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SALIVARY BIOMARKERS OF BONE TURNOVER IN RESPONSE TO BIOMATERIALS

Dissertação apresentada à Universidade Católica Portuguesa
para obtenção do grau de Mestre em Medicina Dentária

Por:
Francisco Gabriel Fernandes Andrade

Viseu, 2024

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Orientador: Professora Doutora Ana Sofia Duarte
Coorientador: Professora Doutora Rita Noites e Professor Doutor Tiago Miguel Marques

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Membros do Júri das Provas Públicas

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Arguente: Professora Doutora Ana Peixoto Gomes

Orientadora: Professora Doutora Ana Sofia Duarte

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Abstract

Introduction: Bone regeneration involves osteoblasts, osteocytes, and osteoclasts, playing a vital role in healing processes. Saliva, a non-invasive sample for PCR, contains biomarkers for monitoring bone turnover markers (BTM), making it a valuable tool for enhancing diagnosis and treatment in periodontics through non-invasive analysis.

Goal: This thesis aims to detect and quantify osteogenic biomarkers in vitro, creating a monitoring panel to validate these biomarkers as reliable indicators of bone remodelling, with the intent to apply this approach to saliva samples.

Materials and Methods: Study Design: Ethical approval was granted in October 2023, the longitudinal study collects data at 0, 3, and 6 months with informed consent.

Inclusion Criteria: Participants include those aged 18+ from the post-graduation course of Implantology at the Faculty of Dental Medicine, Universidade Católica Portuguesa, needing grafts or substitute treatments as identified by orthopantomography. Data collection involved the analysis of orthopantomographic images, administration of questionnaires, and saliva sampling using the passive drooling technique. The relative expression levels of *OPG*, *RANK*, *RANKL,* and *GAPDH* in MC3T3 cells, treated for 7 days with OSSIX® Plus Ossifying Collagen Barrier Membrane, were quantified using the commercial kit NZYSpeedy qPCR Green Master Mix 2x (MB22401), from NZYtech. Additionally, the expression levels of *Alpl*, *Runx2*, *Col1a1*, and *Bglap* genes were quantified in untreated MC3T3 cells.

Results: The study involved 11 patients (6 females, 5 males) average age 51.82 years), revealing reduced radiolucency at treatment sites, indicating bone regeneration. RT-PCR assays from MC3T3 treated cells with collagen membranes confirmed overexpression of *OPG*, *RANK*, *RANKL genes*, while differential expression of *GAPDH* was not detected. *In vitro* gene expression conditions for *Alpl*, *Runx2*, *Col1a1*, and *Bglap* were optimized in untreated MC3T3 cells.

Conclusion: These findings indicate that these genes could serve as salivary biomarkers for non-invasive bone regeneration, warranting further research.

Keywords: Bone regeneration, bone grafts, RT-PCR, gene expression.

Resumo

Introdução: A regeneração óssea, envolve osteoblastos, osteócitos e osteoclastos e desempenha um papel vital nos processos de cura. A saliva, uma amostra não invasiva para RT-PCR, contém biomarcadores para monitorização de marcadores de remodelação óssea, tornando-a numa ferramenta valiosa para melhorar o diagnóstico e tratamento em periodontologia, através de análises não invasivas.

Objetivo: Detetar e quantificar biomarcadores osteogénicos *in vitro*, para criar um painel de monitorização e validar estes biomarcadores como indicadores fiáveis de remodelação óssea, com o intuito de aplicar esta abordagem a amostras de saliva.

Materiais e métodos: O estudo longitudinal, com aprovação ética em outubro de 2023, envolveu a recolha de dados aos 0, 3 e 6 meses, com participantes com 18+ anos do curso de pós-graduação em Implantologia da Universidade Católica Portuguesa, necessitando de enxertos ou tratamentos substitutivos identificados por ortopantomografia. A recolha de dados inclui a análise de imagens ortopantomográficas, questionários e recolha de saliva utilizando a técnica de gotejamento passivo. A análise da expressão génica envolveu a quantificação de *OPG, RANK, RANKL e GAPDH* em células MC3T3 tratadas com a Membrana de Barreira de Colagénio Ossificante OSSIX® Plus usando o o kit comercial NZYSpeedy qPCR Green Master Mix 2x (MB22401), da NZYtech. Adicionalmente, os níveis de expressão de *Alpl, Runx2, Col1a1* e *Bglap* foram quantificados em células MC3T3 não tratadas.

Resultados: O estudo envolveu 11 pacientes (6 mulheres, 5 homens, idade média 51,82 anos), revelando radiolucência reduzida nos locais de tratamento, indicando regeneração óssea. Os ensaios de RT-PCR confirmaram a sobrexpressão de *OPG, RANK e RANKL* em células MC3T3 tratadas, sem deteção de expressão diferencial de *GAPDH*. As condições de expressão génica in vitro para *Alpl, Runx2, Col1a1* e *Bglap* foram otimizadas em células MC3T3 não tratadas.

Conclusões: Estes resultados sugerem o potencial desses genes como biomarcadores salivares para monitorização não invasiva da regeneração óssea, justificando mais pesquisas.

Palavras-Chave: Regeneração óssea, enxertos ósseos, periodontal, RT-PCR, expressão génica.

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1. Introduction

Bone regeneration is the healing of the bone, this regrowth is dependant of the processes that lead to the formation and absorption of bone. Bone can be formed by two distinct routes that rely heavily on three predominant cell types, the osteoblasts, that are derived from osteoprogenitor cells and whose main function is forming and depositing bone. A second cell type is the osteocytes, they serve to form intricate networks connecting each other through various cytoplasmic processes and enabling the transportation and exchange of calcium and phosphorus between the bone. The last cell type is the osteoclasts which is responsible for the bone resorption, enabling the breakdown of the organic matrix composing the bones. The first route to bone formation is the endochondral ossification and the second one is the intramembranous ossification. The latter is of greater importance to this analysis due to its specific role in the formation of the mandible and maxilla (1).

The intramembranous ossification is defined by the differentiation of the progenitor cells directly into osteoblasts which can deposit an organic bone matrix. This matrix will later mineralize into bone being, not only the main way that the bones from the cranium are formed in the embryonic stages, but also, playing a crucial role in bone regeneration, serving as the principle means to primary bone healing (1). This pathway of bone regeneration happens naturally, due to continuous processes of bone remodulation or by injury inflicted by trauma or disease. Even if the body is particularly good at healing itself optimizing these processes is paramount. Effective bone regeneration is crucial for the success of treatments, such as implants, where maximizing bone regeneration is key to achieving the best possible outcome (2).

These optimizations can be achieved by the employment of procedures involving biomaterials, either individually, or in various combinations. Those combinations can be with guided tissue regeneration, growth and differentiation factors, enamel matrix proteins and by the insertion of diverse types of bone grafts or bone substitutes (3). Bone grafts, specifically autogenous grafts, are defined as being the benchmark material, due to its capabilities of either causing

a minute immune response or none, since its origin is from the patient itself. The graft contains cells like osteoblasts as well as osteoprogenitor stem cells that can induce osteogenesis and signalling of various molecules that are helpful to the process of healing, meaning the graft possesses the three optimal properties: osteogenesis, osteoinduction and osteoconduction, hence being the golden standard of biomaterials (1,3).

These treatment options are important for certain conditions, like periodontitis and peri-implantitis, that, in some cases, can affect the oral environment leading to the reduction of bone density, as well as a reduction of the overall bone amount, they can also affect periodontal insertion and tooth-supporting alveolar bone, leading to overall declined oral health (4). It is important to resalt that these intricated diseases are characterized by multiple causative factors and that individuals affected by these conditions typically exhibit one or more associated risk factors (5).

Aggregatibacter actinomycetemcomitans and *Porphyromonas gingivalis* are some of the pathogens found in aggressive periodontitis but low levels of these species can be found in disease-stable sites therefor, the breakdown in specific periodontal sites cannot be solely attributed to bacterial specificity or plaque accumulation and immunopathology. In lieu, an active combined herpesvirus–bacterial infection, as disease-active periodontitis often contains high loads of reactivated herpesviruses, can induce an immunosuppression response. Thus, bacterial overgrowth occurs and potentially can trigger the conversion from gingivitis (disease characterized by the inflammation of only the gingiva, and not the inflammation of all four periodontal tissues like periodontitis) to periodontitis in an individual, or from stable to progressive periodontal disease (5).

Periodontal disease progression may recur following immunosuppressive events or new infections with unrelated herpesviruses. Periodontitis-inactive sites exhibit ongoing inflammation due to the presence of persistent subgingival bacteria (5). The periodontitis and peri-implantitis are ultimately mediated by chronic inflammation and immunosuppression, associated with a microbial and viral dysbiosis and are currently clinically diagnosed with the use of a manual instruments (probes) and radiography’s for assessing periodontal status. This

evaluation includes the measurement of parameters such as bleeding on probing (BOP), plaque index (PI), periodontal pocket depth (PPD), and periodontal bone loss (5,6).

Those pathologies have the potential to harm multiple crucial structures and influence numerous factors related to optimal oral health. Factors like compromised chewing function resulting from tooth loss, the reabsorption of the alveolar bone, and the potential consequences associated with the reduction of the patient's soft tissue aesthetics can be problematic and affect the patient's overall quality of life (4,7). In addressing these challenges, the field of periodontics plays a crucial role, since periodontal treatment focuses on maintaining and restoring the health of the supporting structures around the teeth, (like the alveolar bone, gingiva, root cementum and the periodontal ligament) by employing all the previously mentioned techniques (8).

Periodontitis and peri-implantitis can be more accurately diagnosed by a combination of clinical examination and through the application of advanced testing methods, such as conventional cultures, dark field microscopy, flowcytometry and immunofluorescence, etc. Being the Polymerase Chain Reaction (PCR) approach superior in many ways. It facilitates a more nuanced comprehension of the functioning of these diseases and enhances our understanding of effective treatment strategies, while also permitting the identification of susceptibility to the condition. This technique has multitude of advantages compared to its counterparts such as being able to identify specific specimens that are of clinical interest even if only one copy of the target DNA is present, having simplicity of quantification, doing a fast analysis that has repeatability, having a low contamination probability, having possibility to examinate large samples in one time and because it needs small amounts of genetic material to work. In turn, PCR enables genetic analysis and gene expression studies, making possible, diagnoses of the genetic aspects of the disease in question (9).

The PCR technique works by facilitating the amplification of genes and RNA transcripts obtained from multiple sources. Every PCR test needs the presence of the DNA polymerase enzyme, an extracted DNA sample, primers,

and nucleotides. In the PCR process, the template extracted DNA (or cDNA obtained by reverse transcription from extracted RNA) represents the specific target sequence to be amplified and the DNA polymerase enzyme plays a pivotal role in replicating the target DNA sequences by connecting individual nucleotides, ultimately forming the PCR product. Primer molecules are short, single-stranded DNA or RNA sequences designed to selectively bind to the intended nucleic acid target. Typically, forward and reverse primer pairs consist of 18 to 22 base pairs. To initiate PCR amplification, DNA is extracted from the target, combined with primers, PCR buffer, deoxynucleotides (dNTP), MgCl2, and DNA polymerase enzyme in a reaction mix tube. The reaction tube is then inserted into a thermocycler, which executes repetitive cycles of DNA replication (9).

The PCR reaction encompasses the subsequent stages:

1. Denaturation: Step that heats the reaction tube to 94°C, causing the double-stranded DNA to separate into two single strands.
2. Annealing: At temperatures ranging from 50 to 65°C, forward and reverse primers attach to specific sites on each single-stranded DNA template. The annealing temperature is determined by the melting temperature (Tm) of the primer pairs.
3. Extension or Elongation: At 68-72°C, new complementary DNA strands are synthesized through the elongation of primers using the DNA polymerase enzyme.

Frequently, the procedure is repeated 30-40 cycles, resulting in the generation of approximately $1*(2^{30})$ molecules of the desired PCR product within the reaction tube assuming the process was started from only 1 copy of DNA (9).

Different methods are available for assessing the amplified PCR product. The standard approach involves identifying the product based on its size after undergoing electrophoresis on either an agarose gel or a polyacrylamide gel. These gels are then stained with substances like SYBR Gold, SYBR Green, or ethidium bromide. Upon exposure to ultraviolet light, the PCR products are illuminated, and the amplified DNA sequence can be visualized as a distinct band, the size of which corresponds to the specific DNA fragment that has been

amplified. Depending on the objective, several types of PCR procedures can be employed. The Reverse Transcription PCR (RT-PCR) is the prime procedure for the purposes of this thesis, since it enables the detection of RNA expression by converting RNA molecules into cDNA which can then be used as templates for a PCR test (9,10).

PCR tests are adept at discerning several pathogens associated with periodontitis and peri-implantitis. These tests go beyond mere pathogen identification, extending their capabilities to include the detection and quantification of immune and inflammatory markers by RT-qPCR. These markers encompass microbial antigens, extracellular matrix proteins, and cytokines, shedding light on the intricate molecular landscape of these conditions. Furthermore, PCR facilitates the identification of microbial perio-pathogens and the assessment of viral prevalence, with a specific focus on viruses like the herpes simplex virus. Beyond pathogen identification, PCR also enables comprehensive gene expression studies, providing a holistic approach to understanding the underlying molecular mechanisms in periodontitis and peri-implantitis diseases as well as the bone loss that accompanies these conditions (5,10).

One medium that shows promise for usage with PCR is the saliva, this material has various ways of storage and a multitude of standardized protocols for its safe collection(11). It has also been demonstrated that regarding several markers, such as IL-6, an inflammatory marker, there is correlation between the saliva concentration of the marker and the blood concentration, and the same can be said in relation to certain DNA methylation markers, both at the gene specific and in a global(hydroxyl)methylation tier. Moreover, there are plethora of other molecules, pathogens, proteins and other biomarkers that can be found in saliva such as the ones encompassed in the standard PCR testing (6,11,12).

Genes related to bone regeneration and inflammatory responses expression can also be quantified through saliva and PCR testing(13). Bone turnover markers (BTM) can, through non-invasive methods, also gauge the estimation of bone resorption and formation since many different types of proteins and biomarkers are produced during the remodelling of bone by the cells

responsible (osteoblasts, osteoclasts). More specifically, markers like the N-terminal collagen extension propeptide type I (PINP), the osteocalcin, and the bone alkaline phosphatase can serve as markers, indicating the progression of bone formation. While for assessing bone resorption the crosslinking C-terminal collagen telopeptide type I (bCTX-I), N-terminal telopeptide of collagen type I (NTX), and the tartrate-resistant acid phosphatase isoform 5b (TRAP5b) can be used as indicators (14).

The PCR that uses saliva can be used to assess the effectiveness and the mechanisms behind the methods used to regenerate bone, for example, in the use of biomaterials like grafts. By the analysis of specific pro-inflammatory and regulatory genes present in the saliva, it's possible to determine their genetic expression as well as learning clues into the mechanisms that enable the regeneration of the bone due to the treatments being employed (such as grafts) and also helping in the creation of a base genetic expression profile that could serve as a means for diagnostic and evaluation of patient status (14).

In this sense the objectives of this research, is to not only detect and quantify the chosen osteogenic biomarkers in saliva but also to establish a robust monitoring panel. The goal is to validate these biomarkers as reliable indicators of salivary bone remodelling. By delving into the intricate details of salivary composition, this study aims to contribute valuable insights that can enhance the effectiveness of monitoring and pave the way for advancements in the field of bone regeneration.

2. Materials and Methods

Study Design

The study gained the approval of the ethical committee in October 2023 (Attachment 1). It was designed as a longitudinal study, with patient information collected at three distinct moments: initially, then three months later, and finally at the six-month mark. This process was conducted only after obtaining the acceptance and signature of an informed consent from each participant (Attachment 2). The study was scheduled to take place between October 2023 and May/June 2024.

To be included in this study, an individual needed to meet specific criteria. Firstly, the participant had to be a patient enrolled in the post-graduation course of Implantology at the Faculty of Dental Medicine, Universidade Católica Portuguesa. Additionally, the participant had to be 18 years of age or older and could be of any gender. Another requirement was having an orthopantomography in the Romexis system (Romexis version: 5.1.0.R/ 23/03/2018 build 1411) of the university. Finally, the participant needed to have a bone-related problem that could potentially be improved by the application of a bone graft or a bone substitute, such as a collagen membrane.

Diagnostics

The mouth is divided into four quadrants, with Quadrant 1 (upper right) having teeth numbered from the Central Incisor (11) to the Third Molar (18), while Quadrant 2 (upper left) follows with teeth numbered from the Central Incisor (21) to the Third Molar (28), and in the lower jaw, Quadrant 3 (lower left) includes teeth numbered from the Central Incisor (31) to the Third Molar (38), while Quadrant 4 (lower right) consists of teeth numbered from the Central Incisor (41) to the Third Molar (48) **(Attachment – 5)**. This system allows for precise identification of each tooth based on its position. (15)

Initial screening of patients was performed using the Basic Periodontal Examination (BPE), a rapid screening tool designed to identify patients requiring further diagnostic assessment. The BPE involved "walking" a probe around each

tooth and recording the highest score (ranging from 0 to 4) in each sextant. Scores of 3 or 4 indicated the need for a more detailed periodontal assessment, including radiographic examination to determine bone loss.

For patients with a BPE score of 3 or 4, a detailed periodontal examination was conducted. This included collecting comprehensive medical and dental histories to identify potential risk factors and predisposing conditions, performing a detailed intraoral examination to visually assess periodontal health, taking radiographs to assess the extent of bone loss, and conducting full mouth pocket depth charting to measure probing pocket depths (PPD) and clinical attachment loss (CAL).

The diagnosis and classification of periodontal disease involved several steps. First, patients were categorized into one of the following types based on clinical findings: periodontal health (no signs of inflammation or periodontitis), gingivitis (inflammation without loss of attachment), periodontitis (loss of attachment and bone), or other conditions (including necrotizing periodontal diseases and periodontitis as a manifestation of systemic disease). (16)

Next, the extent of periodontal involvement was determined as localized (≤ 30% of teeth affected), generalized (> 30% of teeth affected), or following a molar/incisor pattern (specific pattern affecting molars and incisors). The severity of periodontitis was then staged based on interdental CAL and bone loss: Stage I (initial) with interdental CAL 1-2 mm and bone loss in the coronal third (<15%) with no tooth loss due to periodontitis; Stage II (moderate) with interdental CAL 3-4 mm and bone loss in the coronal third (15-33%) with no tooth loss due to periodontitis; Stage III (severe) with interdental CAL ≥5 mm and bone loss extending to the middle third of the root and beyond with ≤4 teeth lost due to periodontitis; and Stage IV (advanced) with interdental CAL ≥5 mm and bone loss extending to the middle third of the root and beyond with ≥5 teeth lost due to periodontitis. (Attachment 4)

The rate of disease progression and risk factors were assessed to assign a grade: Grade A (slow) with no loss over 5 years, <0.25% bone loss/age, and heavy biofilm deposits with low levels of destruction; Grade B (moderate) with <2

mm loss over 5 years, 0.25-1.0% bone loss/age, and destruction commensurate with biofilm deposits; and Grade C (rapid) with ≥2 mm loss over 5 years, >1.0% bone loss/age, and destruction exceeding expectations given biofilm deposits and risk factors such as smoking or diabetes. (Attachment 3)

Finally, the current health status of the periodontium was determined based on BOP and PPD: periodontal health (no BOP, PPD ≤ 3 mm, no CAL), gingival inflammation in remission (BOP ≥ 10%, PPD ≤ 4 mm, no CAL), periodontitis currently stable (BOP < 10%, PPD ≤ 4 mm, no BOP at 4 mm sites), and periodontitis currently unstable (PPD ≥ 5 mm or PPD 4 mm with BOP). (Attachment 4)

A comprehensive diagnostic statement was formulated for each patient, incorporating the type, extent, stage, grade, and current status of the periodontal disease, along with relevant risk factors. (16)

Clinical Procedure

The protocol for surgical intervention was carried out using the following materials: 2% lidocaine with 1:200000 epinephrine, surgical instruments including a 12D blade scalpel, periosteal elevator, Gracey curettes, and titanium curette, Bio-Oss® (deproteinized bovine-derived xenograft) as graft material, Bio-Gide® (bioabsorbable collagen membrane) for the collagen membrane, 3-0 silk sutures, sterile saline, and chlorhexidine digluconate solution (0.2%). (17)

In the pre-surgical preparation, informed consent was obtained from the patient, oral hygiene instructions were provided, and prophylactic antibiotics were administered if indicated. A comprehensive periodontal examination was conducted, and radiographs (Orthopantomography and Intra-oral periapical radiographs) were taken.

During the surgical procedure, local anaesthesia was administered using a 2% lidocaine with 1:200000 epinephrine alveolar nerve block. An intracrevicular incision was made, and a full thickness mucoperiosteal flap was raised using a

periosteal elevator to expose the underlying bone. All granulation tissue was thoroughly removed from the defect, and scaling and root planning were performed on the exposed root surfaces using Gracey curettes.

The Bio-Oss® graft material was moistened in sterile saline before placement, then used to fill the intrabony defect. The Bio-Gide® collagen membrane a material with the same properties as OSSIX® Plus Ossifying Collagen Barrier Membrane, that was used for the gene expression study of these thesis, was cut to fit the defect morphology using a template and placed and adapted over the entire defect, ensuring it covered 2-3 mm of the surrounding alveolar bone. The mucoperiosteal flaps were repositioned coronally to prevent membrane exposure and secured using interrupted 3-0 silk sutures from the buccal to the lingual surface.

In the post-surgical care, the patient was instructed to avoid brushing the surgical area, rinse with 0.2% chlorhexidine solution twice a day for two weeks and apply ice intermittently on the first day to reduce swelling. A follow-up visit was scheduled after one week for suture removal, with continued monitoring and cleaning of the surgical site with chlorhexidine at two weeks. Supragingival scaling was performed every month for the first six months.

For maintenance, oral hygiene instructions were reinforced at each visit, and probing or subgingival instrumentation was avoided during the first six months post-surgery. (17)

Data recovery

For each individual who consented to participate in the study, the main investigator collected data through several methods.

Initially, the analysis of the orthopantomography was conducted. The health review concerning the dentition and bone density of the subjects was performed through the analysis of the orthopantomography during the first, second, and third visits.

Additionally, participants completed a questionnaire (Attachment 4).

Furthermore, the saliva collection method was employed. Saliva samples were gathered at the onset of the dental appointment, after informing the patient and obtaining their consent for participation in the study. Using the passive drooling method, unstimulated saliva was collected following a straightforward and non-invasive protocol established at SalivaTec (CIIS platform) and conducted in accordance with guidelines approved by the National Health Authorities. Patients were instructed to accumulate saliva in their mouth and discharge it into a collection tube. Following this procedure, the collection tube was sealed, placed on ice, and transported to the SalivaTec laboratory at the Faculty of Dental Medicine at UCP. The gathered saliva samples were divided into aliquots of 200 µL within 1.5 mL tubes, for each patient there where two aliquots, one only containing 200 µL of saliva, while the other in addition to the saliva also contained 250 µL RNALater, which were then preserved at -80 °C until the time of analysis.

Gene expression Analysis

MC3T3 Cell Culture

The MC3T3 cells were cultivated in Minimum Essential Medium Eagle (MEM), which was enriched with 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic (A/A), and 1% glutamine. The cells were maintained at a temperature of 37°C in a humidified atmosphere containing 5.5% CO2. When the cells had grown to confluence, the medium was carefully removed, and the cells were gently washed with PBS. To detach the cells, trypsin-EDTA was added, and the flask was incubated for 7 minutes. After this period, medium was added to halt the trypsin's action. The cells were then counted and seeded into 6-well plates, with each well receiving 1.5 x 10^5 cells.

RNA extraction

After obtaining the cell pellet, Buffer NR was added and vortexed vigorously to ensure thorough mixing. The lysate was applied to an NZYSpin Homogenization column placed in a 2 mL collection tube. The column was then centrifuged at 11,000g for 1 minute. The flow-through was transferred to a new 1.5 mL microcentrifuge tube. A volume of 350 µL of 70% ethanol was added to the flow-through and mixed immediately by pipetting up and down. The mixture was loaded onto an NZYSpin Binding column, centrifuged at 11,000g for 30 seconds, and the flow-through was discarded. The column was placed in a new microcentrifuge tube. Buffer NI (350 µL) was added to the column, centrifuged at 11,000g for 30 seconds, and the flow-through was discarded.

The Digestion Mix was prepared by combining 10 µL of DNase I with 90 µL of Digestion Buffer. Digestion Mix (95 µL) was applied directly to the centre of the silica membrane of the column and incubated for 15 minutes at room temperature. Then, 200 µL of Buffer NWR1 was added to the column, centrifuged at 11,000g for 1 minute, and the flow-through was discarded.

Next, 600 µL of Buffer NWR2 was added to the column, centrifuged at 11,000g for 1 minute, and the flow-through was discarded. This step was repeated with 250 µL of Buffer NWR2, centrifuging at 11,000g for 1 minute. The flow-through was discarded again to ensure the column membrane was dry. The NZYSpin Binding column was placed in a new RNase-free microcentrifuge tube. 60 µL of RNase-free water was added directly to the column membrane, and the column was centrifuged at 11,000g for 1 minute to elute the RNA.

The RNA concentration was measured using 2 µL of the sample on a microdrop plate. RNase-free water was used as a control in the first well of the microdrop plate to ensure a reading of 0 RNA concentration. The RNA sample was placed in the second well to determine the concentration and purity ratios (18).

cDNA synthesis

All reaction components and the RNA sample were kept on ice to prevent RNA degradation. In a microcentrifuge tube, the following components were

combined: 10 µL of NZYRT 2x Master Mix, 2 µL of NZYRT Enzyme Mix, the RNA sample (volume as required), and DEPC-treated water to make up the final volume. The NZYRT 2x Master Mix contained oligo(dT)18, hexamers, $MgCl_2$, and dNTPs. The oligo(dT) primer bound to the Poly(A) tail of the mRNA, and the hexamers increased the sensitivity of the reaction. The NZYRT Enzyme Mix included NZY Reverse Transcriptase and NZY Ribonuclease Inhibitor. The reverse transcriptase initiated the synthesis of the cDNA strand, while the ribonuclease inhibitor protected the RNA templates from degradation.

The reaction components were gently mixed and incubated at 25°C for 10 minutes to allow the oligo(dT) primers to bind to the RNA template. The mixture was then incubated at 50°C for 30 minutes to promote the activity of the reverse transcriptase and facilitate cDNA synthesis from the RNA template. Following this, the mixture was incubated at 85°C for 5 minutes to inactivate the reverse transcriptase.

Afterwards, 1 µL of RNase H was added to the mixture, which was then incubated at 37°C for 20 minutes to remove the RNA bound to the cDNA, ensuring RNA-free DNA for subsequent RT-qPCR reactions. The synthesized cDNA product was stored at -20°C until further use.(18)

Gradient PCR for primer testing

Twelve cDNA samples converted from mRNA of MC3T3 cells, and twelve cDNA samples converted from non-stimulated saliva mRNA were prepared for the PCR reactions. In a PCR tube, the reaction mixture was prepared by adding the following components in the given order: 5 µL of NZYSpeedy qPCR Green Master Mix (2x), 0.4 µL of Forward primer, 0.4 µL of Reverse primer, 2.2 µL of PCR water, and 2 µL of diluted cDNA.

The gradient PCR machine was set to the following thermal cycling conditions: initial denaturation at 95ºC for 2 minutes, followed by 40 cycles of denaturation at 95ºC for 5 seconds, and annealing at gradient temperatures (69ºC, 65.4ºC, and 59ºC) for 20 seconds. The genes tested included *RANKL, RANK, OPG, RunX2, ALPL, Col1A1, Bglap* and *GAPDH*. After the PCR cycles, the amplification results were analysed to determine the optimal annealing

temperature for each primer set based on the quality and specificity of the amplified products.

3. Results

Sample characterization

A total of 11 patients from the Faculty of Dental Medicine of Viseu participated in the study. Of the 11 individuals, 6 (55%) are female and 5 (45%) are male (**Figure 1**).

Table 1 presents the age and gender distribution of the study participants, categorized into five distinct age groups: 18-30, 31-41, 42-52, 53-63, and 64-74 years. It includes the frequency of participants within each age group, along with the corresponding gender breakdown. The age group 42-52 years has the highest representation, constituting 27% of the total participants, with 67% males and 33% females. Both the age groups 18-30 years and 64-74 years represent 9% of the total participants each. The 18-30 group is exclusively female, while the 64-74 group has a balanced gender distribution. The age groups 31-41 years and 53-63 years each account for 18% of the participants. The 31-41 age group is composed entirely of females, whereas the 53-63 age group has an equal distribution between males and females.

Table 1- Age and Gender Distribution of Study Participants

Age Groups	**Frequency**	**Gender**		**Age Percentage by Age Group**	**Gender Percentage by Age Group**
18 to 30	1	Male	0	9%	0%
		Female	1		100%
31 to 41	2	Male	0	18%	0%
		Female	2		100%
42 to 52	3	Male	2	27%	67%
		Female	1		33%
53 to 63	2	Male	1	18%	50%
		Female	1		50%
64 to 74	3	Male	2	27%	67%
		Female	1		33%

Table 2 illustrates the prevalence of hypertension among the study participants, broken down by gender. Among the participants, 4 individuals (36%) reported having hypertension, while 7 individuals (64%) did not have hypertension. Among those with hypertension, 2 were female and 2 were male. This represents 18% of the total participant population for each gender.

Overall, 36% of the participants reported having hypertension. The gender distribution shows that both males and females are equally represented among participants with hypertension, each accounting for 18% of the total participants. This indicates that the prevalence of hypertension is equally distributed between males and females, with no significant gender disparity.

Table 2 - Hypertension Prevalence Among Study Participants and Correlation with Gender

Question	**Answer**		**Gender**		**Hypertension percentage**	**Patients with hypertension gender percentage**
Do you have hypertension?	Yes	4	Female	2	36%	18%
	No	7	Male	2	64%	18%

Among the participants represented in **Table 3**, 1 individual (9%) reported having diabetes, while 10 individuals (91%) did not have diabetes. The participant with diabetes was male, representing 9% of the total participant population.

Overall, 9% of the participants reported having diabetes. The gender distribution shows that diabetes was only present in a male participant, accounting for 100% of those who reported having diabetes. There were no female participants with diabetes, highlighting a male-specific occurrence of diabetes. Among the participants who did not have diabetes, 4 were male (36%) and 6 were female (55%).

Table 3 - Diabetes Prevalence Among Study Participants by Gender

Question	Answer		Diabetes percentage	Gender		Patients with diabetes gender percentage
Do you have Diabetes?	Yes	1	9%	Male	1	9%
				Female	0	0%
	No	10	91%	Male	4	36%
				Female	6	55%

About the participants on **Table 4**, 36% reported having hypertension, while 64% did not. For diabetes as shown in **Table 3**, 9% of the participants reported having the condition, while 91% did not. Heart problems were reported by 9% of the participants, while 91% did not have heart problems. There were no participants with blood diseases or liver diseases, as 100% of the participants responded "No" to these conditions. Stomach diseases were reported by 9% of the participants, while 91% did not have them. Similarly, 9% of the participants reported having intestinal diseases, and 91% did not. Cancer was reported by 9% of the participants, with 91% not having the condition. There were no participants with kidney diseases or allergies, as 100% responded "No" to these conditions.

Other diseases were reported by 27% of the participants, while 73% did not report any other diseases. Additionally, 9% of the participants reported having undergone radiotherapy or chemotherapy treatment, while 91% had not.

Key observations include that hypertension is the most prevalent condition reported, affecting 36% of the participants. Both diabetes and heart problems are present in 9% of the participants. Notably, none of the participants reported having blood or liver diseases. Other diseases were reported by 27% of the participants, making it the second most prevalent category after hypertension.

Furthermore, 9% of participants have undergone radiotherapy or chemotherapy treatments.

Table 4 - Distribution of Pathological Conditions Among Study Participants

Pathology Distribution	**Yes**	**No**	**Yes (%)**	**No (%)**
Do you have hypertension?	4	7	36%	64%
Do you have diabetes?	1	10	9%	91%
Do you have or have you had any of these conditions? - Heart problems	1	10	9%	91%
Do you have or have you had any of these conditions? - Blood diseases	0	11	0%	100%
Do you have or have you had any of these conditions? - Liver diseases	0	11	0%	100%
Do you have or have you had any of these conditions? - Stomach diseases	1	10	9%	91%
Do you have or have you had any of these conditions? - Kidney diseases	0	11	0%	100%
Do you have or have you had any of these conditions? - Intestinal diseases	1	10	9%	91%
Do you have or have you had any of these conditions? - Cancer	1	10	9%	91%
Do you have or have you had any of these conditions? - Allergies	0	11	0%	100%
Do you have or have you had any of these conditions? - Other diseases	3	8	27%	73%
Have you undergone any radiotherapy or chemotherapy treatment?	1	10	9%	91%

Among the 11 patients, diagnoses of other pathologies not covered in the standard questions include 1 (9%) with gout, 1 (9%) with hypothyroidism and migraines, 1 (9%) with a malignant intestinal tumour, and 1 (9%) with hypothyroidism (**Figure 1**).

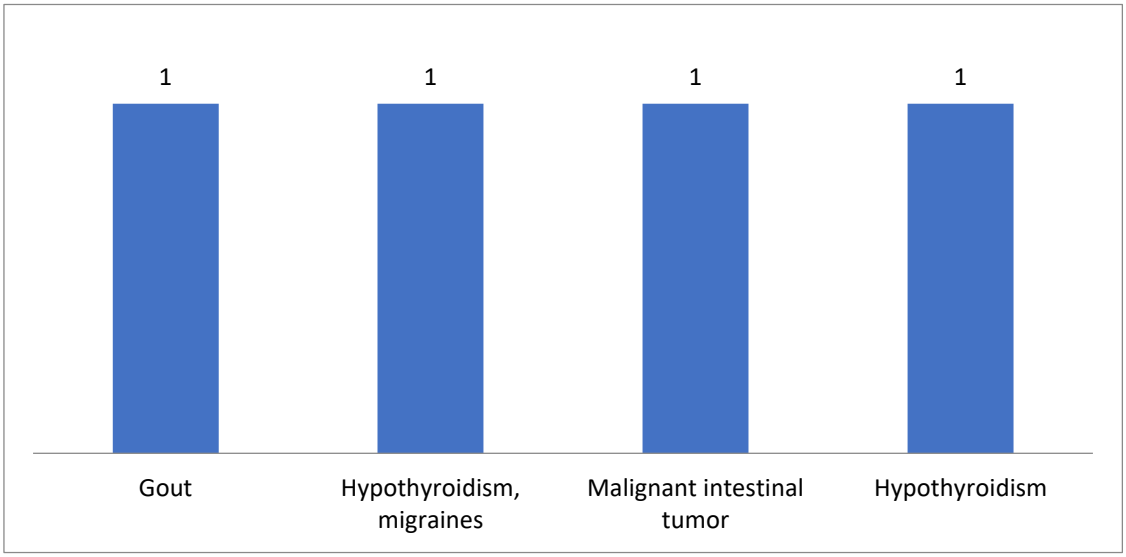

Figure 1 - Distribution of other Pathologies

Family history of diseases among the patients shows that 5 (45%) have no family history of diseases, 2 (18%) have a family history of cancer, 2 (18%) have a family history of heart diseases, 1 (9%) have a family history of diabetes, and 1 (9%) have a family history of both heart diseases and diabetes (**Figure 2**).

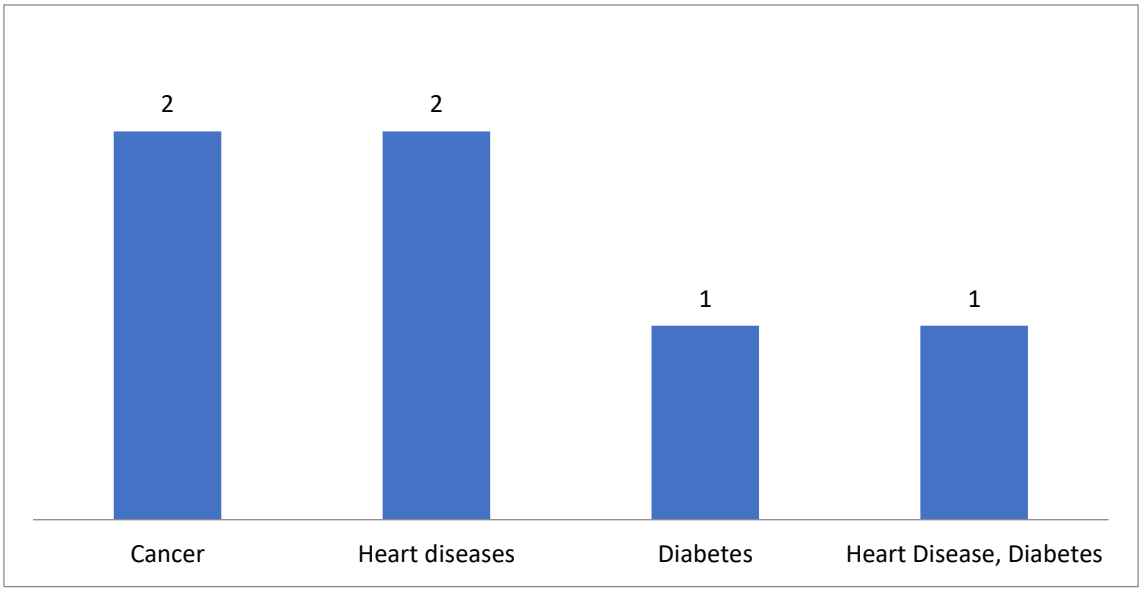

Figure 2 - Distribution of diseases in the family history

Within the group of participants on **Table 5**, 8 individuals (73%) reported undergoing medical treatment or taking medication in the last 30 days, while 3 individuals (27%) did not. Additionally, 4 individuals (36%) reported taking antibiotics in the last 3 months, whereas 7 individuals (64%) did not.

A significant majority of participants (73%) have undergone medical treatment or taken medication recently, indicating a high prevalence of ongoing medical management within the study cohort. Furthermore, over one-third of the participants (36%) have taken antibiotics in the last 3 months, reflecting the use of antibiotics among a notable portion of the cohort. The remaining 64% of participants have not taken antibiotics in this period, suggesting variability in the necessity or prescription of antibiotics among the group.

Table 5 - Medication prevalence among participants

Medication Distribution

Questions	**Response**		**Percentage**
Have you undergone any medical treatment or medication in the last 30 days?	Yes	8	73%
	No	3	27%
Have you taken any antibiotics in the last 3 months?	Yes	4	36%
	No	7	64%

Specified treatments among the patients are as follows: 1 (9%) using simvastatin, Zyloric, 1 (9%) using Euthyrox, Zonegran, 1 (9%) using simvastatin, 1 (9%) using Pentasa suppository, 1 (9%) using tryptophan, cholestyramine, 1 (9%) using simvastatin, Zartan, and 1 (9%) using Euthyrox (**Figure 3**).

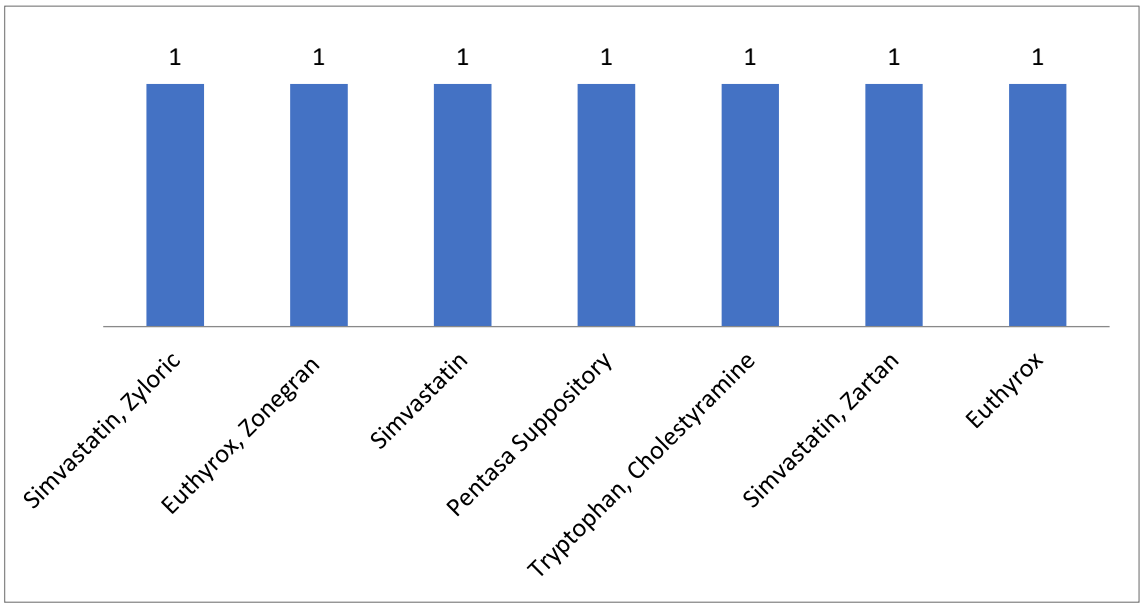

Figure 3 - Specified regular medical treatments or medications

The antibiotics used include 2 (18%) taking amoxicillin 1g, 1 (9%) taking metronidazole 250mg and clavamox 875mg/125mg, and 1 (9%) taking metronidazole 250mg (**Figure 4**).

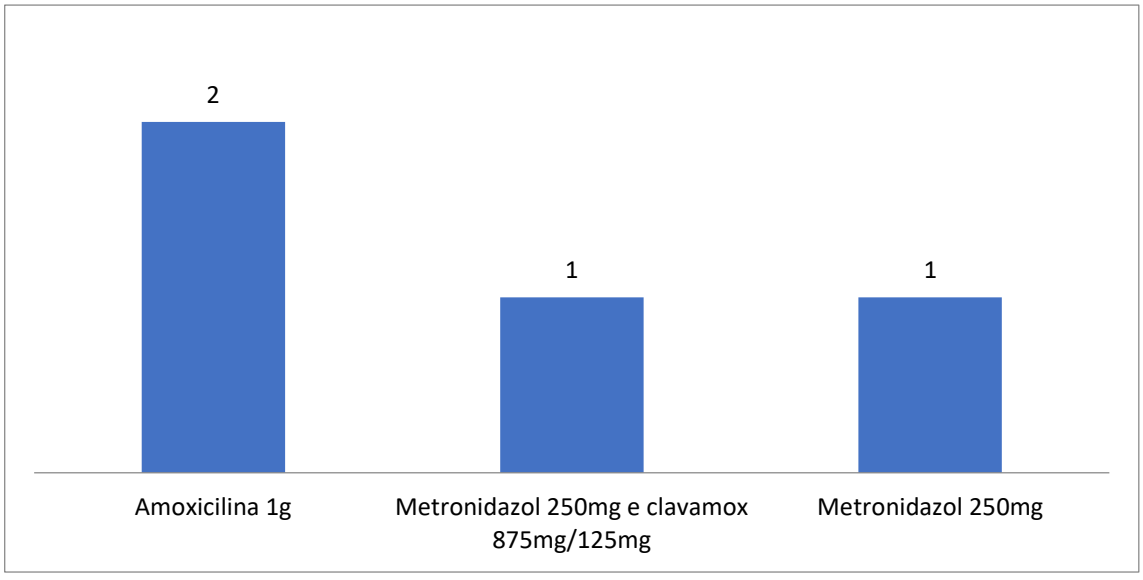

Figure 4 - Distribution of antibiotics taken

The majority of participants on **Table 6**, comprising 73% (8 out of 11), reported that they do not smoke or have never smoked. A smaller segment, accounting for 27% (3 out of 11), identified themselves as former smokers.

Among those who have smoked, 18% (2 out of 11) began smoking at the age of 12, while 9% (1 out of 11) started smoking at the age of 18. Regarding the number of cigarettes smoked per day, 9% (1 out of 11) of the participants smoked more than 10 cigarettes daily, whereas 18% (2 out of 11) limited their smoking to up to 10 cigarettes per day.

For those who have quit smoking, 9% (1 out of 11) quit smoking 40 years ago, another 9% (1 out of 11) ceased smoking 30 years ago, and 9% (1 out of 11) stopped smoking 20 years ago.

Table 6 - Smoking Distribution

Smoking Distribution

Questions	Answers		Percentage
Do you smoke or have you ever smoked?	No	8	73%
	Former smoker	3	27%
If you smoke/smoked: - At, what age did you start smoking?	12	2	18%
	18	1	9%
How many cigarettes do you smoke/smoked (per day): Note: a pack equals 20 cigarettes.	More than 10	1	9%
	Up to 10	2	18%
If you are a former smoker, how many years ago did you quit smoking?	40	1	9%
	30	1	9%
	20	1	9%

Table 7 reveals several key insights into the diagnosis and treatment of periodontitis among the patients. The majority of the patients are diagnosed with Stage II, Grade B periodontitis, with a few cases of Stage IV, Grade C, and Stage I, Grade A periodontitis.

In terms of treatment types, Horizontal Regeneration, Alveolar Preservation, and Maxillary Sinus Elevation are the most common. Notably, Periodontal Regeneration is specifically used for more severe cases, such as those diagnosed with Stage IV periodontitis.

The treatments are distributed across various teeth, with some procedures involving multiple teeth, such as Maxillary Sinus Elevation. Certain teeth, like 26 and 36, are involved in more than one case, indicating a trend in the locations requiring treatment.

Table 7 - Periodontal Treatment Plans and Materials Used

Patient	Periodontal Diagnosis	What treatment will be performed?	Which tooth (teeth) will be treated?	What material will be used?
SP1	Stage II, Grade B	Horizontal Regeneration	11	Bovine xenograft and reticulated collagen membrane
SP2	Stage II, Grade B	Maxillary Sinus Elevation	14, 13, 12, 11, 21, 22	Bovine xenograft
SP3	Stage II, Grade B	Alveolar Preservation	26	Bovine xenograft
SP4	Stage IV, Grade C	Periodontal Regeneration	14, 15, 13	Bovine xenograft and Endogain
SP5	Stage II, Grade B	Horizontal Regeneration	26	Bovine xenograft and reticulated collagen membrane
SP6	Stage II, Grade B	Alveolar Preservation	16	Bovine xenograft and reticulated collagen membrane
SP7	Stage I, Grade A	Maxillary Sinus Elevation	26	Reticulated collagen membrane
SP8	Stage II, Grade B	Alveolar Preservation	22	Bovine xenograft
SP9	Stage II, Grade B	Horizontal Regeneration	36	Bovine and autologous xenograft
SP10	Stage I, Grade A	Maxillary Sinus Elevation	14, 15	Bovine xenograft and reticulated collagen membrane
SP11	Stage IV, Grade C	Periodontal Regeneration	16, 46	Bovine xenograft and Endogain

Table 8 provides insights into the female health status and reproductive history of the participants. None of the participants are currently pregnant, as indicated by 0% answering "Yes" to the question "Are you pregnant?" while 55% answered "No."

Regarding menopause, 18% of the participants are currently in menopause, while 36% are not.

For those who are in menopause, the duration varies: one participant (9%) has been in menopause for 5 years, another participant (9%) for 20 years, and one participant (9%) has been in menopause for less than a year.

Concerning the timing of the last menstruation, the responses are distributed as follows: one participant (9%) reported their last menstruation was 4 days ago, another participant (9%) said 7 days ago, and yet another participant (9%) mentioned 20 days ago

Table 8 - Female Health Status and Reproductive History

Female Health Status and Reproductive History

Questions	Answers		Percentage
Are you pregnant?	Yes	0	0%
	No	6	55%
Are you in menopause?	Yes	2	18%
	No	4	36%
If yes, for how long (years)?	5	1	9%
	20	1	9%
How long ago was your last menstruation (days)?	0	1	9%
	4	1	9%
	7	1	9%
	20	1	9%

Table 9 summarizes the oral hygiene practices and experiences of the participants, revealing several key insights. All participants (100%) reported that they brush their teeth daily, demonstrating a strong adherence to basic oral

hygiene practices. The frequency of brushing varies, with 27% brushing once a day, 45% brushing twice a day, and 27% brushing three times a day.

When it comes to using dental floss, 45% of participants use it regularly, 36% use it sometimes, and 18% do not use it at all. This indicates that while a majority engage in flossing, there is room for improvement in making this a consistent habit among all participants.

All participants (100%) had visited a dentist within the last year, reflecting a commendable level of dental care maintenance. This high rate of dental visits suggests a proactive approach to oral health care among the group.

Only 9% of participants reported experiencing pain in the face or inside the mouth, suggesting that most participants do not suffer from oral discomfort. This low incidence of pain is a positive indicator of overall oral health.

Eighteen percent of participants feel that their mouth is "dry." Among those who experience dry mouth, half (9%) try to compensate by drinking more water, indicating an awareness and proactive approach to managing oral dryness. This issue, while not affecting the majority, is still significant and warrants attention.

All participants (100%) reported not feeling any changes in taste, indicating no recent issues with taste perception among the group.

Table 9 - Oral Hygiene Status

Oral Hygiene Status

Questions	**Answers**		**Percentage**
Do you brush your teeth daily?	Yes	11	100%
	No	0	0%
If yes, how many times a day?	1	3	27%
	2	5	45%
	3	3	27%
Do you use dental floss?	Yes	5	45%
	Sometimes	4	36%
	No	2	18%
When was the last time you visited a dentist?	More than a year ago	0	0%
	Less than a year ago	11	100%

Do you feel any pain in the face or inside the mouth?	Yes	1	9%
	No	10	91%
Do you feel that your mouth is "dry"?	Yes	2	18%
	No	9	82%
If yes, do you try to compensate for this by drinking more water?	Yes	1	9%
	No	1	9%
Do you feel any changes in taste?	Yes	0	0%
	No	11	100%

Patient orthopantomographys Data

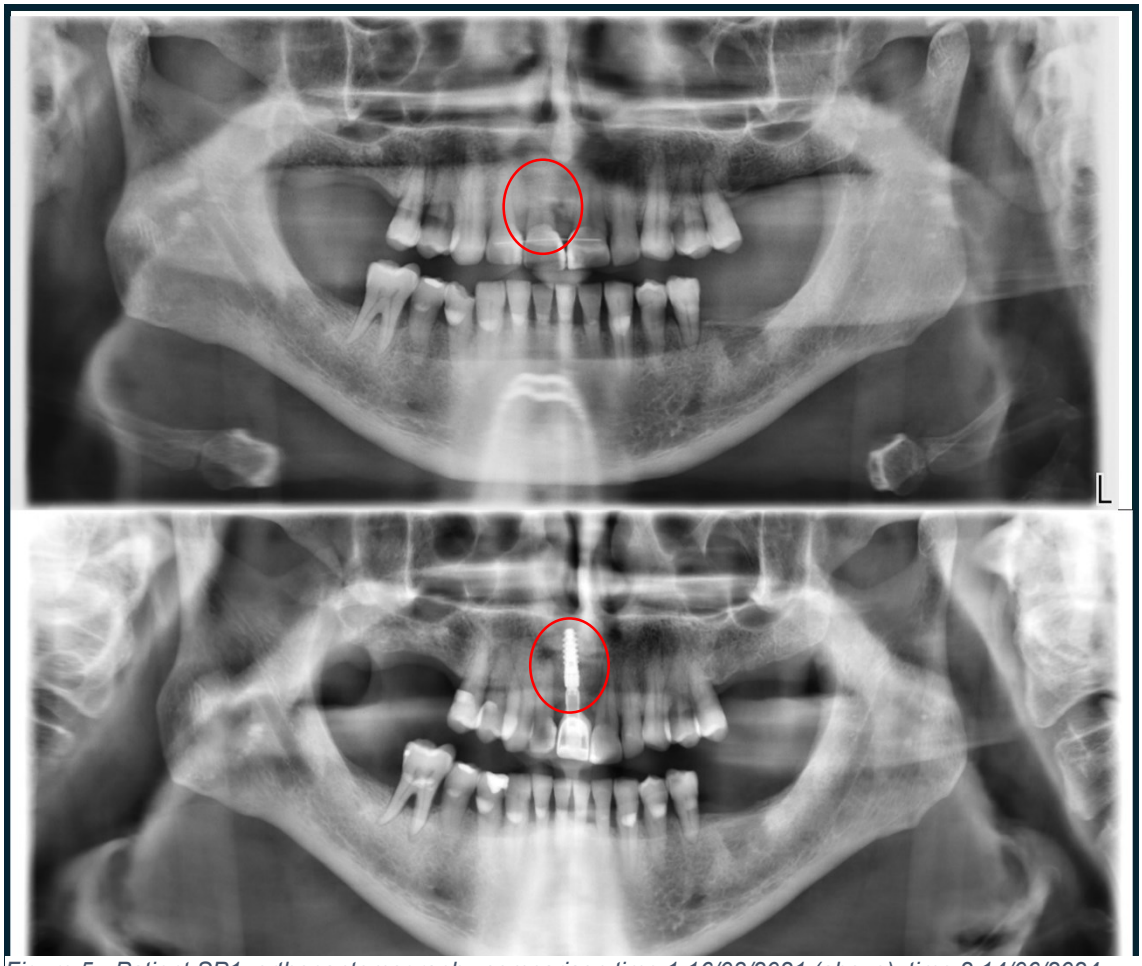

Figure 5 - Patient SP1, orthopantomography comparison time 1 16/03/2021 (above), time 2 14/06/2024 (below)

An analysis of the orthopantomographys from time 1 and time 2, as shown in **Figure 5**, reveals a reduction in radiolucency at the treatment site (tooth 11) in the second image. This finding, highlighted in the figure, suggests potential bone regeneration or an increase in local bone density.

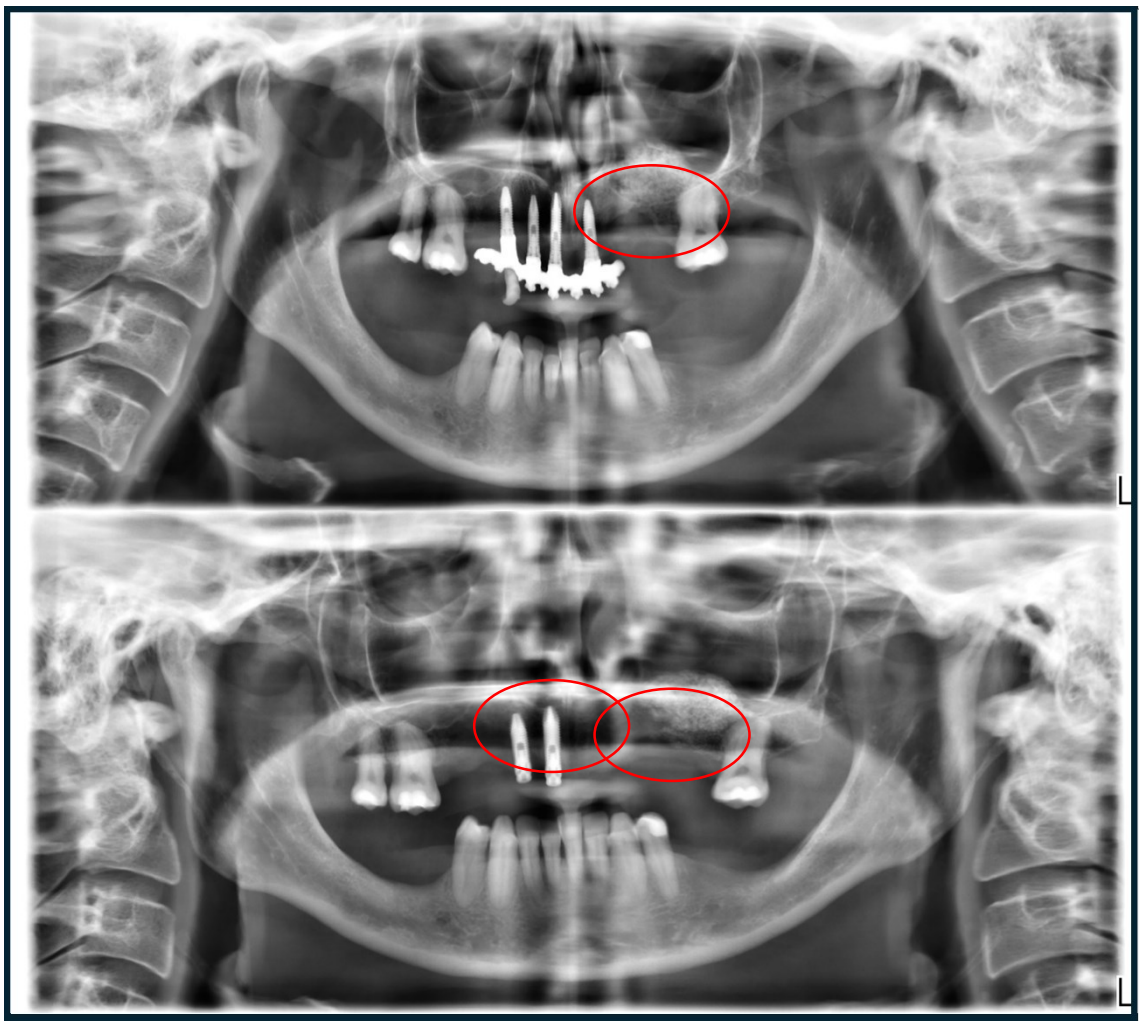

Figure 6 - Patient SP2, orthopantomography comparison time 1 28/10/2023 (above), time 2 03/07/2024 (below)

An evaluation of the orthopantomographys from time 1 and time 2, shown in **Figure 6**, reveals reduced radiolucency at the treatment site in the second image. This area, where a maxillary sinus lift was performed using a bovine xenograft, is highlighted with a red circle. The site corresponds to the location where teeth 14, 13, 12, 11, 21, and 22 would be.

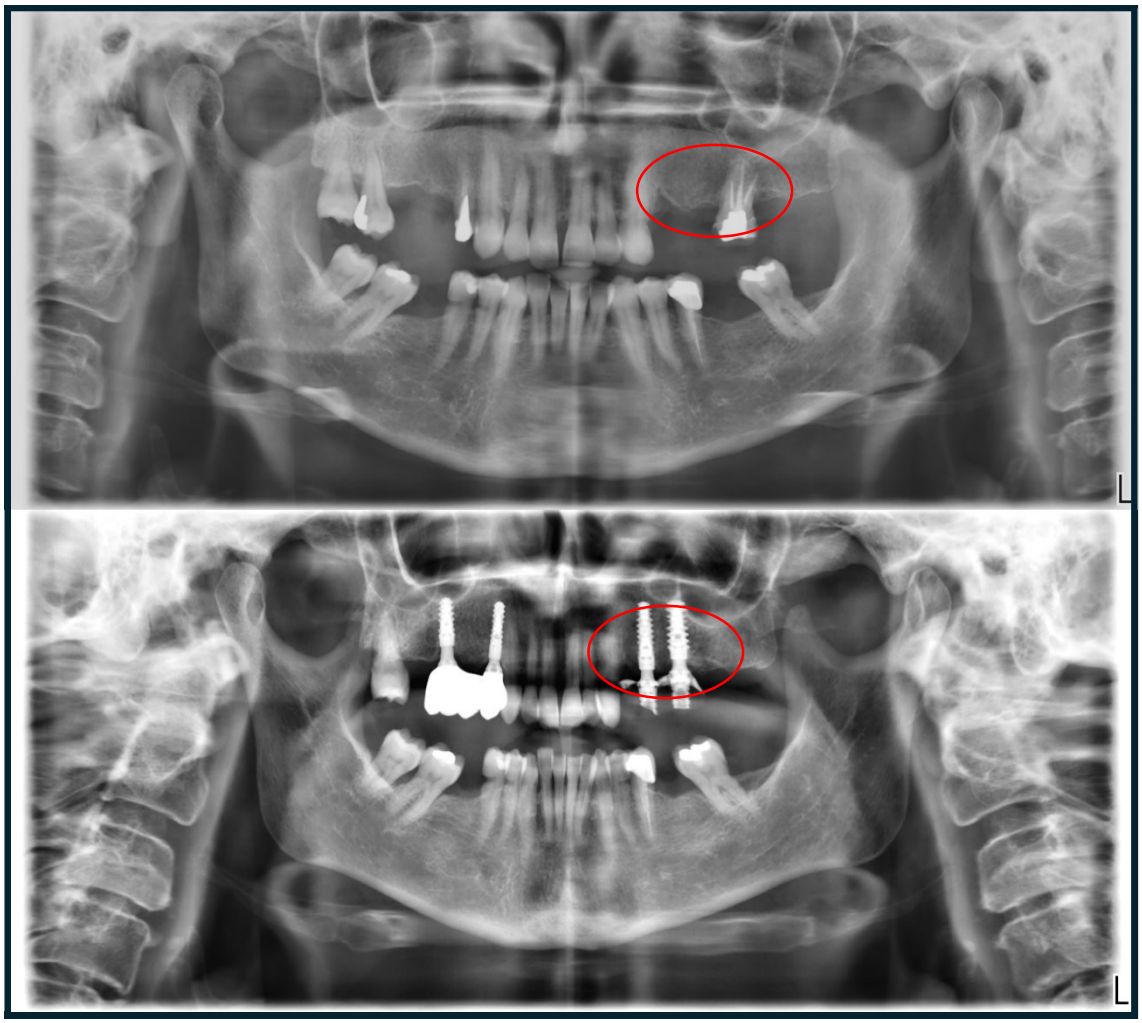

Figure 7 - Patient SP3, orthopantomography comparison time 1 11/12/2020 (above), time 2 03/07/2024 (below)

By contrasting the orthopantomographys from time 1 and time 2 on **Figure 7**, it is evident that there is reduced radiolucency in the second image at the treatment site (tooth 26), where the alveolar preservation was made by using a bovine xenograft, which is highlighted with a red circle. This suggests possible bone regeneration or an increase in local bone density.

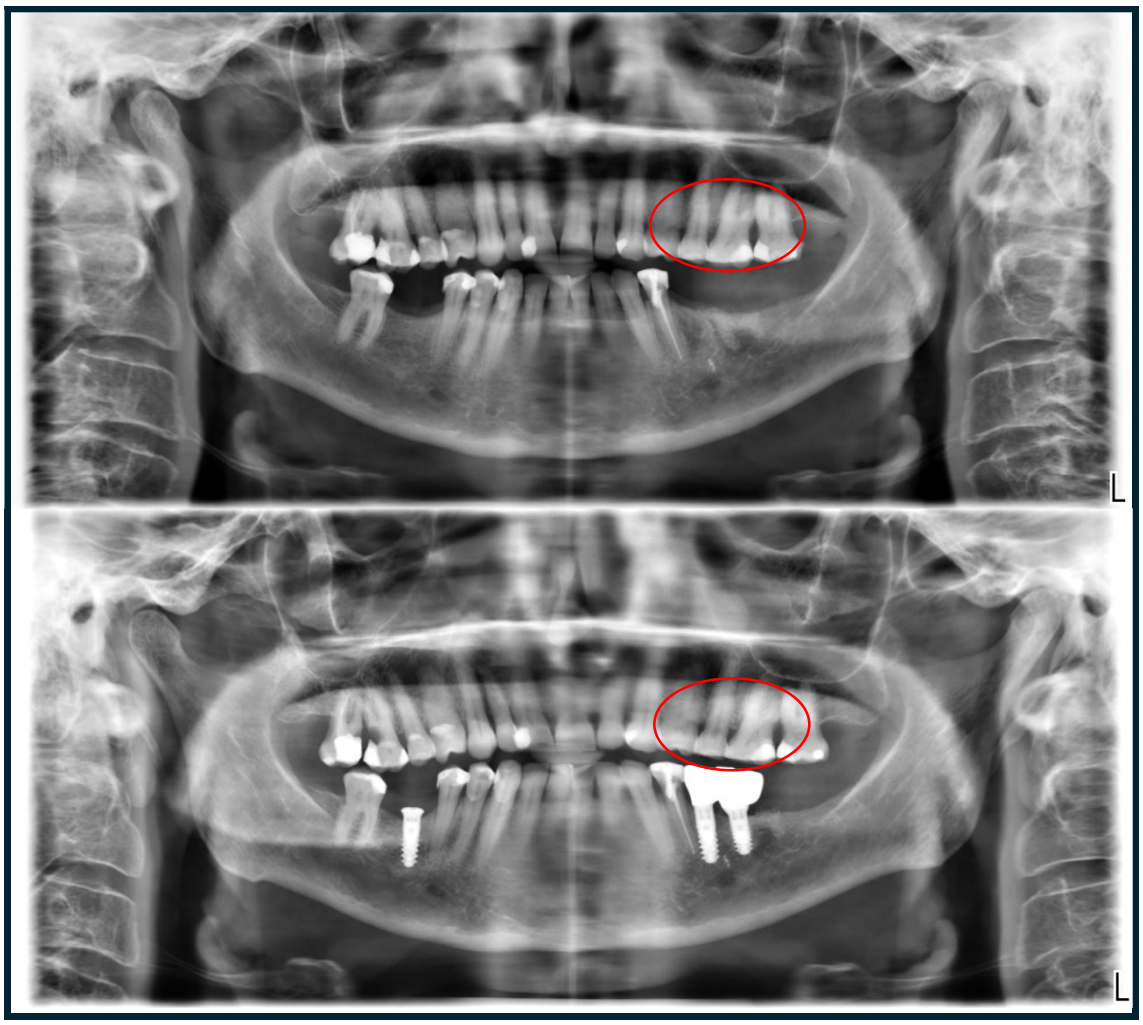

Figure 8 - Patient SP5, orthopantomography comparison time 1 24/10/2023 (above), time 2 25/06/2024 (below)

Comparing the orthopantomographys from time 1 and time 2, as shown in **Figure 8**, reveals reduced radiolucency in the second image at the treatment site (tooth 26), where horizontal regeneration was applied. This area, highlighted in the image with a red circle, suggests possible bone regeneration or an increase in local bone density.

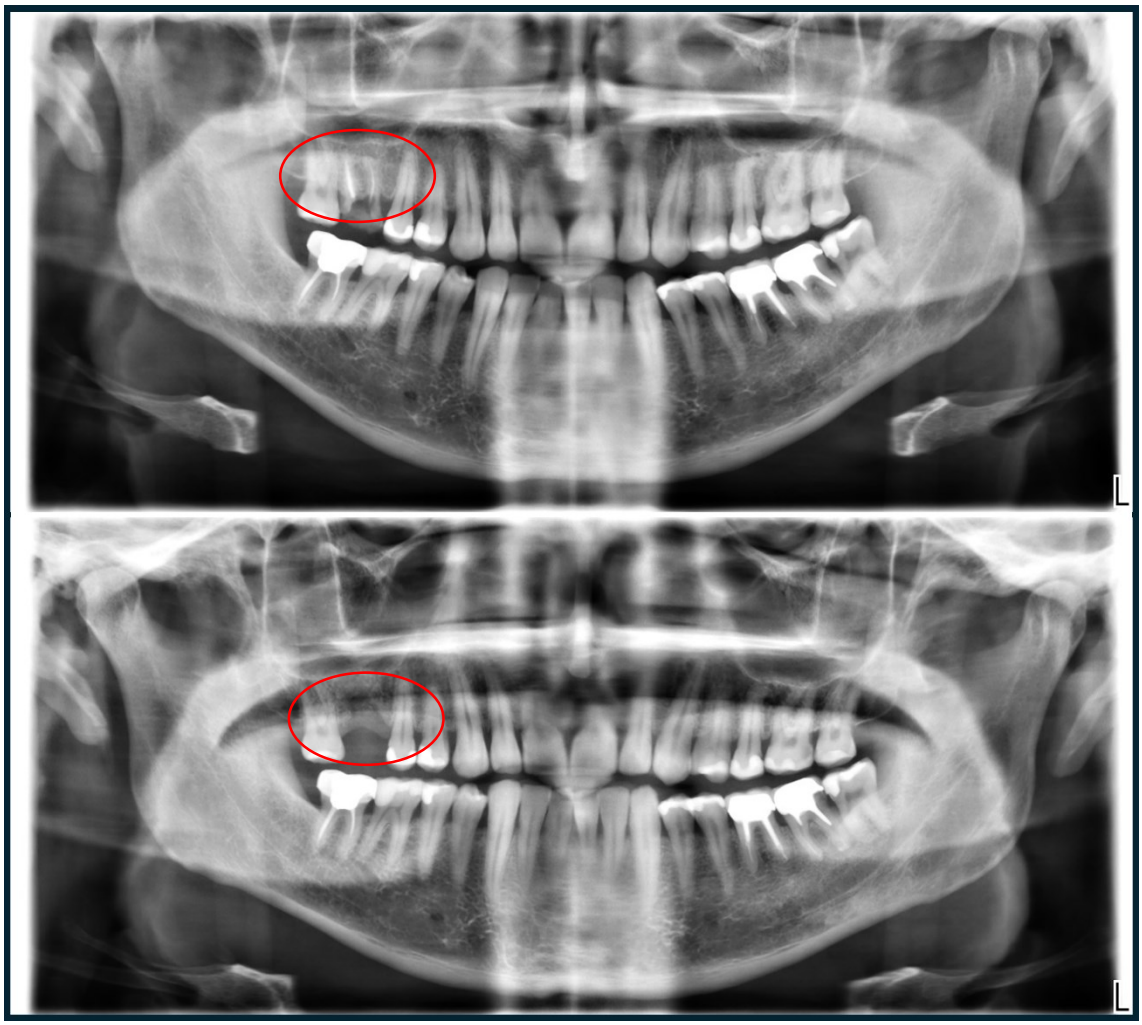

Figure 9 - Patient SP6, orthopantomography comparison time 1 19/10/2023 (above), time 2 28/06/2024 (below)

Through comparison of the orthopantomographys from time 1 and time 2 on **Figure 9**, it is evident that there is reduced radiolucency in the second image at the treatment site (tooth16) where the alveolar preservation was made by using bovine xenografts and cross-linked collagen membranes after the removal of the teeth, highlighted with a red circle. This indicates potential bone regeneration or an increase in local bone density.

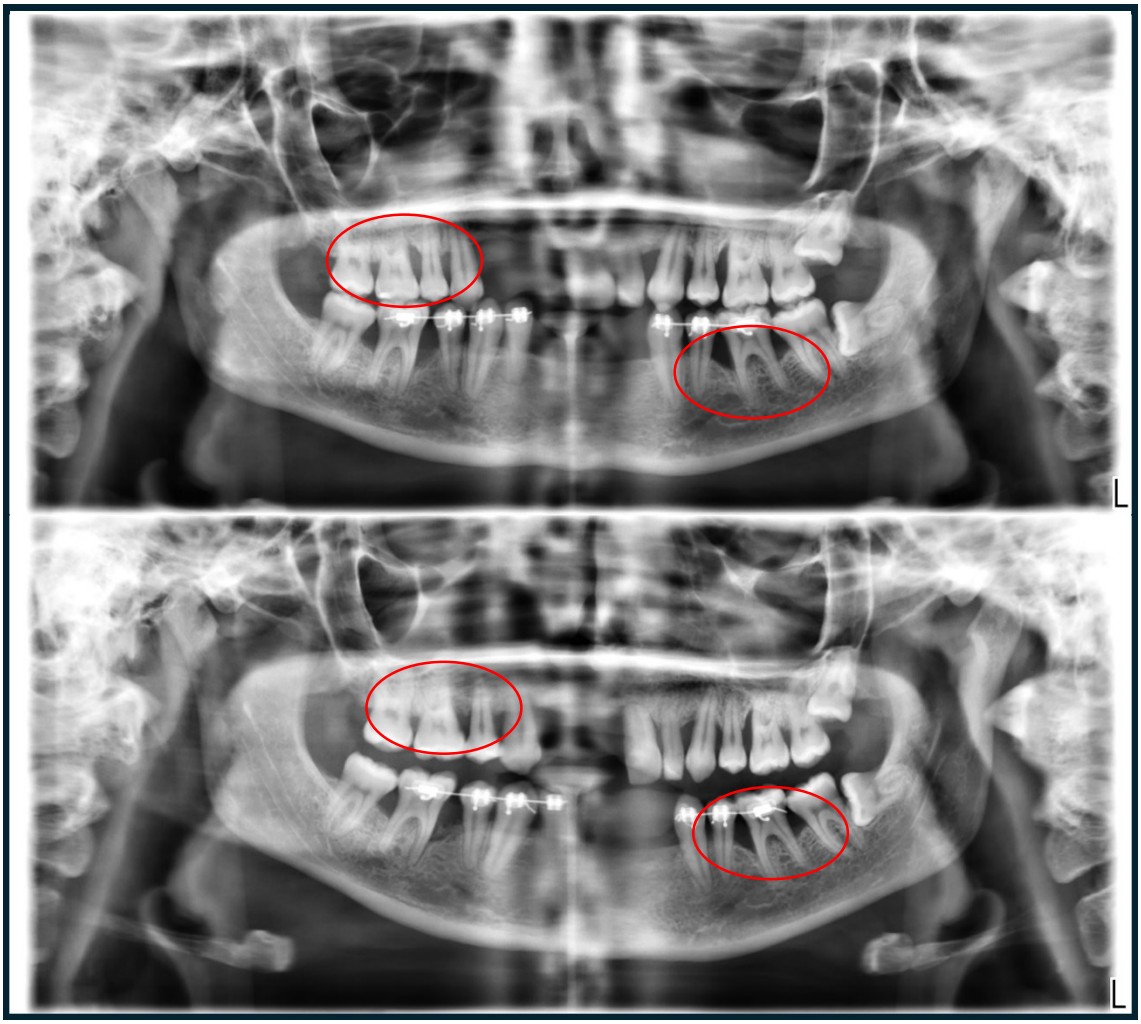

Figure 10 - Patient SP11, orthopantomography comparison time 1 16/12/2023 (above), time 2 28/06/2024 (below)

As well, by comparing the orthopantomographys from time 1 and time 2, as shown in **Figure 10**, reveals reduced radiolucency in the second image at the treatment sites (tooth's 16, 46), where the periodontal regeneration treatment was applied, in this case by using bovine xenograft and Endogain as biomaterials. The treatment site is highlighted with red circles. This also suggests possible bone regeneration or an increase in local bone density.

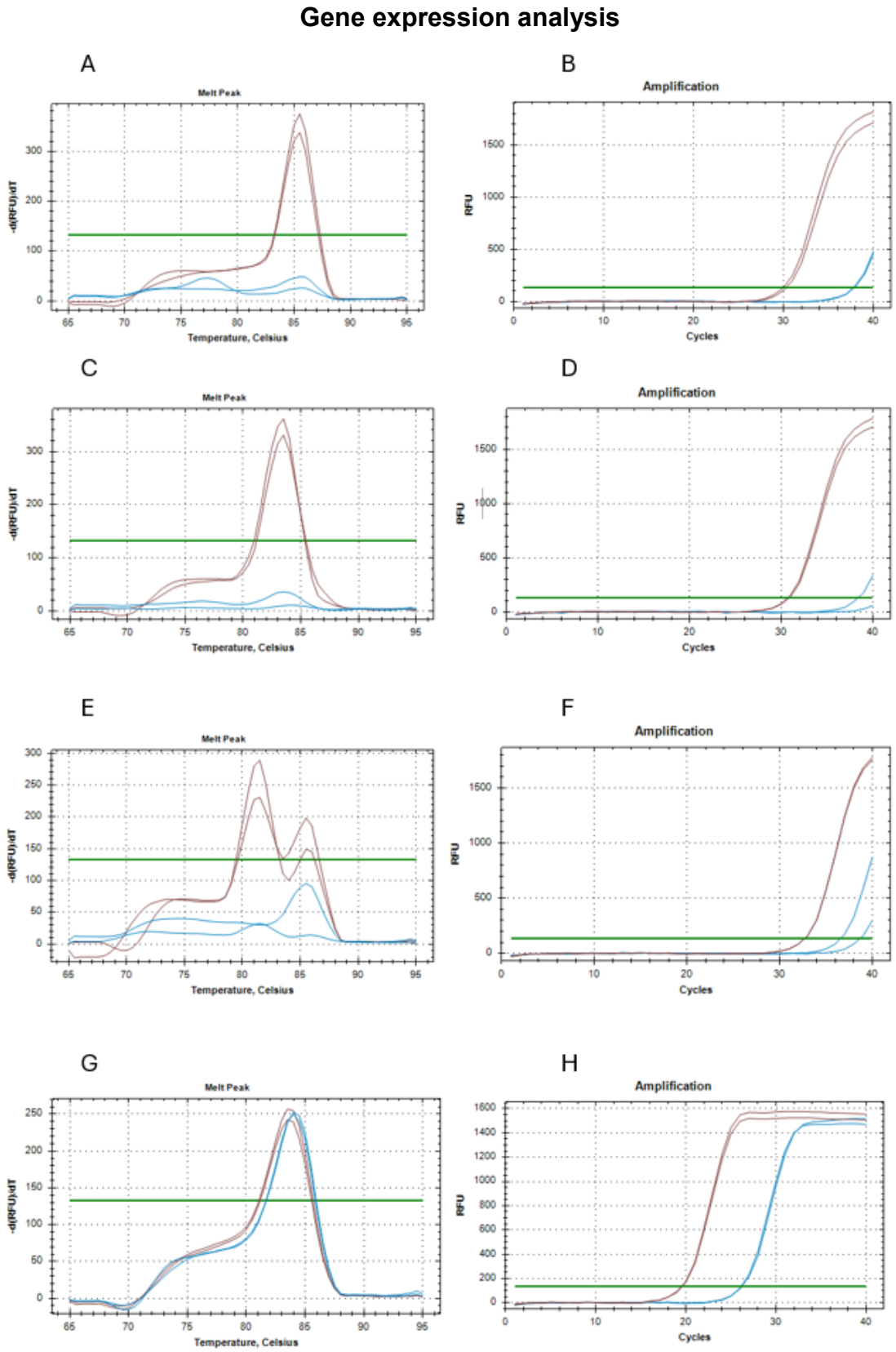

Figure 11 - Melting curve diagrams and amplification graphs depicting the relative expression levels of OPG mRNA (A and B), RANK mRNA (C and D), RANKL mRNA (E and F), and GAPDH mRNA (G and H) in MC3T3 cells on day 7 using the commercial kit NZYSpeedy qPCR Green Master Mix (NZYtech) (performed in duplicate). Red and blue lines represent treated and untreated MC3T3 cells, respectively, with a 4x4 mm OSSIX® Plus Ossifying Collagen Barrier Membrane placed in transwell inserts.

The *RANKL/RANK/OPG* signalling pathway is crucial for osteoclast maturation, bone modelling, and remodelling. To assess its activity, we measured the mRNA expression levels of *OPG, RANK*, and *RANKL* in the presence and absence of the OSSIX® Plus Ossifying Collagen Barrier Membrane **(Figure 11, A-F)**. After 7 days of culture, there was a significant upregulation of *OPG, RANK,* and *RANKL* genes in the presence of the collagen membrane, indicating its potential impact on bone remodelling processes.

In the melting curves **(Figure 11,A, C, E, G)**, a distinct melt peak confirms the specificity of the amplified products, with the treated group (red) showing a shift in peak compared to the untreated group (blue), particularly for OPG and *RANKL*, suggesting altered gene expression dynamics due to the collagen barrier.

The amplification plots **(Figure 11, B, D, F, H)** demonstrate successful amplification of the target genes, with treated cells exhibiting higher fluorescence intensity, indicative of increased gene expression. Notably, the *RANKL* mRNA expression profile **(Figure 11, E-F)** reveals the necessity for further optimization to achieve a clearer amplification peak.

The *GAPDH* expression **(Figure 11, G-H)** remained stable in both treated and untreated conditions, confirming its suitability as a housekeeping control gene for future studies, as indicated by consistent amplification curves and melt peaks across samples.

Overall, the results suggest that the OSSIX® Plus Ossifying Collagen Barrier Membrane modulates the expression of key genes in the *RANKL/RANK/OPG* pathway, which could enhance osteogenic activity and bone regeneration.

Future studies should focus on optimizing conditions for *RANKL* amplification to better understand its role in conjunction with collagen membranes.

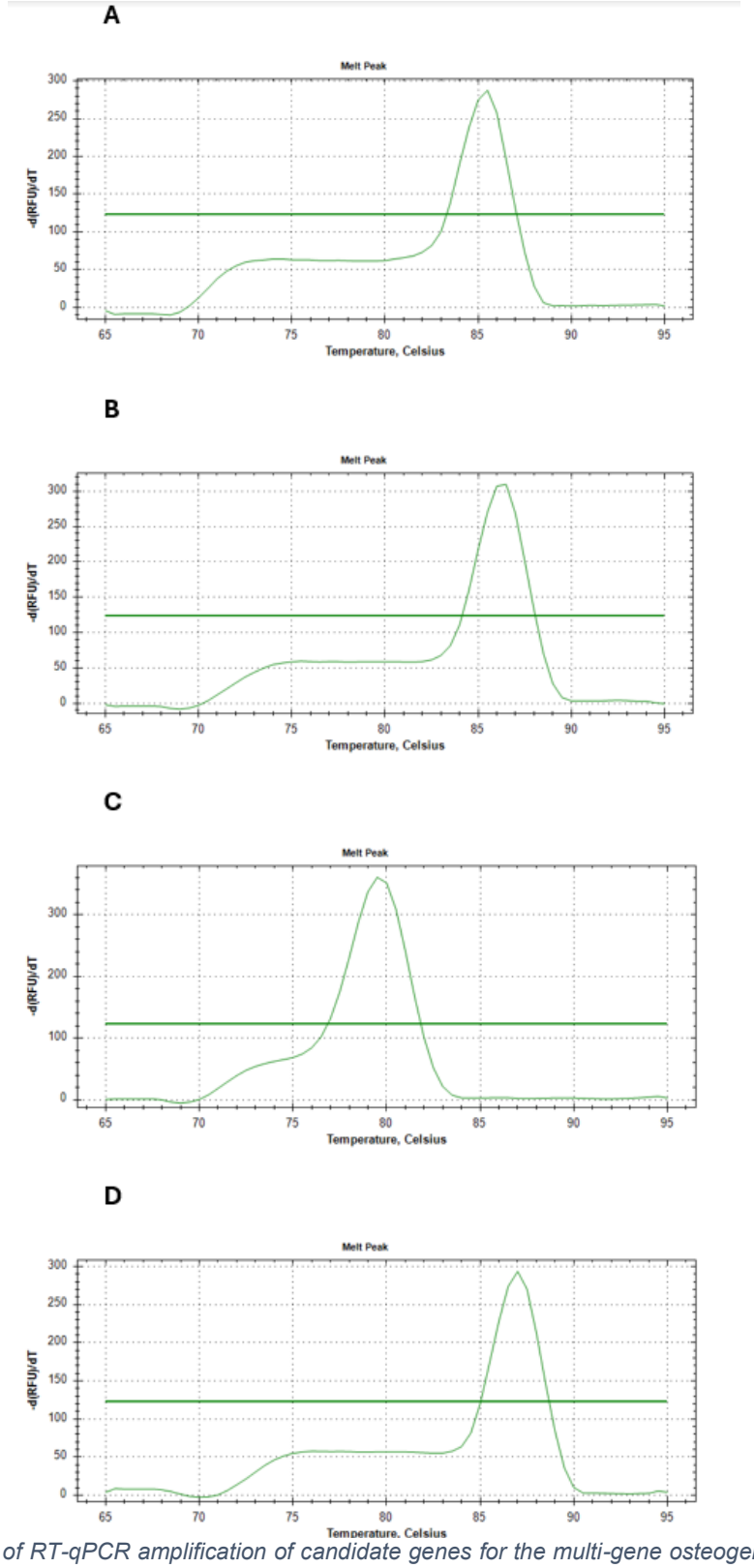

Figure 12 - Melt curves of RT-qPCR amplification of candidate genes for the multi-gene osteogenic biomarker panel: Alpl mRNA (A), Bglap mRNA (B), Col1A1 mRNA (C), and Runx2 mRNA (D) in MC3T3 cells on day 7, using the commercial kit NZYSpeedy qPCR Green Master Mix (NZYtech). Thermal cycling conditions: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 5 seconds and 59°C for 30 seconds.

Figure 12 shows the melt curve analysis of RT-qPCR amplification for different candidate genes associated with osteogenic differentiation in MC3T3 cells. The genes analysed are *Alpl* mRNA (A), *Bglap* mRNA (B), *Col1A1* mRNA (C), and *Runx2* mRNA (D). The graphs provide insights into the specificity and efficiency of the PCR reactions performed after 7 days of untreated MC3T3 cell cultures.

The melt curve for *Alpl* **(Figure 12, A)** shows a single, distinct peak at around 85°C. This indicates a specific and efficient amplification of the *Alpl* mRNA, with no significant off-target amplifications or primer-dimer formations. Similarly, the melt curve for *Bglap* **(Figure 12, B)** shows a single, distinct peak at approximately 85°C. The specificity of the *Bglap* amplification is confirmed, demonstrating efficient and accurate PCR conditions.

The melt curve for *Col1A1* **(Figure 12, C)** presents a single peak at around 80°C. This peak confirms the specific amplification of *Col1A1* mRNA. Lastly, the melt curve for *Runx2* **(Figure 12, D)** shows a single peak at approximately 85°C. This peak indicates the specific and efficient amplification of Runx2 mRNA.

The melt curves for *Alpl, Bglap, Col1A1*, and *Runx2* **(Figure 12, A, B, C, and D, respectively)** illustrate the specificity and efficiency of the RT-qPCR amplifications performed on MC3T3 cells after 7 days of culture. Each graph shows a single, well-defined peak, indicating that the chosen primers and PCR conditions (59°C annealing temperature) are appropriate for these target genes. This validation is essential for ensuring the reliability of the multi-gene osteogenic biomarker panel, confirming that 59°C is an effective annealing temperature for all four genes, thereby supporting their use in further experiments.

4. Discussion

Patient Demographics and Health Status

With a nearly equal gender distribution (55% female and 45% male), the study can analyse potential gender differences in bone regeneration. Hormonal differences between males and females can influence bone density and healing processes. As highlighted by *Ortona et al. 2023* (19)**,** significant gender differences in bone health and healing shown in epidemiological evidence and clinical studies, there are consistent variations in bone fracture healing outcomes between males and females. For instance, males tend to exhibit more rapid fracture healing, while females are more prone to atrophic non-unions and delayed healing processes, often influenced by estrogen levels and inflammatory responses. These differences underscore the necessity of analysing gender-specific data in studies like ours to uncover potential disparities in bone regeneration. Including both genders helps in understanding these variations and improving treatment approaches.

Regarding the age range of the participants was between 29 and 73 years, with an average age of 51.82 ± 14.82 years, this allows for the assessment of bone regeneration across a broad spectrum of adult life stages. Bone healing and regeneration capabilities can differ significantly between younger and older adults. As *Korzh et al. 2021* (20) emphasized that, bone healing is influenced by various factors, including hormonal changes, inflammatory responses, and cellular mechanisms, which can vary with age. Younger adults tend to have more robust bone regeneration capabilities, while older adults may experience slower healing processes and a higher risk of complications due to factors like reduced hormone levels and increased prevalence of osteoporosis. These differences underscore the importance of including a diverse age range in studies to fully understand the variations in bone regeneration.

Family history data in this study provides significant insight into the potential genetic and environmental factors influencing bone health and regeneration in the patient cohort. Among the 11 participants, 45% reported no family history of diseases, while 55% had a family history of conditions such as cancer (18%), heart diseases (18%), diabetes (9%), and a combination of heart diseases and diabetes (9%). As *Oton-Gonzalez et al. 2022* (21), outlined there is significance in the genetic and environmental factors on the development of metabolic bone diseases, emphasizing the importance of genetic predispositions in bone health and regeneration. For example, various bone diseases such as osteoporosis, osteogenesis imperfecta, and osteopetrosis have well-established genetic underpinnings that affect bone turnover and integrity. The genetic basis of these diseases, often involving multiple modes of inheritance and gene mutations, highlights the necessity of considering familial history in understanding bone health. However, this does not imply that the presence of any of these diseases in a family history will influence bone regeneration during the performed treatments.

The presence of comorbidities such as hypertension (36% of participants) and type II diabetes (9% of participants) were considered in the study, but their impact on bone regeneration needs further exploration. As highlighted in *Hu et al. 2021* (22), hypertension is associated with lower levels of bone metabolism markers such as osteocalcin and 25-hydroxy vitamin D, which are crucial for bone formation and mineralization. The study indicates that hypertensive patients, particularly females, tend to have lower osteocalcin levels, suggesting reduced bone turnover. This correlation suggests that hypertension may negatively affect bone health. Additionally, medications used to manage hypertension may have effects on bone metabolism as well. Regarding diabetes type II as highlighted in *Romero-Díaz et al. 2021* (23) showed that, patients with type II diabetes suffer from an increased risk of fractures due to impaired bone quality. This impairment is attributed to low bone turnover, the incorporation of advanced glycation end-products, and microvascular complications, which all contribute to reduced bone strength and increased fracture risk. These factors indicate that diabetes can negatively affect bone regeneration.

Addressing Cancer data, like a malignant intestinal tumour (present in 9% of participants), can metastasize to bones, further complicating regeneration processes. As highlighted in *Santos et al. 2021* (24), mechanically stimulated osteocytes are crucial for bone anabolism and regeneration. The presence of metastatic cancer cells in bones significantly disrupts these regenerative activities. Mechanically stimulated osteocytes can limit the progression of metastatic cancer and support bone regeneration through direct remodelling of the extracellular matrix and activation of signalling pathways linked to genome integrity. However, the impact of metastatic cancer on these processes is detrimental.

Hypothyroidism, reported by 18% of participants, can lead to decreased bone turnover and delayed bone healing. Thyroid hormones are critical for bone growth and remodelling; therefore, insufficient levels can impair the bone regeneration process and lead to osteoporosis. As evidenced in the review by *Wu et al. 2023* (25), thyroid hormones play an essential role in bone metabolism by regulating osteoclast and osteoblast activities. The deficiency of thyroid hormones, as seen in hypothyroidism, results in reduced osteoclastic bone resorption and decreased osteoblastic activity, leading to low bone turnover-mediated bone loss.

Among the participants in this study, 73% were non-smokers, suggesting a generally healthier oral environment within this group. Given that smoking is a well-documented risk factor for periodontal diseases and bone loss, the high percentage of non-smokers may indicate a lower prevalence of these conditions in the study population, as suggested by *Donos et al. 2023* (26).

Conversely, 27% of the participants were ex-smokers, with cessation periods ranging from 20 to 40 years. This extensive duration since quitting smoking suggests that these individuals may have experienced substantial recovery in both oral and systemic health. Prolonged cessation potentially allows for partial reversal of the adverse effects of smoking, thereby improving conditions for bone regeneration and overall treatment outcomes.

The study *Donos et al. 2023* (26)**,** also underscores the critical consideration of past smoking habits on bone turnover and treatment efficacy. Smoking's adverse effects on healing and bone regeneration are primarily due to its vasoconstrictive properties, which reduce oxygen supply to tissues. Additionally, smoking disrupts the proliferation and differentiation of osteoblasts and fibroblasts, essential cells in bone regeneration, by modulating the expression of genes crucial for bone metabolism.

Nicotine, a primary toxic component of cigarette smoke, further exacerbates these issues by increasing platelet adhesiveness, leading to the formation of microclots. These microclots reduce microvascular perfusion, resulting in tissue ischemia. This compromised vascularization is particularly detrimental to graft revascularization and integration, which are vital for the long-term success of bone regeneration procedures.

Medications like Simvastatin, a statin, are known to influence bone metabolism positively. As suggested by study (27) *Jin et al. 2021***,** the use of Simvastatin among several study participants may have contributed positively to bone regeneration outcomes. Simvastatin, a widely prescribed HMG-CoA reductase inhibitor, is known not only for its cholesterol-lowering effects but also for its beneficial impact on bone metabolism. This medication has been extensively studied for its ability to promote osteogenesis and angiogenesis, which are crucial for bone healing and regeneration. It achieves these effects by inducing the expression of key osteogenic genes such as *BMP-2*, *VEGF*, alkaline phosphatase, and type I collagen, while simultaneously inhibiting osteoclast activity. These molecular mechanisms enhance the proliferation and differentiation of osteoblasts, vital for new bone formation. Additionally, pathways such as *Ras/Smad/Erk/BMP-2* signalling and the *Wnt/β-catenin* pathway are activated by Simvastatin, further promoting osteogenic differentiation and bone repair. Given these properties, participants using Simvastatin are likely to benefit from its anabolic effects on bone tissue, potentially leading to improved outcomes in bone regeneration and overall treatment efficacy.

Euthyrox, a thyroid hormone replacement therapy, is important for overall metabolism, including bone health. The study of *Heim et al. 2022* (28)**,** referred to the use of Euthyrox, a thyroid hormone replacement therapy, among study participants showed its critical role in overall metabolism and bone health, by helping to maintain proper thyroid hormone levels, which are vital for various metabolic processes. Both hypothyroidism and hyperthyroidism can negatively impact bone mineral density, increasing the risk of fractures, therefore, managing thyroid hormone levels is essential for preserving skeletal integrity and supporting overall metabolic functions. The significance of maintaining adequate thyroid hormone levels extends to bone health due to thyroid hormones playing a pivotal role in bone remodelling, meaning that this can have a positive effect on patient's outcomes.

The administration of antibiotics such as Amoxicillin and Metronidazole, either individually or in combination, within the last three months may not affect bone regeneration outcomes as suggested by the study *Payer et al. 2020* (29), which examined the impact of systemic antibiotics on clinical outcomes and patient-reported measures in oral implant therapy with simultaneous guided bone regeneration and found no significant benefits in terms of post-surgical complications or patient experiences, even presenting some risks associated with antibiotic resistance and disruption of the microbiome. Further supporting this, a prospective cohort study by *Menon et al. 2019* (30)**,** demonstrated that the use of amoxicillin significantly alters the oral microbiome, with notable changes in the relative abundance of various bacterial taxa and an increase in antibiotic resistance genes such as TEM-1. This prolonged impact on the oral microbiome exposes the potential risks of antibiotic prophylaxis.

Clinical Evaluation

Comparisons between the initial and subsequent orthopantomography imaging sessions revealed significant findings. Patient 1, 2, 3, 5, 6 and 11 showed reduced radiolucency at the treatment sites, suggesting possible bone regeneration or increased bone density. For Patients 4, 7, 8, 9, and 10, (attachments 6 to 10) only initial imaging was available, making comparison

impossible. These results suggest a trend towards bone regeneration in patients treated with biomaterials, with some variability based on individual conditions and treatment specifics.

The orthopantomography results provide preliminary evidence of the effectiveness of different biomaterials in promoting bone regeneration. Most patients showed a reduction in radiolucency at treatment sites, indicating increased bone density and regeneration. This aligns with the established benefits of using biomaterials such as bovine xenografts and collagen membranes. Research has shown that these materials are widely used and effective in periodontal and peri-implant regeneration due to their osteoconductive properties and ability to support bone formation (31)**.**

However, the variability in outcomes suggests that individual patient factors, such as age, health status, and specific treatment protocols, play a significant role in the effectiveness of these biomaterials. It is well-documented that factors related to surgery, the design and surface properties of implants, and patient-specific conditions like systemic health and oral hygiene can significantly influence the success of bone regeneration procedures (31).

The positive outcomes in terms of bone regeneration were evident in most patients, as shown by reduced radiolucency in orthopantomography images. Reduced radiolucency at treatment sites is a strong indicator of bone regeneration or increased bone density. In dental imaging, radiolucency represents areas where bone density is lower, making them appear darker on the radiograph. A reduction in radiolucency signifies that the bone is becoming denser, indicating successful regeneration. This positive outcome was observed in patients 1, 2, 3, 5, 6 and 11. This finding is supported by *Lubis et al. 2023* (32), where significant increases in bone density were observed over time, confirming that reductions in radiolucency on radiographs correlate with increased bone density and regeneration.

While most patients showed positive outcomes, the degree of bone regeneration varied among individuals. *Lubis and colleagues* (32)**,** observed significant variability in bone healing responses, which supports the claim that factors such

as age, gender, overall health status, and specific comorbidities influence the degree of bone regeneration. For instance, older patients or those with chronic conditions like diabetes or hypertension may exhibit slower or less complete bone regeneration compared to younger, healthier individuals.

Taken together, the clinical outcomes and patient characterization in a longitudinal approach will be valuable for supporting and validating the molecular data from salivary samples in future studies.

Gene expression analysis

The primary aim of this study was to develop a non-invasive method for monitoring bone turnover by detecting and quantifying osteogenic biomarkers in saliva. One important goal of this research was to evaluate the expression levels of *OPG, RANK, and RANKL* mRNA in MC3T3 cells. These genes are pivotal in the RANK–RANKL–OPG signalling pathway, as demonstrated by *Leon-Oliva et al. 2023* (33). They are known for their role in bone remodelling and osteoclast maturation. The commercial kit NZYSpeedy qPCR Green Master Mix (NZYtech) was used to measure mRNA levels, revealing a significant upregulation of *OPG, RANK*, and *RANKL* genes in the presence of the OSSIX® Plus Ossifying Collagen Barrier Membrane (used to treat the patients). As expected, these findings indicate that the collagen membrane modulates the expression of these key genes, potentially enhancing osteogenic activity and bone regeneration, moreover, the differential gene expression was monitored in the developed *in vitro* approach, using MC3T3 cells treated with OSSIX® Plus membrane.

In the melting curves depicted in **Figure 11**, a distinct melt peak confirms the specificity of the amplified products. The amplification plots demonstrate successful amplification of the target genes, with treated cells exhibiting higher fluorescence intensity, indicative of increased gene expression. Notably, the *RANKL* mRNA expression profile highlights the necessity for further optimization to achieve a clearer amplification peak. It is worth restating that *GAPDH* expression remained stable in both treated and untreated cells, confirming its

suitability as a housekeeping control gene for future studies. Additionally, the research into *in vitro* conditions for detection and quantification of *Runx2*, *Col1a1*, *Bglap* and *Alpl* mRNAs were also carried out. These genes were selected due to their critical roles in osteogenesis, making them essential targets for understanding bone regeneration processes.

The optimization of protocols for detection of these genes represents a significant milestone. The analysis of melting curves and peak results indicated specific amplification, validating their use in future biomarker comparisons. The melting curves results for *Runx2*, *Col1a1*, *Bglap* and *Alpl* showed specific amplification at 59°C, enabling their simultaneous detection in future assays.

These findings underscore the potential clinical applications of salivary biomarker analysis in monitoring bone regeneration. By combining this approach with orthopantomography, a comprehensive method for assessing the effectiveness of bone grafts and other biomaterials can be established. The OSSIX® Plus membrane's impact on gene expression shows its role in bone remodelling processes, which benefit patients with bone diseases or those requiring bone regeneration.

However, this study has limitations. As indicated by *Lubis et al. 2023* (32), the small sample size and short study duration necessitate further research with larger cohorts and extended follow-up periods to confirm these findings and establish robust clinical protocols. Additionally, variability in saliva sample quality and factors such as diet, oral hygiene, and systemic health may influence salivary biomarkers, requiring careful management in future studies.

Technical challenges encountered during the optimization of PCR protocols, such as ensuring the specificity and sensitivity of primers, highlight the need for ongoing refinement of laboratory techniques. Addressing these challenges will be crucial for the successful implementation of salivary diagnostics in clinical practice. Future studies should focus on optimizing conditions for *RANKL* amplification to better understand its role in conjunction with collagen membranes.

5. Conclusion

The study aimed to evaluate bone regeneration processes through a comprehensive analysis of patient demographics, health status, and advanced biomarker diagnostics. This multifaceted approach provided valuable insights into how various factors such as gender, age, family history, comorbidities, lifestyle choices, and medications influence bone healing.

The evaluation of clinical results demonstrated promising outcomes with biomaterials such as bovine xenografts and collagen membranes in promoting bone regeneration. Most patients exhibited reduced radiolucency at treatment sites, indicating increased bone density and successful regeneration. However, variability in outcomes highlighted the influence of individual patient factors, such as systemic health and treatment protocols, on the effectiveness of these materials. However, the variability in results underscores the impact of individual patient factors—such as systemic health and differing treatment protocols—on the efficacy of these materials. To establish the robustness of these findings, further research involving larger patient cohorts and extended follow-up periods is essential.

The gene expression results, particularly the optimization of PCR protocols for osteogenic biomarkers, represented a significant advancement in non-invasive monitoring of bone turnover. The successful amplification of key genes (*Runx2, Col1a1, and Bglap*) underscores the potential of salivary biomarker analysis in clinical practice. The study also demonstrated the impact of the OSSIX® Plus collagen membrane on gene expression, supporting its role in modulating bone remodelling processes. The findings also suggest that the *RANK/RANKL/OPG* system should be incorporated into the biomarker panel for a more comprehensive assessment.

In summary this study contributes to the growing body of knowledge on bone regeneration by highlighting the intricate interplay of demographic, genetic, health, and lifestyle factors. The findings emphasize the need for personalized treatment approaches, considering the unique characteristics of each patient, to

optimize bone regeneration outcomes. The successful development of non-invasive salivary biomarker analysis offers a promising avenue for future research and clinical applications, potentially revolutionizing the monitoring and management of bone health and regeneration.

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7. Attachments

Attachment 1 – Parecer

UNIVERSIDADE CATÓLICA PORTUGUESA

Parecer sobre o projeto nº 272
Comissão de Ética para a Saúde da Universidade Católica Portuguesa
Mandato 2019/2023

Projeto de Investigação Na reunião do dia 19 de julho de 2023 a CES-UCP esteve reunida e apreciou do ponto de vista ético os elementos submetidos pela investigadora principal. Após apreciação redige o parecer que agora se apresenta.
Título: Salivary bioMarkers of bOne turnOver in response to biomaterials for restorative dentistry - a Tool for salivary monitoring (SMOOT) / Biomarcadores salivares de remodelação óssea em resposta a biomateriais para restauração odontológica - uma ferramenta para monitoração salivar (SMOOT).
Investigadora Principal: Ana Sofia Direito dos Santos Duarte, Prof. Auxiliar/Investigadora do Centro de Investigação Interdisciplinar em Saúde (CIIS) da Faculdade de Medicina Dentária (FMD) da UCP. **Equipa de Investigação:** Ana Peixoto Gomes, Bruna Correia, Karina Mendes, Maria Bartolomeu, Raquel Silva, Miguel Cardoso e Rita Noites.
Resumo: Este estudo fundamenta-se nos seguintes argumentos: Os defeitos ósseos dentários são problemas comuns que podem surgir de infeções, de traumas, de síndromes congénitos, de neoplasias e do envelhecimento. Neste contexto, implantes ou materiais restauradores são necessários para substituir o tecido danificado, mas a sua conexão com o tecido ósseo hospedeiro deve ser assegurada. Os materiais utilizados nesses procedimentos devem fornecer o melhor ambiente estrutural para o desenvolvimento celular durante o processo da cicatrização óssea. Na medicina dentária, as técnicas baseadas em raios-X são valiosas para avaliar o sucesso do tratamento e a condição dos dentes tratados internamente e dos tecidos periapicais. No entanto, esses não são eficazes para prever uma eventual falha do tratamento endodôntico, em estágios iniciais. No corpo humano, o sucesso dos implantes ou tratamentos endodônticos (quando os dentes naturais podem ser salvos/preservados) está diretamente relacionado à biocompatibilidade e à resposta osteoimune. Por exemplo, as células T ativadas podem expressar na superfície o ativador do recetor do ligante do fator nuclear kappa B (RANKL) para estimular a produção de osteoclastos e a reabsorção óssea. O RANKL liga-se ao recetor ativador do fator nuclear κβ (RANK) na superfície dos pró-osteoblastos, ativando a via de sinalização RANKL/RANK e promovendo diretamente a formação de osteoclastos e de diferenciação. Várias dessas moléculas de formação/regulação óssea são liberadas durante os processos de remodelação óssea, o que pode permitir o seu uso como biomarcadores de remodelação óssea. Vários componentes salivares, como imunoglobulinas, citocinas pró-inflamatórias e proteínas, estão a ser estudados como biomarcadores para a triagem dos casos com alterações de tecidos moles e tecidos duros devido à periodontite, o que corrobora a nossa estratégia. A deteção e quantificação dos biomarcadores osteogénicos selecionados na saliva, permitirá que eles se combinem para construir um painel de monitoração eficaz para a sua validação como biomarcadores de remodelação óssea salivar. Os objetivos apresentados são: SMOOT propõe um painel de biomarcadores salivares para orientar os clínicos no trabalho com a Medicina Dentária. Iremos reunir informação molecular sobre o efeito dos biomateriais na remodelação óssea, que irá apoiar a decisão médica. Espera-se que o SMOOT permita a identificação de biomarcadores salivares com a associação mais forte à regeneração óssea. Isso apoiará a proposta destes como futuros biomarcadores salivares para monitorizar, não apenas a eficácia do tratamento endodôntico, mas também em diferentes áreas da odontologia onde a geração da linhagem osteogénica é essencial (por exemplo: cirurgia buco-maxilo-facial, periodontia e implantodontia). Estudo clínico sem intervenção. Os dados recolhidos serão demográficos e clínicos. A saliva como amostra biológica também será recolhida. Os dados demográficos, clínicos e saliva serão recolhidos na Clínica Dentária Universitária – (CDU-UCP). A análise da saliva será realizada no SalivaTec – Centro de Investigação Interdisciplinar em Saúde, UCP.

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Quanto à participação: os pacientes da CDU-UCP que necessitam de tratamento endodôntico serão relevantes para o projeto. No entanto, outros pacientes que necessitem de tratamentos odontológicos, envolvendo regeneração óssea também serão incluídos. Caso seja manifestado o desejo de participar no estudo, será apresentado o Termo de Consentimento Livre e informado, que deverá ser aceite e assinado pelos participantes. Não serão envolvidos menores nem grupos vulneráveis. Anexo III

Relativamente à recolha de dados será aplicado um questionário

Quanto aos procedimentos o SMOOT usará amostras de saliva de pacientes que necessitam de tratamento endodôntico ou outras intervenções odontológicas que envolvam a aplicação de materiais e/ou procedimentos para promover a regeneração óssea. As amostras de saliva serão recolhidas de acordo com o protocolo descrito no Anexo III, e analisadas no SalivaTec, utilizando abordagens de biologia molecular e imunologia.

O procedimento de recolha de amostras não causará nenhum desconforto ao paciente e não exigirá uma abordagem clínica adicional.

Durante o projeto não se espera que os pacientes participantes beneficiem deste estudo. No entanto, os doentes acabarão por beneficiar em consultas futuras, quando for definido um painel de biomarcadores salivares que permita monitorizar os tratamentos.

O investigador principal tem acesso à correspondência entre os códigos gerados para o questionário e amostras biológicas e o número do processo clínico.

É apresentado o modelo de consentimento informado, bem como o procedimento que será adotado pelo investigador principal. Anexo IV

Também se apresenta uma declaração da Investigadora que refere a procedimentos institucionais que asseguram a concordância com RGPD na recolha de dados pelos médicos dentistas.

CV da Investigadora e da Equipa de Investigação

Cronograma de 01/07/2023 a 30/06/2026.

Projeto financiado pelo Centro de Investigação Interdisciplinar em Saúde (CIIS) I&D – UIDP/04279/2020 – Programático. O Anexo VI

Declaração de não existência de conflitos de interesses.

Não foi submetido a outra Comissão de Ética.

Existe a autorização da Direção da instituição onde vai decorrer o estudo. Anexo VII

Apresentado o termo de responsabilidade.

Estiveram presentes na reunião nº 49 da CES-UCP

Presidente: Doutora Mara de Sousa Freitas

Doutor Jerónimo Santos Trigo

Doutor Pedro Garcia Marques

Dr. Eugénio Fonseca

Doutora Ana Mineiro Zaky

Mestre Ivone Gaspar

Conclusão

Ouvido o Relator, e o plenário da reunião de 19 de julho de 2023, realizada por videoconferência, esta CES delibera, por unanimidade, emitir p**arecer favorável.**

Esta CES solicita à Investigadora Principal que, aquando da conclusão do estudo, lhe seja enviada uma síntese dos resultados obtidos e respetivas conclusões, via eletrónica, para o correio eletrónico da CES UCP

A Presidente,

Mara de Sousa Freitas

Mara de Sousa Freitas

19/07/2023

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Attachment 2 - Informed Consent

CATÓLICA FACULDADE DE MEDICINA DENTÁRIA VISEU

Annex VIII - SMOOT

CONSENTIMENTO INFORMADO, ESCLARECIDO E LIVRE PARA PARTICIPAÇÃO EM ESTUDOS DE INVESTIGAÇÃO

(de acordo com a Declaração de Helsínquia e a Convenção de Oviedo)

Título do estudo: Biomarcadores de regeneração óssea na saliva para avaliar a resposta a biomateriais em medicina dentária - uma ferramenta para monitorização salivar (SMOOT)

Objetivo: O objetivo deste estudo é propor um painel de biomarcadores de regeneração óssea, na saliva, que possam auxiliar os clínicos na gestão/monitorização de tratamentos de restauração e reabilitação da saúde oral.

Descrição do estudo: Defeitos ósseos dentários são problemas comuns que podem surgir como resultado de infeções, traumas, doenças genéticas e cancerígenas ou na sequência do envelhecimento. Neste contexto, implantes ou materiais restauradores são necessários para substituir o tecido danificado. Os materiais utilizados nestes procedimentos devem fornecer as melhores condições para o desenvolvimento celular durante o processo de cicatrização óssea, mas se não for atingida uma boa ligação dos materiais aos tecidos que envolvem o dente, o tratamento pode ficar comprometido. No corpo humano, o sucesso dos implantes ou tratamentos endodônticos (quando os dentes naturais podem ser preservados) está diretamente relacionado com a biocompatibilidade e com resposta imunitária, pelo que a deteção precoce de moléculas específicas pode ajudar a identificar situações de insucesso da intervenção clínica, permitindo a criação de soluções mais atempadas e dirigidas ao paciente.

Com este estudo pretendemos analisar amostras de saliva. A recolha de saliva é um processo indolor e não requer nenhum procedimento clínico adicional. O único incómodo será o tempo adicional da consulta devido à entrevista que os investigadores tudo farão para minimizar.

Vantagens e riscos na participação solicitada: Este estudo não envolve procedimentos que não se enquadrem na prática clínica normal nem pretende testar novos produtos ou medicamentos. As amostras recolhidas destinam-se apenas a ser analisadas neste estudo. A participação neste estudo é totalmente voluntária e anónima, não acarretando quaisquer custos. É fundamental que entenda que pode retirar o seu consentimento em qualquer etapa do estudo. Não precisa para tal de apresentar explicações aos responsáveis pela investigação, nem terá qualquer prejuízo, assistenciais ou outros, caso não queira participar. Ao decidir participar pode colocar todas as questões que considerar necessárias para o seu esclarecimento. Mesmo depois de assinado o documento de consentimento esclarecido e informado, pode em qualquer altura solicitar a sua exclusão do estudo. Para tal, basta contactar a investigadora principal

deste estudo: asduarte@ucp.pt ou através do número de telefone 232419500. A participação não implica qualquer remuneração ou encargo económico para o participante. Os participantes colaboram de forma voluntária, livre e esclarecida.

Medidas de Mitigação dos Riscos Reais ou Potenciais: Uma vez que neste estudo não existem riscos para o paciente não estão previstas medidas de mitigação. Ainda assim, é importante referir que os investigadores responsáveis garantem aos participantes o exercício dos seus direitos em relação aos dados recolhidos (como o acesso, a retificação ou a eliminação), bastando o mesmo ser solicitado ao Encarregado da Proteção de Dados deste estudo (contactos no final do documento). Para além do referido, o participante pode efetuar uma reclamação junto do Encarregado de Proteção de Dados (DPO - Data Protection Officer) da UCP, que a encaminhará para a Comissão Nacional de Proteção de Dados (CNPD), caso considerem que existe um incumprimento legal à proteção de dados por parte equipa de investigação (contactos no final do documento).

Confidencialidade e anonimato: Os investigadores garantem o anonimato e a confidencialidade dos dados recolhidos. A informação é recolhida apenas pelo Investigador Principal, num momento único de observação, em ambiente de privacidade, não permite a identificação do participante e é usada apenas para os fins científicos do presente estudo. Os dados são registados e armazenados no computador pessoal do Investigador, com acesso protegido e apenas durante o estudo. Os dados e as amostras armazenadas serão preservados até ao término do projeto, período após o qual os dados serão apagados e as amostras serão destruídas por incineração. Será garantido que a identificação do participante nunca se torne pública."

Medidas de Partilha de Benefícios: Os resultados deste estudo serão partilhados com a comunidade científica através de publicações em revistas com revisão por pares e constituirão parte do corpo de informação e conhecimento científico que permite desenvolver novas formas de diagnóstico precoce e monitorização da saúde com a utilização de amostras não invasivas.

Recolha de Dados: Os dados a recolher neste estudo são de duas naturezas: dados da sua história clínica, que serão recolhidos do seu processo clínico confirmados por entrevista e amostras biológicas de saliva. Os dados recolhidos são totalmente anonimizados e os investigadores (para além do investigador principal) terão apenas acesso à informação codificada não sendo possível identificar a que indivíduo pertence. Os dados e as amostras armazenadas serão preservados até ao término do projeto, período após o qual os dados serão apagados e as amostras serão destruídas por incineração.

Este documento pretende informá-lo(a) e propor-lhe a sua participação neste estudo.

O(a) participante **é livre de aceitar, ou não**, participar no estudo proposto, podendo mudar de opinião e revogar o seu consentimento, abandonando o estudo se assim considerar oportuno, sem qualquer tipo de penalização ou represália.

Confirmo que expliquei à pessoa abaixo indicada, de forma adequada e inteligível, os procedimentos necessários ao ato referido neste documento. Respondi a todas as questões que me foram colocadas e assegurei-me de que houve um período de reflexão suficiente para a tomada da decisão. Também garanti que, em caso de recusa, serão assegurados os melhores cuidados possíveis nesse contexto, no respeito pelos seus direitos.

ASSINATURA

_____

Nome do profissional responsável pela recolha:

_____

Contacto Institucional: _____

Data:___/___/_____

Eu,

_____

Fui informado(a) pelo profissional responsável pela recolha _____

acerca do estudo clínico no qual me é proposto participar.

Fui ainda informado(a) dos riscos possíveis deste estudo.

Pude colocar todas as perguntas ou dúvidas que achei necessárias e entendi todas as explicações que me foram proporcionadas.

Dou o meu consentimento por escrito para participar neste estudo e doar saliva

ASSINATURA

_____

(nome completo do participante)

Consentimento informado do representante legal

Declaro ter lido e compreendido este documento, bem como as informações verbais que me foram fornecidas pela pessoa que acima assina. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Desta forma, como representante legal do (a) _____ aceito a sua participação neste estudo e permito que os dados e amostras biológicas recolhidas sejam usados para fins

de investigação/publicação científica, desde que a minha identidade e da pessoa a meu encargo como seu representante legal seja mantida confidencial.

SE NÃO FOR O PRÓPRIO A ASSINAR POR IDADE OU INCAPACIDADE
Nome: _____
BI/CC nº: _____
Data ou validade _____ /_____ / _____
Grau de parentesco ou tipo de representação: _____
Assinatura: _____

Contacto do Encarregado de Proteção de Dados (DPO - Data Protection Officer) da UCP:

Data Protection Officer - UCP

Dra. Frederica Campos de Carvalho

Contacto telefónico: +351 217214179

E-mail: compliance.rgpd@ucp.pt

Contacto do Encarregado de Proteção de Dados (DPO - Data Protection Officer) da FMD-UCP:

Data Protection Officer FMD- UCP

Dr. Paulo Alexandre de Oliveira Castro Ribeiro

Contacto telefónico: +351 232 419 500

E-mail: pribeiro@ucp.pt

Attachment 3 - Questionnaire

1. Dados de Investigação

Estas informações devem ser preenchidas pelo Médico Dentista ou pelo Investigador Responsável.

Médico Dentista Responsável pela Consulta

Investigador Responsável pela Recolha

Data de Preenchimento (dd/mm/aaaa)

5/30/2023

2. Dados da amostra de saliva

O grupo de informações que se segue devem ser preenchidas pelo Investigador Responsável.

Código da amostra

Amostragem:

- ○ 1ª amostragem
- ○ 2ª amostragem (follow-up)
- ○ 3ª amostragem (follow-up 2 meses)
- ○ 4ª amostragem (follow-up 6 meses)

Hora de Recolha: --:--

Data da recolha (dd/mm/aaaa)

5/30/2023

3. Dados do paciente

O grupo de questões que se segue destinam-se a recolher dados gerais acerca do paciente.

Data de Nascimento (dd/mm/aaaa)

Género

- ○ Feminino
- ○ Masculino

Etnia

- ○ Caucasiana
- ○ Africana
- ○ Oriental
- ○ Cigana
- ○ Outra
- ○ Não respondeu

4. Informações gerais da saúde do paciente

O grupo de questões que se segue destina-se a recolher informação geral acerca da sua saúde.

Tem hipertensão?

- ○ Sim
- ○ Não
- ○ Não sabe

Tem Diabetes?

Tem ou já teve alguma destas patologias?

	Sim	Não	Não sabe
Problemas cardíacos	○	○	○
Doenças de sangue	○	○	○

	Sim	Não	Não sabe
Doenças de fígado	○	○	○
Doenças de estômago	○	○	○
Doenças renais	○	○	○
Doenças intestinais	○	○	○
Cancro	○	○	○
Alergias	○	○	○
Outras doenças	○	○	○

Especifique o tipo (diagnóstico) da patologia:

Foi sujeito a algum tratamento de radioterapia ou quimioterapia?

- ○ Sim

○ Não

Se sim, há quanto tempo? (anos)

0 5 10 15 20 25 30 35 40 45 50 55 60

Anos

História Familiar - Existem doenças na família como?

☐ Doenças Cardíacas
☐ Diabetes
☐ Cancro
☐ Outra
☐ Não tem

Qual ?

5. Medicação

O grupo de questões que se segue pretende recolher informações relevantes relativas à sua medicação.

Faz algum tipo de tratamento médico ou medicação com regularidade?

○ Sim
○ Não
○ Não sabe

Se sim, refira-o:

Fez algum tipo de tratamento médico ou medicação nos últimos 30 dias?

- ○ Sim
- ○ Não

Se sim, refira-o :

Tomou algum antibiótico nos últimos 3 meses?

- ○ Sim
- ○ Não

- ○ Não sei

Se Sim, refira o(s) nome(s) do(s) antibiótico(s):

6. Hábitos tabágicos

O grupo de questões que se segue pretende avaliar os seus hábitos tabágicos.

Fuma ou já fumou?

- ○ Sim
- ○ Não
- ○ Ex-fumador

Se fuma/fumou:

○ [ ] Com que idade começou a fumar?

○ Não sabe

Quantos cigarros fuma/fumava (por **dia**):

Nota: um maço equivale a 20 cigarros.

○ Até 10

○ Mais do que 10

○ Não sabe

Se é ex-fumador há quantos **anos** deixou de fumar?

○ [ ] Sabe

○ Não sabe

7. Consumo de álcool

O grupo de questões que se segue pretende avaliar o seu consumo de álcool.

Bebe ou já bebeu, regularmente, bebidas alcoólicas?

○ Sim

○ Não

Se bebe/bebeu; com que idade começou?

○ [ ] Idade

○ Não sabe

Se bebe ou já bebeu, preencha o seguinte quadro:

	Até 14	Mais de 14	Não sabe	Não bebe
Nº copos vinho (por **semana**)	○	○	○	○
Nº cervejas (por **semana**)	○	○	○	○
Nº digestivos/bebidas brancas (por **semana**)	Até 7 ○	Mais de 7 ○	Não sabe ○	Não bebe ○

Deixou de beber? Se sim com qual idade?

○ [ ] Sim

○ Não

8. Alterações hormonais

O grupo de questões que se segue pretende avaliar possíveis alterações hormonais.

Está grávida?

○ Sim

○ Não

Se sim, de quantas semanas?

0 4 8 12 16 20 24 28 32 36

Nº semanas [ ]

Encontra-se na menopausa?

○ Sim

○ Não

Se sim, há quanto tempo (anos)?

Há quanto tempo teve a última menstruação (dias)?

Toma anticoncecionais?

- ○ Sim
- ○ Não

9. Hábitos e comportamentos de higiene oral

O grupo de questões que se segue pretende avaliar os seus hábitos e comportamentos de higiene oral.

Costuma escovar os dentes diariamente?

- ○ Sim
- ○ Não

Se sim, quantas vezes por dia?

- ○ 1
- ○ 2
- ○ 3
- ○ > 3

Costuma utilizar fio dentário?

- ○ Não
- ○ Sim, às vezes
- ○ Sim, diariamente

- ○ Não sei o que é o fio dentário.

Quando foi a última vez que visitou um dentista?

- ○ Há menos de 1 ano
- ○ Há 1 anos
- ○ Há 2 anos
- ○ Entre 2 e 5 anos
- ○ Há mais de 5 anos
- ○ Nunca

Sente alguma dor na região da face ou no interior da boca?

- ○ Sim
- ○ Não

Sente que a sua boca esta "seca"?

- ○ Sim
- ○ Não

Se sim, tenta compensar este facto com maior consumo de água?

- ○ Sim
- ○ Não

Sente alguma alteração no paladar?

- ○ Sim
- ○ Não

10. Reabilitação protética

O grupo de questões que se segue pretende avaliar a sua prótese e devem ser preenchidas pelo Médico Dentista.

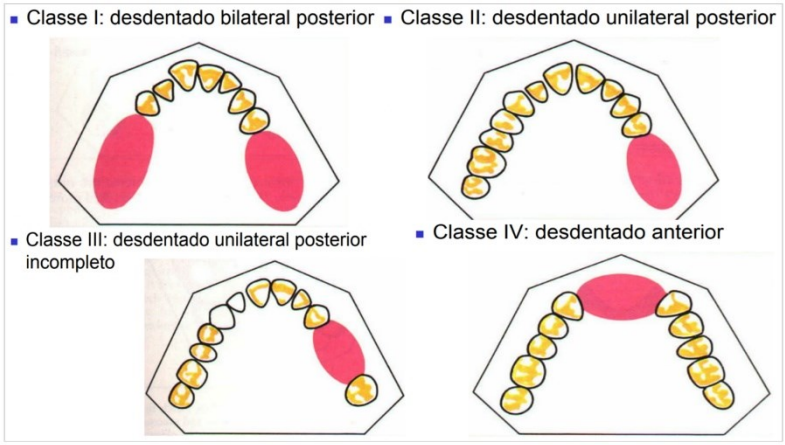
Utiliza prótese?

○ Sim
○ Não

Em qual das arcadas ?

Qual o tipo da prótese ?

	Total	Parcial acrílica	Parcial esquelética
Superior	○	○	○
Inferior	○	○	○



A que tipo de classificação de Kennedy corresponde a prótese que utiliza?

	Classe I	Classe II	Classe III	Classe IV	Sem classificação
Superior	○	○	○	○	○
Inferior	○	○	○	○	○

	Classe I	Classe II	Classe III	Classe IV	Sem classificação

Quando utiliza a(s) prótese(s) ?

Como faz a higienização da sua prótese?

- ☐ Não higienizo
- ☐ Só com água
- ☐ Com água e escova
- ☐ Pastilhas de limpeza
- ☐ Fio dentário
- ☐ Escovilhão
- ☐ Produto dentário (qual?)

Quantas vezes por dia é feita essa higienização?

Costuma retirar a prótese para dormir?

Há quanto tempo utiliza uma prótese dentária? (anos)

Há quanto tempo tem a atual prótese dentária? (anos)

Qual a frequência de consultas de manutenção protética?

11. Saúde oral

O grupo de questões que se segue pretende avaliar a sua saúde oral e devem ser preenchidas pelo Médico Dentista.

Condição atual de cada elemento dentário

	Ausente	Cariado	Tratamento endodôntico	Restaurado	Implante	Raiz Residual	Hígido
11	○	○	○	○	○	○	○
12	○	○	○	○	○	○	○
13	○	○	○	○	○	○	○
14	○	○	○	○	○	○	○
15	○	○	○	○	○	○	○
16	○	○	○	○	○	○	○

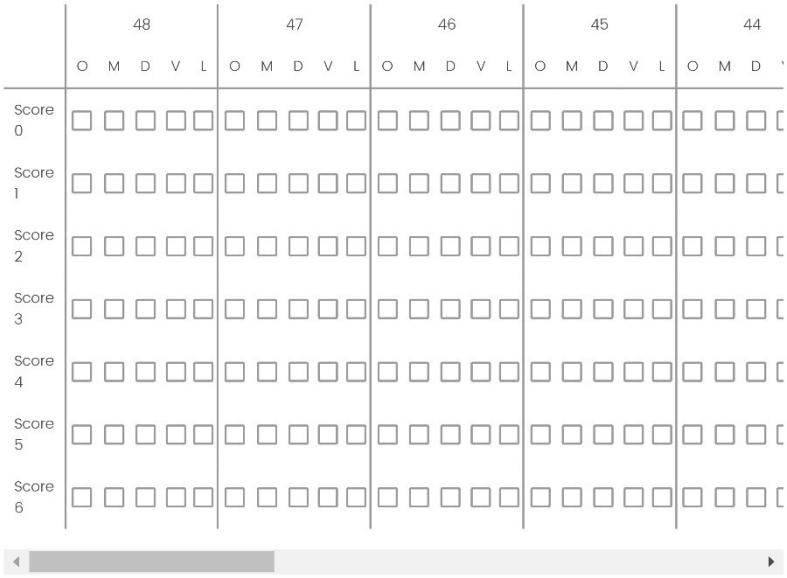
	Ausente	Cariado	Tratamento endodôntico	Restaurado	Implante	Raiz Residual	Hígido
17	○	○	○	○	○	○	○
18	○	○	○	○	○	○	○
21	○	○	○	○	○	○	○
22	○	○	○	○	○	○	○
23	○	○	○	○	○	○	○
24	○	○	○	○	○	○	○
25	○	○	○	○	○	○	○
26	○	○	○	○	○	○	○
27	○	○	○	○	○	○	○
28	○	○	○	○	○	○	○
31	○	○	○	○	○	○	○
32	○	○	○	○	○	○	○
33	○	○	○	○	○	○	○
34	○	○	○	○	○	○	○
35	○	○	○	○	○	○	○
36	○	○	○	○	○	○	○

	Ausente	Cariado	Tratamento endodôntico	Restaurado	Implante	Raiz Residual	Hígido
37	○	○	○	○	○	○	○
38	○	○	○	○	○	○	○
41	○	○	○	○	○	○	○
42	○	○	○	○	○	○	○
43	○	○	○	○	○	○	○
44	○	○	○	○	○	○	○
45	○	○	○	○	○	○	○
46	○	○	○	○	○	○	○
47	○	○	○	○	○	○	○
48	○	○	○	○	○	○	○

ICDAS - Arcada superior

	18					17					16					15					14		
	O	M	D	V	P	O	M	D	V	P	O	M	D	V	P	O	M	D	V	P	O	M	D
Score 0	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Score 1	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Score 2	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Score 3	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Score 4	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Score 5	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Score 6	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

ICDAS - Arcada Inferior



	48					47					46					45					44			
	O	M	D	V	L	O	M	D	V	L	O	M	D	V	L	O	M	D	V	L	O	M	D	
Score 0	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	[
Score 1	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	[
Score 2	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	[
Score 3	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	[
Score 4	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	[
Score 5	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	[
Score 6	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	[

Como questões a seguir referem-se ao diagnóstico periodontal (nova classificação).
Preencher Perio Chart e copiar informações para o questionário.

https://www.periodontalchart-online.com/uk/

Diagnóstico Periodontal (marcar uma resposta ou mais)

- ☐ Saúde periodontal
- ☐ Periodonto reduzido
- ☐ Gengivite
- ☐ Periodontite localizada (menor que 30%)
- ☐ Periodontite generalizada (maior que 30%)
- ☐ Padrão Molar Incisivo
- ☐ Estadio I
- ☐ Estadio II
- ☐ Estadio III
- ☐ Estadio IV
- ☐ Grau A
- ☐ Grau B
- ☐ Grau C
- ☐ Mucosite Peri-implantar

☐ Peri-implantite

Diagnóstico Oral

○ Estomatite protética Classe I
○ Estomatite protética Classe II
○ Estomatite protética Classe III

Necessidade de tratamento médico-dentário?

○ Sim
○ Não

Necessidade de reabilitação protética?

○ Sim
○ Não

12. Tratamento a ser realizado na consulta

O grupo de questões que se segue pretende avaliar o seu tratamento dentário e devem ser preenchidas pelo Médico Dentista.

Vai ser realizado tratamento em qual destas áreas de medicina dentária?

○ Endodontia
○ Periodontologia
○ Cirurgia
○ Reabilitação Oral (Implantes)
○ [ ] Outra

Que tratamento vai ser realizado?

Em que dente (s) vai ser realizado o tratamento?

Que material será utilizado?

Qual o tipo de desinfetantes/irrigantes utilizados durante o tratamento endodôntico?

Vai ser utilizada medicação intracanal?

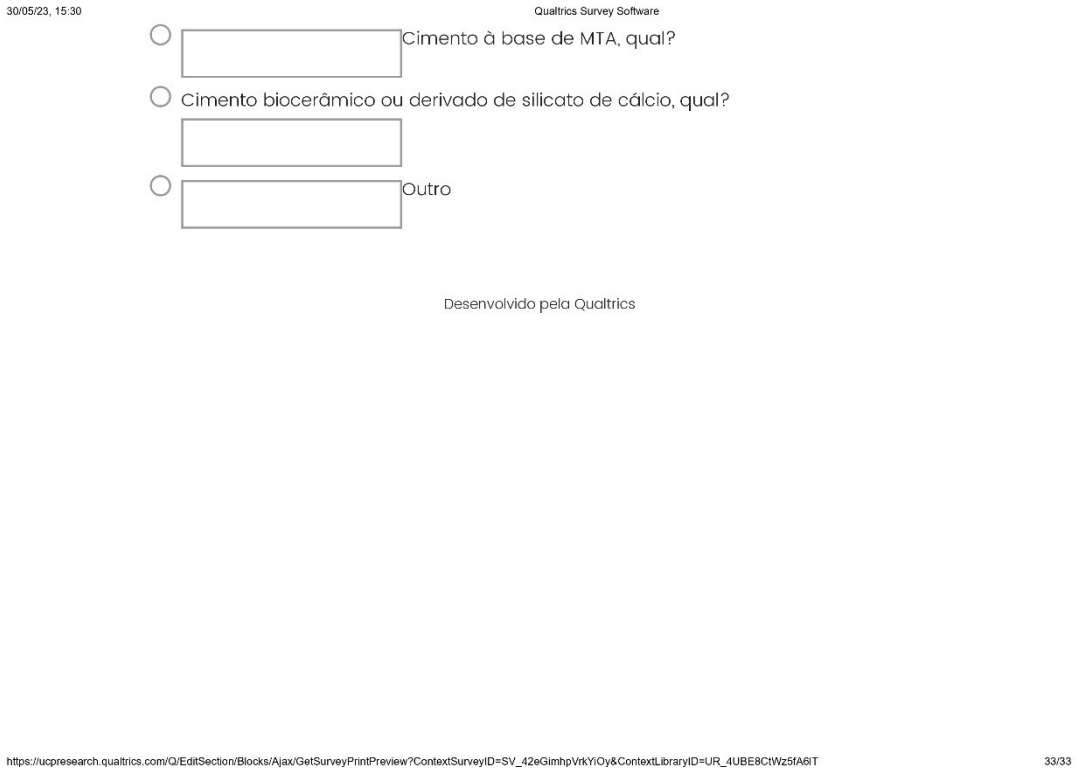
- ○ Sim
- ○ Não

Se Sim, qual a medicação utilizada?

Qual material será utilizado na restauração temporária?

Qual dos seguintes materiais será utilizado na obturação definitiva:

- ○ Cimento à base de resina, qual?



Attachment 4 – Periodontal Diagnostic Table

Aspect	Criteria	Details
Type of Periodontal Disease	Periodontal Health	No signs of inflammation or periodontitis.
	Gingivitis	Inflammation without loss of attachment.
	Periodontitis	Loss of attachment and bone.
	Other Conditions	Necrotizing diseases, periodontitis as a manifestation of systemic disease, etc.
Extent of Disease	Localized	≤ 30% of teeth affected.
	Generalized	> 30% of teeth affected.
	Molar/Incisor Pattern	Specific pattern affecting molars and incisors.
Staging	Stage I (Initial)	Interdental CAL 1-2 mm, bone loss in the coronal third (<15%), no tooth loss due to periodontitis.
	Stage II (Moderate)	Interdental CAL 3-4 mm, bone loss in the coronal third (15-33%), no tooth loss due to periodontitis.
	Stage III (Severe)	Interdental CAL ≥5 mm, bone loss extending to the middle

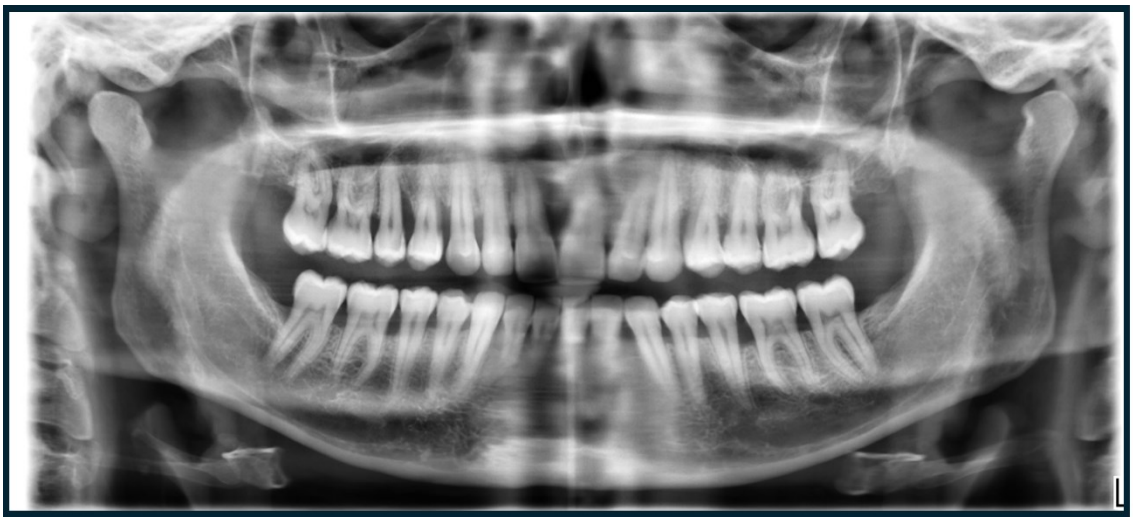
		third of root and beyond, ≤4 teeth lost due to periodontitis.
	Stage IV (Advanced)	Interdental CAL ≥5 mm, bone loss extending to the middle third of root and beyond, ≥5 teeth lost due to periodontitis.
Grading	Grade A (Slow)	No loss over 5 years, <0.25% bone loss/age, heavy biofilm deposits with low levels of destruction.
	Grade B (Moderate)	<2 mm loss over 5 years, 0.25-1.0% bone loss/age, destruction commensurate with biofilm deposits.
	Grade C (Rapid)	≥2 mm loss over 5 years, >1.0% bone loss/age, destruction exceeds expectations given biofilm deposits, risk factors such as smoking/diabetes.
Current Disease Status	Periodontal Health	No BOP, PPD ≤ 3 mm, no CAL.
	Gingival Inflammation (Remission)	BOP ≥ 10%, PPD ≤ 4 mm, no CAL.
	Periodontitis Currently Stable	BOP < 10%, PPD ≤ 4 mm, no BOP at 4 mm sites.
	Periodontitis Currently Unstable	PPD ≥ 5 mm or PPD 4 mm with BOP.

Attachment 5 – Teeth Numbering Table

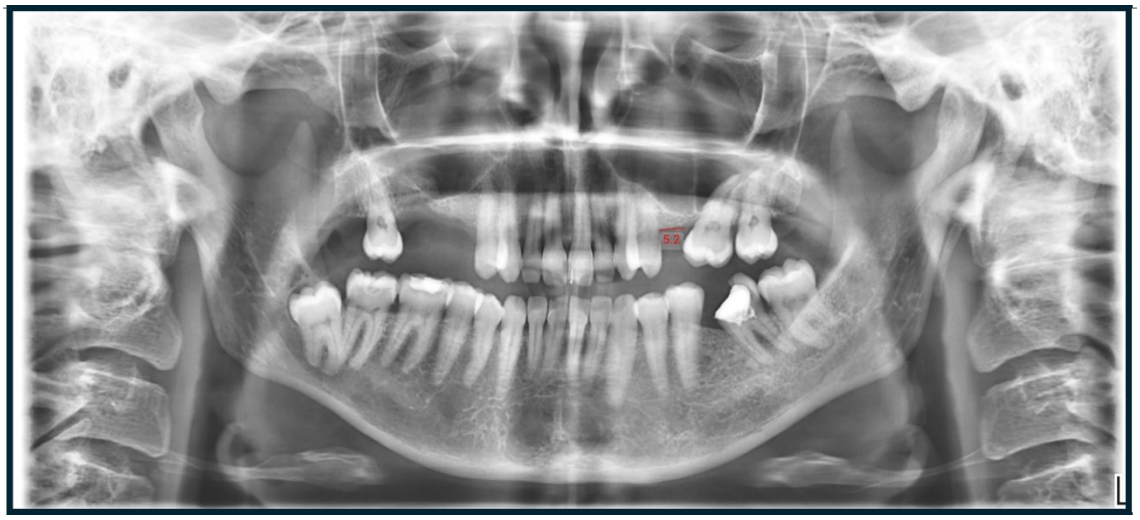
Tooth Name	**Number**	**Quadrant**
Central Incisor	11	Upper Right
Lateral Incisor	12	Upper Right
Canine	13	Upper Right
First Premolar	14	Upper Right
Second Premolar	15	Upper Right
First Molar	16	Upper Right
Second Molar	17	Upper Right
Third Molar (Wisdom)	18	Upper Right
Central Incisor	21	Upper Left
Lateral Incisor	22	Upper Left
Canine	23	Upper Left
First Premolar	24	Upper Left
Second Premolar	25	Upper Left
First Molar	26	Upper Left
Second Molar	27	Upper Left
Third Molar (Wisdom)	28	Upper Left
Central Incisor	31	Lower Left
Lateral Incisor	32	Lower Left
Canine	33	Lower Left
First Premolar	34	Lower Left

Second Premolar	35	Lower Left
First Molar	36	Lower Left
Second Molar	37	Lower Left
Third Molar (Wisdom)	38	Lower Left
Central Incisor	41	Lower Right
Lateral Incisor	42	Lower Right
Canine	43	Lower Right
First Premolar	44	Lower Right
Second Premolar	45	Lower Right
First Molar	46	Lower Right
Second Molar	47	Lower Right
Third Molar (Wisdom)	48	Lower Right

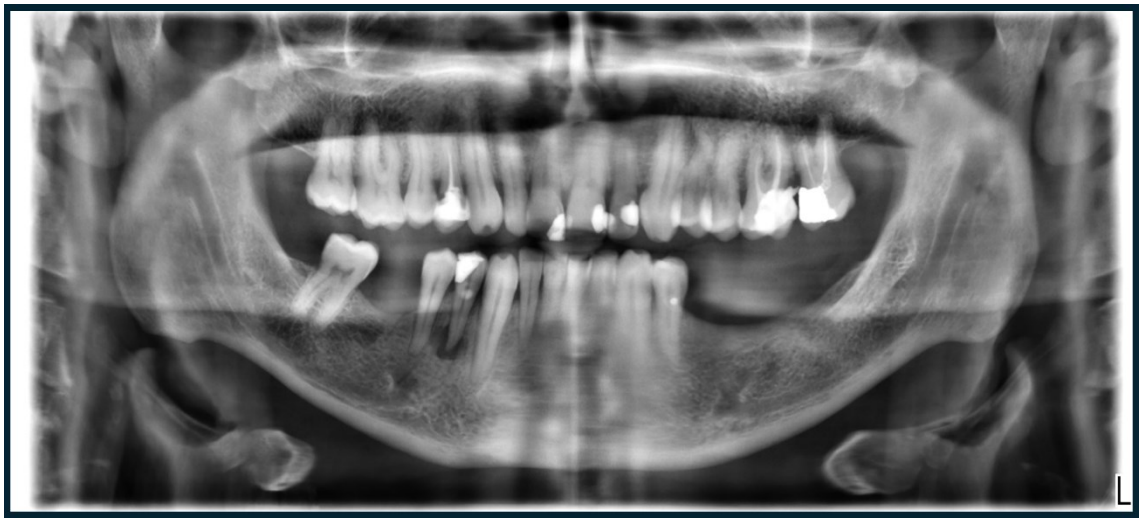
Attachment 6 – Patient SP4, orthopantomography time 1



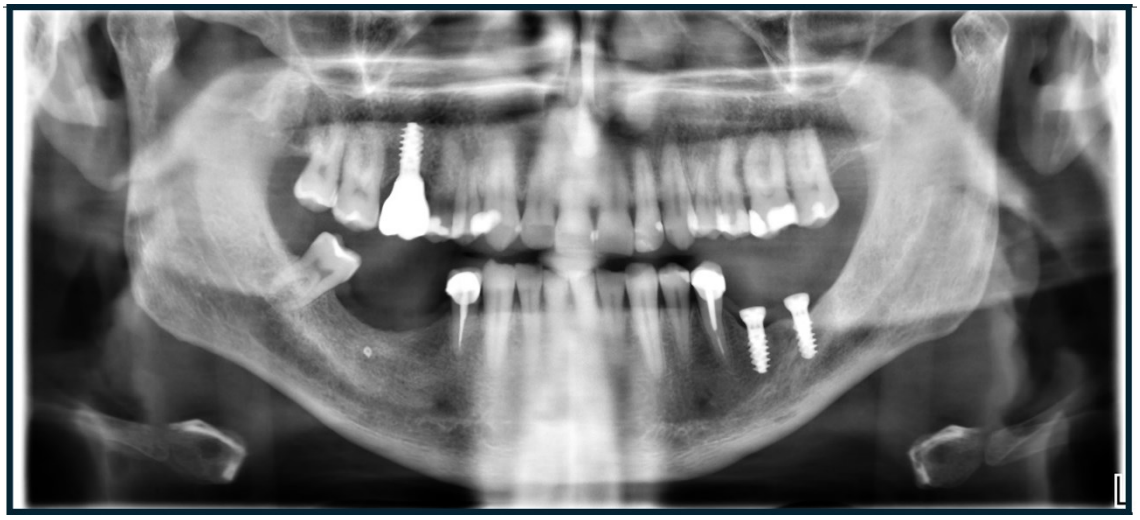
Attachment 7 – Patient SP7, orthopantomography time 1



Attachment 8 – Patient SP8, orthopantomography time 1



Attachment 9 – Patient SP9, orthopantomography time 1



Attachment 10 – Patient SP10, orthopantomography time 1