3 mm[10]. Moreover, periodontal disease can be described by CAL which can be characterized in slight (1-2 mm), moderate (3 to 4 mm) and severe (≥ 5 mm), according to American Academy of Periodontology *Guidelines*[11]. Body Mass Index (BMI) was also calculated for each patient according to the formula BMI=weight (kg)/[height $(m)^2$] (Table V).

1.2. Blood sample collection and glycated hemoglobin determination

Using a 20-guage needle with 30 ml syringes, about 15 ml of venous peripheral blood was collected between 8-10 a.m. by venipuncture into commercially available tubes containing ethylenediamine tetraacetic acid (EDTA) as anticoagulant, at dentistry consultations in Hospital Centre of Coimbra's University (CHUC). Collected samples were immediately transferred to the Center for Neurosciences and Cell Biology (CNC), University of Coimbra, for PBMCs isolation. An aliquot of 3 ml of blood was used to determinate the level of glycated hemoglobin (HbA_{1c}) at Clinical Pathology service, CHUC.

1.3 Quantification of resistin levels

The resistin levels were measured by enzyme-linked immunosorbent assay (ELISA kit from RayBio®) by following the intensity of the fluorescence produced at 450 nm, according to manufacturer's instructions. All the samples were run in duplicates and resistin concentration in plasma samples calculated from a standard curve for resistin.

1.4 Isolation and culture of peripheral blood mononuclear cells

After being collected, 12 ml of blood samples were diluted with 36 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 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 braking and acceleration, and cells resuspended in 10 mL RPMI 1640 (Sigma R4130) culture medium supplemented with: 10 mM Hepes, 12 mM $NaHCO_3$, 2 mM glutamine, 1mM sodium pyruvate plus 10% FBS heat inactivated. Cells were then counted in a hemocytometer by using trypan blue exclusion test currently used to determine the number of viable cells in a cell suspension. The method is based on the principle that live cells with intact cell membranes exclude trypan blue, presenting a clear cytoplasm, whereas a nonviable cell has a blue cytoplasm under an inverted brightfield microscope. Cells were plated in 100 µg/mL poly-*D*-lysine (Sigma P1149; mol wt: 150,000-300,000) precoated XF24 microplate wells at 0.5×10^6 cells/well in a final volume of about 180 µL, and let to adhere for 1.5 - 2h in a humidified incubator chamber with

95% air and 5% CO_2 at 37°C. Cell adherence was checked under an inverted brightfield microscope with 20X magnification.

1.5 Preparation of XF24 microplate wells for oxygen consumption rate by Seahorse analyzer

Adhered lymphocytes (in 180µL RPMI 1640, as described above) were carefully rinsed in order to remove RPMI 1640 culture medium. For this purpose, 100 µL was removed and 100 µL non-buffered DMEM (Sigma 5030) supplemented with 1,85g/L de NaCl, 2g/ L glucose, 1 mM sodium pyruvate and 2 mM glutamine, pH 7.4 at 37ºC were added to the wells. This procedure was repeated and after a mild washout, 450 µL of DMEM, supplemented as described above, was added to the wells. The multi-well plate was then placed for 45 min – 1h in a 37ºC-humidified incubator without CO_2, containing 95% air.

1.6 Analysis of mitochondrial oxygen consumption rate by Seahorse analyzer

Cellular bioenergetics of the isolated cells was determined using the extracellular flux analyzer (Seahorse Bioscience), which measures O_2 and protons in the extracellular *milieu*. This system allows for real time, noninvasive measurement of OCR, which can be correlated with mitochondrial function/oxidative burst. The Seahorse XF Cell Mito Stress Test Kit contains a cartridge lid, a sensor cartridge, a hydro booster and XF calibrant apart the utility plate described above. The day before experiments, seahorse XF24 apparatus was turned on and seahorse XF calibrant was added to the sensor cartridge in order to hydrate the boosters at 37°C in a non-CO_2 incubator, overnight.

On the day of the experiments, the injection ports attached to the wells that allow for injection of inhibitors of mitochondrial respiratory chain (Fig. 2)(Fig. 3), were filled with oligomycin, an inhibitor of ATP synthase (Port A), FCCP, an uncoupler used to measure maximal respiratory activity (Port B) and a mix of rotenone and antimycin A, complex I and complex III inhibitors, respectively (Port C) to determine mitochondrial respiration, as shown in representative trace (Fig. 3). The sequence of addition of these inhibitors and the uncoupler FCCP allows us the determination of basal respiration, proton (H^+) leak, respiratory resting capacity, ATP production, maximal respiration and non-mitochondrial respiration, enabling to understand cellular bioenergetics (Fig. 3).

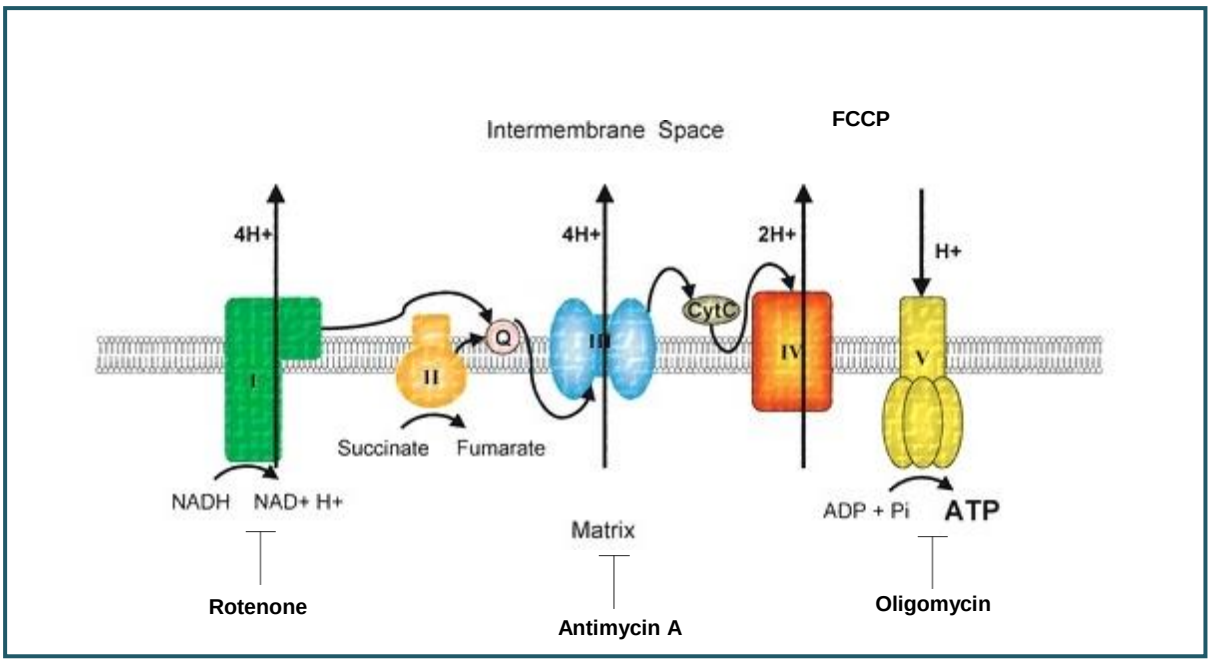

Figure 2. Mitochondria respiratory chain. Complex (Cx) I, II, III, IV and ATP synthase (Cx V) representation at inner mitochondrial membrane (IMM). H^+ flux from matrix to intermembrane space, through Cx I, III and IV as well as the site of action of inhibitors (rotenone, antimycin A and oligomycin) and the uncoupler FCCP, are also shown. *Adapted from:* https://www.researchgate.net/figure/40034270_fig1_Schematic-representation-of-the-mitochondrial-respiratory-chain-After-permeabilization

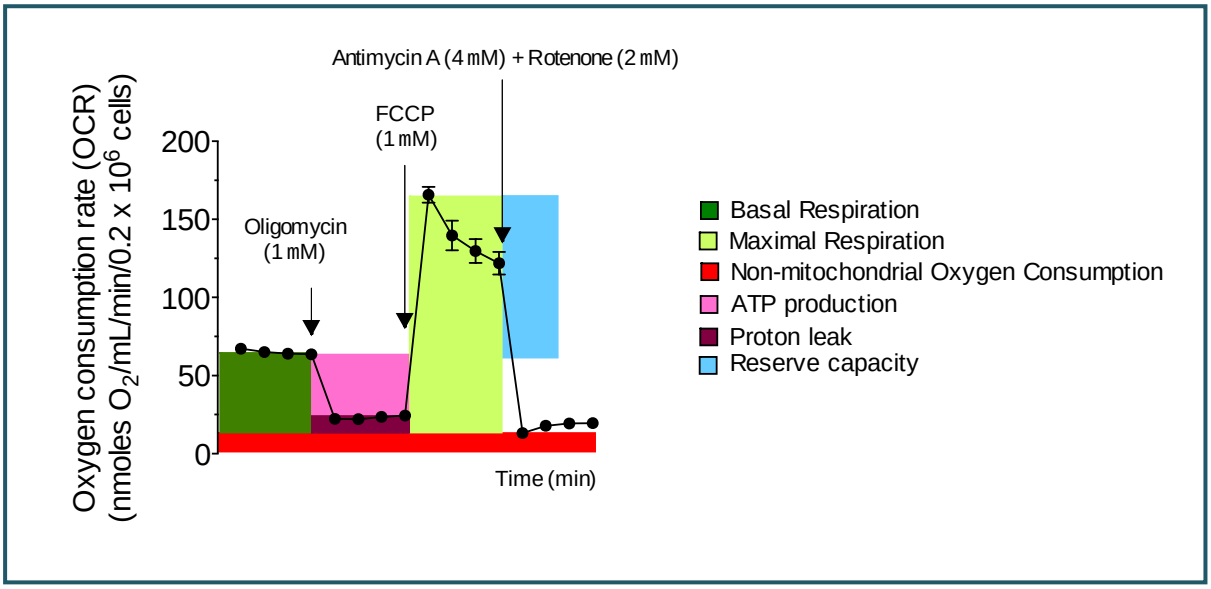

Figure 3. Representative trace of OCR in isolated PBMCs. Parameters of mitochondrial function include basal respiration, ATP production, proton (H^+) leak, non-mitochondrial oxygen consumption, maximal respiration and reserve capacity parameters, depicted by colored areas.

1.7 Measurement of H_2O_2 levels

Briefly, $0.5x10^6$ cells were ressuspended in Na^+ medium containing (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.4 plus Amplex® Red reagent (10 µM) and horseradish peroxidase (0.5 units per mL). The reaction of Amplex® Red (10-acetyl-3.7-dihydroxyphenoxazin) in the presence of peroxidase, occur in 1:1 stoichiometry, producing resorufin, a red-fluorescent oxidation product (Fig. 4)[34]. Fluorescence was followed at 37°C for 20 minutes, at an excitation wavelength of 550 nm and an emission wavelength of 580 nm, using a microplate reader Spectrofluorometer Gemini EM (Molecular Devices, USA). Results were expressed in RFU/minute.

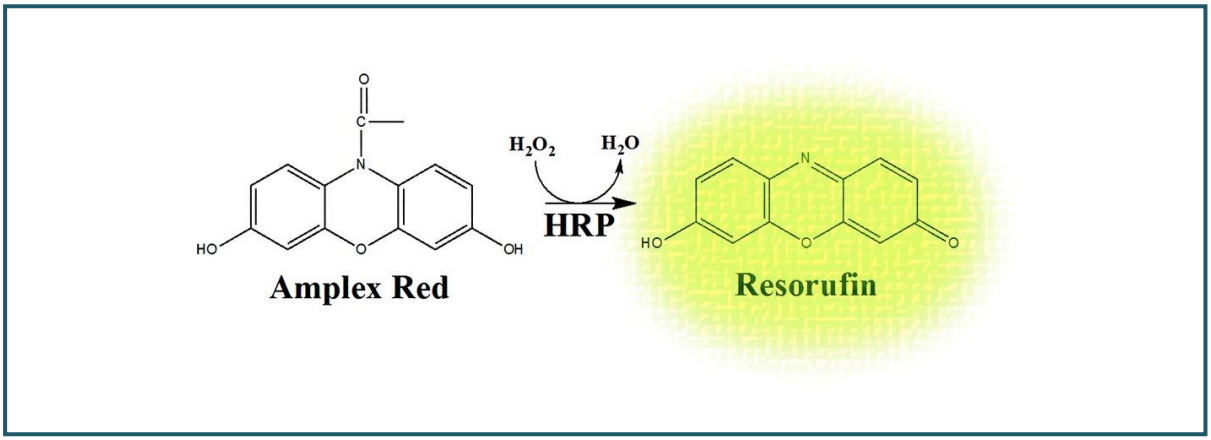

Figure 4. Amplex Red oxidation by H_2O_2. The Horseradish peroxidase (HRP) uses Amplex Red as an electron donor to reduce H_2O_2, resulting in the fluorescent compound resorufin, Changes in fluorescence intensity are directly proportional to the amount of H_2O_2 consumed during the reaction thus reflecting the levels of H_2O_2. *Adapted from*: http://pubs.rsc.org/en/content/articlelanding/2016/lc/c5lc01413a#!divAbstract

Statistical analysis

Data were analyzed by using Excel and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) software and results expressed as the mean ± SEM. Resistin levels and H_2O_2 production were performed in duplicates or quadruplicates, respectively and OCR in duplicates or triplicates in 10 individuals per group. Comparison among groups was performed by one-way ANOVA followed by Tukey's post-hoc test. Comparison between two groups, as described in figure legends, was performed by parametric Student *t*-test. Significance was defined as $p<0.05$.

Results

The clinical parameters were evaluated in all patients that participated in the present study by using a periodontal probe (PD, CAL, BOP and PI). These data and the individuals' BMI and HbA_{1c} levels were organized by diagnostic groups, as shown in Table I, and expressed as mean ± SEM. Patient medications until the day of the blood collection are described in Tables II-V. Data show that antidiabetics are largely prescribed in DM patients, as expected. In addition, antihypertensive drugs were predominantly prescribed in both DM and DM-CP patients. Taking into account that the mean age of the studied populations are about 61.55 years, medications are widely prescribed, which may modify some parameters evaluated in this study. All clinical parameters of periodontal disease (PD, CAL, BOP and PI) were shown to be significantly increased in CP and DM-CP groups, compared to control or DM patients (Fig. 5). HbA_{1c} levels presented an increase in both DM-CP and DM patients, when compared to control individuals. The BMI did not present significant differences between all groups (Fig. 5). Despite a slight tendency to an increase in plasma resistin levels in DM patients, a significant increase was observed in DM-CP patients group only, when compared with the control group (by Student's *t*-test) (Fig. 6-A).

When resistin levels were plotted together with BMI of the same patients for all groups, a correlation seems to occur (r^2=0.4476) (Fig. 6-B). However, no correlation was observed when resistin values were plotted with those obtained for CAL, BOP or HbA_{1c} (Fig. 7).

Table I. Background groups characteristics

Groups	Individuals (n)	Gender		Age (years)	Smokers (n)	PD (mm)	CAL (mm)	PI (%)	BOP (%)	BMI (kg/m^2)	HbA_{1c} (%)
		Male (n)	Female (n)								
Control	10	1	9	58.43 ± 0.03	0	1.21 ± 0.10	1.38 ± 0.10	30.0 ± 3.28	5.7 ± 0.56	26.85 ± 0.96	5.74 ± 0.06
DM	10	4	6	49.60 ± 10.45	0	1.05 ± 0.07	1.25 ± 0.08	26.7 ± 2.61	5.0 ± 0.47	28.11± 1.51	6.41 ± 0.19
CP	10	7	3	56.04 ± 2.06	3	3.67 ± 0.18	4.84 ± 0.25	26.7 ± 2.61	53.2± 7.02	27.2± 1.11	5.72 ± 0.12
DM-CP	10	8	2	53.55 ± 7.66	1	4.07 ± 0.26	5.34 ± 0.41	55.80 ± 767	64.1± 6.59	29.18± 1.40	7.08 ± 0.22

Data are presented as mean ± SEM of age, probing depth (PD), clinical attachment level (CAL), plaque index (PI), bleeding on probing (BOP), body mass index (BMI) and glycated hemoglobin (HbA_{1c}) were assessed in control individuals, and diabetes *mellitus* type II (DM), chronic periodontitis (CP) and diabetes *mellitus* type II and chronic periodontitis (DM-CP) patients.

Table II. Control group medication until the day of blood collection.

Patient	Antidepressants	Anxiolytics	Anti-hypercholes-terolemia	Antihypertensive	NSAI	Anti-aggregants	Others	Antidiabetics
CONT 1	Mirtazapine Sertraline	Cloxazolam	-	-	-	-	Etoricoxib; Tizanidina Glucosamine	-
CONT 2	-	Alprazolam	Sinvastatine	Bisoprolol Enalapril + hidroclorotiazide	-	-	-	-
CONT 3	-	-	Sinvastatine	-	Nimesulide	-	Ciclobenzaprine	-
CONT 4	-	-	-	-	-	-	-	-
CONT 5	Mirtazapine	Alprazolam	-	-	-	Acid acetylsalicylic	-	-
CONT 6	-	-	-	Enalapril + hidroclorotiazide	-	-	-	-
CONT 7	Sertraline	-	Sinvastatine	Losartan	-	-	-	-
CONT 8	-	-	-	-	-	-	Estradiol; Levothyroxine Calcitriol; Calcium	-
CONT 9	-	-	-	Nifedipine; Carvedilol	-	-	Valsartan + Hidroclorotiazide	-
CONT 10	Sertraline	-	-	-	-	-	-	-

Table III. Diabetes *mellitus* type II (DM) patient's medication until the day of blood collection.

Patient	Antidepressants	Anxiolytics	Anti - Hypercholes-terolemia	Antihyper-tensive	NSAI	Anti-aggregants	Others	Antidiabetics
DM 1	-	-	Pravastatine	-	-	Acid acetylsalicylic	Omeprazol	Metformin + Vildagliptine
DM 2	-	-	Sinvastatine	Diltiazem Enalapril + hidroclorotiazide	-	-	-	Metformin + Vildagliptine
DM 3	-	-	-	Amlodipine Olmesartan Medoxomil	-	-	Levothyroxine sodium	Metformin + Sitagliptine
DM 4	-	-	Atorvastatine	Propanolol	-	-	-	Metformin + Linagliptine
DM 5	Sertraline	-	Rosuvastatine	Nifedipine; Bisoprolol	-	Acid acetylsalicylic	Alopurinol; Omeprazol	Metformin + Vildagliptine
DM 6	-	-	Pravastatine + Fenofibrato	Atenolol; Amlodipina + Valsartan	-	-	-	Metformin + Sitagliptine
DM 7	-	-	-	Cilazapril + Hidroclorotiazidel	-	-	Alopurinol; Hesperidine + Ruscus aculeatus+ Ascorbic acid; Pantoprazol	Gliclazide
DM 8	-	-	-	Nimodipine	-	-	-	Metformin + Vildagliptine
DM 9	Sertraline	Alprazolam	Pravastatine	-	-	-	Levothyroxine sodium	Metformin + Vildagliptine
DM 10	Venlafaxine	-	-	Irbesartan + Hidroclorotiazide	-	-	-	Metformin + Vildagliptine

Table IV. Chronic periodontitIs (CP) patient's medication until the day of blood collection.

Patient	Antidepressants	Anxiolytics	Anti Hypercholes -terolemia	Antihyper- tensive	NSAI	Anti-aggregants	Others	Antidiabetics
CP 1	-	-	Sinvastatine	Fosinopril	-	-	Hidroxizine; Omeprazol	-
CP 2	-	-	-	-	-	-	-	-
CP 3	-	-	-	-	-	-	Furosemide	-
CP 4	Sertraline	Mexazolam	-	-	Etodolac	-	Gabapentin	-
CP 5	-	-	-	-	-	-	-	-
CP 6	-	-	-	-	-	-	-	-
CP 7	-	-	Sinvastatine	Lisinopril; Bisoprolol	-	Acid acetylsalicylic; Ticagrelor	Levotiroxine sodium	-
CP 8	-	-	-	Perindopril	-	-	Flutamide	-
CP 9	-	-	-	-	-	-	-	-
CP 10	-	-	-	-	-	-	-	-

Table V. Chronic periodontitis *plus* diabetes *mellitus* type II (DM-CP) patient's medication until the day of blood collection.

Patient	Antidepressants	Anxiolytics	Anti-Hypercholes-terolemia	Antihyper-tensive	NSAI	Anti-aggregants	Others	Antidiabetics
DMCP 1	-	-	-	-	-	-	-	Metformin + Vildagliptine
DMCP 2	-	-	Sinvastatine	-	-	-	-	Gliclazide
DMCP 3	-	Citalopram	Sinvastatine	Irbesartan + Hidroclorotiazide; Furosemide; Amlodipine	-	Acid acetylsalicylic	Trimetazidine; Calcium poliestireno; Sulfonate; Pentoxifiline; Alopurinol; Folic acid; Pantoprazol	Metformin + Sitagliptine
DMCP 4	-	Alprazolam	-	-	-	-	-	Metformin + Vildagliptine
DMCP 5	-	-	Pravastatine	Enalapril + hidroclorotiazide	-	-	Alopurinol	Metformin + Sitagliptine
DMCP 6	-	Escitalopram	Atorvastatine	Indapamide; Valsartan + Hidroclorotiazide	-	-	-	Gliclazide
DMCP 7	-	-	Fenofibrate	Enalapril + hidroclorotiazide; Amlodipine	-	-	-	Metformin + Vildagliptine
DMCP 8	-	-	Atorvastatine	Amlodipine + Telmisartan	-	-	-	Metformin + Sitagliptine
DMCP 9	-	-	Pravastatine	Lisinopril; Bisoprolol	-	-	Lansoprazol	Gliclazide
DMCP 10	-	-	-	-	-	-	Folic acid + *Ferrous* sulfate; Pantoprazol	Gliclazide

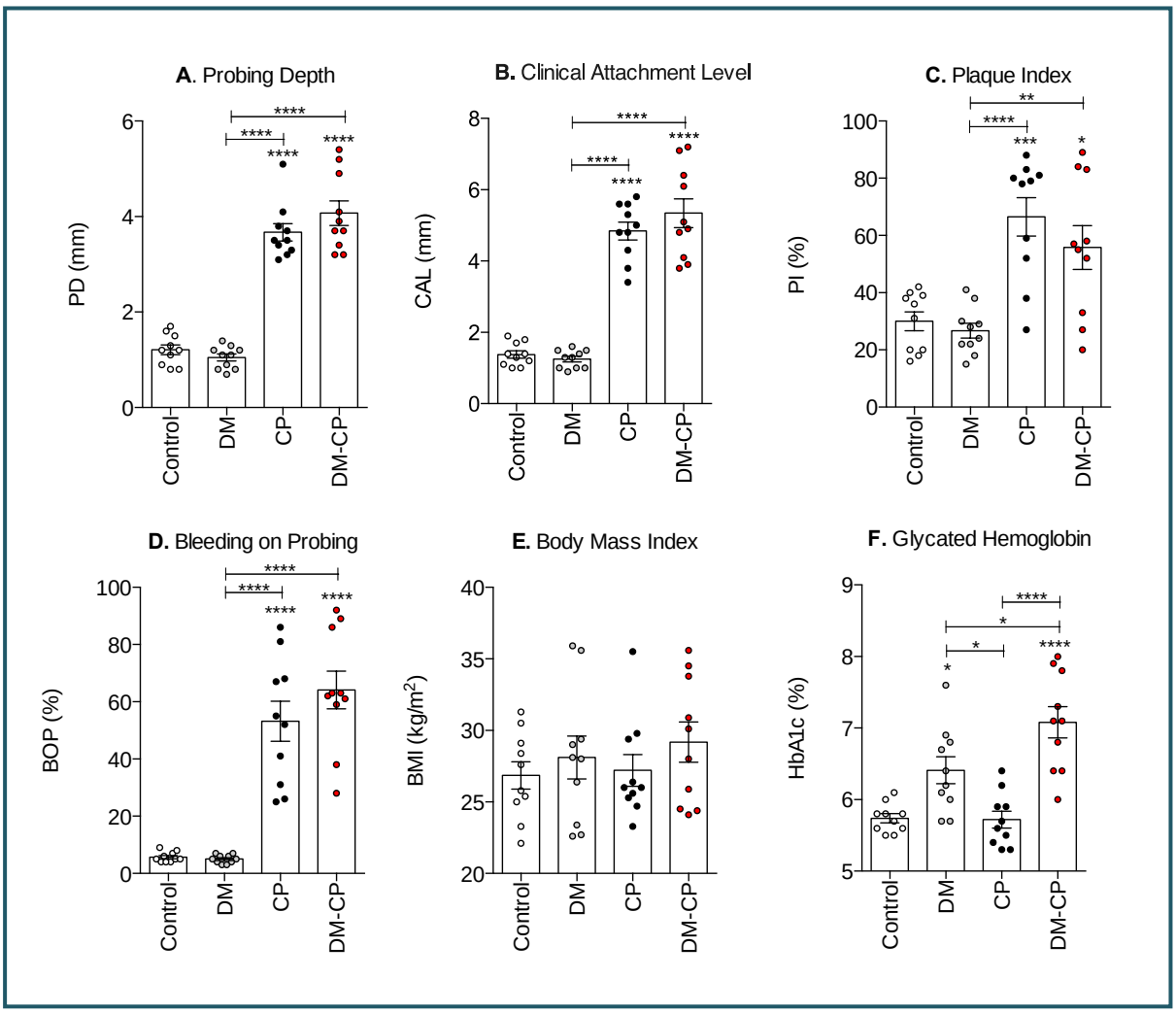

Figure 5. Clinical parameters for periodontitis evaluation. Values of probing depth **(A),** clinical attachment level **(B),** plaque index **(C)**, bleeding on probing **(D)**, body mass index **(E)** and glycated hemoglobin **(F)** in control individuals, diabetes *mellitus* type II (DM), chronic periodontitis (CP) and diabetes *mellitus* type II plus chronic periodontitis (DM-CP) patients. Data are presented in scatter plots as the mean ± SEM from 10 individuals per group. Statistical analysis: *p<0.05; **p<0.01; ****p<0.0001 by One-way Anova followed by Tukey's Multiple Comparison Test.

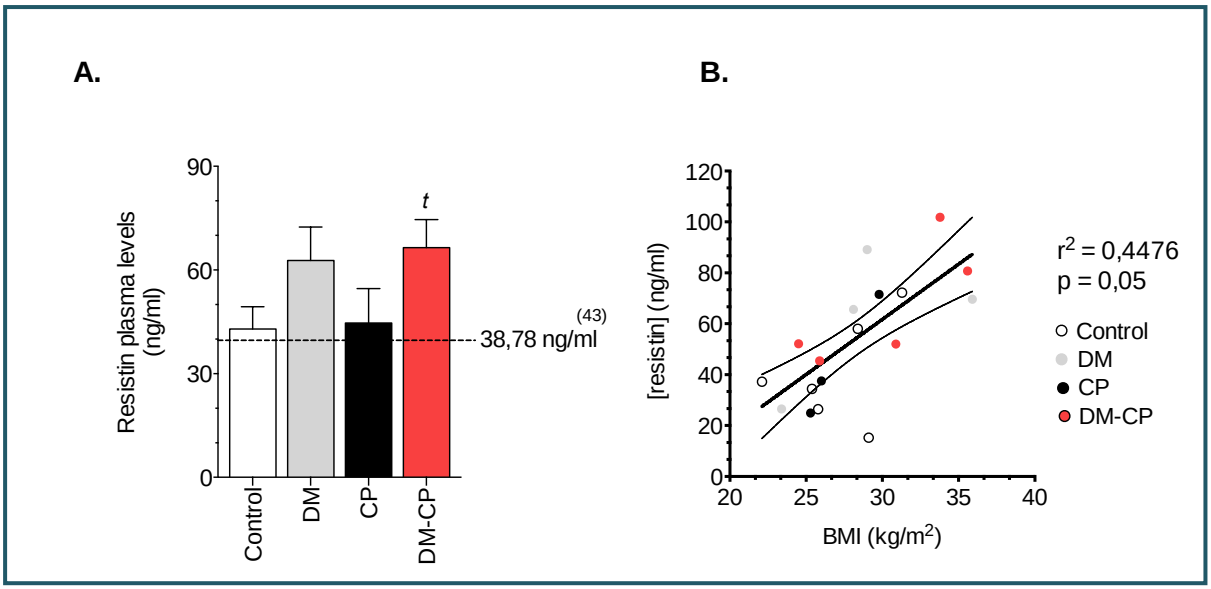

Figure 6. Blood plasma resistin levels and correlation between plasma resistin levels and body mass index (BMI) (A) Resistin levels were evaluated in plasma obtained from control individuals, diabetes *mellitus* type II (DM), chronic periodontitis (CP) and diabetes *mellitus* type II and chronic periodontitis (DM-CP) patients. Control individuals present similar plasma resistin levels as described in literature (38.78 ng/ml)[43].**(B)** Data are presented as mean ± SEM. Statistical analysis: $^{t}p<0.05$ by Studest's *t*-test when compared to the control.

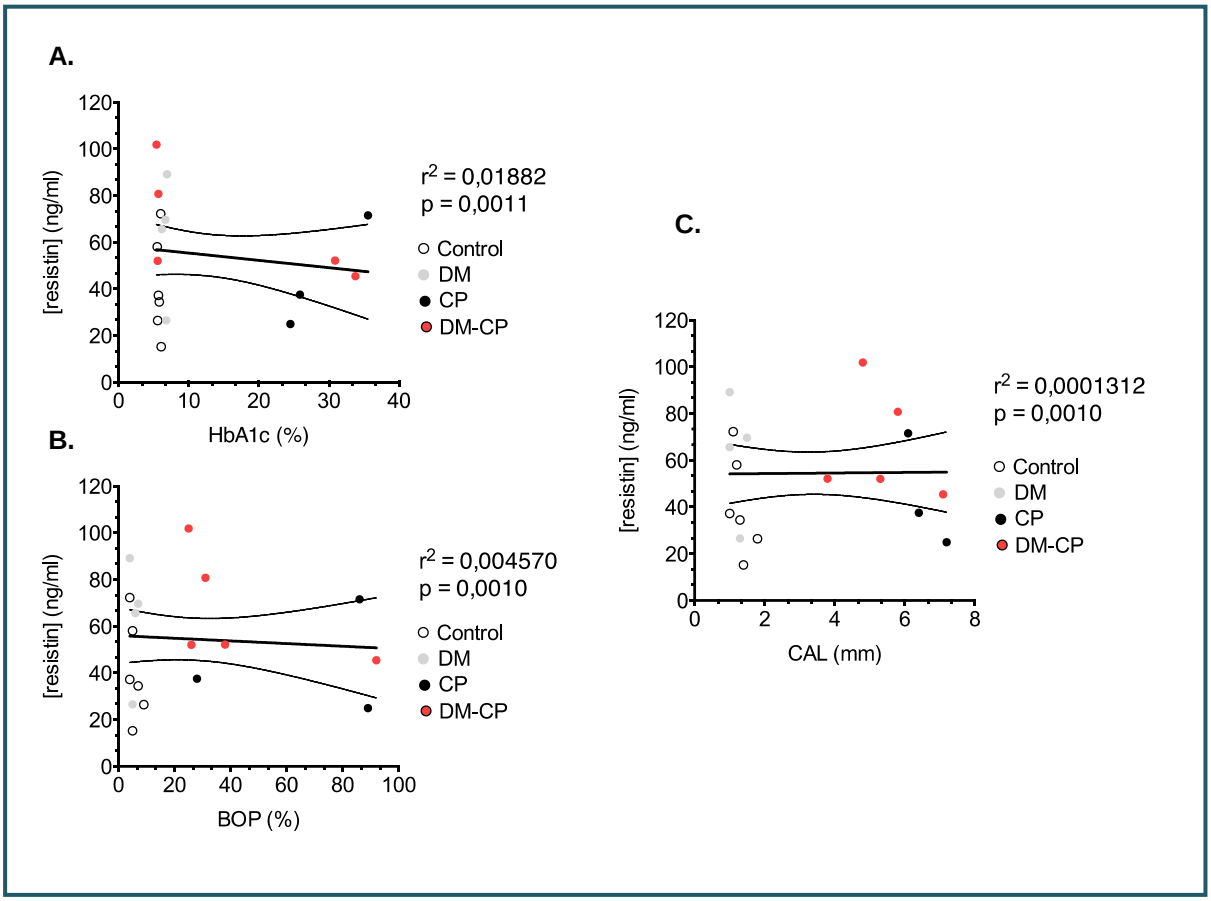

FIgure 7. Correlation between BMI, CAL, BOP and HbA_{1c} with plasma resistin levels. Plasma resistin levels plotted with CAL values **(A)**, BOP percentage **(B)**, and with the percentage of HbA_{1c} **(C)**.

Since periodontitis is considered an inflammatory disease, widely described to be associated with oxidative stress, we next evaluated H_2O_2 production by PBMCs isolated from control individuals (control) or diabetes *mellitus* (DM), chronic periodontitis (CP) or diabetes *mellitus* plus chronic periodontitis (DM-CP) patients. Our results demonstrate increased H_2O_2 production in PBMCs obtained from CP and DM-CP patients when compared with control or DM individuals. No differences were observed between DM and control subjects (Fig. 8).

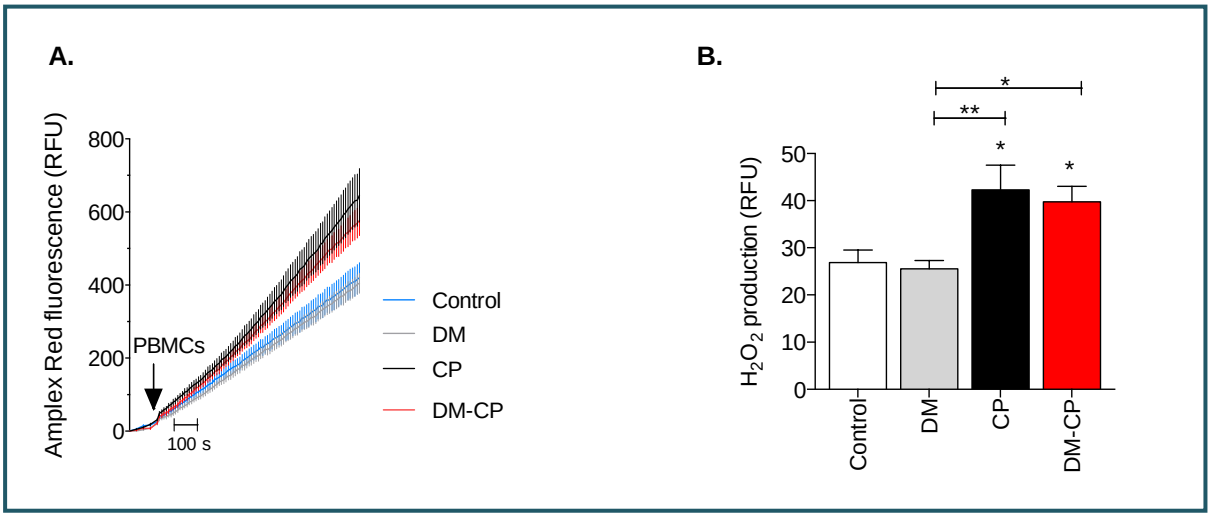

Figure 8. H_2O_2 production by PBMCs. H_2O_2 production by PBMCs isolated from control individuals (control) or diabetes *mellitus* (DM), chronic periodontitis (CP) or diabetes *mellitus* plus chronic periodontitis (DM-CP) patients were evaluated using the Amplex® Red assay, as described in Methods section. **(A)** Representative traces of H_2O_2 production. **(B)** H_2O_2 production calculated as the mean ± SEM of fluorescence increase per minute. Experiments were run in quadruplicates in PBMCs isolated from 10 individuals per group and are expressed in percent change relative to control. Statistical analysis: *p<0.05 and **p<0.01 by one-way Anova, followed by Tukey's Multiple Comparison Test.

Considering that enhanced production of ROS may be a cause or a consequence of modified mitochondrial function, the mitochondrial respiratory activity was evaluated in PBMCs derived from DM, CP or DM-CP patients through the analysis of OCR using the Seahorse analyzer and compared to control. Results presented in Figure 9-A show traces obtained from mitochondrial respiration of all groups studied. The bioenergetic health index (BHI), a parameter that gives information about "mitochondrial health"[30], was calculated and depicted in Figure 9-B. Our data show a significant decrease of BHI in DM-CP when this group only was compared with control group (by Student's *t*-test).

Analysis of OCR parameters was then plotted for basal and maximal respiration, ATP production, H^+ leak, non-mitochondrial respiration, coupling efficiency and reserve capacity. Results depicted in Figure 9 evidence-unaltered levels of all parameters of OCR in DM patients when compared to control individuals. However, despite the slight decrease observed in CP patients for basal respiration and ATP production, a significant decrease was observed for maximal respiration and reserve capacity in these patients (CP) (Fig. 10). Interestingly, when both pathologies were installed in the same patients (DM-CP), basal and maximal respiration, ATP production and H^+ leak were shown to be significantly increased, when compared to patients that only have chronic periodontitis (CP). No major differences

were observed for non-mitochondrial respiration, coupling efficiency or reserve capacity in DM-CP patients when all groups were compared (Fig. 10).

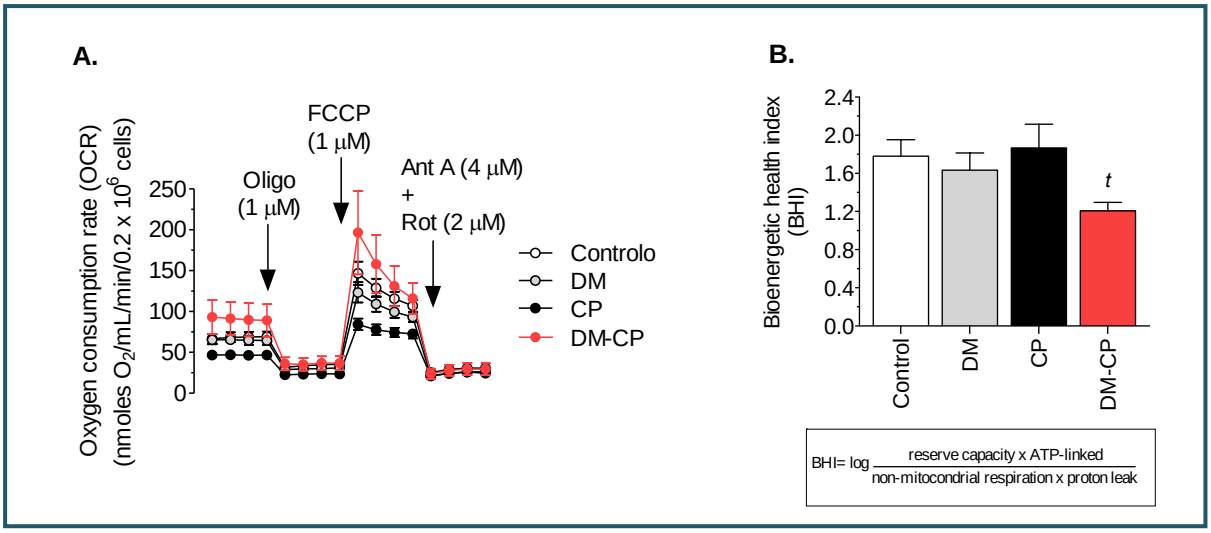

Figure 9. Oxygen Consumption Rate (OCR) in PBMCs. Traces of mitochondrial respiration before and following oligomycin, FCCP, and antimycin plus rotenone injections are represented (**A).** Bioenergetic health index was calculated accordingly to the formula: BHI=log [(reserve capacity x ATP-linked)/non-mitochondria respiration x proton leak)]. **(B)** Results are presented as the mean ± SEM of independent experiments, performed in PBMCs isolated from 10 individuals per group, run in duplicates or triplicates. Statistical analysis: ^{t} p<0.05 Student's *t* test.

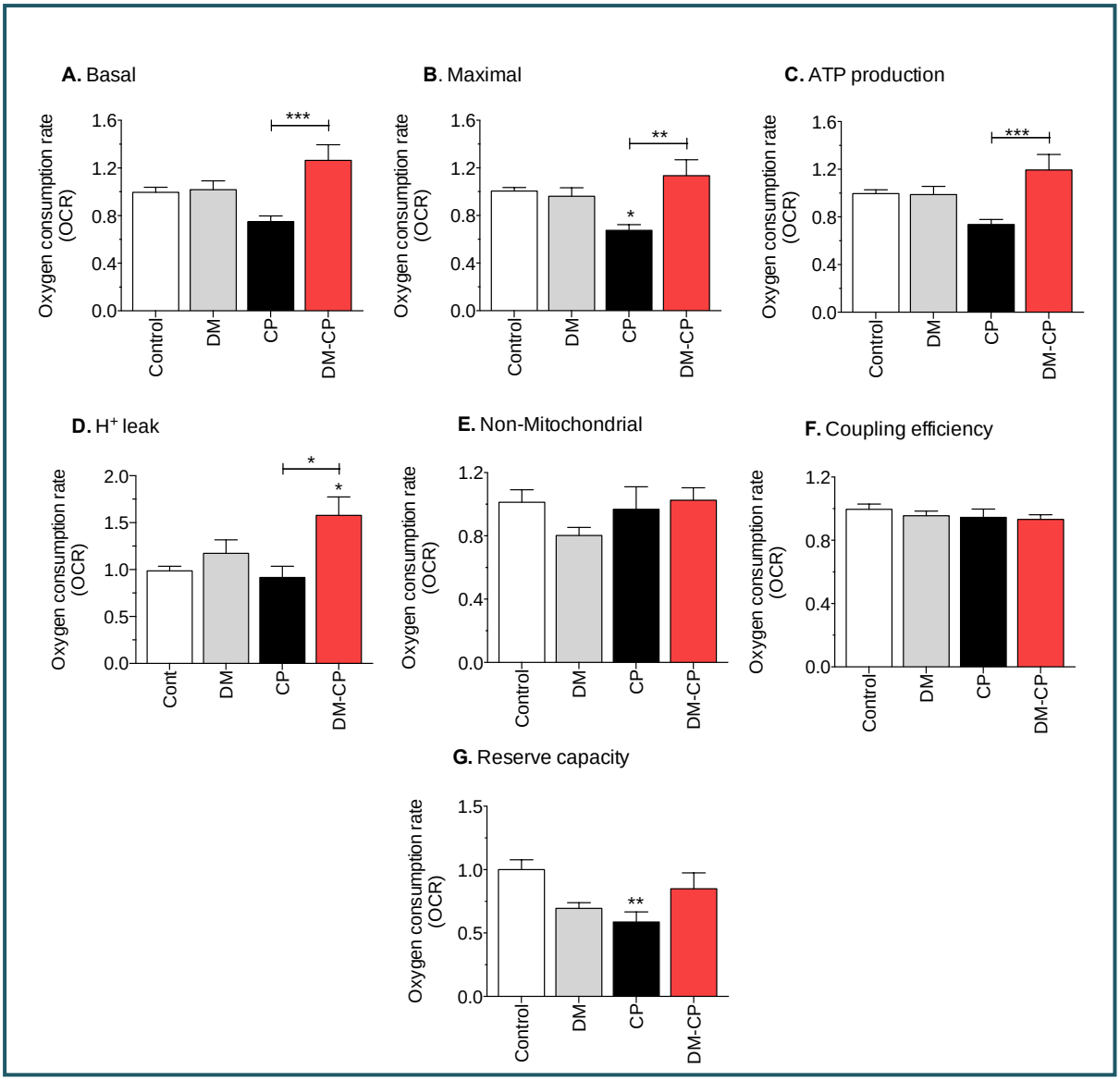

Figure 10. Oxygen Consumption Rate (OCR) in PBMCs. Data present a percent change in basal **(A),** maximal **(B)**, ATP production **(C)**, proton leak **(D)**, non-mitochondrial respiration **(E),** coupling efficiency **(F)** and reserve capacity **(G)** OCR in patients PBMCs compared to controls. The results are presented as the mean ± SEM of independent experiments, performed in PBMCs isolated from 10 individuals per group, run in duplicates or triplicates. Statistical analysis: *p<0.05; **p<0.01 and ***p<0.001 by one-way Anova followed by Tukey's Multiple Comparison Test.

Discussion

In this study we evaluated the influence of diabetic conditions on periodontitis by analyzing plasma resistin levels, oxidative stress and mitochondrial function in PBMCs isolated from patients with chronic periodontitis (CP), diabetes *mellitus* type II (DM) and chronic periodontitis plus diabetes *mellitus* type II (DM-CP) *versus* healthy controls (Control).

Clinical characterization of the subjects was made accordingly to American Academy of Periodontology (AAP) guidelines. Our results demonstrated an increase of probing depth (PD), clinical attachment level (CAL), plaque index (PI) and bleeding on probing (BOP) in patients with CP and patients with DM-CP when compared to healthy (control) or DM individuals allowing us to include this population in our study. No major differences were observed in body mass index (BMI) between all groups studied. Since a positive correlation between periodontal disease and glycemic control levels have been found in the literature, HbA_{1c} was evaluated in the participants of this study[1][16]. According to our results, HbA_{1c} levels were increased in DM and further increased in DM-CP, but not in CP patients, when compared to control subjects (reference value ≤ 6.5%). Therefore, these results support previous hypothesis presented in literature, indicating that chronic periodontitis can deregulate the control of HbA_{1c} levels[1][16].

Resistin was detected in plasma obtained from all individuals, being significantly increased in DM-CP patients when compared to controls. Moreover, we evaluated the putative correlation between resistin levels and parameters of metabolic disease (*e.g.* BMI and periodontal disease). Our data showed a positive correlation between resistin levels and BMI, which is in accordance with the literature showing a positive correlation between resistin levels and obesity or metabolic syndrome[35]. However, no correlation was found when resistin levels were plotted against the periodontal disease parameters, BOP and CAL. Periodontal disease is characterized by an increase of local immune-inflammatory factors, being a cause of increased resistin expression[36], or a consequence of high resistin levels in PBMCs[37]. Indeed, some authors defend that resistin plays an important role in the regulation of metabolic processes like adipogenesis and inflammatory reactions, while others pointed out increased resistin levels in CP when compared to control individuals [10][38]. Despite the controversy concerning the relationship between resistin and diabetes [23], a relationship has been suggested between obesity-diabetes-periodontitis[37]. However, other findings defend that resistin levels in CP are comparable to those observed in control patients, suggesting conflicting findings regarding the role of resistin in periodontitis disease[38].

Lymphocytes, the main population in isolated PBMCs, are a heterogeneous cell

population that largely dependents on mitochondria to meet energetic demands[31]. Considering the relevance of defining redox changes related with mitochondrial function and bioenergetics in PBMCs in periodontitis and DM conditions, we evaluated oxidative stress and mitochondrial function in PBMCs isolated from DM, CP, DM-CP and control groups. Our results evidenced that H_2O_2 production was significantly increased in PBMCs-derived from CP and DM-CP, but not in DM patients, when compared with the control. Opposing data was found by Mor-Li Hartman *et al*. (2014) suggesting that increased ROS production in PBMCs can indicate that diabetes *mellitus* might be associated with highly uncoupled oxygen consumption and thus, higher production of ROS[27]. This apparent discrepancy may due to the fact that DM patients in our study are controlled for diabetes as shown in Table II. It has been described that oxidative stress is associated with the development of insulin resistance and diabetes complications, such as periodontitis[39]. In accordance, our results evidence that the observed increase in ROS production in DM-CP patients can be associated with the increased resistin levels in these patients. In addition, and taking into account that patients evaluated in this study, namely DM and DM-CP patients, are under treatment with antihypertensive agents, which were shown to reduce ROS production[40], the fact that DM-CP present higher ROS production compared with DM patients suggest that chronic periodontitis induce *per se* an increase in ROS levels above DM patients and controls.

Cellular oxygen consumption is recognized as an indicator of mitochondrial function[27]. Our data indicate that when OCR were evaluated in PBMCs derived from DM-CP patients, an increase in both basal and maximal respiration along with increased ATP production, H^+ leak were observed, when compared with CP PBMCs. However, PBMCs derived from DM patients, similarly as shown for H_2O_2 analyses, did not exhibit any changes in all parameters evaluated by seahorse analysis. Interestingly, an increase in basal and maximal respiration, as well as ATP production was observed in DM-CP patients, in relation to CP patients, whereas H^+ leak also increase in the same experimental group (DM-CP PBMCs), when compared to both CP and controls conditions. The reserve capacity, defined as the capability of the cell to respond to an energetic demand, was shown to be slightly (although non-significantly) decreased in both DM and DM-CP patients, being significantly decreased in CP individuals[41]. In our isolated PBMCs, no differences were observed in non-mitochondrial respiration defined as oxygen consumption due to a subset of cellular enzymes that consume oxygen in conditions of blockade of cellular respiration achieved by complex I and complex III inhibition[27]. Decreased maximal respiration observed in CP-derived PBMCs, seems to be in accordance with decreased reserve capacity in the same patients. These data suggest that incremental mitochondrial activity, linked to enhanced H^+ leak, observed in

DM-CP PBMCs might reflect mitochondrial impairment due to metabolic or oxidative stress associated with periodontal or diabetic inflammation condition.

Recently, mitochondrial bioenergetics has been highlighted as a key to understand pathological mechanisms associated with metabolic diseases like diabetes[31]. Accordingly to these authors, the bioenergetics health index (BHI) (BHI=log [(reserve capacity x ATP-linked)/non-mitochondria respiration x proton leak)]) is a parameter that might reflect a potential function biomarker to study pathologies influenced by oxidative stress[31], such as diabetes and chronic periodontitis. Our results show a decrease in BHI in PBMCs obtained from DM-CP patients, as compared to control individuals, suggesting compromised mitochondria in patients with both pathologies. These results seem to be associated with increased H^+ leak observed in the PBMCs obtained from DM-CP group and with the fact that these patients present a slight tendency, although not significant, for increased values of BMI. In addition, high caloric intake, which is associated with obesity and thus BMI values, and also with high resistin levels, markers of the DM-CP patients selected for this study, can influence oxidative response and mitochondrial deregulation observed in this work. Data obtained in the present study corroborate the hypothesis that BHI might be considered as a new potential biomarker for assessing patient's health with prognostic and diagnostic values, as reported elsewhere[31].

Conclusion

Periodontitis has been largely associated with high local inflammation levels and oxidative stress. On the other hand, diabetes *mellitus* has been shown to be associated with obesity and increased resistin levels. Importantly, two-way relationship between these inflammatory diseases has been described in the literature. Data evidence increased plasma resistin levels in DM-CP patients that correlate with body mass índex, along with an increase in glycated hemoglobin. Whether obesity, diabetes mellitus and chronic periodontitis are related to each other through increased resistin levels and H_2O_2 production, as well as mitochondrial dysfunction remains unclear. Our study unveils a novel relationship between DM and CP by defining that DM-CP patients show exacerbated, but still bioenergetically abnormal, mitochondrial activity that may impact on increased ROS levels. Indeed, we do not know whether ROS are being produced by mitochondria and/or if the antioxidant levels/activity or the activity of mitochondrial respiratory complexes are altered in PBMCs of these patients. Overall, promoting basic research in this field may help to explain how DM and CP are interconnected through molecular and cellular events that may culminate in aggravated disease prognosis.

Acknowledgements

À Professora Doutora Ana Cristina Rego, orientadora deste trabalho, por me ter recebido de braços abertos, por me dar a oportunidade de entrar num projeto como este.

À Professora Doutora Isabel Poiares Baptista, co-orientadora deste trabalho, por me ajudar, incentivar e apoiar em todas as etapas do trabalho.

À Doutora Luísa Ferreira, a minha "mestre" de laboratório. Obrigada pela paciência, amizade, apoio incondicional. Obrigada por tudo o que ensinou ao longo de todo o percurso. Sem si, nada disto seria da mesma forma.

Obrigada às três por me ajudarem a superar todas as dificuldades. Obrigada pela dedicação. Tudo isto fez com que este projeto se concretizasse.

Ao Doutor Francisco Marques, pela disponibilidade e paciência que mostrou.

Às enfermeiras da Área de Medicina Dentária, pelo carinho, por me ajudarem com o material e por estarem sempre presentes.

À Isabel Dantas (CNC), pela simpatia que demonstrou ao longo de todas as minhas visitas e por me ajudar em algumas etapas laboratoriais.

A todos os elementos do CNC com quem contactei, por me receberem de braços abertos.

A todos os doentes pela autorização na realização da recolha de sangue e análise da saúde periodontal.

Ao João, pela amizade, paciência, motivação, alegria, amor e apoio. Elementos essenciais ao longo de todo o meu percurso académico que me ajudaram a crescer e a tornar-me mais forte.

Aos meus pais e à minha irmã, por me apoiarem em todos os momentos, por serem os meus pilares. Pessoas que me viram crescer e que estão sempre do meu lado. Obrigada pela dedicação e pela paciência. Sem o amor incondicional deles, não teria conseguido conquistar tantas pequenas grandes coisas.

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Attachments

Attachment 1. Informed Consent

FMUC FACULDADE DE MEDICINA
UNIVERSIDADE DE COIMBRA

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 plasmásticos 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.

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:_____/_____/____