Microbiological background of the Medical-Related Osteonecrosis of the Jaw

Doctoral (PhD) thesis

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1. INTRODUCTION

Bisphosphonate (BP)-induced osteonecrosis of the mandible and maxilla was first described as a diagnosis and disease in 2003 as induced vascular necrosis of the jaw. In their publication, Marx R.E. *et al.* drew attention to a previously unrecognized and unreported severe side effect of these agents and the need for caution when using these drugs (Marx R.E. *et al.,* 2003).

Bisphosphonates are chemically stable analogues of inorganic pyrophosphates (PPi). The observation in human medicine that PPi and BPs delay not only bone growth but also the dissolution of hydroxyapatite crystals has prompted studies on inhibiting bone resorption. Although PPi could not do so, BPs were highly effective in inhibiting bone resorption by inhibiting osteoclast activation in both *in vitro* and *in vivo* experimental systems, this effect was eventually confirmed in humans as well. The aim was to prevent pathological fractures, inhibit intraosseous tumour enlargement, reduce bone pain and control hypercalcaemia.

The first geminal bisphosphonate used in human therapy was etidronate (Smith R. *et al.*, 1976). Following the success of this first treatment, bisphosphonates in human medicine have become increasingly popular and more and more patients are now receiving this therapy. The main indications are Paget's disease, multiple myeloma, primary breast and prostate metastases in bone and osteoporosis (Reid IR. and Hosking DJ. 2011; Coleman RE. and McCloskey EV. 2011; Eastell R. *et al.*, 2011; Guarneri V. *et al.*, 2010). Bisphosphonates have a broad spectrum of uses and can provide a good quality of life for a long time. However, new side effects have been reported as their therapeutic use has become more common and combination and sequential therapies have emerged. The most severe late side effect is Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ), which was renamed **MRONJ** (Medication-Related Osteonecrosis of the Jaw) by the American Association of Oral and Maxillofacial Surgeons (AAOMS) in 2014 (Ruggiero SL. et al., 2014). In 2022, the AAOMS made the most recent change to the MRONJ recommendation (Ruggiero SL. et al., 2022). Due to frequent use and therapeutic combinations, osteonecrotic side effects have also become more frequent and severe. The pathophysiology and aetiology of MRONJ are still not fully understood, so it is important to identify and better understand the underlying and maintaining causes of the pathology. Changes in the qualitative and quantitative composition of the oral microbiome, which is mainly composed of anaerobic bacteria, and thus inflammation induced by bacterial infection of the jaw bones, may play an important role in the development of MRONJ. Based on current research, the role of changes and transformations of the oral microbiome in the pathology is unclear. Some literature suggests that the pathogenic role of Actinomyces and Actinomyces-like Organisms (ALOs) strains, which are members of the anaerobic microbiota, is particularly prominent in the development and exacerbation of the disease (Cerrato A. et al., 2021). If it is confirmed that Actinomyces species are present and present in higher germ numbers in affected tissues and that their local inflammation plays a role in the clinical course and prognosis of MRONJ; it would be important to consider this for prevention and therapy. The microbiological cultivation used so far can confirm the presence of the pathogen. However, this requires a properly equipped microbiological laboratory background, where the specific

culture requirements (anaerobic atmosphere, appropriate media, incubation for at least 14 days) can be met. Nowadays, using modern molecular diagnostic methods (Polymerase Chain Reaction: PCR, 16S rRNA sequencing, whole genome analysis: WGS), species-level quantitative detection is possible (Könönen E. and Wade WG. 2015, Zhao K. et al. 2014; Panya S. et al., 2017). Recent research has revealed a possible link between bisphosphonates and their induced immune dysfunction, leading to increased susceptibility to oral infections (Roato I. et al., 2023). This new perspective challenges the previous belief that the oral microbiome directly causes MRONJ. Other factors, such as systemic diseases like rheumatoid arthritis (RA) and diabetes mellitus (DM), may affect the immune system's resistance and the body's ability to respond to infections and inflammation (Chang J. et al. 2018). It is hypothesized that patients with MRONJ have reduced immune resistance, which may affect their ability to cope with N-BP-induced immunological stress (Kalyan S. et al., 2015).

2. LITERATURE REVIEW

2.1. Medication-Related Osteonecrosis of the Jaw (MRONJ)

2.1.1. Aetiology and pathomechanism of the disease

Bone necrosis is the condition when bone cells die due to any effect that may affect part or all the bone. Osteonecrosis of the jaw occurs when there is a denuded bone surface in any area of the jawbone. The lesion persists within 8 weeks of the first follow-up, and the affected area shows no tendency to heal. There is no history of head and neck radiotherapy (Lončar Brzak B. et al. 2019). In terms of aetiological factors, the pathology can be divided into osteoradionecrosis due to radiotherapy, traumatic and non-traumatic jawbone death, idiopathic jawbone death, which is also considered a rare case in jawbone literature, and drug-induced jawbone death (Lončar Brzak B. et al. 2023). The incidence of MRONJ varies considerably depending on the class of drug, dosage and route of administration, ranging from 0.4% to 21% (Kim HY. et al., 2024) Currently, five main factors, namely immune dysfunction, drug class-dependent effects of bisphosphonate (BP), bone remodelling, inflammation/infection and the oral microbiome are thought to play a role in the development of MRONJ (Jelin-Uhlig S. et al. 2024) (Figure 1). The clinical picture suggests that osteonecrosis and MRONJ can only be clearly distinguished if an accurate medical history is known (Studer G. et al., 2016).

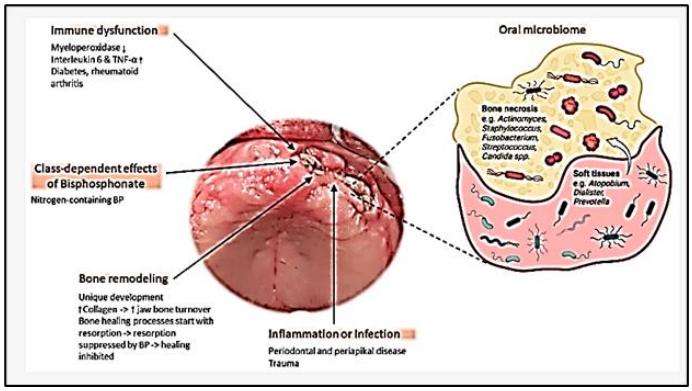

Figure 1. Five major factors involved in the pathophysiology of osteonecrosis of the jaw (Jelin-Uhlig S. *et al*. 2024).

2.1.2.Staging of the disease

In addition to bisphosphonates, osteonecrosis in the jawbone can be provoked by vascular endothelial growth factor inhibiting factors (VEGF), including bevacizumab (Guarneri V. et al., 2010) and aflibercept (Mawardi H. et al., 2017), by sunitinib, a tyrosine kinase inhibitor (Soós B. et al., 2015), by sorafenib and cabozantinib (Sánchez López JD. et al. 2021) and monoclonal antibodies such as denosumab.

MRONJ criteria:

1. The patient has had or is currently undergoing antiresorptive therapy - alone or combined with immunomodulators- and/or antiangiogenic therapy.
2. Denuded bone surface, or maxillofacial region of the jawbone area, that can be examined through an extra- and/or intra-oral fistula, with a lesion present for more than 8 weeks.
3. No history of radiotherapy to the jawbone or metastatic bone metastases.

In MRONJ, different disease progression stages are distinguished and classified using the "staging" system. The AAOMS recommendation is not the only classification system in force; other international organisations have also developed a consensus (Campisi G. et al., 2020). In this paper, I will describe the AAOMS recommendation for the classification of MRONJ according to the five groups of pathological severity (risk group, stages 0-III):
Risk group refers to patients who had received or are receiving intravenous or oral antiresorptive therapy and are not currently diagnosed with jaw necrosis.

Stage 0: Patients classified in this stage are those with no evidence of necrotic bone surface on clinical examination but with nonspecific complaints (tooth pain without tooth origin; dull jaw pain radiating to the temporomandibular joint; facial pain; altered neurosensory function), clinical (tooth loss, intra- and extraoral swelling) and/or radiological (alveolar bone loss; alteration of trabecular structure, periodontal ligament thickening) abnormalities. Considering that the chance of progression from stage 0 to stage 1 is close to 50%, the AAOMS recommends that particular attention should be paid to the accurate recognition of symptoms, as this will allow early diagnosis of the disease (Fedele S. et al., 2010).

Stage I: The jawbone shows denuded and necrotic bone surface or probing fistulae without inflammatory symptoms. Radiological abnormalities may be consistent with those of the processus alveolar described in stage 0.

Stage II: The jawbone is denuded, and necrotic bone surface or probing fistulae are seen with inflammatory signs. Radiological abnormalities are limited to the processus alveolaris, as described in stage 0.

Stage III: In addition to the symptoms of stage II, at least one of the following criteria must be met:
- the necrosis extends beyond the processus alveolaris (ramus mandibulae, sinus maxillaris, zygoma)
- pathological fracture
- extraoral fistula
- oronasal or oroantral fistula
- osteolysis extending to the base of the mandible or the base of the maxillary sinus.

2.1.3 Diagnosis, differential diagnosis

Clinically, the most important sign is a denuded necrotic bone surface, which has been present for more than 8 weeks without the tendency to heal and can be examined through an/extra-oral or intra-oral fistula. This area has no history of head and neck radiotherapy or bone metastasis. The patient's past and or current medications include antiresorptive therapy, possibly combined with immunomodulators or antiangiogenic therapy. The diagnosis can be made if all three criteria are met (Ruggiero SL et al., 2022). For differential diagnosis, it is important to distinguish it from osteonecrosis of the jaw caused by other impacts (trauma, radiotherapy, etc.), as well as from other cases of osteitis or even malignant bone diseases (sarcoma), chronic sclerotic osteomyelitis, and fibro-osseous lesions with varied symptoms and appearance (Kün-Darbois JD. and Fauvel F. 2021).

2.1.4. Risk factors

The development of MRONJ is a multifactorial process which can be influenced by several factors simultaneously. Nothing demonstrates its multifactorial origin better than the fact that the incidence of the disease has a variable spectrum of 0.4%-21% worldwide (Faiman B. et al. 2013; Khan AA. et al. 2015; Kos M. 2015; Owosho AA. et al. 2016; Bagan J. et al. 2016; Limones A. et al. 2020; Soares AL. et al. 2020; Hajeri S. and Alturkistany Y. 2022; Amigues C. et al. 2023; Kim HY. et al. 2024). The pathogenesis of the disease can be profoundly influenced by the properties of the therapeutic agent used and its route of administration, its quantity, duration, co-morbidities and drug regimens, and other additional local and systemic factors (Kos M., 2015). Nowadays, the number of therapeutic agents used, their indications and possible combination therapies have increased the incidence of ORJ, and the presence of risk factors cannot be avoided. Therefore, knowledge and systematisation of these factors can provide useful guidance in therapy and managing and possibly preventing adverse effects.

2.1.5. Therapeutic options

Distinct stages of the disease can be classified according to the advanced nature of stages; thus, different stages may require varied treatments. The most important thing is to inform and educate patients about the disease process. The main task of the treating physician is to prevent deterioration in the quality of life, which can be prevented by pain relief and by preventing the spread of superinfection and necrosis. Surgical care, antibiotic therapy, which can be used as pre-, post- and conservative therapy, maintenance of adequate oral hygiene, mouthwash and frequent dental check-ups, proper control of DM, and avoidance of smoking can all form part of the broad therapeutic and preventive spectrum of MRONJ (Kunchur R. and Goss AN. 2008; Lodi G. et al., 2010; Bonacina R. et al., 2011).

2.1.6. Prevention

Primary prevention is defined as dental decontamination: Patients undergo a mandatory, thorough dental check-up before starting bisphosphonate and other drug therapy for jaw necrosis, where dental decontamination is performed if necessary (Nicolatou-Galitis, O. et al. 2019). The aim is to eliminate oral foci, which, combined with education, can ensure good oral hygiene and reduce additional pathological risk factors (Soares AL. et al. 2020), thus

ensuring the patient has the lowest chance of developing MRONJ. Primary prevention is recommended to include not only a dental check-up before therapy but also continuous monitoring during and after drug therapy.

Secondary prevention, or early diagnosis, is an important pillar of the disease and includes avoidance or reduction of smoking and drug and alcohol use. Patients can be categorised as high risk and low risk based on the factors influencing the risk of jaw necrosis (Veréb T. et al., 2020; Kammerhofer G. et al., 2022) (Table 1).

Low-risk patients	High-risk patients
Systematic	
Benign osteologic disease	Malignant disease
Single RA or VEGF therapy	Combined/Concomitant AR+VEGF therapy
Per os administration (p.o)	Intravenous administration (i.v.)
Low cumulative dose, short-term therapy	High cumulative dose, long-term therapy
Lack of comorbidity (DM, AR)	Presence of concomitant disease (DM, AR)
Absence of drug treatments	Other drug treaatments: steroid, immuntherapy
Local	
No previous MRONJ in patient's history	History of previous MRONJ
Adequate compliance	Inappropriate compliance
Good oral hygiene	Inappropriate oral hygiene
Well fixed denture	Inappropriate denture

Table 1. "Low and high-risk" factors (Veréb T. *et al.* 2020; Kammerhofer G. *et al.* 2022).

2.2. Relationship between MRONJ and the oral microbiota

2.2.1 Role of the microbiota in MRONJ

The oral microbiota represents various species/subspecies of microorganisms in dynamic and polymicrobial communities. The oral microbiota is a physiological part of the oral cavity with important functions such as protection against pathogenic bacteria that can be detrimental to overall health; together, these microbial communities are the main drivers of homeostasis and dysbiosis in health and disease (Gao L. et al., 2018; Baker JL. et al., 2024). Alteration of this highly susceptible ecosystem, the proliferation of pathogenic microorganisms with different additive virulence factors leading to ecological imbalances, up to a dysfunctional immune system, can significantly impact both local and systemic health. The oral microbiota also has a significant impact on the immune system of the human host and thus plays an important role not only in oral health but also in systemic health. A widely held hypothesis (Sedghizadeh PP. et al., 2008; 2009; Street (J. et al., 2002). Bones maintain their health if not

injured or infected by pathogenic oral bacteria. The combination of infection with BP-induced bone resorption loss and blood vessel damage may inhibit the process of new bone formation, thus promoting the development of MRONJ, suggesting that bacterial colonisation in MRONJ may originate from the oral microbiome, influenced by BP treatment and subsequent changes in oral/bone health (Aspenberg P. 2002, Bi Y. et al. 2010; Migliorati CA. et al. 2005). Whether the oral microbiome plays a causal role in MRONJ is complex and has several implications. Several studies show that the oral microbiome cannot be considered a direct causative agent of BRONJ, as individuals treated with BP typically have an immune deficiency due to their underlying disease. Therefore, additional immune stress caused by BP treatments may increase the risk of developing BRONJ (Kalyan S. et al., 2015). Other studies show that the bacteria associated with MRONJ-related bone infections may differ from those found in other oral bone infections (Sedghizadeh PP. et al. 2008, 2009), which include various microorganisms found in MRONJ lesions that are responsible for opportunistic infections affecting bones, joints and teeth (Wei X. et al., 2012; Jabbour Z. et al., 2016).

2.2.2. Microbiology of actinomycetes

2.2.3. Role of actinomyces in dental pathologies

The Actinomyces species belong to the family "Actinomycetaceae", together with other genera such as Actinomyces, Actinotignum, Arcanobacterium, Schaalia and Varibaculum and "Actinomyces-like organisms" (ALO). The genus Actinomyces currently consists of 46 species and two subspecies, which have been characterized by molecular and phenotypic methods.

Members of Actinomyces spp. are branched, coryneform Gram-positive rods, except for a few clinically relevant species, are facultative anaerobic bacteria. Filamentous, microscopic colonies of Actinomyces spp. strains can multiply within 2-4 days after incubation in an anaerobic environment at 37°C, but usually, 7-14 days are required for colony culture (Könönen E., 2020). Actinomyces infections can be divided into head and neck (including central nervous system), abdominal, thoracic (lung), pelvic and skin infections, the most clinically common type being cervicofacial infections. Infections caused by Actinomyces strains are endogenous infections that arise after damage to standard physiological barriers, such as the oral mucosa, which allow microorganisms to enter soft tissues. The characteristic lesion of actinomycosis is an indurated area of several small communicating abscesses surrounded by granulation tissue (Bojanova L. et al., 2015). The lesions contain so-called "sulphur" granules, which are sulphur-free, so called because of their yellowish appearance; they consist of a tangled mass of branched

filaments of Actinomyces bacteria. Damage to the physiological barrier in the oral cavity can occur due to trauma, such as tooth extraction, jaw fracture or other traumatic injuries, periodontal surgery, but also due to deep periodontal pockets or infected root canals. Most Actinomyces infections are polymicrobial, i.e. several aerobic-anaerobic microorganisms with different virulence factors play a pathogenic role in the infection (Brook I., 2008). In general, members of the genus Streptococcus are the most associated organisms (Doran A. et al., 2004), which act synergistically by inhibiting host defence mechanisms and significantly reducing the oxygen potential in the tissue, thereby increasing the proliferation of anaerobic bacteria. To understand the virulence factors of pathogenic Actinomyces strains, actinomycosis infection experiments have been performed in mice (Jordan HV. et al., 1984), where histological evidence showed that polymorphonuclear leucocytes are unable to penetrate the centre of granulomatous bacterial lesions, thus failing to reach the bacteria within the granules and that these surviving bacteria promote Actinomyces cell viability and proliferation by creating an enhanced anaerobic environment. Thus, the accompanying co-pathogenic bacteria can produce toxins and enzymes and inhibit the host's defences. Some research suggests that Actinomyces species play a role in oral biofilm formation (D'Amore F. et al., 2020).

2.2.4. Relationships between actinomycetes and MRONJ

In MRONJ, the accumulation of bisphosphonates in tissues is thought to be the primary cause of osteonecrosis, as bisphosphonates inhibit bone repair and lead to vascular necrosis. However, observations have challenged this hypothesis, suggesting that bisphosphonates may promote actinomycosis infection, leading to osteonecrosis. Several preclinical and clinical studies suggest that infection plays a significant role in pathogenesis, the so-called "infection hypothesis". However, there is not enough conclusive evidence to support this hypothesis. There is no clear evidence of whether the colonisation of *Actinomyces* species strains and significant changes in their qualitative and quantitative ratios contribute to the inflammation that causes bone necrosis or whether they colonise already dead bone. Nevertheless, available research in the literature shows a strong correlation between local infection and the development of MRONJ.

2.3. Drugs resulting in osteonecrosis of the jaw

2.3.1. Antiresorptive drugs

A: Bisphosphonates: zoledronic acid, alendronic acid

The main reason for the clinical human therapeutic use of BPs was that BPs have been shown to inhibit bone resorption (Russell RG. *et al.*, 2008). Bisphosphonic acids are compounds containing two phosphonic acid groups and a P-C-P bond, like the P-O-P structure of pyrophosphate, an important building block of bone. The diverse structure influences the potential for antiresorptive activity and binding to hydroxyapatite. The molecular structure of bisphosphonate may influence several physical factors including solubility and membrane penetration (Drake MT. *et al.*, 2008) These synthetic pyrophosphate analogues can be divided into two groups based on R2 side chain: one group is nitrogen-containing amino-bisphosphonates (N-BP): zoledronic acid, ibadronic acid, pamidronic acid, alendronate, risedronate, the other group is non-nitrogen-containing bisphosphonates (etidronic acid). The simple BPs are like PPi (e.g. clodronate, R2=Cl) and were the first absorption inhibitors developed for clinical use. Nitrogen-containing bisphosphonates (N-BPs) inhibit bone resorption *in vivo* more than simple bisphosphonates.

Bisphosphonates not only affect the osteoclast-osteoblast-osteocyte system but also directly affect the local vasculature. In addition, *in vitro* studies on the effects of bisphosphonates on progenitor cells are ongoing. Experiments with an endothelial cell line differentiated from placental mesenchymal stem cells have demonstrated that nitrogen-containing bisphosphonates (N-BP) have inhibitory effects on endothelial cell migration, differentiation, function and morphogen properties (Sharma D. *et al.,* 2016). Low therapeutic doses of BPs increase the proliferative capacity of fibroblasts but reduce the expression of genes required for their growth and differentiation, thus limiting the functional capacity of fibroblasts and, in combination with other factors, increasing the risk of MRONJ development (Manzano-Moreno FJ. *et al.*, 2019). Experiments on oral mucosa of healthy humans and cultured human oral keratinocytes have demonstrated that BPs affect epithelial cell adhesion, differentiation, proliferation, migration, senescence and promote apoptosis (Oike A. *et al.* 2022). Bisphosphonates have several routes of administration: alendronate is given orally (po), neridronate intramuscularly (im) and zoledronic acid intravenously (iv). The inhibition of bone remodelling by BPs is one of the key factors that provoke MRONJ (Tetradis S. *et al* 2023). The effect of BPs on bone remodelling and the different developmental mechanisms of the jaw bones compared to long bones may explain the predominance of MRONJ in the jaw. While the

maxilla and mandible are formed by intramembranous bone development, long bones undergo endochondral ossification. This difference in developmental pathways leads to significant anatomical differences, including differences in bone density and the balance of cortical and spongy bone and bone marrow spaces. Interestingly, human mandibles have higher collagen content and lower levels of hydroxylysine enzyme than long bones, which may contribute to the higher rates of osteonecrosis in the jawbone (Sasaki M. *et al.*, 2010). In addition, BPs have anti-angiogenic properties: they inhibit proliferation, adhesion and migration of human endothelial cells and suppress angiogenesis (Vincenzi B. *et al.*, 2005). These studies emphasize the multifactorial pathophysiology of MRONJ, which is triggered by a combination of immune dysfunction, altered gene expression and anti-angiogenic effects.

B: Monoclonal antibodies (MAB): denosumab

Even though bisphosphonate and denosumab therapy have the same indication, their mechanisms of action are different (Malan J. *et al.*, 2012). While bisphosphonates act at the cellular level, i.e. via osteoclasts, denosumab acts in the extracellular space (Baron R. *et al.*, 2011). Denosumab is a monoclonal antibody (IgG2) that acts along the RANK-RANKL-osteoprotegerin axis (Jakab L., 2014). The antiresorptive molecule binds to RANKL with high affinity and selectivity, preventing the activation of its receptor (RANK) on the surface of osteoclast precursors and adult osteoclasts, and therefore reducing osteoclast maturation, activity and bone resorption (Taylor KH. *et al.*, 2010; Tofé VI. *et al.*, 2020).

2.3.2. Antiangiogenic agents

A. Vascular endothelial growth factor (VEGF) inhibitors

Bevacizumab: The human monoclonal antibody G1, which belongs to the group of anti-angiogenic drugs, inhibits binding to the so-called VEGF receptor (Morita Y. *et al.*, 2020). VEGF is one of the factors responsible for angiogenesis and vasculogenesis, and bevacizumab thus reduces tumour vascularisation and inhibits tumour growth.

Aflibercept: Aflibercept is a recombinant fusion protein in which the extracellular domains of the human VEGF receptor fuse with the Fc portion of human immunoglobulin G1 (IgG1) (Clarke JM. and Hurwitz HI. 2013). VEGF-A and PlGF are members of the VEGF family of angiogenic factors, where the P1GF factor acts synergistically with VEGF-A, a

relationship responsible for vascular permeability. Inhibition of the receptors blocks tumour angiogenesis and vascular permeability.

B. Tyrosine kinase inhibitors: sunitinib, sorafenib and cabozantinib

Sunitinib: The tyrosine kinase receptor family has been shown to affect tumour growth, pathological angiogenesis, and metastasis spread (Soós B., 2015; Ramírez L. *et al.*, 2015). Sunitinib inhibits members of the tyrosine kinase receptor family (PDGFRD, PDGFRE, VEGFR1-2-3, KIT, FLT+, CSF-1R, RET) and thus signal transduction pathways that influence the induction of angiogenesis and tumour progression (Hoefert S. *et al.*, 2010).

Sorafenib: Sorafenib is a multikinase inhibitor inhibiting tumour vascularisation (CRAF, VEGFR-2, VEGFR-3 and PDGFR-ß), reducing tumour cell proliferation and angiogenesis. Its main indications are hepatocellular and renal carcinoma.

Cabozantinib: Cabozantinib is most used to treat renal carcinoma pancreatic and gastrointestinal stromal tumours. It inhibits several tyrosine kinase receptors, so it may interfere with signalling processes that stimulate tumour growth and vascularisation, reduce metastasis progression, and inhibit pathological bone remodelling.

3. OBJECTIVES

Our research aims to:

- compare the medical history of patients with medical related osteonecrosis of the jaws in our patient database: mean age, gender, underlying diseases, predisposing factors, therapy received, its mode of administration, duration of treatment, with international data.

- confirm the presence of **anaerobic** bacterias, including ***Actinomyces*** species, and to characterise the quality and quantity of the oral microbiota, which can be detected by culture from bone fragments surgically removed from patients.

- confirm, both by culture and PCR, the higher prevalence of *Actinomyces* species in the MRONJ patient material compared to the control group and the other bacterial species that co-occur in the bone microbiota.

- randomly selected 5-5 cases will be analysed for differences in the composition of the bone microbiome, alpha and beta diversity.

- investigate whether there is a correlation between the clinical status of patients, risk factors for disease and the microbiota composition as detected by culture of bone biofilms.

- long-term (1 month, 3 months, 6 months) follow-up of the patients' condition, observing the course of the disease and the incidence of possible recurrences after postoperative conservative therapy.

4. PATIENT MATERIAL AND TEST METHODS

4.1 Selection of patients and control groups

The study included 35 patients with various stages of osteonecrosis of the jaws who received bisphosphonate or other drug therapy either per os or intravenously, before the present surgery. The control group consisted of 35 otherwise healthy individuals who had primarily undergone wisdom tooth extraction or other tooth extraction with bone removal. In our study, five patient samples were randomly selected from patients with MRONJ and five samples from patients in the control group, already selected for qualitative and quantitative microbiological analysis based on clinical parameters using conventional culture techniques and whole genome sequencing in order to compare differences in the microbiomes of the two groups.

MRONJ patients

Patients included in the study were clinically classified as Stage I, II or III. At these stages, the denuded bone surface is already visible, from which appropriate sampling is possible, and only conservative therapy cannot be used in the long term given the complaints, clinical symptoms and advanced stage of the process. Due to the extent of bone loss, all these patients required surgical treatment. The denudation in the oral cavity, which can maintain a permanent inflammation in the body, can be a source of infection and cannot be eliminated by other therapies. The process also resulted in tooth loss in all patients, further increasing the nutritional difficulties and deterioration of quality of life. Patients were further classified according to both high and low MRONJ risk factors. The patient's gender, age, use of stimulants (alcohol, smoking, etc.), and oral hygiene status were considered. In addition, we analysed the type of osteonecrosis drug(s) used, the duration of therapy used, the intravenous or per os use of the medication, the underlying disease justifying the therapy and other underlying diseases.

The surgical intervention performed for curative purposes in all patients during the study period involved the removal of the necrotic area. The sampling for the study did not involve any other repeated intervention, pain or stress (physical or psychological) for the patient, as the necrotic bone fragment, which would have been removed under local anaesthesia or

anaesthesia, was sampled. In all cases, the operation aimed to remove the necrotic bone fragment, and the particle sent for analysis was taken from this resected bone for microbiological analysis, the size of the bone fragment removed being determined by the degree of necrosis.

Control group

The control group consisted of otherwise healthy individuals without malignant disease, immunosuppressed, non-gravid, not receiving bisphosphonate or other drug or radiological therapy for maxillary necrosis, who had undergone tooth extraction for any reason. Tooth extraction and microbiological sampling were performed in parallel with the patient group and during the same period. In some cases, the necessary tooth extraction involved bone removal, such as wisdom teeth and other teeth or tooth roots removed. Again, the bone particles for microbiological analysis were taken from bone fragments already necessarily removed during surgery.

Ethical approval

The study design and the ethical approval of the patient consent form and the patient information leaflet were approved by the University of Pécs Regional and Institutional Scientific and Research Ethics Committee, ethical approval number 9503-2023 University of Pécs (PTE)/2023. The design of the investigation was in line with data protection standards and the Helsinki Declaration.

4.2. Microbiological sampling

Microbiological sampling was performed during surgery for the patients and the control group. Five minutes before the procedure, patients and control group members were disinfected intraorally by washing the affected area and the whole oral cavity with a 0.2% chlorhexidine solution for approximately 1-2 minutes. Subsequently, a piece of necrotic bone (approximately 2-3 mg), representative of the necrosis, was removed from the patients using a sterile instrument (bone pliers, drill, etc.) and placed in the anaerobic transport medium using sterile forceps. For the control group, alveolar bone fragments that had been removed were placed similarly in the anaerobic transport medium.

4.3. Microbiological processing of samples

4.3.1. Conventional culture method

Bone samples received by the laboratory were processed immediately after receipt. Necrotic bone fragments to be removed, or alveolar bone fragments for the control group, were placed in 1.0 ml of reduced BHI (Brain Heart Infusion pH 7.2) broth (Oxoid, Bakingstoke, UK) using sterile forceps and homogenised in a Stomacher (Stomacher 80, Labsystem) for 30 seconds. The bone fragments placed in the test tube were pre-weighed, and the results were expressed as CFU/milligram (CFU/mg). A dilution series was prepared from the stock solution to a dilution of 10^{-1}-$10^{-6.}$ Fastidious Anaerobic Agar medium (FAA) supplemented with 5% (v/v) beef blood, haemins and vitamin K1 was used to isolate and enumerate all cultivable anaerobic bacteria. Cultured bacteria and fungi were enumerated by accurate germ counting, and strains with different telepmorphology were identified at the species level by mass spectrometry based on matrix-assisted laser desorption, ionization, time-of-flight (MALDI-TOF MS; (Bruker Daltonik, Bremen, Gr) identification method.

4.3.2. PCR method

DNA extraction

Total genomic DNA was extracted using the E.Z.N.A® Bacterial DNA Kit (D3350-00, Omega Bio-tek) following the "difficult to lyse microbes" protocol from 1.5 ml of liquid anaerobic culture grown in anaerobic medium (FAB) for 5 days at 37°C, according to the manufacturer's recommendation.

PCR amplification

The amplification was performed using the method described by Xia T. and Baumgartner J.C. in 2003. DNA isolated from *Actinomyces oris* type strain (VPI 12593/CDC W1544/) and aerobic cultures of *Enterobacter cloacae* strain were used as *Actinomycetales*-PCR positive and negative controls. After analysis, amplified PCR products were stored at -20°C until further processing.

4.3.3 DNA isolation, 16S rRNA gene library construction and MiSeq sequencing

DNA isolation was performed using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA). DNA concentrations of samples were measured using a Qubit fluorimeter with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Bacterial DNA was amplified with labelled primers covering the V3-V4 region of the bacterial 16S rRNA gene. PCR and DNA purifications were performed according to Illumina protocol. PCR product libraries were evaluated using DNA 1000 Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Gr.). Equimolar concentrations of the libraries were pooled, and next-generation sequencing (NGS) was performed with MiSeq® Reagent Kit vs3 (600 cycles) in an Illumina MiSeq System (Illumina, San Diego, CA, USA).

5. RESULTS

5.1. Statistical analysis of data from the patients' and control group's medical histories.

Patient group

Gender/ age

Of the 35 individuals selected as patients, 54% were female (19 patients) and 46% were male (16 patients). The youngest patient at the time of MRONJ diagnosis was a 40-year-old female patient, and the oldest was an 87-year-old male patient, with a median age at diagnosis of 67 years (67.4).

Indications for antiresorptive therapy

Antiresorptive treatment was required for 11 female patients with breast tumours (31%), 14 male patients with prostate tumours (40%), two male and one female patient with kidney tumours (8.5%), three female patients with lung tumours (8.5%), one female patient with multiple myeloma (3%), one female patient with colon tumours (3%), representing 33 cases (94%) of underlying malignancy with bone metastases. In two patients (6%), the osteonecrosis of the jaw was not related to a malignancy: 1 female patient (3%) was treated for Langerhans histiocytosis and one female patient (3%) for osteoporosis.

Co-morbidities, underlying diseases

In the patient group, 24 (68.5%) patients had a history of hypertension, and the patient was also receiving concomitant antihypertensive therapy. There was one obese patient in the patient group (BMI ≥35). DM was also investigated as another important underlying disease based on anamnestic data: 11 patients (31%) mentioned diabetes mellitus at admission, 10 patients in the study had a coexistence of both mentioned conditions, and 28.5% of the patients in the patient group had hypertension and diabetes mellitus together.

Stimulants

Our research assessed alcohol consumption, drug use and smoking habits. None of the patients reported drug use; 12 out of 35 patients (34%)/eight women (23%) and four men (11%)/were daily active smokers or had a history of smoking. Regular or occasional alcohol consumption was present in 6 male patients (17%), and in 5 cases, both stimulants were used (Table 2).

The therapeutic agents, the route and duration of administration:

Our study included patients treated with bisphosphonates (ibadronic acid, zoledronic acid) and denosumab. Most patients received intravenous zoledronic acid 32 (91%), intravenous ibadronic acid one patient, per os denosumab one patient and per os ibadronic acid one patient primarily at the diagnosis of bone lesions. For initial therapy, most patients, 33 patients (94%), received intravenous therapy, while only two (6%) received oral therapy. Based on the anamnesis, 26 (74%) cases had a change in the drug class causing jaw necrosis during treatment: this included a single drug change in 24 cases and a triple-drug change in two cases. A switch from intravenous zoledronic acid to per os ibadronic acid was made in 8 cases, to intravenous denosumab therapy in 18 cases, and in two cases, intravenous denosumab therapy was ordered again after the switch to per os ibadronic acid. In our patient population, the earliest onset of osteonecrosis of the jaw during therapy was 8 months in two patients: one male patient with renal cancer and one female patient with breast cancer. In the male patient, primary intravenous zoledronic acid therapy was ordered, and neither decontamination nor drug replacement was performed during the initiation of therapy. However, in the female patient, the primary IV zoledronic acid ordered was changed to per os ibadronic acid after two therapeutic doses, followed by subsequent tooth extraction, and then the non-healing denuded bone surface alerted us to MRONJ. The most extended treatment in the study was given to a female breast cancer patient, who was changed from primary intravenous ibadronic acid therapy to per os ibadronic

acid therapy 58 months later and presented to our department 162 months later with MRONJ-like complaints. The mean time between the start of therapy and primary oral maxillary osteotomy in our patients was 43 months (median 36 months). Thirteen patients were diagnosed with a primary malignancy at the time of diagnosis of bone metastasis. They were started on bisphosphonate, ibadronic acid or denosumab therapy in conjunction with oncological treatment (surgical or medication therapy).

5.2. Localisation and staging of MRONJ in the patient population

Twelve (34%) patients developed primary maxillary necrosis, one case involved both the mandible and the maxilla, and most of our patients (22 patients, 63%) had MRONJ in the mandibular region. Patients classified as **stage I, II or III** according to the classification were included in the patient group. In this study, three patients were classified as stage I in the internationally accepted classification, while most patients (19 patients 54.3%) were classified as stage II and 13 patients as stage III; however, despite the clear textbook definition of stages, the exact classification of patients in the clinic may be affected by the variable, overlapping, individual presentation of symptoms. From the point of view of patient care, the therapeutic recommendation varies from stage to stage, based on the international guidelines that we use. In the three stage I patients, conservative therapy was applied without any major surgical intervention, but bone sampling was possible during status check. In addition to thorough oral hygiene, restorative revision was recommended when necessary and these patients were followed up monthly under close supervision.

Conservative surgical therapy was used in all but 5 of the stage II and III patients. The general condition of the five patients (anaesthesia difficulties due to expected severe complications) did not allow adequate surgical treatment despite the advanced stage, and they were treated with additional conservative therapy in addition to antibiotic therapy. Sampling of necrotic bone was performed under local anaesthesia with asepsis before starting antibiotic therapy. Twenty-seven patients underwent adequate surgical intervention, most often for pathological fracture, bone necrosis beyond the alveolus, intra- or extraoral fistula causing permanent complaint, or oroantral or oronasal fistulae in patients with maxillary involvement. Alveolar correction, window resection, mandibular resection and partial maxillary resection were performed in addition to removing sequesters, which were often already mobile. The importance of the "drug holiday" (temporary suspension of bisphosphonate therapy) was

considered, and the treating physician who prescribed bisphosphonate and denosumab therapy was consulted before oral procedures.

All (100%) of the patients in the cohort required a shorter to longer (1-3 weeks) hospitalisation. Adequate intravenous antibiotic therapy was initiated at the appropriate dose and duration during the stay: most commonly, amoxicillin-clavulanic acid, supplemented with metronidazole if necessary, was used intravenously or occasionally per os, clindamycin or erythromycin was ordered in case of known penicillin drug allergy (1 patient). After discharge, 25 patients (71%) received additional per os antibiotic therapy at home due to disease recurrence.

Control group

Gender/age

The control group comprised 18 men (51%) and 17 women (49%). The gender distribution was selected to be like that of the patient group, so there was no significant difference between the patient and control groups ($p=0.632$). The control group included cases with no history of antiresorptive or antiangiogenic treatment, malignant neoplasm and its therapy, osteoporosis, head and neck radiotherapy, osteomyelitis or any other form of jaw necrosis. According to the inclusion criteria, no patients in the control group were gravid and had received antibiotic treatment 2 months before the operation. The average age of patients in the control group was 35 years (34.5 years at the time of the intervention). The youngest patient was a 17-year-old boy, and the oldest was a 78-year-old man. Since these patients were selected because they did not have severe underlying disease, often detected at a late age, but "only" for extraction due to dental indication, the average age was much lower than that of the patient group.

Co-morbidities

Five (14%) patients in the control group were treated for hypertension, while DM occurred in 8%. We found a significant difference between the control and patient groups for the two underlying conditions studied: diabetes $p=0.004$, whereas hypertension $p<0.001$. The apparent explanation for this could be the age difference. There were no obese patients (BMI ≤ 35) in the control group.

Stimulants

In the medical history, 54% (19) of patients reported that they had previously smoked but had quit or were still smoking, a higher prevalence than the patient group. Regarding alcohol consumption, a total of 9 (26%) patients reported regular or occasional consumption. Although members of the control group were younger than those in the patient group, none of the patients reported drug use based on their medical histories.

Causes of tooth extractions

In the control group, we selected patients who underwent tooth extraction with bone removal: 26 (74%) patients underwent the procedure for impacted mandibular wisdom tooth removal, in which the tooth extraction was not performed by conventional extraction but often required flap extraction, bone removal and tooth sectioning. In the remaining (9) cases, the reason for removal was root canal treatment, removal for orthodontic reasons, or prosthetic indication. The microbiological sample of the bone to be removed was approximately 0.5-1 cm. We examined the association between the **two groups (patient-control)** and the other relevant categorical variables (sex, smoking, etc.), with the sex ratio being the same, which was also one of the criteria for inclusion. Subjects in the control group were younger, as they were otherwise healthy, so this age difference is also due to the nature of the underlying disease in the patient group. There is no significant difference in the proportion of alcohol drinkers in the patient and control groups and no significant difference in the proportion of smokers in the patient and control groups, as the number of cases is statistically low, so we have considered the 10% level where there is already, more smokers in the control group. There is a significant difference in the proportion of diabetics in the patient and control groups, with significantly more diabetics in the patient group and a significant difference in the proportion of people with hypertension in the patient and control groups.

5.3. Microbiological results

5.3.1. Results of conventional culture methods

Conventional aerobic-anaerobic microbiological **culture** procedures yielded a mixed aerobic-anaerobic complex bacterial microbiota with a high bacterial count in all but one of the 35 patients. In the 66-year-old female patient, only aerobic bacteria could be isolated by culture. The proportion of strains of different anaerobic species cultured per patient ranged from 0-14

(mean: 5.6), for aerobic bacteria from 0-4 (mean: 1.3), and two patients had fungal colonies of spores and moulds. In the bone samples of healthy patients in the control group, 11 patients (31.4%) had no anaerobic bacteria to grow. The number of anaerobic strains isolated ranged from 0 to 6 (mean: 2.2). The number of isolated aerobic bacteria ranged from 0 to 3 (mean: 1.3), with 1 sample showing spores of fungi.

In the patient group, 185 anaerobic strains belonging to 65 different species were cultured. In contrast, 72 anaerobic bacterial strains of 27 species were cultured in the control group, so the species richness was much higher in the patient group. In the patient group, 23 (65.7%) patients had one or more ***Actinomyces*/ALO** species strains **isolated by culture** (detection limit: ca. 10^4 CFU/mg), compared to 6 patients: 17.1% in the control group, a highly significant difference between the two groups ($p<0.001$). Nine of the twenty-three MRONJ patients were cultured with one strain of *Actinomyces*/ALO species, 13 with two strains, and one patient's sample was cultured with three different strains of *Actinomyces*/ALO species (36 strains in total, 20% of the isolated strains), with germination rates of the cultured strains ranging from10^5 to 10^9 CFU/mg. Of the six cases in the control group that were positive in culture, four were one strain of *Actinomyces*/ALO species, and two were two different strains. Germination rates of the cultured strains ranged from 10^4 to 10^5 CFU/mg. In addition to Actinomyces, strains of *Fusobacterium* spp.*, Prevotella* spp.*, Veillonella* spp. and GPAC species were the most frequently isolated in the patient group in anaerobic culture with high germ counts. Compared to the control group samples, strains of *Fusobacterium* spp *were isolated.* (22 vs. 7; p=0.001), *Prevotella* spp. (22 vs. 6; p=0.034). Moreover, GPAC (30 vs 9; p=0.016) species was significantly more frequent among MRONJ patient samples; however, there was no significant difference in the isolation rate of *Veillonella* spp. (30 vs. 25; $p > 0.05$) between the two groups. In both the MRONJ patient and control group, the most common aerobic bacteria were the α-hemolytic oral streptococci *(S. anginosus, S. constellatus, S. mitis, S. oralis* and *S. sanguis);* surprisingly, Gram-negative bacteria of the *Enterobacterales* order *(Escherichia coli, Klebsiella pneumoniae, Citrobacter* spp. and *Morganella morganii)* and *Acinetobacter* spp. were among the species detected in both groups (Figures 2, 3).

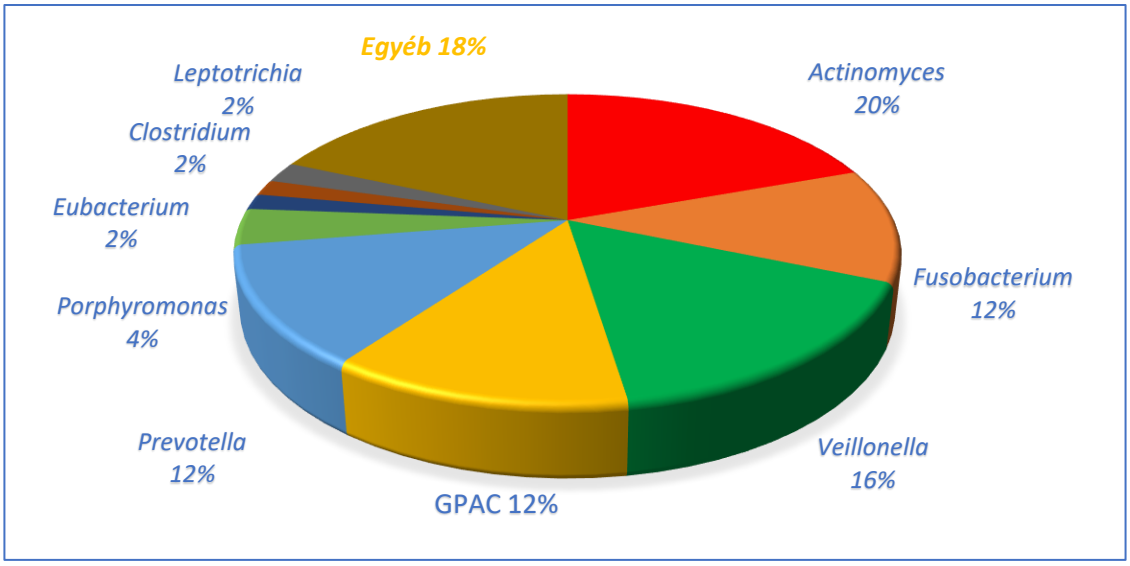

Figure 2. % distribution of 185 anaerobic strains belonging to 65 different species isolated from the patient group

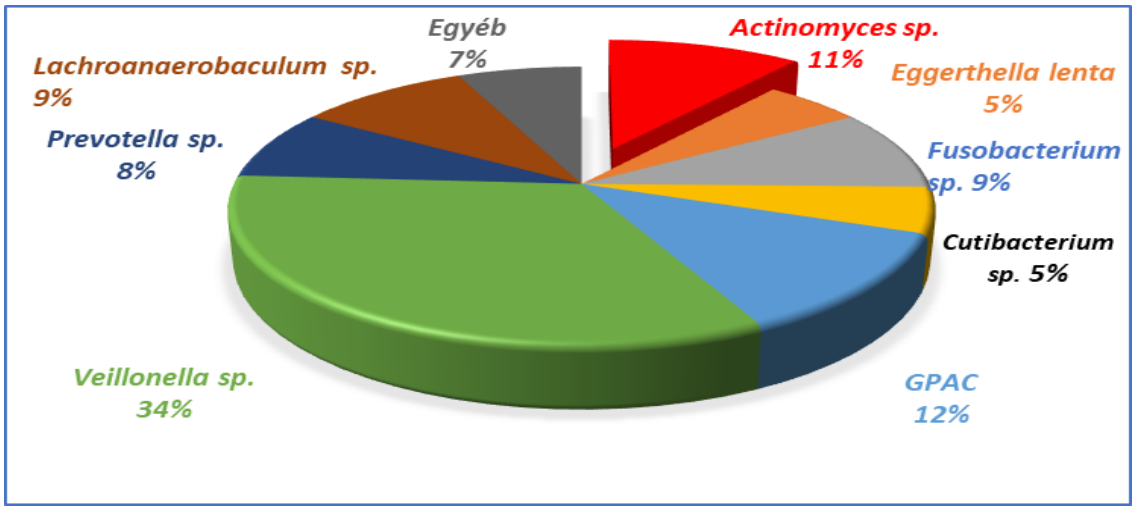

Figure 3. Distribution of 72 anaerobic bacterial strains belonging to 27 species from the control group samples

The total number of isolated Gram-positive anaerobic rods/coccobacillus strains was 54 in the patient group and 25 in the control group, with an even more significant difference in the number of isolated Gram-negative anaerobic rods between the patient group and controls: 71 strains vs. 15 strains. There was a very significant difference in the isolation rate of Gram-positive anaerobic cocci (GPAC): 30 strains in the patient group and 9 strains in the control group, but a less significant difference in the number of Gram-negative anaerobic cocci isolated: 30 vs. 25 strains.

5.3.2. PCR test results

The PCR tests/culture results agreed in the **patient group** cases where *Actinomyces*/ALO strain was cultured from patient samples in high germ counts ($\geq 10^5$ CFU/mg). From samples of the MRONJ patient group, *Actinomyces* strain(s) were isolated from 23 patients by conventional culture for 12 days, of which nine patient samples had one strain, and 13 patient samples had two strains of different species. One patient sample had three strains of different *Actinomyces* species with 10^4-10^9 CFU/mg germ counts. All these samples (23 samples) were positive by species-specific PCR using universal *Actinomyces* primers. Of the 12 patient samples that were negative by culture (*Actinomyces* strain not cultured after 12 days of incubation), five were also negative by PCR detection, and one was doubtful negative (+), for a total of 6 patient samples (17.5%) that were negative by both culture and PCR. Three patient samples (8.5%) were PCR + positive (10^2-10^3 CFU/mg), another three patient samples (8.5%) were strongly positive, corresponding to a high bacterial count (10^4-10^6 CFU/mg), but culture failed to detect the bacterium in 17% of patients. Twenty-nine patient samples were positive by *Actinomyces*-specific 16SrRNA PCR detection (82.9%), no samples were found to have cultured *Actinomyces* strain but were PCR negative. According to the classification of the stages, none of the 3 patients with stage I was confirmed by culture to have *Actinomyces* (0%), while one was negative by PCR and the other two were positive (66%). Of the 19 patients in stage II, 14 (73.7%) were positive for *Actinomyces* by culture, while PCR was able to detect *Actinomyces* in 1 additional patient (15 patients, 79%). 13 patients in stage III were positive for Actinomyces by culture in 9 patients (69.2%), while PCR was able to detect *Actinomyces* in 3 additional patients (12 patients: 92.3%). Of the six (17.1%) *Actinomyces* positive samples in the **control group**, four were cultured with one strain of *Actinomyces* sp. and two with two different strains, with germ counts ranging from10^4 to 10^5 CFU/mg, all of which were positive by PCR. Of the 29 (82.9%) culture-negative samples, 7 (24.1%) were detected by PCR for *Actinomyces* genus, giving a detection rate of 37.1% for all control patient samples (Table 4). The difference in PCR positivity was significant between MRONJ and control samples (82.9% vs. 37.1%; $p < 0.001$).

5.3.3. Results of sequence analysis

As part of our study, 5-5 patient samples were randomly selected (labelled K1-K5 for controls and T1-T5 for MRONJ patients) before the results of conventional culture were

obtained. From these, 16S rRNA gene sequencing was performed to analyse possible differences in the microbiome of the samples. The average size of the 16S rRNA PCR products was 654 bp. Neither DNA isolation nor 16S rRNA PCR resulted in measurable amounts of DNA from the simultaneously processed transport buffers of the samples compared to the negative controls. 2.88 million valid sequences were obtained, resulting in 2.1 million high-quality reads; the median number of reads per sample was 212. 000.

When comparing the results of bacterial 16S rRNA gene sequencing with the Chao1 alpha diversity results from bone samples, no significant difference was found between control and MRONJ patients ($p>0.05$). Furthermore, no significant difference was observed when comparing Bray-Curtis PCoA beta diversity results between control and MRONJ samples ($p>0.05$). For the five patients and five controls, samples sent for sequencing were selected "randomly" before the culture results were known and sent for analysis simultaneously. Controls had significantly fewer bacterial species in bone samples than patients with MRONJ. Among the control samples, we observed dominance of *Streptococcus* sp. (in samples K2 and K5) and *Veillonella* sp. (K1, K3 and K4), whereas bacterial abundance was significantly more heterogeneous in MRONJ bone samples. Except for sample T5, where *Actinomyces* dominated the bone microbiome (relative abundance: 38.5%), no significant differences in the relative abundance of *Actinomyces* were observed between samples K (0-0.19%) and T1-T4 (0.05-0.46%). The T5 sample was a sample from a patient with stage III MRONJ who was receiving long-term antiresorptive treatment for prostate carcinoma and associated bone metastases. Note that samples K1-K3 and K5 were negative for *Actinomyces/ALOs* by both conventional culture and PCR-based assays; in contrast, samples T1, T2 and T5 were positive by both methods (Table 2).

Number	***Actinomyces* culture**	***Actinomyces* PCR**	**Sequence analysis *Actinomyces* %**
K1	Negative	-	0,05
K2	Negative	-	0,0
K3	Negative	-	0,19
K4	*Actinomyces oris* 10^5 CFU/g	+	0,05
K5	Negativ	-	0,01
B1	*Schalia odontolytica* 10^6 CFU/g	++	0,05
B2	*Actinomyces naeslundii* 10^6CFU/g	++	0,2
B3	Negative	++++	0,46
B4	Negative	++	0,06
B5	*Schalia odontolytica* *Actinomyces oris*	++++	38,5

Table 2. Comparison of culture, Actinomyces-specific PCR detection and gene sequence analysis of 5 randomly selected patient and control samples

6. FOLLOW-UP OF PATIENTS: experiences, results

6. 1. Follow-up, monitoring

In the patient group, the first follow-up after surgery was usually on day 10 at the time of suture removal, with further follow-up appointments at 1-2-3 months and six-month intervals, depending on the complaints. Several of our patients who participated in the study have already reached the 1-year check-up interval. Of course, in the case of acute complaints, we advised the patient to present as soon as possible. At the follow-up visits, we also performed imaging scans when necessary, including OP, CBCT, conventional, and SPECT CT scans. In 27 patients, recurrence was seen in 4 patients (15%) 3 months after surgical therapy and six patients (22%) at the six-month follow-up. The recurrences developed in the mandible in 7 cases (70%) and in the maxilla in 3 cases (30%). The recurrences were 9 cases (33%) of denuded bone surface primarily associated with fistula and/or pus discharge, where repeated surgical therapy with hospitalisation and antibiotic therapy was indicated. In three (30%) cases, a partial mandibular resection with reconstruction plate osteosynthesis was performed instead of the previous window resection, and in one case (10%), the previously placed reconstruction plate was removed due to denuded bone surface and extraoral fistula. In two cases (20%), repeated alveolar correction was performed in the mandibular area with appropriate antibiotic therapy. No denuded bone surface was visible in one case (10%), but submandibular swelling developed. Imaging diagnostics did not confirm bone lesion; however, due to the extent of the abscess, an incision was performed under anaesthesia, and 3x1.2 intravenous amoxicillin + clavulanic acid therapy was initiated during one week of hospitalisation. In 1 case (10%) of the maxilla, a first premolar and canine tooth were mobile in the premolar region compared to previous controls, where extraction was performed with appropriate antibiotic protection and the post-extraction area was covered with PRF and closed with suture. In 1 additional case (10%), further alveolar correction was also performed in the maxillary area using PRF and the area was closed with suture. In the remaining 1 (10%) case of recurrence involving the maxilla, we performed a partial maxillectomy and Luc-Caldwell surgery. During the follow-ups, several patients mentioned complaints of uncertain origin characteristic of stage 0; in these cases, imaging was indicated, and per os antibiotic therapy was initiated.

Within 1 year of the start of the study, six patients (17%) had dropped out due to their underlying disease. The earliest patient to die was a female patient with MRONJ stage 3 who was being cared for breast cancer 2 months after her first presentation to our clinic. We chose conservative therapy because of her general condition despite the extent of the lesion. Of the

patients who died, one patient (17%) had MRONJ stage 1, two patients (33%) had stage 2, three patients (50%) had stage 3, three patients (50%) had breast cancer, and three patients (50%) had prostate cancer and were treated at the oncology. Among the deceased, 2 cases (33%) underwent primary conservative therapy, and 4 cases (67%) underwent surgical therapy for MRONJ. At follow-up visits, patients were educated to maintain proper oral hygiene.

7. DISCUSSION

Medication-Related Osteonecrosis of the Jaw (MRONJ) due to bisphosphonates and other antiresorptive drugs associated with the death of jawbone tissue is a profound side effect of these drugs and affects an increasing number of patients worldwide. The complex aetiology of the condition and its limited therapeutic options make it a significant challenge for clinicians in clinical care. A thorough knowledge of the pathophysiological and patient-related factors contributing to the development of the disease is essential. Recently, several studies have explored the relationship between the oral microbiome and MRONJ. Although modern genomic sequencing methods have provided a wealth of new data on the microbial composition of MRONJ lesions, the role of individual species in the disease process remains questionable. Tooth extraction, surgical procedures and high doses of bisphosphonates are the main risk factors for MRONJ, but changes in the oral microbiome play a significant role in disease progression. The oral microbiome certainly plays a role in the development and progression of MRONJ, as the risk of developing MRONJ is significantly increased by the presence of oral infections and inflammation. The relationship between the oral microbiome and the development of MRONJ is mediated through several mechanisms. Disbalance of the oral microbiome can lead to a reduction in the species richness and number of "protective" microorganisms; dysbiosis, i.e. disruption of the healthy microbial balance, can lead to an overgrowth of pathogenic bacteria that cause inflammation and may contribute to bone death. The oral microbiome regulates the host immune response, so when bisphosphonates reduce immune activity, pathogenic members of the oral microbiome may colonise tissues more efficiently, contributing to the development of MRONJ. Immunosuppression and reduced blood flow are also important factors in bone death. Metabolites produced by pathogenic oral bacteria, e.g. certain enzymes such as proteases, in addition to lipopolysaccharides (LPS), degrade bone tissue, stimulate osteoclasts that cause bone resorption and may contribute to bone tissue damage and enhance the inflammatory response. This process is particularly harmful in patients receiving bisphosphonates, as these drugs act by inhibiting the activity of osteoclasts, resulting in a paradoxical situation where microbial effects nevertheless enhance bone resorption. Oral

pathogens also produce inflammatory mediators, e.g. cytokines and chemokines, which promote the inflammatory response in the oral cavity and jawbone tissue. Pathogenic bacteria can also produce toxins that directly induce apoptosis of cells, especially osteoblasts and osteocytes, which can lead to the death of jawbone tissue, a significant feature of MRONJ. Inflammatory mediators and microbial toxins can damage blood vessels and reduce microcirculation in the maxilla and mandible. This reduced blood circulation impairs tissue oxygenation and nutrient supply, which contributes to the proliferation of other pathogenic anaerobic bacteria and bone necrosis.

The use of bisphosphonates and other antiresorptive drugs can reduce the effectiveness of the host's immune response, and the weakened immune response, with reduced defences, makes the body less able to fight infections, further increasing the risk of MRONJ. Members of the microbiome can form a biofilm on the jawbone and tooth surface that provides a protective barrier for bacteria against antibiotics and the immune system, allowing infections to become chronic and contribute to the maintenance of bone loss. These biofilm-associated microbial communities adhere to both living and inanimate surfaces, showing differences in reproduction rates and gene expression compared to their planktonic, floating state. Biofilms allow microorganisms to interact with each other, the host, and resist external conditions, antibiotics and other environmental challenges by forming a protective barrier around themselves.

Demographic factors related to the disease, including age and gender, have been shown to occur in individuals aged between 50 and 70 years, with a slight predominance of female patients (Sedghizadeh PP. *et al.*, 2013; AlRowis R. *et al.*, 2022). Our studies confirm the same finding, where the **average age of patients was 67 years**, and we **did not observe a female predominance** due to the smaller number of cases. According to the same publications and a study published by Ewald, F. and his team in 2021, the mandible is more often affected by MRONJ than the maxilla, which is **confirmed by our results**: 12 patients (34.3%) had maxillary, 22 patients (62.85%) had mandibular, one patient had both (Ewald F. *et al.*, 2021). These studies also suggest that MRONJ has a **multifactorial aetiology**, in which both the type of drug and the route of administration, patient demographics and local involvement of the oral cavity, as well as changes in the microbiome, play a role in the development and progression of the disease. Our research supports this multifactorial aetiology. The riskiest period for the development of MRONJ is between the 2nd and 3rd year of therapy. In the present study, the **mean time** between the start of therapy and primary oral chin lesion **in our patients was 43**

months (median 36 months). The incidence of MRONJ is associated with the dose of antiresorptive drug, the duration of administration and its type. **This was also the case in our study**: 94% of patients received intravenous therapy at the onset of their therapy, while only two patients received oral therapy, and in 26 (74%) cases, switching occurred within the class of drugs provoking osteonecrosis of the jaw during therapy. Based on the literature, the indication, i.e. whether the patient receives the prescribed therapy for malignancy or osteoporosis, osteopenia or other reasons, also influences the development of MRONJ, as **confirmed by our patient database**, where 94% of our patients had an underlying malignancy with bone metastases. We found a significant difference in the proportion of diabetic patients among the patients and the control group, with significantly more diabetic patients in the patient group and a significant difference in the proportion of hypertensive patients in the patient and control groups.

Some research suggests that bacterial strains of the genus ***Actinomyces*** may play a significant role in the development and progression of MRONJ. Several research groups have studied the interaction between the oral microbiome and MRONJ. RE Marx et al. published a paper on the relationship between bisphosphonates and MRONJ development in 2005. They reported 119 patients with comorbidity, clinical bone exposure and mean time to symptom induction with a similar distribution to our findings (Marx RE *et al.*, 2005). Rassmueller G. *et al.* (2006) also found that MRONJ is very often associated with the presence of *Actinomyces* species: in Austria, histological evaluation of necrotic bone samples from 111 patients over 65 years of age showed *Actinomyces* spp. in 99 (89%) of 111 patients. Bródy A. 2022, in his PhD dissertation, examined samples from 39 patients and found only 2 (5.13%) of them to be positive for *Actinomyces* strain by culture. These two samples were also positive by histological evaluation. Out of the 39 samples examined, two were negative by both microbiological and histological testing, and a comparison of their microbiological results with their re-evaluation by three staining methods showed high specificity and very low sensitivity. Their routine microbiological test had a negative predictive value of 0.054. *Fusobacterium* sp., *Prevotella* sp.*, Eikenella* sp. and *Enterobacterales* spp. were also cultured from the two *Actinomyces* positive samples, and the microbiological culture results from the other 35 histologically positive samples only showed a similar composition of co-bacteria. In patients undergoing MRONJ surgery, *Actinomyces* was detected in more than 94% of the samples using appropriate histological procedures (PAS, Gram and Grocott staining) (Bródy A., 2022). Hansen T. et al. conducted studies from 2006 to investigate whether the role of strains of *Actinomyces* spp. in

MRONJ has been underestimated: 31 patients, all of whom had clinical and radiological signs of MRONJ, were histologically examined for *Actinomyces* spp. In addition, a PCR method was used to detect the *Actinomyces-specific*16S ribosomal RNA gene. 20 of the 31 patients (61.5%) were histologically typified by *Actinomyces* morphology, and PCR confirmed the presence of *A. israelii* strains. These results confirm the results of our present study, where we detected the presence of Actinomyces in **62.9% of the patient group** by conventional culture methods. Interestingly, *Actinomyces* strains were found almost exclusively adherent to necrotic bone. The authors found that these organisms are involved in the chronic, non-healing inflammatory processes and purulent exudation characteristic of MJORN. Otto S. et al. conducted a critical review in 2010 to gather the most reliable evidence on the relationship between local infection and the pathogenesis of MRONJ (Otto S. *et al.* 2010). They concluded that the pathogenesis of MRONJ usually starts near infected areas or, in the absence of appropriate preventive measures, near infected areas of dentoalveolar surgery. The increased colonization of tissue samples from MRONJ patients studied by bacterial species as a triggering event emphasizes a probable link to local infection. Known risk factors for MRONJ, such as poor oral hygiene, smoking, steroid intake, immunosuppression and diabetes mellitus, contributed to the higher risk of infection in the patient population studied, a conclusion **consistent with our findings**. Ibrahim et al. studied actinomycosis of the jaw from 2022 to 2022 concerning the underlying disease: they showed that 43 (93.5%) of a total of 45 patients diagnosed with actinomycosis of the jaw had MRONJ (58.7%) or IORN (35.6%), three patients (6.7%) had not received antitumour treatment. In all cases, a direct correlation was found between the histological presence of *Actinomyces* and the degree of bone destruction. Specific PCR was also performed, and *A. israelii* was detected in seven cases analysed using this method. The authors concluded that actinomycosis of the jaws is a complication in patients with MRONJ or IORN (Ibrahim N. *et al.*, 2022).

Our studies confirm the fact that, among the anaerobic copathogens and members of the oral microbiome of the genus *Streptococcus*, fusobacteria are the most associated organisms with actinomycetes and, when coexisting in the biofilm, they act synergistically. This is supported by studies demonstrating that infections caused by *F. nucleatum* in extraction cavities of mice following high doses of BP treatment result in delayed wound healing, leading to bone exposure.

Similar to our studies, the bacterial genera typically found include *Actinomyces*, *Fusobacterium* and *Streptococcus members.* In exploring the relationship between a healthy oral cavity and systemic bone conditions, studies by Novince et al. have focused on the oral

cavity's unique anatomical and physiological features: the distinct blood supply and specific bone structure (Novince CM *et al.*, 2009). Modern metagenomic research has revealed new findings in the MRONJ microbiota, suggesting a close relationship between altered microbial diversity and disease progression. Emerging research demonstrates the role of microbial colonisation, which can be of both bacterial and fungal origin, and the high prevalence of *Actinomyces* is a critical factor in the development of MRONJ. (In contrast, the incidence of MRONJ in other regions of the skeletal system is significantly lower, highlighting the unique susceptibility of the jawbone (Boff RC. *et al.,* 2019). Sedghizadeh PP. et al. have validated the multispecies microbial biofilm theory in affected bone in patients with jaw osteonecrosis due to bisphosphonate therapy. Conventional histopathological methods and scanning electron microscopy examined the biofilm on bone samples from four patients with active MRONJ. Bone samples from all patients formed biofilms containing different bacterial strains and a minor proportion of fungi embedded in an extracellular polymer matrix. **Similar to our** results, the number of bacterial morphotypes in the biofilms varied between 2 and 15 and included species from the genera *Fusobacterium, Bacillus, Actinomyces, Staphylococcus, Streptococcus, Selenomonas* and *Treponema.* They observed co-aggregation between different species within biofilms and concluded that their results may indicate a key role of microbial biofilms in the disease process of MRONJ (Sedghizadeh, PP. et al. 2008). In 2021, Cerrato et al. published a retrospective study reporting the prevalence of Actinomyces among MRONJ cases receiving antiresorptive and antiangiogenic therapy. They processed data from 114 patients, of which 101 oncology patients were confirmed to have MRONJ, and 83 samples showed the presence of Actinomyces infection histopathologically (82.18%), which also agreed with our data.

Research highlights a bidirectional relationship between the oral microbiome and MRONJ lesions: strains of *Streptococcus, Lactobacillus, Bifidobacterium* and other saccharolytically active bacteria create an anoxic, acidic microenvironment, which is further exacerbated by the effects of dental infections, invasive procedures and BP (Kim HY. *et al.*, 2024). Another significant component of the pathophysiology of the disease is the presence of Gram-negative bacteria; these have a significant impact on the pathophysiology by promoting osteoclast differentiation and activity; in addition, BP treatment can lead to increased adhesion of bacteria to bone surfaces, which can alter the local microbiome and create an environment that promotes osteonecrosis. Based on detailed genomic analysis of the microbiome of patients with MRONJ, the bacterial profiles obtained clearly show significant differences compared to those observed in typical jawbone infections such as dental caries and periodontal disease (Kos

M. and Luczak K. 2009, Allen MR. and Burr DB. 2009). MRONJ lesions show a much higher number of microbial morphotypes, much more diverse than non-bisphosphonate-associated osteomyelitis (Mawardi H. et al., 2011).

8. CONCLUSION

Our present research results confirm the hypothesis that the aetiology of MRONJ is multifactorial, where the oral microbiome plays a crucial role but is not exclusively causal, interacting with other factors (immune responses and BP treatment), leading to the development of MRONJ. We reviewed the clinical risk factors for MRONJ and presented the treatment options. While the causal pathophysiological mechanisms are unknown, recent studies have found a significant association between microbial colonization and MRONJ progression. In agreement with this, we have been able to identify risk factors in our patient population, such as trauma: tooth extraction, dentoalveolar surgery (see three patients described separately), DM, smoking and alcohol consumption, hypertension therapy and specific patient demographics, which contribute to a better understanding of the predisposition to the disease. Current treatment strategies include conservative and surgical approaches and depend on the stage of the disease, with an increasing emphasis on understanding microbial dynamics and dysbiosis for treatment. Research should focus on elucidating the interaction between BPs, the oral microbiota and the immune response to develop targeted therapies that reduce the risk of developing MRONJ and improve prognosis. In conclusion, maintaining the balance of the oral microbiome may be key to the prevention and treatment of MRONJ.

Our study aimed to compare the data of patient assessments and medical histories of patients with drug-induced jawbone death in the patient population of the University of Tartu Medical School: mean age, gender, underlying diseases, predisposing factors, therapy received, route of administration, duration of therapy, and to compare them with international data. The average age of the 35 MRONJ patients included in our study was 67 years, similar to the literature data, and we did not observe a female predominance due to the smaller number of cases. Studies have shown that the mandible is more frequently affected by MRONJ than the maxilla, which was supported by our results: 12 patients (34.3%) had maxilla involvement, two patients (62.85%) had mandibular involvement, and one patient had both. The riskiest period for the development of MRONJ is between the 2nd and 3rd year of therapy, and our data support this: an average of 43 months elapsed between the start of therapy and primary oral chin lesion

in our patients. The development, exacerbation and staging of MRONJ were associated with the dose, duration and type of antiresorptive drug: this was also the case in our study, with 94% of patients receiving intravenous therapy at the onset of therapy, while only two patients received oral therapy, and 26 (74%) patients changed the group of drugs causing jaw necrosis during therapy. The indication also influences the development of MRONJ, whether the patient receives therapy for a malignant tumour or osteoporosis, osteopenia or other conditions, as evidenced by our patient database, where 94% of patients had an underlying malignant disease with bone metastases.

The presence of anaerobic bacteria, including *Actinomyces* species, was confirmed in the patients' surgically removed bone fragments by conventional and molecular diagnostic methods. By culture in an anaerobic atmosphere for 12 days, a bacterial strain belonging to *Actinomyces* species was isolated in 65.7% of patients, whereas by culture and PCR, *Actinomyces* was detectable in 82.9% of the patient group.

In the MRONJ patient material, a significantly higher proportion of *Actinomyces* species were found compared to the control group, and these species were most frequently found in association with oral streptococci, fusobacteria, veillonellae, prevotellae and GPAC species. In the control group, streptococci, veillonellae and lactobacilli were the most common species. We investigated whether there was a correlation between the clinical status of the patients, the risk factors of the disease and the composition of the microbiota detected in the bone biofilm and found that there was a significant correlation between the clinical stage classification of the disease and the detection of *Actinomyces* strains, with a higher proportion of *Actinomyces* being detected as the stage classification progressed with disease progression and severity. In 3 patients with stage I disease, none were confirmed by culture for *Actinomyces* (0%), while only one was negative by PCR. Of the 19 patients in stage II, 14 (73.7%) were confirmed by culture, while PCR detected *actinomycosis* in 1 additional patient (15 patients, 79% in total). 13 patients in stage III were positive in 9 (69.2%). In comparison, PCR detected actinomycosis in 3 additional patients (12 patients, 92.3% in total). In the patient group, 24 patients (68.5%) suffer from hypertension and receive antihypertensive therapy; all these patients were detected for actinomycosis. Eleven patients (31%) suffered from DM at admission, and in all these patients, we could detect the different strains of *Actinomyces* by culture. In 10 (28.5%) of the patients in the study, these two pathologies coexisted, and Actinomycetes were cultured in all these patients.

Long-term follow-up of patients (1 month, 3 months, 6 months, and sometimes even 1 year) after postoperative conservative therapy was performed to monitor the course of the disease and the incidence of recurrence. Three months after surgical therapy, four patients (15%) and six patients (22%) had recurrences at the six-month follow-up; seven patients had recurrences in the maxilla of the mandible, and three patients had recurrences in the maxilla. Nine patients were found to have denuded bone surfaces primarily associated with fistula formation and/or pus discharge; in these patients, repeated surgical and antibiotic therapy was indicated. One patient developed submandibular swelling, but no denuded bone surface was seen. No evidence of bone lesion was confirmed by imaging, but due to the extent of the abscess, an incision was made, and the patient received antibiotic therapy. Another patient underwent further alveolar correction in the maxillary region using PRF and suture closure, and one patient underwent a partial maxillectomy and Luc-Caldwell surgery for recurrence in the maxilla. During follow-up, patients reported several non-specific complaints characteristic of stage 0. Unfortunately, in the 1 year since the study, six patients have expired due to their underlying disease.

Research on the association between the presence of *Actinomyces* and MRONJ and our present study indicate that Actinomyces are present in higher proportions in affected bones of patients with MRONJ. In our opinion, they may play a significant role in the disease's development, exacerbation and progression. *Actinomyces* strains are important early members of the oral biofilm, and infections resulting from this colonisation cause chronic inflammation, contributing to the necrosis of the jawbone tissue. Early detection of *Actinomyces* infections is of utmost importance for effective treatment and prevention of MRONJ. Current therapeutic approaches are disease stage-specific, and more effective treatment strategies are needed. To prevent and treat MRONJ, it is important to maintain the health of the oral microbiome and improve oral hygiene.

9. NEW OBSERVATIONS

The present study aimed to demonstrate the role of *Actinomyces* spp. in the development and progression of MRONJ. We investigated the presence of *Actinomyces* in bone samples from MRONJ patients in clinical and microbiological contexts, compared to healthy control subjects, using conventional culture-based and molecular biology methods. To our knowledge, this is the first such comprehensive microbiological study of MRONJ patients in Hungary.

As part of our study, we also presented the **patients' medical history** related to the development of MRONJ:

The age range corresponded to the age at which the condition is most seen, with a slight female predominance also previously reported, which was not confirmed in our case; however, the study's sample size could explain this. Most patients (94.0%) were affected by malignant tumours, and most patients also had some underlying chronic disease. Lifestyle factors did not differ significantly between MRONJ patients and control subjects. MRONJ more often involves the mandible, which is consistent with our experience. The median time from initiation of antiresorptive therapy to diagnosis of MRONJ was 36 months on average, which is consistent with data from abroad, we highlighted that the symptoms most often occurred in the second to third year of therapy.

Using a variety of **microbiological methods,** our study has shown that ***Actinomyces*** spp. are more frequently found in necrotic bone tissue from patients receiving antiresorptive drugs than in samples from unaffected controls, indicating that they may play a role in the clinical course and prognosis of MRONJ:

In the population included in the study (35 MRONJ patients and 35 healthy controls), the culture of bone samples by conventional culture method in an anaerobic atmosphere for at least 12 days was 65.8% vs. 17.1% for *Actinomyces*/ALOs, while the PCR-based detection rate was 83.9% vs. 37.1% for MRONJ vs. control samples. PCR increased the detection of bacterial presence by an additional 25.9% for MRONJ samples and 216.9% for control samples compared to using culture-based methods alone.

We confirmed that **the disease stage** also had a significant effect, as the detection rate of *Actinomyces*/ALOs increased in the more advanced stages of MRONJ.

Actinomyces/ALOs were isolated most frequently in association with oral **Streptococci and *Fusobacterium*** spp. confirming synergistic interactions between these microorganisms in the oral biofilm.

Modern metagenomic research has revealed new findings in the MRONJ microbiome that suggest a strong link between altered microbial diversity and disease progression. Based on these findings, we complemented our studies by sequencing the 16S rRNA gene to randomly selected bone samples. Although in this context, our results are only cross-sections - and not representative of the entire population - they provide important insights into the microbiome of MRONJ and healthy bone tissue. Although no significant differences were identified (based on alpha and beta diversity measurements), the contrast in the number of taxa represented shows that **MRONJ bone samples had higher microbial diversity in their taxa** than those of controls. Our study supports the role of microbial colonization and the high prevalence of *Actinomyces* as a critical factor in the development of MRONJ.

The results of our current study, together with the clinical experience of follow-up of patients after treatment, show that changes in the oral microbiome, of which *Actinomyces* and related species are the most important, play a key role in the development of MRONJ, which undoubtedly interact with other additive factors. These results confirm the "infection

hypothesis" of MRONJ formation, according to which PP treatment-induced infections due to *Actinomyces* colonization of tissues lead to chronic inflammation, resulting in osteomyelitis and subsequent necrosis of the jawbone tissue.

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