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Medication-Related Osteonecrosis of the Jaw (MRONJ) Risk after Tooth Extraction:
A Randomized Controlled Trial in a Rat Model

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Abstract

Medication-related osteonecrosis of the jaws (MRONJ) is a potentially severe adverse effect associated with antiresorptive and antiangiogenic medications, which can negatively impact the quality of life of affected patients.

Tooth extraction is recognized as one of the main local triggering events, and several preventive surgical protocols, such as alveoloplasty with primary closure, have been proposed. However, despite advances in clinical management, MRONJ still poses significant challenges due to its complex pathogenesis and the limited strength of existing evidence.

Experimental animal models offer a valuable opportunity to investigate MRONJ under controlled conditions, reproducing high-risk therapeutic regimens and standardized surgical procedures. In this context, the present research focused on a rat model of oncological zoledronic acid therapy combined with maxillary molar extraction.

The study was designed to evaluate whether intraoperative alveoloplasty with primary closure can modify post-extraction healing dynamics and influence the onset of MRONJ, compared with standard extraction and secondary intention healing. A further objective was to explore the possible development of osteonecrotic changes in the jaws of zoledronic acid-treated animals even in the absence of dentoalveolar surgery, thereby assessing the potential spontaneous component of the disease. This research investigated the link between surgical techniques, drug-induced bone changes, and MRONJ development through combined clinical and histopathological analysis, with the objective of improving preventive strategies and supporting evidence-based oral surgical protocols for patients receiving high-dose antiresorptive therapy.

Summary

MRONJ has emerged over the last two decades as a significant complication of oncological and osteoporotic treatments, profoundly affecting oral function, nutrition and overall quality of life. Its onset reflects a complex interaction between systemic pharmacological effects and local oral factors and remains difficult to predict in individual patients.

Clinical experience has underscored the role of dentoalveolar surgery, particularly tooth extractions, in precipitating MRONJ, but has also highlighted that not all exposed patients behave in the same way, suggesting an underlying biological susceptibility of the jawbones. Against this background, there is a growing need for experimental models capable of reproducing high-risk therapeutic settings and of isolating the contribution of specific surgical procedures. The present doctoral project was conceived within this perspective. Using Wistar rats treated with zoledronic acid at oncological dosing, a controlled framework was created in which maxillary molar extraction could be performed according to different surgical approaches, allowing direct comparison between standard extraction and extraction combined with alveoloplasty and primary closure. Particular attention was given not only to the clinical course of the extraction sites, but also to the microscopic features of bone remodeling and vitality, which are rarely accessible in human studies.

In this doctoral research, a randomized controlled rat model was used to assess the impact of alveoloplasty under zoledronic acid treatment, with the broader aim of clarifying how surgical management of extraction sites may interact with drug-induced alterations of jawbone biology and of providing experimental support for more rational preventive strategies in patients at risk of MRONJ.

CHAPTER 1 Background Rationale and Objectives

1.1 Definition of MRONJ

Medication-related osteonecrosis of the jaw (MRONJ) is a drug-related adverse reaction that represents a clinically important, potentially painful and debilitating condition. It is characterized by the progressive destruction of bone of the mandible or maxilla that occurs in subjects currently or previously exposed to treatment with drugs associated with an increased risk of osteonecrosis and in absence of previous radiation treatment or metastatic disease to the jaw [1,2].

The historical evolution and the gradual recognition of specific etiopathogenetic and clinical features of MRONJ have led to the development of various definitions and classification systems, from the initial reports to the present day [1,2].

According to the most recent definition by the Italian Societies of Oral Pathology and Medicine (SIPMO) and of Maxillofacial Surgery (SICMF), patients should be considered affected by MRONJ if the following criteria are met [1]:

- current or previous treatment with antiresorptive (AR) and/or antiangiogenic (AA) drugs;
- clinical and radiological evidence of progressive bone destruction

and necrosis;

- absence of primary bone malignancy and/or metastases affecting the jawbones.

MRONJ is mainly associated with bone-modifying agents (BMAs) such as bisphosphonates (BPs) and denosumab (DMB), that are ARs that prevent or treat osteometabolic diseases or reduce the risk of skeletal-related events in patients with bone metastases. BPs inhibit osteoclastic activity and proliferation, reducing bone resorption, while DMB inhibits the formation, function, and survival of osteoclasts [3,4].

Furthermore, several studies have suggested that MRONJ may be associated not only with ARs but also with AAs such as angiogenesis inhibitors, tyrosine kinase inhibitors and mTOR inhibitors, which affect angiogenesis but do not directly impact bone resorption. AAs are used in the treatment of solid tumors by inhibiting neovascularization, thereby slowing tumor progression and pathological angiogenesis [5–7].

Clinically, various signs and symptoms such as swelling, abscess formation and dental mobility may raise suspicion of MRONJ. Bone exposure, previously considered the reliable indicator of osteonecrosis, is now regarded as possible but not essential [8,9].

1.2 Epidemiology

Currently, there are no sufficiently robust epidemiological data on MRONJ, as the lack of definitive prospective studies in the literature makes the true incidence of the disease uncertain. Available estimates of its frequency in different at-risk populations show considerable variability. Epidemiological data indicate a higher risk for oncology patients with metastases or multiple myeloma, likely due to simultaneous exposure to a substantial number of risk factors associated with the adverse reaction [10]. At present, the scientific literature lacks accurate studies on MRONJ due to several challenges, including the heterogeneity of the exposed population, diagnostic uncertainty, variability in the collection of clinical data for suspected and confirmed cases, temporal changes in therapeutic protocols for oncology patients, and discrepancies between data sources, such as pharmacovigilance reports versus clinical trials and multicenter studies [11].

1.3 MRONJ Classification Based on SIPMO–SICMF Criteria

A MRONJ classification system, based on clinical and radiological features, has been approved by the Italian Society of Oral Pathology and Medicine (SIPMO) and Maxillofacial Surgery (SICMF) in 2013, and validated by numerous publications over the past 10 years; it allows the staging based on clinical and radiological findings describing three stages: focal, diffused, and complicated [1,12].

Early forms of MRONJ are classified as focal disease (stage I), established forms as diffuse disease (stage II), and complex cases as complicated disease (stage III). Stage III MRONJ may present with clinical signs such as extraoral fistula, nasal discharge, abnormal mandibular mobility, and radiographic findings including mucocutaneous fistula, pathological fracture, osteolysis extending to the maxillary sinus, and osteosclerosis of the zygoma and/or hard palate [1,12].

1.4 Pathogenesis of MRONJ

MRONJ is a multifactorial disease with an incompletely understood etiology. Its development likely results from the combined effects of multiple mechanisms, which may act at different stages. These include local infection and inflammation, inhibition of osteoclast-mediated bone resorption by ARs drugs such as BPs and DMB, impaired bone remodeling, and antiangiogenic effects. Additional factors include direct toxicity on non-osteoclastic cells, alterations of the local immune environment, accumulation and release of drugs from bone tissue, repeated microtrauma, and nutritional deficiencies. Genetic predisposition and systemic conditions, such as diabetes or corticosteroid therapy, may further increase susceptibility, ultimately leading to progressive necrosis of the jawbone in patients exposed to high-risk medications [12–14].

The almost exclusive localization of MRONJ in the jaws appears to be related to several anatomical and physiological factors. The jaws exhibit a physiologically higher bone turnover compared to the rest of the skeleton,

and the mandible has a terminal vascularization pattern. Additionally, the thin mucoperiosteal covering protecting the underlying bone is easily susceptible to trauma, while the distinctive oral microflora and biofilm further contribute to local vulnerability. The dento-alveolar interface may also predispose the bone to exposure, particularly in the presence of periodontal disease or following oral surgical procedures [15,16].

1.5 Risk Factors

The variability of MRONJ is primarily influenced by risk factors associated with the patients' underlying conditions, whether oncological or osteometabolic. Although conclusive data on risk factors are currently lacking, those reported in the literature to date can be categorized as follows: drug-related, systemic, or local [1,12].

Drug-related risk factors primarily involve ARs and AAs. The likelihood of developing MRONJ depends on factors such as the type of drug, route of administration, cumulative dose, treatment duration, and concomitant therapies [12].

BPs due to their strong affinity for hydroxyapatite crystals, induce osteoclast apoptosis and suppress their activity, leading to delays and impairments in bone healing, particularly following dento-alveolar surgical procedures. The inhibition of bone remodeling disrupts the delicate balance between bone resorption and formation, playing a crucial role in the initiation and progression of this drug-related adverse event. The combination of osteoclast suppression, induction of apoptosis, and

compromised osteoblast differentiation creates a microenvironment conducive to the development of MRONJ. Additionally, BPs are known to interfere with angiogenesis by inhibiting the formation of new blood vessels within the jawbones. This impairment of blood supply can hinder bone healing, increase tissue susceptibility to infection, and promote the onset of adverse reactions [17,18].

Systemic risk factors for MRONJ are mainly related to the patient's underlying conditions. Oncology patients are particularly susceptible, especially when treated with high-risk drugs intravenously and at high cumulative doses, with comorbidities and concomitant therapies further increasing their vulnerability. In contrast, patients with osteometabolic disorders receiving oral antiresorptive therapy have a lower risk due to reduced cumulative exposure [12,19].

Chronic dento-periodontal inflammatory conditions increase the risk of MRONJ, as inflammation induces tissue damage through the release of chemical mediators and ischemic effects caused by edema and increased intramedullary pressure. This risk is further exacerbated both by the chronicity of the inflammatory process in bone with altered metabolism and by the need for dento-alveolar surgical interventions [20,21].

The association between dento-alveolar surgery, particularly tooth extractions, and MRONJ is well documented, with studies reporting an incidence of up to 55% in patients undergoing extractions. Evidence suggests that the primary trigger of MRONJ is not the surgical trauma itself but the preexisting infectious-inflammatory environment, especially in oncologic patients treated with BPs or other high-dose ARs agents [22].

Although dento-alveolar surgery is recognized as a common predisposing

event for MRONJ onset, there is a lack of randomized controlled studies, both in humans and in animal models, aimed at identifying effective preventive strategies for individuals at risk undergoing dento alveolar surgery [23].

1.6 Prevention of MRONJ

The management of MRONJ remains controversial, as no consensus exists on a “gold standard” treatment. Current literature reports a range of therapeutic approaches, from conservative to radical interventions, with unpredictable success rates, resulting in substantial costs to the healthcare system without clear benefits for patients [24].

Therefore, in the absence of standardized and effective treatments, the most reliable approach remains the adoption of preventive oral surgical protocols aimed at preserving the oral health of patients who are about to begin, are currently receiving, or have previously undergone therapy with drugs associated with an increased risk of MRONJ [8].

Primary prevention is specifically aimed at controlling the risk factors that contribute to the development of MRONJ. Its purpose is to maintain an optimal state of oral health, thereby pursuing two main objectives: to limit or contain the onset of infectious and inflammatory processes affecting the oral tissues, and to perform dentoalveolar surgical procedures when unresolved infectious conditions are present, even though such procedures are still currently regarded local risk factors for MRONJ [8].

1.7 Surgical Protocols for Tooth Extractions in Patients at Risk

According to multiple authors, the application of structured preventive protocols during tooth extractions plays a crucial role in lowering the incidence of MRONJ in patients at risk [8,25].

Several authors emphasize that dentoalveolar surgical procedures should be performed using an appropriate surgical protocol to prevent bacterial colonization of the alveolar bone and the development of subsequent infections. The demonstrated effectiveness of these measures further underscores the central role of infection in the pathogenesis of MRONJ [9,26].

Diniz-Freitas et al. propose antiseptic rinses, antibiotic prophylaxis, atraumatic extractions, alveoloplasty with primary closure and suturing, the possible use of autologous fibrin concentrates or ozone therapy, and limiting the number of extractions per session, although many of these measures are based on clinical experience and expert consensus [27].

Clinical evidence supports these approaches: Lodi et al. reported no cases of MRONJ after 38 extractions in patients receiving intravenous BPs, using professional oral hygiene, mucoperiosteal flap closure with alveoloplasty and suturing, cavity revision, and prolonged antibiotics [28]. Similarly, Ferlito et al. observed no cases after 102 extractions with perioperative antibiotics, smoothing of alveolar bone, primary closure, and antiseptic rinses [29]. Heufelder et al. further recommend minimally traumatic techniques with antibiotic coverage and tension-free closure [30].

Alveoloplasty is a widely used surgical procedure in dentistry that involves

the smoothing or reshaping of irregular bony edges at the post-extraction site, with the aim of promoting optimal healing of the surrounding soft tissues. Smoothing the bone margins not only reduces the risk of secondary flap perforation but also facilitates wound closure, thereby minimizing the likelihood of infections and other postoperative complications. High success rates have been reported in preventing MRONJ when tooth extractions are followed by careful alveoloplasty and meticulous mucosal closure of the surgical site [31–33].

A systematic review has suggested that the risk of developing MRONJ in oncological patients receiving high doses of BPs and undergoing tooth extraction may be reduced through a surgical protocol involving alveoloplasty performed at the time of tooth extraction [31].

Several studies indicate that the rodent model constitutes a reliable, widely used, and well-validated system for investigating the pathophysiology of MRONJ and for developing effective preventive strategies to reduce the risk of this condition [34,35].

1.8 Rationale for the experimental model

In recent years, many studies have explored MRONJ pathogenesis and its clinical management, but robust evidence from randomized controlled studies on preventive surgical strategies in high-risk settings is still lacking. Most of the available information comes from retrospective series or expert opinion, which are difficult to reproduce and often affected by selection bias. For this reason, a controlled experimental model is needed to better

understand how surgical procedures, such as alveoloplasty and primary closure, may influence the onset of MRONJ in subjects exposed to high dose antiresorptive therapy. The present study was conceived to address this gap by providing standardized and reproducible data from a randomized animal model, with the aim of supporting the development of evidence-based preventive protocols for patients at risk.

1.9 Aim

This randomized controlled animal trial in a rat model, including four experimental arms, was designed to identify preventive strategies aimed at improving primary prevention measures for MRONJ.

The primary aim of this doctoral project was to test the hypothesis that intraoperative alveoloplasty, combined with suturing of the surgical site, could reduce the risk of MRONJ in Wistar rats treated with zoledronic acid (ZA) following tooth extraction, compared with standard extraction procedures. Control groups consisted of rats undergoing tooth extraction without ZA administration.

A secondary aim was to investigate whether MRONJ could already be present before tooth extraction in ZA-treated rats, based on systematic sampling of the extraction sites and non-extracted controls.

These objectives were intended to clarify the potential preventive role of combining medical therapy with alveoloplasty in the development of MRONJ. Through a randomized experimental design and a statistically robust cohort of four rat groups, this study assessed the effectiveness of the

integrated therapeutic approach. Should the findings be interpreted as favorable, they may support the standardization of alveoloplasty within clinical protocols for patients receiving ZA, ultimately contributing to the establishment of a gold standard for the prevention of MRONJ.

CHAPTER 2 Materials and Methods

2.1 Ethics and Animal Welfare

The study was conducted following the recommendations of the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines from the National Center for the Replacement, Refinement and Reduction of Animals in Research [36]. The study protocol was reviewed and approved by the Institutional Organism for Animal Welfare (OPBA) of the University of Palermo and subsequently authorized by the Italian Ministry of Health (approval #06/2017). The OPBA ensured that all procedures complied with the principles of the 3Rs (Replacement, Reduction, Refinement) and monitored the welfare and care of the animals throughout the study [37].

All personnel involved in the study were trained in the species-specific behavior and in the recognition of behavioral changes indicative of stress, pain, or distress. This knowledge allowed for the rapid identification of pre-defined experimental endpoints, supported by their experience. Animal observations and health assessments were conducted collaboratively by the research team throughout the study.

2.2 Animal Groups

The study involved Wistar rats divided into two main groups: Group **A** (BP-treated) and Group **B** (saline-treated).

Group **A** received intravenous ZA at a dose of 0.04 mg in saline solution (0.2 mg/mL) once weekly for 5 weeks. Group **B** received intravenous saline solution for 5 weeks.

According to Biasotto et al. the dose of 0.04 mg intravenous zoledronic acid used in this study replicated the oncological dose in humans and is associated with the development of MRONJ following tooth extraction in 100% of cases [34].

The Wistar rats were randomly allocated to four experimental subgroups (**A1**, **A2**, **B1**, **B2**) using a computer-generated random sequence to ensure balanced group sizes. Group assignment was performed sequentially by an investigator blinded to the upcoming treatment procedures.

The operator performing tooth extractions and alveoloplasty was not blinded to treatment allocation, as the surgical protocols differed visibly between standard extraction (**A1**, **B1**) and alveoloplasty (**A2**, **B2**) groups.

Clinical follow-up and MRONJ scoring were performed by examiners blinded to group allocation. Histopathological evaluation of hematoxylin and eosin-stained (H&E) sections was carried out by an experienced oral pathologist who assessed slides in a blinded fashion, without knowledge of treatment group or clinical findings, to minimize observer bias in semiquantitative endpoints.

2.3 Sample Size Determination

The minimum number of animals required per group to achieve statistically valid results was determined through a power analysis, assuming a statistical power of 80%, a significance level (α) of 0.05, and a medium *effect size* of $f > 0.6$. Based on these parameters, the minimum *sample size* for each group was calculated to be 9 animals (Figure 1).

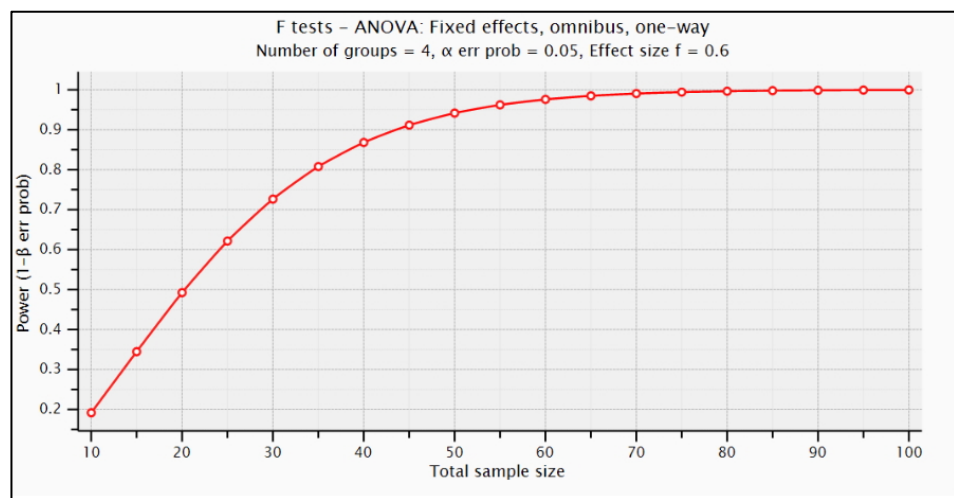


Figure 1: Power analysis parameters showing the *effect size* ($f > 0.6$), $\alpha = 0.05$, and 80% statistical power.

2.4 Animals Husbandry and Care

The animals were housed for the entire duration of the study in the Animal Research Facility of the University of Palermo, Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STeBiCeF), with ad libitum access to food and water and were maintained under a 12-hour light/12-hour dark cycle.

Upon arrival, rats underwent a quarantine period during which their health status was monitored. Animals were housed individually in ventilated cages with High-Efficiency Particulate Air (HEPA) filters, under standardized temperature and humidity conditions, and provided with nesting material and enrichment items to promote species-typical behaviors. The housing system included a remote alarm for environmental deviations.

All handling and procedures were performed by trained personnel with expertise in animal care and dental surgery. Pre-experimental handling reduced stress, and animals were transferred using cylinders, avoiding tail suspension. Welfare was continuously assessed using standardized evaluation sheets and facial *grimace scales* to detect subtle signs of pain or distress. Cage changes and experimental procedures were conducted in HEPA-filtered stations and under laminar flow hoods to further minimize stress.

2.5 Surgical Procedures

2.5.1 Anesthesia and Preoperative Preparation

Each animal was anesthetized with a combination of ketamine (100 mg/kg), diazepam (8 mg/kg), and atropine (0.4 mg/kg) prior to performing the tooth extraction procedures. This anesthetic protocol also provided prolonged analgesic effects for several hours after recovery, offering an advantage in terms of animal welfare. Postoperative analgesia was

administered using meloxicam (1 mg/kg) subcutaneously for the first three days following tooth extraction.

The animals were placed in a prone position to allow optimal visibility for maxillary molar extraction. Each animal was secured to the surgical table at the extremities using adhesive tape, and the depth of anesthesia was confirmed by checking the plantar reflex. Oral opening was maintained with a specialized mouth gag, and steel spatulas bent at a 90-degree angle were placed at the lateral commissures to retract the surrounding tissue, allowing access to the extraction site up to the last molar without forcing the jaw muscles.

As a prophylactic measure, dental cleaning was performed using a 0.12% chlorhexidine solution.

2.5.2 Tooth Extraction Procedures

Tooth extractions of the right first maxillary molar were performed according to a standard protocol 2 weeks after the last administered dose in groups **A1** (BP treated) and **B1** (saline treated). In groups **A2** (BP treated) and **B2** (saline treated), the extraction of the right first maxillary molar was followed by alveoloplasty and soft tissue suturing, carried out after the same two-week latency period (**Figure 2** and **3**).

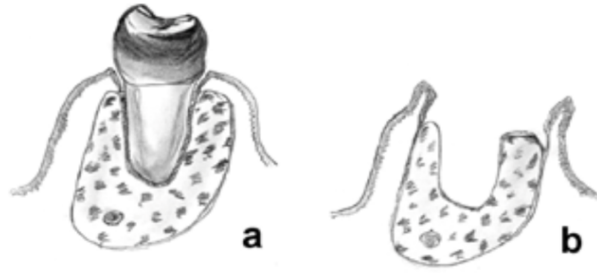


Figure 2: Simple extraction (without alveoloplasty) and healing by secondary intention for the groups **A1** and **B1**.

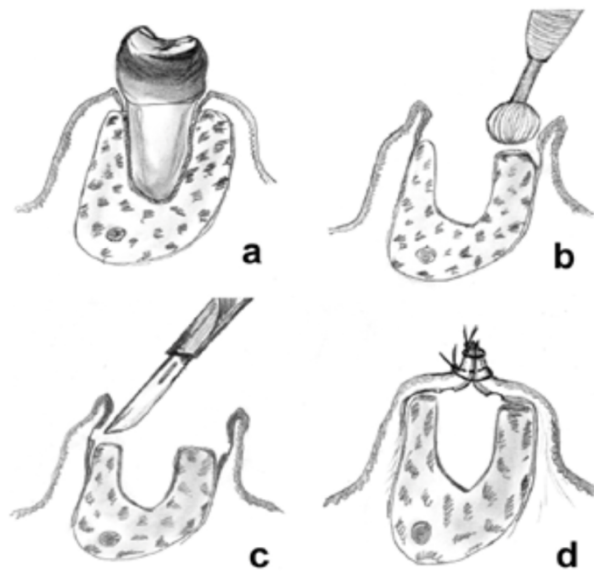


Figure 3: Stages of the alveoloplasty procedure (a, b, c and d) for the groups **A2** and **B2**.

The right first maxillary molars were extracted using a combination of a periosteal elevator, a thin dental elevator (2 mm), and surgical forceps.

In the groups undergoing standard extraction (**A1** and **B1**), the socket was left to heal by secondary intention. In the groups assigned to the surgical protocol with alveoloplasty (**A2** and **B2**), extraction was followed by contouring of the alveolar bone and soft tissue suturing using 8-0 Vicryl sutures.

The decision to perform the surgical extraction on the first maxillary molar was based on considerations related to animal welfare. Extraction of the upper molar allows easier access to the oral cavity, facilitating the surgical procedure and resulting in a less painful postoperative course. This approach also minimizes the risk of trauma or dislocation of the temporomandibular joint, which is more likely when procedures are performed on the mandible. Furthermore, selecting the maxillary molar reduces the likelihood of mandibular pathological fractures associated with MRONJ.

2.5.3 Monitoring and Evaluation

Throughout the entire duration of the study, the animals were subjected to close monitoring from their arrival and housing until euthanasia.

During the 5-week drug administration period and the subsequent 2-week latency period, Wistar rats were monitored for changes in body weight, food and water intake, signs of pain (*grimace scale*), and the jaws were examined clinically (visually and with dental probes) and photographed to assess the potential development of MRONJ as a direct consequence of BP therapy, prior to the surgical phase.

The potential development of MRONJ after 5 weeks of BP therapy was assessed clinically and histologically through analysis of standardized alveolar bone samples (2–3 mm³). These samples were systematically collected from the right maxillary quadrant prior to tooth luxation and avulsion during the surgical procedure. Samples included both the extraction site before tooth elevation and adjacent non-extracted areas, then fixed in 4% formaldehyde for histopathological analysis.

Following the surgical procedure, animals in all four groups were examined clinically and photographed daily for 8 weeks postoperatively and were euthanized at week 16 of the experiment, after which the jaws were collected for histological evaluation (H&E staining) (**Figure 4**).

Euthanasia was performed across all experimental groups via a single intraperitoneal injection of sodium pentobarbital at a dosage of 200 mg/kg.

Clinical signs of MRONJ were assessed using a standardized clinical scoring system, evaluating the following features:

- **Exposed bone**
- **Incomplete mucosal coverage of the surgical site**
- **Mucosal or cutaneous fistulae**
- **Tooth mobility of teeth adjacent to the surgical site**
- **Swelling or erythema of the surgical site mucosa**
- **Abscess formation**
- **Bone sequestration**

Histopathological analysis of H&E-stained sections in two predefined regions of interest (ROIs) from pre-extraction alveolar bone samples (2–3 mm³, collected prior to tooth luxation) and post-extraction jaws (socket + adjacent perialveolar bone) was performed by an experienced histopathologist using light microscopy at ×40 magnification, with the following parameters semiquantitatively assessed:

- **Osteonecrosis or necrotic foci** – at least 8–10 adjacent empty lacunae in the alveolar bone [38]

- **Degree of alveolar bone regeneration** – evaluated quantitatively using a four-point scale based on the percentage of newly formed bone: 0 = absent (0%), 1 = mild (1–30%), 2 = moderate (30–60%), 3 = good (>60%) [39].
- **Inflammatory response** – assessed based on the density and distribution of inflammatory cells within the sample, scored using the same four-point scale: 0 = absent (0%), 1 = mild (1–30%), 2 = moderate (30–60%), 3 = marked (>60%) [40].

The experimental timeline was structured as follows:

- Week 0: start of treatment with ZA or saline; 5 weeks.
- Week 5: last dose administered; 2 weeks of latency.
- Week 7: tooth extraction performed (start of 8-week postoperative follow-up)
- Week 15: end of 8-week postoperative follow-up.

Euthanasia at week 16 (including pre-surgical phases).

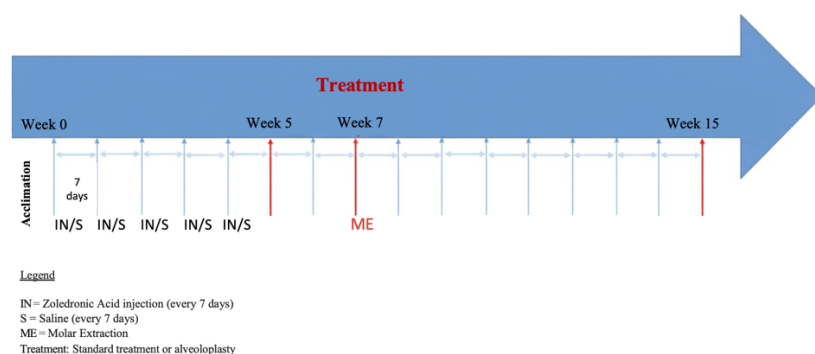


Figure 4: Experimental timetable.

2.6 Statistical analysis

All data were processed using *GraphPad InStat*, version 3.10 (GraphPad Software Inc., San Diego, CA, USA). The main histological outcome was the presence or absence of osteonecrosis of the jaws, which was compared between groups using *Fisher's exact test*. Secondary histological variables (alveolar bone regeneration and inflammatory infiltrate), recorded on a four-point ordinal scale, were summarized as medians and interquartile ranges (IQR) and compared between groups using the *Kruskal–Wallis test*, with post hoc pairwise comparisons where appropriate. A two-sided p value < 0.05 was considered statistically significant.

CHAPTER 3 Results

3.1 Animal Characteristics and Group Allocation

A total of 36 Wistar rats, with a mean age of 80 days and a mean body weight of 450 g, were included in the study and underwent surgical interventions.

The animals were randomly and evenly allocated into two main experimental groups, each comprising 18 rats. Group **A** consisted of 18 rats treated with intravenous ZA (BP-treated) at a dose of 0.04 mg in saline solution (0.2 mg/mL) once weekly for 5 weeks, while Group **B** included 18 rats that received an equivalent volume of intravenous saline (saline-treated) for the same period.

To assess the effects of the surgical procedure on post-extraction healing, each main group was further divided into two experimental cohorts of 9 animals, resulting in four groups: **A1**, **A2**, **B1**, and **B2**.

- Group **A1**, consisting of 9 rats that received intravenous ZA, underwent extraction of the right first maxillary molar according to the standard procedure, following the designated latency period (2 weeks after the last administered dose).

- Group **A2**, consisting of 9 rats that received intravenous ZA, underwent extraction of the right first maxillary molar according to alveoloplasty and suturing of the surgical site, following the designated latency period (2 weeks after the last administered dose).
- Group **B1**, consisting of 9 rats that received saline, underwent extraction of the right first maxillary molar according to the standard procedure, following the designated latency period (2 weeks after the last administered dose).
- Group **B2**, consisting of 9 rats that received saline, underwent extraction of the right first maxillary molar according to alveoloplasty and suturing of the surgical site, following the designated latency period (2 weeks after the last administered dose).

During the period of drug and placebo administration, no evident differences were observed between the groups, and no signs of distress, aggression, abnormal behavior, or post-dose symptoms (such as flu-like signs) were detected.

Body weight, food, and water intake remained within normal ranges throughout the study period, with no significant differences observed between groups prior to surgery.

During anesthesia, two animals, one from group **A1** and one from group **A2**, died.

Therefore, 34 rats (8 from **A1**, 8 from **A2**, 9 from **B1** and 9 from **B2**) completed the experimental procedures and were included in the subsequent analyses (**Figure 5** and **6**).

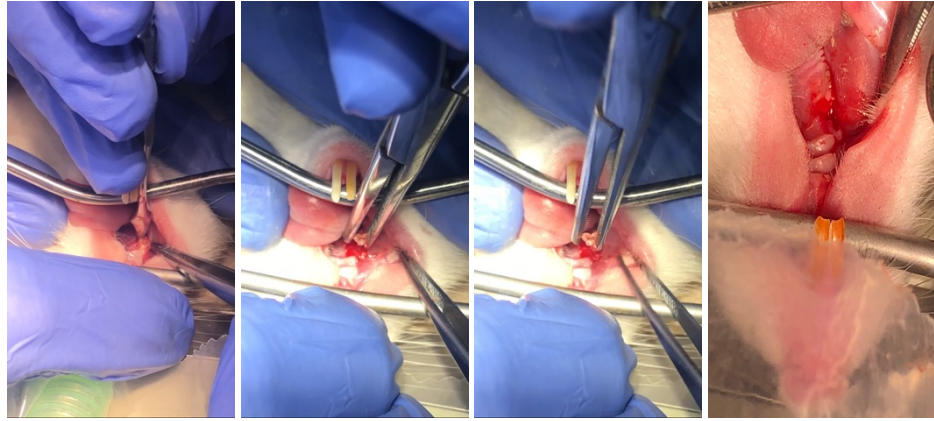


Figure 5: Standard procedure of the right first maxillary molar performed in groups **A1** and **B1**.

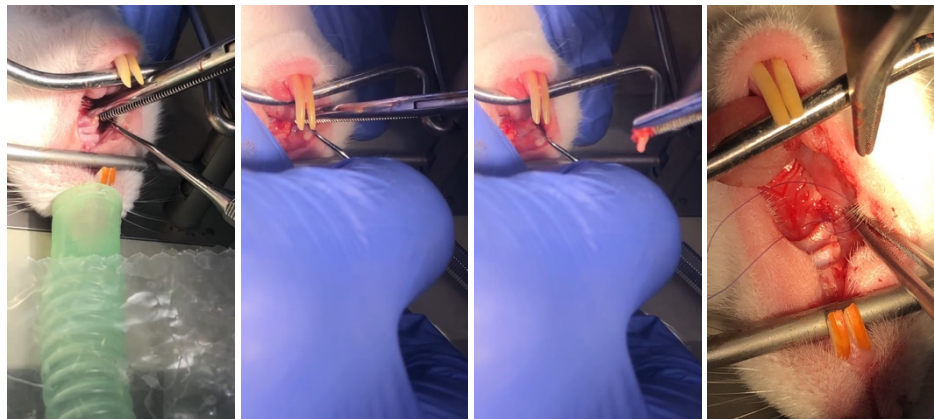


Figure 6: Tooth extraction of the right first maxillary molar followed by alveoloplasty and suturing of the surgical site in groups **A2** and **B2**.

An elevator was carefully inserted between the crown and the gingiva at an approximately 45° angle to detach the crown from the surrounding gingival tissue and separate the mucosa around the cervical portion of the tooth. Additional leverage could be applied on the mesial surface of the second molar or the buccal surface of the first molar to insert the

instrument between the roots, applying vertical pressure against the alveolar bone to facilitate proper extraction. Once luxated, the molar was grasped with forceps and removed.

During the surgical procedures on groups **A1** and **A2**, standardized alveolar bone samples (approximately 2–3 mm³) were systematically collected from the right maxillary quadrant prior to tooth luxation and avulsion. Samples were obtained from both the alveolar extraction site before tooth elevation and adjacent non-extracted areas to evaluate possible presence of MRONJ independent of extraction trauma, as per the secondary aim in section 1.9 (**Figure 7**).



Figure 7: Representative macroscopic images of alveolar bone specimens harvested from rats in groups **A1** and **A2**. The images illustrate the samples collected from surgical sites prior to and during fixation in a 4% formaldehyde solution for the assessment of MRONJ.

Bone biopsy samples were subsequently decalcified using a decalcifying solution, whose main component is disodium EDTA, through a chelation process in an acidic environment.

Eight weeks after tooth extraction of the right first maxillary molar (at week 16 of the experiment), the 34 rats from all groups were euthanized. Their jaws, together with those of the 2 rats that died during anesthesia,

were removed and cleared of soft tissues and prepared for histological evaluation.

3.2 Clinical Outcomes

Groups **A1** and **A2** were clinically evaluated, both visually and with dental probes, daily for eight weeks following tooth extractions, with particular attention given to the identification of any early clinical signs of MRONJ. During the postoperative observation period, no clinical signs of MRONJ were detected in the ZA-treated rats (**Table 1**).

Table 1: Presence or absence of clinical signs associated with MRONJ in ZA-treated rats.

Clinical Features	Yes	No
Exposed bone		X
Incomplete mucosal coverage of the surgical site		X
Mucosal or cutaneous fistula		X
Tooth mobility (teeth adjacent to the surgical site)		X
Swelling or erythema of the surgical site mucosa		X
Abscess		X
Sequestrum		X

Groups **B1** and **B2** were clinically evaluated, both visually and with dental probes, daily for eight weeks following tooth extractions for changes in body weight and food and water intake, but no significant variations were observed.

No major differences were observed between groups **A1** and **A2** or between groups **B1** and **B2**. However, post-extraction sites treated with alveoloplasty and suturing demonstrated faster and more favorable healing, with closure of the extraction socket occurring approximately one week earlier compared with sites left without surgical intervention.

3.3 Histopathological Outcomes

The bone samples and jaw sections were evaluated by a histopathologist using H&E staining and examined by light microscopy observed at magnifications of 200× and 400×.

Histological osteonecrosis of the jaw was observed in all ZA-treated rats (17/17, 100%) and in none of the saline-treated animals (0/17, 0%).

Fisher's exact test confirmed the significant difference in osteonecrosis between groups ($p < 0.001$) [34].

Regeneration and inflammation scores showed floor effect (score 0: 0% newly formed bone, 0% inflammatory cells) across all groups and both pre-extraction alveolar bone ROI and post-extraction socket/perialveolar ROI, consistent with ZA-induced suppression of bone remodeling and absence of significant inflammation. This precluded inferential statistical analysis for these secondary outcomes.

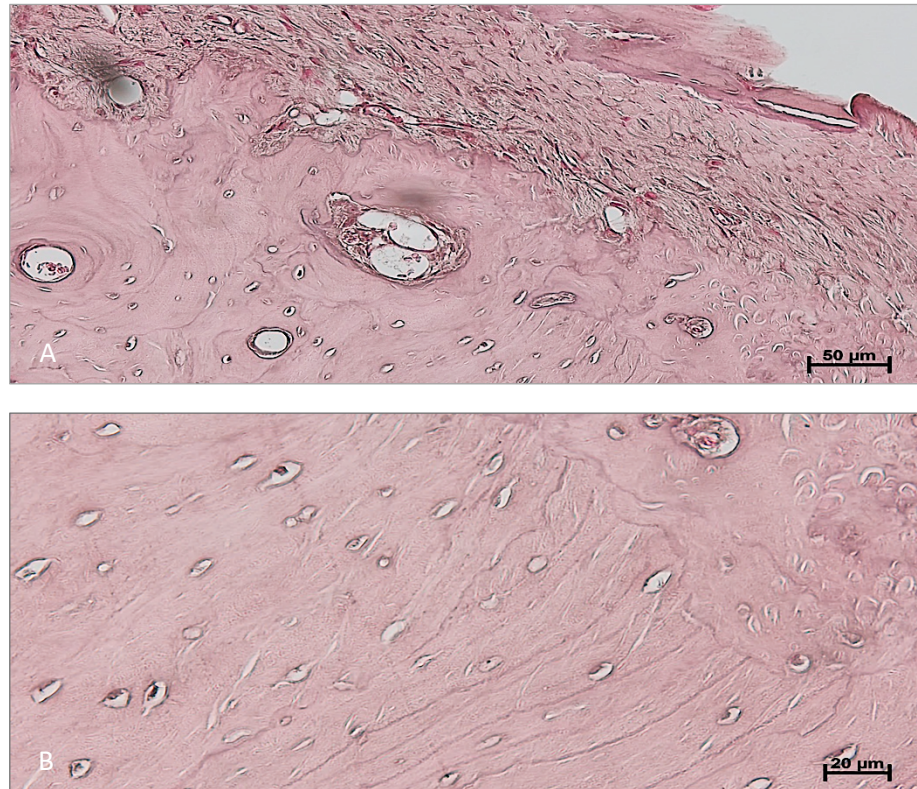
Quantitative endpoints (micro-CT, TRAP/osteoclasts, osteoblast surface) were not performed; semiquantitative H&E scoring served as primary histopathological measure (**Table 2**). This clinical–histological dissociation may reflect the limited sensitivity of clinical scoring in

detecting early or subclinical lesions, the relatively short 8-week postoperative follow-up relative to the slow progression of MRONJ in experimental models, or the stricter histological criteria (≥ 8 –10 empty osteocyte lacunae) compared to clinical endpoints [34].

Table 2: Histological MRONJ findings in each experimental group.

Group	<i>n</i> animals	Osteonecrosis, <i>n</i> (%)	Median alveolar bone remodelling score (IQR) (0–3)	Median inflammatory infiltrate score (IQR) (0–3)
A1 Standard tooth extraction	8	8 (100%)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
A2 Tooth extraction with alveoloplasty and suturing	8	8 (100%)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
B1 Standard tooth extraction	9	0 (0%)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
B2 Tooth extraction with alveoloplasty and suturing	9	0 (0%)	0.0 (0.0–0.0)	0.0 (0.0–0.0)

Histological analysis of samples from groups **A1** and **A2** did not reveal significant differences, showing in both groups a comparable degree of osteonecrosis, absence of alveolar bone regeneration, and lack of an inflammatory response. It was also possible to identify both the absence of osteocytes within the lacunae and the absence of osteoblasts along the endosteal surface (**Figure 8 and 9**).



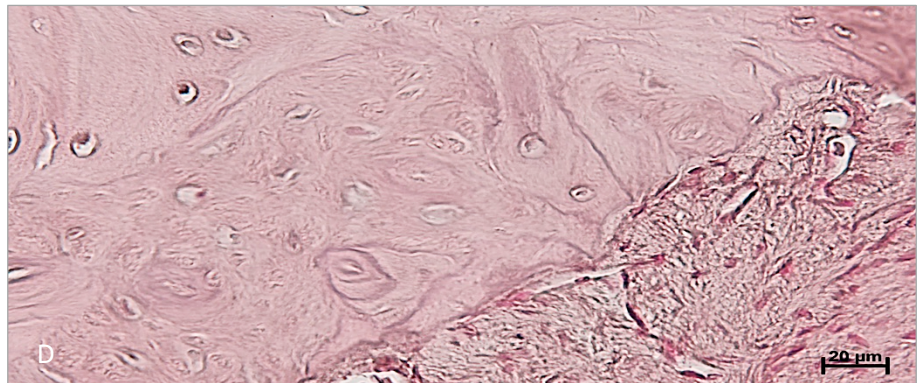
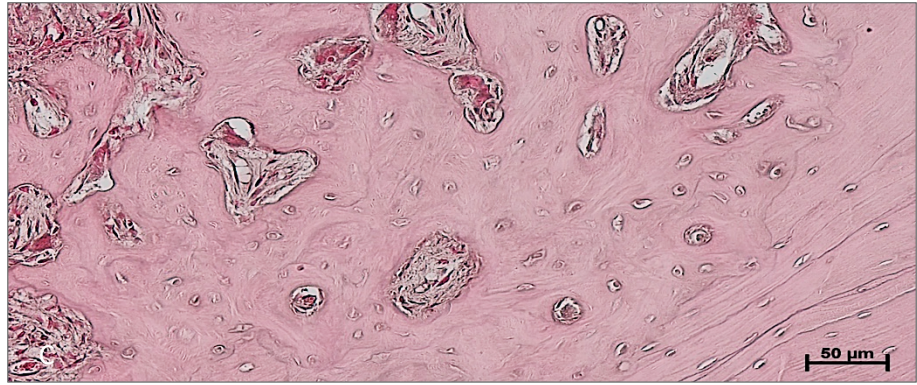
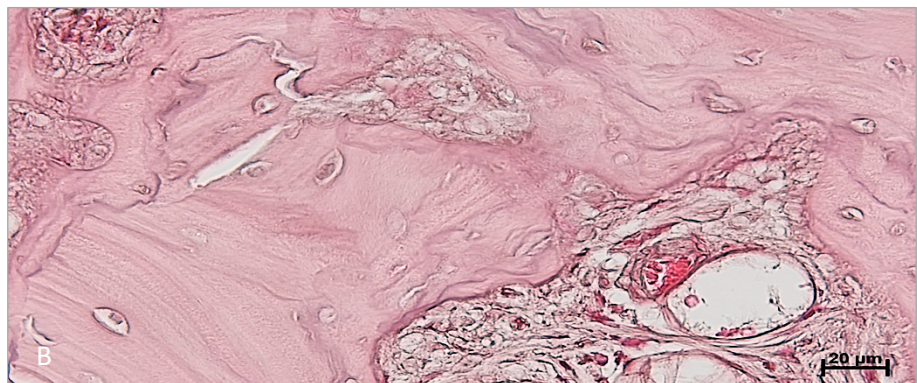
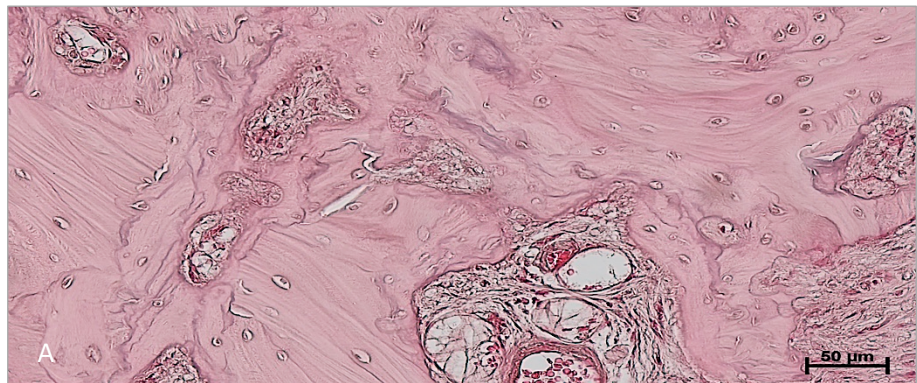


Figure 8: Perialveolar bone in subjects from group A1 showing marked MRONJ. MRONJ is evident from the death of osteocytes within the lacunae, which appear pale and empty. Images shown at 200× (A and C) and 400× (B and D) magnification.



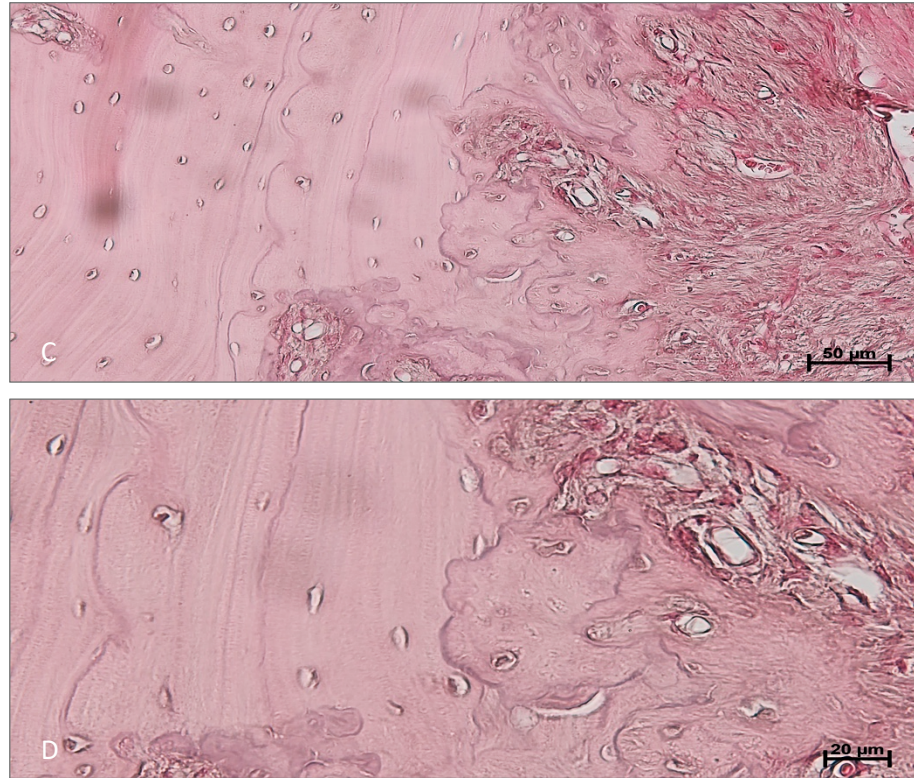


Figure 9: Perialveolar bone in subjects from group **A2**. MRONJ is evident from the death of osteocytes within the lacunae. The bone exhibits a lack of identifiable osteoblasts along the margins of the bone trabeculae. Images shown at 200× (A and C) and 400× (B and D) magnification.

Conversely, histological analysis of tissue samples collected from subjects not treated with the BP (**B1** and **B2**) showed well-organized bone tissue, with well-preserved osteocytes within the lacunae and osteoblasts arranged along the margins of the bone trabeculae, near the endosteal surface and the intertrabecular space, indicating vital and metabolically active bone tissue (**Figure 10**).

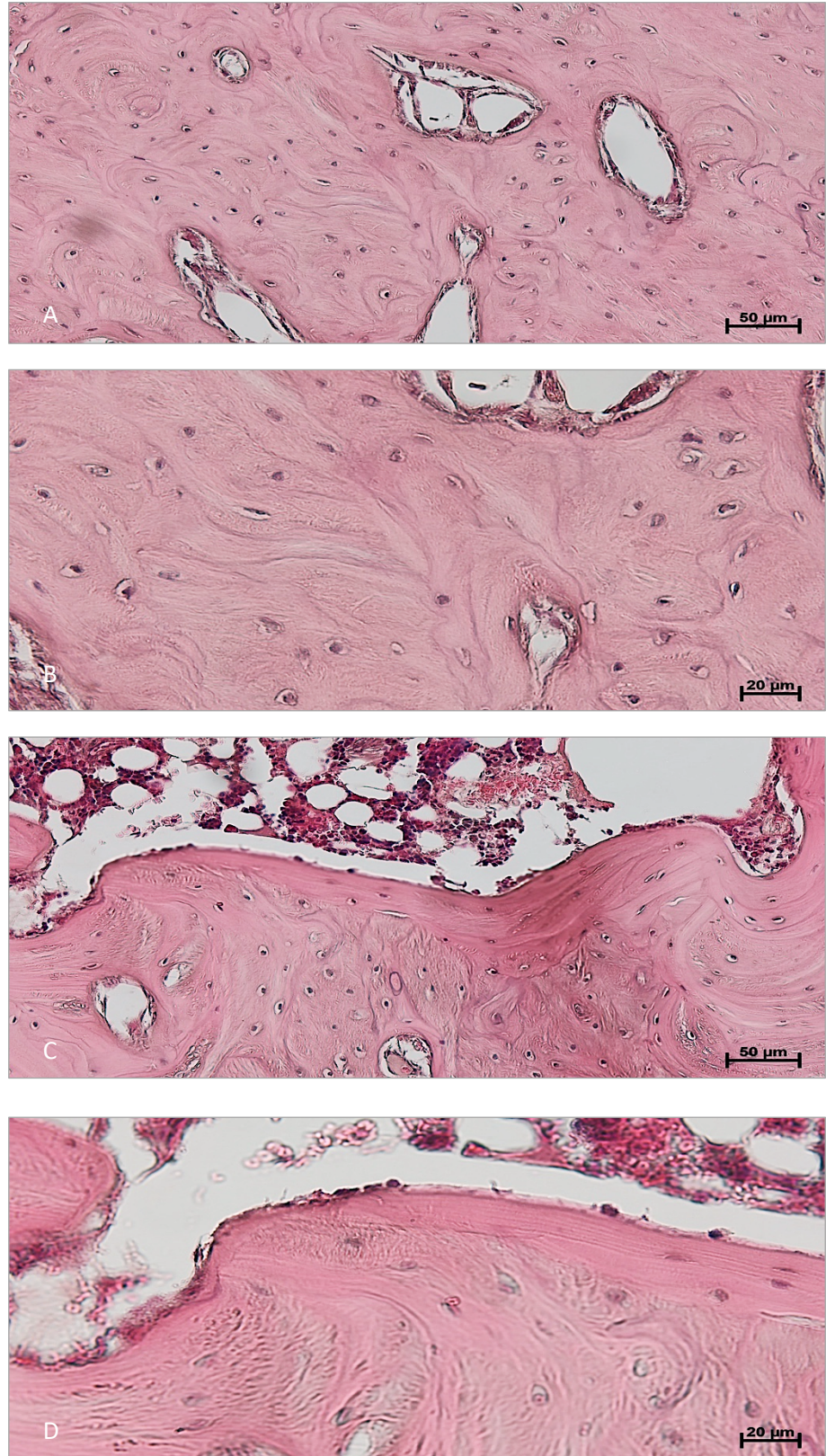
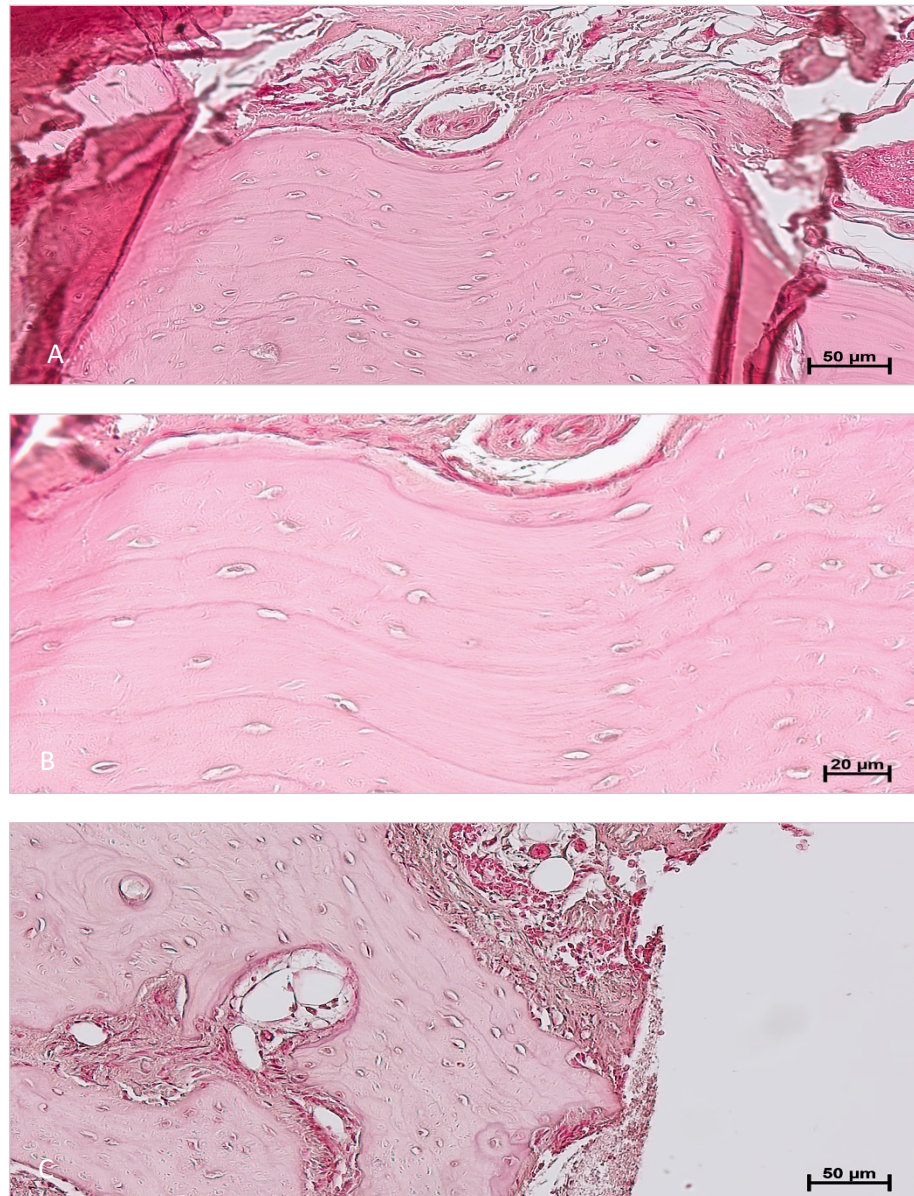


Figure 10: Bone tissue from untreated samples (**B1** and **B2**) showing normal histological architecture. Osteocytes are visible within the lacunae, and osteoblasts are present near the edges of the bone trabeculae. Images shown at 200× (A and C) and 400× (B and D) magnification.

The degree of alveolar bone regeneration and the inflammatory response were evaluated histologically but were found to be absent. Bone remodeling was not observed in the test groups (**A1** and **A2**). Likewise, no inflammatory infiltrate was detected in the area surrounding the extraction site in either group.

Differences in H&E staining were observed between the test and control groups: bone tissue samples from the test groups (**A1** and **A2**) appeared less intensely stained than those from the control groups (**B1** and **B2**) (**Figure 11**).



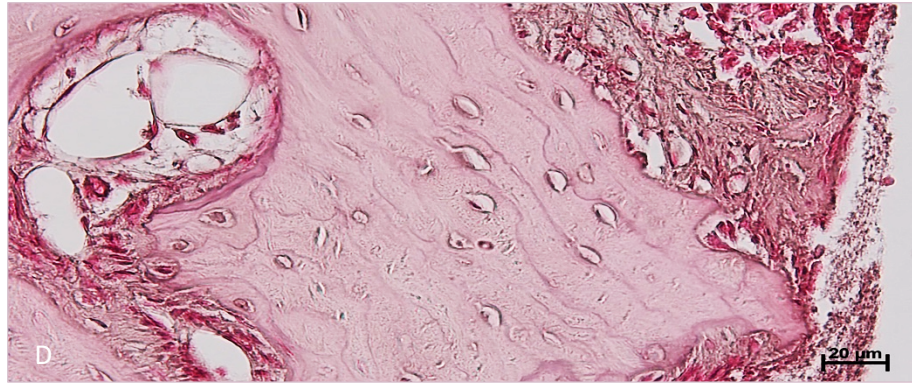
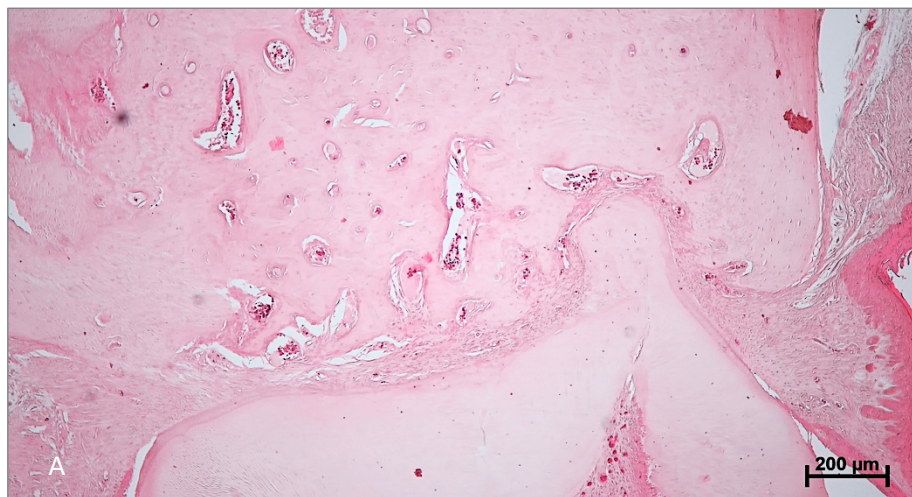


Figure 11: Differences in H&E staining between the test (A and B) and control groups (C and D). Bone tissue samples from the test group (**A1**) appear less intensely stained compared with those from the control groups (**B1**). Images shown at 200× (A and C) and 400× (B and D) magnification.

A preliminary time-0 control (n=2) from the BP-treated rats in group **A1** and **A2** that died during anesthesia (prior to tooth extraction) showed histological osteonecrotic changes suggestive of MRONJ development following drug administration alone. This hypothesis-generating observation requires more robust pre-extraction sampling for confirmation (**Figure 12**).



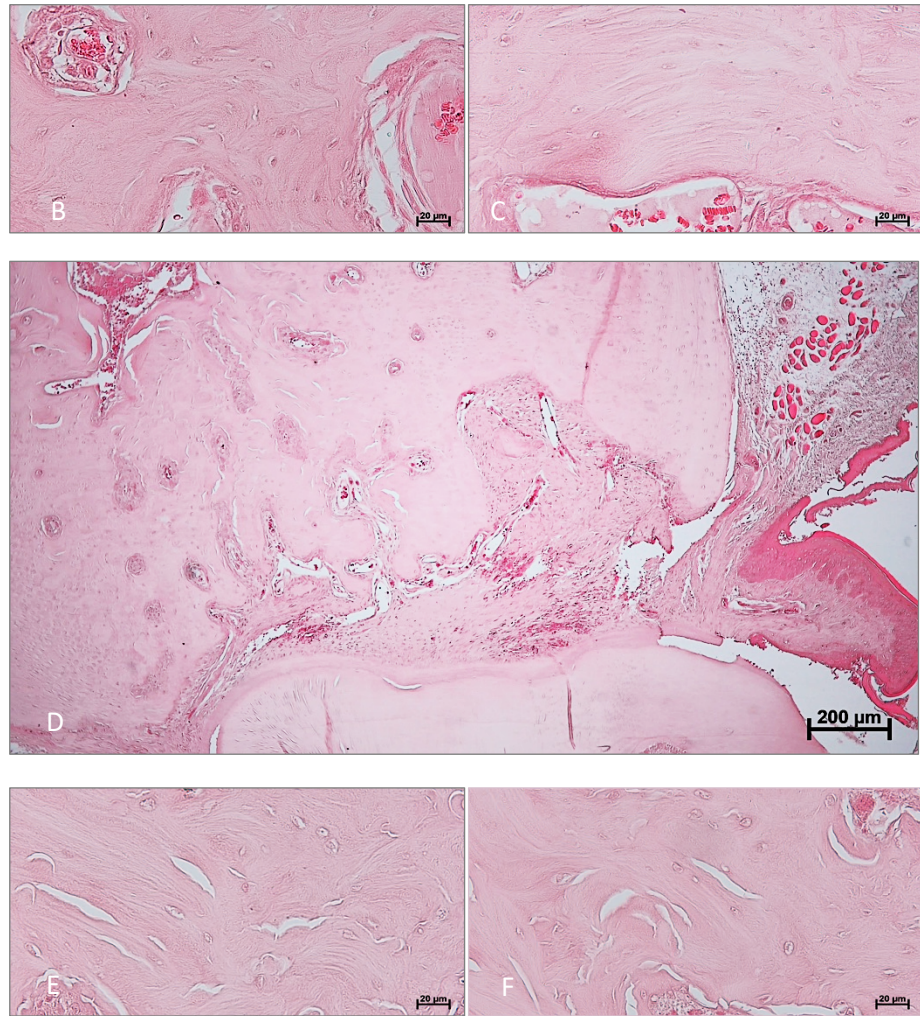


Figure 12: Diffuse osteonecrosis is evident prior to tooth extraction in the jaw of the rats died during anesthesia. (A) Unextracted dental tissue and adjacent alveolar bone tissue near the tooth root are visible. (B)(C) Detailed view of at higher magnification (400×). (D) Marked osteosclerosis with numerous empty lacunae, normally occupied by osteocytes, is observed. (E)(F) Detailed view at higher magnification (400×).

CHAPTER 4 Discussion

4.1 Main Findings and Interpretation

The present randomized controlled animal study investigated the impact of alveoloplasty and primary closure on the development of MRONJ in ZA-treated rats.

The main findings can be summarized as follows:

- histological MRONJ of the jaw was observed in all BP-treated animals after tooth extraction, whereas no saline-treated animal showed MRONJ;
- no clinical signs of MRONJ were detected in any group during the 8-week postoperative follow-up;
- alveoloplasty with primary closure improved clinical soft-tissue healing but did not alter the presence or degree of histological MRONJ;
- MRONJ-like histological observations were also noted in the jaws of the 2 ZA-treated rats that died during anesthesia (before tooth extraction), suggesting possible osteonecrotic predisposition under high-dose ZA therapy independent of surgical trauma.

These results are consistent with previous experimental work showing that ZA at oncological doses can induce MRONJ-like lesions in rodents, particularly when combined with dentoalveolar surgery [41,42].

The dose and schedule used in this study were based on Biasotto et al., who reported a high incidence of MRONJ following tooth extraction in ZA-treated animals [34].

Our findings confirm the high sensitivity of the rat jaw to bisphosphonate-induced bone damage and support the validity of this model for reproducing key histological features of MRONJ [35,43].

Importantly, the present study extends previous observations by demonstrating that MRONJ can develop histologically even in the absence of tooth extraction, reinforcing the concept that drug exposure alone may be sufficient to initiate bone damage in susceptible sites such as the jaws [9].

4.2 Clinical–histological Dissociation and Implications

The dissociation between clinical and MRONJ-like histological phenotype observed in this study deserves particular attention.

Despite the presence of histological osteonecrosis in all ZA-treated animals, none exhibited exposed bone, fistulae, persistent mucosal defects or other clinical signs used in MRONJ diagnosis (SIPMO-SICMF criteria) [12].

Several explanations may account for this discrepancy. First, the follow-up period, although relatively long for an animal experiment, may still be insufficient for histological changes to progress to overt clinical lesions. Second, the robust capacity for soft tissue healing in rodents, particularly

under carefully controlled surgical conditions and with appropriate analgesia, may mask underlying bone pathology. Third, the most widely adopted clinical MRONJ definitions, according to the American Association of Oral and Maxillofacial Surgeons (AAOMS), relies primarily on macroscopic bone exposure, which may represent late-stage disease and therefore underestimate earlier, clinically silent forms.

4.3 Role of Alveoloplasty within MRONJ Pathogenesis

The lack of effect of alveoloplasty on histological MRONJ represents another important finding of this study.

Alveoloplasty with primary closure is widely advocated in preventive protocols for patients at risk of MRONJ, with the rationale of smoothing sharp bony margins, facilitating mucosal coverage and reducing the risk of secondary dehiscence and infection [44,45].

In our model, this procedure effectively improved the speed and quality of soft-tissue healing, with earlier closure of the extraction socket compared with sites left to heal by secondary intention.

However, it did not reduce the presence of MRONJ or promote bone regeneration at the histological level. All ZA-treated animals, irrespective of the surgical technique, showed extensive areas of empty osteocytic lacunae and absence of osteoblasts along bone trabeculae. These observations suggest that, in the context of high-dose ZA therapy, surgical optimization of the extraction site may be able to modulate clinical healing but not to prevent or reverse the underlying drug-induced bone toxicity.

The pathogenesis of MRONJ is multifactorial and involves suppression of bone turnover, antiangiogenic effects, local infection and inflammation, microtrauma and patient-related systemic factors. Alveoloplasty primarily addresses local mechanical and soft-tissue aspects but cannot counteract the profound alterations in bone remodeling and vascularity induced by BPs. This may explain why, in our experiment, all ZA-treated animals developed histological osteonecrosis regardless of the surgical protocol.

4.4 Translational Implications for Prevention

The finding of histological MRONJ in ZA-treated rats that did not undergo tooth extraction further supports the concept that MRONJ may, at least in part, represent an expression of systemic bone toxicity in a site with high remodeling demands and specific anatomical characteristics.

The jaws are characterized by intense bone turnover, thin mucosal coverage and proximity to the oral microbiota, which may render them particularly vulnerable to ARs and AAs drugs. In this scenario, tooth extraction and other dentoalveolar surgeries may act as additional triggers, precipitating or exacerbating lesions in bone that is already compromised, rather than being the sole causative factors.

From a translational perspective, these results have several implications. First, they highlight the limitations of relying exclusively on clinical criteria, such as bone exposure, to define and diagnose MRONJ. Subclinical or early histological stages may go undetected, which could contribute to the apparent variability in reported incidence and outcomes.

Second, the study suggests that preventive strategies should not focus solely on surgical technique but should instead integrate optimized medical management, strict control of oral infections, and careful risk–benefit assessment of ARs and AAs therapy. Third, the rat model used here provides a robust platform for testing combined preventive approaches, including pharmacological modulation of bone remodeling, angiogenesis or inflammation in addition to refined surgical protocols.

4.5 Limitations and Future Perspectives

This study also has limitations. The use of a single ZA regimen, although representative of oncological practice, does not allow evaluation of dose–response relationships or comparison with other antiresorptive or antiangiogenic agents.

The follow-up period, while adequate to document histological MRONJ, may not have been sufficiently long to capture the full spectrum of clinical manifestations of MRONJ. Furthermore, the *sample size*, determined a priori to detect medium effect sizes, limited the possibility of exploring subtler differences between surgical groups or performing more complex statistical analyses. Finally, caution is needed when extrapolating findings from an animal model to human patients, given interspecies differences in bone biology, oral environment and systemic comorbidities.

Despite these limitations, the present work makes several contributions. It confirms, in a controlled experimental setting, the association between high-dose ZA and histological MRONJ. It demonstrates that alveoloplasty

with primary closure, while beneficial for soft-tissue healing, does not prevent or attenuate the underlying MRONJ in this context. It provides evidence that osteonecrotic changes may arise independently of tooth extraction, supporting a broader view of MRONJ pathogenesis as drug-driven bone disease modulated by local events. Finally, it establishes a reproducible rat model that can be used to investigate new preventive strategies and to refine current clinical guidelines.

Moreover, the study was conducted using a single antiresorptive agent administered according to one oncological dosing regimen and relied primarily on a binary histological endpoint without integrating molecular markers or advanced imaging techniques; these aspects may limit the depth of mechanistic insight and the generalizability of the findings to other drugs or therapeutic protocols.

Future studies should focus on extending the follow-up period to assess the progression from histological osteonecrosis to clinically overt MRONJ, comparing different antiresorptive and antiangiogenic regimens, and testing combined medical and surgical interventions. Particular attention should be paid to the role of infection, immune response and microvascular alterations, which may represent potential targets for intervention. The integration of imaging, molecular and biomechanical analyses could further enhance the translational value of the model.

CHAPTER 5 Conclusion

This doctoral research showed that all ZA-treated rats developed histological MRONJ after tooth extraction, whereas no MRONJ was detected in saline-treated controls. Despite this, no clinical signs of MRONJ were observed in any group during follow-up, revealing a clear dissociation between histological damage and clinical presentation.

Alveoloplasty with primary closure accelerated and improved soft-tissue healing but did not reduce the occurrence or extent of histological MRONJ compared with standard extraction. Moreover, osteonecrotic changes were also identified in ZA-treated rats that did not undergo tooth extraction, indicating that jawbone damage can arise spontaneously under oncological BP regimens and that dentoalveolar surgery acts as a promoting factor rather than the sole cause.

Overall, these findings support a multifactorial, drug-driven model of MRONJ pathogenesis and suggest that preventive strategies cannot rely on surgical technique alone. The experimental model developed in this thesis provides a robust platform for future studies aimed at testing integrated medical and surgical approaches and at refining clinical recommendations for patients at risk of MRONJ.

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2022

I. Oral Malignant Melanoma of the Palatal Mucosa: A Case Report

Seminara G., Pometti O. S., Buttacavoli F., Bazzano M., Lo Muzio E., Spirito F., Togni L., Oteri G., Campisi G.

Simposio internazionale SIPMO in associazione con European Association of Oral Medicine: La saliva e le patologie salivari in odontoiatria (Parma 25-26 novembre 2022)

Proceedings submitted per Special issue of *Medicina Oral, Patologia Oral y Cirugia Bucal*

II. Oral Manifestation of Langerhans Cell Histiocytosis Recurrence: A Case Report

Pometti O. S., **Seminara G.**, Coppini M., La Mantia G., Santarelli A., Lo Muzio E., Mauceri R., Oteri G., Campisi G.

Simposio internazionale SIPMO in associazione con European Association of Oral Medicine: La saliva e le patologie salivari in odontoiatria (Parma 25-26 novembre 2022)

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2023

III. Level of Depression and Anxiety in Patients with Oral Potentially Malignant Disorders

Bazzano M., Buttacavoli F., **Seminara G.**, Coppini M., Santarelli A., Spirito F., Caponio V.C.A., Panzarella V., Di Fede O., Campisi G.

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IV. An Abnormal Sized Mucoepidermoid Carcinoma: A Rare Case Report

Tisci A., Spirito F., Kim B.C., Memè L., Caroprese M., Buttacavoli F., **Seminara G.**, Santarelli A., Di Cosola M.

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V. OCT-Trained Learning Algorithms for Oral Carcinogenesis: Project Phase I

Buttacavoli F., **Seminara G.**, Coppini M., Bazzano M., Dioguardi M., Zhurakivska K., Tozzo P., Campisi G., Panzarella V.

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WINNER OF THE BEST POSTER AWARD IN THE ORAL PATHOLOGY AND MEDICINE CATEGORY

- VI. Combining Deep Learning and Case-Based Reasoning for Early Detection of OSCC: A Pilot Study

La Mantia G., Coppini M., Bazzano M., **Seminara G.**, Togni L., Lo Muzio L., Caponio V.C.A., Mauceri R., Campisi G., Di Fede O.

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- VII. Synchronous Oral Squamous Cell Carcinoma and High-Grade Dysplasia: A Case Report

Seminara G., Tozzo P., La Mantia G., Pometti O.S., Troiano G., Lo Muzio E., Santarelli A., Oteri G., Di Fede O., Campisi G.

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- VIII. Mandibular Angle Metastasis from Breast Cancer: A Rare Case Report

Ambrosino M., Spirito F., Memè L., Santarelli A., Coppini M., Buttacavoli F., **Seminara G.**, Dioguardi M., Lo Muzio L.

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- IX. Use Of Integra® Dermal Regeneration Template for Mucosal Defects after Oral Cancer Surgery
Fammilume M., Togni L., Consorti M., Monarchi M., Balercia P., Zhurakivska K., **Seminara G.**, Santarelli A., Mascitti M.

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- X. Analysis of salivary microbiota composition in OSCC patients using ONT technology.

R. Mauceri, G. Campisi, V. Panzarella, M. Coppini, F. Buttacavoli, G. La Mantia, **G. Seminara**, O. Di Fede

Project proposal - Closed Meeting contest 2023 Clinical research and innovation

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Taormina, June 15-19, 2023

WINNER OF THE SPECIAL MENTION AWARD CATEGORY: Oncological predictive models and new technologies in oral medicine

- XI. SYMILIS OCT: SYsteMatical trained learning aLgorithms for oral carcinogenesis interpretation by Optical Coherence Tomography
Panzarella V., Campisi G., Buttacavoli F., Mauceri R., Di Fede O., La Mantia G., Coppini M., **Seminara G.**
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WINNER OF THE SPECIAL MENTION AWARD CATEGORY: Advanced Diagnostic Systems in Oral Cavity Neoplasms
- XII. DoctOral: a smartphone-based decision support tool for the early diagnosis of Potentially Malignant Oral Disorders
Gaetano La Mantia, Giuseppina Campisi, Martina Coppini, Fortunato Buttacavoli, **Giuseppe Seminara**, Rodolfo Mauceri, Vera Panzarella, Olga Di Fede.
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TAOBUK - DA VINCI AWARD
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- XIII. Gestione Odontoiatrica in Paziente con Pregressa Osteonecrosi Delle Ossa Mascellari Da Farmaco Non Anti-Riassorbitivo
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Buttacavoli F., Coppini M., **Seminara G.**, Bazzano M., Panzarella V.
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ADR in odontoiatria (ADR-O), Reazioni avverse a farmaci: Stato dell'arte e prospettive
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- XV. Angiotensin Receptor Blockers-Induced Gingival Hyperplasia: A Case Report
Coppini M., **Seminara G.**, Buttacavoli F., La Mantia G., Mauceri R.
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- XVI. Oral Lichenoid Bullous Reaction Induced By β -blockers: A Case Report

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XXI. Accuracy Of Different Large Language Models in Pediatric Dentistry Meta-Analysis

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XXII. Imatinib-Induced Mucosal Hyperpigmentation Of The Hard Palate: A Case Report

Iuso S., Lo Muzio E., Buttacavoli F., **Seminara G.**, Togni L., Mascitti M., Musella G., Zhurakivska K.
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- XXIII. Ultra-High Frequency Ultrasound in Oral Squamous Cell Carcinoma: A Pilot Diagnostic Study
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- XXIV. Efficiency Of SIPMO-SICMF MRONJ Prevention Protocol in High-Risk Cancer Patients
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- XXX. Field Cancerization in Women Without Well-Known Risk Factors
Coppini M., **Seminara G.**, La Mantia G., Mauceri N., Panzarella V., Mauceri R., Di Fede O., Campisi G.
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- XXXI. Primary Intraosseous Squamous Cell Carcinoma: A Rare Malignancy of The Jaws
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- XXXII. Synchrotron Phase Contrast Microtomography and Artificial Intelligence Algorithm: An Imaging Method for the Deep Learning Study of the Oral Cancer Microenvironment
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