FELIPE RICARDO NUNES DE MORAES

Perfil proteômico de bactérias presentes em canais radiculares de dentes com periodontite apical sintomática ou assintomática.

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**UNIVERSIDADE ESTADUAL PAULISTA
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Campus de Araçatuba

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Dissertação apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para obtenção do título de Mestre em Endodontia.

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Resumo Geral

MORAES,FRN **Perfil proteômico de bactérias presentes em canais radiculares de dentes com periodontite apical sintomática ou assintomática.** Dissertação de (Mestrado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2020.

RESUMO

As infecções endodônticas são causadas por uma comunidade multiespécie de bactérias. Com os métodos de identificação microbiológica e quantificação de endotoxinas, é difícil inferir a fisiologia, a função e a patogenicidade microbiana. Assim, a análise proteômica é uma técnica que pode revolucionar o estudo da patogênese das infecções endodônticas. O presente estudo tem por objetivo analisar o perfil proteômico de infecções endodônticas relacionadas a dentes com periodontite apical sintomática ou assintomática utilizando cromatografia líquida associada à espectrometria de Massas. A análise deste perfil proteômico visa a proporcionar a compreensão dos aspectos ecológicos e patogênicos do comportamento de comunidades bacterianas endodônticas através da identificação de proteínas expressas no referido ambiente no momento da coleta e a determinação da função dessas proteínas. Foram coletadas amostras de 18 pacientes encaminhados para tratamento de canal radicular ou tratamento de emergência na Faculdade de Odontologia de Araçatuba FOA – UNESP. Foram incluídos dentes com infecção endodôntica primária, sintomáticos ou assintomáticos. A identificação dos peptídeos foi feita num sistema nanoACQUITY UPLC-Xevo QTof MS system (Waters), a identificação das proteínas foi obtida utilizando o software Protein Lynx Global Server (PLGS) versão 3.0, utilizando o banco de dados de proteínas UniProtKB. A diferença de expressão entre os grupos foi obtida através do mesmo software, considerando-se $p<0,05$ para as proteínas subreguladas e $1-p>0,95$ para as proteínas suprareguladas. Foram identificados um total de 2181 números de acessos entre fragmentos, isoformas e proteínas completas humana e 51 proteínas bacterianas e ambas foram classificadas quanto a sua função biológica, em relação às proteínas exclusivas de cada grupo, 347 proteínas foram identificadas no grupo sintomático. As funções biológicas mais prevalentes foram comunicação celular e transdução de sinais, seguidas pela resposta imune, observou-se diversas proteínas exclusivamente

expressas no grupo sintomático, indicando a influência direta da periodontite sintomática na resposta do hospedeiro.

Palavras-chave: endodontia, bactérias, proteômica.

Moraes,FRN **Proteomic profile of bacteria present in root canals of teeth with symptomatic or asymptomatic apical periodontitis.** 2020**.** Dissertation (Masters degree) - School of Dentistry, Paulista State University, Araçatuba, 2020.

ABSTRACT

Endodontic infections are caused by a multispecies community of bacteria. With microbiological identification methods and endotoxin quantification, it is difficult to infer physiology, function and microbial pathogenicity. Thus, proteomic analysis is a technique that can revolutionize the study of the pathogenesis of endodontic infections. The present study aims to analyze the proteomic profile of endodontic infections related to teeth with symptomatic or asymptomatic apical periodontitis using liquid chromatography associated with mass spectrometry. The analysis of this proteomic profile aims to provide an understanding of the ecological and pathogenic aspects of the behavior of endodontic bacterial communities by identifying proteins expressed in the said environment at the time of collection and determining the function of these proteins. Samples were collected from 18 patients referred for root canal treatment or emergency treatment at the School of Dentistry of Araçatuba FOA - UNESP. Teeth with primary endodontic infection, symptomatic or asymptomatic were included. The identification of peptides was made in a nanoACQUITY UPLC-Xevo QTof MS system (Waters) system, the identification of proteins was obtained using protein lynx global server (PLGS) software version 3.0, using the UniProtKB protein database. The difference in expression between the groups was obtained through the same software, considering $p < 0.05$ for subregulated proteins and $1-p > 0.95$ for the superregulated proteins. A total of 2,181 access numbers were identified between human fragments, isoforms and complete proteins and 51 bacterial proteins, and both were classified as their biological function, in relation to the exclusive proteins of each group, 347 proteins were identified in the symptomatic group. The most prevalent biological functions were cellular communication and signal transduction, followed by the immune response, and several proteins were observed exclusively expressed in the symptomatic group, indicating the direct influence of symptomatic periodontitis on the host response.

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Sumário

Introduction

Apical periodontitis (AP) is a consequence of endodontic infections, which is caused by multispecies microbial communities organized as biofilms attached to the root canal walls [1–3]. Root canals of teeth with symptomatic AP

tend to harbor a larger number of bacteria and a more complex anaerobic bacterial community than asymptomatic AP [1,4,5]. In addition, spontaneous pain may be the result of an increase in the virulence of microorganisms in the root canal [4,6]. Another relevant association in symptomatic cases is the presence of viruses in endodontic infection, present in the most severe forms of AP [7,8].

Clinical symptoms have been associated with increased pathogenicity of the endodontic infection as a consequence of complex interactions with the host, resulting in an increased inflammatory response and oxidative damage [9,10]. Within this context, proteomic techniques provide relevant data on the host's response to specific clinical situations, allowing a broad view of the host's physiology and strategies to fight endodontic infections, through descriptive and quantitative analysis regarding protein expression [11].

The identification of human proteins expressed in endodontic infections was addressed for the first time by Provenzano et al. [12] in samples collected from teeth with asymptomatic apical periodontitis and acute apical abscesses, describing the host behavior and potential biomarkers in the activity of endodontic diseases. Qualitative analysis was employed in subsequent studies that evaluated samples from post-treatment AP infections [13,14] and acute apical abscesses [11,12], and represents the most recurrent analytical proteomic technique in endodontic studies.

More recently, a quantitative approach was used to describe the proteomic changes in the progression of endodontic pathogenesis, allowing a more accurate interpretation of the host's inflammatory response by comparing the protein expression of normal, inflamed and necrotic pulp tissues [15]. To date, however, no study has quantitatively compared the protein expression of symptomatic and asymptomatic AP, in order to determine the biological function of these proteins within the complex pathogen-host relationship. Therefore, the present study aimed to quantitatively and qualitatively compare the host`s proteomic profile in samples of symptomatic and

asymptomatic AP using nano Liquid Chromatography Electron Spray Tandem Mass Spectrometry (nLC-ESI-MS/MS).

2 Materials and Methods

2.1 Patient Selection

Samples were obtained from 18 patients referred for root canal treatment or emergency treatment at Araçatuba dental school. The selection of patients considered the medical record, anamnesis, and clinical/radiographic examinations. The study was submitted and approved by the Research Ethics Committee of Araçatuba dental school (Nº 91331518.7.0000.5420), and all patients who agreed to participate in the research signed an informed consent form. The following characteristics were observed for each patient: age, gender, dental and pulp condition, nature of pain, history of previous pain, spontaneous pain, tenderness to percussion, pain on palpation, mobility, presence of fistula and its origin, presence of swelling of the periodontal tissues, and depth of the periodontal pocket. Only teeth with primary endodontic infections of patients of both genders between 18 and 60 years were included. The selected teeth could be upper or lower, belonging to any dental group, as long as they had at least one root canal that allowed the sample collection. The inclusion criteria for the asymptomatic group (n = 9) were the presence of an asymptomatic tooth with necrotic pulp (negative response to cold sensitivity test) and an apical radiolucency regardless of its size. Clinically, the tooth could be completely asymptomatic or present a slight sensitivity to percussion or palpation, while in the symptomatic group (n = 9) the inclusion criteria involved necrotic teeth (negative response to sensitivity cold test) with spontaneous pain and/or pain when biting, eating and tenderness to percussion. Radiographic findings included thickening of the periodontal ligament or presence of an apical radiolucency. The exclusion criteria comprised the use of antibiotics in the past three months, systemic diseases, teeth that could not be isolated, teeth with visibly exposed root canals, fistula, edema in periapical tissues, advanced periodontal disease, incomplete root formation, and history of dental trauma.

2.1 Sample Collection

The collection was conducted according to the protocol previously described by Jacinto et al. [16] and Loureiro et al. [15]. The endodontic clinical procedures were

performed by an endodontist (CL). Initially, the patient's face was decontaminated with Povidone-Iodine (Riodeine, Rioquímica, São José do Rio Preto, SP, Brazil) and local anesthesia was applied to the region of the involved tooth with 2% lidocaine and epinephrine at 1:100,000 (Alphacaine, Nova DFL Industria e Comercio S/A, Curicica, RJ, Brazil). The tooth was isolated with rubber dam to avoid saliva contamination. Following, the crown/rubber dam interface was sealed with a light-cured gingival barrier (TopDam-FGM, Joinville, SC, Brazil). Antisepsis of the operative field was performed with 30% H_2O_2 (Merck KGaA, Darmstadt, Germany) and 2.5% sodium hypochlorite (Rioquímica, São José do Rio Preto, SP, Brazil) for 30 s each. Next, neutralization with 5% sodium thiosulphate solution (Merck KGaA, Darmstadt, Germany) was performed using sterilized cotton swabs [17]. During access cavity preparation, the water inside the equipment was interrupted, and cooling was performed manually with sterile saline solution using sterile high-speed diamond burs 1012HL, 1014HL, or 1016HL (Microdont, Socorro, SP, Brazil). Coronal contaminants (*i.e.*, restorations and carious tissue) were removed, and further disinfection was performed before accessing the pulp chamber.

Complete access to the root canal was performed with a new sterile diamond bur. Disinfection of the operative field was verified through the evaluation of the negative culture of the specimens collected from the crown with a sterilized cotton swab. In teeth with more than one root canal, specimens were collected only from the broadest and the rectilinear canal (minimum initial diameter of file #15 was established, otherwise, the tooth was excluded from the study), whereas the other canals were sealed with Coltosol (Vigodent, Rio de Janeiro, RJ, Brazil). Initially, a sterile K-type file was introduced with minimal instrumentation, without the use of any irrigant to disrupt biofilms of the canal wall; then, three sterile paper points were introduced into the apparent length of the canal determined on diagnostic radiographs and held in place for 60 s each. If the canal was completely dry, a drop of sterile saline was placed before removing the paper point. After the collection, the paper points and tissue samples were stored in sterile, DNA-free and RNA-free cryotubes, which were frozen at -80° C until used for proteomic analysis.

2.3 Sample preparation for proteomic analysis

The protocol of protein extraction was based on a previous study described by Ventura et al. [18] and validated in a study using samples of infected root canals [15]. The paper points were cut, and samples corresponding to each group were pooled in biological triplicate. In the tubes containing the paper points, an extraction solution containing 6 M urea, 2 M thiourea in 50 mM NH_4HCO_3 pH 7.8 was added until the papers were covered. The samples were then vortexed for 10 min at 4 °C, followed by sonication for 5 min and centrifugation at 20,817 × g for 10 min at 4 °C. The supernatant was collected, and this procedure was repeated once. The papers were placed in filter tubes (Corning® Costar® Spin-X® Plastic Centrifuge Tube Filters Sigma-Aldrich, New York, USA) and centrifuged at 20,817 × g for 10 min at 4 °C. The supernatant was collected and added to the previously collected supernatant. After, 50 mM NH_4HCO_3 (volume corresponding to 1.5 × the sample volume) was added to the samples, which were placed in Falcon Amicon Ultra-4 10k tubes (Merck Millipore, Ireland) and centrifuged at 4,500 × g at 4° C to approximately 150 µL. Then, 5 mM dithiothreitol (DTT) was added to the samples and they were incubated at 37° C for 40 min. Afterward, 10 mM iodoacetamide (IAA) was added and the samples were incubated for 30 min in the dark. After the incubations, 100 µL of 50 mM NH_4HCO_3 were added, followed by the tryptic digestion for 14 h at 37 °C by the addition of 2% (w/w) trypsin (Promega, Madison, USA). Subsequently, 5% formic acid was added to stop the action of trypsin and the procedures were performed with the C18 spin column (Thermo Scientific, United States) for desalting and purifying the samples. An aliquot of each sample (1 µL) was removed, and protein quantification was performed by the Bradford method (Bio-Rad Bradford Assays). The remnants were dried to approximately 1 µL in SpeedVac (Thermo Scientific, United States). After drying, the samples were resuspended in 3% acetonitrile and 0.1% formic acid for the application to the nLC-ESI-MS/MS.

2.4 Shotgun label-free quantitative proteomic analysis and protein classification

Peptides identification was performed on a nanoACQUITY UPLC-Xevo QTof MS system (Waters, Manchester, New Hampshire, UK). The nanoACQUITY UPLC was equipped with nanoACQUITY HSS T3, analytical reverse phase column (75 µm× 150 mm, 1.8 µm particle size (Waters, Manchester, New Hampshire, UK). The column was equilibrated with mobile phase A (0.1% formic acid in water). Then, the peptides

were separated with a linear gradient of 7-85% mobile phase B (0.1% formic acid in acetonitrile) for 70 min. at a flow rate of 0.35 μL/min. The column temperature remained at 55 °C. The Xevo G2 Q-TOF mass spectrometer was operated in positive nanoelectrospray ion mode and data were collected using the MSE method in elevated energy (19-45 V), which allows data acquisition of both precursor and fragment ions, in one injection. Source conditions used included capillary voltage, 2.5 kV; sample cone, 30 V; extraction cone, 5.0 V and source temperature at 80 °C. Data acquisition occurred over 70 min. and the scan range was 50–2000 Da. The lock spray, used to ensure accuracy and reproducibility, was run with a [Glu1] fibrinopeptide solution (1 pmol/μL) at a flow rate of 1 μL/min., as a reference ion in positive mode at m/z 785.8427. Protein Lynx Global Server (PLGS) version 3.0 was used to process and search the LC-MSE continuum data. Proteins were identified with the embedded ion accounting algorithm in the software and a search of the *Homo sapiens* database (UniProtKB/Swiss-Prot) downloaded on January 2020 from UniProtKB (http://www.uniprot.org/). For label-free quantitative proteome, three MS raw files of symptomatic and asymptomatic groups were analyzed using the Protein Lynx Global Service (PLGS, v3.0, Waters Co., Manchester, UK) software. All the proteins identified with a score with confidence greater than 95% were included in the quantitative statistical analysis embedded in the PLGS software. Identical peptides from each technical triplicate by sample were grouped based on mass accuracy (<10 ppm) and on time of retention tolerance <0.25 min, using the clustering software embedded in the PLGS. Differences in expression between the symptomatic and asymptomatic groups was calculated using Monte-Carlo algorithm, considering $p < 0.05$ for down-regulated proteins and $1-p > 0.95$ for up-regulated proteins. Proteins expressed with a ratio between 0.5 and 0.95 were disregarded. The identified proteins were classified according to their biological functions using *Homo sapiens* database (UniProtKB/Swiss-Prot).

3 RESULTS

A total of 2,181 accession numbers were identified, comprising fragments, isoforms, and complete proteins. To analyze different human proteins, a clustering was made between complete proteins and protein fragments from the same gene; thus, the repeated proteins identified were removed, resulting in 853 different human proteins.

The exclusive proteins in each group, as well as the expression levels between common proteins and the subcellular location of the proteins identified in all samples are shown in Figure 1. Most of the proteins identified derived from the cytoplasm (29.5%) and the nucleus (23.9%), followed by plasma membrane (15.4%), extracellular matrix (11.4%), and unknown locations (9.0%).

In the quantitative analysis, common proteins to both symptomatic and asymptomatic groups accounted for 143 proteins. The difference in expression when the symptomatic group was compared with the asymptomatic group resulted in 51 up-regulated proteins in the symptomatic group, including *Alpha-1-antitrypsin, Protein S100-A8, Myeloperoxidase, Peroxiredoxin,* and *Lactotransferrin* (Table 1). This group also had 38 down-regulated proteins, comprising *Immunoglobulin, Neutrophil defensin, Pyruvate kinase,* and *Alpha-enolase* (Table 2). Figure 2 presents the interactions between up- and down-regulated common proteins. The most prevalent biological functions among these proteins were immune response at both levels of expression, followed by transport function in the up-regulated proteins, and structural function in the down-regulated proteins (Table 3).

Regarding the exclusive proteins of each group, 347 proteins were identified in the symptomatic group, such as *Dedicator of cytokinesis protein, Intersectin, Prostaglandin, Phospholipase DDHD2,* and *Superoxide dismutase*. The most prevalent biological functions were cell communication and signal transduction, followed by immune response (Table 3). For the asymptomatic group, 363 proteins were identified, including *Azurocidin, C-reactive protein, Collagen alpha, Cathepsin, Heat Shock,* and *Laminin*, with the most prevalent functions related to immune response and structural function (Table 3).

Bacterial proteins

A total of 51 bacterial proteins were found in both groups. Among them we can highlight: 3-dehydroquinate dehydratase, D-aminoacyl-tRNA deacylase, Endoribonuclease, Ribosomal RNA small subunit methyltransferase, tRNA pseudouridine synthase. These proteins were expressed by 32 different species of microorganisms, with a higher incidence of gram-negative (19) and facultative anaerobic (22), among them: Actinomyces sp, Prevotella sp, Bacteroidetes oral sp, Parvimonas sp, Staphylococcus aureus, among others. The most prevalent biological

functions of bacterial proteins were related to catalytic activity, amino acid biosynthesis, protein dephosphorylation, oxidation/reduction process, transcription regulation, intracellular transport, cell division, among others (Table 4).

4 Discussion

The imbalance between endodontic infections and the host's response may result in the onset of symptoms associated with AP. Several studies report changes in the expression of proteins, metalloproteinases and inflammatory cytokines related to endodontic clinical symptoms [19,21]. In this context, proteomic analysis has been used as a valuable tool to identify and quantify protein expression, allowing a better understanding of the bacterial metabolism and the host's immune response under different clinical conditions [11,12,22].

This study analyzed the proteomic profile of endodontic infections of 18 tooth samples with symptomatic and asymptomatic apical periodontitis. The number of patients per group (n=9) allowed the analysis in biological and technical triplicate, aspects necessary to increase the sensitivity of the method. In addition, it represents a reliable sample number for this type of analysis, being superior to several other proteomic studies of endodontic infections [13,22,11]. The high sensitivity of the proteomic analysis method through the nano LC-ESI-MS/MS system allowed the identification of 853 human proteins and 51 bacterial proteins. This method allows protein identification in small amounts of samples, such as samples of endodontic infections obtained through the use of paper cones inside the root canal [23]. It is important to highlight that this is the first among proteomic studies of endodontic infections that uses a control group (asymptomatic) as a reference for comparing the difference in protein expression in relation to the clinical interest group (symptomatic).

The evaluation of protein expression was made through two approaches, qualitative analysis and quantitative analysis. Qualitative analysis is characterized by direct identification and description of proteins, representing the most recurrent type of analysis adopted among proteomic studies of endodontic infections (Alfenas et al., 2017; Francisco et al., 2019). While quantitative analysis allows to interpret more accurately the inflammatory response of the host, since it is possible to compare the

difference of protein expression in a given condition. This methodology was previously validated by a pilot study that qualitatively and quantitatively analyzed human proteins differentially expressed in normal, inflamed and necrotic pulp tissue. In this study, 465 human proteins were identified, classified according to their biological function in order to understand the pathogen-host relationship in the progression of pulp diseases [15]

The methodology proposed in this study was effective in the identification and comparison of human proteins, presenting robust and quantitative data of the groups studied. On the other hand, the identification of bacterial proteins presented two technical limitations, the infeasibility of performing a specific protocol for the protein extraction of bacterial cells and the difficulty in obtaining a complete database of oral bacterial proteins, as already punctuated by Provenzano et al. [13]. Such limitations made it impossible to quantitatively analyze bacterial proteins, however, descriptive analysis of proteins and their biological functions was performed.

Bacterial proteins

Studies have linked the incidence of clinical symptomatology to increased bacterial virulence and complexity of the endodontic microbiome and its products potentially aggressive to periapic tissues [4,3]. The microbial composition of endodontic infection is significantly different in symptomatic and asymptomatic cases [5]. In symptomatic cases, gram-negative species are predominant and their toxic products are present in higher concentrations, this leads to an increase in the pathogenicity of the infection and consequent exacerbated host response [5]. Studies on the bacterial proteomic profile of endodontic infections detected several proteins involved with virulence, cell asion and antibiotic resistance in secondary infections and periapical abscesses [12,13,14]. However, no study addressed the bacterial proteomic profile comparing symptomatic and asymptomatic cases of apical periodontitis.

In this study, the bacterial proteins identified in the total root canal samples were mostly related to two biological processes: metabolism and energy pathways and DNA/RNA regulation and repair. The high incidence of proteins related to the metabolic processes of the cell evidences bacterial cell activity at the time of canal collection. As an example, 3-dehydroquinate dehydratase represents a protein of catalytic activity related to amino acid biosynthesis pathways. While proteins with DNA/RNA regulation

and repair function suggest that bacteria have a maintenance mechanism, surviving possible cellular damage [14].

Human proteins

<u>Immune Response/Inflammation</u>

The host response is activated from bacterial products, in case of gram-negative bacteria, by the presence of lipopolysaccharide (LPS) and in gram-positive, lipothecoic acid (ATL). Bacterial invasion initiates an inflammatory process through the innate immune response with the recruitment of phagocytes and release of pro-inflammatory cytokines, in addition to the activation of the complement system. Bacterial LPS activates adaptive immunity by stimulating TCD8+ and TCD4+ cells with consequent specific antibody production, and at high concentrations stimulate B cells in the nonspecific response. Inflammation mediators stimulate the maturation of osteoclastic cells leading to the resorption of periapical tissue[21].

A large number of host defense proteins involved with the innate and adaptive immune response were identified in this study, corroborating previous studies that analyzed human proteins in cases of asymptomatic apical periodontitis, persistent infection or abscesses [11,12,14]. This means that regardless of the clinical characteristics of periapicopathies, there is intense activity in the defense of the host against bacterial infection. The study of human proteins expressed in the face of symptomatic apical periodontitis is of great importance to identify differences in host response that can lead to the onset of clinical symptomatology.

It was observed that proteins involved with the inflammatory response and acute phase were more identified in the symptomatic group, among them were: Endoribonuclease ZC3H12A, Scavenger receptor cysteine-rich type 1 protein (CD163), Apolipoprotein A-II, cGMP-dependent 3'_5'-cyclic phosphodiesterase. Endoribonuclease ZC3H12A or Regnase-1 suppresses unnecessary inflammatory reactions in unstimulated conditions, playing a key role in regulating inflammatory response in response to interleukin 17 (Tanaka et al., 2019). CD163 is a receptor present in macrophages, its expression is induced by bacteria and results in the

production of pro-inflammatory cytokines, having an immunomodulatory activity related to the maintenance of periapic lesions [25,26].

The symptomatic group had the presence of proteins involved in the positive regulation of aaquidonic acid secretion, 85/88 kDa calcium-independent phospholipase A2 and Prostaglandin E2 receptor EP3 subtype. Clinical symptomatology in apical periodontitis has already been associated with higher levels of PGE2 highlighting its pro-inflammatory and immunomodulatory effect against a bacterial LPS stimulus [27,28].

Virus response

There is a relevant association between symptomatic cases and the presence of viruses in endodontic infections, being Epstein-Barr and human cytomegalovirus the most common species identified [29]. Viruses have a great pathogenic potential in endodontic infections, stimulating the increase in the expression of pro-inflammatory and immunoregulatory cytokines by host cells [30]. Thus, the joint action of bacterial and viral pathogens is associated with the most severe forms of periapical disease, including larger lesions and painful symptoms [7,31]. The present study identified several unique proteins in the symptomatic group, whose biological function was related to the host-virus interaction that may occur due to direct infection of a host cell, replication of the viral genome, or constitution of the viral progeny. *Protein FAM111A, Protein Red, NLR family member X1, 3 variations of Intersectin-1,* and *Intersectin-2* were some of the proteins identified in the symptomatic group, with evidence of interactions between *Intersectin-1* and *Intersectin-2* with the *Latent membrane protein 1* encoded by the Epstein-Barr virus [32]. Besides, the *FAM111A protein* plays a critical role in the host-virus interaction, and small differences in the levels or activity of this protein may affect viral replication. In addition, it has a serine-peptidase domain suggesting that it can act as a specific peptidase [33].

Oxidative Stress/Bone Remodeling

The role of oxidative stress in the pathogenesis and progression of apical periodontitis has been demonstrated, especially when it is associated with pain and bone destruction [34,35]. The main antioxidant reactions involve primary enzymes that act directly on reactive oxygen species, such as peroxidases, peroxiredoxins,

superoxide dismutase, and catalase [36]. In this context, *Superoxide dismutase* protein was exclusively found in the symptomatic group, while *Myeloperoxidase, Peroxiredoxin-1, Peroxiredoxin-2* proteins were over-regulated in this group. These findings indicate that antioxidant proteins are increased in symptomatic cases in response to reactive oxygen species. Nevertheless, pro-oxidant proteins were found in both groups, including *Arachidonate 5-lipoxygenase, Flavin reductase,* and *Lysine-specific demethylase* in the symptomatic group, and *Nicotinate phosphoribosyl transferase, Sulfite oxidase,* and *Malic enzyme* in the asymptomatic group. A group of proteins involved with oxidative stress was identified exclusively in the asymptomatic group (Heat shock proteins – HSP - including the HSPA1A, HSPA1B, HSPA1L, HSPA2, HSPA4L, HSPA6, and HSPA8 genes). Despite being induced by cell stress during inflammation or infection, HSPs have a protective role in these conditions [37].

Bone remodeling involves resorption and formation mechanisms, which are dependent on the activity of bone cells (osteoclasts, osteoblasts, and osteocytes) in conjunction with cells and defense mediators [38]. Proteins with an important role in bone remodeling activity were unique to the symptomatic group, including Carbonic anhydrase 2, Carbonic anhydrase 12, and Protein-tyrosine kinase 2-beta. Furthermore, Carbonic anhydrase 1, common to both groups, showed higher levels in the symptomatic group. These proteins promote the differentiation and potentiation of the resorptive activity of osteoclasts, in addition to inhibiting the differentiation and activity of osteoprogenitor cells [39]. On the other hand, proteins that act in the differentiation of osteoblasts and organization of the extracellular matrix were identified exclusively in the asymptomatic group. Collagen alpha-1 (I) chain and Collagen alpha-2 (I) chain were the most relevant proteins identified, which are essential in the process of collagen maturation and tissue repair. Overall, structural proteins were quantitatively more expressed in the asymptomatic group, including 8 types of Actins and 2 types of Profilins, indicating greater cytoskeletal structure in this group.

Another mechanism that can be mediated by ROS is the formation of Neutrophil Extracellular Traps (NETs), a complex of extracellular strands of non-condensed DNA, histones, and granular proteins. In this study, all proteins that comprise NETs were identified. In the quantitative analysis, the myeloperoxidase, Lactotransferrin (LTF), 3 types of histones, and the protein S100-A8 (a component of calprotectin) were more expressed in the symptomatic group, while Neutrophil Elastase, Histone H4, and the

protein S100-A9 were less expressed. Defensin-1 was present at similar levels in both groups. Besides, LTF performs antimicrobial functions, inhibiting bacterial oral biofilms due to its bacteriostatic and bactericidal functions [40] and can also influence immune and inflammatory processes by regulating the production of cytokines, such as TNF-alpha. In addition, LTF has anabolic and anti-apoptotic effects on osteoblasts that are differentiating and may inhibit osteoclastogenesis, thus playing a possible role in regulating bone growth [41]. Although NETs have a protective role in the organism, studies have shown that when produced in excess they can stimulate inflammatory and cytotoxic molecules [42]. This chronic inflammatory effect is potentially destructive to tissues and can contribute to the progression of pulp diseases. Thus, NETs can represent new markers and/or therapeutic targets for endodontic pathologies [43].

Proteases and Protease Inhibitors

Proteolytic enzymes or proteases are proteins released by several immune cells during the inflammatory response and participate in biological processes with potential damage to host tissues. In contrast, protease inhibitors act by blocking the catalytic site of proteases, thus limiting their degradative action on the host. The imbalance between these enzymes has a role in the progression of the pathological process [44]. Several exclusive proteins with proteolytic function were found in the symptomatic group, such as *Aminoacylase-1, ABHD14A-ACY1 readthrough, Endothelin-converting enzyme 1, Cytosolic carboxypeptidase 6, A disintegrin* and *metalloproteinase with thrombospondin motifs 4. Endothelin-converting enzyme 1* is a metalloendopeptidase involved in the release of biologically active peptides, specifically in the hydrolysis of bradykinin [45,46], which is one of the main inflammatory mediator in the activation of pain pathways for sensitizing nociceptors [47].

On the other hand, *Alpha-1-antitrypsin* (SERPINE1) and *Alpha-2-macroglobulin* (α2M) - protease-inhibiting proteins - were up-regulated in the symptomatic group. The presence of these protease inhibitors in the pulp tissue and the increase in their levels in inflammation indicate that these proteins play an important role in the pathogenesis of endodontic diseases [48]. SERPINE 1 participates in the acute phase, a body's response to infections, immune reactions or inflammatory processes, protecting tissues against proteolytic enzymes [49]. In addition, it is involved in the healing process as it promotes adhesion, cell migration, and collagen deposition, which are

essential steps for a successful repair of periapical injuries [50]. In turn, α2M has a superior action to SERPINE1 in inhibiting proteases, being effective in inactivating all protease groups. The increase in its concentration is directly related to the severity of inflammatory responses. Thus, the high levels of α2M in the symptomatic group might be related to the increased demand for protease inactivation due to the exacerbated response of the host [15,48].

Finally, *Pyruvate kinase* and *Alpha-enolase* - proteins related to metabolic processes - were down-regulated in both groups. *Pyruvate kinase* is a glycolytic enzyme whose function is to generate energy for the cell and stimulate transcriptional activation, playing a general role in cell apoptosis [51]. *Alpha-enolase*, on the other hand, has varied functions, such as participation in glucose metabolism, cell growth control, allergic responses, and tolerance to hypoxia. It acts on the intravascular and pericellular fibrinolytic system as a receptor and activator of plasminogen on the surface of various types of cells, such as leukocytes and neurons [52], in addition to stimulating the production of immunoglobulin [53].

5 Conclusion

The results found provide quantitative and qualitative data of protein expression in symptomatic apical periodontitis, highlighting the response of the host to the different diagnoses studied. In this study, several proteins were observed exclusively expressed in the symptomatic group, in addition to significant differences in the level of expression between the groups, indicating the direct influence of symptomatic periodontitis on the host response.

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Figure Captions

Figure 1. Venn diagram of exclusive and common human proteins identified in both groups (A); up- and down-regulated common proteins (B) and description of subcellular locations of all proteins (C).

Figure 2. Interactions of common proteins between the symptomatic and asymptomatic groups (STRING database). * The line thickness indicates the strength of the data support. A. Up-regulated proteins. A2M: Alpha-2-macroglobulin; ADA:

Adenosine deaminase; ANXA3: Annexin A3; APOA1: Apolipoprotein A-I; CA1: Carbonic anhydrase 1; DCTN1: Dynactin subunit 1; DES: Desmin; GPI: Glucose-6-phosphate isomerase; HBA2: Hemoglobin subunit alpha 2; HBB: Hemoglobin subunit beta; HBD: Hemoglobin subunit delta; HBE1: Hemoglobin subunit epsilon; HBG1: Hemoglobin subunit gamma 1; HBG2: Hemoglobin subunit gamma-2; HBZ: Hemoglobin subunit zeta; HIST1H2BA: Histone H2B type 1-A; HIST1H2BC: Histone H2B type 1-C; HIST1H2BL: Histone H2B type 1-L; HIST1H2BM: Histone H2B type 1-M; HIST1H2BN: Histone H2B type 1-N; HIST2H2BE: Histone H2B type 2-E; HIST2H2BF:Histone H2B type 2-F; HP: Haptoglobin; LTF: Lactotransferrin; MPO: Myeloperoxidase; NUMA1: Nuclear mitotic apparatus protein 1; PRDX1:Peroxiredoxin-1; PRDX2: Peroxiredoxin-2; PRPH: Peripherin; S100A8: Protein S100-A8; SERPINA1: Alpha-1-antitrypsin; SPG11: Spatacsin; TF: Serotransferrin; TTR: Transthyretin; TBB4A: Tubulin beta-4A chain; VIM: Vimentin; B. Down-regulated proteins. ACTA1: Actin alpha skeletal muscle; ACTA2: Actin aortic smooth muscle; ACTB: Actin cytoplasmic 1; ACTBL2: Beta-actin-like protein 2; ACTC1: Actin alpha cardiac muscle 1; ACTG1: Actin cytoplasmic 2; ACTG2: Actin gamma-enteric smooth muscle; AKAP13: A-kinase anchor protein 13; ALB: Serum albumin; DEFA1: Neutrophil defensin 1; DEFA3: Neutrophil defensin 3; ENO1: Alpha-enolase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HIST1H2BB: Histone H2B type 1-B; HIST1H2BD: Histone H2B type 1-D; HIST1H2BH: Histone H2B type 1-H; HIST1H4F: Histone H4; IGLL5: Immunoglobulin lambda like polypeptide 5; MYH9: Myosin-9; ORM1: Alpha-1-acid glycoprotein 1; PFN1: Profilin-1; PIP: Prolactin induced protein; PKM: Pyruvate kinase; RETN: Resistin; S100A9: Protein S100-A9;

Tables

Table 1. Description and biological function classification of up-regulated human proteins in the symptomatic group in relation to the asymptomatic group.

Access	Description	Biological function	Score	Ratio Symp:Asymp
P53804	E3 ubiquitin-protein ligase TTC3[A]	Catabolic process	56	1
P06744	Glucose-6-phosphate isomerase [A]	Glycolytic process	855	0.96
Q9BXU1	Serine/threonine-protein kinase 31[A]	Catabolic process	315	1
P00813	Adenosine deaminase [B]	Inflammatory response	107	1
P01009	Alpha-1-antitrypsin [B]	Acute phase response	129	1
P12429	Annexin A3 [B]	Inflammatory response	661	0.99
D6RA82	Annexin [B]	Inflammatory response	532	0.98
P00915	Carbonic anhydrase 1 [B]	Interleukin signaling pathway 12	445	1
H0Y300	Haptoglobin [B]	Acute inflammatory response	602	1
Q96A08	Histone H2B type 1-A [B]	Inflammatory response	148	0.99
P62807	Histone H2B type 1-C/E/F/G/I [B]	Response to Gram-positive bacteria	873	0.97
Q16778	Histone H2B type 2-E [B]	Response to Gram-positive bacteria	873	0.98
P01876	Immunoglobulin heavy constant alpha 1 [B]	Adaptive immunity	92	1
P01877	Immunoglobulin heavy constant alpha 2 [B]	Adaptive immunity	1016	1
P01857	Immunoglobulin heavy constant gamma 1[B]	Response to bacteria	407	1
P01860	Immunoglobulin heavy constant gamma 3 [B]	Response to bacteria	169	0.99
E7ER44	Lactotransferrin [B]	Response to bacteria	156	1
P05109	Protein S100-A8 [B]	Inflammatory response	6738	0.98
P02787	Serotransferrin [B]	Response to bacteria	1128	1
P08670	Vimentin [B]	Response to lipopolysaccharide	911	1
P01023	Alpha-2-macroglobulin [B]	Protease inhibitor	89	1
P02647	Apolipoprotein A-I [C]	Transport	175	1
A0A2R8Y7X9	GLOBIN domain-containing protein [C]	Oxygen transport	822	1
G3V1N2	HCG1745306_ isoform CRA_a [C]	Transport	3991	1
P69905	Hemoglobin subunit alpha [C]	Transport	6719	1
P68871	Hemoglobin subunit beta [C]	Transport	14638	1
P02042	Hemoglobin subunit delta [C]	Transport	1324	1
P02100	Hemoglobin subunit epsilon [C]	Transport	822	1
P69891	Hemoglobin subunit gamma-1 [C]	Transport	822	1
P69892	Hemoglobin subunit gamma-2 [C]	Transport	822	1
P02008	Hemoglobin subunit zeta [C]	Transport	134	1
Q9BXS9	Solute carrier family 26 member 6 [C]	Transport	309	0.99
P02766	Transthyretin [C]	Transport	833	1
P17661	Desmin [D]	Cytoskeleton organization	318	0.96
P41219	Peripherin [D]	Cytoskeleton organization	170	1
Q5ST81	Tubulin beta chain [D]	Cytoskeleton organization	771	0.97
P04350	Tubulin beta-4A chain [D]	Cytoskeleton organization	818	0.96

Access	Description	Biological function	Score	Ratio
U3KQK0	Histone H2B [E]	DNA binding	873	1
Q99880	Histone H2B type 1-L [E]	DNA binding	873	0.99
Q99879	Histone H2B type 1-M [E]	DNA binding	873	0.96
Q99877	Histone H2B type 1-N [E]	DNA binding	873	0.99
Q5QNW6	Histone H2B type 2-F [E]	DNA binding	873	0.99
Q8WXA9	Splicing reg glutamine/lysine-rich protein 1 [E]	mRNA processing	65	0.99
B7ZM87	SLIT-ROBO Rho GTPase-activating protein [F]	Signal transduction	82	1
Q14203	Dynactin subunit 1 [G]	Cell division	102	0.99
Q14980	Nuclear mitotic apparatus protein 1 [G]	Cell division	145	0.99
Q96JI7	Spatacsin [I]	Apoptosis	309	1
P05164	Myeloperoxidase [J]	Response to oxidative stress	1718	1
A6NIW5	Peroxiredoxin 2_ isoform CRA_a [J]	Response to oxidative stress	206	1
Q06830	Peroxiredoxin-1 [J]	Response to oxidative stress	539	1
P32119	Peroxiredoxin-2 [J]	Response to oxidative stress	1003	1

Proteins were classified according to the Uniprot database: A- Metabolism and energy pathways; B- Immune response; C- Transport; D- Structural; E - Regulation and repair of DNA/RNA; F- Cellular communication and signal transduction; G- Cell growth and maintenance; H- Differentiation of neural cells; I- Apoptosis; J- Stress response; K- Unknown.

Table 2. Description and classification of down-regulated human proteins in the symptomatic group in relation to the asymptomatic group.

Access	Description	Biological function	Score	Ratio Symp:Asymp
P06733	Alpha-enolase [A]	Glycolytic process	1252	0.01
P62805	Histone H4 [A]	Metabolic process	632	0.01
P02763	Alpha-1-acid glycoprotein 1 [B]	Acute phase response	1415	0
P01859	Immunoglobulin heavy constant gamma 2 [B]	Adaptive immunity	305	0
P01861	Immunoglobulin heavy constant gamma 4 [B]	Adaptive immunity	191	0
P01834	Immunoglobulin kappa constant [B]	Adaptive immunity	784	0
P0CG04	Immunoglobulin lambda constant 1 [B]	Adaptive immunity	1529	0
P0DOY2	Immunoglobulin lambda constant 2 [B]	Response to bacteria	1529	0.03
P0DOY3	Immunoglobulin lambda constant 3 [B]	Response to bacteria	1529	0.03
B9A064	Immunoglobulin lambda-like polypeptide 5 [B]	Response to bacteria	1529	0.01
P59665	Neutrophil defensin 1 [B]	Response to lipopolysaccharide	3061	0
P59666	Neutrophil defensin 3 [B]	Response to lipopolysaccharide	3061	0
P12273	Prolactin-inducible protein [B]	Immune response	1718	0
P06702	Protein S100-A9 [B]	Inflammatory response	6768	0
Q9HD89	Resistin [B]	Degranulation of neutrophils	1127	0
C9JKR2	Albumin_ isoform CRA_k [C]	Transport	6882	0
Q8TCU4	Alstrom syndrome protein 1 [C]	Endosomal transport	46	0
P02768	Serum albumin [C]	Transport	14626	0
P68032	Actin_ alpha cardiac muscle 1 [D]	Structural component	1319	0
P68133	Actin_ alpha skeletal muscle [D]	Structural component	1319	0
P62736	Actin_ aortic smooth muscle [D]	Structural component	1319	0

P60709	Actin_ cytoplasmic 1 [D]	Structural component	2154	0
P63261	Actin_ cytoplasmic 2 [D]	Structural component	2154	0
P63267	Actin_ gamma-enteric smooth muscle [D]	Structural component	1319	0
Q562R1	Beta-actin-like protein 2 [D]	Structural component	173	0
K7EJ44	Profilin [D]	Cytoskeleton organization	786	0
P07737	Profilin-1 [D]	Cytoskeleton organization	157	0
Q9BYX7	Putative beta-actin-like protein 3 [D]	Structural component	82	0
P33778	Histone H2B type 1-B [E]	DNA binding	873	0
P58876	Histone H2B type 1-D [E]	DNA binding	873	0
Q93079	Histone H2B type 1-H [E]	DNA binding	873	0
O94818	Nucleolar protein 4 [E]	Binding to RNA	194	0.03
Q12802	A-kinase anchor protein 13 [F]	Signal transduction	79	0.01
P35579	Myosin-9 [G]	Cell adhesion	82	0.02
P22105	Tenascin-X [G]	Cell adhesion	128	0
B4DNK4	Pyruvate kinase [I]	Programmed cell death	2059	0.02
P04406	Glyceraldehyde-3-phosphate dehydrogenase [J]	Oxidoreductase activity	3029	0
Q5THK1	Protein PRR14L [K]	Unknown	57	0.01

Proteins were classified according to the Uniprot database: A- Metabolism and energy pathways; B- Immune response; C- Transport; D- Structural; E - Regulation and repair of DNA/RNA; F- Cellular communication and signal transduction; G- Cell growth and maintenance; H- Differentiation of neural cells; I- Apoptosis; J- Stress response; K- Unknown.

Table 3. Classification of exclusive and common human proteins between groups according to their biological functions (%).

Biological Function	Exclusive Proteins (%)		Common Proteins (%)	
	Symptomatic	**Asymptomatic**	**Up-regulated**	**Down-regulated**
Metabolism and energy pathways	10.4	12.7	5.9	5.3
Immune response	**14.7**	10.7	**33.3**	**34.2**
Transport	8.9	6.9	**23.5**	7.9
Structure	9.5	**14.0**	7.8	**26.3**
Regulation and repair of DNA/RNA	9.8	11.6	11.8	10.5
Cellular communication and signal transduction	**20.5**	**16.5**	2.0	7.9
Growth and/or cell maintenance	7.8	9.6	5.9	0.0
Differentiation of neural cells	4.3	2.8	0.0	0.0
Apoptosis	4.3	3.6	2.0	2.6
Stress response	4.9	5.2	7.8	2.6
Unknown	4.9	6.3	0.0	2.6

Tabela 4 . Identification of the total number of bacterial proteins and their biological functions
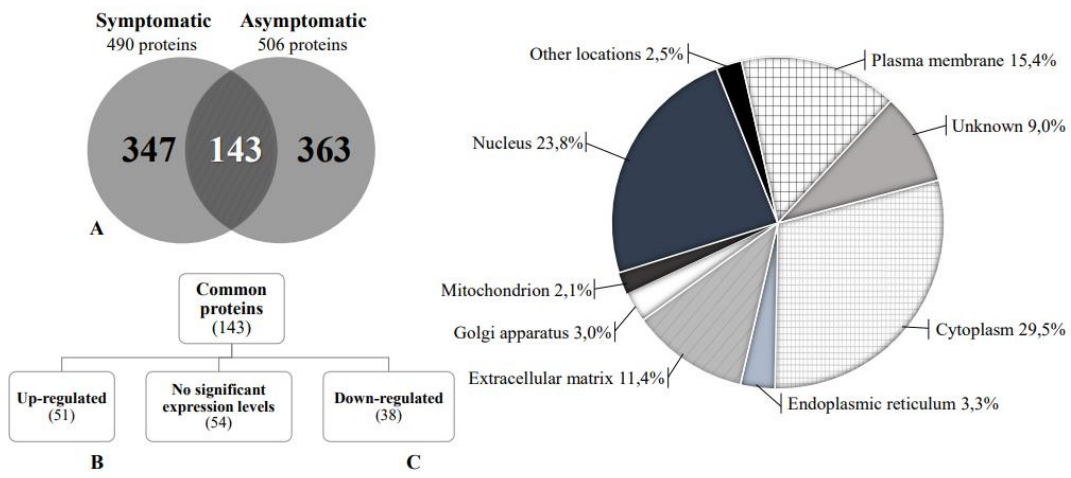
Acess	Description	Biological function	Score

33

Q7V9F9	30S ribosomal protein S6 [A]	Processo metabólico	798
A4G8P1	3-dehydroquinate dehydratase[A]	Biossíntese de aminoácido	911
Q46XJ2	3-methyl-2-oxobutanoate hydroxymethyltransferase[A]	Transferase	556
B9DTB9	D-aminoacyl-tRNA deacylase [A]	Atividade catalítica	656
Q9KBI9	Putative 4-hydroxy-4-methyl-2-oxoglutarate aldolase [A]	Atividade catalítica	925
Q8CTR0	Putative acetyl-CoA C-acetyltransferase VraB [A]	Transferase	114
Q5HRH3	Putative acetyl-CoA C-acetyltransferase VraB [A]	Atividade catalítica	114
Q7VUD2	Urease subunit beta [A]	Atividade catalítica	1440
P00924	Enolase 1 [A]	Processo glicolítico	3827
A0A4Y9FKK4	ATP-binding protein [A]	Ligação ATP	169
A4ST21	Protein-export protein SecB [C]	Transporte	617
E8JIW7	ABC superfamily ATP binding cassette transporter [C]	Transporte	643
A8MJJ8	10 kDa chaperonin [D]	Estrutural	869
B2SG11	Endoribonuclease YbeY [E]	Biogenese de ribossomo	457
Q54087	Leucine-rich protein [E]	Ligação de DNA	499
Q2YVV2	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
Q5HII3	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
A6TYW7	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
A5IQ45	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
Q6GJH8	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
Q6GBZ5	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
A7WYP0	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
Q932G1	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
P66663	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
P66662	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
Q2G0T0	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
A8Z0Y8	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
Q2FJE9	Ribosomal RNA small subunit methyltransferase A [E]	Processamento de rRNA	623
D7JDQ2	Transcriptional regulator_ XRE family [E]	Ligação de DNA	791
A0A4Y9H4S0	tRNA pseudouridine synthase A [E]	Processamento de rRNA	203
A0A2S0LZ40	30S ribosomal protein S15 [E]	Ribonucleoproteína	335
A4TIN3	Putative phosphoenolpyruvate synthase regulatory protein [F]	Desfosforilação de proteínas	471
Q1C752	Putative phosphoenolpyruvate synthase regulatory protein [F]	Desfosforilação de proteínas	471
Q1CII7	Putative phosphoenolpyruvate synthase regulatory protein [F]	Desfosforilação de proteínas	471
Q8ZDY4	Putative phosphoenolpyruvate synthase regulatory protein [F]	Desfosforilação de proteínas	471
Q66A13	Putative phosphoenolpyruvate synthase regulatory protein [F]	Desfosforilação de proteínas	471
B2K5K4	Putative phosphoenolpyruvate synthase regulatory protein [F]	Desfosforilação de proteínas	471
A7FHI3	Putative phosphoenolpyruvate synthase regulatory protein [F]	Desfosforilação de proteínas	471
B1JJ39	Putative phosphoenolpyruvate synthase regulatory protein [F]	Desfosforilação de proteínas	471

P45596	Phosphocarrier protein HPr [F]	Regulação da transcrição	1528
Q043U6	Segregation and condensation protein B [G]	Divisão celular	682
P00330	Alcohol dehydrogenase 1 [J]	Atividade oxidorredutase	3453
Q8E9H2	Protein CyaY [K]	Ligação a metal	807
A0KS62	Protein CyaY [K]	Ligação a metal	807
Q0HN97	Protein CyaY [K]	Ligação a metal	807
Q0HQI9	Protein CyaY [K]	Ligação a metal	807
A7MF54	UPF0145 protein ESA_02470 [K]	Desconhecida	588
A0A2G6FPF4	Uncharacterized protein [K]	Desconhecida	502
C9PWA6	Uncharacterized protein [K]	Desconhecida	486
A0A1B3WTS1	Phage antirepressor protein [K]	Desconhecida	287
F9PSJ9	Ribosomal protein L7/L12 C-terminal-like domain protein [K]	Desconhecida	189

Proteins were classified according to the Uniprot database: A- Metabolism and energy pathways; B- Immune response; C- Transportation; D- Structural; E - REGULATION and repair of DNA/RNA; F- Cellular communication and signal transduction; G- Cell growth and maintenance; H- Differentiation of neural cells; I- Apoptosis; J- Response to oxidative stress; K- Unknown or others.

Figure 1

A

Symptomatic — 490 proteins

Asymptomatic — 506 proteins

347 | 143 | 363

Common proteins (143)

B — Up-regulated (51)

No significant expression levels (54)

C — Down-regulated (38)

Other locations 2,5%

Plasma membrane 15,4%

Nucleus 23,8%

Unknown 9,0%

Mitochondrion 2,1%

Golgi apparatus 3,0%

Cytoplasm 29,5%

Extracellular matrix 11,4%

Endoplasmic reticulum 3,3%

Figure 2

Anexos

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[5] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).

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