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ANALYSIS OF NRF2-TARGET GENES IN PERIODONTITIS AND DIABETES SUBPOPULATIONS OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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ABBREVIATIONS

ADA - American Diabetes Association

AGEs - Advanced glycation end-products

ANOVA - One-way analysis of variance

BOP - Bleeding on probing

CAL - Clinical attachment level

cDNA - Complementary DNA

CHUC - Centro Hospitalar e Universitário de Coimbra

CNC - Center for Neurosciences and Cell Biology

DM - Diabetes mellitus

EDTA - Ethylenediamine tetraacetic acid

FACS - Fluorescence activated cell sorting

GCLc - Glutamate-cysteine ligase catalytic subunit

GST - Glutathione S-transferase

HbA1c - Glycated hemoglobin

HO1 - Heme oxygenase 1

mtROS - Mitochondrial reactive oxygen species

Nrf2 - Nuclear factor erythroid 2-related factor 2

PBMCs - Peripheral blood mononuclear cells

PBS - Phosphate-buffered saline

PDT - Periodontitis

PI - Plaque index

PMNs - Polymorphonuclear leukocytes

PPD - Periodontal pocket depth

qRT-PCR - Quantitative Real Time PCR

ROS - Reactive oxygen species

RT - Room temperature

SEM - Standard error of mean

SOD1 - Superoxide dismutase 1

T1D - Type-1 diabetes mellitus

T2D - Type-2 diabetes mellitus

RESUMO

A periodontite (PDT) é uma doença inflamatória crónica, resultante de um biofilme disbiótico subgengival que afeta os ligamentos periodontais e o osso em redor dos dentes, conduzindo à perda dentária. A diabetes mellitus tipo 2 (DM2) é uma síndrome metabólica caraterizada por hiperglicemia, sendo considerada um fator de risco para o desenvolvimento de PDT. Têm sido observados biomarcadores de stresse oxidativo no sangue periférico de pacientes com PDT e DM2, porém a sua associação ainda não é clara. Assim, o objetivo deste estudo foi avaliar a análise da expressão de genes alvo relacionados ao fator nuclear eritróide 2 (Nrf2, do inglês "*nuclear factor erythroid 2-related factor 2*"), envolvidos na resposta ao stresse oxidativo, em células mononucleares do sangue periférico (PBMCs, do inglês "*peripheral blood mononuclear cells*") e subpopulações derivadas destas células, nomeadamente linfócitos CD3+CD8+, de pacientes com PDT, DM2 e PDT-DM2 versus indivíduos controlo. Os nossos resultados evidenciam que pacientes com PDT e aqueles com as duas condições (PDT-DM2) apresentam marcadores clínicos periodontais alterados, enquanto indivíduos com DM2 e PDT-DM2 apresentam níveis aumentados de hemoglobina glicada (HbA1c). As PBMCs foram isoladas em quatro subpopulações celulares, nomeadamente linfócitos CD3+CD4+, linfócitos CD3+CD8+, linfócitos "não CD3+" e monócitos, por FACS (do inglês "*fluorescence activated cell sorting*"). O mRNA das PBMCs e das suas subpopulações foi isolado para avaliar a expressão génica de diferentes alvos do Nrf2, particularmente SOD1, GCLc, GST e HO1. Os nossos resultados revelaram níveis reduzidos de SOD1, GST e GCLc e uma tendência, embora sem significado estatístico, de diminuição nos níveis de mRNA de HO1 em pacientes com PDT, quando comparados com indivíduos controlo. Além disso, os níveis de mRNA de SOD1 e GCLc estavam diminuídos em doentes com PDT-DM2. Curiosamente, os níveis de mRNA de SOD1 e GST também estavam reduzidos, enquanto os níveis de mRNA de HO1 não sofreram alteração na subpopulação de linfócitos CD3+CD8+ em pacientes com PDT e PDT-DM2. Os nossos resultados também sugeriram um aumento significativo na expressão de HO1 e uma tendência para níveis aumentados de mRNA de GST nas PBMCs de pacientes com PDT-DM2, em comparação com indivíduos com PDT. Em suma, os nossos dados revelam uma diminuição da expressão de genes alvo do Nrf2 em pacientes com PDT associada ou não a DM2, tanto em PBMCs como em linfócitos CD3+CD8+, sugerindo um fator comum subjacente ao dano tecidular causado por PDT. Assim, a relação bidirecional entre a desregulação oxidativa e as características patológicas da PDT parece ser relevante na procura de novos alvos terapêuticos.

Palavras-chave: Diabetes mellitus; Nrf2; stresse oxidativo; periodontite; células mononucleares do sangue periférico

ABSTRACT

Periodontitis (PDT) is a chronic inflammatory disease, resulting of a dysbiotic subgingival biofilm's that affects both the periodontal ligaments and bone surrounding teeth, leading to consequent tooth loss. Type-2 diabetes mellitus (T2D) is a metabolic syndrome characterized by hyperglycemia, and a well-recognized risk factor for the development of PDT. Oxidative stress biomarkers have been observed in peripheral blood of PDT and T2D patients, however, their association is still not clear. Therefore, the aim of this study was to analyze the expression of nuclear factor erythroid 2-related factor 2 (Nrf2)-target genes, involved in oxidative stress response, in peripheral blood mononuclear cells (PBMCs) and derived subpopulations, including CD3+CD8+ lymphocytes, from PDT, T2D and comorbid PDT-T2D patients *versus* control individuals. Our results evidence that PDT patients and those with the two conditions exhibit altered periodontal clinical markers, while T2D and T2D-PDT individuals display augmented levels of glycated hemoglobin (HbA1c). PBMCs were isolated in four cellular subpopulations, namely CD3+CD4+ lymphocytes, CD3+CD8+ lymphocytes, non CD3+ lymphocytes and monocytes by fluorescence activated cell sorting (FACS). mRNA was further isolated from both whole PBMCs and its subpopulations in order to evaluate the gene expression of different Nrf2 targets, namely SOD1, GCLc, GST and HO1. Our results reveal reduced levels of SOD1, GST and GCLc and a tendency, although without statistical significance, of decreased mRNA levels of HO1 in PDT patients, when compared to control individuals. Moreover, SOD1 and GCLc mRNA levels were diminished in PDT-T2D. Interestingly, SOD1 and GST mRNA levels were also decreased while HO1 mRNA levels were unaltered in CD3+CD8+ lymphocyte subpopulation derived from PDT and PDT-T2D patients. Our results also suggest a significant increase in HO1 expression and a tendency for increased GST mRNA levels in PBMCs from PDT-T2D patients in comparison to PDT. Altogether, our data reveal decreased expression of Nrf2-target genes in PDT with or without T2D in both PBMCs and CD3+CD8+ lymphocytes, suggesting a common factor underlying PDT-associated tissue damage. Thus, bi-directional relationship between oxidative deregulation and PDT pathological features might be relevant when exploring new therapeutical targets.

Keywords: Diabetes mellitus; Nrf2; oxidative stress; periodontitis; peripheral blood mononuclear cells

INTRODUCTION

Periodontitis (PDT) is a multifactorial, chronic inflammatory disease that, if untreated, can lead to the non-reversible damage of periodontium, which comprises a set of supportive tissues surrounding the teeth (gingiva, periodontal ligament, cementum, and alveolar bone), with consequent tooth loss (1,2). It is often associated with, and possibly caused by, an altered dynamic interaction between specific pathogenic bacteria, exaggerated inflammatory and immune responses, environmental stressors and individual genetic predisposition. The major triggering factor starts with inadequate oral hygiene accompanied by an imbalance of micro-organisms forming the dental plaque (1). In a susceptible individual, the accumulation and maturation of a sub-gingival dysbiotic biofilm containing predominantly gram-negative anaerobic bacteria, results in clinical attachment level (CAL) loss and radiographically assessed alveolar bone loss, presence of periodontal pocketing and gingival bleeding (3,4). Progression of untreated PDT can result in pain, edentulism, masticatory dysfunction, nutritional compromise, altered speech, aesthetic problems, low self-esteem and a poorer overall quality of life (3,5,6). PDT is an important public health problem due to its high prevalence, affecting about 50% of the adult population worldwide (2,7–9). During the last decades, there has been recognized a significant association between periodontal disease and other chronic systemic disorders where there is low grade inflammation (6). In fact, PDT was stated the sixth complication of diabetes (10).

Diabetes mellitus (DM) is a clinical syndrome that encompasses a heterogeneous group of metabolic diseases distinguished by a hyperglycemic state secondary to limitations in insulin action and/or production (11,12). There are two major forms of this disorder: type 1 DM (T1D) and type 2 DM (T2D) (13). T1D is due to the autoimmune destruction of pancreatic β-cells with consequent absolute insulin deficit. T2D is usually caused by the association between insulin resistance and progressive loss of satisfactory β-cells insulin production (14). American Diabetes Association (ADA) has recommended HbA1c with a cut-point ≥6.5% for diagnosing DM. It is madly becoming a pandemic concern with 537 million people worldwide suffering from this disease (International Diabetes Federation 2021). In adults, T2D is the most frequent form, accounting about 90-95% of all diabetes diagnosed cases (13). In all forms of diabetes, chronic elevated glycemia results in protein, carbohydrate and fat metabolism dysfunction. These systemic alterations upraise the risk for developing irreversible complications like cardiovascular diseases, nephropathy, retinopathy, neuropathy and delayed healing (11). T2D is associated with oral complications as well, showing a higher risk of infection and to develop PDT (15).

The link between T2D and PDT is not yet been fully understood. However, through the years many explanations have been purposed such as (a) the increase of oxidative stress, (b)

the accumulation of advanced glycation end-products (AGEs), and (c) a dysregulation in collagen metabolism with decrease of collagen production and raise of collagenase activity (16,17). Recently, the oxidative stress-inflammatory pathway has gained importance in the pathogenesis of PDT and T2D. Given the pro-oxidative state common to these two diseases, this appears to be a possible explanation to their synergistic interactions (12). In fact, recent findings have shown that patients with DM and PDT reveal mitochondrial dysfunction and enhanced production of reactive oxygen species (ROS) in peripheral blood mononuclear cells (PBMCs) and endothelial cells (18–20). Lymphocytes, the major population in isolated PBMCs, are substantially dependent on mitochondria in order to achieve energetic demands (21). Elevated levels of ROS are crucial in the activation of lymphocytes and act as powerful triggers to the production of pro-inflammatory cytokines like IFN-γ and TNF-α. IFN-γ has an important role in the regulation of the adaptative as well as the innate immunity, leading to both direct and indirect oxidative harm to periodontal structures (12,19). Leading the inflammatory cascade during bacterial or viral infections, this cytokine can activate macrophages and consequently the secretion of additional pro-inflammatory mediators, such as TNF-α. TNF-α also has an essential role in systemic inflammation, being possibly involved in insulin-resistance regulation. Although the precise relationship between glycemic control and inflammation is still not clear, it can certainly be assumed that glucose metabolism is majorly regulated in mitochondria (19). In this regard, when the nutrients availability exceeds the demand for ATP, there is a reduction in the rate of electron flow, extending the reactive intermediates lifespan at mitochondrial complexes I and III, ultimately generating excessive amount of ROS (19,22). Besides, higher levels of ROS will act as a key factor for insulin-resistance worsening, possibly initiating a vicious cycle (19,23). Still, chronic hyperglycemia may compromise antioxidant capacity (12). Decreased antioxidant status and excessive oxidative stress can be identified in blood of patients with PDT (24). These findings sustain the use of PBMCs picked up from human blood to evaluate the mitochondrial (dys) function and oxidative stress associated to inflammatory diseases like PDT and DM (21).

One potential mechanism that may contribute for the exacerbation of PDT by T2D is the downregulation of key local antioxidant transcription factors, like nuclear factor erythroid 2-related factor 2 (Nrf2) (25). Nrf2 is a redox-sensitive transcription factor that controls nuclear genes, such as superoxide dismutase 1 (SOD1), heme oxygenase 1 (HO1), glutathione S-transferase (GST) and glutamate-cysteine ligase catalytic subunit (GCLc), consequently regulating the expression of various antioxidant and detoxifying enzymes like SOD1, HO1, GST and GCLc. Its activation represents an important cellular defense mechanism against oxidative damage (7,25). Downregulation of Nrf2 and consequent deficiency of antioxidant production results in increased ROS level that have been associated with aggravated

periodontal destruction and hyperglycemia. Thus, this pathway may contribute to explain the synergetic pathogenesis of PDT and T2D (25).

Our previous study, has shown increased ROS levels in both PDT and PDT-T2D PBMCs, associated with increased Nrf2 protein levels (author´s unpublished data). In that regard, the present study aimed to investigate oxidant markers in PBMCs and sorted T cell populations, linking the inflammatory response and oxidative stress to Nrf2 regulated gene expression in patients with both PDT and T2D, when compared with patients only with T2D or PDT and healthy controls.

MATERIAL AND METHODS

1. Material

Lymphoprep™ was from Stemcell™Technologies (Alere Technologies AS, Oslo, Norway). FITC mouse anti-human CD3, PE mouse anti-human CD4 and PerCP-Cy 5.5 Mouse Anti-Human CD8 were obtained from BD Pharmingen®. NZYol reagent and NZY first-strand cDNA synthesis kit were from NZYTech (Lisbon, Portugal) and iQ SYBR Green supermix were purchased from BioRad (Hercules, USA)**.** All other reagents were of analytical grade.

2. Subject selection

2.1 Study Population

Sixteen subjects (age range: 38 to 71 years) presenting with at least 15 teeth were selected from the population referred to Dentistry appointments in Centro Hospitalar e Universitário de Coimbra (CHUC). Detailed dental and medical records were obtained and patients who fulfilled the following inclusion–exclusion criteria were invited to participate in the study. All eligible subjects were rigorously informed of the nature and potential risks/benefits of their participation in the study, and all provided written informed consent (attachment 1). This study protocol was previously approved by the institutional Ethics Committee.

The study's participants were categorized as follows:

- **Group 1:** 4 Controls – Systemically and periodontally healthy subjects.
- **Group 2:** 4 patients with T2D without PDT.
- **Group 3:** 4 patients with PDT without T2D.
- **Group 4:** 4 patients with PDT and T2D.

2.2 Exclusion criteria

Exclusion criteria included pregnancy/lactation, HIV or hepatitis (B, C), uncontrolled systemic diseases (except T2D) or neoplasms, medical condition that prevents an oral exam, chronic antibiotic use or requiring antibiotic coverage for dental procedures, undergoing therapy with corticosteroids and/or immunosuppressive treatment in the 3 months prior to periodontal evaluation, chronic use of non-steroidal anti-inflammatory drugs and long-term treatment with medications known to affect periodontal status (phenytoin, cyclosporine).

2.3 Evaluation of periodontal status

All study participants were clinically evaluated by one trained and calibrated examiner. Clinical parameters were assessed using a Williams probe, including periodontal pocket depth (PPD) and CAL at six different sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual) per tooth at all teeth. The plaque index (PI), presence of gingival bleeding on probing (BOP), furcation involvement and tooth mobility were also recorded. PDT was diagnosed in case of interdental CAL is detectable at ≥2 non-adjacent teeth, or buccal/oral CAL ≥3mm with pocketing ≥3mm is detectable at ≥2 teeth, according to 2017 World Workshop for Classification of Periodontal Diseases and Conditions (3).

3. Blood sample collection and glycated hemoglobin determination

All samples were collected the following morning after an overnight fast, during which participants were requested not to eat or drink anything in the morning (except water). About 10 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 at CHUC. Collected samples were immediately transferred to the Center for Neurosciences and Cell Biology (CNC), University of Coimbra, for PBMCs isolation. For all diabetic patients, extra 3 mL of blood were collected to determinate the level of glycated hemoglobin (HbA1c) at Clinical Pathology service, CHUC.

4. Isolation of peripheral blood mononuclear cells

After being collected, 10 mL of blood samples were carefully layered onto 8 mL Lymphoprep™ solution in 50 mL Falcon tubes and then centrifuged at 2,500 rpm for 20 minutes at 18ºC in a swing-out rotor without brake using the Eppendorf Centrifuge 5810R (Fig. 1). After centrifugation, the lymphocyte-containing ring was removed with a Pasteur pipette, collected in another 50 mL Falcon tube, and further diluted with 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 to a final volume of 45 mL. The tubes were then centrifuged at 1,500 rpm for 10 min at 18°C with maximum braking and acceleration, and pelleted cells (peripheral blood mononuclear cells - PBMCs) resuspended in 500 µL of PBS. About 200 µL of cell suspension was immediately transferred to the Laboratory of the Institute of Immunology at FMUC to perform fluorescence activated cell sorting (FACS). The remaining suspension was divided for RNA extraction (in 800 µL of NZYol and stored at -20°C) or frozen as pellets for future studies at -80°C.

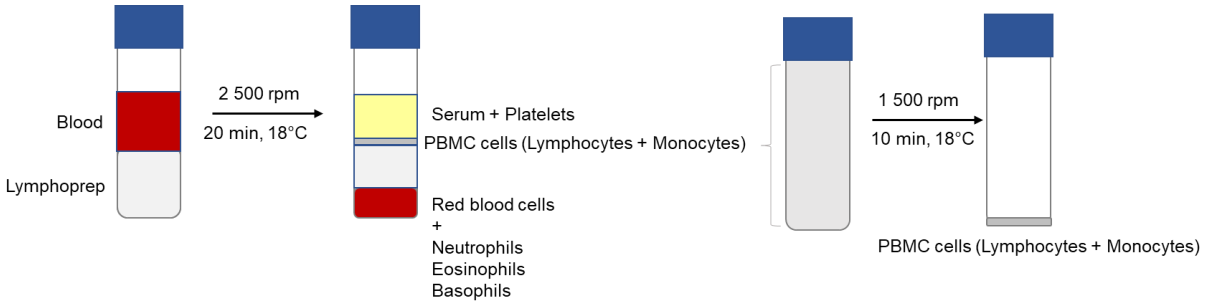

Figure 1 – PBMCs isolation procedure. After being collected, blood was layered onto Lymphoprep solution and then subjected to density gradient centrifugation at 2,500 rpm for 20 min at 18°C. At the end four layers were visible namely, the yellow plasma layer on the top, the PBMCs ring, the Lymphoprep layer, and the red blood cells plus granulocytes on the bottom. The PBMCs-containing ring was then collected as described in Methods section.

5. Fluorescence activated cell sorting (FACS)

The PBMCs absolute numbers were determined using DXH 500 (Beckman Coulter, CA, USA) equipment in order to adjust the volume of each antibody. FITC mouse anti-human CD3, PE mouse anti-human CD4 and PerCP-Cy 5.5 Mouse Anti-Human CD8 (BD Pharmingen, CA, USA) antibodies were added per $1x10^6$ cells according to manufacturer`s instructions. The contents were mixed and then incubated for 15 min at RT (room temperature) in the dark. Cells were further washed in 2 mL of PBS, centrifuged for 5 min at 450 xg and pelleted cells resuspended in PBS until sorting using a FACSAria III™ from Becton Dickinson, CA, USA.

About 10000 events were acquired to define gating strategy using morphologic and fluorescence parameters. Using a dot-plot from FSC (forward light scatter) versus SSC (Side scatter) lymphocytes were gated and, after excluding doublet, lymphocytes subsets were identified by gating CD3+CD4+ and CD3+CD8+ and non CD3+. Monocytes were initially selected by morphologic parameters, then doublets were excluded, and monocytes identified by CD4 positivity, since we did not use a specific monocyte marker. Cell sorting was performed to separate CD3+CD4+, CD3+CD8+, non CD3+ cells and monocytes.

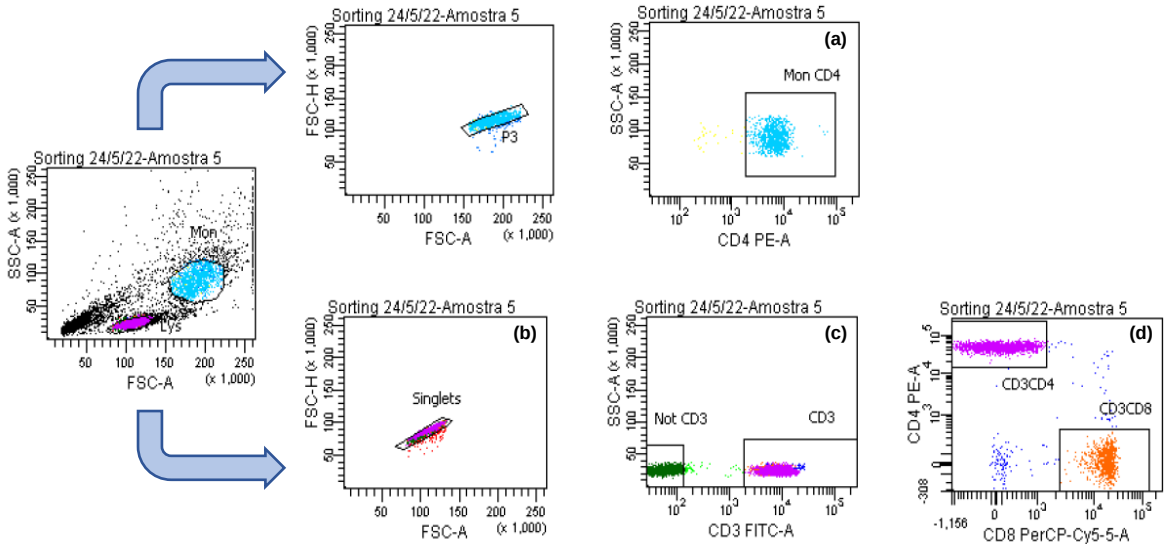

Figure 2 – Gating strategy used for identification of PBMCs subpopulations by Fluorescence activated cell sorting (FACS): (a) monocytes, (b) singlets, (c) non CD3+ lymphocytes, (d) CD3+CD4+ and CD3+CD8+ lymphocytes.

6. RNA isolation and quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted from sorted and total PBMCs using NZYol (0.8 mL/ 90-300x10^3 cells), according to the instructions of the supplier. Briefly, cell lysates were incubated for 5 min at RT and chloroform (200 µL per 1 mL NZYol used) was added and mixed vigorously before incubating for another 2 min. After centrifugation at 12,000 ×*g* using an Eppendorf Centrifuge 5417R for 15 min at 4°C, the clear upper aqueous layer was transferred to a new tube, isopropanol (500 µL per 1 mL NZYol) was added and the samples incubated for 10 min at -20°C before centrifugation at 12,000 ×*g* for 10 min, at 4°C. The RNA precipitate was washed with 75% ethanol and the samples centrifugated at 12,000 ×*g* for 10 min, at 4°C. The pellet was dried at RT and then solubilized in 20 µL of DEPC-treated water. RNA concentration was determined with NanoDrop 2000c spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was then synthesized from 500 ng of total extracted RNA using the NZY First-Strand cDNA Synthesis Kit, following the manufacturer instructions. PCR reactions were performed in 10 µL volumes containing 5 µL of iQ SYBR Green Supermix, 300 nM of each primer (as described below) and 50 ng of cDNA template in a Bio Rad CFX96 Real-Time PCR Detection System using the following cycling conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 55-61.7°C for 45 sec. At the end, samples were subjected to a melting curve analysis in order to confirm the absence of unspecific amplification products and primers dimers. Samples containing no template were included as negative controls in all experiments. Reactions were run in duplicates. Analysis of gene expression was performed using the ΔΔCT method. GAPDH was

used as an internal control for all samples. PCR primer sequences used were as follows (forward primer/reverse primer):

SOD1	F:GGTGGGCCAAAGGATGAAGAG	R:CCACAAGCCAAACGACTTCC	61,7°C
HO1	F: CCTGAGTTTCAAGTATCC	R:AACAACAGAACACAACAA	55°C
GST	F: GGAGGCAAGACCTTCATT	R:ATGGATCAGCAGCAAGTC	55°C
GCLc	F: AACTCTTCATCATCAACTA	R:AACTCCATCTTCAATAGG	55°C
GAPDH	F: ATTCCACCCATGGCAAATTC	R:GGGATTTCCATTGATGACAAGC	58°C

7. Statistical analysis

Data were analyzed by GraphPad Prism 9 software and expressed as the mean of independent experiments ± standard error of mean (SEM). Statistical significance was assessed using the Mann-Whitney test, unpaired Student's *t*-test or One-way analysis of variance (ANOVA) followed by uncorrected Fisher's LSD multiple comparison test. Sample normality was tested using Shapiro-Wilk test. Statistical significance was defined as *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

RESULTS

The demographic variables and diabetic parameters of the study groups namely, individuals' distribution by age, gender, HbA1c levels, body mass index (BMI) and smoking status was organized according to diagnostic groups (Table 1). No significant difference in glycated hemoglobin (HbA1c) was observed between T2D and PDT-T2D patients. The BMI was shown to be significantly increased in T2D patients (p=0.057) in comparison with the control group. None of subjects of all groups was a smoker.

Table 1 – Demographic and clinical characteristics of the study group

Subject	Age *(years)*	Males/Females *(number)*	HbA1c *(%)*	BMI *(kg/m2)*	Smokers *(number)*
Control	47.8 ± 3.9	1/3	n.a.	23.35 ± 1.69	0
PDT	56.8 ± 2.1	1/3	n.a.	28.00 ± 1.22	0
T2D	49.6 ± 10.4	2/2	6.75 ± 0.41	29.68* ± 1.13	0
PDT-T2D	53.6 ± 7.7	3/1	6.98 ± 0.56	28.68 ± 2.35	0

Subjects were categorized per age, gender, glycated hemoglobin (HbA1c), body-mass index (BMI) and smoking status and classified in accordance with clinical evaluation for periodontitis (PDT), type 2 diabetes (T2D) and type 2 diabetes plus periodontitis (PDT-T2D) (*see Figure 3 for details of clinical periodontal evaluation*). Statistical analysis: *p=0,057 for T2D vs Control by Mann Whitney test; n = 4 per group. n.a.= not applicable.

Patient medications until the day of the blood collection, grouped according to the Anatomical Therapeutic Chemical classification system, are described in Table 2. As expected, data show that T2D or PDT-T2D patients are prescribed with drugs to lower blood glucose and blood pressure. None of the control group subjects were taking any medications, but in the other studied populations drugs are widely prescribed, which may modify some parameters evaluated in this study.

Table 2. Characterization of experimental groups to medication.

Subject	Alimentary Tract and Metabolism[1] *(antidiabetics)*	Nervous System[2] *(antidepressants + anxiolytics)*	Cardiovascular System[3]	Other Classes[4]
Control	0%	0%	0%	0%
PDT	0%	25%	50%	50%
T2D	75%	50%	75%	25%
PDT-T2D	100%	25%	100%	50%

Percentages reflect the number of subjects in a group taking at least a compound from the indicated class. Medication nomenclature in accordance with the Anatomical Therapeutic Chemical (ATC) classification system and presented as subject percentage (%). [1]Dapagliflozin, Empagliflozin, Insulin, Metformin, Metformin+Sitagliptin; [2]Alprazolam, Bromazepam, Diazepam, Fluoxetine, Lorazepam, Pregabalin, Trazodone, Venlafaxine; [3]Acetylsalicylic acid, Amiodarone, Atorvastatin, Bisoprolol, Chlorthalidone, Clopidogrel, Edoxaban, Fenofibrate, Furosemide, Methyldopa, Metoprolol, Perindopril, Pitavastatin, Pravastatin, Propranolol, Rilmenidine, Rosuvastatin, Rosuvastatin+Ezetimibe, Sinvastatin, Telmisartan; [4]Alopurinol, Cyclobenzaprine, Levothyroxine, Montelukast sodium, Tramadol, Ursodiol. [PDT: periodontitis; T2D: type 2 diabetes; PDT-T2D: periodontitis plus type 2 diabetes].

The clinical periodontal parameters (PD, CAL, PI and BOP) were evaluated in all patients that participated in the present study and presented in Fig. 3. As expected, PD levels were shown to be significantly increased in all groups, compared to control patients (Fig. 3A). PDT-T2D subjects also revealed a significant difference with respect to PD (Fig. 3A) and CAL (Fig. 3B) in contrast to T2D group. Regarding CAL, PDT and PDT-T2D groups exhibited higher values in comparison to control individuals (Fig. 3B). Moreover, PI (Fig. 3C) and BOP levels (Fig. 3D) were significantly raised in PDT-T2D group when compared with control individuals.

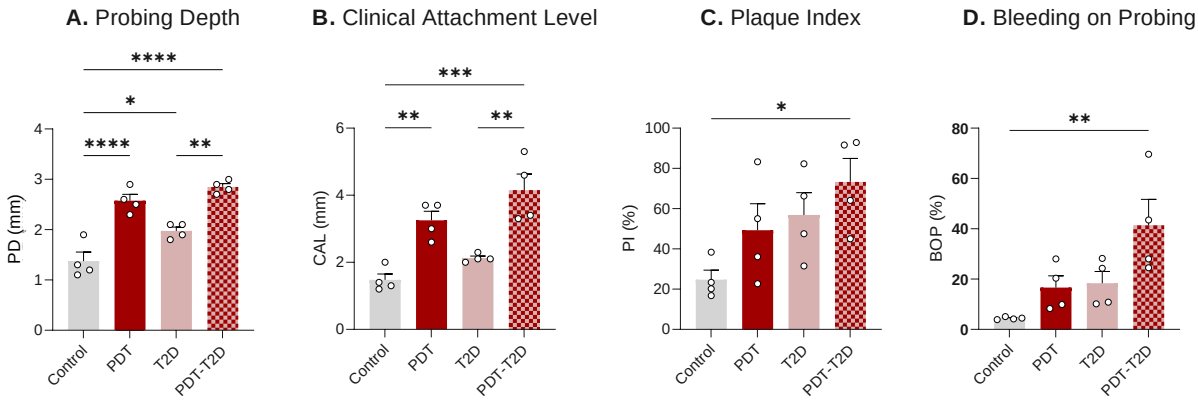

Figure 3 - Clinical parameters for periodontal evaluation. Values of probing depth [PD] (A), clinical attachment level [CAL] (B), plaque index [PI] (C) and bleeding on probing [BOP] (D) in control individuals, PDT, T2D and PDT-T2D patients. Data are presented in scatter plots as the mean ± SEM from 4 individuals per group. Statistical analysis: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 by one-way ANOVA followed by uncorrected Fisher's LSD multiple comparison test.

Since previous data from our lab evidenced increased nuclear factor erythroid 2-related factor 2 (Nrf2) protein levels in T2D and in PDT-T2D comorbid patients (author´s unpublished data), mRNA was further isolated from PBMCs in order to evaluate the gene expression of different Nrf2 targets, namely SOD1, GCLc, GST and HO1, and to verify the effect of Nrf2 to activate its transcription targets. Our data show a significant reduction in SOD1 (Fig. 4A), GST (Fig. 4B) and GCLc (Fig. 4C) mRNA levels, as well as a tendency for decreased expression of HO1 (Fig. 4D) in PBMCs from PDT patients, as compared to control. Interestingly, both SOD1 (Fig. 4A) and GCLc (Fig. 4C) mRNA levels were also diminished in PDT-T2D. Moreover, a significant increase in HO1 (Fig. 4D) expression and a tendency to increase in GST (Fig. 4B) mRNA levels were observed in PBMCs from PDT-T2D patients in comparison to PDT. Also, the expression of Nrf2 target genes evaluated in this study were not significantly altered in T2D patients' PBMCs when compared to control. Our results suggest that increased Nrf2 previously observed by us in PBMCs from PDT-T2D patients (author´s unpublished data) may not be sufficient to activate the Nrf2 pathway and the transcription of all its target genes.

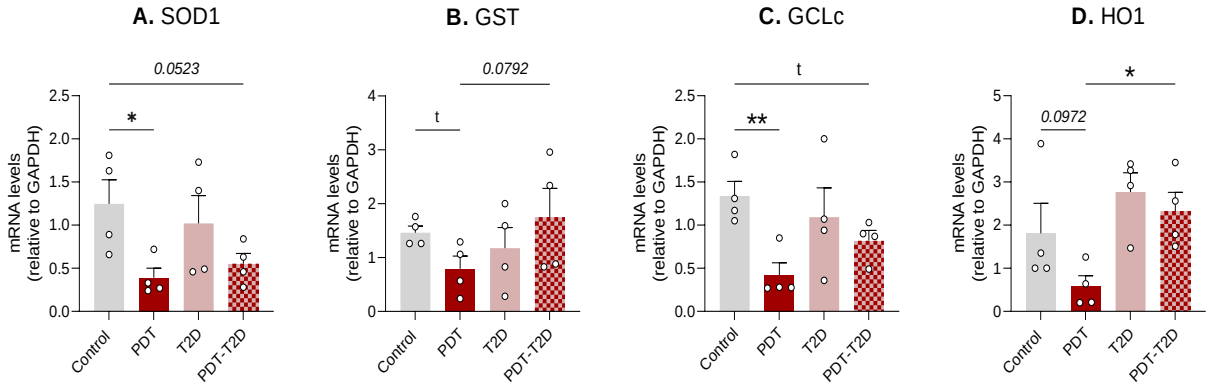

Figure 4 – mRNA levels of Nrf2 target genes in PBMCS. Relative expression of SOD1 (A), GST (B), GCLc (C) and HO1 (D) mRNA in PBMCs from control individuals, PDT, T2D and PDT-T2D patients. GAPDH was used as the housekeeping messenger. Data are presented in scatter plots as the mean ± SEM from 4 individuals per group. Statistical analysis: *p<0.05; **p<0.01 by one-way ANOVA followed by uncorrected Fisher's LSD multiple comparison test or unpaired Student's *t*-test.

PBMCs were then subjected to FACS in order to quantify and separate different cell populations by using various combinations of antibodies for detection of specific surface and intracellular molecules. At the end, cell sorting led to a set of 4 populations: CD3+CD4+ lymphocytes, CD3+CD8+ lymphocytes, non CD3+ lymphocytes and monocytes. No differences were observed in the percentage of lymphocyte subpopulations and monocytes obtained among the groups (Fig. S1). mRNA from the different populations were isolated and further converted to cDNA. Although both concentration and purity of mRNA and cDNA were similar in the PBMCs populations (Fig. S2), we could not detect DNA amplification for Nrf2 target genes, as evaluated by qRT-PCR, in CD3+CD4+ lymphocytes, non CD3+ lymphocytes and monocytes, which could be accounted for reduced expression of these genes in these cell

populations. Therefore, we investigated whether the alterations observed in PBMCs from patients with both comorbidities were observed in CD3+CD8+ lymphocytes. Our results show that SOD1 (Fig. 5A) and GST (Fig. 5B) mRNA levels were reduced in both PDT and PDT-T2D patients in comparison with control individuals. However, mRNA levels of HO1 were unaltered (Fig. 5C). Besides, CD3+CD8+ lymphocytes from T2D patients didn't reveal a significant difference in the expression of SOD-1 and GST target genes.

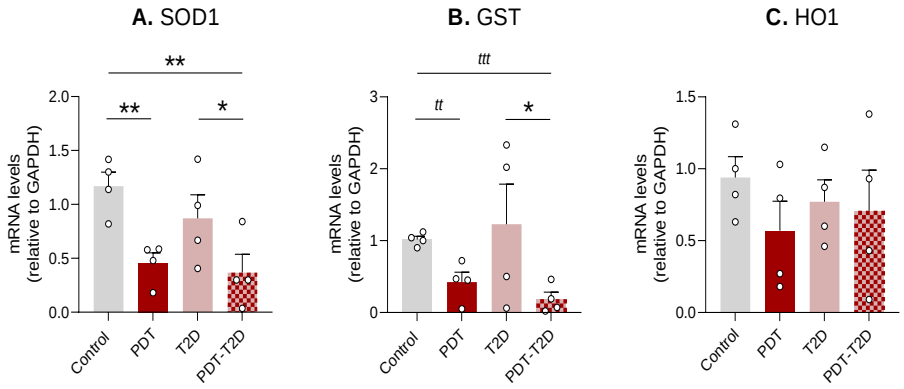

Figure 5 – mRNA levels of Nrf2 target genes in CD3+CD8+ lymphocytes. Relative expression of SOD1 (A), GST (B) and HO1 (C) mRNA in CD3+CD8+ lymphocytes from control individuals, PDT, T2D and PDT-T2D patients. GAPDH was used as the housekeeping messenger. Data are presented in scatter plots as the mean ± SEM from 4 individuals per group. Statistical analysis: *p<0.05; **p<0.01; ***p<0.001 by one-way ANOVA followed by uncorrected Fisher's LSD multiple comparison test or unpaired Student's *t*-test.

DISCUSSION

The high prevalence and severity of PDT in T2D populations have become a serious worldwide health problem. So, there is an urgent need to clarify the mechanism that may explain the bi-directional deleterious effects of these two diseases. Most likely, there is a multifactorial explanation probably related to chronic systemic inflammation, compromised host defense, and increased oxidative stress (4,26,27).

We confirmed that PDT patients and those with the two conditions (PDT-T2D) displayed periodontitis markers, while T2D and PDT-T2D individuals showed augmented levels of HbA1c. According to our results, HbA1c levels were increased in T2D and further increased in PDT-T2D (considering the reference value ≤ 6.5) supporting previous hypothesis presented in literature, indicating that PDT can deregulate the control of HbA1c levels (28,29). Systemic inflammation has been considered a potential mediator of this association, with elevated mitochondrial reactive oxygen species (mtROS) and a dysregulated immune-inflammatory response in patients with PDT and T2D reported in literature (12,19).

Increased production of mtROS is an essential step in lymphocytes' activation, the main population present in isolated PBMCs (19). In fact, recent findings have noted the importance of studying mitochondrial (dys)function in human immune cells, such as lymphocytes, derived from inflammatory diseases like T2D and PDT (30). Lymphocytes are heterogeneous cell population responsible for organism's defense against pathogenic agents, assuming important functions during bacterial infections like the one that occurs in PDT, specially the CD3+CD4+ and CD3+CD8+ subsets. Early studies in cells extracted from PDT lesions revealed a 1:1 CD3+CD4+/CD3+CD8+ ratio in PDT (Cole et al. 1987; Stoufi et al. 1987), demonstrating an important increase of CD3+CD8+ T cells compared to gingivitis, whereas the CD3+CD4+/CD3+CD8+ ratio is approximately 2:1 (Seymour et al. 1988; Berglundh et al. 2002a; Zitzmann et al. 2005). CD3+CD8+ cytotoxic T cells produce inflammatory cytokines that participate in both adaptative and innate immune responses, and play an important role in the lysis of tissues and cells infected or damaged by bacteria (31). Given the importance of CD3+CD8+ lymphocytes in PDT's inflammatory pathways, this might be a possible explanation for the exclusive expression of Nrf2 target genes, in this PBMCs' subpopulation.

To minimize the oxidative damage induced by periodontal/diabetic inflammation the transcription factor Nrf2 is responsible for inducing hundreds of antioxidant and detoxifying enzymes, such as SOD1, GCLc, GST and HO1 (25). Our data evidence that in PBMCs from PDT patients a significant reduction in SOD1, GST and GCLc mRNA levels, while a tendency for decreased expression of HO1 was found. Accordingly are findings obtained by Sima and co-authors (2016) (32) showing that levels of SOD1 in oral polymorphonuclear leukocytes (PMNs) were decreased in PDT patients compared to controls. Our results also show that both

SOD1 and GCLc mRNA levels were diminished in PDT-T2D's PBMCs. In fact, total SOD activity was shown to be decreased in the serum of PDT-T2D rats when compared with the control animals (25). Similar results were observed in our study in subpopulation of CD3+CD8+ lymphocytes where SOD1 mRNA levels were reduced in PDT and PDT-T2D patients, in comparison to control individuals. In accordance with our findings, studies performed by Patil and co-authors (2016) (15) in red blood cells, showed a significant reduction of SOD activity in PDT and PDT-T2D patients. Opposing data was found by Trivedi and co-authors (2013) (4), showing an increase in SOD in red blood cells from PDT-T2D patients, suggesting that higher ROS production in PDT may enhance the antioxidant defense system, counterbalancing the pro-oxidant environment. Additionally, contradictory results in respect of GCLc activity and protein levels were found in our previous study (author´s unpublished data), revealing an increase in PDT-T2D PBMCs compared to controls, suggesting a compensatory mechanism. Our data also suggest a significant increase in HO1 expression and a tendency to increase GST mRNA levels in PBMCs from PDT-T2D patients in comparison to PDT. One possible explanation for these results might be an attempt to afford an extra biological protection against superoxide generation observed in hyperglycemic states (26).

Unfortunately, we could not detect DNA amplification for GCLc in CD3+CD8+ lymphocytes, in order to compare with its mRNA levels in PBMCs, which might be explained by a diminished expression of this gene in this subpopulation.

The present study had a few limitations. Firstly, the sample size was relatively modest to take strong conclusions. Secondly, the patients included were taking medications that might influence cellular function and confound the results, like metformin, ACEI/ARB or statins (33,34).

CONCLUSIONS

In light of recent findings, a new research field is emerging bridging redox deregulation in the study of mechanisms related to T2D and PDT. The data presented in this study evidence reduced SOD1, GST and GCLc mRNA levels in PBMCs and CD3+CD8+ lymphocytes derived from PDT and PDT-T2D patients. In addition, enhanced Nrf2-dependent targets, as HO1 and GST in cells from PDT-T2D patients may act to balance the cellular antioxidant profile. In conclusion, data corroborate the hypothesis that ROS play a role in periodontal complications, which may occur through the impairment of antioxidants responsible for decreasing ROS levels and maintaining systemic health. Determining the mechanisms that contribute for the increase in oxidative stress in patients with PDT and T2D (including PDT-T2D) may help identifying new therapeutic targets that allow a better control of PDT's progression in these patients.

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REFERENCES

1. Liccardo D, Cannavo A, Spagnuolo G, Ferrara N, Cittadini A, Rengo C, et al. Periodontal disease: a risk factor for diabetes and cardiovascular disease. Vol. 20, International Journal of Molecular Sciences. MDPI AG; 2019.
2. Nazir M. Prevalence of periodontal disease, its association with systemic diseases and prevention. Int J Health Sci (Qassim). 2017;1(2):72–80.
3. Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, et al. Periodontitis: consensus report of workgroup 2 of the 2017 world workshop on the classification of periodontal and peri-implant diseases and conditions. J Periodontol. 2018 Jun 1;89:S173–82.
4. Trivedi S, Lal N, Mahdi AA, Mittal M, Singh B, Pandey S. Evaluation of antioxidant enzymes cctivity and malondialdehyde levels in patients with chronic periodontitis and diabetes mellitus. Journal of Periodontology. 2014 May;85(5):713–20.
5. Allen EM, Matthews JB, O'Halloran DJ, Griffiths HR, Chapple IL. Oxidative and inflammatory status in type 2 diabetes patients with periodontitis. Journal of Clinical Periodontology. 2011 Oct;38(10):894–901.
6. Sanz M, Ceriello A, Buysschaert M, Chapple I, Demmer RT, Graziani F, et al. Scientific evidence on the links between periodontal diseases and diabetes: consensus report and guidelines of the joint workshop on periodontal diseases and diabetes by the International Diabetes Federation and the European Federation of Periodontology. Journal of Clinical Periodontology. 2018 Feb 1;45(2):138–49.
7. Sun X, Mao Y, Dai P, Li X, Gu W, Wang H, et al. Mitochondrial dysfunction is involved in the aggravation of periodontitis by diabetes. Journal of Clinical Periodontology. 2017 May 1;44(5):463–71.
8. Eke PI, Zhang X, Lu H, Wei L, Thornton-Evans G, Greenlund KJ, et al. Predicting periodontitis at state and local levels in the United States. Journal of Dental Research. 2016 May 1;95(5):515–22.
9. Sanders AE, Sofer T, Wong Q, Kerr KF, Agler C, Shaffer JR, et al. Chronic periodontitis genome-wide association study in the hispanic community health study / study of latinos. Journal of Dental Research. 2017 Jan 1;96(1):64–72.
10. Kassebaum NJ, Bernabé E, Dahiya M, Bhandari B, Murray CJL, Marcenes W. Global burden of severe periodontitis in 1990-2010: a systematic review and meta-regression. Vol. 93, Journal of Dental Research. SAGE Publications Inc.; 2014. p. 1045–53.

11. Mohamed HG, Idris SB, Ahmed MF, Åstrøm AN, Mustafa K, Ibrahim SO, et al. Influence of type 2 diabetes on local production of inflammatory molecules in adults with and without chronic periodontitis: a cross-sectional study. BMC Oral Health. 2015 Jul 27;15(1).

12. Allen E, Matthews J, O'Connor R, O'Halloran D, Chapple I. Periodontitis and type 2 diabetes: is oxidative stress the mechanistic link? Scott Med J. 2009;54(2):41–7.

13. Zhou X, Zhang W, Liu X, Zhang W, Li Y. Interrelationship between diabetes and periodontitis: role of hyperlipidemia. Vol. 60, Archives of Oral Biology. Elsevier Ltd; 2015. p. 667–74.

14. American Diabetes Association. Classification and diagnosis of diabetes: standards of medical care in diabetes-2020. Diabetes Care. 2020 Jan 1;43:S14–31.

15. Patil VS, Patil VP, Gokhale N, Acharya A, Kangokar P. Chronic periodontitis in type 2 diabetes mellitus: oxidative stress as a common factor in periodontal tissue injury. Journal of Clinical and Diagnostic Research. 2016 Apr 1;10(4):BC12–6.

16. Ritchie CS. Mechanistic links between type 2 diabetes and periodontitis. Vol. 37, Journal of Dentistry. Elsevier Ltd; 2009.

17. Sudhakara P, Gupta A, Bhardwaj A, Wilson A. Oral dysbiotic communities and their implications in systemic diseases. Vol. 6, Dentistry Journal. MDPI Multidisciplinary Digital Publishing Institute; 2018.

18. Hartman ML, Shirihai OS, Holbrook M, Xu G, Kocherla M, Shah A, et al. Relation of mitochondrial oxygen consumption in peripheral blood mononuclear cells to vascular function in type 2 diabetes mellitus. Vascular Medicine (United Kingdom). 2014 Feb;19(1):67–74.

19. Masi S, Orlandi M, Parkar M, Bhowruth D, Kingston I, O'Rourke C, et al. Mitochondrial oxidative stress, endothelial function and metabolic control in patients with type II diabetes and periodontitis: a randomised controlled clinical trial. International Journal of Cardiology. 2018 Nov 15;271:263–8.

20. Costa A, Ferreira I, Baptista I, Rego A. Mitochondrial function analysis in diabetes and chronic periodontitis-derived human blood cells: role of resistin. University of Coimbra; 2017.

21. Holmström MH, Iglesias-Gutierrez E, Zierath JR, Garcia-Roves PM. Tissue-specific control of mitochondrial respiration in obesity-related insulin resistance and diabetes. Am J Physiol Endo-crinol Metab [Internet]. 2012;302:731–9. Available from: http://www.ajpendo.org

22. Murphy MP. How mitochondria produce reactive oxygen species. Vol. 417, Biochemical Journal. 2009. p. 1–13.

23. Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature. 2006 Apr 13;440(7086):944–8.

24. Tóthová L, Celec P. Oxidative stress and antioxidants in the diagnosis and therapy of periodontitis. Vol. 8, Frontiers in Physiology. Frontiers Media S.A.; 2017.

25. Li X, Sun X, Zhang X, Mao Y, Ji Y, Shi L, et al. Enhanced oxidative damage and Nrf2 downregulation contribute to the aggravation of periodontitis by diabetes mellitus. Oxidative Medicine and Cellular Longevity. 2018;2018.

26. Monea A, Mezei T, Popsor S, Monea M. Oxidative stress: a link between diabetes mellitus and periodontal disease. International Journal of Endocrinology. 2014;2014.

27. Polak D, Shapira L. An update on the evidence for pathogenic mechanisms that may link periodontitis and diabetes. Journal of Clinical Periodontology. 2018 Feb 1;45(2):150–66.

28. Wang X, Han X, Guo X, Luo X, Wang D. The effect of periodontal treatment on hemoglobin A1c levels of diabetic patients: a systematic review and meta-analysis. PLoS ONE. 2014 Sep 25;9(9).

29. Bascones-Martínez A, Muñoz-Corcuera M, Bascones-Ilundain J. Diabetes and periodontitis: a bidirectional relationship. Vol. 145, Medicina Clinica. Ediciones Doyma, S.L.; 2015. p. 31–5.

30. Nicholas D, Proctor EA, Raval FM, Ip BC, Habib C, Ritou E, et al. Advances in the quantification of mitochondrial function in primary human immune cells through extracellular flux analysis. PLoS ONE. 2017 Feb 1;12(2).

31. Seymour GJ, Berglundh T, Trombelli L. Pathogenesis of gingivitis and periodontitis. In: Berglundh T, Giannobile W v, Lang NP, Sanz M, editors. Lindhe's Clinical Periodontology and Implant Dentistry. Seventh. John Wiley & Sons Ltd; 2022. p. 235–56.

32. Sima C, Aboodi GM, Lakschevitz FS, Sun C, Goldberg MB, Glogauer M. Nuclear factor erythroid 2-related factor 2 down-regulation in oral neutrophils is associated with periodontal oxidative damage and severe chronic periodontitis. American Journal of Pathology. 2016 Jun 1;186(6):1417–26.

33. Jia L, Xiong Y, Zhang W, Ma X, Xu X. Metformin promotes osteogenic differentiation and protects against oxidative stress-induced damage in periodontal ligament stem cells via activation of the Akt/Nrf2 signaling pathway. Experimental Cell Research. 2020 Jan 15;386(2).

34. Yang L, Li X, Jiang A, Li X, Chang W, Chen J, et al. Metformin alleviates lead-induced mitochondrial fragmentation via AMPK/Nrf2 activation in SH-SY5Y cells. Redox Biology. 2020 Sep 1;36.

ATTACHMENTS

Attachment 1. Informed Consent

Attachment 2. Supplemental data

TÍTULO DO PROJETO DE INVESTIGAÇÃO:

Analysis of nrf2-target genes in periodontitis and diabetes subpopulations of human peripheral blood mononuclear cells

PROMOTOR:

Instituto de Periodontologia - FMUC

INVESTIGADOR COORDENADOR/ORIENTADOR:

Isabel Cláudia Masson Poiares Baptista

CENTRO DE ESTUDO CLÍNICO:

Área de Medicina Dentária, Faculdade de Medicina da Universidade de Coimbra

INVESTIGADOR:

Isabel Cláudia Masson Poiares Baptista

MORADA:

Av. Bissaya Barreto, Bloco de Celas, 3000-075 Coimbra

CONTACTO TELEFÓNICO:

967602143

NOME DO PARTICIPANTE:

É convidado(a) a participar voluntariamente neste estudo enquanto participante no grupo de estudo e/ou grupo de controlo. Este estudo vai contribuir para esclarecer a relação entre a diabetes mellitus e a periodontite.

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 as alterações celulares da resposta inflamatória na relação da periodontite 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.

As informações que se seguem destinam-se a esclarecê-lo acerca da natureza, alcance, consequências e risco do estudo, de modo a permitir que, depois de esclarecido, se encontre capaz de decidir participar, ou não, neste estudo.

Caso não tenha qualquer dúvida acerca do mesmo, deverá tomar a decisão de participar ou não. Se não quiser participar não sofrerá qualquer tipo de penalização. 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, que deve guardar.

1. INFORMAÇÃO GERAL E OBJETIVOS DO ESTUDO

Trata-se de um estudo clínico observacional, pelo que não será feita nenhuma alteração na sua medicação ou tratamentos habituais. Este estudo não terá intervenção terapêutica, sendo só necessário recolher 2oml de sangue venoso periférico e líquido gengival.

Este estudo irá decorrer na Área de Medicina Dentária da Faculdade de Medicina da Universidade de Coimbra em colaboração com o Centro de Neurociência e Biologia Molecular da Universidade de Coimbra e o Laboratório de Imunologia da FMUC, tendo como objetivo(s) explorar as alterações da função mitocondrial e do perfil imunoinflamatório de doentes diabéticos e com periodontite.

Desde há alguns anos, têm sido desenvolvidos estudos que relacionam a diabetes mellitus tipo 2 e a periodontite. A periodontite é uma patologia crónica não transmissível, das mais prevalentes no mundo e que implica a perda dos tecidos que sustentam os dentes nos maxilares, devido à presença de microorganismos que se acumulam no contorno gengival. É uma doença ainda sem resolução completa. A periodontite grave é uma das principais causas de perda dentária, comprometimento nutricional, fala alterada, baixa autoestima e pior qualidade de vida geral. Os principais fatores de risco que favorecem o desenvolvimento de periodontite incluem o tabagismo, a obesidade e o sedentarismo.

A diabetes mellitus tipo 2 é um distúrbio metabólico bem conhecido caracterizado por aumento de açucar no sangue e foi recentemente implicada numa relação bidirecional com a periodontite, a qual pode ser considerada uma complicação direta, sendo mais prevalente e grave em pacientes diabéticos. Sendo uma doença cada vez mais frequente na população mundial, reveste-se de grande interesse a investigação sobre as alterações moleculares que possam explicar a relação entre a diabetes com a periodontite. Neste estudo, em específico, serão estudadas algumas alterações das células inflamatórias presentes no sangue e nos tecidos gengivais.

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

2. PLANO E METODOLOGIA DO ESTUDO

A população em estudo irá incluir doentes com idades compreendidas entre os 40 e os 70 anos que apresentem no mínimo 15 dentes em boca. Para além destes critérios, a história clínica médica geral deverá reportar um diagnóstico de Diabetes Mellitus tipo 2 e/ou Periodontite. Não serão incluídos os doentes que apresentem os seguintes critérios: gravidez ou aleitamento; condição médica que impeça um exame oral; HIV ou hepatite (B, C); indivíduos com doenças sistémicas não controladas ou neoplasias; a realizar terapêutica com antibióticos sistémicos, corticosteróides e/ou terapia imunossupressora nos 3 meses anteriores à avaliação periodontal; uso crónico de antiinflamatórios não esteróides; com necessidade de cobertura de antibiótica para procedimentos de medicina dentária; tratamento crónico com medicamentos conhecidos por afetarem a saúde periodontal (fenitoína, ciclosporina); histórico de uso de álcool ou drogas.

Os participantes serão recrutados na população que frequenta as consultas de medicina dentária do Centro Hospitalar da Universidade de Coimbra (CHUC), pelos investigadores da Área de Medicina Dentária; após confirmados os critérios de inclusão e de exclusão, formaliza-se, quer presencialmente quer por via telefónica, um convite aos doentes para participarem no estudo. O recrutamento far-se-á de forma sucessiva, alocando o participante a cada um dos três grupos em estudo (Diabetes tipo 2 sem Periodontite; Periodontite sem Diabetes tipo 2; Diabetes tipo 2 e Periodontite) e o grupo de controlo (sem Diabetes nem Periodontite) até se obter o número previsto de 15 indivíduos por grupo, perfazendo um total de 60 participantes.

Após ter aceite o convite para participar no estudo, cada participante será sujeito a uma atualização da história clínica médica geral e a um exame periodontal (avaliação de parâmetros clínicos como o indíce de placa, profundidade de sondagem e hemorragia à sondagem),

procedimentos realizados por rotina nas consultas de Periodontologia. Para além destes, serão também realizadas colheitas de líquido crevicular e de sangue, específicos para este estudo. A recolha de sangue será efectuada por enfermeiras que respeitarão o manual de boas práticas. A colheita de líquido crevicular não é um procedimento invasivo, nem provoca dor ou desconforto. As amostras recolhidas serão imediatamente transferidas para o Centro de Neurociências e Biologia Celular (CNC) da Universidade de Coimbra e Laboratório de Imunologia da FMUC, para se proceder ao isolamento e processamento laboratorial das células em estudo. A análise dos parâmetros clínicos e bioquímicos será efectuada de acordo com os testes estatísticos mais adequados ao desenho do estudo. Cada participante deve vir às consultas de Medicina Dentária entre uma ou duas vezes. Prevê-se que a totalidade dos procedimentos clínicos devam ser realizados em 60 minutos. Este estudo irá decorrer durante o ano de 2022, sendo que a colheita de dados e amostras (consultas) deverá decorrer no primeiro trimestre do ano.

3. PROTEÇÃO DE DADOS DOS PARTICIPANTES

3.1 Responsável pelos dados

Isabel Poiares Baptista

3.2 Recolha de dados

A recolha de dados é realizada pelo investigador principal, presencialmente ou por via telefónica, e indiretamente, recorrendo ao Processo Clínico Interno e Arquivo de imagens radiográficas intra-orais da Área de Medicina Dentária (FMUC).

3.3 Categorias de dados

Dados Identificativos: Nome, idade e contacto

Dados relativos à saúde: história clínica médica geral, com especial pormenor no caso de doentes diabéticos (tipo, duração da doença desde o diagnóstico, terapêutica) e presença específica de comorbilidades diagnosticadas e as terapêuticas instituídas.

3.4 Tratamento de dados

A partir do momento em que cada participante entre no estudo, ser-lhe-á atribuído um código de participante. Os dados dos doentes serão guardados numa base criada para este estudo, com código de acesso, sendo acessível apenas aos médicos dentistas/médicos da equipa de investigação. A base de dados que permite a identificação do participante com base no seu código será mantida na posse exclusiva do investigador principal, num ficheiro do Promotor, com palavra-chave de acesso. Os dados serão tratados estatisticamente em bloco, sem identificação individual. A divulgação dos resultados do estudo, não permitirá a identificação individual dos participantes.

3.5 Medidas de proteção adotadas

Se os resultados deste estudo forem publicados a identidade dos participantes manter-se-á confidencial.

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.

3.6 Prazo de conservação dos dados

10 anos, findo os quais se procederá ao apagamento e destruição dos registos digitais e físicos.

3.7 Informação em caso de publicação

A sua identidade não será revelada em quaisquer relatórios ou publicações resultantes deste estudo.

4. RISCOS E POTENCIAIS INCONVENIENTES PARA O PARTICIPANTE

O exame periodontal, realizado por um médico dentista, 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, utilizando uma sonda periodontal. Este procedimento não implica quaisquer riscos nem inconvenientes para o doente. Este procedimento demora cerca de 20 minutos, dependendo do número de dentes presentes em boca.

A recolha de 20 ml de sangue será efectuada por enfermeiras treinadas no procedimento e que respeitarão o manual de boas práticas.

A colheita de líquido crevicular não é um procedimento invasivo, nem provoca dor ou desconforto; é efectuada pelo médico dentista, em quatro pontos, selecionados aleatoriamente entre as bolsas periodontais com maior profundidade. Para a recolha, tiras de papel absorvente específicas são inseridas na bolsa periodontal durante 30 segundos. As tiras com marcas de sangue ou saliva são descartadas.

5. POTENCIAIS BENEFÍCIOS

Este estudo permitirá confirmar a relação entre as duas patologias, periodontite e diabetes, através da identificação de alterações serão estudadas algumas alterações das células inflamatórias presentes no sangue e nos tecidos gengivais. Assim, este estudo irá permitir um melhor conhecimento da progressão e complicações associadas às duas doenças. Além disso, a informação que será recolhida irá contribuir para uma melhor informação dos médicos de forma a melhorar os cuidados clínicos a prestar aos doentes com situações idênticas à sua.

6. 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.

7. RESPONSABILIDADE CIVIL

Não há constatação de seguro, uma vez que se trata de um estudo observacional e que não requer deslocações específicas.

8. PARTICIPAÇÃO / RETIRADA DO CONSENTIMENTO

É inteiramente livre de aceitar ou recusar participar neste estudo. Pode retirar o seu consentimento em qualquer altura, através da notificação ao investigador, sem qualquer consequência, sem precisar de explicar as razões, sem qualquer penalização ou perda de benefícios e sem comprometer a sua relação com o investigador que lhe propõe a participação neste estudo.

O consentimento entretanto retirado não abrange os dados recolhidos e tratados até a essa data.

O investigador do estudo pode decidir terminar a sua participação neste estudo se entender que não é do melhor interesse continuar nele. A sua participação pode também terminar se o plano do estudo não estiver a ser cumprido. O investigador notificá-lo-á se surgir uma dessas circunstâncias.

9. CONFIDENCIALIDADE

Será garantido o respeito pelo direito do participante à sua privacidade e à proteção dos seus dados pessoais; devendo ainda ser assegurado que será cumprido o dever de sigilo e de confidencialidade a que se encontra vinculado, conforme disposto no artigo 29.º da Lei n.º 58/2019, de 08/08.

10 – DIREITO DE ACESSO E RETIFICAÇÃO

Pode exercer o direito de acesso, retificação e oposição ao tratamento dos seus dados. Contudo, este direito pode ser sujeito a limitações, de acordo com a Lei.

11. REEMBOLSO E/OU RESSARCIMENTO DO PARTICIPANTE

Este estudo é da iniciativa do investigador e, por isso, solicita-se a sua participação sem uma compensação financeira para a sua colaboração.

12. COMPENSAÇÃO DO CENTRO DE ESTUDO / INVESTIGADOR

O Centro de Estudo não receberá uma compensação financeira pela realização do estudo. Os investigadores não receberão uma compensação financeira pelo seu trabalho na realização deste estudo.

13. CONTACTOS

Se tiver questões sobre este estudo deve contactar:

Investigador	Isabel Cláudia Masson Poiares Baptista
Morada	AV. BISSAYA BARRETO, BLOCO DE CELAS, 3000-075 COIMBRA
Telefone	967602143
Email	icbaptista@fmed.uc.pt

Se tiver dúvidas relativas aos seus direitos como participante deste estudo, poderá contactar:

Presidente da Comissão de Ética da FMUC

Universidade de Coimbra • Faculdade de Medicina

Pólo das Ciências da Saúde • Unidade Central Azinhaga de Santa Comba, Celas

3000-354 COIMBRA • PORTUGAL

Tel.: +351 239 857 708 (Ext. 542708) | Fax: +351 239 823 236

E-mail: comissaoetica@fmed.uc.pt | www.fmed.uc.pt

NÃO ASSINE O 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

Título do Projeto de Investigação

Analysis of nrf2-target genes in PDT and diabetes subpopulations of human peripheral blood monuclear cells

Nome do Participante:	
BI / CC:	**Contactos:**
Nome do Investigador:	

No âmbito da realização do Projeto de Investigação acima mencionado, declaro que tomei conhecimento:

a. do conteúdo informativo anexo a este formulário e aceito, de forma voluntária, participar neste estudo;
b. da natureza, alcance, consequências, potenciais riscos e duração prevista do estudo, assim como do que é esperado da minha parte, enquanto participante;
c. e compreendi as informações e esclarecimentos que me foram dados. Sei que a qualquer momento poderei colocar novas questões ao investigador responsável pelo estudo;
d. que o investigador se compromete a prestar qualquer informação relevante que surja durante o estudo e que possa alterar a minha vontade de continuar a participar;
e. e aceito cumprir o protocolo deste estudo. Comprometo-me ainda a informar o investigador de eventuais alterações do meu estado de saúde que possam ocorrer *(quando aplicável);*
f. e autorizo a utilização e divulgação dos resultados do estudo para fins exclusivamente científicos e permito a divulgação desses resultados às autoridades competentes;
g. que posso exercer o meu direito de retificação e/ou oposição, nos limites da Lei;
h. que sou livre de desistir do estudo a qualquer momento, sem ter de justificar a minha decisão e sem sofrer qualquer penalização. Sei também que os dados recolhidos e tratados até a essa data serão mantidos;
i. que o investigador tem o direito de decidir sobre a minha eventual saída prematura do estudo e se compromete a informar-me do respetivo motivo;
j. que o estudo pode ser interrompido por decisão do investigador, do promotor ou das autoridades reguladoras.

Local e data:	***Assinaturas***
	Participante:
	Representante legal:
	Representante legal:

	Investigador (*):

(*) confirmo que expliquei ao participante acima mencionado a natureza, o alcance e os potenciais riscos do estudo acima mencionado***.***

Attachment 2. Supplemental data

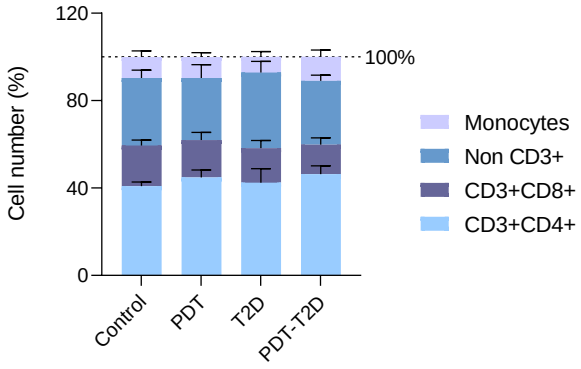

Figure S1 – PBMCs subpopulations. PBMCs derived from control individuals, PDT, T2D and PDT-T2D patients were sorted into CD3+CD4+ lymphocytes, CD3+CD8+ lymphocytes, non CD3+ lymphocytes and monocytes and the number of cells counted by FACS. Data are presented in percentage (%) of cells evaluated as the mean ± SEM from 4 individuals per group.

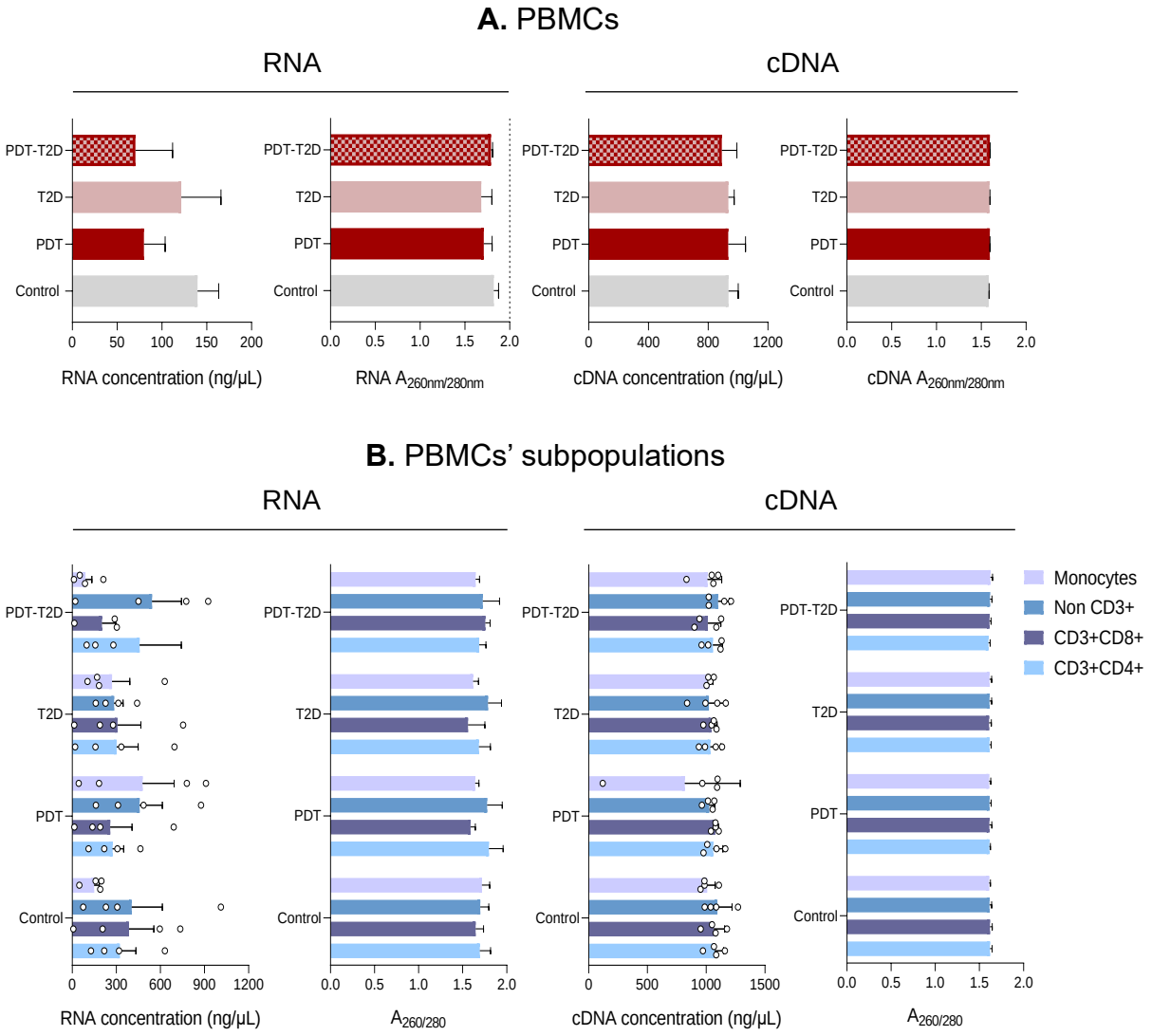

Figure S2 - Characterization of mRNA and cDNA in PBMCs and its subpopulations. Concentration and purity of isolated mRNA and cDNA in PBMCs (A) and in CD3+CD4+ lymphocytes, CD3+CD8+ lymphocytes, non CD3+ lymphocytes and monocytes (B) from control individuals, PDT, T2D and PDT-T2D patients. Data are presented in scatter plots as the mean ± SEM from 4 individuals per group.