Clinical outcome analysis of medication-related osteonecrosis of the jaw (MRONJ) after surgical treatment from 2011 to 2019

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1 Hypothesis and objectives

1.1 Hypothesis

The most common and recommended treatment in many cases of MRONJ in stages II and III includes the surgical removal of necrotic bone, a procedure performed under general anesthesia. However, the various influences on the clinical outcome parameters have not yet been conclusively elucidated. Since the pathophysiology of the disease is not comprehensively understood, possible correlations to the mechanisms affecting the healing period after intervention are to be reviewed. On the one hand, general factors such as the underlying disease itself and its antiresorptive therapy, and on the other hand, local factors such as the microbiological colonization of the necrotic bone, are often considered as initiating factors, but their role in the rehabilitation process remains to be investigated. The individual species in the specimen and even the range of kingdoms, especially an additional fungal infestation, are only fragmentarily examined. Simultaneously, antibiotic treatment is considered to play an important role and is often adjusted or expanded after surgery to improve the outcome. The aim of this study was to provide insight into the potential factors influencing the clinical outcome in patients with established MRONJ after surgical intervention. Critical assessment of postoperative healing and recurrence of osteonecrosis were the primary outcome measures. The results may contribute to preventing the progression of the condition, substantiating early diagnosis, decreasing the number of required interventions, and allowing for more specific treatment options for individual patients in the future.

1.2 Objectives

i) Evaluation of the impact of the underlying diagnosis and its antiresorptive therapy on the recurrence and healing of the disease.

ii) Examination of the colonization in the necrotic bone specimens and correlations to the primary outcome measures.

iii) Investigation of the role of the antibiotic treatment regimen during and after surgical intervention, considering alterations and additions.

iv) Determination of the challenging impact of fungal colonization.

2 Introduction

2.1 MRONJ

Medication-related osteonecrosis of the jaw (MRONJ) is considered a multifactorial disease (Aghaloo et al., 2015, Ruggiero et al., 2022), that occurs as a severe side effect during or after a period of antiresorptive medication alone or in combination with antiangiogenic agents or immune modulators (Galis et al., 2017, Marx, 2003, Ruggiero et al., 2022). Bisphosphonates (BPs), either administered orally (e.g., alendronate) or parenterally (e.g., zoledronate), as well as receptor activator of nuclear factor-κB ligand (RANKL) inhibitors (e.g., denosumab), hormonal therapies (e.g., Enzalutamide) or angiogenesis inhibitors (e.g., bevacizumab, sunitinib), are currently in frequent use. Indications for these therapies are a range of conditions, including neoplastic diseases, such as tumor-associated hypercalcemia (Carter et al., 2005), multiple myeloma (Boonyapakorn et al., 2008), and skeletal metastatic carcinomas (e.g. breast cancer, prostate cancer) (Body, 2006, Carter et al., 2005), and non-neoplastic diseases, such as osteoporosis (Pazianas et al., 2007), osteitis deformans (Paget's disease) (Carter et al., 2005) and arthritis (Magopoulos et al., 2007). Antiresorptive medications are well known to show significant success in improving the quality of life, by reducing pain and preventing skeletal-related events (SREs) such as fractures (Body et al., 2004, Moll et al., 2021, Mücke et al., 2011, Ruggiero et al., 2022, Vassiliou et al., 2007). However, the number of patients exposed to these drugs with the potential of resulting in MRONJ cannot be dismissed as an increasing healthcare issue in recent years, particularly in countries of a demographic shift (Chan et al., 2018, Häussler et al., 2007, Wade et al., 2014).

Clinical symptoms of MRONJ include exposed necrotic bone in the oral cavity, loosening of teeth, odontalgia, or gingival swelling. Radiographic images, presenting with alveolar bone resorption, osteolysis, or persisting extraction sockets, can support the diagnosis in the asymptomatic stages of the disease (Boff et al., 2014, Ruggiero et al., 2022).

Management of MRONJ is often long-term and a strenuous process for the patient concerned. In the early stages, it can be treated conservatively, whereas in an advanced stage, therapy involves minor to extensive surgeries under general

anesthesia, along with costly hospitalizations. Due to the refractory pattern, repeated interventions can remain a lifelong issue.

Apart from the ongoing treatment of a diagnosed MRONJ, the underlying diseases of those affected are already an enormous burden on the healthcare system (Burge et al., 2007). The population of patients treated with antiresorptive medications is particularly at a high risk of suffering from SREs (Aghaloo et al., 2015, Koch et al., 2012). Up to 70% of patients with advanced breast or prostate cancer suffer from bone metastases (Roodman, 2004). The cumulative incidence of SREs in bone metastasis patients was described with over 45% (Hong et al., 2020), while the event of osteoporotic fractures was estimated to occur in about 33% of postmenopausal women and 20% of men over the age of 50 years (Häussler et al., 2007). Possible consequences are disability, severe pain, and even increased mortality (Center et al., 1999, Coughlan and Dockery, 2014). Antiresorptive medications aim to increase bone density, compromise bone turnover, reduce bone metastasis, and prevent any of the correlated consequences. SREs demonstrated a decrease of 30% to 40% in correlation to prolonged drug administration (Body, 2006). The improvement in quality of life and substantial analgetic effects have a high clinical value for affected individuals (Body et al., 2004). The positive effects are broadly required in various medical fields. Underlying diseases (e.g., osteoporosis, breast cancer) are widely undertreated and underdiagnosed, which leaves us anticipating numerous emerging cases of MRONJ in the years to come (Apantaku, 2000, Coughlan and Dockery, 2014, Häussler et al., 2007).

The risk of developing MRONJ remains low but undeniable with an estimated cumulative incidence between 0.001% and 21.26%, depending on the underlying disease, the antiresorptive drug regimen, and the duration of administration (Assaf et al., 2013, Galis et al., 2017, Khan et al., 2015, Ruggiero et al., 2022, Walter et al., 2008). In the group of neoplastic diseases, current data show a considerably higher incidence than in the group of non-neoplastic diseases (Bone et al., 2017, Boquete-Castro et al., 2016, Gnant et al., 2015, Grbic et al., 2010, Papapoulos et al., 2012, Saag et al., 2017, Valachis et al., 2013). Other factors contributing to the difficulty in precisely quantifying incidence and prevalence may be related to the various comorbidities that are present in the population of MRONJ patients (McGowan et al., 2019, Ruggiero et al., 2022, Wang et al., 2007).

The enormous burden associated with this uncommon but serious disease is its difficult-to-manage and often recurring nature. The result is severe impairment in the quality of life and the decline correlates with disease stage (Beth-Tasdogan et al., 2022, Miksad et al., 2011). Physical pain and functional limitations were frequently assessed as highly restricting factors for patients (Caminha et al., 2020, Capocci et al., 2017, Kyrgidis et al., 2012), with increasing impact during the progression of the disease (Miksad et al., 2011). Further, it is increasingly recognized that the psychological discomfort caused by concern and stress has a significant impact on quality of life (Caminha et al., 2020). Serious social and emotional distress have previously been reported in numerous studies (Capocci et al., 2017, Miksad et al., 2011). The lives of patients with severe underlying diseases are only further compromised by the burdens of MRONJ.

Evidence suggests that antiresorptive medications, in combination with inflammation or infection, play an essential role in the development of MRONJ (Aghaloo et al., 2015, Ruggiero et al., 2022). Influencing factors such as underlying disease, duration of medication, and type of treatment have been reported (Chen et al., 2021, Klingelhöffer et al., 2016, Mücke et al., 2011). Further, microorganisms seem to play an essential role in the etiopathogenesis and severity of the disease (Boff et al., 2014, Kim et al., 2018, Soma et al., 2021). Conditions creating access for pathogens (e.g., tooth extraction, periodontal disease, trauma) are frequently considered dental risk factors (Boff et al., 2014, Kim et al., 2018, Kyrgidis et al., 2008, McGowan et al., 2018, Soma et al., 2021). Other risk factors are inconsistently associated with an increased incidence of MRONJ, including medical factors, like diabetes and anemia (Aghaloo et al., 2015, Allen and Burr, 2009, Saad et al., 2012, Zhang et al., 2015), or the use of tobacco (Aparecida Cariolatto et al., 2018, Sánchez-Gallego Albertos et al., 2021, Wessel et al., 2008).

The etiology and pathogenesis encompass a multifactorial complex of causalities, which has not yet been conclusively clarified. Different approaches suggest a link to the inhibition of bone remodeling and angiogenesis, as well as to infection by microorganisms (Allen and Burr, 2009, Boff et al., 2014). Providing the overall pathophysiology of this rare medication-related complication remains a subject to further investigation (Aghaloo et al., 2015, Ruggiero et al., 2022). There may be potential mechanistic overlap in the development of the frequent wound healing disorders and recurrences of the disease, associated with MRONJ (Allen and Burr,

2009, Chen et al., 2021). By deepening the understanding of the factors influencing surgical outcomes, we aim to explore and reduce the frequency and extent of interventions needed in the management of the disease.

2.2 Bone structure and remodeling

The musculoskeletal system consists primarily of mineralized bones and connective tissue structures such as cartilage, ligaments, joints, and tendons, which are moved by the skeletal muscles. The bone is a complex tissue with mechanical functions and metabolic activity. It serves as the support of the human body and protects inner organs. Additional features are the storage of calcium, phosphorous and other minerals, as well as different growth factors, hormones, proteins, and fatty acids (Aumüller et al., 2014, Boff et al., 2014, Buckwalter and Cooper, 1987, Lüllmann-Rauch, 2006, Matthies, 2018). Bone tissue contains numerous cells, including osteoprogenitor cells, osteoblasts, osteocytes, and osteoclasts. Hematopoiesis occurs in different areas of the skeleton throughout a lifetime. It contributes fundamentally to cellular regeneration by providing multipotent stem cells (Buckwalter and Cooper, 1987). The maturation of osteoblasts and osteocytes arises from the mesenchymal stem cell to the osteoprogenitor cell, which is the precursor to the more specialized osteoblasts. Osteocytes are mature cells, developing from osteoblasts and representing a major part of bone cells (Bonewald, 2011). Osteoclasts differentiate in a different line from monocytes (Buckwalter and Cooper, 1987). The main components of the extracellular matrix are collagen and hydroxyapatite crystals, giving it tensile strength and resistance to compression, with little elasticity (Buckwalter and Cooper, 1987).

Cortical or compact bone consists of anatomical and functional units known as osteons, illustrated in Figure 1. It consists of concentrically arranged bone lamellae surrounding a central Haversian canal (Datta et al., 2008), which connects via perpendicularly oriented Volkmann’s canals to other parts of the system and provides the vessels for nutrition, nerves, and connective tissue. Osteocytes are contained in small, irregular lacunae within the lamellae. The outer surface of the bone is covered by the periosteum, and the inner surface by the endosteum (Datta et al., 2008). Skeletal progenitor cells deriving from the periosteum play a crucial role in bone remodeling and fracture healing (Roberts et al., 2015). The cancellous

or trabecular bone is the tightly meshed interior of the bone, which consists of trabeculae. These can adapt their orientation and density to the current load and reduce the substance and weight with undiminished stability (Schmidt et al., 2021). Bone marrow serves the hematopoiesis and can be found in the medullary spaces of the cancellous bone (Datta et al., 2008).

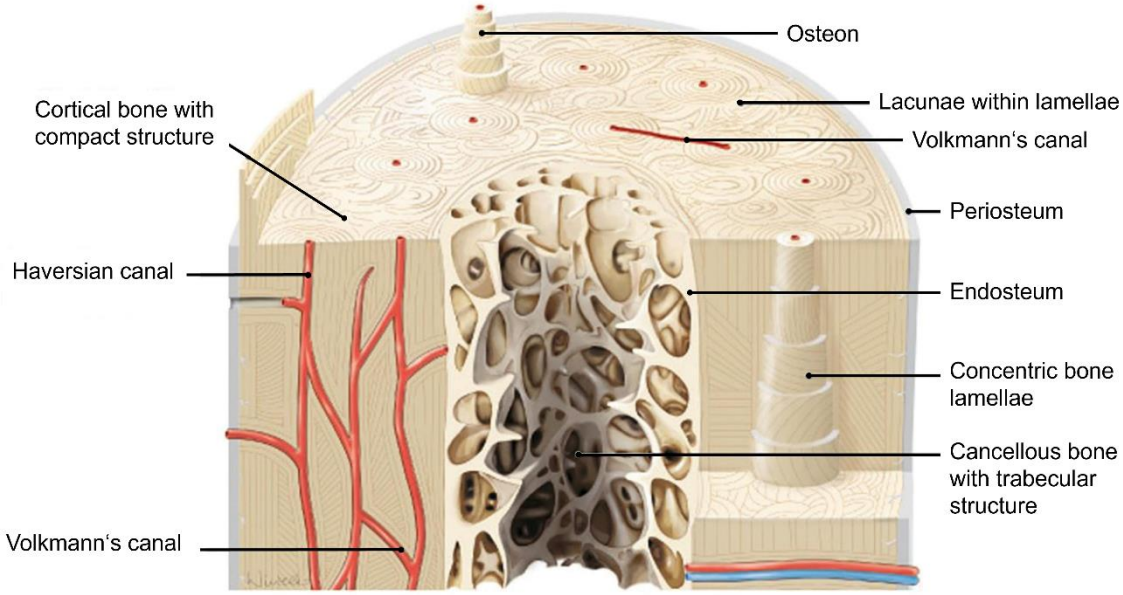

Figure 1: Cortical and cancellous bone structure. Schematic diagram of the anatomy of long bones. Modified from Sobotta Atlas of Anatomy (Paulsen and Waschke, 2011).

The coordinated dynamic state of building up and breaking down of the skeleton requires the interaction between resorption by osteoclasts and deposition by osteoblasts. Bone matrix is being remodeled not only for the replacement of immature woven bone, regulation of calcium homeostasis, and fracture healing but also for functional adaptation (Aumüller et al., 2014, Buckwalter and Cooper, 1987, Matthies, 2018). The activity of the involved cells is orchestrated by several different local and systemic factors. Mechanical loading can trigger an adjustment to the stress applied to the bone tissue. The receptor activator of nuclear factor-κB (RANK) on osteoclasts and osteoclast precursor cells is activated by the released RANKL of osteoblasts, initiating their differentiation into activated osteoclasts, and preventing their apoptosis (Boyce and Xing, 2008, Hanley et al., 2012, Suda et al., 1992, Yasuda, 2021). To suppress the resorption process, osteoblasts can release

osteoprotegerin (OPG) to counter-regulate the RANK/RANKL system, as illustrated in Figure 2. OPG binds to RANK to prevent the activation of osteoclasts and excessive resorption (Boyce and Xing, 2008, Hanley et al., 2012, Simonet et al., 1997, Yasuda, 2021).

Other mechanisms include the paracrine regulation by growth factors released during bone matrix resorption, which can initiate osteoblast differentiation, and cytokines that stimulate osteoclast formation (Kenkre and Bassett, 2018). Endocrine regulation includes several hormones such as the parathyroid hormone (PTH) to stimulate bone resorption and physiological calcium homeostasis, and sex hormones (e.g., estrogen, androgens) to inhibit bone resorption and increase bone formation (Kenkre and Bassett, 2018, Lüllmann-Rauch, 2006). The volume and shape of remodeled bone physiologically correspond to the bone resorbed by osteoclasts. Under normal circumstances, this process is very precisely controlled, but imbalances result in conditions of high bone density (e.g., osteopetrosis, osteosclerosis) or bone loss, most commonly osteoporosis (De Luna et al., 2018, Sobacchi et al., 2013, Wu et al., 2017). In the case of altered bone metabolism (e.g., osteoporosis or bone metastases) the increase in growth factors released from the matrix can stimulate cancer cells to proliferate and release further bone-resorbing factors (e.g., PTHrP, IL-6) (Yasuda, 2021). High RANKL activity, with its severe influence on bone turnover, is associated with malignant disorders such as multiple myeloma (Pearse et al., 2001) or other oncologic diseases (e.g., breast or prostate cancer) with osteolytic metastases (Morony et al., 2001). All these diseases threaten bone homeostasis and therefore potentially require antiresorptive medications.

2.3 Associated medications

In a variety of skeletal disorders, including osteoporosis, arthritis, multiple myeloma, or bone metastasis, antiresorptive agents (e.g., bisphosphonates, denosumab) are commonly administered to reduce bone resorption, prevent SREs, and relieve bone pain (Coleman and McCloskey, 2011, Koch et al., 2012, Rachner et al., 2011).

Alendronate and zoledronate are some of the most widely used BPs to this date. They are analogues of the pyrophosphate, which occurs physiologically in the human body, and replace it by covalently binding to the hydroxyapatite of the bone surface. During bone turnover, the main targets are osteoclasts, as they internalize

BPs during the resorption process (Figure 2), which leads to cell death (Boff et al., 2014). Diverse mechanisms lead to apoptosis of osteoclasts, inhibition of osteoclast activity, and impede with reactivation of osteoblasts (Fisher et al., 2000, Kwak et al., 2009, Roelofs et al., 2010, Weinstein et al., 2009). The result is a higher bone density with compromised vascularization, and bone metastases are prevented by the reduced release of growth factors from the bone matrix (Aghaloo et al., 2015, Roelofs et al., 2010, Santini et al., 2003, Wood et al., 2002). Intravenous (e.g. zoledronic acid) or oral application (e.g. alendronic acid) is possible. Even after months or years, depending on the active ingredient, the form of application, and the duration of treatment, BPs are not fully metabolized and remain embedded as part of the matrix.

BPs can also impair physiological bone turnover, and apoptosis of osteoclasts can lead to necrosis. Since the jaws show high metabolic activity for bone remodeling and repair, they are greatly affected. Especially in the setting of dental disease and inflammatory processes in the oral cavity, the bone can be exposed to an unsuitable environment of bacteria, cytokines, and oxidative stress (Aghaloo et al., 2011, Marx et al., 2005, Russell et al., 1999). Further, antiangiogenic effects and vascular disruption have been observed in multiple studies with BPs, and are hypothesized to be possible influences on the pathophysiology and development of MRONJ (Allen and Burr, 2009, Fournier et al., 2002, Marx et al., 2005, Vincenzi et al., 2005, Wood et al., 2002). Considering the compromised wound healing in MRONJ patients, direct toxic effects of the accumulated BPs on the oral epithelium have been discussed as triggers (Reid et al., 2007). Persisting bone exposure instead of soft tissue healing in the affected areas may lead to further progression of the disease and persisting infection of the underlying bone (Boff et al., 2014, Reid et al., 2007).

More novel treatment approaches include denosumab, a monoclonal antibody (mAb) against RANKL (Fusco et al., 2022). Blocking the binding to its original receptor, RANK, as shown in Figure 2, results in the inhibition of osteoclast differentiation and activity (Rachner et al., 2011, Stopeck et al., 2010). Stimulation of cancer cells by growth factors, released during bone resorption, is interrupted, and a direct effect on RANK-expressing tumor cells has been reported to reduce metastatic growth (Blake et al., 2014, Yasuda, 2021). Denosumab can be administered in a low-dose form of 60 mg every six months (Prolia®), usually in cases of osteoporosis with a high risk of fractures, or a high-dose form of 120 mg

every month (Xgeva®), in cases of skeletal metastasis or multiple myeloma. Denosumab is effective in delaying or preventing SREs with great therapeutic potential (Bone et al., 2017, Gnant et al., 2015, Ruggiero et al., 2022, Wu et al., 2018). The effect on the bone remodeling by the RANKL inhibitor can be reversed within six months after the discontinuation of the treatment, since it does not incorporate into the bone matrix, unlike BPs (Aghaloo et al., 2015, Ruggiero et al., 2022).

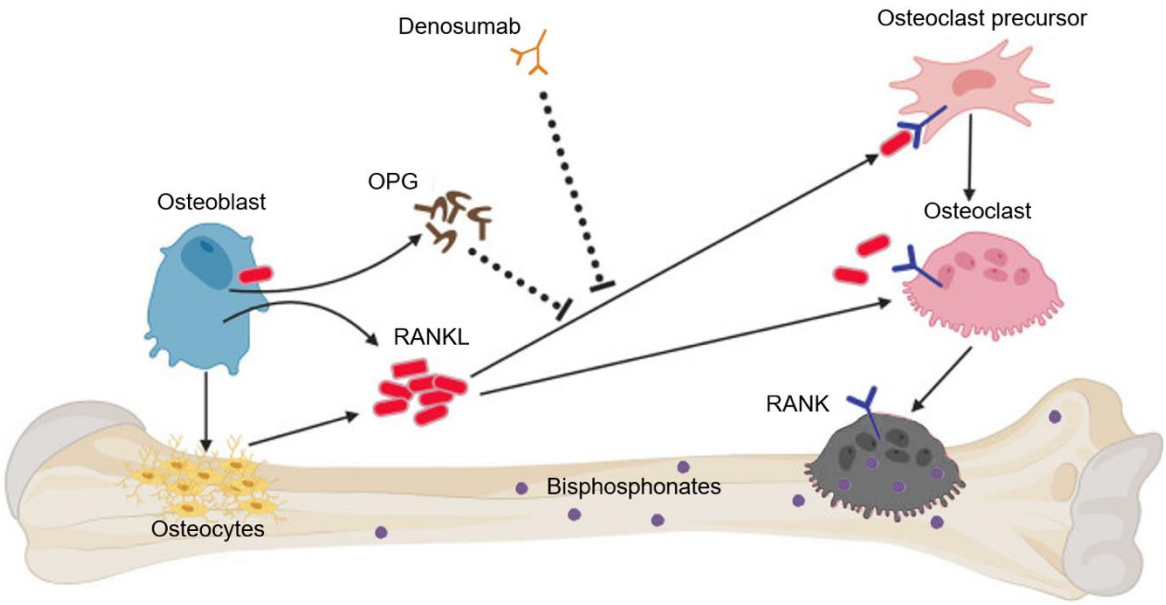

Figure 2: Interaction of antiresorptive medications with bone homeostasis. The interaction of RANK/RANKL/OPG during deposition and resorption of bone is illustrated. Osteoblasts can release RANKL and OPG. RANKL binds to RANK on osteoclast precursor cells and osteoclasts, leading to bone resorption. OPG interrupts the signaling pathway, by binding to RANKL, and inhibits the resorption process. Antiresorptive medications interfere with bone turnover. Bisphosphonates bind to the bone matrix and are internalized by osteoclasts, causing inhibition of activity or apoptosis, and therefore reduced bone resorption. Denosumab interacts with RANKL, interrupting the signaling pathway with RANK, and decreasing bone resorption in this way. Modified from (Ming et al., 2020).

Other supportive co-medications in cancer treatment that have also been associated with MRONJ include antiangiogenic medications, such as other mAb therapies and vascular endothelial growth factor (VEGF) inhibitors (e.g., Bevacizumab), chemotherapies, such as tyrosine kinase inhibitors (TKIs) (e.g., Sunitinib),

immunomodulatory drugs (e.g., Infliximab, corticoids), or hormonal therapies (e.g., enzalutamide, leuprorelin) (Eguia et al., 2020, Guarneri et al., 2010, Ruggiero et al., 2022). The effects of BPs show a decrease in vascular regeneration, which is amplified by supportive therapy (Santini et al., 2003, Wood et al., 2002). VEGF inhibitors interrupt the signaling cascade that depends on the binding of VEGF to its receptor, thereby inhibiting angiogenesis. The dysregulated formation of new blood vessels in oncologic patients serves to supply the tumor tissue with nutrients and oxygen, allowing it to grow excessively (Carmeliet, 2005). Restricted blood supply to the tumor enables clinically noticeable regression or arrest in tumor growth (Melincovici et al., 2018). Simultaneously, impaired vascularization also compromises osseous wound healing after extractions or surgical interventions (Carmeliet, 2005). TKIs interrupt various signaling pathways and their cellular processes by binding to tyrosine kinases (e.g., epidermal growth factor (EGF) receptor, VEGF receptor). The anti-angiogenic and anti-proliferation effects can stop the progression of the tumor or even shrink it in size (Qin et al., 2019, Zhu et al., 2020). Direct inhibition of angiogenesis and reduction of vascularity also affect the development of necrosis (Vallina et al., 2019) and further the healing process of affected lesions after surgical intervention (Akita et al., 2018). Osseous wound healing showed a negative influence by the decrease in blood vessels and therefore reduction of recruited immune cells for regeneration (Akita et al., 2018, Vallina et al., 2019). Mechanisms to defend against infections may also be compromised by suppressed angiogenesis, which can promote necrosis (Vallina et al., 2019). Patients with additional medical conditions that compromise the immune system (e.g., metastatic or primary bone malignancies, diabetes, rheumatoid arthritis) or with a history of medications that interfere with the immune system (e.g., chemotherapy, corticoids, infliximab) show a higher prevalence or severity of MRONJ (Hayano et al., 2020, Kabilova et al., 2014, Marx et al., 2005, Ruggiero et al., 2022, Zhang et al., 2015). Immune cells such as leukocytes and macrophages play a central part in wound healing by eliminating invading bacteria and promoting immune responses (Hayano et al., 2020). By intruding into the immune system, these effects may be impaired and contribute to the progression of the disease.

Recent literature further described an increased onset of MRONJ in correlation to co-medications, such as hormonal therapy (Wick et al., 2022). The goal of this treatment is to stop tumor progression by depriving the body of its circulating hormones or by preventing the tumor from producing excessive hormones itself.

Hormones are part of a regulating system with a multitude of effects on the human body, which is often altered in patients with various oncological diseases (e.g., prostate cancer, and breast cancer). Many of these effects are associated with the progression of tumor growth, including an increase in transcription of genes, upregulation of EGF, VEGF, and endothelial proliferation, leading to higher angiogenesis, while apoptosis decreases (Student et al., 2020). Hormonal therapy aims to reduce the signaling mechanisms within the affected tissue (Student et al., 2020). However, the treatment also shows an immunomodulatory effect. Hormones, such as estrogen, have an anti-inflammatory influence on several cells of the immune system, promoting the development of tumor cells (Huang et al., 2021). The deprivation of those hormones has a beneficial effect on the treatment of the underlying disease. Simultaneously, it may be related to the elevated risk of MRONJ onset, which is accompanied by infection and inflammation (Huang et al., 2021, Wick et al., 2022).

2.4 Diagnostics and disease classification

The diagnostic criteria for MRONJ include a present or preceding treatment with antiresorptive medication alone or in combination with antiangiogenic agents or immune modulators, exposed bone in the oral cavity persisting over eight weeks, and no history of radiation in the maxillofacial region (Galis et al., 2017, Ruggiero et al., 2014, Ruggiero et al., 2022). The occurrence of extraoral or intraoral fistulae that can be probed through to the bone as an alternative to directly exposed bone expands the required criteria of the American Association of Oral and Maxillofacial Surgeons (AAOMS) Position Paper since the modifications in 2014 (Ruggiero et al., 2014).

Non-specific features, like odontalgia, sinus pain, or jaw bone pain, loosening of teeth, and gingiva swelling, can occur in combination with radiographic findings (e.g., alveolar bone resorption without correlation to periodontal disease, or persisting extraction sockets) in a nonexposed bone variant (stage 0). The lesions showing exposed necrotic bone in the jaws, or bone that can be probed through a fistula, can remain asymptomatic without signs of infection and inflammation (stage I) for long periods. Radiographic findings may occur in this stage. The symptomatic stage (stage II) shows additional clinical symptoms and confirmed

infection of the lesion, as demonstrated in Figure 3. Further progression into stage III is reported by the evidence of extensive necrosis and osteolysis beyond the alveolar bone, extraoral fistula, or pathological fractures. Even after successful surgical or non-surgical treatment with no apparent necrotic bone left and no signs of any symptoms, the patient remains at risk for MRONJ, solely due to the history of antiresorptive medications. (Boff et al., 2014, Ruggiero et al., 2022)

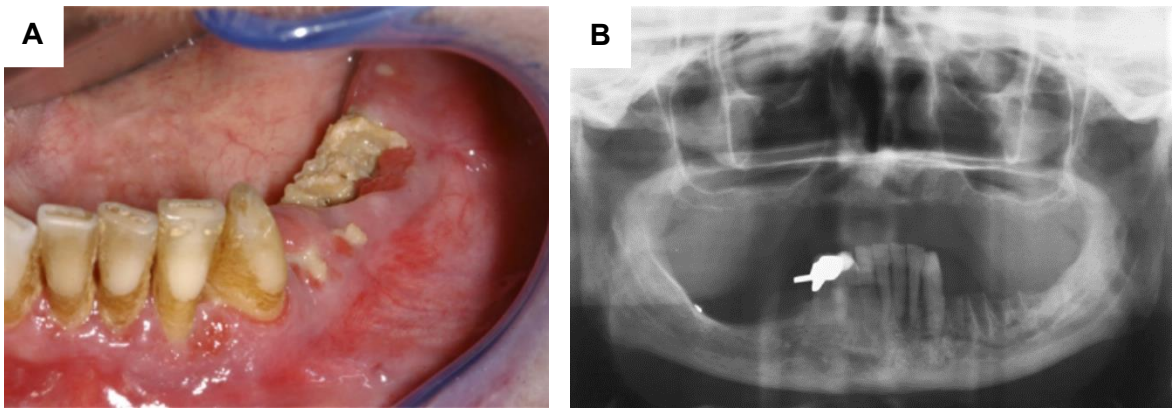

Figure 3: Clinical and radiologic presentation of MRONJ stage II. (A) Left mandible of a 69-year-old female, showing exposed bone and signs of infection; stage II MRONJ. (B) Panoramic view of the same patient. Visible extraction sockets and osteonecrosis 13 months after tooth extraction. Modified from (Pautke et al., 2011).

An additional classification system was implemented in 2007 to address lesion size (Weitzman et al., 2007). There are four grades described, ranging from a lesion size of under 0.5 cm (Grade 1) to 0.5 – 0.99 cm (Grade 2), and 1 – 2 cm (Grade 3) up to over 2 cm (Grade 4). Each grade is further subcategorized into a single lesion (e.g., Grade 1A) or multiple lesions (e.g., Grade 1B) with the largest lesion being the decisive factor (Weitzman et al., 2007). This classification can help monitor the healing process and recurrences of the disease.

Diagnostic procedures include clinical examination, X-ray analysis, such as panoramic view and computed tomography (CT), magnetic resonance imaging (MRI), and bone scintigraphy (Assaf et al., 2015, Baba et al., 2018, Otto et al., 2018). The advantages of different imaging modalities can be implemented for specific observations, such as morphological extent, disease-related complications, and vascularization of the bone tissue (Assaf et al., 2015, Baba et al., 2018). Non-

specific symptoms and possible trigger factors in the medical history must be evaluated in patient anamnesis.

2.5 Colonization

The human body is widely colonized by a physiological bacterial flora. The number of bacteria and the type of species in different areas of the body varies greatly. While the colon is home to a particularly large number of microorganisms, including Enterobacteria, Enterococci, and other anaerobes, the stomach is rather sparsely populated due to its acidic environment. Streptococci, Staphylococci, Actinomyces, and Neisseria are frequently found in the oral cavity and the pharynx (Hellwege, 2003). Small numbers of yeast are often represented in the physiological and stable system of the microbial flora (Cannon and Chaffin, 1999). The oral mucosa constantly acts as a barrier between this diverse microbiome and the jaw bones, resisting mechanical and immunological triggers (Moutsopoulos and Konkel, 2018). Even the direct access for pathogens to healthy bone due to dental procedures, ill-fitting dentures, trauma, or periodontal diseases seems to leave it unaffected (Ewald et al., 2021).

However, the presence of microorganisms in the necrotic bone of patients treated with antiresorptive medications is causing infection and inflammation, potentially perpetuating the course of the disease. They have not only been described in the characteristics of the more severe stages II and III of MRONJ (Ruggiero et al., 2022) but also associated with the etiopathogenesis of the condition itself (Boff et al., 2014, De Bruyn et al., 2018, Ewald et al., 2021). Various mechanisms have since been suggested to lead to the progression of the disease. These include the impairment of bone matrix synthesis, the release of acids and proteases with direct damage to the bone, or the stimulation of osteoclast differentiation and activity (Boff et al., 2014, De Bruyn et al., 2018, Ewald et al., 2021, Henderson and Nair, 2003). Infections have consistently been reported in histopathological examinations of the affected necrotic bone with a variety of species (Aftimos et al., 2014, Ewald et al., 2021, Reid, 2009). Although some have been detected more frequently, such as Actinomyces or Streptococci (Boff et al., 2014, Ewald et al., 2021, Reid, 2009, Thumbigere-Math et al., 2009), the importance of individual colonization in the development and severity of this multifactorial disease is not determined to this date. Possible origins

of microorganisms are numerous, for example, odontogenic or periodontal infection with access to the underlying bone, with or without traumatic mucosal and dentoalveolar events (Kumar et al., 2010). The compromised vascularization, inflammatory response, and epithelial covering of the tissue provide for continued bacterial growth (Boff et al., 2014, Conte Neto et al., 2013). The frequent fungal colonization in the specimens was given little attention in wider research analyses (Aftimos et al., 2014), though the contribution of fungi to impaired healing of wounds and the subsequent complications were described in different contexts before (Kalan et al., 2016, Kalan and Grice, 2018). Dental prophylaxis and the maintenance of oral hygiene are part of prevention strategies, while eradication of biofilm, antimicrobial mouth rinses, and rational antibiotic therapies are important parts of the management of a flared disease (Ruggiero et al., 2022). Interkingdom colonization impacts the outcome and prognosis of the patient population and becomes a target in the management of MRONJ.

2.6 Management strategies

Preventive strategies and early diagnosis play a leading role in the management of MRONJ and need to be approached multidisciplinary. Prior to initiating antiresorptive therapy, medical conditions should be addressed proactively, and proper oral hygiene must be instituted (Aparecida Cariolatto et al., 2018, Campisi et al., 2020, Chan et al., 2018). Overall patient health is to be optimized (Ruggiero et al., 2022). Any acute infections or dental diseases must be managed, and necessary dental procedures should be performed preventively (Beth-Tasdogan et al., 2022, Campisi et al., 2020, Chan et al., 2018). During the treatment, possible trauma to the oral mucosa and dentoalveolar trauma is to be avoided or minimized (Weitzman et al., 2007). The awareness of medical practitioners towards key symptoms and triggers of the disease is important to decrease delayed diagnoses and increase the chance of effective treatment (Otto et al., 2018, Ruggiero et al., 2004). Patient education on the risk associated with antiresorptive medications, and motivation regarding preventive measures are to be ensured, whereby the positive effects of the therapy are not to be compromised (Ruggiero et al., 2022).

In established MRONJ, antimicrobial mouth rinses (e.g., chlorhexidine), pain control medications, and antibiotics (e.g., amoxicillin and clavulanic acid, clindamycin,

moxifloxacin) are effective tools in the conservative nonoperative management (Bermudez-Bejarano et al., 2017, Varoni et al., 2021). The recommended duration of initial conservative therapy is typically two to eight weeks before a reassessment of the clinical situation. Therapeutic success, surgical capability, and general condition of the patient are included in the evaluation. Based on this, the decision is made as to whether conservative therapy should be continued, or to what extent surgical therapy is advisable. Orally applied antibiotics can be useful in all stages and their use may be prolonged in patients who are unsuitable for surgical interventions (Ruggiero et al., 2022). Local wound care for exposed lesions and the removal of sequestrum is recommended at any time (Ruggiero et al., 2022). Generally desired outcomes in conservative therapy are stabilization, moderation in severity, or even resolution of disease (Ruggiero et al., 2022).

The combination of initial conservative therapy with an additional surgical treatment, ranging from minimally invasive local debridement or sequestrectomy, to complete resection of the necrotic lesion, has demonstrated beneficial outcomes (Carlson and Schlott, 2014, Chan et al., 2018, Ristow et al., 2019, Varoni et al., 2021). In these cases, the oral administration of antibiotics begins one week before the intervention and is switched to intravenous administration of systemic antibiotics one day preoperatively. Antibiotics are continued for four to five days intravenously after the surgery and orally until suture removal (between 14 and 21 days postoperatively). The efficient delivery of the antimicrobial agent to the hypovascular necrotic bone might play an important role, especially in the reduction of disturbed wound healing and refractory diseases (Ewald et al., 2021). Marginal or segmental resections are recommended in patients with progressive MRONJ, patients with advanced stages at first presentation, or after insufficient conservative therapy (Ruggiero et al., 2022). Local flap coverage is to be performed tension-free. Resections with continuity defects requiring reconstruction and tissue replacement are to be avoided but can become necessary (Ruggiero et al., 2022). Benefits must be vigilantly weighed against the risks of operative therapy under general anesthesia in each individual case.

The leading goals of treatment are the prevention of disease outbreaks, minimizing progression, and preservation of quality of life (Ruggiero et al., 2022). The improvement of wound healing and decrease of refractory diseases are aspired while prioritizing bone health and the continuation of the underlying oncologic

treatment to ensure its benefits (e.g., prevention of skeletal complications, controlling of symptoms, and preservation of the quality of life) (Mücke et al., 2011, Ruggiero et al., 2014, Ruggiero et al., 2022). However, the lack of clinical data leaves incomplete knowledge of the factors that contribute to the onset of disease and the increase in severity. Influences on the frequency of wound healing disorders and recurrences of the disease are not fully clarified. This leads to inconsistent antibiotic regimens and the repetition of surgical procedures. While the importance of prevention is becoming increasingly recognized by healthcare providers, the management and resolution of this complex disease remain challenging.

3 Material and methods

3.1 Study design and cohort

3.1.1 Data collection

This is a monocenter, retrospective study including 148 participants, treated at the University Medical Center Hamburg-Eppendorf between January 2011 and November 2019. The data were collected in the Department of Oral and Maxillofacial Surgery using the software Soarian Clinicals® (Oracle Cerner, Kansas City, MO, USA) and Evident® (Evident GmbH, Bad Kreuznach, Germany). These included the medical history, as well as detailed observations on interventions and measures applied during the treatment and control period. The results of microbiological examinations were incorporated. Using the software ViewPoint™ (GE HealthCare, Solingen, Germany), radiographic images were considered in the evaluation of bone statuses and dentition. A standardized scale allowed for a consistent interpretation. All information about the patient population was compiled in tabular form using Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA). Written reports on medical history and observations during the treatment and control period had to be converted into measurable criteria. Therefore, only uniformly recorded information was included in the evaluation. Documentation of the intraoperative procedure followed a structured and uniform protocol scheme, therefore allowing direct comparison. Any selected written files were revised for reliability and applicability. To visualize the results and processes, graphical representations were created using Microsoft PowerPoint® (Microsoft Corporation, Redmond, WA, USA).

3.1.2 Cohort characteristics

All participants were diagnosed with and treated for MRONJ in clinically and histopathologically confirmed stages II or III according to the criteria in the AAOMS position paper on MRONJ from 2014/2022 and national treatment guidelines (Ruggiero et al., 2014, Ruggiero et al., 2022). Individuals gave informed consent before study participation. Any patients with a history of radiation in the jaws or head region were excluded.

3.2 Surgical debridement and antibiotic treatment algorithm

According to the current national treatment guidelines, surgical intervention at stages II and III is indicated (Schiegnitz et al., 2018). This includes the complete removal of the necrotic bone, smoothening of sharp bone edges, and local flap coverage of the wounds under antibiotic therapy. More precisely, the procedure involves elevation of a full-thickness mucoperiosteal flap, extending beyond the margins of the affected region, and resection of the entire necrotic bone reaching into a disease-free area of vascularized, vital bone. The closure of the soft tissue must be accomplished without tension. In total, 290 surgical interventions were performed under general anesthesia, ranging from one (n=75) to nine (n=1) times in each patient. Additional procedures under local anesthesia, such as surgical smoothening of extraction sockets, sequestrectomy, and secondary wound closures, were conducted 45 times, but not included in the quantitative analysis.

Antibiotics were administered seven days before surgical intervention and continued or adjusted postoperatively until suture removal. Antiseptic mouthwashes (e.g. chlorhexidine) were used for oral decontamination. Preoperative treatment schedules were 875/125 mg twice daily for amoxicillin and clavulanic acid, 400 mg once daily for moxifloxacin, and 300 mg three times daily for clindamycin. All treatments were switched to intravenous administration of antibiotics at admission one day before surgery, except for moxifloxacin, which was continued orally. Postoperatively the antibiotics were adjusted to the results from the microbiological examination or due to other reasons (e.g., intolerance). Additional application of other antibiotic agents (e.g., ciprofloxacin) or antifungal treatment (e.g., amphotericin B) for various reasons was possible.

The follow-up appointments for reevaluation and suture removal were scheduled between seven and 21 days after surgery. Further, signs of disease progression were monitored, and adequate oral hygiene was revised in intervals of three, six, and twelve months, considering the individual risk profile.

3.3 Intraoperative sampling for microbiological assessment

Considering the entire collective of participants, 290 cases of surgical intervention were performed and a total of 222 bone samples were harvested intraoperatively for microbiological assessment. Therefore, at least one sample of necrotic bone was collected from 97.97% of all 148 participants (Figure 4). To prevent contamination of the bone specimens, a topical antiseptic was applied at the beginning of the procedure. The sample was harvested from a non-superficial layer during the debridement, while saliva was continuously aspirated, and any contact with surrounding soft tissue or medical instruments was avoided. The microbiological examination to differentiate on species level was available for 89.86%, using routine culture methods (Columbia blood agar, chocolate agar, and Schaedler agar – all Thermo Fisher, Bremen, Germany) and whole-cell mass spectrometry (Biotyper®, Bruker, Bremen, Germany). Further susceptibility testing (Vitek® II system – Biomerieux, Marcy L'Étoile, France) was subjected to 43.24% to identify bacterial resistance.

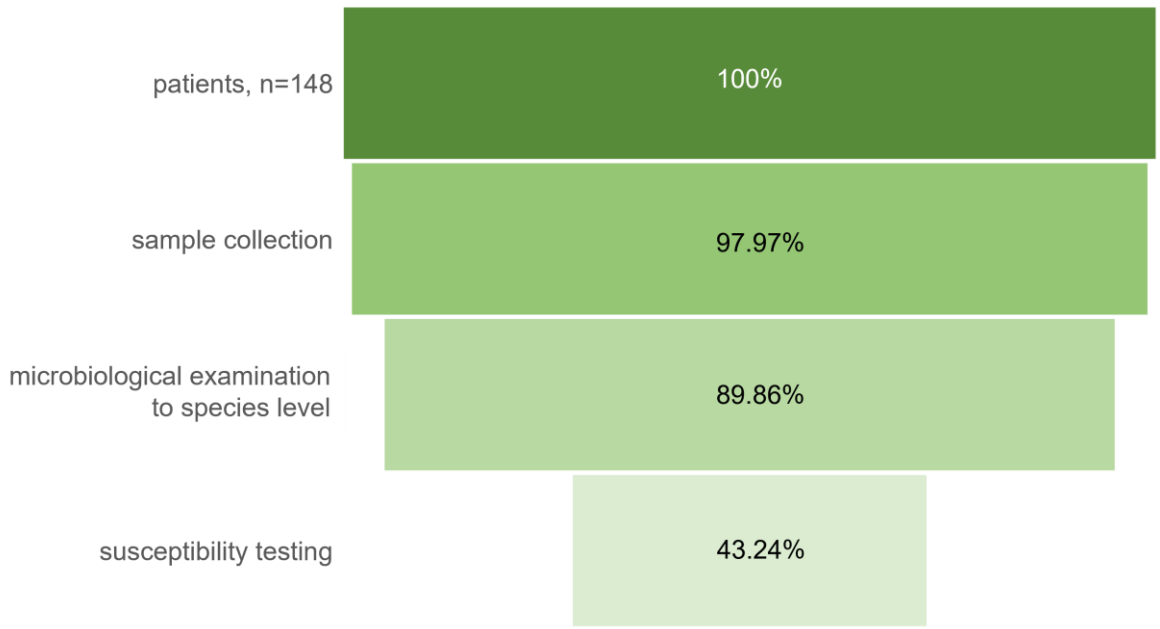

Figure 4: Percentage of patients with intraoperative sample collection, microbiological examination, and susceptibility testing. Across the cohort of 148 participants, 97.97% had at least one necrotic bone sample collected during a surgical intervention. The microbiological examination of the colonization to species level was available for 89.86% of all participants, while susceptibility testing was subjected to 43.24%.

3.4 Definition of the primary outcome measures: healing and recurrence

As wound healing disturbances and recurrences of the disease impose major challenges for both the surgeon and the patient alike, the following primary outcome measures were defined: i) *healing* – full mucosal healing at six weeks postoperatively, and ii) *recurrence* – recurrence of the disease requiring subsequent surgical intervention.

The usual healing time after surgical intervention of the extent described above was estimated to be completed after six weeks, implying no wound healing disorder. No further information concerning the outcome of i) could be provided for the cases with dismissed follow-up appointments at the six-week mark (n=21). Since the recurrence of the disease was defined as requiring another surgical intervention after a previous procedure, a healing disorder was not classified as a recurrent disease. The absence of recurrence by this definition may also include cases in which patients failed to return to an appointment, changed the clinic for their treatment, or passed away. Possible locations of the recurrent lesions were either the same as the previous one (maxilla, mandible, or both), a new one that was different from the previous one, or the same and a new one simultaneously.

3.5 Statistical analysis

The collected data were analyzed descriptively, continuous variables were expressed as mean and their standard deviation (SD), while categorical variables were expressed as number and percentage (%). The median was computed as an alternative measure of central tendency when the mean was deemed to be heavily influenced by outliers. To compare the influences on the healing process and the recurrence of the disease in terms of categorical variables, the Chi^2 or Fisher's exact tests were applied. Where possible, odds ratios (OR) with a 95% confidence interval (CI) were calculated for all variables. The statistical analyses were performed utilizing the statistical program IBM® SPSS® Statistics (Version 29 – IBM, Armonk, NY, USA) and GraphPad Prism (Version 9.5.0 – Dotmatics, Boston, MA, USA) for Windows. The illustrations were created using Microsoft® Excel® and PowerPoint® (Office 365®) for Windows. Statistical significance was set at a two-tailed p-value of 0.05.

4 Results

4.1 Characteristics of the study cohort

4.1.1 Demographics

The distribution of gender in the collective of 148 participants showed a slight predominance of females with 57.43% (n=85), while 42.57% (n=63) were male (Table 1). The mean age was 71.3 years (SD 9.53) at the date of surgery, ranging from 40 to 94 years, with half of the cohort falling within the limits of 67 to 78.

Table 1: Characterization of the study population, including demographic and medical data. The table includes the history of administrated antiresorptive drugs and the underlying diagnoses of each participant at the initial presentation. *Others < 1%* comprise diagnoses, which were mentioned only once each, representing less than one percent of the population (carcinoma of the oropharynx, the thyroid, the parotid, or the uterus, systemic mastocytosis, chondrosarcoma, and rheumatoid arthritis).

	n	%
Male	63	42.57
Female	85	57.43
Age at surgery	⌀ 71.3	
Antiresorptive drug regimen		
Bisphosphonates	84	56.76
i.v.	68	45.95
p.o.	16	10.81
Denosumab	67	45.27
High-dose, Xgeva®	54	36.49
Low-dose, Prolia®	13	8.78
Other mAb therapy	11	7.43
Hormonal therapy	8	5.41
Chemotherapy	19	12.84
Underlying diagnosis		
Breast cancer	45	30.41
Prostate cancer	35	23.65
Osteoporosis	34	22.97
Multiple myeloma, MDS	22	14.86
Renal cancer	13	8.78
CUP	13	8.78
Lung cancer	7	4.73
Bladder cancer	2	1.35
GI tumors	2	1.35
Others < 1%	7	4.73
Oncological diagnosis	144	97.30
Non-oncological diagnosis	36	24.32

4.1.2 Antiresorptive medications

In addition to each patient's medication at initial presentation before MRONJ treatment (Table 1), we monitored the current involved antiresorptive medications for each case of intervention (Figure 5). Prior to the 290 cases of surgical intervention, 50.34% (n=146) of the cases had exposure to intravenous (i.v.) BPs (zoledronate, pamidronate), whereas 7.93% (n=23) of the cases had received BPs per os (p.o.) (alendronate), shown in Figure ***5***. A total of 38.28% (n=111) had been treated with the high-dose form of denosumab (Xgeva®), 6.90% (n=20) with the low-dose form (Prolia®), and 6.12% (n=18) with other mAb therapies (nivolumab, bevacizumab, pertuzumab, trastuzumab, rituximab, daratumumab). On the other hand, 5.17% (n=15) of the cases had received hormonal therapy (enzalutamide, goserelin acetate, tamoxifen, leuprorelin acetate, fulvestrant) and 19.66% (n=57) received other forms of chemotherapy (sunitinib, pazopanib, cabozantinib, erlotinib, osimertinib, bortezomib, everolimus, capecitabin, docetaxel, carfilzomib, chlorambucil, cyclophosphamide, ixazomib, paclitaxel). The combination of drugs occurred in this study cohort.

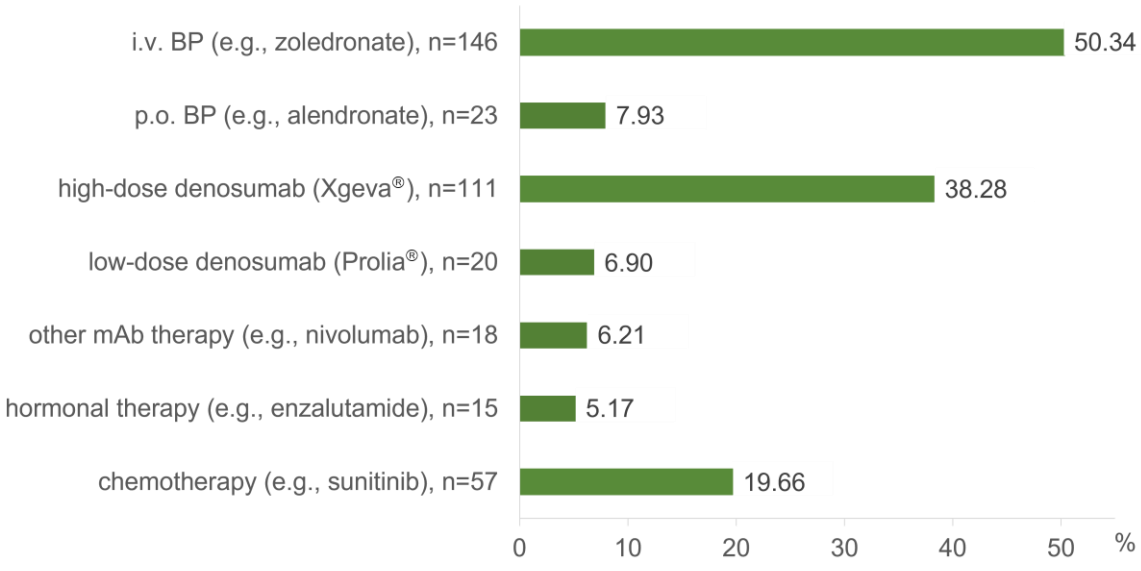

Figure 5: Drug regimen at surgical intervention. BPs were administered intravenously in 50.34%, and orally in 7.93% of the cases of surgical intervention. Denosumab was received in the high-dose form, Xgeva®, in 38.27%, while it was received in the low-dose form, Prolia®, in 6.90%. Other mAb therapies (e.g., nivolumab) were administered in 6.21%, hormonal therapy (e.g., enzalutamide) in 5.17%, and other forms of intensive chemotherapy (e.g., sunitinib) in 19.66% of the cases.

4.1.3 Medical condition

The underlying diagnosis, being the target of the antiresorptive treatment, was predominantly a neoplastic disease (n=144). There were 13 subcategories of oncological diagnoses found in the collective, leading to a total of 97.30% of oncological cases across the study cohort. Diagnoses that occurred only once across all participants accounted for a share of less than one percent of the collective (carcinoma of the oropharynx, the thyroid, the parotid, or the uterus, systemic mastocytosis, chondrosarcoma, and rheumatoid arthritis, indicated in Table 1 and Figure 6 as *others < 1%*). Up to three diagnoses were listed simultaneously at the admission of the included cases. Breast cancer (n=45) and prostate cancer (n=35) formed the biggest groups of oncological conditions (Figure ***6***), followed by multiple myeloma and myelodysplastic syndrome (MDS) (n=22), renal cancer (n=13) and cancer of unknown primary (CUP) (n=13). Further conditions of the oncological participants were lung cancer (n=7), bladder cancer (n=2), and gastrointestinal tumors (GIT) (n=2). In contrast, 36 participants (24.32%) were additionally or solely treated for non-neoplastic diseases (osteoporosis, rheumatoid arthritis, systemic mastocytosis), including 34 cases of osteoporosis. Only four participants (2.70%) were solely diagnosed with a non-neoplastic disease.

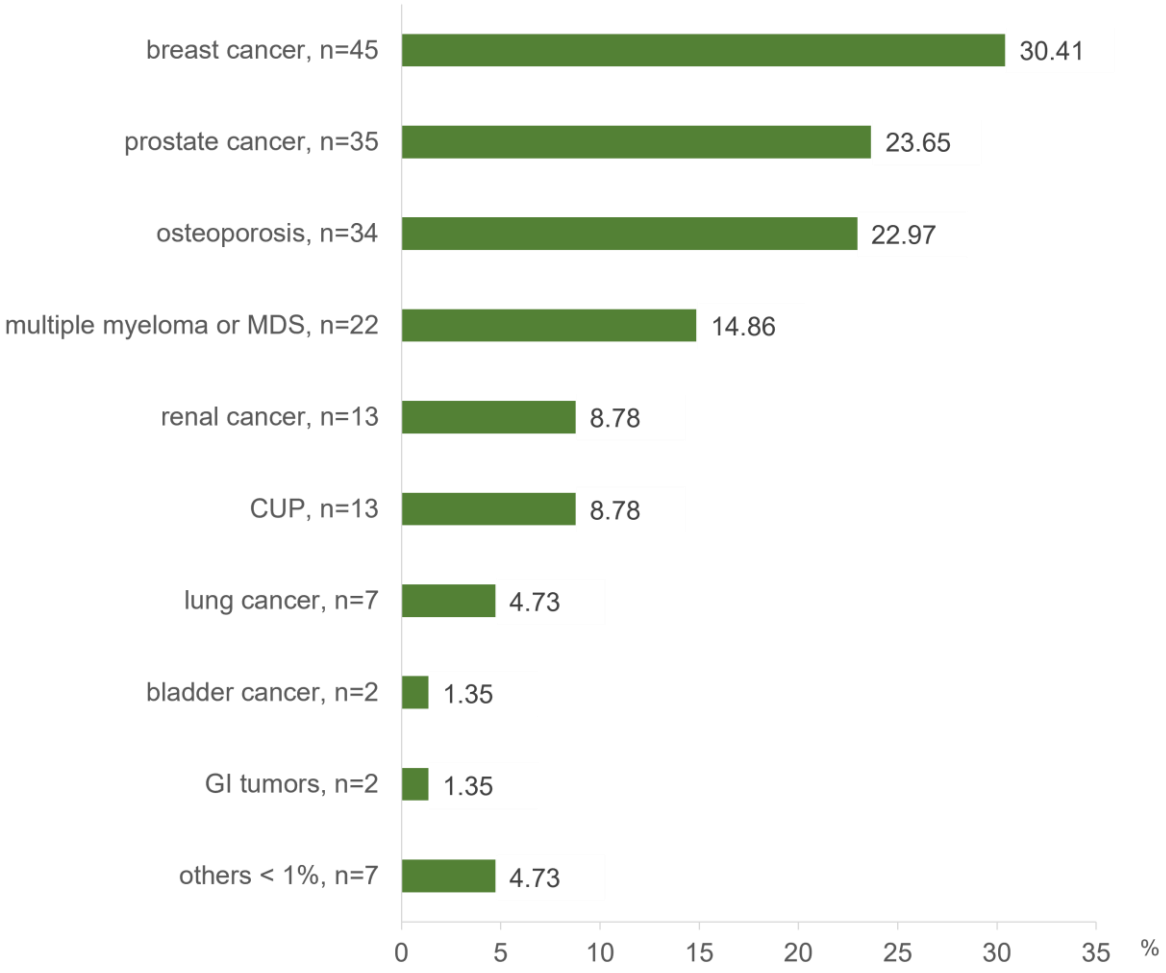

Figure 6: Percentage of study cohort with underlying diagnosis. In the cohort of 148 participants, oncological and non-oncological underlying diagnoses were reported. Up to three diagnoses were listed simultaneously in the same participant. The most frequent oncological diseases were breast cancer at 30.41% and prostate cancer at 23.65%. Osteoporosis was the largest group of the non-oncological diagnoses at 22.97%, however, only 2.70% of the study cohort were solely diagnosed with a non-neoplastic disease.

4.1.4 Trigger factors

For the onset of MRONJ, seven different trigger factors were identified (Table 2): extraction (n=73), pressure sores (e.g., from ill-fitting dentures; n=34), periodontal disease (n=29), abscess or other infection (n=21), implant or related trigger factor (n=15), other forms of oral surgery (n=7) and the unclear factor to which no specific cause could be assigned (n=22). For 64.19% of the participants, only one factor was involved (n=95), while two factors were involved in 35.81% (n=53).

Table 2: Characteristics and staging of the osteonecrosis. For the onset of MRONJ, seven different categories of trigger factors were distinguished, with an extraction being the most frequently reported. In 35.81% there were two factors involved. The location of the necrotic lesion was detected in either the maxilla (upper jaw), the mandible (lower jaw), or both. The staging at the initial presentation refers to the AAOMS position paper (Ruggiero et al., 2022) and the classification of the lesion size refers to Weitzman (Weitzman et al., 2007).

	n	%
Trigger factor for MRONJ onset		
Extraction	73	36.32
Pressure sores	34	16.92
Periodontal disease	29	14.43
Infection or abscess	21	10.45
Implant or related	15	7.46
Oral surgery	7	3.48
Unclear trigger	22	10.95
Two factors involved	53	35.81
Location		
Maxilla	32	21.62
Mandible	97	65.54
Both	19	12.84
MRONJ stage at initial presentation		
Stage II	113	76.35
Stage III	35	23.65
Weitzman classification at initial presentation		
1A	15	10.14
1B	4	2.70
2A	16	10.81
2B	4	2.70
3A	30	20.27
3B	7	4.73
4A	52	35.14
4B	20	13.51

4.1.5 Anatomic factors

The location of the lesions appeared most frequently only in the mandible, with 65.54% (n=97), as shown in Table 2. The maxilla was affected alone in 21.62% (n=32), while both jaws were involved in 12.84% (n=19).

4.1.6 Staging

All participants were staged at admission and only included in this research if at stages II or III of the AAOMS position paper (Ruggiero et al., 2022). The distribution showed a predominance of participants in stage II with 76.35% (n=113), whereas

23.65% (n=35) were classified as stage III (Table 2). Considering the further grading of the lesion size (Weitzman et al., 2007), lesions of stage 4A made up the largest group in the study cohort with 35.14% (n=52) (Table 2).

4.1.7 Antibiotic regimen

The antibiotic treatment was received preoperatively in the form of amoxicillin and clavulanic acid in 69.7% of all surgical cases, while 22.4% were initially treated with moxifloxacin and 4.8% with clindamycin (Figure 7). Postoperatively, 36.0% of treatments were also changed to moxifloxacin and 18.0% to clindamycin. The most common addition to the antibiotic regimen was used to treat fungal infections: in 40.0% the addition was amphotericin B and in 10.0% fluconazole.

Initial antibiotic treatment (n=290 ≙ 100%)	
69.7%	amoxicillin
22.4%	moxifloxacin
4.8%	clindamycin
2.4%	doxycycline
2.1%	ciprofloxacin
1.7%	cefuroxime
0.3%	flucloxacillin
0.3%	none
2.8%	combinations

Change in the antibiotic treatment (n=50 ≙ 17.24%)	
36.0%	moxifloxacin
18.0%	clindamycin
12.0%	ampicillin/amoxicillin
12.0%	ciprofloxacin
8.0%	cefuroxime
4.0%	cotrimoxazole
2.0%	flucloxacillin
2.0%	piperacillin/tazobactam
2.0%	ceftriaxone
4.0%	antibiotic treatment stopped

Addition to the antibiotic treatment (n=20 ≙ 6,9%)	
40.0%	amphotericin B
10.0%	moxifloxacin
10.0%	cefuroxime
10.0%	fluconazole
5.0%	doxycycline
5.0%	clindamycin
5.0%	ciprofloxacin
5.0%	erythromycin
5.0%	cotrimoxazole
5.0%	oxytetracycline

Figure 7: Antibiotic treatment algorithm including postoperative change and additions. In 69.7% of all cases, the initial antibiotic treatment was amoxicillin. A postoperative change of agent was implemented in 50 cases, which corresponds to 17.24% of the study cohort. Moxifloxacin was implemented in 36.0% of the cases of changed treatment. An addition was postoperatively introduced in 20 cases, representing 6.9% of the cohort, with amphotericin B as the most common agent.

4.1.8 Surgical data

Between the first diagnosis of MRONJ and the start of surgical treatment, the mean time was 2.59 months with a SD of 6.54 (Table 3). In total, 88.51% started their treatment at the University Medical Center Hamburg-Eppendorf within the first three months of diagnosis. Only 4.73% were subjected to prolonged conservative management for over twelve months. The number of performed surgeries under general anesthesia per participant varied between one and nine times (Table 3). In 76.36% the patient underwent one (n=75) or two (n=38) surgeries. Over five interventions were executed in four patients.

Table 3: Surgical data, including the primary outcome measures. In total, 290 surgical interventions were performed, with one to nine surgeries in the same participant. Additional surgeries under local anesthesia were executed in 20.00%. The mean time between the first diagnosis and the start of surgical treatment was 2.59 months. The primary outcome measures of the study were healing and recurrence. Six weeks after the intervention, 46.90% of the cases showed not healed lesions. A recurrence of the disease, requiring another surgical intervention, occurred in 61.38% of the cases, including 44.83% in the same location.

	n	%
Time between first diagnosis and start of surgical treatment in months	⌀ 2.59	
≤ 3 months	131	88.51
3 to 6 months	7	4.73
6 to 12 months	3	2.03
> 12 months	7	4.73
Surgeries under general anesthesia	290	
1 surgery	75	50.68
2 surgeries	38	25.68
3 surgeries	18	12.16
4 surgeries	8	5.41
5 surgeries	5	3.38
> 5 surgeries (max. 9)	4	2.70
Additional surgery under local anesthesia	45	20.00
Healing, six weeks postoperatively		
Healed cases	133	45.86
Not healed cases	136	46.90
No follow-up	21	7.24
Recurrence of the disease		
Cases with recurrence	178	61.38
Same location	130	44.83
New location	28	9.66
Same and new location	20	6.90
Cases with no recurrence of the disease	112	38.62

4.1.9 Primary outcome measures in the study population

Considering the 290 cases of surgical intervention, 45.86% (n=133) showed full mucosal healing at the six-week follow-up appointment, whereas 46.90% (n=136) of the cases examined exhibited incompletely healed lesions. Due to missed follow-up appointments in 21 cases, 7.24% of the population could not be evaluated for their healing status after six weeks. Further investigations on the healing process were unavailable in those cases. A total of 269 surgical interventions were investigated at the six-week appointment, resulting in a ratio of healed lesions at 49.44% (n=133) and not healed lesions at 50.56% (n=136) (Figure 8A). Across the 290 surgical interventions, a recurrence of the disease occurred in 61.38% (n=178), whereas 38.62% (n=112) did not show a recurrence and therefore had no further interventions under general anesthesia performed (Figure ***8***B).

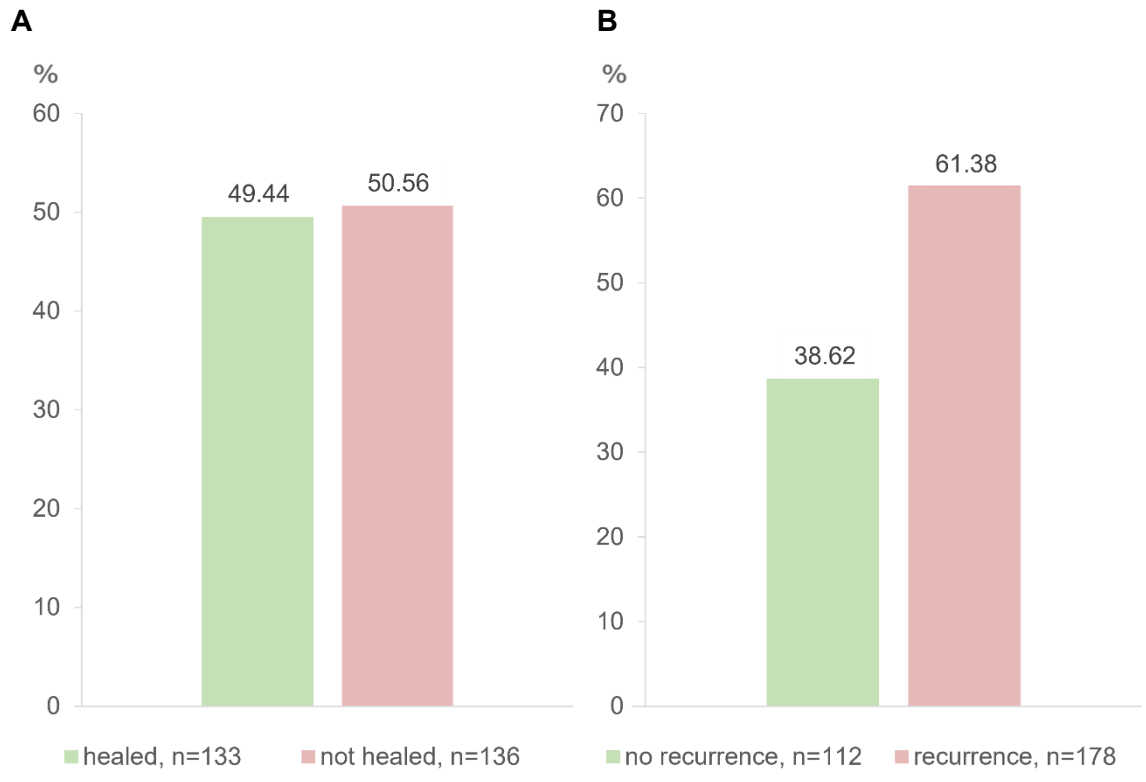

Figure 8: Healing and recurrence across the study population. (A) Healing process across the study population at six weeks after surgical intervention. The distribution of cases with healed and not healed lesions at the six-week mark showed 49.44% of the cases as healed, while 50.56% were not healed. (B) Recurrence of the disease across the study population. A refractory disease was evident in 61.38% of the cases in this study. No further surgical interventions were performed in 38.62% of the cases.

4.1.10 Location of recurrence

The refractory disease appeared in 84.27% in the same location as the previously treated lesion (Table 3). In 15.73% the lesion of the recurrence only occurred in a different location to the former one, while a different location and the previous location were simultaneously afflicted in 11.24% of the refractory diseases.

4.2 Limited influence of the underlying diagnosis

The primary outcome measures, healing after six weeks and recurrence of the disease, were analyzed for the influence of various aspects. The influence of the oncological or non-oncological category of underlying diagnosis on healing and recurrence rate was non-significant, as indicated by the p-values (healing: $p>0.999$, OR 0.989 [95% CI 0.557-1.751]; recurrence: $p=0.474$, OR 1.227 [0.706-2.164]). Multiple simultaneous diseases were prevalent in many patients and an overall high rate of oncological diagnoses (97.30%) was present – although these did not always warrant the antiresorptive medication. The cases of intervention attending the follow-up appointment with an oncologic underlying diagnosis (n=241) showed a healing rate of 50.62% after six weeks, very similar to the cases with a non-oncologic underlying diagnosis (n=57), which showed a healing rate of 50.88% at checkup (Figure 9A). The oncological cases (n=259) were subjected to another intervention in 61.39%, whereas the non-oncological cases (n=62) developed fewer recurrent forms of the disease in 56.45%, requiring, although not statistically significant, fewer surgical interventions in this group (Figure 9B).

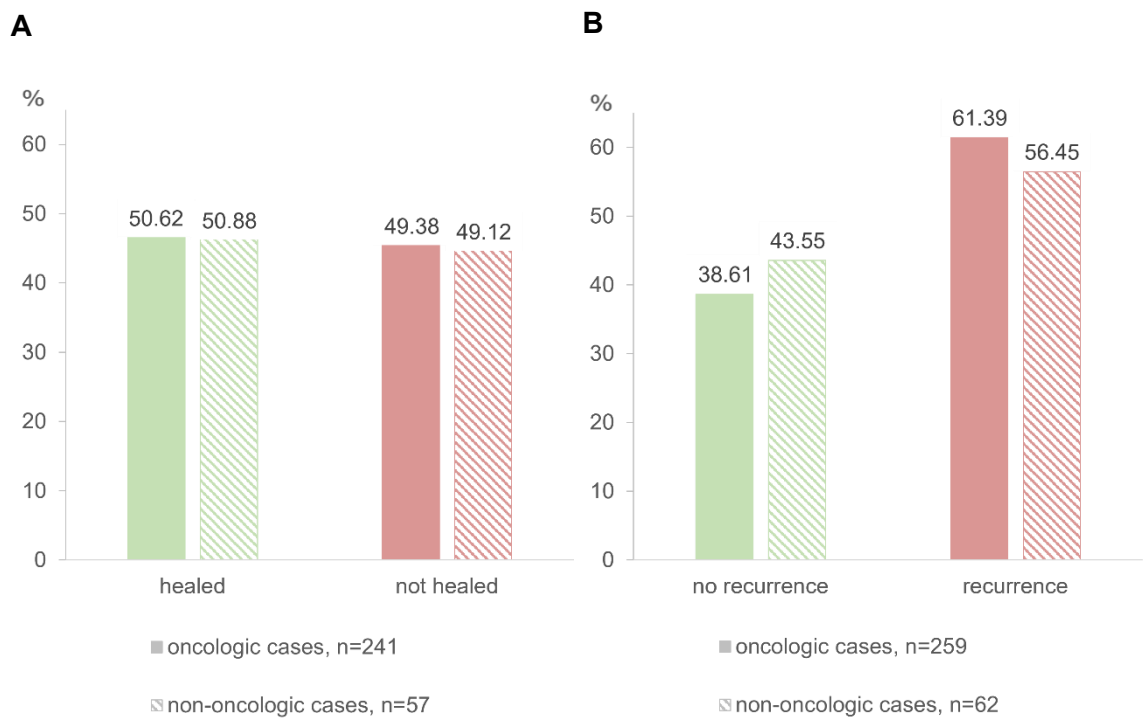

Figure 9: Healing and recurrence in oncologic and non-oncologic cases. Oncologic and non-oncologic cases are presented by uniformly colored or striped columns. (A) Healing in oncologic and non-oncologic cases. Six weeks after the intervention, 50.62% of the oncologic cases and 50.88% of the non-oncologic cases showed healed lesions. (B) Recurrence in oncologic and non-oncologic cases. A recurrence of the diseases occurred in 61.39% of the oncologic cases and 56.45% of the non-oncologic cases.

4.3 Increased recurrence under chemotherapy

We hypothesized, that the antiresorptive drug regimen the recurrence process of our patients influences. Across the 146 cases with a history of i.v. BPs, 97 cases presented with a recurrence of the disease, while 49 cases were not required to undergo another intervention under general anesthesia (Figure 10). The recurrence rate of the disease, at 66.44%, was found to be higher in comparison to the cases treated with p.o. BPs, which had a recurrence rate of 47.83%. This result was statistically non-significant with a p-value of 0.084, and an OR of 2.160 [0.933-5.120], but it indicated an increased risk of recurrence for cases treated with i.v. BPs. The cases receiving the high-dose form of denosumab, Xgeva®, also showed a refractory disease more often (62.16%) than the cases receiving the low-dose form, Prolia® (40.00%) with an OR of 2.464 [0.938-6.538] and a p-value of 0.064. The OR between the BPs and denosumab was 1.242 [0.785-1.963], and the

p-value was 0.365, indicating no significant associations. Patients treated with other mAb therapies suffered from a recurrence in 38.89%. A total of 60.00% of the cases treated with hormonal therapy resulted in recurrent disease. The highest recurrence rate appeared in the cases of chemotherapy (78.95%). Comparing chemotherapy to the cases of hormonal therapy, the p-value was 0.132 and the OR was 2.500 [0.667-7.736], showing a non-significant tendency of a higher risk of recurrence. The comparison of cases treated with chemotherapy to the cases with other mAb therapies shows a p-value of 0.001 with an OR of 5.893 [1.891-18.040], indicating a significantly higher risk of recurrence in the group of chemotherapy, with a vastly increased OR and high CI. Comparing the three drug regimens, other mAb therapies, hormonal therapy, and chemotherapy, a p-value of 0.005 was shown, indicating significant differences in the recurrence rates between these therapeutic regimens.

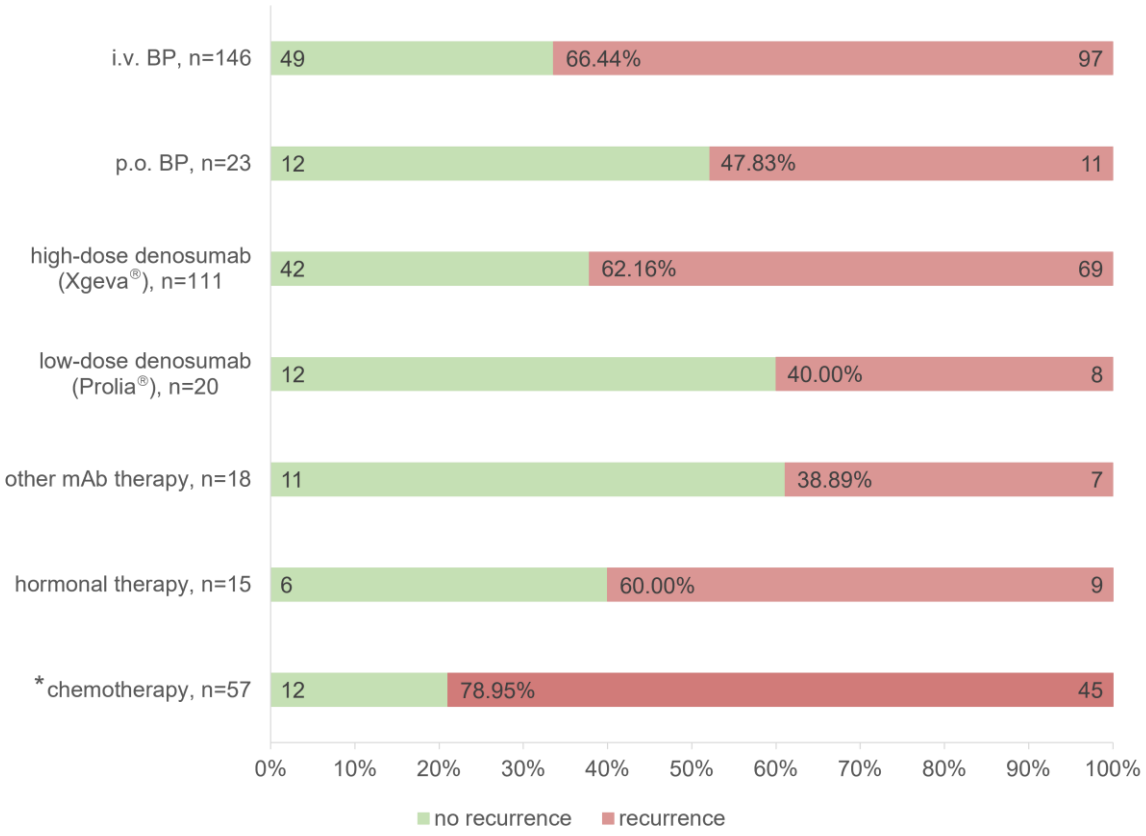

Figure 10: Influences of the underlying antiresorptive drug regimen on the recurrence of the disease. The seven different underlying drug regimens influenced the recurrence rate of the disease to various degrees. The number of affected cases is shown on either end of the bars. The recurrence rates are displayed within the ends of the bars of recurrence. The lowest recurrence rate was presented in cases treated with the low-dose form of denosumab, Prolia®, (40.00%) or other mAb therapies (38.89%). Chemotherapy displayed a significant (*) negative impact on the recurrence rate with a p-value of 0.001, indicated by the bar in a darker red, presenting a refractory disease in 78.95% of the cases.

4.4 Effect of antiresorptive medications on the healing process

The influence of the antiresorptive drug regimen on the healing process only showed a non-significant tendency of a positive impact in cases treated with the low-dose form of denosumab, Prolia®, displaying a p-value of 0.134 and an OR of 0.405 [0.143-1.113] between the groups of the high-dose and the low-dose form. The value of the OR indicates a less detrimental effect associated with the low-dose denosumab. Patients who received the low-dose form showed a higher percentage of healed lesions at six weeks after surgical intervention at 68.42%, whereas the patients with the high-dose form, Xgeva®, appeared as healed

in 46.73% (Figure 11). Between the intravenous and orally received BPs, no difference in the healing process was observed (*p*=0.821, OR 1.146 [0.483-2.910]). Cases receiving i.v. BPs presented in 48.85% and the cases receiving p.o. BPs in 45.45% as healed after six weeks. The p-value between the groups treated with BPs and denosumab was *p*=0.811, and the OR was 0.937 [0.589-1.489]. The healing rate of patients with other mAb therapy was 50.00% and therefore indicated no tendencies. The patients receiving hormonal therapy showed the lowest healing rate (30.77%). Only 13 patients were affected by hormonal therapy and attended the follow-up examination. Although chemotherapy showed no negative influence on the healing process (51.79% healed), compared to BPs, denosumab, and other mAb-based drugs, the need for further surgical interventions was still high (Figure ***10***). The p-value for the healing rates of chemotherapy compared to other mAb therapy was >0.999, and the OR was 1.074 [0.369-3.104], implying no imbalances in the risk of a healing disorder. The p-value for chemotherapy compared to hormonal therapy was 0.224, and the OR was 2.417 [0.735-7.665], which indicates no significance, but a higher healing tendency for the group of chemotherapy. Between chemotherapy, hormonal therapy, and other mAb therapy, the p-value was 0.389.

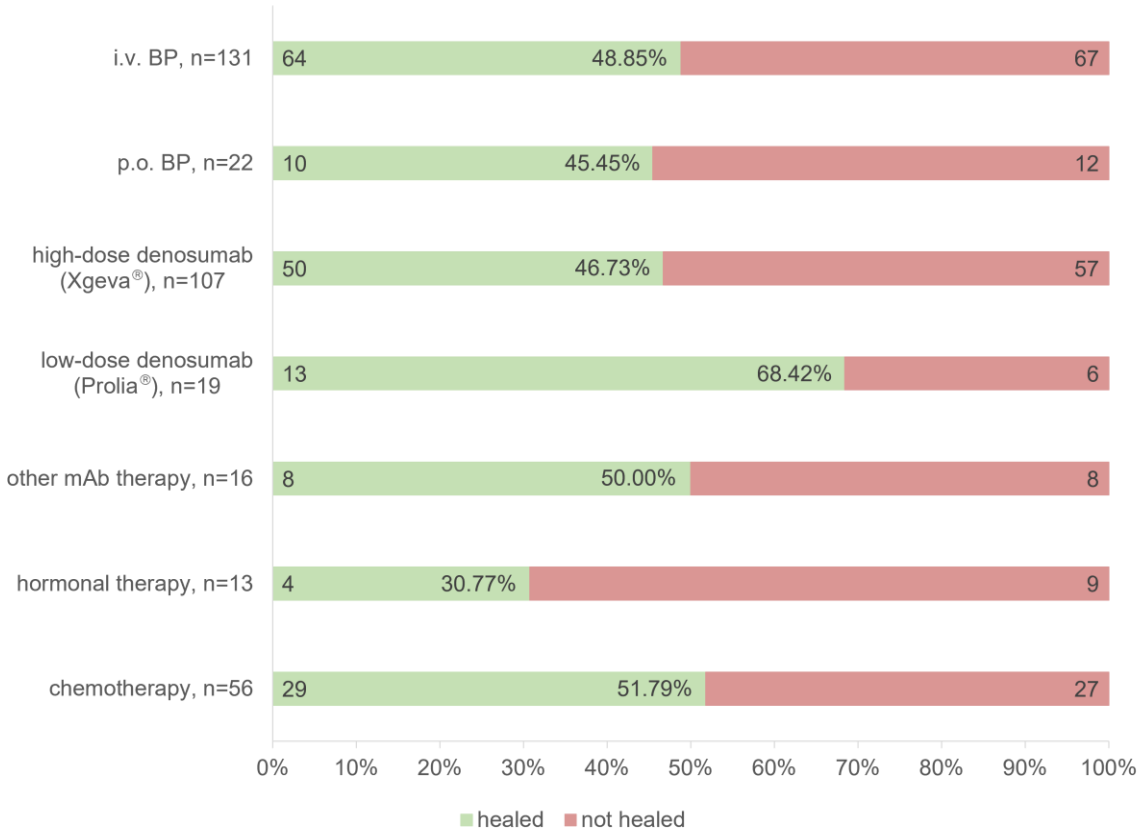

Figure 11: Influences of the underlying antiresorptive drug regimen on the healing process. The seven different underlying drug regimens influenced the healing process as shown by the number of affected cases on either end of the bars. In the group of cases, treated with the low-dose denosumab, Prolia®, the highest percentage of lesions presented as healed at the six-week appointment (68.42%). Cases administered with hormonal therapy showed the lowest percentage of healed lesions (30.77%).

4.5 Diverse colonization of the necrotic bone

During the 290 surgical interventions, 222 specimens of necrotic bone were harvested, of which 186 were identified to species level. Across the total of 449 isolates found during the study of this population, 48 different species of bacteria and seven species of fungi were detected. Of the examined bone specimens, 24.19% were colonized by *Streptococcus anginosus*, the most frequently identified pathogen (Figure 12). Other common species found in the necrotic bone (besides Streptococci) were *Neisseria* spp. (14.52%), *Lactobacillus* spp. (12.37%) and *Candida albicans* (11.83%). *Actinomyces* spp. were identified in 8.60% of the specimens. No specific pathogens were found in 5.38%, here labeled as *sterile*.

Various species were detected in under one percent of all specimens, however, accumulated to a total of 6.45%, representing the diversity of the microbiome.

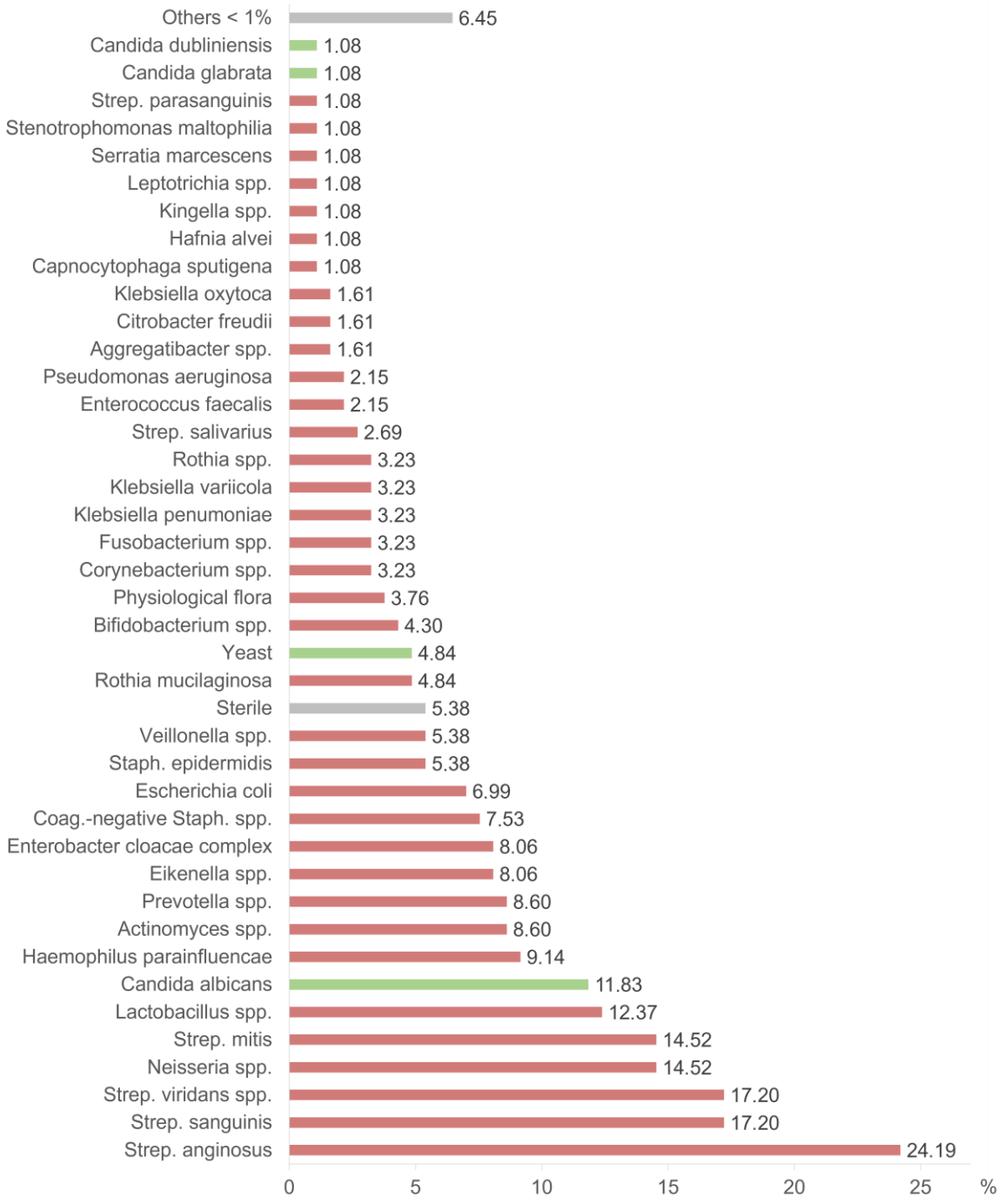

Figure 12: Percentage of necrotic bone specimens colonized by the detected species. Bacteria are indicated by red bars; fungi are indicated by green bars. In the 186 examined necrotic bone specimens from the study population, various species of Streptococci were identified. A total of 24.19% were colonized by *Streptococcus anginosus*. The most common fungal species was *Candida albicans*, found in 11.83%. In 5.38% no pathogens were detected, here indicated by *sterile* and a grey bar. Species found in < 1% of the specimens added up to 6.45%, also illustrated with a grey bar.

Altogether, 91.76% of the 449 individual isolates were identified as bacteria, while 8.24% were fungi. All isolates of the genus Streptococcus added up to a total of 32.29% (n=145) (Figure 13, Figure 14). *Neisseria* spp. were detected in 6.01% (n=27), coag.-negative *Staph.* spp. in 5.35% (n=24), and *Lactobacillus* spp. in 5.12% (n=23) across all detected isolates. Both gram-positive and gram-negative isolates were present in most bone samples. They revealed 60.73% gram-positive and 39.27% gram-negative bacteria.

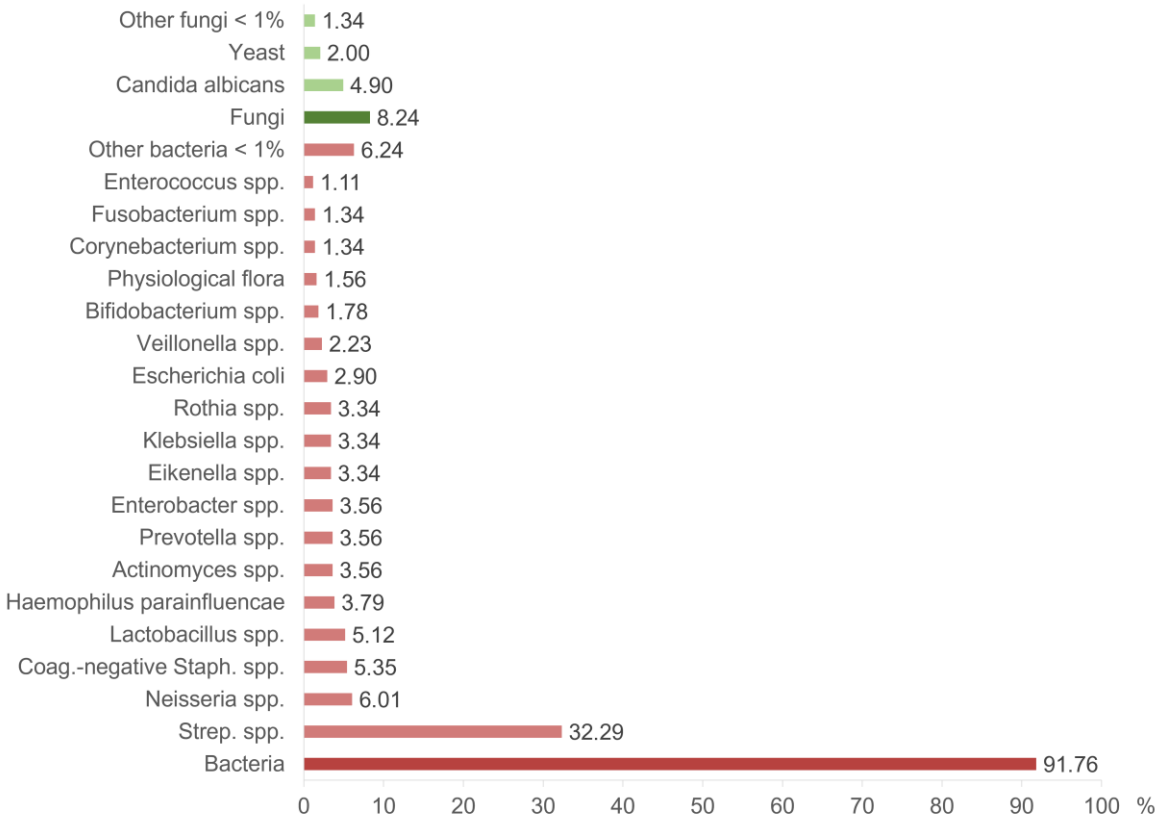

Figure 13: Percentage of isolates detected as listed species. Considering all 449 isolates, that were identified in the microbiological examination, 91.76% were bacteria, illustrated with red bars, while 8.24% were fungi, illustrated with green bars. Streptococci made up the largest group of bacterial species with 32.29% of all isolates, while *Candida albicans* was the most frequently detected fungal species with 4.90%. The wide range of the microbiome included a total of 55 species, many of them only represented in < 1% of the isolates.

To provide an overview of the various species detected in the specimens of the study population, twelve groups were subdivided in a microbiologically appropriate manner. Some isolates have been grouped according to their genus, family, order, class, or kingdom. Species of the genus Streptococcus formed the largest group with 145 isolates detected. The order Enterobacterales was represented by 54 isolates, the class of Actinomycetia by 45 isolates, and a total of 44 isolates were identified as part of the family Neisseriaceae. The fungal kingdom was represented by 37 isolates. Staphylococcus was the genus of 25 isolates and Lactobacillus was the genus of 23 isolates. In ten cases, no pathogens were detected, which is indicated as *sterile*. The order Fusobacterales included eight isolates, a physiological flora (normal oropharyngeal flora) was identified in seven cases, and the genus Enterococcus was represented by five isolates. Other remaining species from various genera, families, and orders added up to a total of 56 isolates. These groups and species are visualized in Figure 14 and utilized in subsequent group analyses.

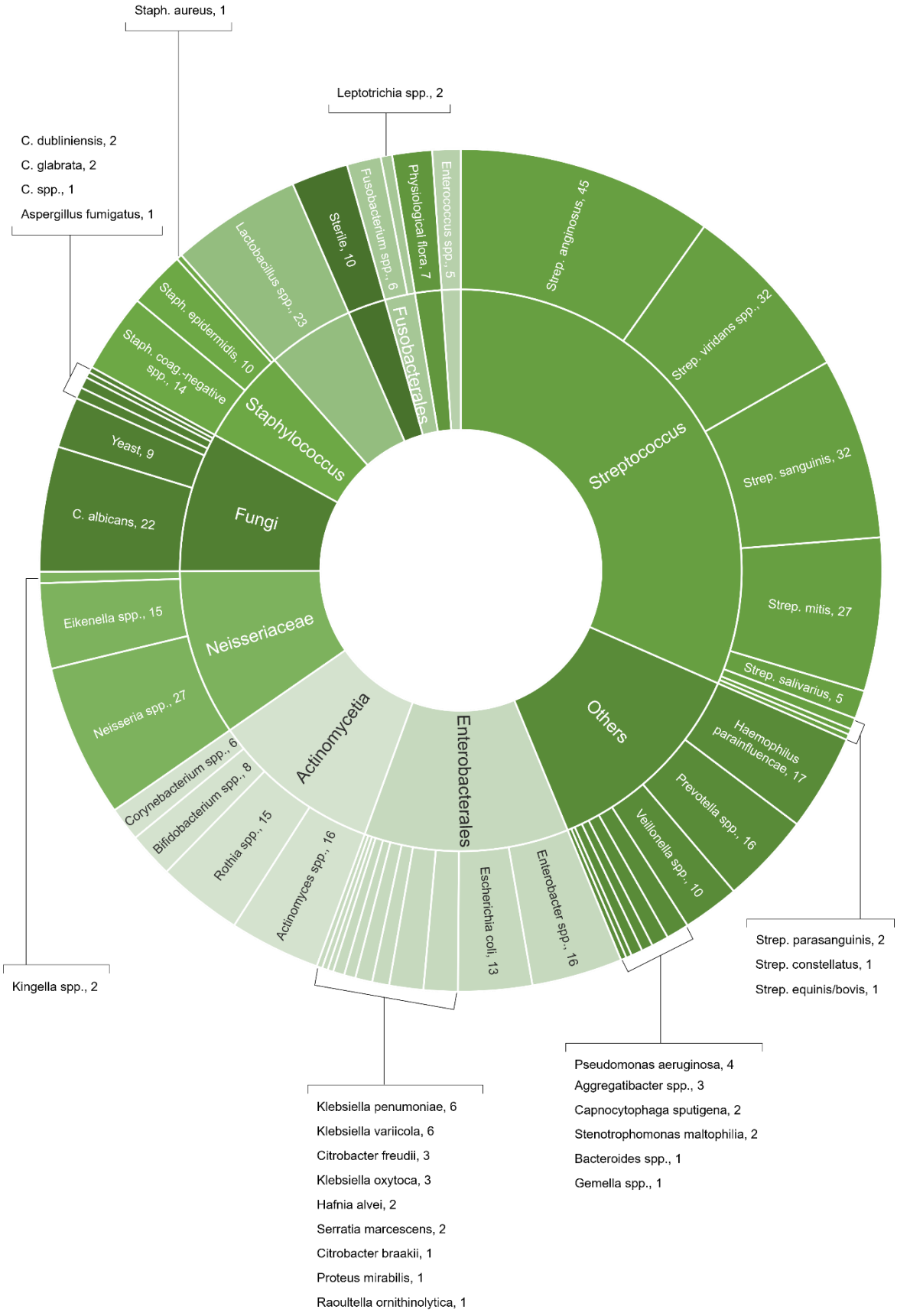

Figure 14: Overview of species distribution across all isolates. The pie chart is divided into twelve groups that comprise the various isolates. The largest group of 145 isolates included all species of the genus Streptococcus.

Candida albicans (n=22) was found to be the most frequently identified species of fungi in the necrotic bone with a percentage of 59.46% across all fungal isolates, followed by yeast (n=9) with 24.32% (Figure 15). Fungi of the genus Candida were collectively represented by 27 isolates, whereas the genus Aspergillus was detected once. Considering the whole collective of 449 isolates, *Candida albicans* accounted for a total of 4.90% (Figure 13), while being present in 11.83% across all specimens (Figure 12).

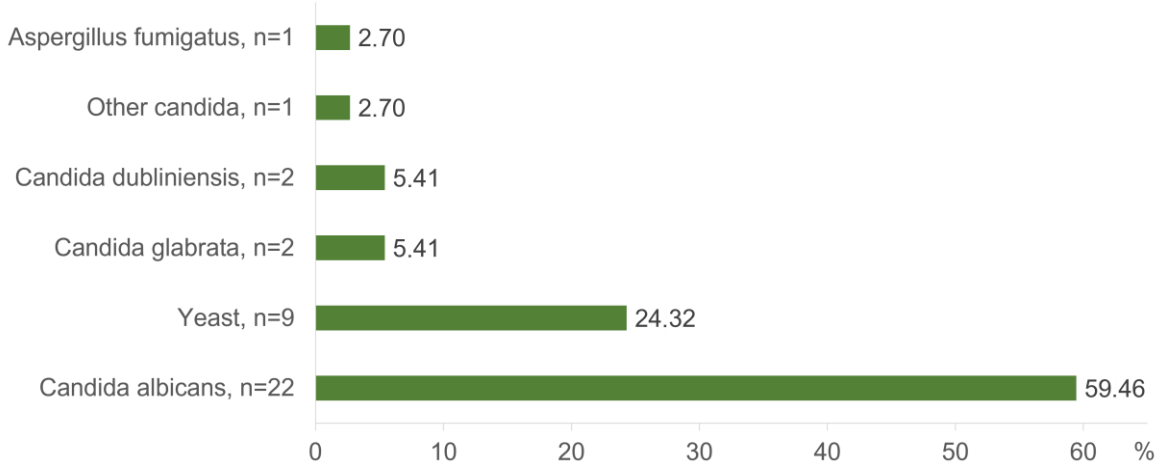

Figure 15: Distribution of fungal species. Across the 37 isolates of fungal species, the most frequently identified was *Candida albicans* (n=22) in 59.46%. The presence of yeasts accounted for 24.32% of all fungal species, represented by nine isolates.

4.6 Healing and recurrence remain unaffected by the number of species

The number of bacterial and fungal species detected in the necrotic bone specimens varied between zero and five. In 5.38% of the microbiologically examined specimens, no pathogenic bacterial or fungal growth was revealed (Figure 16). In 16.67% only one species was detected. In most cases, the presence of two (31.72%) or three species (25.81%) was identified. In 13.98%, there were four species, and in 5.38% were five species found. The number of species had no significant influence on the healing (p=0.332) and the recurrence rate (p=0.266).

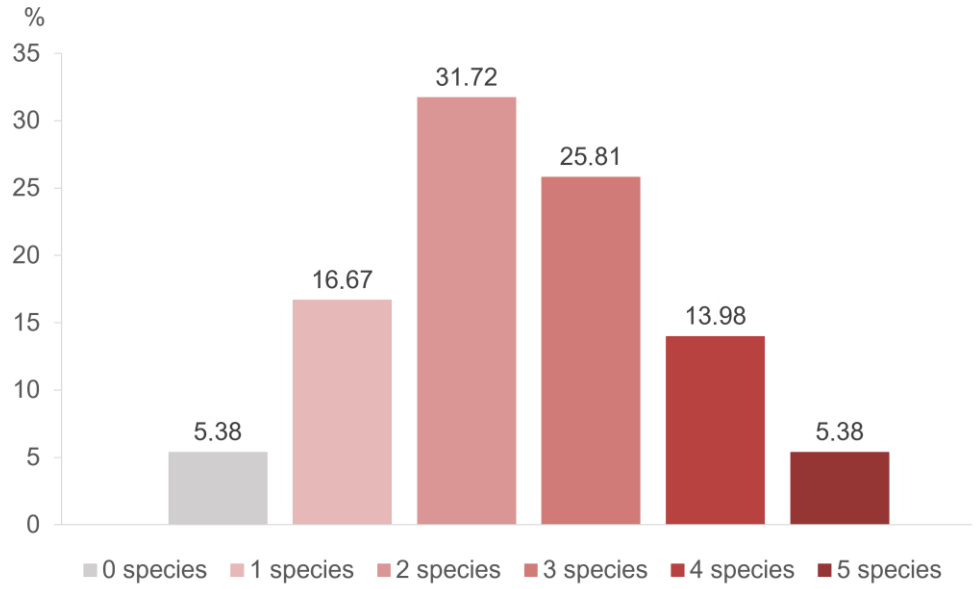

Figure 16: Number of species detected in necrotic bone specimens. Between zero and five different species were detected per specimen. The most common scenario was two species (31.72%). The presence of one species was found in 16.67%, three species in 25.81%, and four species in 13.98%. A total of five various species was present in only 5.38% of the cases, which was equally rare as finding zero pathogenic species in this cohort. Bacterial and fungal species are included.

4.7 Fungal colonization impairs healing

For the comparison of the healing process of lesions, we considered the colonization of 27 different species. Only the species with a minimum incidence in the necrotic bone specimens of five times in our collective (4.5 was the median number of incidences per species) and participants, attending the follow-up appointment (21 cases of intervention were lost to follow-up), were selected for this subsequent group analysis. The number of isolates per species varied between five and 41 times in the included cases. The effects on the healing process were analyzed by comparing the cases that were colonized by a particular species with the cases that were colonized exclusively by other species from this study. Within the 20 specimens colonized by *Candida albicans*, 65.00% were not healed within six weeks after the surgical intervention (Figure 17). Further, 66.67% of the specimens colonized by forms of yeast were also not healed within six weeks. In addition to the fungal colonization, two bacterial species also indicated a negative healing tendency: *Klebsiella pneumoniae* (n=5) revealed a slower healing process after the

surgery or a wound healing disorder in 60.00% and physiological flora (n=7) in 57.14%. The four species mentioned are the only ones with a lower healing rate six weeks after the surgical procedure than the overall study population (49.44% healed, Figure 8). In cases of no detected pathogens (n=10, indicated as *sterile*) no influence on the healing process was assessed.

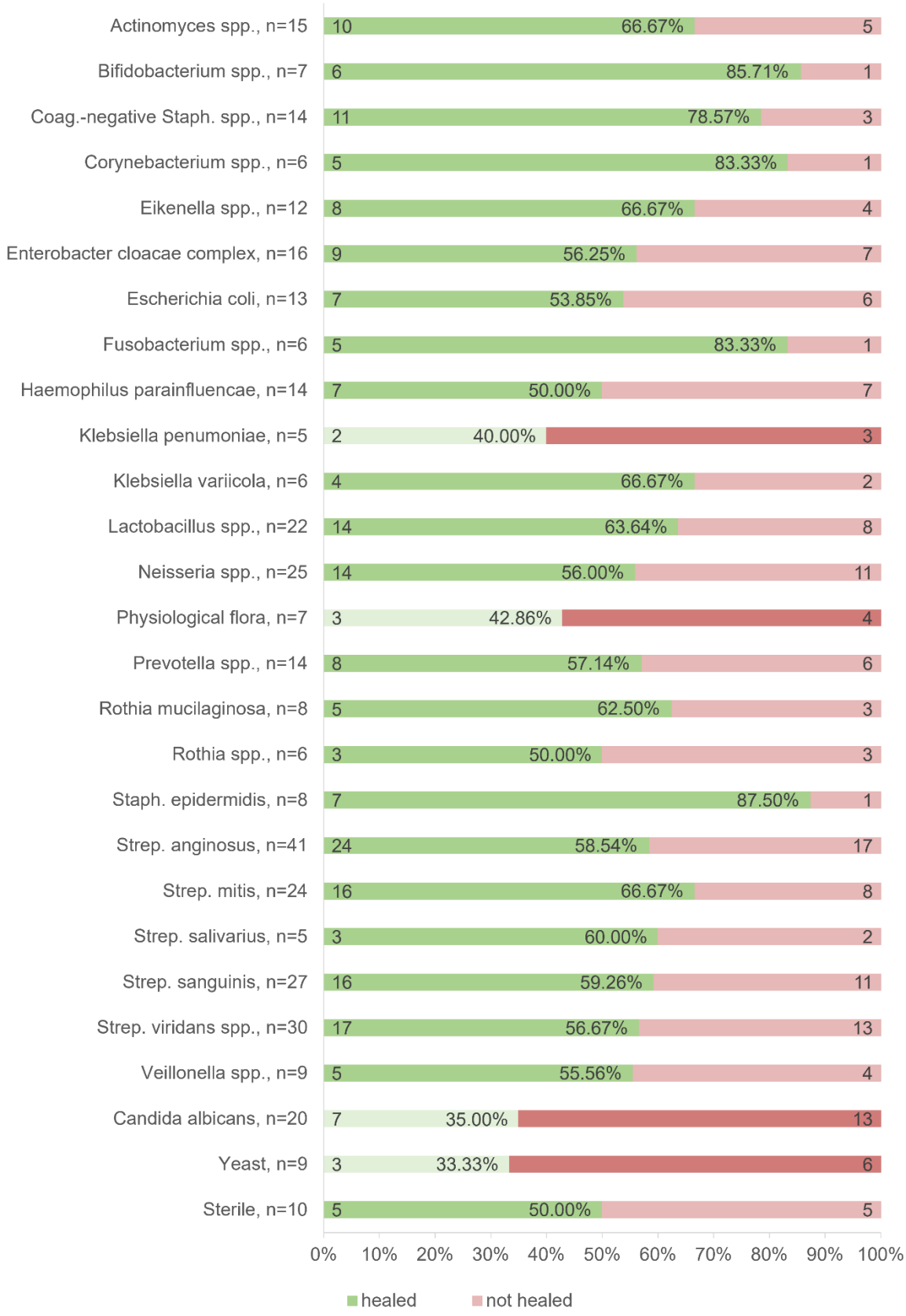

Figure 17: Influence of colonization on the healing process. The species with a negative influence on the healing process, compared to the cases that were colonized exclusively by the other species from this study, are indicated by the darker red bars.

For the statistical analysis we categorized the species into twelve groups, earlier displayed in Figure 14, and here shown in Figure 18. A positive influence on the healing process (77.27% healed, 22.73% not healed after six weeks) was implied the most in cases colonized by species of the genus Staphylococcus (n=22) with a p-value of 0.089 and an OR of 2.361 [0.872-5.942]. Although healing rates of 74% and 88% were observed for Actinomycetia and Fusobacteriales, respectively, no statistical significance was calculated (Actinomycetia: n=34; *p*=0.093, OR 1.950 [0.888-1.499]); Fusobacteriales: n=8; *p*=0.109, OR 4.770 [0.660-54.070]). A significant negative influence on the healing process, compared to other cases with microbiological examination to species level, was found in the cases of fungal colonization (n=35). They appeared as healed after six weeks in only 37.14% and indicated a high risk of impaired healing (*p*=0.004, OR 0.357 [0.178-0.876]). The cases of no detected pathogens (n=10, indicated as *sterile*) appeared to have no influence on the healing process, showing 50.00% healed and 50.00% not healed lesions.

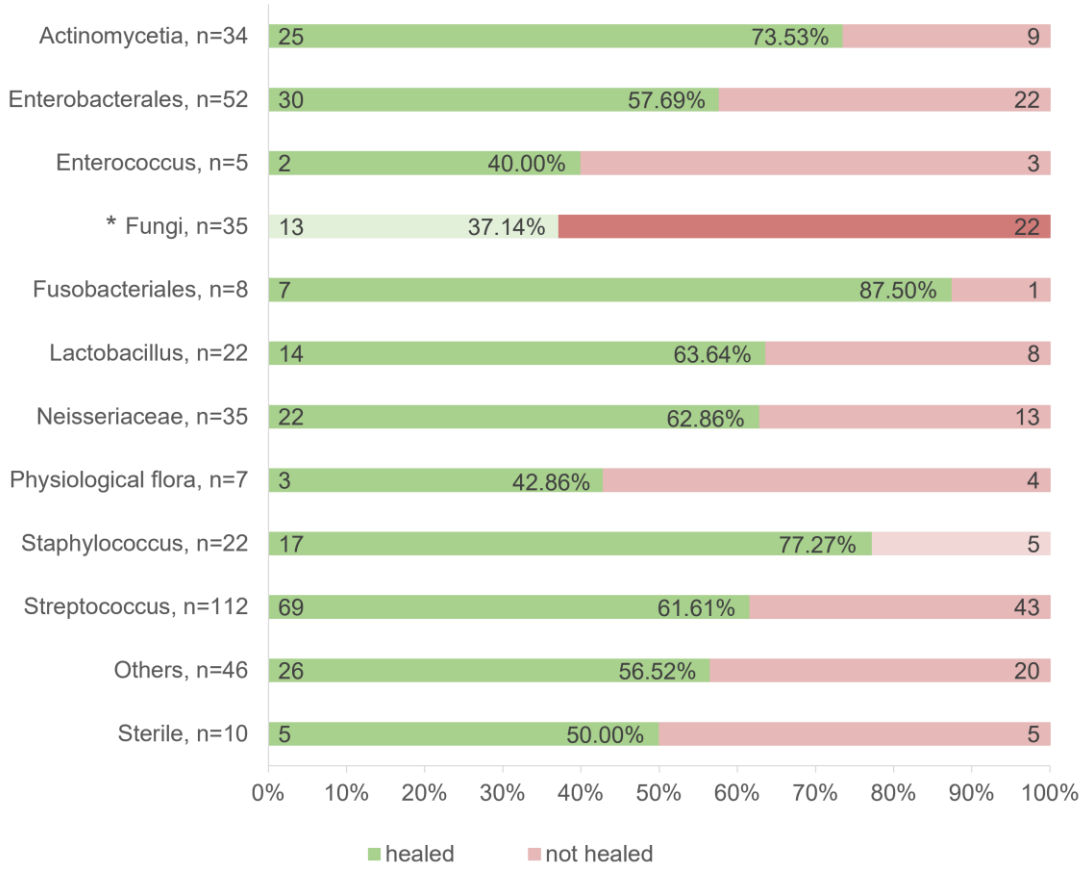

Figure 18: Influence of different microbiological groups on the healing process. Considering the twelve microbiological groups of species we specified in section 4.5, fungal colonization presented as a significant (*) negative impact on the healing process with a p-value of 0.004.

4.8 Recurrence rate is affected by microbiological colonization

The investigation of the same 27 species from above (Figure 17) was continued, to consider their influence on the refractory pattern of MRONJ. Species occurrence varied from six to 45 times in the specimens. The impact on the recurrence rate was evaluated by comparing the cases colonized by a particular species with the cases colonized exclusively by other species from this study. After the surgical interventions, the recurrence of the disease was negatively influenced by fungal colonization (*Candida albicans* and yeast) (Figure 19). Due to the relapse, 63.64% of the 22 cases detected with *Candida albicans* had to undergo another surgical intervention. Further, 66.67% of the cases detected with forms of yeast also showed a recurrence. In addition to the fungal colonization, seven species of bacteria also influenced the recurrence rate negatively: *Enterobacter cloacae complex* (n=16),

Escherichia coli (n=13), *Neisseria* spp. (n=27), *Rothia mucilaginosa* (n=9), *Veillonella* spp. (n=10), *Klebsiella variicola* (n=6), and the physiological flora (n=7), revealing a recurrence rate ranging from 55.56% up to 69.23%. Of the nine species mentioned, six species (*Candida albicans*, yeast, *Enterobacter cloacae complex*, *Escherichia coli*, *Neisseria* spp., and *Klebsiella variicola*) have a higher recurrence rate than the overall study population (61.38% with recurrence, Figure 8). All negatively influenced cases manifested in the recurrence of a necrotic bone lesion solely in the same location as the previously treated bone, except for the cases colonized by *Klebsiella variicola*. In 50.00% of *Klebsiella* cases, the recurrence appeared in a new location. The specimens with no pathogens detected (n=10, indicated as *sterile*) showed no influence on the recurrence of the disease (Figure 19).

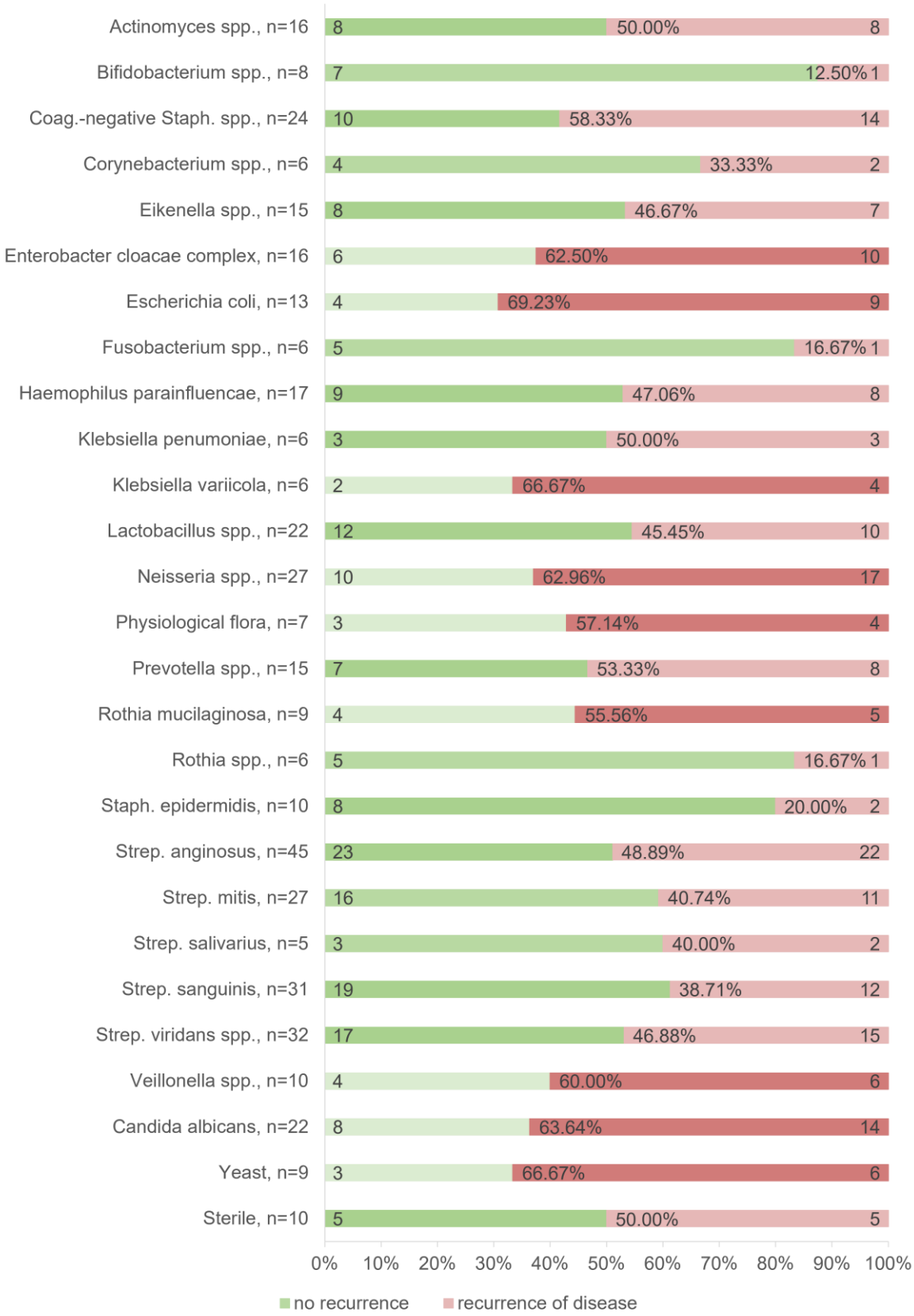

Figure 19: Influence of colonization on the recurrence of the disease. The species with a negative influence on the recurrence rate, compared to the cases that were colonized exclusively by the other species from this study, are indicated by the darker red bars.

Considering the same twelve groups of species as in Figure 18 for the statistical analysis of the influences on the recurrence of the disease (compared to other cases with microbiological examination to species level), a significant negative influence, with a p-value of 0.034 and an OR of 2.116 [1.082-4.341], was revealed in cases of fungal colonization (Figure 20). A total of 64.86% of the 37 cases detected with fungi relapsed after the intervention, leading to another surgery. The cases colonized by Staphylococci showed a significantly lower recurrence rate with a p-value of 0.019 and an OR of 0.338 [0.138-0.825]. Only 25.00% of the 24 cases in the group of Staphylococci were affected by a recurrent lesion. The specimens with no identified pathogens (n=10, indicated as *sterile*) showed no influence on the recurrence of the disease.

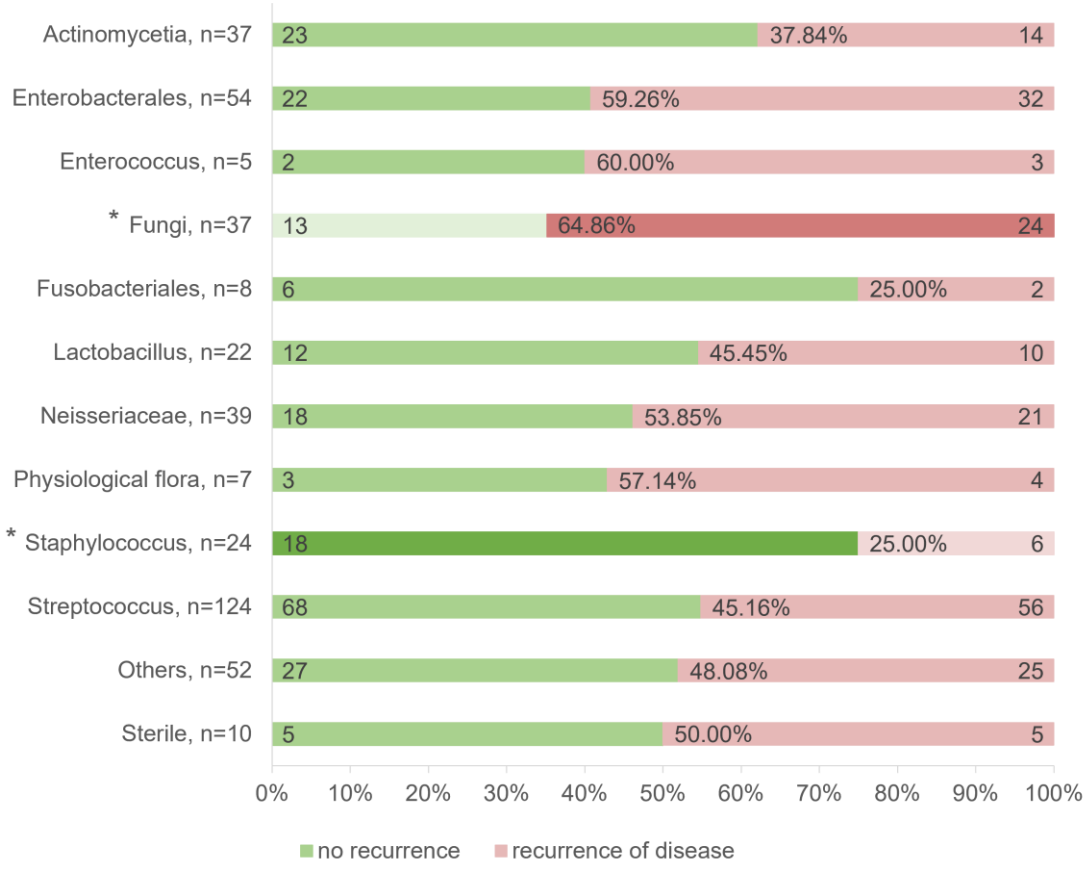

Figure 20: Influence of different microbiological groups on the recurrence of the disease. Considering the twelve microbiological groups of species, fungal colonization presented as a significant (*) negative impact on the recurrence of the disease with a p-value of 0.034. Staphylococcus species showed a significant (*) positive influence in the recurrence rate with a p-value of 0.019.

4.9 Species impact on recurrence-free interval

To compare the impact of different species on the recurrence-free interval, defined as the time between the surgical intervention and the occurrence of a refractory necrotic lesion, nine species were chosen for the analysis. Included cases were colonized by species with a negative influence on the recurrence rate and an occurrence of five times or more in the necrotic bone specimens (*Candida albicans*, yeast, *Neisseria* spp., *Enterobacter cloacae complex*, *Escherichia coli*, *Veillonella* spp., *Rothia mucilaginosa*, physiological flora, and *Klebsiella variicola*).

The time between the surgery and the recurrence of the disease showed a mean time of 4.43 months, a median time of three months (SD 4.52), and a range from one to 25 months. To avoid the results being influenced by occasional outliers, we used the median for the individual calculations. The recurrence of the disease developed rather early in cases of fungal colonization (n=31) and cases of physiological flora (n=7): the median time of the recurrence-free interval was two months with a SD of 0.71 in these cases (Figure 21). The recurrence developed rather late in cases of *Escherichia coli* (n=13; median 4 months, SD 7.05) and cases of *Neisseria* spp. (n=27; median 4 months, SD 4.19). The cases colonized by *Veillonella* spp. (n=10) presented with the longest recurrence-free interval: the median time was 7.5 months (SD 5.68).

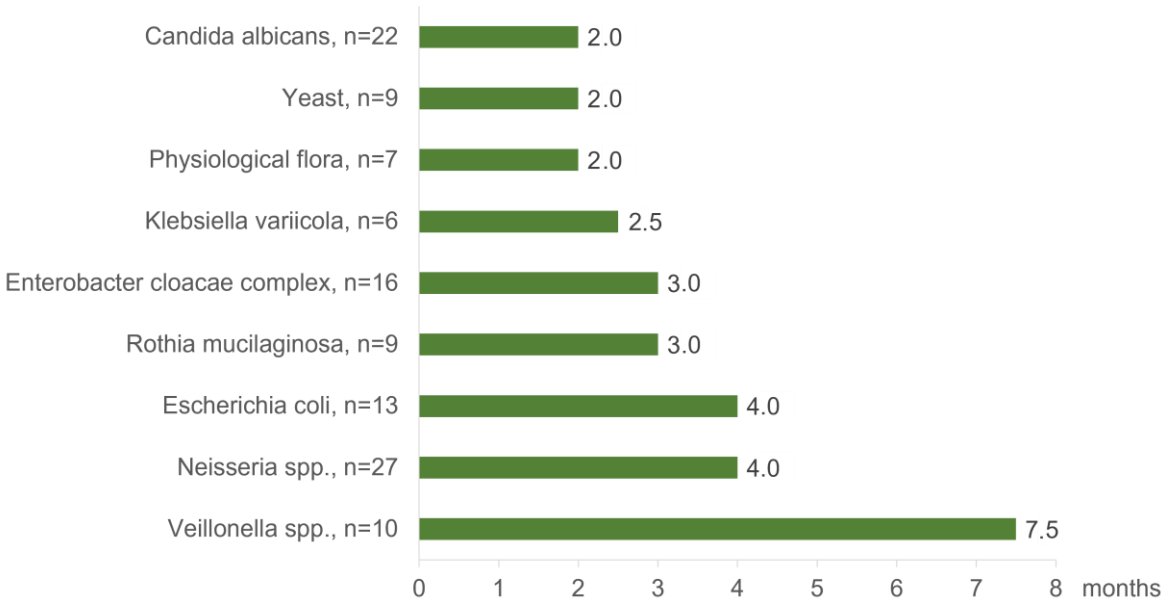

Figure 21: Median time of the recurrence-free interval per species in months. Species that showed a negative influence on the recurrence rate and occurred five or more times in the specimens were considered. The median time of the recurrence-free interval ranged from two to 7.5 months. Fungal and physiological colonization showed the shortest time until the onset of refractory disease and the need for further surgery. The presence of *Veillonella* spp. presented the longest recurrence-free interval.

4.10 Role of the antibiotic treatment

The consensus on prolonged antibiotic treatment in all patients with stage II or III MRONJ has led to further emphasis on indicated agents. In 17.24% (n=50) of the 290 surgical interventions, the patients received a postoperative change in the antibiotic treatment (Figure 22). In 32 cases the change was warranted by susceptibility testing, whereas 18 cases were adapted due to other reasons, e.g., intolerance. The antibiotic agent remained unchanged in 82.76% (n=240) across all cases. Susceptibility testing was subjected in 40 cases, while no testing was subjected and no other indications for a change were found in 200 cases.

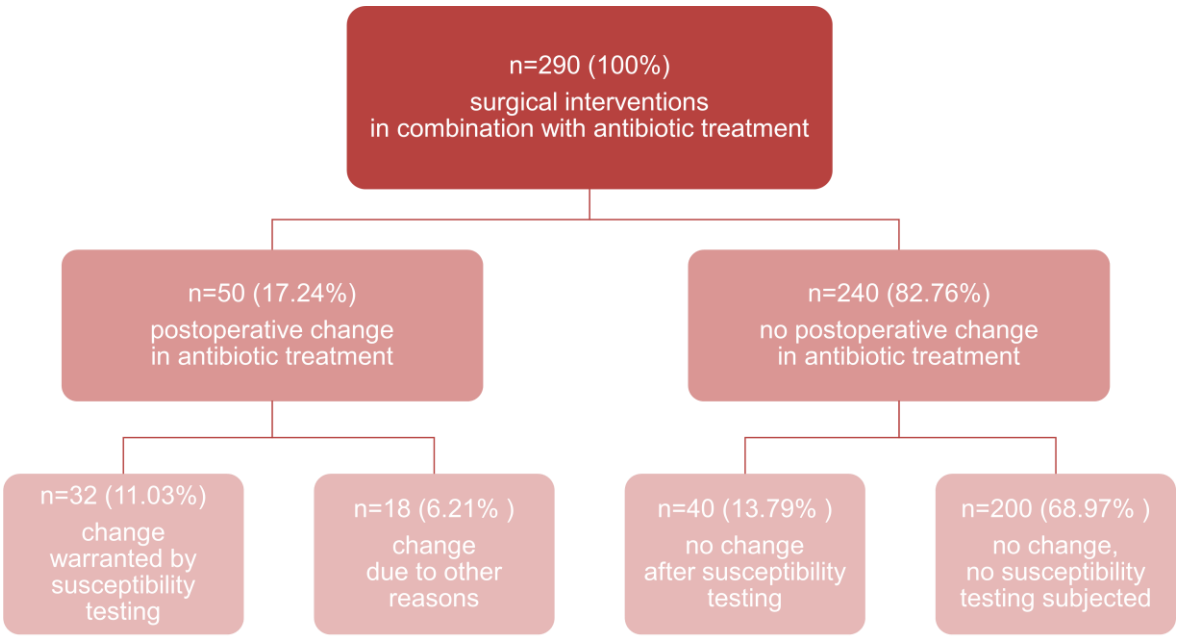

Figure 22: Influences on the antibiotic treatment regimen. Across the 290 cases of surgical intervention with perioperative antibiotics, the treatment was postoperatively changed in 50 cases (17.24%). For 32 cases (11.03%) the change was warranted by susceptibility testing, while 18 cases (6.21%) were changed due to other reasons. The treatment of the remaining 240 cases (82.76%) was not changed postoperatively. In 40 cases (13.79%) susceptibility testing was subjected, while 200 cases (68.97%) had a consistent antibiotic treatment with no susceptibility testing.

The influence of the postoperatively changed antibiotic treatment on the healing process was non-significant (p=0.739, OR 1.108 [0.593-1.988]): with 52.00% (n=26) of healed lesions (Figure 23B), the outcome was marginally improved compared to the 49.44% (n=133) of healed lesions regarding the whole collective (Figure 23A). The attendance to the follow-up appointments was a hundred percent in the patients of adjusted treatments. The recurrence of the disease was unaffected by the postoperative change (p=0.934, OR 1.027 [0.550-1.876]): 38.00% (n=19) remained without recurrence (Figure 24B), compared to 38.62% (n=112) regarding the whole collective (Figure 24A).

An addition to the initial antibiotic treatment showed a non-significant tendency towards a decreased healing rate (p=0.701, OR 0.837 [0.348-2.025]): at the six-week mark, nine cases (45.00%) presented as healed, whereas eleven cases (55.00%) demonstrated a delayed healing process (Figure 23C). All patients with additional treatments attended the follow-up appointment. A non-significant tendency of an increased recurrence rate was established in the cases requiring an

addition to their treatment (p=0.747, OR 1.169 [0.483-3.007]): a total of 13 cases (65.00%) depended on further surgical interventions under general anesthesia (Figure 24C).

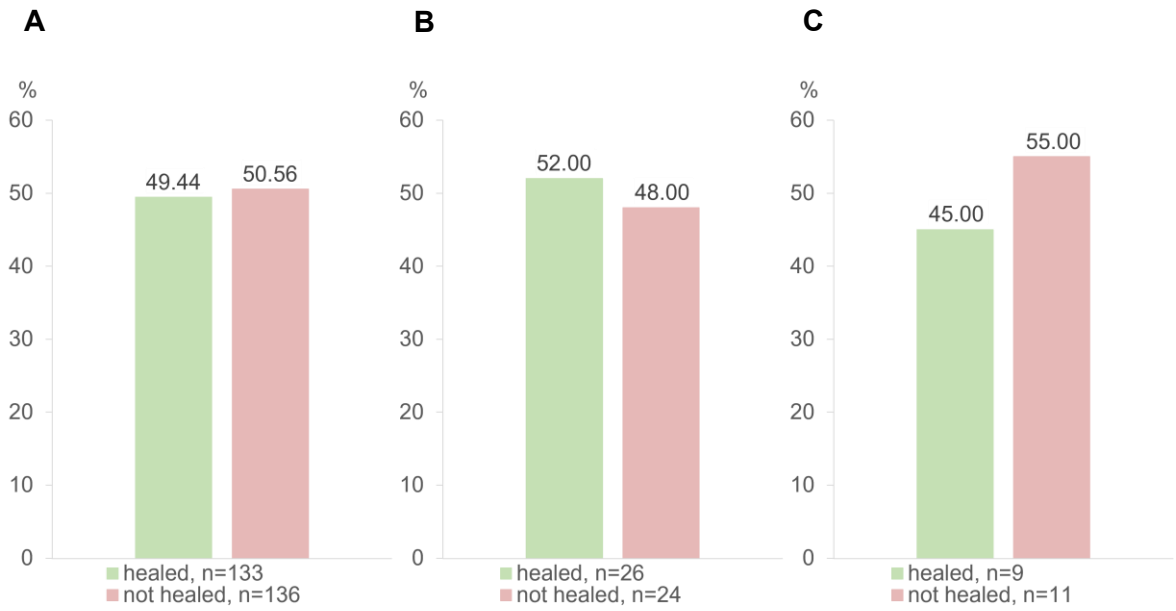

Figure 23: Influences of the antibiotic regimen on the healing process. (A) Distribution of healed and not healed cases six weeks after surgical intervention across the whole study collective. (B) The cases of a postoperatively changed antibiotic regimen showed a slight increase in the healing rate. (C) In the cases, requiring an addition to the treatment, the healing rate was decreased.

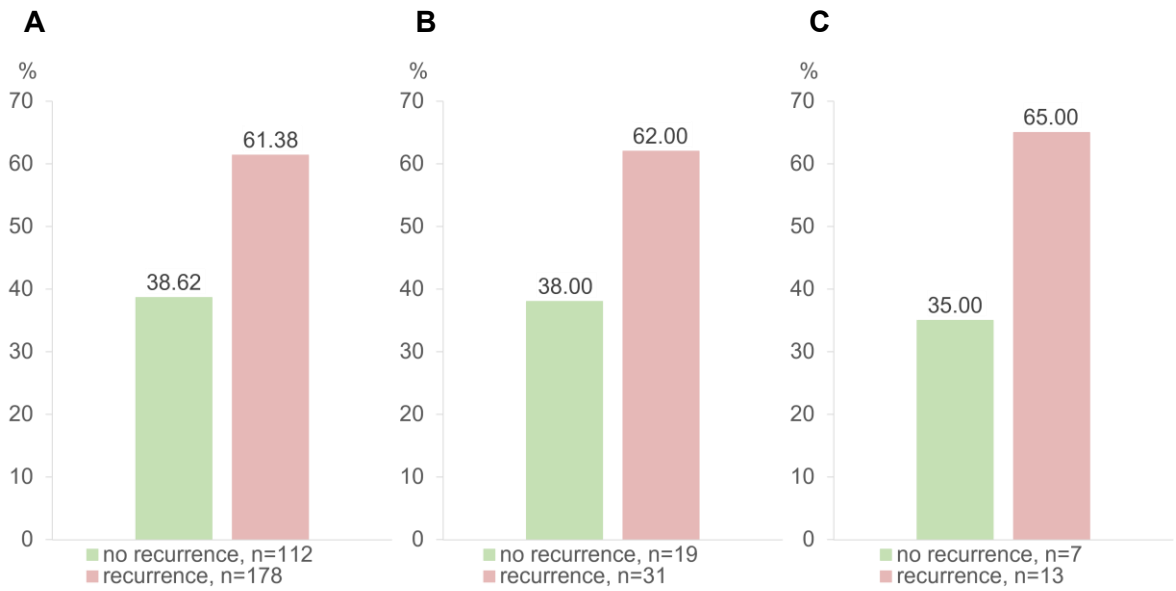

Figure 24: Influences of the antibiotic regimen on the recurrence of the disease. (A) Distribution of recurrences of the disease, leading to another surgical intervention, across the whole study collective. (B) The cases of a postoperatively changed antibiotic regimen showed a similar recurrence rate. (C) Across the cases requiring an addition to the treatment, the recurrence rate was slightly increased.

4.11 Fungal colonization as a challenging factor

The cases treated with additional treatment (n=20) were affected to a large extent by fungal colonization (50.00%; n=10) and received amphotericin B or fluconazole. Out of the 41 cases colonized by fungi, 31 cases did not receive additional antifungal treatment, and two cases did not appear for the follow-up appointment at six weeks. Comparing the two groups, cases with antifungal treatment against cases without antifungal treatment, in their healing process, a non-significant influence was presented by the addition in the treatment (p=0.908, OR 1.091 [0.294-4.334]): a total of 40.00% (n=4) of the cases with antifungal treatment, compared to 37.93% (n=11) of the cases without the additional treatment, attended the six-week evaluation with healed lesions (Figure 25A).

The recurrence rate of the disease in cases colonized by fungi was not influenced positively by the antifungal treatment either. Instead, a non-significant tendency of a higher recurrence rate was displayed (p=0.751, OR 1.283 [0.318-5.256]): the cases additionally receiving the antifungal treatment remained refractory to the surgical treatment in 70.00% (n=7), whereas the recurrence occurred in only

64.52% (n=20) of the cases colonized by fungi without the additional treatment (Figure 25B).

In the cohort of this study, a negative influence of fungal colonization on the healing process and the recurrence of the disease was observed. This was independent of the addition of antifungal treatment.

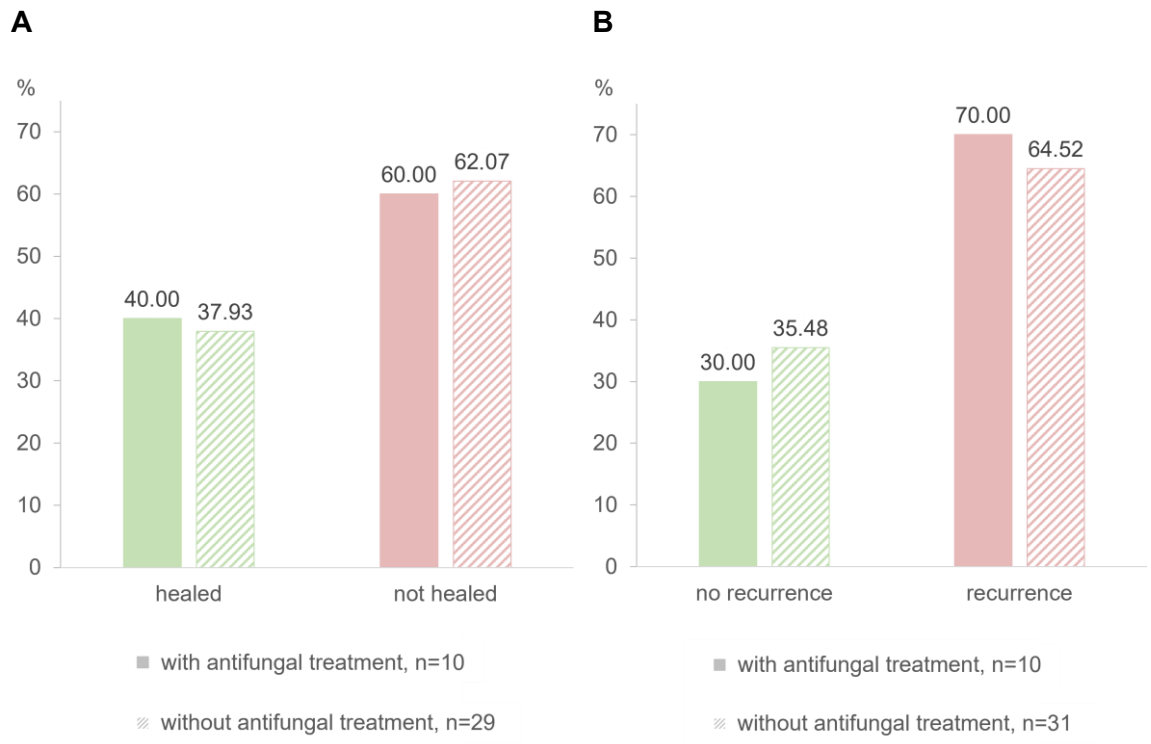

Figure 25: Influence of the additional antifungal treatment on the healing process and the recurrence of the disease. Cases treated with and without antifungal treatment are presented by uniformly colored or striped columns. (A) Influence of the additional antifungal treatment on the healing process. The cases treated with an additional antifungal treatment showed a positive impact on the healing process to a small extent. Both groups presented with a comparatively low healing rate. (B) Influence of the additional antifungal treatment on the recurrence of the disease. The recurrence rate was not impacted positively by the antifungal treatment. Cases receiving the additional treatment showed an even higher percentage of refractory lesions. Both groups had a high recurrence rate compared to cases colonized by other species or the overall study population.

5 Discussion

The intricate interplay between microbial colonization and wound healing has been a longstanding puzzle in medical research. Although previous studies have outlined the enduring difficulties encountered in treating fungal infections within the bone environment and other contexts before (Aftimos et al., 2014, Kalan et al., 2016, Kalan and Grice, 2018), the associated mechanisms of the multi-kingdom microbiome found in MRONJ patients are an ongoing challenge in research (Boff et al., 2014, De Bruyn et al., 2018, Henderson and Nair, 2003). The presence of fungi in the oral cavity does not imply a pathological flora (Cannon and Chaffin, 1999), but the prevalence of *Candida* and yeast in the collected necrotic bone samples was remarkable. A total of 11.83% across all specimens were colonized by *Candida albicans,* an additional 2.70% with further *Candida* species, and 4.84% with yeast. As a fungal infection can occur symptom-free for the person affected, it often remains unnoticed and without sampling of the local tissue (Millsop and Fazel, 2016). However, the negative influence on the primary outcome measures in the affected cases of this study was significant. Cases detected with fungi showed impaired healing and had to undergo another surgical intervention more frequently, due to a refractory disease. The duration of treatment and the control period are prolonged, causing the quality of life to decline accordingly. Microorganisms are therefore not only associated with the etiopathogenesis of the condition itself but further with the course of disease and subsequent complications.

There is general agreement that antibiotic medication is warranted, and susceptibility testing is important to determine an effective treatment algorithm for MRONJ patients (Bermudez-Bejarano et al., 2017, Ruggiero et al., 2022, Varoni et al., 2021). However, our results did not reveal a significant correlation between a postoperative change in the antibiotic treatment and the healing process or recurrence rate in the cohort of this study. Despite the lack of data for an antibiotic-naïve control group, it is evident that the dominant adverse impact is from fungal colonization, a group of species not covered by initial antibiotic therapy. No statistically significant correlations with a demand for clinical improvement were obtained between any other species and the outcome parameters, with the exception of fungi. Given the microbiological colonization, our data confirm the

validity of an anti-infective regime that begins before surgical intervention and continues prolonged until suture removal, as suggested in the AAOMS position paper (Ruggiero et al., 2022), while the postoperatively adapted treatment remains of secondary relevance.

Regardless of additional antifungal treatment, the healing process and the recurrence of the disease remained negatively influenced in the cases of fungal colonization. No other group of species in this study was associated with a negative influence on both outcome measures of this significance. The early administration of antibiotics that target only the bacterial species in this multi-kingdom microbiome might even implement an opportunity for fungal colonization to spread in the tissue (Kalan et al., 2016, Kalan and Grice, 2018). Recognition of these results may help to draw more attention to clinical and histopathological signs of fungal infection. A thorough oral examination or even a smear test by a practitioner may be considered in preventive care before surgery. Early identification of affected cases and including a specific antifungal treatment concurrent with preoperative antibacterial agents could potentially reduce the number of surgical interventions required and accelerate the healing process (Aftimos et al., 2014, Kalan and Grice, 2018, Ruggiero et al., 2004).

The bacterial genera that were most frequently identified in the examination of necrotic bone (e.g., Streptococcus, Neisseria, Lactobacillus) include several species ranging from typical mucosal flora to more frequent pathogens, from gram-positive to gram-negative, and from aerobic to anaerobic bacteria. This highlights the diversity of involved microorganisms in the development of MRONJ as discussed in the literature (Boff et al., 2014, Ewald et al., 2021). *Actinomyces* spp. have been detected in 8.60% of all specimens, which appears to be a rather low percentage compared to other studies in this field (Boff et al., 2014, O'Ryan et al., 2009, Reid, 2009). The process of cultivation does not eliminate deficient accuracy in the results of the examinations. A more sensitive method of detection for this species might be required for an even more extensive representation of the colonization. In addition to the widely investigated pathogenicity of the highly diverse microbiomes in wounds and the main species responsible for impaired clinical outcomes after surgical interventions (Boff et al., 2014, Ersanli et al., 2023, Ewald et al., 2021, Percival et al., 2015, White and Grice, 2023, Wolcott et al., 2016), the effects of the number of different species found in

the necrotic bone specimens were examined in this study as well. This does not reflect the density of microorganisms or the ability to control their proliferation. However, the number of species detected in the necrotic bone did not influence healing or recurrences. The focus should rather highlight individual pathogens and their possible mechanisms of interaction with the disease, which supports the continuing efforts to research in this field.

The significantly reduced number of cases of refractory disease and the improvement in healing associated with the colonization of *Staphylococcus* spp. do not warrant urgent modification of clinical instructions based on current knowledge. The detection of these non-resident bacteria was formerly also assigned to other inflammatory jaw conditions such as osteomyelitis or osteoradionecrosis (He et al., 2022). Future research will have to assess the extent to which this outcome can be implemented. Furthermore, the information on microbiological colonization is to be considered when scheduling follow-up appointments. To our knowledge, the correlation between these parameters has not been described before. While patients affected by fungi should participate in tighter intervals of evaluation, as patients developed early recurrence, cases colonized by *Veillonella* spp. should commit to extended monitoring.

Other influencing factors on the primary outcome measures evaluated in this study were the underlying diagnosis and the antiresorptive medications. Imbalances in the bone metabolism are present in all participants, with underlying neoplastic or non-neoplastic disease (De Luna et al., 2018, Morony et al., 2001, Pearse et al., 2001, Sobacchi et al., 2013, Wu et al., 2017, Yasuda, 2021). Many of the individuals were affected by several simultaneous diagnoses, aligning with the elderly, multimorbid population of MRONJ patients described in the literature and represented in this cohort (Bräuer et al., 2023, Curtis et al., 2020, Gregson and Compston, 2022). In the patients with established MRONJ (stages II or III), oncological diseases were almost universally represented, with only four exceptions being exclusively diagnosed with a non-neoplastic disease. The causal mechanism affecting the outcome could thereby not always be clearly distinguished from one group to another. The female predominance in the study cohort can partly be related to their higher risk of developing breast cancer and osteoporosis, some of the most common diagnoses associated with MRONJ (Assaf et al., 2013, Hess et al., 2008). The occurrence of osteonecrosis itself is not gender-specific, but the prevalence of the

underlying diagnoses demonstrates an increased emergence in one gender (Assaf et al., 2013, O'Ryan et al., 2009). Concerning the incidence of MRONJ, recent data mark a higher risk in the group of oncologic diseases (Bone et al., 2017, Boquete-Castro et al., 2016, Gnant et al., 2015, Grbic et al., 2010, Papapoulos et al., 2012, Saag et al., 2017, Valachis et al., 2013), which sparks the question of their outcome after surgical intervention, compared to the group of non-oncologic diseases. Here it was found that the healing process and the recurrence rate were not significantly influenced by the underlying diagnoses. Further, the value of the OR being close to one presented no association between the underlying diagnosis and the healing process. That said, the value of the OR for the recurrence rate being greater than one indicated an increased risk for a recurrence of the disease in the cases with an underlying oncological diagnosis. Though considering the investigation of the involved treatment of the underlying disease, this impact appears to be attributed to the route of therapy and medication, rather than to the diagnosis itself.

The required antiresorptive therapy is recognized to be one of the main contributors to the development of MRONJ (Eguia et al., 2020, Kawahara et al., 2021, Pazianas et al., 2007, Ruggiero et al., 2022). Participants diagnosed with neoplastic diseases generally receive a high-dose antiresorptive medication, whereas osteoporosis is commonly addressed with a lower dosage, less frequent administration, or oral medication (Eguia et al., 2020, Hess et al., 2008, Kawahara et al., 2021, Pazianas et al., 2007). BPs are the most widely used antiresorptive medications over the last decades and are very reliable in preventing SREs and improving the quality of life in patients with severe bone loss in a variety of conditions (Body, 2006, Coleman and McCloskey, 2011). Therefore, a high percentage of participants afflicted with intravenous or oral BPs was anticipated and confirmed. Denosumab was first approved for administration in osteoporosis treatment in 2010 and later for further medical conditions (Kendler et al., 2022). Given the shorter history of this medication, it is plausible that a smaller group of patients treated with denosumab were affected by necrosis symptoms in our cohort. However, this is not to be interpreted as a lower risk of developing the disease when exposed to the drug. The incidence of MRONJ for patients treated with denosumab is found to be significantly higher than with bisphosphonates (Boquete-Castro et al., 2016, Limones et al., 2020). The relatively large number of attendees administered with intensive chemotherapy can be attributed to standardized treatment recommendations in oncologic therapies (McGowan et al., 2019). While BPs and co-medications, such

as chemotherapy and hormonal therapy, are described to promote an increased onset of the disease (Allen and Burr, 2009, Fournier et al., 2002, Hayano et al., 2020, Kabilova et al., 2014, Marx et al., 2005, Wick et al., 2022), and the impaired angiogenesis and vascularity with suppressed mechanisms to regenerate and defend pathological infections are suggested to increase the risk of wound healing disorders (Akita et al., 2018, Vallina et al., 2019), the correlation to the development of repeated refractory osseous lesions are given minor attention. This study especially indicates the negative impact on the recurrence rate in cases treated with co-medications used in cancer therapy. This aspect has been mentioned, but rather marginally investigated in recent years (Khan et al., 2015, McGowan et al., 2018, McGowan et al., 2019). Chemotherapy and hormonal therapy showed significant differences in the recurrence rates compared to other mAb therapies (p=0.005). In this study cohort, the high relapse rate of MRONJ patients treated with intensive chemotherapy directly compared to other mAb therapies was demonstrated as statistically significant (p=0.001, OR 5.893 [1.891-18.040]). The data provide a strong correlation between the history of medication application and an increased number of recurrences, while there is no significant correlation with a delayed healing process (p>0.999, OR 1.074 [0.369-3.104]). Affected patients with healed lesions at the six-week mark may continue to be scheduled for prolonged monitoring to allow early detection of refractory signs. The impact on the healing process in the different medication cohorts is generally lower, highlighting impaired healing as an overall symptom associated with the antiresorptive drugs (Eguia et al., 2020, Otto et al., 2018).

The results of this analysis also support further exploration into the benefits of the low-dose form of denosumab, Prolia®, especially in the treatment of osteoporosis (Deeks, 2018, Eguia et al., 2020, Yasuda, 2021). The primary outcome parameters were both positively correlated with this type of antiresorptive drug. Alternative therapies, like mAb therapies, demand additional investigations. The significant difference in the impact on the recurrence rate in the cases treated with mAb therapies indicated a possible improvement for the course of therapy for those treated with this medication. The choice of treatment and their concomitant side effects are subjects of further research, not only in terms of inducing osteonecrosis but also in terms of contributing to additional surgical procedures.

At the six-week appointment, the ratio of healed to not healed lesions demonstrated in the participants was almost equal. Other procedures of comparable extent usually show physiologic mucosal healing by that time (Haj Yahya et al., 2020, Hamzani and Chaushu, 2018). In our collective, over 60% of the interventions demanded additional surgery, which is a much higher rate than previously reported (Mücke et al., 2011, Sánchez-Gallego Albertos et al., 2021, Varoni et al., 2021). This is presumably related to the longer follow-up time frame of this investigation, which enhances the reliability and draws attention to this alarming finding. The performance of more than five required surgeries on a single patient illustrates the extent of the refractory character of the disease (Mücke et al., 2011).

The most frequent location of necrotic lesions is agreed to be in the mandible (Hallmer et al., 2018, Ruggiero et al., 2022, Saad et al., 2012), which is in line with the results of this study. A notable finding on this topic was, that the location of the recurrence was not limited to the previously affected area. Cases have been identified in which the lesion of the refractory disease has spread to a completely new location of the jaws. To our knowledge, this observation has not been conclusively analyzed yet, and further exploration into this is hereby proposed.

Trigger factors for the development of MRONJ, as they are described in recent literature (Hallmer et al., 2018, McGowan et al., 2018, Ruggiero et al., 2022, Saad et al., 2012, Walter et al., 2008), were observed in the same or a similar way in the population of this study. These risk factors emphasize the effect of conditions that create pathways for pathogens to access the bone in the oral cavity as critical triggers for the manifestation of the disease.

Despite interesting findings with this study, limitations arose from the definition of the primary outcome measures: full mucosal healing at six weeks postoperatively and recurrence of the disease. Participants with neglected or delayed follow-up appointments could not be prevented in clinical routine completely. This led to the exclusion of cases from certain evaluation points. In addition, the assessment of refractory diseases may be distorted by participants, who were assumed to be in a stable state of disease, although changing hospital, passing away, or other reasons may have led to the absence of subsequent medical inspections. A tight interval of monitoring and early recognition of individuals, who would benefit from surgical intervention, was generally aimed for. This approach aligns with the suggestions found in the literature (Khan et al., 2015, Klingelhöffer et al., 2016). Surgical

debridement and the antibiotic treatment algorithm followed the suggested guidelines and consensus (Ruggiero et al., 2022, Schiegnitz et al., 2018). The sample collection is part of the intervention routine and allows further examination to differentiate the species in the necrotic bone. Although alternative approaches (e.g., PCR) may allow for a more extensive detection of the microbiome (De Bruyn et al., 2018, Panya et al., 2017), the culture method was applied to detect the colonizing microorganisms in the necrotic tissue and enable susceptibility testing, as recommended by the AAOMS (Ruggiero et al., 2022). Some species that are part of the resident flora of the oral cavity were excluded from further referencing and susceptibility testing, while the potentially relevant pathogens were tested for resistance markers. Despite the greatest efforts, it cannot be completely ruled out that sample contamination and bone biopsy handling may have affected certain results. Technical challenges in the cultivation of some species prospectively may be avoided by using a more sensitive ribonucleic acid (RNA) profiling method. The warranted antibiotic treatment before, during, and after the surgical intervention possibly caused a shift in the present oral flora during specimen collection, as described in recent literature (Ewald et al., 2021). This can lead to advantages for specific species, for example, the ones resistant to antibiotics like penicillin. The comparability of the microbiome detected in the affected bone of our cohort to the results of previous studies remains high (De Bruyn et al., 2018, Ewald et al., 2021). Data collected in the course of this project are available for future sub-group analysis and extended research.

The correlations found in this study between influencing factors and the healing process or recurrences of MRONJ provide important and unique evidence for the highly relevant application of antiresorptive medications, immunomodulators, and angiogenesis inhibitors in our ageing population. These results contribute to the development of a critically evaluated treatment algorithm and the enforcement of a rigorous prevention protocol. The refractory nature of this disease should not lead to a vicious cycle of surgical interventions in already impaired individuals. Instead, the value of preventing the onset and stabilizing a progressive course of the disease is emphasized. Given the significantly larger group of participants with an underlying oncologic disease than a non-oncologic disease, the correlation to the recurrence rate was notable, but not significant. This can also be seen in the associated medication of the patients. Both the group treated with oral BPs and the group with low-dose denosumab showed considerably lower rates of refractory lesions than

their corresponding groups of intravenous BPs and high-dose denosumab. Although a tendency can clearly be recognized, future studies of a larger non-neoplastic population are warranted. To understand the influence of the colonization in the oral cavity, the initial examination could examine the present flora at the initial presentation in more detail. Rational treatment, avoiding the increase of bacterial resistance, is an ongoing goal in the studies of antibiotic treatment algorithms. However, in this study, especially the impact of fungi infestation on healing and recurrence raises questions that could be explored in future research. Perioperative medications may be more precisely adapted to the individual conditions and differentiated according to the microbiome. Subsequent alteration or addition in the treatment shows no considerable positive effects on the outcome, which indicates the importance of an optimized initial application. To objectify the correlations between fungal colonization and the negative impact on surgical outcome parameters regardless of additional postoperative treatment, further studies implementing a control group treated preoperatively with concomitant antifungal agents are suggested. Requirements for the administration of these drugs must be respected. Considering the limitations of current antiresorptive medications in the treatment of oncological and non-oncological diseases, it is desirable to preserve the quality of life in affected patients. It should be noted that co-medications in cancer therapy deserve more attention in terms of their risk for developing osteonecrosis, despite the limited alternatives.

In conclusion, our study cohort presented healed lesions six weeks after surgical intervention only in half of the cases, and recurrences occurred in over 60.00% of the cases, especially in the groups receiving hormonal therapy or intensive chemotherapy. Although the microbiome of the necrotic bone was highly diverse, the surgical outcome was not impaired by the number of different pathogens, but particularly by fungi. Alteration or addition of the therapeutic agent showed minimal effect on disease progression and highlights the impact of fungal colonization.

6 Summary

6.1 English

MRONJ is a rare condition in a large group of patients treated with antiresorptive medications. It severely reduces the quality of life and frequently presents with a refractory behavior after surgical treatment of stage II and III cases. Therefore, postoperative wound healing and recurrence of the diseases were defined as primary outcome parameters.

Since the etiopathogenesis has not yet been conclusively elucidated, different mechanisms are hypothesized to impact the onset and progression of the disease. Antiresorptive agents and their route of administration are considered to influence the development of the disease, while the microorganisms seem to play a leading role in the pathogenesis of symptomatic necrosis in the jaw tissue.

In this retrospective study of 148 patients from 2011 to 2019, medical history and therapeutic regimens were evaluated for their influence on surgical outcome parameters. Microbiological examinations of necrotic bone specimens and radiographic images were included in the descriptive and statistical analysis.

Both healing (p=0.004) and recurrence (p=0.034) were negatively impacted by fungal colonization, while remaining unaffected by the number of species in the necrotic bone. The postoperative change in the antibiotic treatment (influence on healing: p=0.738; influence on recurrence: p=0.934) or an addition of an antifungal agent (influence on healing: p=0.701; influence on recurrence: p=0.747) did not improve the outcome in the affected patients as desired. Co-medications in cancer therapy also demonstrated a significant influence on the recurrence rates. Cases treated with additional chemotherapy compared to cases of other mAb therapies showed a negative influence with a p-value of 0.001 and the comparison of chemotherapy, hormonal therapy, and other mAb therapies showed a p-value of 0.005. To further substantiate these findings, it is suggested to investigate a larger population of non-oncologic participants and their associated medications. Future research on the possible impact of early fungal detection before the surgical intervention could also fundamentally improve the management of MRONJ.

6.2 Deutsch

MRONJ ist eine seltene Erkrankung in einer großen Gruppe von Patienten, die mit antiresorptiven Medikamenten behandelt werden. Sie schränkt die Lebensqualität stark ein und zeigt sich häufig mit refraktärem Verhalten nach chirurgischer Behandlung von Fällen im Stadium II und III. Daher wurden die postoperative Wundheilung und Rezidivrate der Erkrankung als primäre Endpunkte definiert.

Da die Ätiopathogenese noch nicht abschließend geklärt ist, werden verschiedene Mechanismen diskutiert, den Ausbruch und das Fortschreiten der Krankheit zu beeinflussen. Es ist anerkannt, dass die Art und Verabreichungsform der Antiresorptiva den Krankheitsverlauf beeinflussen, während Mikroorganismen bei der Pathogenese der Nekrosen im Kiefergewebe eine bedeutende Rolle zu spielen scheinen.

In dieser retrospektiven Studie von 148 Patienten aus den Jahren 2011 bis 2019 wurden die medizinische Vorgeschichte und der Behandlungsablauf auf ihren Einfluss auf die chirurgischen Ergebnisparameter untersucht. Mikrobiologische Untersuchungen von Proben des nekrotischen Knochens sowie bildgebende Verfahren wurden in die deskriptive und statistische Analyse einbezogen.

Sowohl die Heilung (p=0,004) als auch das Rezidiv (p=0,034) wurden durch die Pilzbesiedlung negativ beeinflusst, während sie von der Anzahl der Spezies im nekrotischen Knochen unbeeinflusst blieben. Die postoperative Anpassung der Antibiotika (Einfluss auf Heilung: p=0,738; Einfluss auf Rezidiv: p=0,934) oder die Zugabe eines Antimykotikums (Einfluss auf Heilung: p=0,701; Einfluss auf Rezidiv: p=0,747) verbesserte die Ergebnisse der Betroffenen nicht wie erhofft. Die Begleitmedikation in der Krebstherapie führte ebenfalls zu einer signifikanten Auswirkung auf die Rezidivrate. Die Chemotherapie zeigte im Vergleich zu Fällen mit anderen mAb-Therapien einen negativen Einfluss (p-Wert von 0,001) und der Vergleich zwischen Chemotherapie, Hormontherapie und anderen mAb-Therapien ergab einen p-Wert von 0,005. Um diese Ergebnisse zu untermauern, wird angeraten eine größere Population mit nicht-onkologischen Diagnosen und der damit verbundenen Medikation zu untersuchen. Zukünftige Forschungen zu möglichen Auswirkungen einer frühzeitig erkannten Pilzbesiedelung vor dem chirurgischen Eingriff könnten außerdem das Management von MRONJ grundlegend verbessern.

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8 List of abbreviations

AAOMS	American Association of Oral and Maxillofacial Surgeons
BP	Bisphosphonate
CT	Computed Tomography
CUP	Cancer of Unknown Primary
EGF	Epidermal Growth Factor
GIT	Gastrointestinal Tumor
i.v.	intravenous
IL-6	Interleukin 6
mAb	Monoclonal Antibody
MDS	Myelodysplastic Syndrome
MRI	Magnetic Resonance Imaging
MRONJ	Medication-related Osteonecrosis of the Jaw
OPG	Osteoprotegerin
p.o.	per os
PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone-related Protein
RANK	Receptor Activator of Nuclear Factor-κB
RANKL	RANK Ligand
RNA	Ribonucleic Acid
spp.	Species
SREs	Skeletal-related Events
TKI	Tyrosine Kinase Inhibitor
VEGF	Vascular Endothelial Growth Factor

9 List of figures

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11 Erklärung des Eigenanteils

Die Arbeit wurde am Universitätsklinikum Hamburg-Eppendorf in der Klinik für Mund-, Kiefer- und Gesichtschirurgie unter der Betreuung von PD Dr. Dr. Alexandre Thomas Assaf durchgeführt.

Die Konzeption der Studie erfolgte in Zusammenarbeit mit Dr. Dr. Levi Matthies, Klinik für Mund-, Kiefer- und Gesichtschirurgie, Vertreter des Betreuers.

Die Datenerhebung, Datenkuration und Datenanalyse wurden von mir eigenständig durchgeführt. Dazu gehören Entwicklung einer Datenbank mit übergreifenden Forschungsparametern der Patientenpopulation, sowie die Auswertung der mikrobiologischen Untersuchungen, bildgebenden Verfahren, medizinischen Vorgeschichten und intraoperativen Protokollen. Die deskriptive Analyse und Visualisierung der Forschungsdaten wurden von mir selbstständig umgesetzt.

Die statistische Auswertung der Ergebnisse erfolgte mit Unterstützung durch Dr. Dr. André Strahl, Klinik für Orthopädie und Klinik für Psychosomatische Medizin und Psychotherapie.

Ich versichere, das Manuskript selbstständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

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12 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe, insbesondere ohne entgeltliche Hilfe von Vermittlungs- und Beratungsdiensten, verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe. Das gilt insbesondere auch für alle Informationen aus Internetquellen.

Soweit beim Verfassen der Dissertation KI-basierte Tools („Chatbots“) verwendet wurden, versichere ich ausdrücklich, den daraus generierten Anteil deutlich kenntlich gemacht zu haben. Die „Stellungnahme des Präsidiums der Deutschen Forschungsgemeinschaft (DFG) zum Einfluss generativer Modelle für die Text- und Bilderstellung auf die Wissenschaften und das Förderhandeln der DFG“ aus September 2023 wurde dabei beachtet.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich damit einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Datum 15.12.2024 Unterschrift 

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