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The role of macrophages in Medication Related Osteonecrosis of the Jaws. An immunohistochemical study

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ΕΘΝΙΚΟΝ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟΝ ΠΑΝΕΠΙΣΤΗΜΙΟΝ ΑΘΗΝΩΝ

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Ευχαριστίες

Ολοκληρώνοντας το δεύτερο κύκλο σπουδών μου θέλω να ευχαριστήσω ιδιαίτερα τον Καθηγητή και Διευθυντή του Εργαστηρίου Στοματολογίας του Πανεπιστημίου Αθηνών και επιβλέποντά μου στο μεταπτυχιακό πρόγραμμα κ. Νικητάκη Νικόλαο για την καθοδήγηση και την στήριξή του σε κάθε επιστημονικό μου βήμα. Θα ήθελα να τον ευχαριστήσω τόσο για τη βοήθειά του στην επιλογή του θέματος όσο και για την καθοδήγησή του στην ολοκλήρωση της διπλωματικής μου εργασίας. Για την εκπόνηση της εργασίας ουσιαστικό ρόλο είχε επίσης ο κ. Γκούβερης Ιωάννης, διδάκτορας του Εργαστηρίου Στοματολογίας καθώς και το εργαστήριο του καθηγητή Τετράδη στο UCLA όπου πραγματοποιήθηκαν τα πειράματα της εργασίας αυτής.

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Περίληψη

Η Φαρμακοεπαγώμενη Οστεονέκρωση των Γνάθων-ΦΟΝΓ είναι μια δυσμενής επίπτωση της μακροχρόνιας χόρηγησης αντιοστεολυτικών, κυρίως διφωσφονικών και δενοσουμάμπης. Παρόλο που έχει αναφερθεί πως τα μακροφάγα είναι ένας σημαντικός μεσολαβητής της ΦΟΝΓ, ο λεπτομερής πιθανός μηχανισμός της παραμένει ασαφής. Ο κύριος σκοπός αυτής της μελέτης ήταν να διερευνηθεί η σχέση μεταξύ της αναλογίας M1/M2 μακροφάγων και της εξέλιξης της νόσου σε ασθενείς με Οστεονέκρωση λόγω λήψης διφωσφονικών και δενοσουμάμπης.

Το υλικό της μελέτης περιλαμβάνει ιστούς από τριάντα (30) περιπτώσεις ασθενών της Κλινικής Στοματικής και Γναθοπροσωπικής Χειρουργικής της Οδοντιατρικής Σχολής του Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών που εμφανίζουν διαφορετικά κλινικά στάδια (I-III) ΦΟΝΓ. Ως ομάδες ελέγχου (control group) χρησιμοποιήθηκαν μονιμοποιημένοι μαλακοί ιστοί ούλων, προερχόμενα από ασθενείς που λαμβάνουν αντιοστεολυτικά φάρμακα αλλά δεν έχουν εμφανίσει ΦΟΝΓ και ασθενείς που δε λαμβάνουν αντιοστεολυτικά φάρμακα.

Για την ταυτοποίηση M1 και M2 μακροφάγων πραγματοποιήθηκαν διπλές χρώσεις ανοσοφθορισμού CD68/iNOS και CD68/CD206 αντίστοιχα. Κάθε τομή εκτιμηθηκε ως προς την ένταση της χρώσης με τη χρήση ειδικού λογισμικού (Image J, NIH) σε υπολογιστή και ύστερα από την εφαρμογή συγκεκριμένων φίλτρων (threshold) για κάθε αντίσωμα. Για τις εναπομένουσες κυτοκίνες (IL6, IL10), επιλέχθηκε η περιοχή ενδιαφέροντος, εφαρμόστηκαν ειδικά φίλτρα και υπολογίστηκε η συνολική έκφραση τους. Η ανάλυση πραγματοποιήθηκε με τη βοήθεια του στατιστικού λογισμικού SPSS .

Η ανάλυση μας έδειξε ότι υπήρξε μια στατιστικά σημαντική αύξηση σε $CD68^{+}/iNOS^{+}$ M1 πληθυσμό μακροφάγων ($p < 0.001$) και στο ποσοστό M1/M2 (p

<0.001) ανάμεσα στα διάφορα στάδια ΦΟΝΓ και στις ομάδες ελέγχου. Βρήκαμε στατιστικά υψηλότερη πυκνότητα Μ1 μακροφάγων και αυξημένο Μ1/Μ2 ποσοστό σε ασθενείς με ΦΟΝΓ σε Στάδια 2 και 3 σε σύγκριση με τις ομάδες ελέγχου που λαμβαναν ή όχι αντιοστεολυτική θεραπεία. (p <0.05 για όλες τις κατά ζεύγη συγκρίσεις). Τα αποτελέσματα μας έδειξαν ότι η πυκνότητα Μ1 και Μ2 μακροφάγων ήταν στατιστικά σημαντικά υψηλότερη σε ασθενείς που λάμβαναν διφωσφονικά σε σύγκριση με αυτούς που λάμβαναν δενοσουμάμπη (p = 0.005, p = 0.002 αντιστοίχως). Παρατηρήσαμε επίσης μια σημαντική αύξηση στην έκφραση της IL-6 σε ασθενείς με ΦΟΝΓ με προχωρημένα στάδια 2 και 3 όπως και σημαντικά υψηλή έκφραση IL-10 σε ασθενείς με στάδιο 1 ΦΟΝΓ σε σύγκριση με τις ομάδες ελέγχου. (p <0.05 για όλες τις συγκρίσεις κατά ζεύγη.

Συνοψίζοντας, η μελέτη μας αποκαλύπτει το ρόλο της αναλογίας των μακροφάγων σε ασθενείς με ΦΟΝΓ σε προχωρημένα στάδια. Καταλήγουμε ότι η αυξημένη πυκνότητα Μ1 μακροφάγων, το αυξημένο ποσοστό Μ1/Μ2 όπως και η αυξημένη έκφραση IL-6 σε μαλακούς ιστούς γύρω από οστεονεκρωτικές περιοχές σχετίζονται με προχωρημένα στάδια ΦΟΝΓ.

Λέξεις κλειδιά: Οστεονέκρωση, πόλωση Μακροφάγων, Διφωσφονικά,

Δενοσουμάμπη, Αντιοστεολυτικά φάρμακα

Summary

Medication-related osteonecrosis of the jaw (MRONJ) is an adverse side effect of long-term administration of antiresorptives, mainly bisphosphonates and denosumab. Although macrophage polarization has been reported to be an important mediator of MRONJ, the detailed potential mechanism of MRONJ remains unclear. The main aim of this study was to investigate the link between M1 and M2 macrophage polarization and disease progression in patients with bisphosphonate-related osteonecrosis of the jaw (BRONJ) and denosumab-related osteonecrosis of the jaw (DRONJ).

The study material comprised mucosal tissues near osteonecrotic areas of 30 MRONJ patients with stage I-III, obtained at the Department of Oral and Maxillofacial Surgery of Dental School, National and Kapodistrian University of Athens (NKUoA), Greece. As controls, inflamed mucosal tissues from participants without MRONJ who received either bisphosphonates or denosumab and participants who did not received antiresorptive therapy were used.

For M1 and M2 macrophage identification, double CD68/iNOS and CD68/CD206 immunofluorescence staining were performed respectively. Each slide was evaluated for the intension of staining with specific software (Image J, NIH) in computer and after the use of specific filters (threshold) for each antibody. For the remaining secreted cytokines (IL6, IL10), the region of interest was selected, a minimal and maximal threshold were set and total expression was calculated. Statistical analysis was performed using the SPSS.

Our analysis showed that there was a statistically significant increase in $CD68^{+}/iNOS^{+}$ M1 macrophage density ($p < 0.001$) and M1/M2 ratio ($p < 0.001$) between the different MRONJ stages and controls. We found a statistically significant higher

M1 macrophage density and increased M1/M2 ratio in MRONJ patients with stages 2 and 3 compared to controls receiving antiresorptive therapy and controls not receiving antiresorptive therapy ($p < 0.05$ for all pairwise comparisons). Our results showed that M1 and M2 macrophage density were statistically significant higher in patients receiving bisphosphonates compared to those receiving denosumab ($p = 0.005$, $p = 0.002$ respectively). We observed a significant increase in expression of IL-6 in MRONJ patients with advanced stages 2 and 3 as well as a significant higher IL-10 expression in MRONJ patients with stage 1 compared to controls ($p < 0.05$ for all pairwise comparisons).

In conclusion, our study reveals the role of macrophage polarization in MRONJ patients with advanced disease. We demonstrate that a higher density of M1 macrophages and increased M1/M2 ratio as well as enhanced expression of IL-6 in mucosal tissues surrounding necrotic bone are associated with advanced stage in both BRONJ and DRONJ patients.

Key words: Osteonecrosis, Macrophages polarization, Bisphosphonates, Denosumab, Antiresorptives

Introduction

1.1 Definition

Medication – Related Osteonecrosis of the Jaw (MRONJ) is the exposed necrotic bone in mandible or maxilla often associated with mucosa swelling, erythema, ulceration, and pain. The American Association of Oral and Maxillofacial Surgeons describes MRONJ as the exposed alveolar bone for >8 weeks in patients who currently receive or have a history of receiving antiresorptive medication (including Bisphosphonates and denosumab) for osteoporosis, Paget's disease, multiple myeloma, and osseous metastases of solid tumors and absence of radiation to the head and neck area.(Yoneda, Hagino et al. 2010; Ruggiero, Dodson et al. 2014) The first case of MRONJ was reported by Marx et al. in 2003 (Marx 2003) and since then, the number of patients with MRONJ has been increasing yearly.

1.2 Etiology – Pathophysiology

Although the first MRONJ case was reported over a decade ago, the pathophysiology of MRONJ has not been fully elucidated and its treatment remains symptomatic.(Marx 2003; Ruggiero, Mehrotra et al. 2004) There is a great debate among clinicians and researchers about the potential mechanisms of MRONJ pathophysiology. (Allen and Burr 2009; Landesberg, Woo et al. 2011) Proposed hypotheses that attempt to explain the unique localization of MRONJ exclusively to the jaws include altered bone remodeling or oversuppression of bone resorption, angiogenesis inhibition, constant microtrauma, suppression of innate or acquired

immunity, vitamin D deficiency and inflammation or infection. (Reid, Bolland et al. 2007; Sonis, Watkins et al. 2009) Theses hypotheses are based on studies showing that the jaw has a high remodeling rate, that bisphosphonates suppress remodeling and that remodeling is considerably higher in the jaw compared with other skeletal sites. (Russell, Xia et al. 2007; Russell, Watts et al. 2008) An animal study showed that the mandible remodeling rates, specifically within the alveolar region, are more than ten times higher than those within the long bones.(Allen and Burr 2008)

Another theory is that MRONJ is caused by dental infection. In an animal study, ligature-periodontal inflammation was induced in rats treated with zoledronic acid. Osteonecrosis was observed associated with periodontitis in the group of rats treated with zoledronic acid, suggesting that periodontal disease and zoledronic acid therapy are sufficient for MRONJ development. (Aghaloo, Kang et al. 2011) It is demonstrated that periodontal disease and antiresortive drugs are sufficient to induce ONJ in rats. Moreover, an ONJ mouse model using high-dose BP treatment in combination with experimentally induced periapical disease (inflammation of the tissues surrounding the apical part of the tooth root), was developed, emphasizing the importance of dental disease in ONJ pathophysiology. (Hadaya, Soundia et al. 2019) (Soundia, Hadaya et al. 2016)

1.3 Risk factors

Risk factors reported for the development of MRONJ include general risk factors such as corticosteroid treatment, chemotherapy, diabetes and smoking as well as local risk factors such as anatomic locations predisposed to trauma (Khan, Morrison et al. 2016); (Otto, Schreyer et al. 2012) (Marx, Sawatari et al. 2005) (Rasmusson and

Abtahi 2014). The trigger in the majority of cases is tooth extraction. (Otto, Schreyer et al. 2012)

The clinical presentation is similar to osteomyelitis (OM) or osteoradionecrosis (ORN) of the jaws. OM is caused by an infection in the bone and ORN by high doses of localized radiation therapy. (Mehrotra and Ruggiero 2006) (Marx, Sawatari et al. 2005) However, MRONJ patients have no history of radiation therapy and may have a secondary bacterial infection, and classic therapies for OM and ORN are usually ineffective. (Bamias, Kastritis et al. 2005) (Ruggiero, Mehrotra et al. 2004)

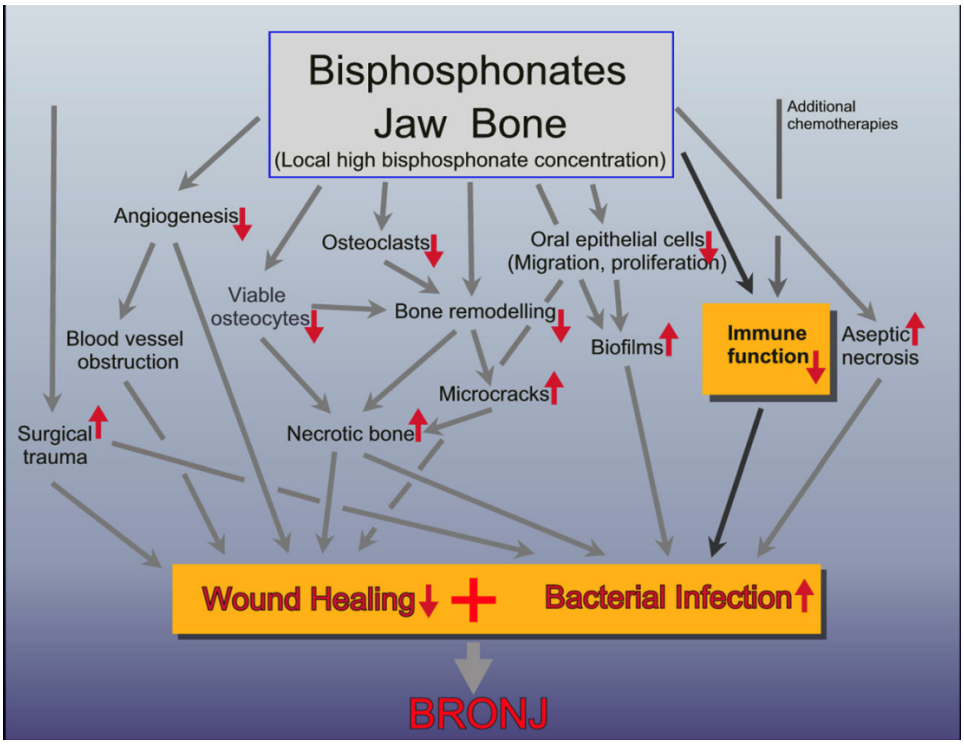

Figure 1

Modified aetiology model of **bisphosphonates related necrosis of jaw** (BRONJ). The immune suppression of macrophages caused by bisphosphonates could also be worsened by additional chemotherapies. A surgical trauma initialises the infection and exposes bone of the compromised jaw. By a possible immune dysfunction, the bacterial infection (by oral cavity) is intensified leading to or reinforces BRONJ in combination with a bated wound healing (Yoneda, Hagino et al. 2010)

1.4 Staging

Several staging systems have been described in the literature (Table 1). According to the recommendations of the AAOMS 2014, MRONJ is staged from 0-3. (Ruggiero, Dodson et al. 2014) Stage 0 is defined as no clinical evidence of necrotic bone but non-specific clinical findings, radiographic changes and symptoms; Stage 1 as exposed necrotic bone or fistulas that could be probed to bone without pain or signs of infection (asymptomatic); Stage 2 as exposed necrotic bone or fistulas that could be probed to bone with pain or signs of infection (symptomatic) and Stage 3 as exposed necrotic bone or fistulas that could be probed to bone with pain or signs of infection and one or more of the following: pathological fracture, oral-cutaneous fistula, involvement of the maxillary sinus or necrosis extending to the inferior border of the ramus of the mandible.

Stage 0 category was added in 2009 to include patients with nonspecific symptoms or clinical and radiographic abnormalities that might be due to exposure to an antiresorptive agent. At that time, the risk of a patient with stage 0 disease advancing to a higher disease stage was unknown. Since then, several cases studies have reported that up to 50% of patients with stage 0 have progressed to stage 1, 2, or 3. (Fedele, Porter et al. 2010) (O'Ryan, Khoury et al. 2009) Therefore, stage 0 seems to be a valid disease category that captures patients with prodromal disease (unexposed variant).

Table 1: Osteonecrosis of the Jaw Staging Proposal by Drugs.

Ruggiero *et al.* 2006 (5)	Stage 1: Exposure of necrotic bone which is asymptomatic. Stage 2: Exposed necrotic bone associated with pain and infection Stage 3: Exposed necrotic bone in patients with pain, infection and pathological fracture, extraoral fistula or osteolysis which extends to the inferior border.
McMahon *et al.* 2007 (6)	Stage 1: Non-exposed / necrotic bone. Moderate and intermittent jaw pain. Dental findings /normal mucosas and X-rays Gammagraphy, CT and MR reveal osteoblast activity but there is no evident infection. Stage 2: Non-exposed / necrotic bone. Moderate and constant jaw pain. Dental findings / normal mucosas but the X-rays reveals sclerotic changes and radiotransparencies The gammagraphy, CT and MR show alterations. There is no evidence of infection. Stage 3: There is no apparent exposed / necrotic bone. Severe, constant jaw pain requiring analgesic drugs. Mucosal edema, erythema with severe pain of the alveolar bone. Dental X-ray, gammagraphy, CT and MR show alterations. There may be infection, although not of dental origin. Stage 4: <2 cm of exposed / necrotic bone without cortical fenestration. Considerable, constant jaw pain requiring potent analgesic drugs. The mucosa surrounding the exposed bone is red and swollen. Swelling of the surrounding tissues, with no clear evidence of infection. Dental XR, CT and MR show alterations. Dental pathology ruled out. Stage 5: > 2 cm of exposed / necrotic bone with or without cortical fenestration. Severe, constant jaw pain requiring analgesic drugs. The mucosa surrounding the exposed bone is red and swollen. Slight to moderate swelling of the peripheral tissues with or without purulent suppuration. Dental XR, gammagraphy, CT and MR show alterations. Dental pathology ruled out. Stage 6: > 4 cm of exposed / necrotic bone with cortical fenestration and infection. Severe and constant jaw pain. Fetid smell, the mucosa surrounding the exposed bone is red and swollen. At least one of the following: pathologic fracture, extraoral fistula, oroantral fistula or osteolysis extending to the inferior mandibular margin. Dental XR, bone gammagraphy with radioisotopes, CT and MR show alterations. Dental pathology ruled out.

(Ruggiero, Gralow et al. 2006) (McMahon, Bouquot et al. 2007)

Ruggiero *et al.* 2009 (7)	*Classification of the AAOMS (American Association of Oral and Maxillofacial Surgeons)* Risk category: There is no apparent necrotic bone in patients who have been treated with intravenous or oral bisphosphonates. Stage 0: There is no clinical evidence of necrotic bone, clinical findings or non-specific symptoms. Stage 1: Exposed necrotic bone in asymptomatic patients who present no evidence of infection. Stage 2: Exposed necrotic bone associated with infection, with pain and erythema in the exposed bone area with or without purulent drainage. Stage 3: Exposed necrotic bone in patients with pain, infection and one or more of the following: exposed necrotic bone extending beyond the region of alveolar bone (that is, the lower border and ramus of mandible, the maxillary sinus and maxillary zygoma) Pathologic fracture, extraoral fistula, oral antral / oral nasal communication or osteolysis extending to the lower border of the mandible of sinus floor.
Mawardi *et al.* 2009 (8)	*Proposal for the modification of the 2009 AAOMS classification, introducing a new stage called 0s.* Stage 0s: "suspected ONJ" No exposed bone. Presence of fistulas, severe tooth movement, deep periodontal pockets, positive radiographic findings. 2 subcategories: Stage 0ss: "suspect" and symptomatic. Stage 0sa: "suspect" and asymptomatic.
Bagán *et al.* 2009 (9)	Stage 1: Presence of exposed necrotic bone or small oral fistula with no exposure of the necrotic bone. Asymptomatic. Stage 2a: Presence of exposed necrotic bone or small oral fistula with no exposure of the necrotic bone. Patient with symptoms controlled by medical treatment. Stage 2b: Presence of exposed necrotic bone or small oral fistula with no exposure of the necrotic bone. Patient with symptoms not controlled by medical treatment. Stage 3: Pathologic fracture, extraoral fistula, osteolysis extending to the inferior mandibular margin.
Yoneda *et al.* 2010 (10)	The same specifications as the AAOMS in its 2009 classification, except: Stage 0 Includes hypoesthesia or anesthesia of the lower lip and/or deep periodontal pockets
Bagán *et al.* 2012 (11)	The same stages as the classification of the AAOMS in 2009, but also: Stage 3: Exposed necrotic bone or oral fistula with no exposed bone, in patients with pain, infection and one or more of the following: radiographic evidence of bone necrosis extending beyond the alveolar bone, pathological fracture, extraoral fistula, oronasal communication, osteolysis extending to the inferior mandibular margin or sinus floor.
Bedogni *et al.* 2012 (12)	Stage 1 - Focal ONJ Clinical signs and symptoms: bone exposure, tooth mobility, no post-extraction healing, fistula, inflammation, abscess formation, trismus, important mandibular deformity and / or lip hypoesthesia. CT findings: increased bone density limited to alveolar bone (trabecular thickening and / or focal osteosclerosis), with or without the following signs: sclerotic and markedly thickened lamina dura, persistent socket space and / or cortical disruption. 1a. Asymptomatic 1b. Symptomatic (pain and purulent secretion) Stage 2 - Diffuse ONJ Clinical signs and symptoms: the same as for stage 1. CT findings: increased bone density extending to the basal layer (diffuse osteosclerosis), with or without the following signs: inferior dental nerve canal prominence, periosteal reaction, sinusitis, bone sequestration and / or oro-antral communication. 2a. Asymptomatic 2b. Symptomatic (pain and purulent secretion) Stage 3 - Complicated ONJ As for stage 2, with one or more of the following: Clinical signs and symptoms: extraoral fistula. Mandibular stump displacement, nasal fluid drainage. CT findings: osteosclerosis of adjacent bone (zygoma, hard palate), pathological mandibular fracture and / or osteolysis extending to the sinus floor.

(Ruggiero, Dodson et al. 2009) (Bagan, Jimenez et al. 2009; Mawardi, Treister et al. 2009; Yoneda, Hagino et al. 2010; Bagan, Hens-Aumente et al. 2012; Bedogni, Fusco et al. 2012)

Patel *et al.* 2012 (13)	*Modification of AAOMS 2009 classification* No exposed bone (NE) Asymptomatic Stage 1NE No clinical evidence of infection; radiographic findings may be present. Symptomatic Stage 2NE Non-exposed necrotic bone; clinical evidence of infection, presence of intraoral sinus tracts, swelling, pain, paresthesia/dysesthesia and radiographic evidence of bone necrosis. Stage 3NE Non-exposed necrotic bone; pain, clinical evidence of infection and symptoms as stage 2 NE, and one or more of: • Radiographic evidence of necrotic bone extending beyond alveolar bone • Pathologic fracture • Extraoral fistula • Oral antral/oral nasal communication • Osteolysis extending to the inferior border of the mandible or sinus floor
Schiodt *et al.* 2014 (14)	*Modification of classification of AAOMS of 2009 and Patel et. al. (2012)* **Criteria for bone exposure (E-ONJ)** - Bone exposure Asymptomatic Name: E-ONJ, Stage 1 - Bone exposure Clinical symptoms of infection Name: E-ONJ, Stage 2 The same criteria as stage 3 of AAOMS Name: E-ONJ, Stage 3 **Criteria for no bone exposure (E-ONJ)** - No bone exposure Asymptomatic Name: NE-ONJ, Stage 1 - No bone exposure Clinical symptoms of infection Name: NE-ONJ, Stage 2 **No bone exposure, with necrosis in patients with pain, infection, and one or more of the following:** - Necrotic bone without exposure, as evidenced by imaging techniques, extending beyond the alveolar bone, that is, inferior border and ramus of the mandible, maxillary, sinus, and zygoma in the superior maxillary. - Pathologic fracture - Extraoral fistula - Oral antral/oral nasal communication - Osteolysis extending to the inferior border of the mandible or sinus floor Name: NE-ONJ, Stage 3
Franco *et al.* 2014 (15)	Clinical and radiological findings. Stage 0: No exposed bone, with non-specific radiographic findings, such as osteosclerosis and non-specific symptoms such as pain. Stage I: Exposed bones and / or radiographic evidence of necrotic bone or persistent socket space >2 cm with or without pain. Stage II: Exposed bones and / or radiographic evidence of necrotic bone, between 2-4 cm in diameter, with pain that responds to NSAIDs and possible abscesses. Stage III: Exposed bones and / or radiographic evidence of necrotic bone, >4 cm in greater diameter, with intense pain that responds or does not respond to NSAIDs, abscesses, maxillary sinus fistulization, or mandibular nerve involvement.

(Patel, Choyee et al. 2012; Franco, Miccoli et al. 2014; Schiodt, Reibel et al. 2014)

Mawardi *et al.* 2015 (16)	Stage 1: Asymptomatic, with bone exposure (stage 1E) or with no bone exposure (stage 1NE). Stage 2: Pain and infection with bone exposure (stage 2E) or with no bone exposure (stage 2NE). Stage 3: Greater impact, with pain and infection with bone exposure (stage 2E) or with no bone exposure (stage 2NE).
Yoneda *et al.* 2017 (17)	*Proposal by the Japanese Committee on Osteonecrosis of the jaw* Stage 0 * Clinical symptoms: no bone exposure or bone necrosis, deep periodontal pocket, loose tooth, oral mucosal ulcer, swelling, abscess formation, trismus, hypoesthesia / numbness of the lower lip (Vincent's symptom), non-odontogenic pain. Image findings: sclerotic alveolar bone, thickening and sclerosis of the lamina dura, remaining tooth extraction socket. **(Care should be taken to avoid over-diagnosis given that half the stage 0 ARONJ cases do not progress to ONJ)* Stage 1 Clinical symptoms: asymptomatic bone exposure / necrosis with no sign of infection, or fistula in which the bone is palpable with a probe. Image findings: sclerotic alveolar bone, thickening and sclerosis of the lamina dura, remaining tooth extraction socket. Stage 2 Clinical symptoms: bone exposure / necrosis associated with pain, infection, fistula in which the bone is palpable with a probe, or at least one of the following symptoms, including bone exposure / necrosis over the alveolar bone (for example, reaching the mandibular inferior border or mandibular ramus, or reaching the maxillary sinus or mandibular ramus), resulting in a pathologic fracture, extraoral fistula, nasal / maxillary sinus fistula formation, or advanced osteolysis extending to the mandibular inferior border or maxillary sinus. Stage 3 Clinical symptoms: bone exposure / necrosis associated with pain, infection or at least one of the following symptoms, or a fistula in which bone is palpable with a probe. Bone exposure / necrosis over the alveolar bone (for example, reaching the mandibular inferior border or mandibular ramus, or reaching the maxillary sinus or mandibular ramus or the cheekbone). As a result, pathologic fracture, or extraoral fistula, nasal / maxillary sinus fistula formation, or advanced osteolysis extending to the mandibular inferior border or maxillary sinus Image findings: osteosclerosis / osteolysis of the surrounding bone, pathologic mandibular fracture and osteolysis extending to the maxillary sinus floor.

MR: Magnetic resonance, **CT**: Computed tomography, **XR**: X-ray, **ONJ**: Osteonecrosis of the Jaw, **NSAIDs**: non-steroidal anti-inflammatory drugs; **ARONJ**: Antiresorptive Agent-related Osteonecrosis of the Jaw.

(Mawardi and Woo 2015; Japanese Allied Committee on Osteonecrosis of the,

Yoneda et al. 2017)

1.5 Treatment

Treatment choice for MRONJ is still controversial and recommendations from AAOMS suggest antibacterial mouth rinses, symptomatic treatment with antibiotics in the early stages and in more severe cases, superficial debridement for long term palliation of infection and pain (Table 2). (Ruggiero, Dodson et al. 2014) (El-Rabbany, Sgro et al. 2017) Conservative treatment include procedures such as minor local debridement with elimination of sharp bone edges, local hygiene of the area of exposed bone, the use of topical antibacterial agents, and systemic antibiotics for infection and pain control. In a study long-term antibiotics resulted in complete or partial healing in 18% of the patients. (Lazarovici, Yahalom et al. 2009) Similar findings were obtained in studies where conservative treatment showed poor treatment outcome with a high recurrence rate of MRONJ. (Magopoulos, Karakinaris et al. 2007) (Zervas, Verrou et al. 2006)

Surgical interventions have previously been reported to be capable of exacerbating bone exposure and a conservative approach was recommended. (Ruggiero, Gralow et al. 2006) (Van den Wyngaert, Claeys et al. 2009) More recent reports suggest a more radical treatment strategy with surgical removal of the necrotic bone and primary closure in combination with antibiotic treatment and in severe cases segmental resection. (Bedogni, Saia et al. 2011) (Pautke, Bauer et al. 2011) (Pichardo and van Merkesteyn 2016) (Stockmann, Burger et al. 2014) (Williamson 2010) In particular, complete removal of the affected region and closure of the wound are considered important to achieve a complete cure. (Khan, Morrison et al. 2015) (Japanese Allied Committee on Osteonecrosis of the, Yoneda et al. 2017)

In terms of surgical treatments, variations in their efficacies may partly be due to the lack of standardized surgical procedures. (Comas-Calonge, Figueiredo et al. 2017)

Necrotic and non-healthy bone should be removed; however, it is not easy to distinguish non-healthy from healthy bone simply from macroscopic appearance. Many surgeons rely on bleeding from the bone cutting surface and the colour of the bone. (Silva, Curra et al. 2016) The bone colour may be a good marker to distinguish necrotic bone, while bleeding may not as sclerotic change could be the result from antiresorptive medication but not due to necrosis. In fact, portions of trabecular bone become wide and extremely sclerotic, and present adjacent to the necrotic bone tissue. Furthermore, as a part of the physiological reaction against inflammation, the ONJ area is usually surrounded by sclerosing bone areas, which are less vascularized. Histological as well as bacteriological analyses of the sclerotic region of ONJ will be essential to determine the resection margin and should lead to better and more stable prognoses postoperatively.

Recently, several case reports have indicated that teriparatide, a recombinant form of parathyroid hormone, is effective in the treatment of BRONJ induced by oral BPs. (Kim, Park et al. 2014) It is also described in the literature a case of MRONJ in an 85-year-old woman who was successfully treated with teriparatide. Teriparatide was administered once per week without any surgical interventions. Compared with most recently reported cases involving daily treatment with teriparatide, once-weekly administration of teriparatide may minimize side effects and patient discomfort. (Kim, Park et al. 2019)

Table 2: Staging and Treatment Strategies.

MRONJ† Staging	Treatment Strategies‡
At risk category No apparent necrotic bone in patients who have been treated with either oral or IV bisphosphonates	• No treatment indicated • Patient education
Stage 0 No clinical evidence of necrotic bone, but non-specific clinical findings, radiographic changes and symptoms	• Systemic management, including the use of pain medication and antibiotics
Stage 1 Exposed and necrotic bone, or fistulae that probes to bone, in patients who are asymptomatic and have no evidence of infection	• Antibacterial mouth rinse • Clinical follow-up on a quarterly basis • Patient education and review of indications for continued bisphosphonate therapy
Stage 2 Exposed and necrotic bone, or fistulae that probes to bone, associated with infection as evidenced by pain and erythema in the region of the exposed bone with or without purulent drainage	• Symptomatic treatment with oral antibiotics • Oral antibacterial mouth rinse • Pain control • Debridement to relieve soft tissue irritation and infection control
Stage 3 Exposed and necrotic bone or a fistula that probes to bone in patients with pain, infection, and one or more of the following: exposed and necrotic bone extending beyond the region of alveolar bone,(i.e., inferior border and ramus in the mandible, maxillary sinus and zygoma in the maxilla) resulting in pathologic fracture, extra-oral fistula, oral antral/oral nasal communication, or osteolysis extending to the inferior border of the mandible of sinus floor	• Antibacterial mouth rinse • Antibiotic therapy and pain control • Surgical debridement/resection for longer term palliation of infection and pain

† Exposed or probable bone in the maxillofacial region without resolution for greater than 8 weeks in patients treated with an antiresorptive and/or an antiangiogenic agent who have not received radiation therapy to the jaws.

‡ Regardless of the disease stage, mobile segments of bony sequestrum should be removed without exposing uninvolved bone. The extraction of symptomatic teeth within exposed, necrotic bone should be considered since it is unlikely that the extraction will exacerbate the established necrotic process.

PAGE 14 Medication-Related Osteonecrosis of the Jaw – 2014 Update

1.6 Antiresorptive medication

1.6.1 Bisphosphonates

Bisphosphonates (BPs) are drugs that inhibit bone resorption, bone metabolism and bone remodeling through inhibition of osteoclasts. (Allen and Burr 2009) (Allen and Burr 2011) (Russell, Watts et al. 2008) BPs have high affinity for hydroxyapatite crystals of the bone and they bind preferentially to bones which have high turnover rates. They are liberated again only when the bone in which they are deposited is resorbed (Drake, Clarke et al. 2008) (Lin 1996) Due to an irreversible binding to the hydroxyapatite crystals in bone mineral, bisphosphonates have a half-life of approximately eleven years. (Lasseter, Porras et al. 2005)

The true mechanism of action of BPs is still unknown. One theory is that bisphosphonates are absorbed by the osteoclasts during bone remodeling, causing apoptosis of the osteoclast and decreasing osteoclast progenitor development and recruitment. (Drake, Clarke et al. 2008) (Hughes, Wright et al. 1995) Another theory is that BPs interfere with and inhibit proteins on osteoclasts' cell surface, necessary for the attachment of the osteoclast to the bone surface. (Drake, Clarke et al. 2008)

BPs can be divided into two groups, non-nitrogen containing BPs (NON-N-BPs) and nitrogen-containing BPs (N-BPs). BPs are chemicals with a non-hydrolysable P-C-P bond and are analogues of PPi, which has a hydrolysable P-O-P bond (Fig. 1). Many derivatives have been synthesized by modifying the central carbon and they are being applied widely in clinical settings (Figure 2). Interestingly, the BPs that have a nitrogen-containing side-chain (N-BPs) exhibit far stronger antibone-resorptive effects than the BPs that lack such a nitrogen-containing side-chain (non-N-BPs).(Dunford, Thompson et al. 2001) The presence of a nitrogen group increases the BPs antiresorptive potency by ten times.(Drake, Clarke et al. 2008) It is mainly nitrogen-

containing BPs that have been associated with the development of MRONJ. The explanation for this is probably that the nitrogen containing side chains increase the potency and perhaps toxicity. (Otto, Pautke et al. 2010; Otto, Schreyer et al. 2012) The two most potent and widely used nitrogen-containing BPs are zoledronate or zoledronic acid and alendronate.

BPs are used in the management of metabolic and malignant bone diseases, as well as osteoporosis. Intravenous (IV) BPs, including zoledronic acid and pamidronate, are standard treatments for patients with bone metastasis from breast, prostate, or lung cancer, patients with hypercalcemia of malignancy, and those with multiple myeloma.(Hortobagyi, Theriault et al. 1998) Oral BPs have been used to treat osteoporosis, Paget's disease, and paediatric osteogenesis imperfecta. (Watts 2003) BPs selectively bind to the bone and inhibit bone resorption by inducing osteoclast apoptosis. (Licata 2005) Newer nitrogen-containing BPs such as zoledronic acid may have a direct effect on tumours via their anti-angiogenic properties, by inducing cell apoptosis and blocking tumour invasion. (Heymann, Ory et al. 2004)

In general, when orally administered the BPs alendronic acid and pamidronic acid are poorly absorbed from the gastrointestinal tract as a result of their poor lipophilicity which prevents transcellular transport across epithelial barriers. This means a low bioavailability of 0.3-0.8%. (Daley-Yates, Dodwell et al. 1991; Gertz, Holland et al. 1995) Intravenously administered BPs on the other hand lead to a rapid uptake in bone tissue. To date, all BPs studied show no evidence of metabolism. Renal excretion is the only route of elimination.(Lin 1996)

After administration of a BP, most of the drug is plasma-bound. About 40% of the dose is excreted in the urine within 24 hours, and the remainder of the dose is presumed to be bound to the bone.(Chae, Seo et al. 2014) This suggests that several

cells could likely expose to BP ranging from immune cells circulating in the blood to cells residing in the bone. It has been estimated that the frequency of MRONJ in patient receiving intravenous BP is up to 21%. (Walter, Al-Nawas et al. 2010)

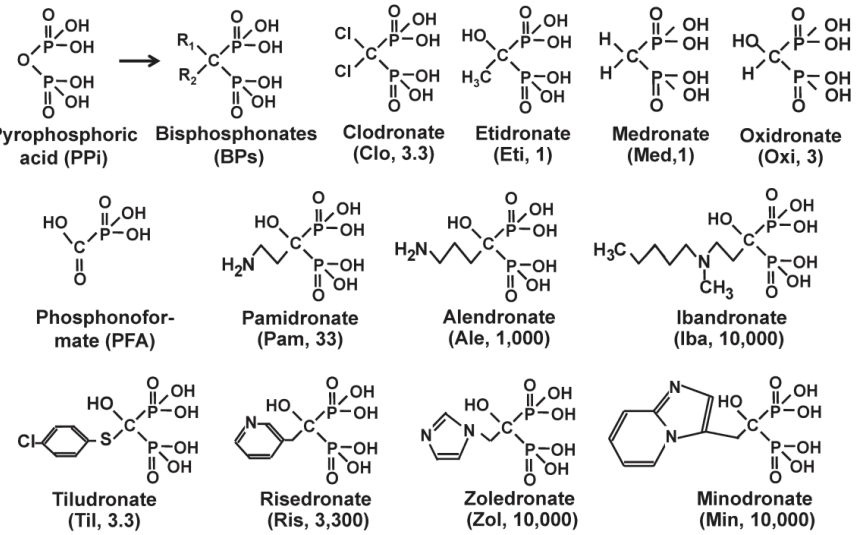

Fig. 1. Structure of Bisphosphonates (BPs) and Related Substances

Bisphosphonates (BPs), with a non-hydrolysable P-C-P structure, are analogs of pyrophosphate (PPi), which has a hydrolysable P-O-P structure. There are two types of BPs, the nitrogen-containing BPs (N-BPs) and the non-nitrogen-containing BPs (non-N-BPs). Phosphonoformic acid (PFA) is an inhibitor of the phosphate transporter SLC34, although at higher concentrations it inhibits SLC20, too (see Section 7). For each BP, its abbreviation and the relative potency of its anti-bone-resorptive effect are shown within parentheses (that of Eti being given the value 1) (refs. 5, 6).

Figure 2

(Dunford, Thompson et al. 2001)

1.6.2 Denosumab

Denosumab is a fully humanized monoclonal immunoglobulin antibody that inhibits the development and activation of osteoclasts by preventing the binding of receptor for nuclear factor kappa β ligand (RANKL) to RANK, a transmembrane receptor that is expressed in the cell membranes of pre-osteoclasts and osteoclasts (Figure 3). (Lewiecki 2010) This antibody therefore promotes osteoclast apoptosis that in turn decreases bone resorption and increases bone density. Denosumab was approved in 2010 by the FDA for the prevention of Skeletal Related Effects (SREs) in patients with bone metastases and in 2011 to prevent endocrine-therapy-induced bone loss in patients taking aromatase inhibitors for breast cancer and in patients with non-metastatic prostate cancer.(Baron, Ferrari et al. 2011; Diz, Lopez-Cedrun et al. 2012; Lee, Higgins et al. 2012)

Various clinical trials have shown that denosumab may be more effective than zoledronic acid in patients with metastatic bone disease. (Stopeck, Lipton et al. 2010) (Fizazi, Carducci et al. 2011; Sun and Yu 2013) Denosumab is administered subcutaneously and cleared by the reticuloendothelial system, thereby preventing nephrotoxicity. The circulatory half-life of denosumab is 26 days, while the half-life of intravenous bisphosphonates (IVBPs) ranges from 10–12 years. Unlike IVBPs, denosumab does not appear to accumulate in the bone. In addition, denosumab has been found to be more cost-effective in the prevention of SREs. Patients on denosumab for metastatic bone disease receive 120 mg subcutaneously every 4 weeks while patients on denosumab for the management of osteoporosis/osteopenia or to increase bone mass receive 60 mg subcutaneously every 6 months. After cessation of denosumab treatment normal osteoclast function can be expected after approximately 12 months (Iranikhah, Deas et al. 2018) (McClung, Wagman et al. 2017)

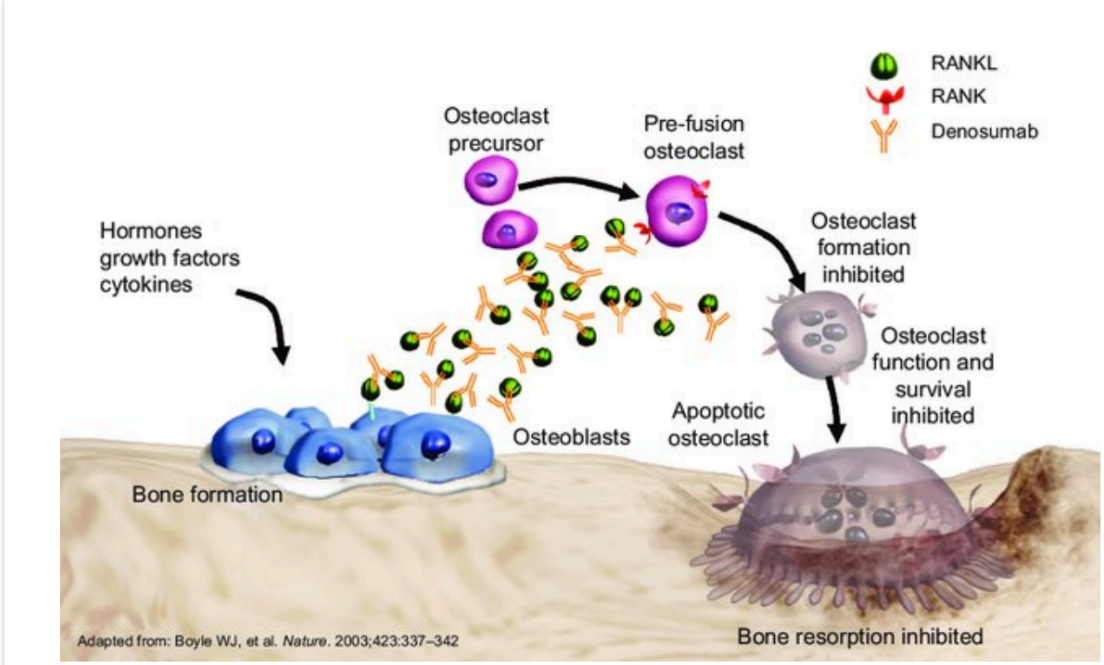

Figure 3: Inhibition of RANK/RANKL interaction decreases bone resorption and increases bone strength (Boyle, Simonet et al. 2003)

1.6.3 Antiangiogenics

Angiogenesis is a critical step in tumor progression and the RANKL system represents the central pathway leading to osteoclast differentiation. (Lumachi, Brunello et al. 2009) The cell-surface receptor vascular endothelial growth factor (VEGF) receptor plays a major role in cancer progression and can be targeted by drugs inhibiting tyrosine kinase activator or other second-line messengers, such as extracellular-signal regulated kinases/mitogen-activated protein kinase, and mammalian target of rapamycin. (Brunello, Borgato et al. 2013) Most angiogenesis inhibitors, such as the monoclonal antibody bevacizumab and the kinase inhibitor sunitinib, target the VEGF signaling pathway. Bevacizumab was the first anti-angiogenetic drug approved for clinical use, initially for the treatment of colorectal cancer and currently also for breast cancer and lung cancer. (Gotink and Verheul 2010) It may compromise microvessel integrity, leading to compromise of the osteon at the jaw, and several studies report the risk of ONJ in patients treated with this drug. (Estilo, Fornier et al. 2008; Brunamonti Binello, Bandelloni et al. 2012; Katsenos, Christophylakis et al. 2012) A metanalysis of data from 3,560 patients with advanced breast cancer treated with bevacizumab alone or in combination with BPs showed that the overall incidence of ONJ in this population was 0.2% and 0.9%, respectively.(Guarneri, Miles et al. 2010) Sunitinib is a multi-targeted receptor tyrosine kinase inhibitor that inhibits cellular signaling by targeting platelet-derived growth factor receptors and VEGF receptors. (Nicolatou-Galitis, Migkou et al. 2012) (Papaetis and Syrigos 2009) It also inhibits KIT (CD117) and other RTKs, including colony stimulating factor-1 receptor. There are only few studies reporting ONJ in patients treated with sunitinib, and thus the incidence of sunitinib-related ONJ is unknown. (Hoefert and Eufinger 2010) (Koch, Walter et al. 2011)

1.6.4 Other Factors

ONJ has also been reported in patients not receiving BPs, denosumab, or antiangiogenics. These cases are rare and are associated with glucocorticoids, infection, trauma, chemotherapy, and coagulation disorders.(Glueck, McMahon et al. 1998; Sung, Chan et al. 2002) Diseases such as rheumatoid arthritis A and the medications used to treat it can present a risk for impaired healing and can present lesions clinically and radiographically identical to ONJ. (Horie, Kawano et al. 2015) The present inciting events are similar to those for MRONJ and include extractions, periodontal disease, trauma, implants, or even spontaneous or unknown.(Lambade, Lambade et al. 2012) (Pogrel and Miller 2003) However, less common medications or disease processes are associated with these cases, including methotrexate, etanercept, prednisone, adalimumab, rituximab, and distant local steroid injections. (Aghaloo and Tetradis 2017)

1.7 Macrophages

Macrophages are derived from monocytes and move out into extravascular tissues under inflammatory or non-inflammatory conditions, playing different roles according to their surrounding environment.(Ariel, Maridonneau-Parini et al. 2012) Oral macrophages also play important roles in the inflammatory response, as well as in signaling to resolve inflammation, and promote healing and regeneration.(Hasturk, Kantarci et al. 2012) Macrophages are divided into M1 and M2 macrophage types. (Solinas, Germano et al. 2009) While investigating the factors that regulate macrophage arginine metabolism, Mills et al. found that macrophages activated in mouse strains with T helper type (Th)1 and Th2 backgrounds differed qualitatively in their ability to respond to the classic stimulation of interferon (IFN)-γ or LPS or both and defined an

important metabolic difference in the pathway. They proposed that these be termed M1 and M2 macrophage responses. (Mills, Kincaid et al. 2000) Macrophages are polarized into the M1 macrophages, when exposed to classical activators such as LPS and IFN-γ. Macrophages are polarized into the M2 from when exposed to alternative activators such as interleukin (IL)-4 or IL-13.(Martinez and Gordon 2014) M1-polarized macrophages produce pro-inflammatory cytokines, such as IL-1β, and infiltrate into injured tissues soon after damage.(Arnold, Henry et al. 2007) M2-polarized macrophages produce IL-10 and TGF-b, and appear at late stages of repair and remodeling in injured tissue. (Biswas and Mantovani 2012)

1.8 Interleukins

Cytokines are small (15–20 kDa) and short-lived proteins important in autocrine, paracrine, and endocrine signaling. Cytokines coordinate the development and the activity of the immune system. (Gandhi, Bennett et al. 2016) Many cytokines belong to the four α-helical class of mediators, which share a common up-up–down-down topology of the four helices. Furthermore, cytokines are grouped into families according to the structure and the specificity and composition of their receptor complexes. Cytokines bind to multimeric receptor complexes in which often one subunit is also found in the receptor complexes for other cytokines. (Spangler, Moraga et al. 2015) . In recent years, many researchers have noticed that differences in cytokine levels (high or low) are associated with certain allelic variants of cytokine genes. These polymorphisms might play an important role in the pathophysiology of various diseases.

Interleukin (IL)-6 family cytokines are defined as cytokines that use the common signaling receptor subunit glycoprotein 130 kDa (gp130). Presently, eight cytokines fulfill this criterion although, as will be explained below, the group of IL-6 family cytokines is still expanding and the definition of gp130-containing complexes needs to be revised. (Rose-John, Scheller et al. 2015) IL-6 family cytokines have been implicated in many functions, including B-cell stimulation and induction of the hepatic acute phase proteins. Moreover, metabolic functions and neurotrophic functions have been ascribed to this group of cytokines. Lately, an IL-6 receptor (IL-6R)-neutralizing monoclonal antibody (tocilizumab) has been approved in more than 100 countries for the treatment of autoimmune diseases, and blockade of IL-6 activity was observed to be at least as efficient as the blockade of tumor necrosis factor α in patients with rheumatoid arthritis. (Tanaka, Narazaki et al. 2014)

Interleukin 10 (IL-10) is an important pleiotropic immunoregulatory cytokine mainly secreted by macrophages, but also by T helper 1 (Th1) and Th2 lymphocytes, dendritic cells, cytotoxic T cells, B lymphocytes, monocytes and mast cells. Some studies have shown that it can be produced also by human carcinoma cell lines. (Gastl, Abrams et al. 1993) IL-10 activity is mediated by the IL-10 receptor (IL-10R) which is a member of the class II cytokine receptor family. IL-10 inhibits the capacity of monocytes and macrophages to present antigen to T cells via an inhibitory effect on expression of major histocompatibility complex (MHC) class II, costimulatory molecules such as CD80 and CD86 and therefore downregulates the expression of IL-1, IL-6, IL-8, IL-12 and tumor necrosis factor – alpha (TNF-α). In B cells, IL-10 prevents apoptosis, enhances cell proliferation and has a role in immunoglobulin (Ig) class switch.

The IL-10 gene is located on chromosome 1 at 1q31-32, spans about 4.7 kb and contains four introns and five exons. (Spits and de Waal Malefyt 1992) There are many genetic variants of IL-10 gene. However, the most studied are two dinucleotide repeats (microsatellites), IL10.G and IL10.R, located 1.2 kb and 4 kb upstream of the transcription start site and three single nucleotide polymorphisms (SNPs) -1082(G/A), -819(C/T) and -592(C/A) which form three predominant haplotypes (GCC, ACC, ATA). Although endogenous and exogenous factors stimulate cells to produce IL-10, its secretion also depends on IL10.R, IL10.G and SNP polymorphisms in promoter region. (Eskdale, Kube et al. 1997)

Aim

The objective of this study was to investigate the relationship between M1- and M2-polarized macrophages in mucosal tissues surrounding necrotic bone and disease progression in patients with MRONJ who underwent treatment with bisphosphonates or denosumab. Given that staging of MRONJ is determined by the progression and manifestations of clinical infection and inflammation in the maxillofacial region, we hypothesized a stage-dependent switch of macrophage polarization, predominantly toward the anti-inflammatory M2 phenotype in patients with early stage of MRONJ and toward the proinflammatory M1 phenotype in patients with advanced stage of disease.

Materials and Methods

2.1 Study population

The study population comprised 30 patients with MRONJ who underwent surgical debridement and biopsy of mucosal tissue surrounding the osteonecrotic area in the lower or upper jaw at the Department of Oral and Maxillofacial Surgery of the School of Dentistry, National and Kapodistrian University of Athens (NKUoA), Greece between 2016 and 2019. Inclusion criteria were: 1) MRONJ patients who received oral bisphosphonates for more than 4 years or intravenous bisphosphonates (e.g. 5 mg zoledronic acid once a year) or denosumab (Prolia 60 mg administered subcutaneous every 6 months for osteoporosis or Xgeva 120 mg administered subcutaneous every 4 weeks for prevention of bone complications in cancer), 2) patients with clinical diagnosis of MRONJ stage 1–3, with 10 patients assigned to each clinical stage, 3) availability of biopsies of sufficient quality for immunofluorescence studies. Exclusion criteria were: 1) history of head and neck radiotherapy, 2) patients with acute or chronic renal or hepatic insufficiency or any hematologic disorder, 3) patients who underwent cardiovascular operation within a year, and 4) recent use of local or systemic corticosteroids. Two groups of participants without MRONJ who underwent biopsy of inflamed oral mucosa adjacent to extraction socket of teeth with periodontal disease were assigned as controls: 1) control group participants who received antiresorptive medication without presenting any clinical or radiographic findings of MRONJ and 2) control group participants who did not receive antiresorptive therapy.

For all eligible patients, clinical characteristics were recorded, including sex, age, primary disease, antiresorptive medication, administration period, site of lesion and MRONJ staging. Diagnosis and assignment of patients into clinical stages of

MRONJ was based on clinical and radiographic examination and according to the proposed staging system of the American Association of Oral and Maxillofacial Surgeons (AAOMS) in 2014 (Ruggiero, Dodson et al. 2014). Radiographic evaluation took place at the Department of Oral Diagnosis and Radiology of the School of Dentistry, NKUoA and included panoramic x-ray and cone-beam computed tomography (CBCT). Histopathologic examination of all surgical specimens was performed for confirming the diagnosis. Written informed consent was obtained from all the patients. The local Institutional Ethics Committee approved this prospective study.

2.2 Immunofluorescence staining

To study M1 and M2 macrophage density and the expression of IL-6 and IL-10, formalin-fixed, paraffin embedded (FFPE) biopsy specimens archived at the Department of Oral Pathology and Medicine of the School of Dentistry, NKUoA, were subjected to standard immunofluorescence analysis. In brief, representative specimens were sectioned into blocks (4 μm thickness). One section was stained with haematoxylin and eosin and adjacent sections were used for immunofluorescence staining. Sections were dewaxed in xylene and then rehydrated in graded alcohols. Sections were washed in water before antigen retrieval with 10 mM sodium citrate buffer (pH 6.0) at 60 °C overnight. UltraCruz Blocking Reagent (Santa Cruz) was used for 1 hour at room temperature. In turn, tissue sections were incubated with the following primary antibodies overnight: CD68 1:200 (M00602, Boster), iNOS 1:100 (ab15323, Abcam), CD206 1:100 (sc-58986, Santa Cruz), IL-6 1:250 (sc-28343, Santa Cruz), IL-10 1:400 (ab34843, Abcam). Sections were incubated with the following secondary antibodies for 45 min: Boster BA1089 TRITC anti-mouse 1:500, Boster

BA1101 FITC anti-mouse 1:500, Invitrogen Alexa Fluor 568 anti-rabbit 1:500. After incubation with secondary antibody and three washes with PBS/T, the slides were mounted using Fluoroshield with DAPI staining to detect nuclei (F6057, SIGMA).

2.3 Assessment of immunofluorescence staining

Immunofluorescence stained slides were digitally scanned utilizing the Aperio AT automated slide scanner and automated image analysis was performed using the Aperio Image Scope software (Aperio Technologies, Inc., Vista, CA, USA). Digital imaging was performed at the Translational Pathology Core Laboratory (TPCL) at David Geffen School of Medicine, University of California, Los Angeles (UCLA), USA. The magnification of the digital images varied continuously as it could be controlled by the computer software. Computer-assisted immunofluorescence quantification of markers was performed. Stained slides were assessed by two independent investigators who evaluated at least two representative image fields at 20x followed by 40x magnification for further verification. For each slide, every marker was digitally evaluated for the intension of staining separately with Aperio ImageScope software and after the use of specific filters (threshold) for each antibody.

For identification of M1 and M2 macrophages, double CD68/iNOS and CD68/CD206 immunofluorescence staining was performed respectively. CD68 ab was labelled with secondary FITC ab while iNOS and CD206 abs with secondary TRITC ab. Every cell expressing CD68, iNOS or CD206 above defined thresholds was considered positive. In turn, CD68 and iNOS as well as CD68 and CD206 images were fused to create the double staining image. All nucleated cells with double positive staining for the phenotype marker M1 ($CD68^+/iNOS^+$) or M2 ($CD68^+/CD206^+$) in each image were counted manually. Density of M1 and M2 macrophages was calculated as

the number of positively stained cells per square millimeter (cells/mm^2) in the region of interest. For IL-6 and IL-10 quantification, marker expression above defined thresholds in the region of interest was considered positive and the percentage of positive IL-6 and IL-10 staining was digitally calculated. The autofluorescence of erythrocytes was manually removed from all quantification. All investigators performing measurements were blinded to patient clinical data.

2.4 Statistical analysis

Categorical data were described with absolute and relative frequencies. Skew data were expressed as median and interquartile range (IQR) and group differences were tested by Mann–Whitney U test or Kruskal–Wallis H test as appropriate. Bonferroni correction was applied to protect from Type 1 error when conducting multiple comparison tests on the same dependent variable. Boxplots depicted the distribution of macrophage densities across MRONJ stages and controls. One way ANOVA was used to assess whether there are differences between the means of two or more independent groups. A Tukey post hoc test was conducted for multiple comparisons. Two-sided p-values <0.05 were considered statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS®, version 26.0; IBM Corp., Armonk, NY).

2.5 Primary Antibodies

ANTIBODIES	COMPANY	ORIGIN	CONCENTRATION
iNOS (M1)	ab15323, abcam	Mouse monoclonal	1:100
CD68 (pan macrophage marker)	M00602, Boster	Mouse monoclonal	1:200
CD206 (M2)	sc-8986, Santa Cruz	Mouse monoclonal	1:100
IL6 (M1)	sc-28343 Santa Cruz	Mouse monoclonal	1:250
IL10 (M2)	Ab34843 abcam	Mouse monoclonal	1:400

Results

3.1 Patient characteristics

Our study cohort comprised 30 patients with histologically confirmed MRONJ following therapy with either bisphosphonates (*n* =15) or denosumab (*n* = 15), 13 control group participants who received either bisphosphonates (*n* =8) or denosumab (*n* = 5), and 6 control group participants who did not receive antiresorptive therapy.

The MRONJ patients were classified as stage 1 (*n* =10, 33.3%), stage 2 (*n* =10, 33.3%) and stage 3 (*n* =10, 33.3%). The median age of the MRONJ patients was 71, ranging from 45 to 82 years and they were predominately female (70%, *n* = 21). The underlying disease was osteoporosis in 9 patients (30%) and malignancy in 21 patients (70%) including 9 patients with breast cancer, 3 with prostate cancer, 1 with lung cancer and 8 with multiple myeloma. Eleven patients (37%) received zoledronic acid (Zometa), 4 patients (13%) received alendronate (Fosamax) and 15 patients received denosumab (5 patients received Prolia (17%) and 10 patients received Xgeva (33%)). The median administration period of antiresorptive therapy was 36 months, while 11 patients (37%) received antiresorptive treatment for more than 36 months. MRONJ was located in the lower jaw in 18 patients (60%) and the upper jaw in 12 patients (40%). Demographic and clinical characteristics of MRONJ patients and control group participants are listed in Table 3.

Table 3

Patient demographic and clinical characteristics.

		MRONJ patients *n* = 30		Cohort antiresorptive *n* = 43		Control no antiresorptive *n* = 6		All patients *n* = 49	
Variable	**Category**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**
Sex	Male	9	(30)	11	(26)	1	(17)	12	(24)
	Female	21	(70)	32	(74)	5	(83)	37	(76)
Age	≤ 60 years	7	(23)	10	(23)	5	(83)	15	(31)
	> 60 years	23	(77)	33	(77)	1	(17)	34	(69)
Primary disease	Osteoporosis	9	(30)	19	(44)				
	Cancer	21	(70)	24	(56)				
Antiresorptive types	Bisphosphonates	15	(50)	23	(54)				
	Denosumab	15	(50)	20	(46)				
Antiresorptive drugs	Zometa	11	(37)	14	(33)				
	Fosamax	4	(13)	9	(21)				
	Prolia	5	(17)	8	(18)				
	Xgeva	10	(33)	12	(28)				
Administration period	≤ 36 months	19	(63)	24	(56)				
	> 36 months	11	(37)	19	(44)				
Site of MRONJ	Upper jaw	12	(40)						
	Lower jaw	18	(60)						
Staging of MRONJ	Stage 1	10	(33.3)						
	Stage 2	10	(33.3)						
	Stage 3	10	(33.3)						

MRONJ=Medication-Related Osteonecrosis of the Jaw.

3.2 Density of M1 and M2 macrophages across MRONJ stages and controls

Immunofluorescence analysis was performed to quantify the density of M1 and M2 macrophages in mucosal tissues surrounding necrotic bone in patients with MRONJ stages 1–3 and controls. In the MRONJ cohort (n = 30), the median density of $CD68^+/iNOS^+$ M1 macrophages was 18 cells/mm^2 (IQR: 11–24), while a median of 12.5 cells/mm^2 (IQR: 8–17) was counted for $CD68^+/CD206^+$ M2 macrophages. Representative examples of $CD68^+/iNOS^+$ and $CD68^+/CD206^+$ immunofluorescence staining are shown in Figure 4 a–b.

The density of $CD68^+/iNOS^+$ M1 macrophages was statistically significant different across MRONJ stages and controls ($\chi^2(4) = 30.575$, $p < 0.001$, Kruskal-Wallis H test; Table 4), with a median $CD68^+/iNOS^+$ M1 macrophage density of 10 cells/mm^2 for stage 1, 25 cells/mm^2 for stage 2, 21 cells/mm^2 for stage 3, 3 cells/mm^2 for control group receiving antiresorptive therapy and 4 cells/mm^2 for control group not receiving antiresorptive therapy. Pairwise comparison of M1 macrophage distribution across MRONJ stages and controls showed a statistically significant higher M1 macrophage density in: i) stage 2 compared to both control groups (antiresorptives: $p < 0.001$, no antiresorptives: $p = 0.006$) and ii) stage 3 compared to both control groups (antiresorptives: $p < 0.001$, no antiresorptives: $p = 0.017$). We also observed a higher M1 macrophage density in stage 1 compared to control group receiving antiresorptive therapy, however, this difference was not statistically significant ($p = 0.069$) (Table 5).

The results showed a statistically significant difference in $CD68^+/CD206^+$ M2 macrophage density across MRONJ stages and controls ($\chi^2(4) = 10.935$, $p = 0.027$; Table 4), with a median $CD68^+/CD206^+$ M2 macrophage density of 18.5 cells/mm^2 for stage 1, 9.5 cells/mm^2 for stage 2, 11 cells/mm^2 for stage 3, 9 cells/mm^2 for control

group receiving antiresorptive therapy and 16 cells/mm^2 for control group not receiving antiresorptive therapy. Pairwise comparison of M2 macrophage distribution across MRONJ stages and controls revealed a statistically significant higher M2 macrophage density in stage 1 compared to stage 2 ($p = 0.024$) (Table 5).

The analysis demonstrated a statistically significant difference in the ($CD68^+/iNOS^+$) M1) / ($CD68^+/CD206^+$) M2 ratio across MRONJ stages and controls ($\chi^2(4) = 29.817$, $p < 0.001$; Table 4). A M1/M2 ratio >1 indicates that there are relatively more M1- than M2-polarized macrophages and vice versa. The results showed a median M1/M2 ratio of 0.56 for stage 1, 2.11 for stage 2, 1.55 for stage 3, 0.2 for control group receiving antiresorptive therapy and 0.19 for control group not receiving antiresorptive therapy. Pairwise comparison of M1/M2 ratio across MRONJ stages and controls showed a statistically significant higher M1/M2 ratio in: i) stage 2 compared to both control groups (antiresorptives: $p < 0.001$, no antiresorptives: $p = 0.002$) and ii) stage 3 compared to both control groups (antiresorptives: $p = 0.002$, no antiresorptives: $p = 0.016$). In contrast, a statistically significant lower M1/M2 ratio was found in stage 1 compared to stage 2 ($p = 0.049$) (Table 5). The boxplots in Figure 5a-c show the densities of $CD68^+/iNOS^+$ M1 macrophages, $CD68^+/CD206^+$ M2 macrophages and the M1/M2 ratio across patients with MRONJ stages 1–3 and controls.

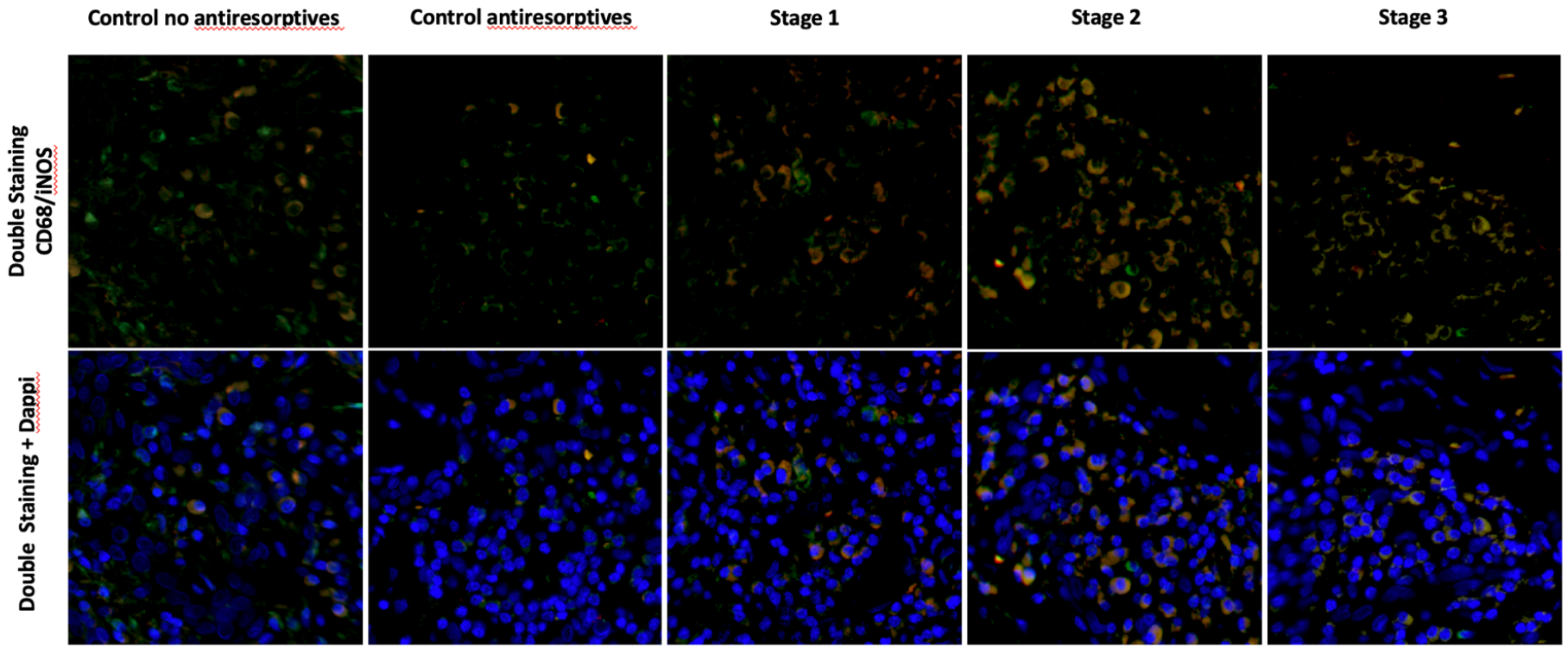

Figure 4a. Representative case of CD68/iNOS immunofluorescence staining (x40). There is a statistically significant difference in M1 macrophage density between the different MRONJ stages and controls, $p < 0.001$. ($CD68^+$:FITC, $iNOS^+$:TRITC)

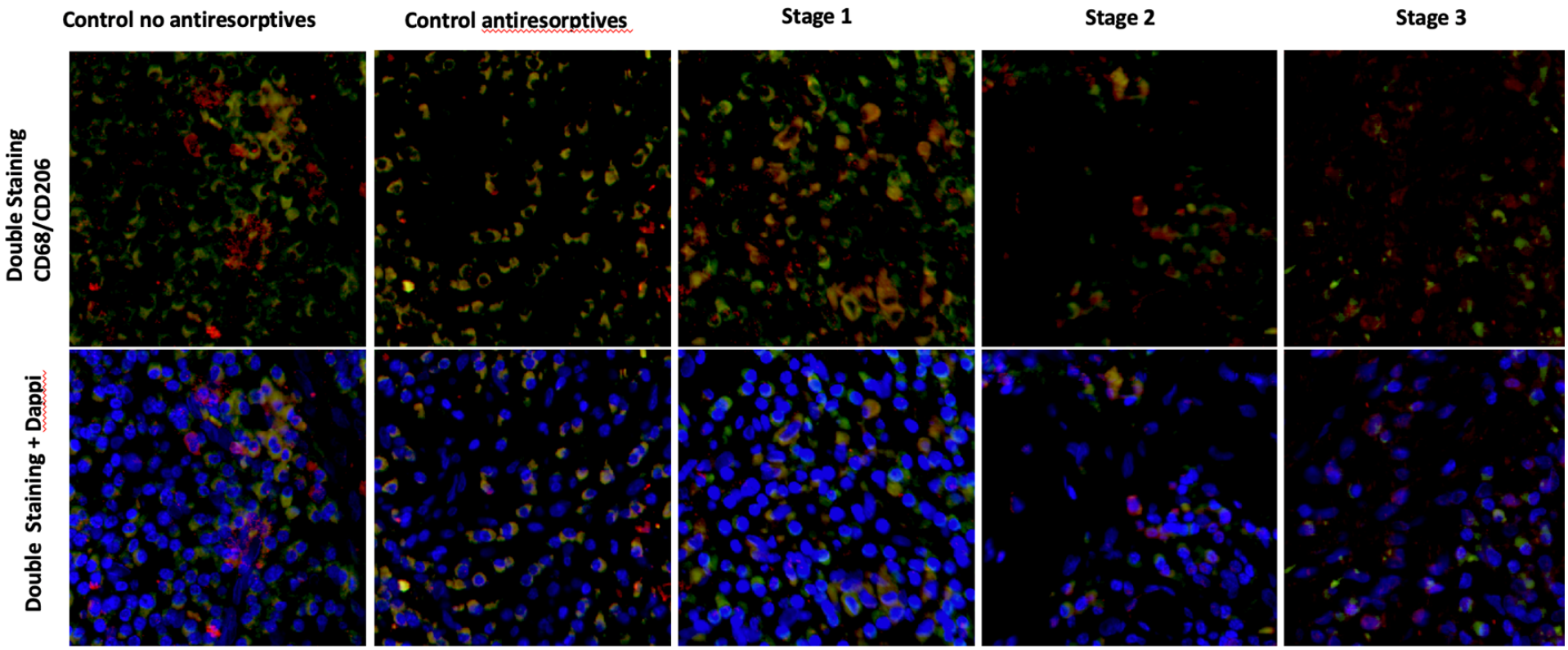

Figure 4b. Representative case of $CD68^{+}/CD206^{+}$ immunofluorescence staining (x40). There is a statistically significant difference in M2 macrophage density between the different MRONJ stages and controls, p =0.027. ($CD68^{+}$:FITC, $CD206^{+}$:TRITC)

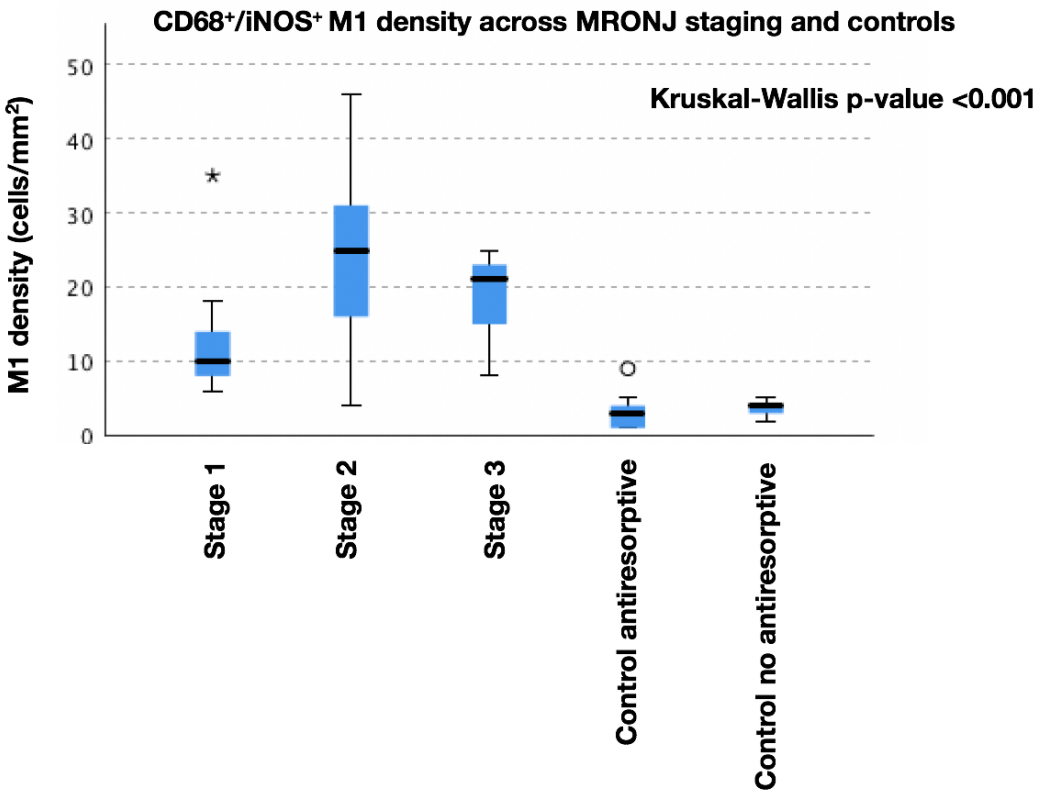

Figure 5a M1 Density across MRONJ and controls

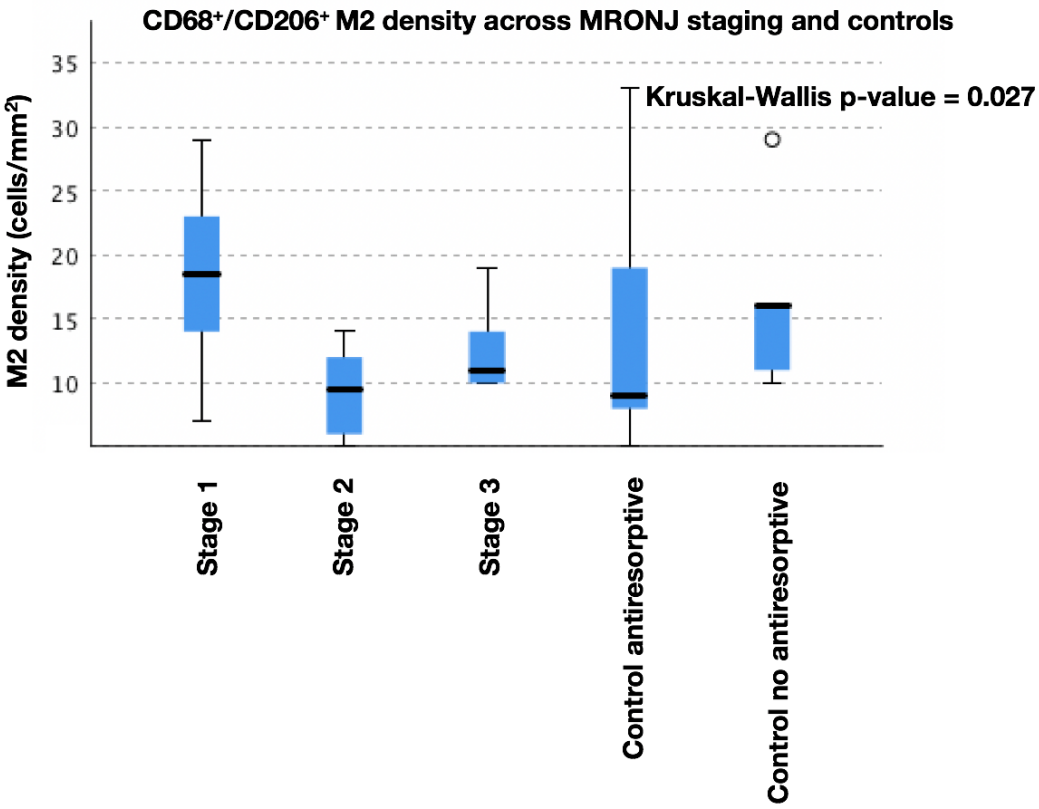

Figure 5b M2 Density across MRONJ and controls

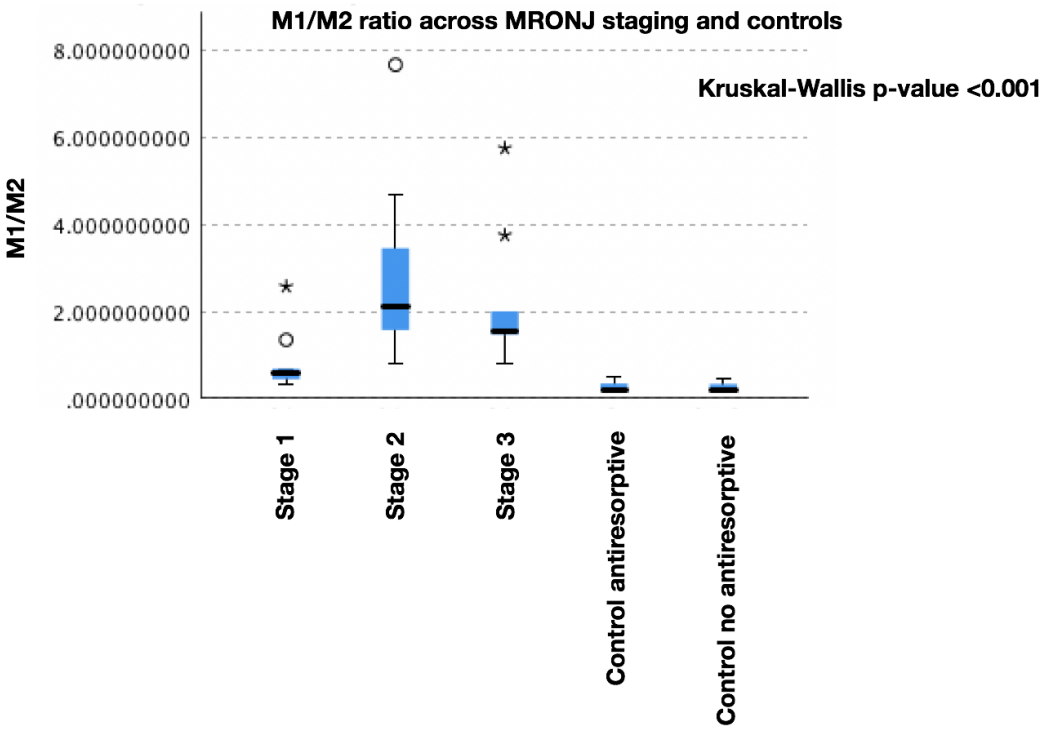

Figure 5c M1/M2 ratio across MRONJ staging and controls

Table 4

Comparison of M1 and M2 macrophage density across MRONJ staging and controls.

	M1–M2 macrophage density		
Variable	M1[a] median (IQR)	M2[b] median (IQR)	M1/M2 median (IQR)
MRONJ stage 1	10 (8–14)	18.5 (14–23)	0.56 (0.44–0.68)
MRONJ stage 2	25 (16–31)	9.5 (6–12)	2.11 (1.57–3.45)
MRONJ stage 3	21 (15–23)	11 (10–14)	1.55 (1.47–2)
Control–antiresorptive	3 (1–4)	9 (8–19)	0.2 (0.15–0.33)
Control–no antiresorptive	4 (3–4)	16 (11–16)	0.19 (0.16–0.32)
p-value[c]	<0.001**	0.027*	<0.001**

[a]$CD68^+/iNOS^+$ M1 macrophage density (cells/mm^2).

[b]$CD68^+/CD206^+$ M2 macrophage density (cells/mm^2).

[c]Kruskal-Wallis H test.

*p <0.05, **p <0.01

MRONJ = Medication-Related Osteonecrosis of the Jaw; IQR= interquartile range.

Table 5

Comparison of M1 and M2 macrophage density across MRONJ stages and controls pairwise

Pairwise comparison	M1[a] p-value[c]	M2[b] p-value	M1/M2 p-value
Control antiresorptive vs. Control_no antiresorptive	1.000	1.000	1.000
Control antiresorptive vs. Stage 1	0.069	0.478	0.732
Control antiresorptive vs. Stage 3	<0.001**	1.000	0.002**
Control antiresorptive vs. Stage 2	<0.001**	1.000	<0.001**
Control no antiresorptive vs. Stage 1	0.473	1.000	1.000
Control no antiresorptive vs. Stage 3	0.017*	1.000	0.016*
Control no antiresorptive vs. Stage 2	0.006**	0.805	0.002**
Stage 1 vs. Stage 3	1.000	0.199	0.357
Stage 1 vs. Stage 2	0.762	0.024*	0.049*
Stage 3 vs. Stage 2	1.000	1.00	1.000

[a]$CD68^+/iNOS^+$ M1 macrophage density (cells/mm^2).

[b]$CD68^+/CD206^+$ M2 macrophage density (cells/mm^2).

[c]Kruskal-Wallis Test, pairwise comparison; p-values have been adjusted by the Bonferroni correction for multiple tests.

*p <0.05, **p <0.01

MRONJ = Medication-Related Osteonecrosis of the Jaw.

3.3 Density of M1 and M2 macrophages according to clinical variables in patients with MRONJ

The comparison of M1 and M2 macrophage density according to clinical variables in patients with MRONJ is shown in Table 6. The analysis showed that density of M1 and M2 macrophages was statistically significant higher in patients receiving bisphosphonates compared to those receiving denosumab ($p = 0.005$ and $p = 0.002$, respectively; Mann-Whitney U test). In particular, with regard to specific antiresorptive agents (zoledronic acid, alendronate, denosumab (Prolia), denosumab (Xgeva) there was a statistically significant difference in the density of M1 and M2 macrophages ($p = 0.018$ and $p = 0.016$ respectively; Kruskal-Wallis H test). Pairwise comparison of M1 macrophage distribution across antiresorptive agents showed a statistically significant higher M1 macrophage density in: i) patients receiving zoledronic acid (Zometa) compared to patients receiving denosumab (Prolia) ($p = 0.008$) and ii) patients receiving alendronate (Fosamax) compared to patients receiving denosumab (Prolia) ($p = 0.007$). Pairwise comparison of M2 macrophage distribution across antiresorptive agents revealed a statistically significant higher M2 macrophage density in: i) patients receiving zoledronic acid (Zometa) compared to patients receiving denosumab (Xgeva) ($p = 0.003$) and ii) patients receiving alendronate (Fosamax) compared to patients receiving denosumab (Xgeva) ($p = 0.033$). M2 macrophage density was statistically significant higher in patients > 60 years and those receiving antiresorptive therapy for > 36 months ($p = 0.021$ and $p = 0.033$, respectively).

Table 6

Comparison of M1 and M2 macrophage density according to clinical variables in patients with MRONJ

		M1–M2 macrophage density					
Variable	Category	M1[a] median (IQR)	p-value[c]	M2[b] median (IQR)	p-value	M1/M2 median (IQR)	p-value
Sex	Male	22 (16–29)	0.226	13 (11–14)	0.504	1.81 (1.26–2.21)	0.625
	Female	15 (9–22)		12 (7–17)		1.5 (0.68–2)	
Age	≤ 60 years	15 (11.5–20.5)	0.606	7 (5.5–8.5)	0.021*	2 (1.15–4.16)	0.291
	> 60 years	20 (12–24.5)		14 (11–17.5)		1.5 (0.66–2)	
Primary disease	Osteoporosis	18 (8–22)	0.422	13 (11–17)	0.625	1.34 (0.8–1.5)	0.125
	Cancer	18 (14–24)		12 (7–17)		1.81 (0.68–2.57)	
Antiresorptive class	Bisphosphonates	22 (17–30)	0.005**	14 (12.5–20)	0.002**	1.53 (0.97–2.11)	0.967
	Denosumab	15 (8–18)		10 (6.5–12.5)		1.5 (0.72–2.28)	
Antiresorptive agents	Zoledronic acid (Zometa)	22 (13.5–30)	0.018*[d]	14 (13–20)	0.016*[d]	1.57 (0.64–2.31)	0.202[d]
	Alendronate (Fosamax)	23.5 (21–30)		15 (12–21.5)		1.5 (1.4–1.7)	
	Denosumab (Prolia)	8 (8–9)		12 (10–14)		0.8 (0.64–0.8)	
	Denosumab (Xgeva)	15.5 (14–20)		7.5 (6–11)		2 (1.5–3.7)	
Administration period	≤ 36 months	15 (10–21)	0.111	10 (6.5–16)	0.033*	1.5 (0.6–3)	0.703
	> 36 months	22 (19–27)		14 (12.5–17)		1.53 (1.4–1.8)	
Site of MRONJ	Upper jaw	19 (10–22.5)	0.832	12 (8.5–17)	0.849	1.52 (0.66–2.2)	0.849
	Lower jaw	17 (13–28)		12.5 (8–18)		1.53 (0.68–2.2)	

[a]$CD68^+/iNOS^+$ M1 macrophage density (cells/mm^2).

[b]$CD68^+/CD206^+$ M2 macrophage density (cells/mm^2).

[c]Mann-Whitney U test, unless otherwise specified; [d]Kruskal-Wallis H test.

*p <0.05, **p <0.01

MRONJ = Medication-Related Osteonecrosis of the Jaw; IQR= interquartile range.

3.4 Density of M1 and M2 macrophages across BRONJ stages and controls

In subgroup analyses, M1 and M2 macrophage density was assessed in samples of patients with Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ) and controls. A Kruskal-Wallis H test showed a statistically significant difference in densities of $CD68^+/iNOS^+$ M1 macrophages, $CD68^+/CD206^+$ M2 macrophages, M1/M2 ratio between the different BRONJ stages and controls ($p < 0.001$, $p = 0.032$, $p < 0.001$) (Table 7).

Pairwise comparison of macrophage distribution across BRONJ stages and controls is shown in Table 8. The results showed a statistically significant higher M1 macrophage density in: i) stage 2 compared to control group receiving bisphosphonates ($p = 0.004$), ii) stage 2 compared to control group not receiving bisphosphonates ($p = 0.004$). Pairwise comparison of M2 macrophage distribution across BRONJ stages and controls revealed statistically significant difference between stage 2 and stage 1 ($p = 0.024$). Pairwise comparison of M1/M2 distribution across BRONJ stages and controls showed a statistically significant higher M1/M2 ratio in: i) stage 2 compared to control group receiving bisphosphonates ($p = 0.018$), and ii) stage 2 compared to control group not receiving bisphosphonates ($p = 0.005$).

Table 7

Comparison of M1 and M2 macrophage density across BRONJ staging and controls

Variable	M1[a] median	M2[b] median	M1/M2 median
BRONJ stage 1	13	23	0.608
BRONJ stage 2	31	12	2.416
BRONJ stage 3	22	14	1.538
Control antiresorptive	4	26	0.154
Control no antiresorptive	3.5	13.5	0.193
p-value[c]	0.001**	0.032*	0.001**

[a]$CD68^+/iNOS^+$ M1 macrophage density (cells/mm^2).

[b]$CD68^+/CD206^+$ M2 macrophage density (cells/mm^2).

[c]Kruskal-Wallis H test.

*p <0.05, **p <0.01

BRONJ=Bisphosphonate-Related Osteonecrosis of the Jaw.

Table 8

Comparison of M1 and M2 macrophage density across BRONJ staging and controls pairwise

Pairwise comparison	M1[a] p-value[c]	M2[b] p-value	M1/M2 p-value
Control Bisphosphonates vs. Control_no Bisphosphonates	1.000	1.000	1.000
Control Bisphosphonates vs. Stage 1	0.554	1.000	1.000
Control Bisphosphonates vs. Stage 3	0.098	1.000	0.254
Control Bisphosphonates vs. Stage 2	0.004**	0.552	0.018*
Control no Bisphosphonates vs. Stage 1	0.527	0.428	1.000
Control no Bisphosphonates vs. Stage 3	0.092	1.000	0.179
Control no Bisphosphonates vs. Stage 2	0.004**	1.000	0.005**
Stage 1 vs. Stage 3	1.000	0.347	1.000
Stage 1 vs. Stage 2	1.000	0.024*	0.134
Stage 3 vs. Stage 2	1.000	1.000	1.000

[a]$CD68^+/iNOS^+$ M1 macrophage density (cells/mm^2).

[b]$CD68^+/CD206^+$ M2 macrophage density (cells/mm^2).

[c]Kruskal-Wallis Test, pairwise comparison; p-values have been adjusted by the Bonferroni correction for multiple tests.

*p <0.05, **p <0.01

BRONJ=Bisphosphonate-Related Osteonecrosis of the Jaw.

3.5 Density of M1 and M2 macrophages across DRONJ stages and controls

M1 and M2 macrophage density was assessed in samples of patients with Denosumab-Related Osteonecrosis of the Jaw (DRONJ) and controls. The results demonstrated a statistically significant difference in densities of $CD68^+/iNOS^+$ M1 macrophages, $CD68^+/CD206^+$ M2 macrophages, M1/M2 ratio between the different DRONJ stages and controls ($p = 0.001$, $p = 0.020$, $p = 0.002$) (Table 9).

Pairwise comparison of macrophage distribution across DRONJ stages and controls is shown in Table 10. The results showed a statistically significant higher M1 macrophage density in: i) stage 3 compared to control group receiving denosumab ($p = 0.009$), ii) stage 2 compared to control group receiving denosumab ($p = 0.024$). Pairwise comparison of M2 macrophage distribution across DRONJ stages and controls revealed no statistically significant difference ($p > 0.05$). Pairwise comparison of M1/M2 distribution across MRONJ stages and controls showed a statistically significant higher M1/M2 ratio in: i) stage 3 compared to control group receiving denosumab ($p = 0.045$), ii) stage 3 compared to control group not receiving denosumab ($p = 0.024$), and iii) stage 2 compared to control group not receiving denosumab ($p = 0.032$).

Table 9

Comparison of M1 and M2 macrophage density across DRONJ staging and controls

Variable	M1[a] median	M2[b] median	M1/M2 median
DRONJ stage 1	8	14	0.461
DRONJ stage 2	16	7	2.000
DRONJ stage 3	15	10	1.818
Control antiresorptive	3	8	0.300
Control no antiresorptive	3.5	13.5	0.193
p-value[c]	0.001**	0.02*	0.002**

[a]$CD68^+/iNOS^+$ M1 macrophage density (cells/mm^2).

[b]$CD68^+/CD206^+$ M2 macrophage density (cells/mm^2).

[c]Kruskal-Wallis H test.

*p <0.05, **p <0.01

DRONJ=Denosumab-Related Osteonecrosis of the Jaw.

Table 10

Comparison of macrophage density across DRONJ staging and controls pairwise

Pairwise comparison	M1[a] p-value[c]	M2[b] p-value	M1/M2 p-value
Control Denosumab vs. Control_no Denosumab	1.000	0.198	1.000
Control Denosumab vs. Stage 1	0.167	0.345	1.000
Control Denosumab vs. Stage 3	0.009**	1.000	0.045*
Control Denosumab vs. Stage 2	0.024*	1.000	0.059
Control no Denosumab vs. Stage 1	0.771	1.000	1.000
Control no Denosumab vs. Stage 3	0.070	0.263	0.024*
Control no Denosumab vs. Stage 2	0.158	0.187	0.032*
Stage 1 vs. Stage 3	1.000	0.448	1.000
Stage 1 vs. Stage 2	1.000	0.327	1.000
Stage 3 vs. Stage 2	1.000	1.000	1.000

[a]$CD68^+/iNOS^+$ M1 macrophage density (cells/mm^2).

[b]$CD68^+/CD206^+$ M2 macrophage density (cells/mm^2).

[c]Kruskal-Wallis Test, pairwise comparison; p-values have been adjusted by the Bonferroni correction for multiple tests.

*p <0.05, **p <0.01

DRONJ=Denosumab-Related Osteonecrosis of the Jaw.

3.6 Expression of IL-6 and IL-10 across MRONJ stages and controls

Figure 6 a–b shows representative examples of IL-6 and IL-10 immunostaining. The mean percentages of positive IL-6 and IL-10 staining across patients with MRONJ stages 1–3 and controls are presented in Figure 7. There was a statistically significant difference in the mean percentages of positive IL-6 staining across MRONJ stages and controls ($F(4,40) = 32.244$, $p < 0.001$; one-way ANOVA), with a mean percentage of positive IL-6 expression of 3.25 for stage 1, 7.71 for stage 2, 8.50 for stage 3, 1.47 for control group receiving antiresorptive therapy and 1.24 for control group not receiving antiresorptive therapy (Table 11). Pairwise comparison of IL-6 expression across MRONJ stages and controls showed a statistically significant higher IL-6 expression in: i) stage 2 compared to both control groups (antiresorptives: $p < 0.001$, no antiresorptives: $p < 0.001$), ii) stage 3 compared to both control groups (antiresorptives: $p < 0.001$, no antiresorptives: $p < 0.001$), and iii) stages 2 and 3 compared to stage 1 (both $p < 0.001$) (Table 12).

The analysis showed a statistically significant difference in the mean percentages of positive IL-10 staining among MRONJ stages and controls ($F(4,40) = 37.975$, $p < 0.001$; one-way ANOVA), with a mean percentage of positive IL-10 expression of 5.29 for stage 1, 1.73 for stage 2, 1.06 for stage 3, 2.14 for control group receiving antiresorptive therapy and 1.77 for control group not receiving antiresorptive therapy (Table 11). Pairwise comparison of IL-10 expression across MRONJ stages and controls demonstrated a statistically significant higher IL-10 expression in: i) stage 1 compared to both control groups (antiresorptives: $p < 0.001$, no antiresorptives: $p < 0.001$) and ii) stage 1 compared to stages 2 and 3 (both $p < 0.001$)(Table12).

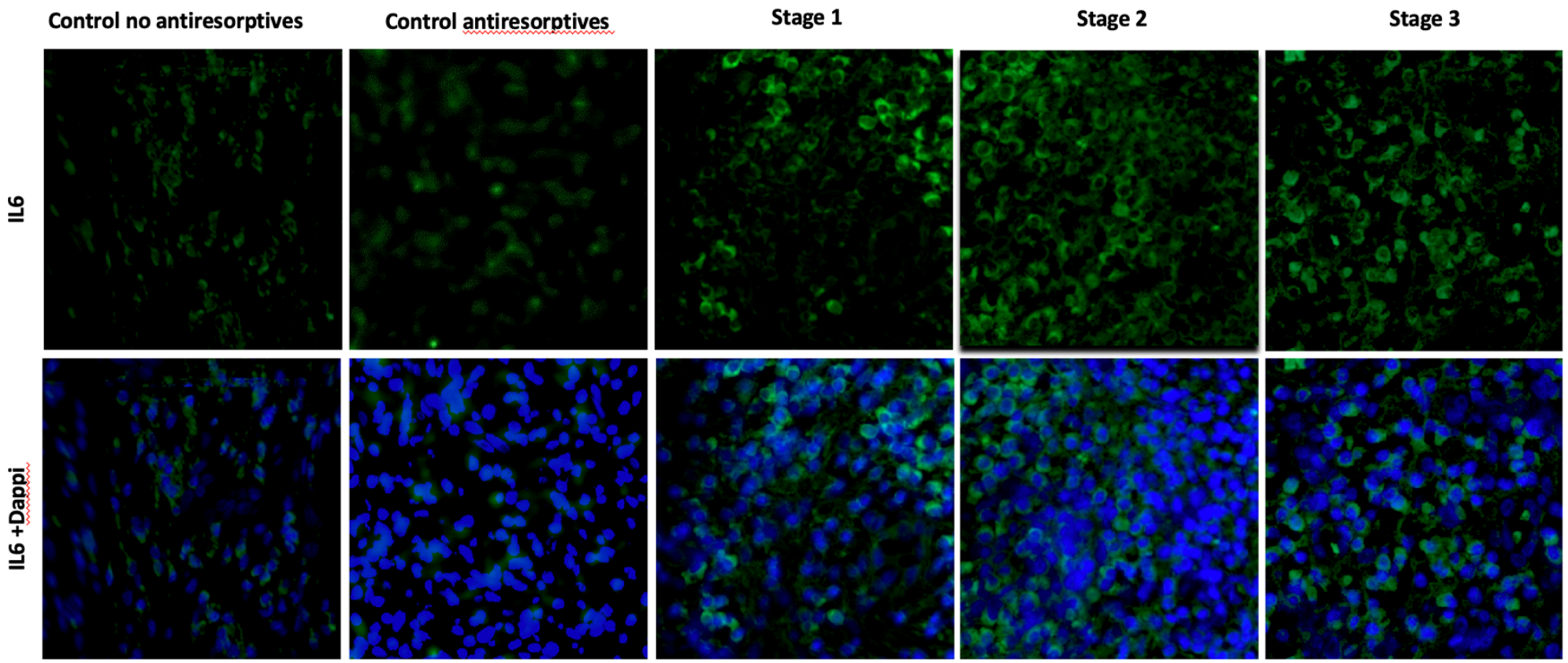

Figure 7A. Representative case of IL-6 immunofluorescence staining (x40). There was a statistically significant difference in the mean percentages of IL-6 expression among different MRONJ stages and controls $p < 0.001$. (IL6 :FITC)

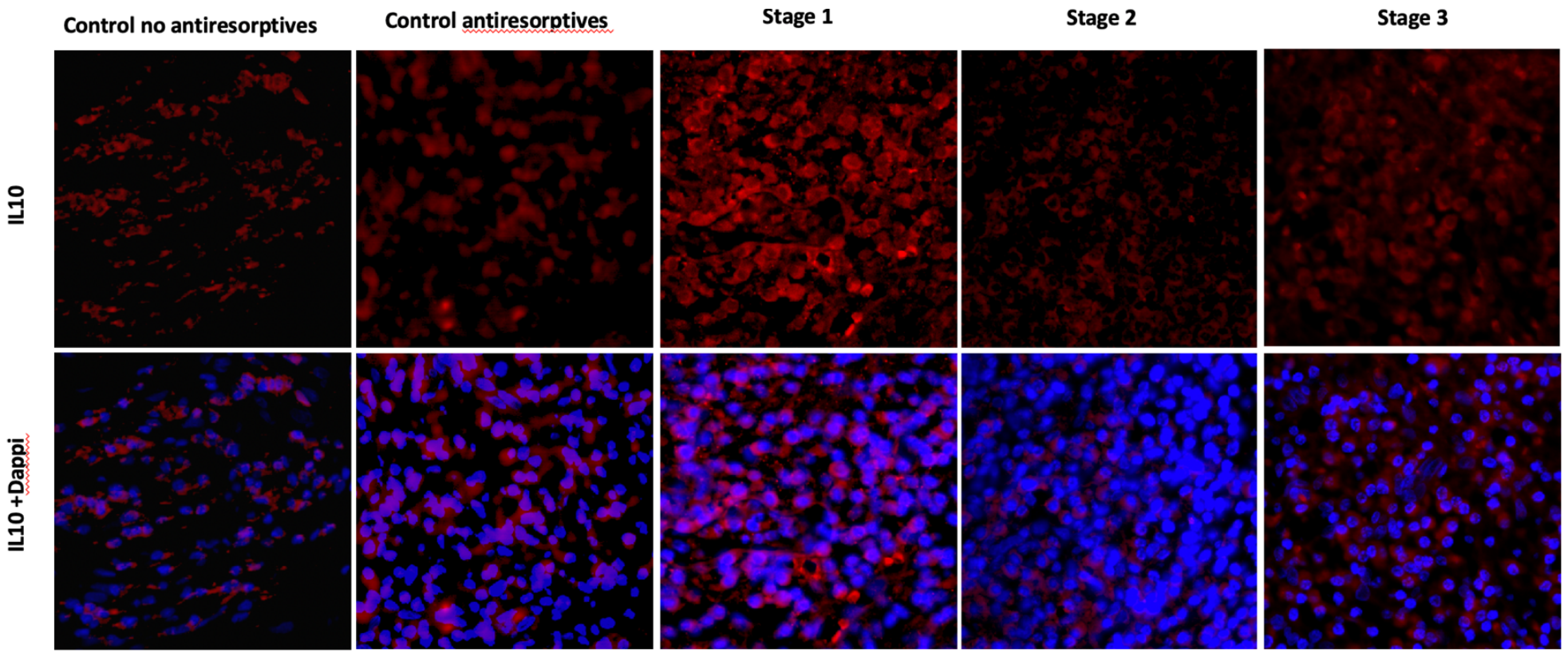

Figure 7B. Representative case of IL10 immunofluorescence staining (x40). There was a statistically significant difference in the mean percentages of IL-10 expression among different MRONJ stages and controls p <0.001. (IL10 :AF568)

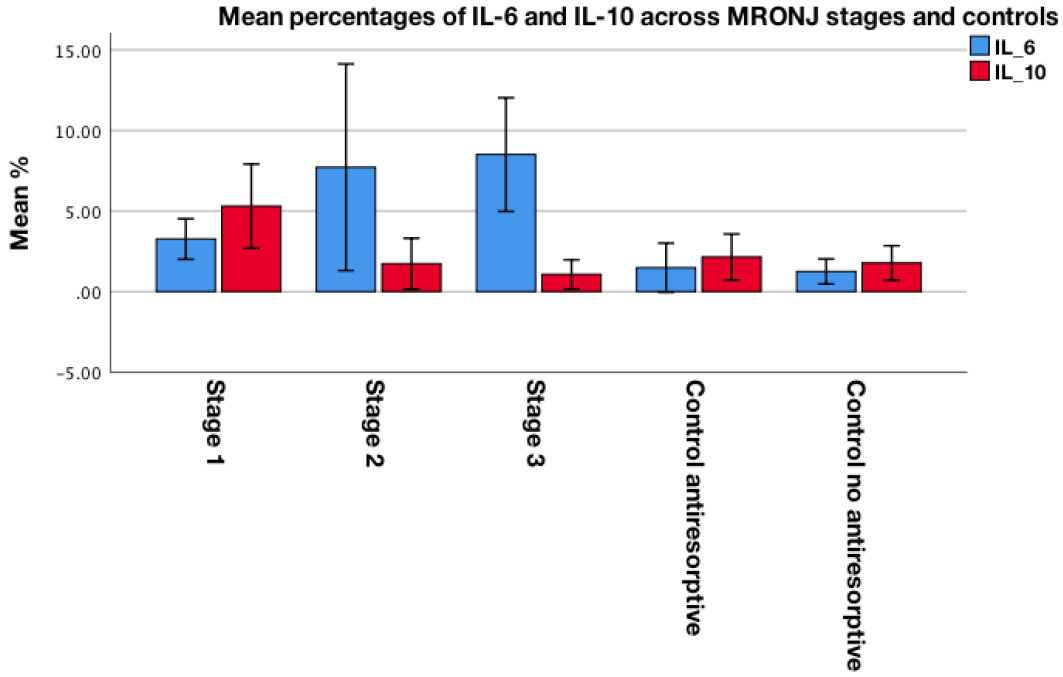

Figure 7. Clustered bar for IL-6 and IL-10 across MRONJ stages and controls

Table 11

Comparison of mean percentages of positive IL-6 and IL-10 staining across MRONJ stages and controls

Variable	IL-6 mean % (SD)	IL-10 mean % (SD)
MRONJ stage 1	3.25 (0.62)	5.29 (1.31)
MRONJ stage 2	7.71 (3.21)	1.73 (0.78)
MRONJ stage 3	8.5 (1.76)	1.06 (0.45)
Control–antiresorptive	1.47 (0.76)	2.14 (0.71)
Control–no antiresorptive	1.24 (0.38)	1.77 (0.53)
p-value[a]	<0.001**	0.027*

[a]One-Way ANOVA.

*p <0.05, **p <0.01

MRONJ=Medication-Related Osteonecrosis of the Jaw; SD=Standard Deviation.

Table 12

Comparison of IL-6 and IL-10 expression across MRONJ stages and controls pairwise

Pairwise comparison	IL-6 p-value[a]	IL-10 p-value
Control antiresorptive vs. Control_no antiresorptive	1.000	0.930
Control antiresorptive vs. Stage 1	0.198	<0.001**
Control antiresorptive vs. Stage 3	<0.001**	0.050
Control antiresorptive vs. Stage 2	<0.001**	0.810
Control no antiresorptive vs. Stage 1	0.266	<0.001**
Control no antiresorptive vs. Stage 3	<0.001**	0.548
Control no antiresorptive vs. Stage 2	<0.001**	1.000
Stage 1 vs. Stage 3	<0.001**	<0.001**
Stage 1 vs. Stage 2	<0.001**	<0.001**
Stage 3 vs. Stage 2	0.864	0.410

[a]p-values have been calculated by Tukey's post-hoc multiple comparison test.

*p <0.05, **p <0.01

3.7 Expression of IL-6 and IL-10 across BRONJ stages and controls

In subgroup analyses, expression of IL-6 and IL-10 was assessed in samples of patients with BRONJ and controls. One-Way ANOVA showed that there was a statistically significant difference in the mean percentages of IL-6 expression among different BRONJ stages and controls ($F(4,20) = 44.598$, $p < 0.001$), with a mean percentage of IL-6 expression of 3.50 for stage 1, 10.29 for stage 2, 9.45 for stage 3, 1.89 for control group receiving antiresorptive therapy, and 1.24 for control group not receiving antiresorptive therapy (Table 13).

The results showed a statistically significant difference in the mean percentages of IL-10 expression among different BRONJ stages and controls ($F(4,40) = 47.292$, $p < 0.001$), with a mean percentage of IL-10 expression of 6.16 for stage 1, 1.83 for stage 2, 0.87 for stage 3, 2.61 for control group receiving antiresorptive therapy, and 1.77 for control group not receiving antiresorptive therapy (Table 13).

Table 13

Comparison of IL-6 and IL-10 expression across BRONJ staging and controls.

Variable	IL-6 (%) mean	IL-10 (%) mean
BRONJ stage 1	3.50	6.16
BRONJ stage 2	10.29	1.83
BRONJ stage 3	9.45	0.87
Control antiresorptive	1.89	2.61
Control no antiresorptive	1.24	1.77
p-value	**<0.001**	**<0.001**

One-Way ANOVA. Significant p-values are presented bold.

BRONJ=Bisphosphonade-Related Osteonecrosis of the Jaw.

3.8 Expression of IL-6 and IL-10 across DRONJ stages and controls

In subgroup analyses, expression of IL-6 and IL-10 was evaluated in samples of patients with DRONJ and controls. There was a statistically significant difference in the mean percentages of IL-6 expression among DRONJ stages and controls ($F(4,20) = 67.005$, $p < 0.001$), with a mean percentage of IL-6 expression of 3.01 for stage 1, 5.13 for stage 2, 7.54 for stage 3, 1.06 for control group receiving antiresorptive therapy, and 1.24 for control group not receiving antiresorptive therapy (Table 14).

The results showed a statistically significant difference in the mean percentages of IL-10 expression among different DRONJ stages and controls ($F(4,20) = 15.592$, $p < 0.001$), with a mean percentage of IL-10 expression of 4.43 for stage 1, 1.62 for stage 2, 1.25 for stage 3, 1.67 for control group receiving antiresorptive therapy, and 1.77 for control group not receiving antiresorptive therapy (Table 14).

Table 14

Comparison of IL-6 and IL-10 expression across DRONJ staging and controls.

Variable	IL-6 (%) mean	IL-10 (%) mean
DRONJ stage 1	3.01	4.43
DRONJ stage 2	5.13	1.62
DRONJ stage 3	7.54	1.25
Control antiresorptive	1.06	1.67
Control no antiresorptive	1.24	1.77
p-value	**<0.001**	**<0.001**

One-Way ANOVA. Significant p-values are presented bold.

MRONJ=Medication-Related Osteonecrosis of the Jaw.

Discussion

In this study, we show that the M1–M2 macrophage polarization status in mucosal tissues adjacent to necrotic bone correlates with progression of MRONJ as manifested by clinical stage. Our data suggest that early stage MRONJ patients without clinical evidence of infection show a shift predominantly toward M2-polarized macrophages, as indicated by the higher density of $CD68^+/CD206^+$ M2 macrophages and the decreased M1/M2 ratio compared to patients with advanced stage, as well as the significant overexpression of IL-10 compared to patients with advanced stage and controls. In contrast, late stage MRONJ patients who developed clinical infection demonstrate a switch primarly toward M1-polarized macrophages, as revealed by the significantly higher density of $CD68^+/iNOS^+$ M1 macrophages, the increased M1/M2 ratio and the upregulation of IL-6 expression compared to controls. Furthermore, our results show a significantly higher density of both M1 and M2 phenotypes in MRONJ patients undergoing therapy with either zoledronic acid or alendronate compared to those receiving denosumab.

It is well-established that in response to changes in the local microenvironment, macrophages can be differentiated from monocyte precursors and polarized toward classically activated M1 or alternatively activated M2 macrophages (Geissmann, Manz et al. 2010; Biswas and Mantovani 2012; Mantovani and Locati 2013). The molecular networks orchestrating M1–M2 macrophage reprogramming are yet not fully understood and include signaling pathways, such as toll-like receptors (TLR)/nuclear factor-κB (NF-κB), peroxisome proliferator-activated receptor-γ (PPAR-γ)/NF-κB and janus kinase (JAK)/signal transducers and activators of transcription (STAT), and post-transcriptional regulation by microRNAs (miRNAs) (Wang, Liang et al. 2014). MRONJ is a multi-step disease progressing over time from an early phase characterized

by exposed necrotic bone without clinical signs of infection to a late phase with diverse manifestations of infection (Ruggiero, Dodson et al. 2014). Given this background, we sought to investigate the relationship between macrophage polarization status in samples of patients with MRONJ and disease progression as determined by clinical stage. Our results suggest that early stage 1 MRONJ patients demonstrate a shift of macrophage polarization primarly toward the M2 population, as evidenced by the increased density of $CD68^+/CD206^+$ M2 macrophages. Although a higher $CD68^+/iNOS^+$ M1 macrophage density was also observed in stage 1 compared to controls, this difference did not reach statistical significance ($p = 0.069$). Nevertheless, a M1/M2 ratio <1 was found, suggesting relatively more M2- than M1-polarized macrophages in early phase of MRONJ. Considering that M2-polarized macrophages produce anti-inflammatory cytokines that are associated with tissue repair and homeostasis regulation, we next investigated the expression of IL-10 in tissues of MRONJ patients with early and late stages of disease. IL-10 is an anti-inflammatory cytokine that plays a central role in the regulation of immune responses by suppressing the production of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1, and IL-6, and downregulating the expression of major histocompatibility complex (MHC) class II on macrophage surface, thereby suppressing the ability of activated macrophages to stimulate antigen-specific $CD4^+$ T cells (Ip, Hoshi et al. 2017). IL-10 also has an inhibitory effect on osteoclastogenesis, directly by suppressing osteoclast formation and indirectly by upregulating the expression of osteoprotegerin (OPG) and downregulating the expression of RANKL (Evans and Fox 2007). Our data showed a significant upregulation of IL-10 in MRONJ patients with stage 1 compared to patients with advanced stages and controls.

Our analysis indicates that MRONJ patients with advanced stage show a shift of macrophage polarization toward the M1 phenotype, as evidenced by the increased density of CD68+/iNOS+ M1 macrophages and the M1/M2 ratio >1 indicating comparatively more M1- than M2-polarized macrophages. Macrophage transition toward the M1 population in late MRONJ stages may be facilitated by the positive modulatory effects of antiresorptive agents on proinflammatory signaling pathways or induced by the progression of bacterial infection in oral tissues (Russmueller, Seemann et al. 2016; Wehrhan, Moebius et al. 2017; Kaneko, Okinaga et al. 2018). Zhang et al. reported that zoledronic acid mediates enhanced expression of IL-17, which in turn promotes IFN-γ–induced M1 polarization in the mucosal tissues bordering extraction sockets of BRONJ patients (Zhang, Atsuta et al. 2013). Recent evidence suggests that bacterial infection of the oral mucosa, periodontium or alveolar bone may play a central role in the development and progression of MRONJ (Russmueller, Seemann et al. 2016; Morita, Iwasaki et al. 2017). Pathogen-associated molecular pattern molecules (PAMPs), such as LPS, interact with IFN-γ to switch macrophages toward the M1 phenotype. Given that M1-polarized macrophages secrete proinflammatory cytokines, we subsequently assessed the expression of IL-6 in samples of MRONJ patients with early and advanced stages of disease. IL-6 is a pleiotropic cytokine that plays a critical role in the regulation of immune and proinflammatory responses and is involved in organ development and modulation of metabolism (Garbers, Heink et al. 2018). Il-6 is also actively involved in osteoclastogenesis by inducing osteoblasts to increase the expression of RANKL, a mediator of osteoclast formation and differentiation, thus leading to excessive bone resorption (Kang, Tanaka et al. 2019). Our results suggest that MRONJ patients with advanced stages show a significant overexpression of IL-6 compared to early stage 1 patients and controls, which may further exacerbate oral

inflammation and impair bone tissue homeostasis, thus contributing to the progression of MRONJ.

Accumulating evidence indicates that in response to stimuli from the local microenvironment, macrophages show substantial plasticity and are capable of polarization changes from the M1- to the M2-phenotype and vice versa (Galli, Borregaard et al. 2011; Wang, Liang et al. 2014). Our analysis suggests that the M1–M2 macrophage polarization status is associated with clinical staging and may determine progression of MRONJ. Thus, inhibition of the proinflammatory M1 phenotype and suppression of the IL-6, IL-1β and TNF-α signaling pathways might be beneficial strategies for patient with advanced stages of MRONJ. Furthermore, MRONJ patients who received antiresorptive treatment for benign diseases might benefit from modulatory agents inducing macrophage reprogramming to the anti-inflammatory M2 phenotype, including peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists, Vitamin D and statins (Zhang, Shao et al. 2017; Wasnik, Rundle et al. 2018; Yao, Liu et al. 2018).

Evidence from in vitro studies and animal models suggests that bisphosphonates induce macrophage polarization toward the M1 phenotype (Zhang, Atsuta et al. 2013; Kaneko, Okinaga et al. 2018). Zhu et al. reported that zoledronic acid administration increases TLR-4 expression, which leads to activation of the NF-κB pathway, and subsequently enhanced M1 phenotype both in vitro and in vivo (Zhu, Xu et al. 2019). To date, no study has been conducted to assess the potential effects of denosumab on macrophage polarization. Nevertheless, we performed an exploratory subgroup analysis to investigate the relationship between M1–M2 macrophage phenotypes and progression of BRONJ and DRONJ. We found that patients with early stage BRONJ and DRONJ show a switch primarly toward M2-polarized macrophages, while

advanced stage BRONJ and DRONJ patients demonstrate a shift toward the M1 phenotype (Tables 7-10). The density, however, of both M1 and M2 populations was significantly enhanced in patients receiving bisphosphonates compared to those receiving denosumab.

This prospective study is limited by its relatively small sample size. However, our eligibility criteria were strict and the investigation was based on a formally approved study protocol that was exactly followed. Moreover, we tried to avoid bias resulting form cutoff point determination, therefore marker expression was considered as continuous variable.

Conclusions

To the best of our knowledge, this is the first study investigating the correlation between M1–M2 macrophage polarization in mucosal tissues surrounding necrotic bone and disease progression in patients with MRONJ who underwent treatment with bisphosphonates or denosumab. We demonstrate that early stage MRONJ patients without evidence of clinical infection show a switch toward the M2 phenotype, as indicated by the higher density of M2 macrophages, the decreased M1/M2 ratio and the significant upregulation of IL-10. We also reveal that late stage MRONJ patients with established infection show a shift toward M1-polarized macrophages, as implied by the higher density of M1 macrophages, the increased M1/M2 ratio and the significant overexpression of IL-6. Thus, therapeutic molecules targeting the inflammatory microenvironment via the regulation of either M1 or M2 macrophage polarization may represent a novel strategy for treatment of MRONJ. Well-designed prospective studies are warranted to validate our findings and widen our understanding of the M1–M2 paradigm of macrophage polarization in MRONJ.

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