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Fluorescence-guided bone resection: A histological analysis in medication-related osteonecrosis of the jaw



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ABSTRACT

Purpose: Surgical treatment of medication-related osteonecrosis of the jaw (MRONJ) consists of necrotic bone removal followed by dense mucosal closure. Fluorescence-guided surgery has become a promising tool to intraoperatively distinguish between healthy and necrotic bone. Until now, there has been a lack of histopathological studies correlating the intraoperative fluorescence situation to histopathological analyses of the respective bone areas in order to further validate this method.

Materials and methods: Histopathological sections from intraoperatively detected fluorescence- and non-fluorescence-labeled bone were analyzed detecting osteocyte and collagen content, RANK(L) and TRAP expression as well as proportion of immature bone regeneration. Samples were compared with viable-looking bone areas according to the intraoperative clinical situation.

Results: Staining revealed a significant decrease of osteocytes and collagen type-I fibers in necrotic, non-fluorescing areas compared to fluorescing bone (R/RGB [%]: 0.56 ± 0.38 (fluorescence positive) vs. 3.18 ± 2.22 (fluorescence negative), $p = 0.041$). Furthermore, the number of osteocytes was higher in fluorescing, clinically viable bone samples (cell/mm²: 151.26 ± 95.77 (fluorescence positive) vs. 0.56 ± 0.38 (fluorescence negative), $p = 0.028$). Additionally, the amount of immature bone was substantially increased in luminescent jaw bone (proportion of red [%]: 6.78 ± 7.00 (fluorescence positive) vs. 2.24 ± 1.36 (fluorescence negative), $p = 0.442$). RANK(L) and TRAP expression did not differ between the investigated areas, resembling a generalized decrease in osteocyte-osteoclast function all over the jaw (RANK(L) –positive cells per mm²: 8.97 ± 7.85 (fluorescence positive) vs. 7.76 ± 6.41 (fluorescence negative), $p = 0.793$; TRAP-positive cells per mm²: 0.36 ± 0.38 (fluorescence positive) vs. 0.33 ± 0.41 (fluorescence negative), $p = 0.887$).

Conclusion: Intraoperative fluorescence-guided surgery might be more precise in identifying and resecting the necrotic bone compared to previous indicators like bone bleeding, which could be useful to further improve surgical therapy in MRONJ patients.

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1. Introduction

Medication-related osteonecrosis of the jaw (MRONJ), primarily caused by anti-resorptive drugs such as bisphosphonates or

humanized monoclonal antibodies against RANK(L) (Denosumab), still poses a therapeutic challenge in daily clinical treatment (Ruggiero et al., 2014). During the last few years, numerous cases have been reported and presented in the literature, indicating an increasing number of affected patients (Allen and Ruggiero, 2014; Khan et al., 2015). Therapeutically, surgical treatment in MRONJ patients, irrespective of the selected approach, requires the removal of as much bone as necessary and as little as possible (Mucke et al., 2011; Ristow et al., 2017). Conservative surgery including sequestrectomy, local debridement accompanied by antibiotic therapy resulted in stable mucosal healing in 75% of

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patients. Furthermore, surgical bone resection extending to bleeding, viable bone margins, and total removal of necrotic bone followed by mucosal closure reached healing rates in terms of stable mucosal closure at a minimum of 3 months' follow-up in 85% of patients (Rupel et al., 2014).

Unfortunately, the intraoperative differentiation between necrotic and healthy bone, especially with regard to the extension of bony resection, is difficult to determine and depends on the surgeon's individual assessment. In this context, fluorescence-guided bone resection has become a potential tool in order to distinguish more objectively between necrotic and viable jaw bone intraoperatively. During the last few years, several cases of fluorescence-guided bone resection with tetracycline labeling in osteonecrosis of the jaw have been reported (Pautke et al., 2005; Pautke et al., 2006; Pautke et al., 2010; Otto et al., 2013; Assaf et al., 2014; Otto et al., 2016). The incorporation of calcium-binding fluorochromes, such as doxycycline, in bony tissue is dependent on active mineralization processes and sufficient blood supply. Therefore, doxycycline is predominantly incorporated in viable bone, showing a greenish fluorescence signal when illuminated with blue light. In contrast, necrotic bone presents a significantly lower fluorescence intensity (Fig. 1) (Pautke et al., 2006).

A prospective pilot study by Pautke et al. indicated the effectiveness of this surgical technique in a clinical setting (Pautke et al., 2011). However, an analysis correlating the fluorescence-labeled and non-fluorescence-labeled bone areas in the intraoperative situation with histopathological criteria for necrotic and healthy bone in order to further validate this promising operative tool is still missing. According to the literature, healthy compared to necrotic jaw bone is characterized by an increased number of osteocytes accompanied by areas of immature, newly created bone and a high content of collagen type-I fibers (Matsuura et al., 2014; Mitsimponas et al., 2014; Rentsch et al., 2014). As bone remodeling does not only depend on the number but also on the activity of osteoclasts, the presence of active, tartrate-resistant acid phosphatase (TRAP)-expressing osteoclasts is reported to be associated with bone turnover (Kim et al., 2016). With special regard to MRONJ-affected bone samples, the histological presence of RANK(L)-expressing osteocytes is also of great importance as bisphosphonates are known to decrease RANK(L)-positive osteocytes in necrotic jaw bone (Wehrhan et al., 2011; Nagaoka et al., 2015; Kim et al., 2016).

The aim of this analysis was to histologically compare the fluorescence-labeled and non-fluorescence-labeled bone areas in MRONJ patients with regard to pathomorphological characteristics for healthy and necrotic jaw bone. Additionally, we compared the fluorescence-guided situation with bone samples detected as clinically viable without fluorescence.

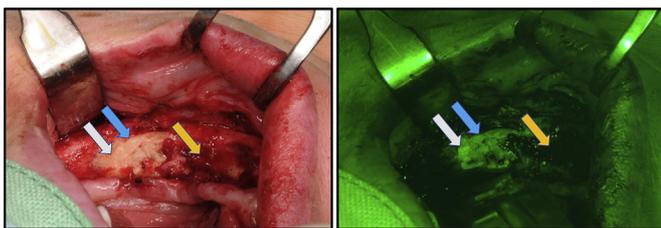


Fig. 1. Intraoperative situation presenting necrotic, non-fluorescing (orange arrow), fluorescing (grey arrow) and clinically viable-looking but non-fluorescing bone (blue arrow) in the corresponding views (left: without fluorescence detection, right: with VELscope system).

2. Materials and methods

2.1. Patients

Ten patients with MRONJ stage III were included in this analysis (Table 1). Patients presented to the Department of Oral and Maxillofacial Surgery, University Hospital Erlangen (Germany), with exposed necrotic bone over a period of more than 8 weeks. Written and oral informed consent was obtained from all patients prior to enrollment. Operative indication was posed according to current guidelines. Preoperatively, all patients received doxycycline p.o. 100 mg twice daily for 7 days to achieve adequate incorporation into mineralizing, vital bone. Intraoperatively, the VELscope system (VELscope, Atlanta, GA, USA) inducing doxycycline bone fluorescence by emitting blue light (400–460 nanometers [nm]) was applied under sterile conditions.

Operative procedure was performed according to standardized techniques: a mucoperiosteal flap was raised, exposing the necrotic bone area. After documenting bone fluorescence via VELscope, osteotomy was performed until clinical vital bone in terms of bleeding and unaltered color became visible. Bone fluorescence marked viable bone with a greenish fluorescence signal, whereas necrotic bone returned no signal as described in the literature (Pautke et al., 2011). During this procedure, the routinely taken histopathological biopsies were attributed to the respective fluorescing signals detected previously. Samples were categorized as fluorescing and non-fluorescing bone areas as well as according to localizations occurring clinically viable with no signal when using the VELscope tool. At the end of the operative treatment, sharp edges were removed and wound closure was performed without tension using resorbable material for interrupted sutures (Vicryl 5-0, Ethicon, Norderstedt, Germany) (Stockmann et al., 2010). Biopsy samples were routinely investigated in the local pathological department examination to exclude malignancy and used for this analysis subsequently (Ref. No. 2017-100).

Postoperatively, oral food intake was not allowed for 5 days while supplied with a naso-gastric tube, and antiseptic mouth rinses (CHX, Pfizer Pharma GmbH, Karlsruhe, Germany) were performed three times a day. Ampicillin 1.0 g with Sulbactam 0.5 g three times a day was administered during the postoperative course intravenously starting from the day of surgery.

2.2. Histopathological analysis

The formalin-fixed, decalcified, paraffin-embedded tissue samples were sliced in sections of 2 μ m thickness via rotation microtome (Leica, Nussloch, Germany). Then the sections were processed by dewaxing in xylene and rehydrating in graded alcohol before immunohistochemical staining.

For the anti-RANK(L) staining process, we used the LSAB (labeled-streptavidin-biotin) method in an automated staining device (Autostainer plus, Dako Cytomation, Hamburg, Germany) according to the manufacturer's protocol. Specific anti-RANKL antibody 1:50 (ab9957, abcam, Cambridge, UK) was used as primary antibody, incubating the sections in the autostainer (21 °C, 30 minutes). Biotinylated immunoglobulins were applied for all sections. Finally, they were processed in hematoxylin (Dako Cytomation) for counterstaining the nucleus. Each immunohistochemical staining was done with positive as well as negative controls. Staining for detection of tartrate-resistant acid phosphatase (TRAP) was performed on dewaxed sections according to the manufacturer's protocol (MK 300 TRAP, Takara, CA, USA). Briefly, sections were incubated with 200 μ l TRAP solution for 5 hours at room temperature; counterstaining was conducted with Hemalaun solution, incubated for 5 minutes.

Table 1

Patients included in this study, with their age, gender, the applied bisphosphonate due to the original diagnosis and the site of treated jaw necrosis.

Patient no.	Age (yr)	Gender	Drug	Initial Diagnosis	Location
1	73	m	Zoledronate	Prostate cancer	right maxilla
2	82	m	Zoledronate	Prostate cancer	right maxilla
3	83	m	Zoledronate	Prostate cancer	right maxilla
4	75	f	Zoledronate	Breast cancer	left maxilla
5	74	m	Zoledronate	Lung cancer	right mandible
6	61	f	Zoledronate	Breast cancer	left mandible
7	72	m	Zoledronate	Prostate cancer	left mandible
8	69	m	Zoledronate	Multiple myeloma	right mandible
9	57	m	Zoledronate	Prostate cancer	left mandible
10	55	m	Zoledronate	Prostate cancer	left mandible

Cell count for osteocytes situated in bony lacunae was performed by randomly choosing different regions of interest (ROI) and identifying the cells via hematoxylin and eosin (H&E) staining, conducted according to the manufacturer's protocol (GHS116, Sigma–Aldrich, Germany).

All samples used in this study were scanned and digitalized completely by using the method of whole slide imaging. All scanned samples were virtually microscopied on PC (Pannoramic MIRAX viewer, Zeiss, Jena, Germany). Quality controls were performed with a bright-field microscope (Zeiss Axioskop and AxioCam 5, magnification: 5–400 ×). Three visual fields per section at magnification of 200 × were selected for each sample. The visual fields were imported into Optimas 6.5 (Media Cybernetics, Rockville, MD, USA) to perform cell counting by three independent observers. Quantitative analysis for RANK(L)-expressing osteocytes situated in bony lacunae and TRAP-expressing osteoclasts localized on the bone surface as well as for the total number of osteocytes (H&E staining) was performed by comparing the number of positive cells per area, sample and study group.

Collagen-I content was determined by Sirius red stainings in polarization microscopy as previously published (Mitsimponas et al., 2014). Briefly, tissue samples were processed and stained with a picric Sirius red working solution according to the manufacturer's protocol (abcam, Cambridge, UK). Slices were observed under a polarization microscope (Axioscope, Zeiss, Jena, Germany) detecting collagen type-I fibers in randomly chosen region of interest by measuring the intensity grade of red within the whole red, green, blue (RGB) spectrum. Quantification was performed by measuring red, green and blue intensity in every pixel included in the selected ROI. Collagen-I content was estimated by the ratio R to the arithmetic mean of RGB values.

Goldner's Masson trichrome staining was used to distinguish between mature bone matrix staining green and newly formed, immature bone presented in red in the selected jaw samples. Slices were processed and stained according to the manufacturer's protocol with Fuchsin (2 minutes), phosphomolybdic acid (7 minutes) and phosphotungstic acid (7 minutes) solution. Quantification was performed by determining the number of red pixels in the selected ROI under standardized conditions. The ratio of red pixels to total amount of pixels in the ROI was used for comparing the different groups.

2.3. Statistical analysis

Statistical testing was performed by using the Student *t*-test and analysis of variance (SPSS 22.0, IBM Inc., Armonk, NY, USA) comparing the following three groups: 1) clinically viable, 2) fluorescence positive, and 3) fluorescence negative. A *p*-value ≤ 0.05 were defined as significant. Results are expressed as mean \pm standard deviation.

3. Results

3.1. Fluorescence-labeled areas consist of significantly more osteocytes and collagen fibers

Investigations on osteocyte content revealed significantly more osteocytes per area in the doxycycline-fluorescence-labeled bone areas compared to the non-fluorescence areas ($p = 0.028$) (Fig. 2a–c). Furthermore, the number of osteocytes in bone areas well perfused and viable-looking was also substantially reduced ($p = 0.050$) compared to the fluorescence-labeled bone [osteocytes per mm^2 : 151.26 ± 95.77 (fluorescence positive) vs. 42.25 ± 32.21 (fluorescence negative) vs. 65.00 ± 30.22 (clinically viable)] (Fig. 3).

The amount of detected collagen type-I fibers in Sirius red stainings (Fig. 2g–j) was significantly elevated in fluorescence-positive bone areas compared to fluorescence-negative and clinically viable bone ($p = 0.041$). Comparing non-fluorescing and clinically viable areas, collagen content did not differ ($p = 0.216$) [R/RGB [%]: 3.18 ± 2.22 (fluorescence positive) vs. 0.56 ± 0.38 (fluorescence negative) vs. 1.23 ± 0.98 (clinically viable)] (Fig. 4).

Trichrome staining (Fig. 2d–f) revealed a higher amount of immature, unmineralized bone, resembling a higher degree of bone turnover in fluorescence-positive bone compared to the other two investigated areas ($p = 0.442$) [proportion of red [%]: 6.78 ± 7.00 (fluorescence positive) vs. 2.24 ± 1.36 (fluorescence negative) vs. 5.55 ± 9.09 (clinically viable)] (Fig. 5).

3.2. RANK(L) and TRAP expression did not significantly differ between the investigated groups

RANK(L) expression (Fig. 2j–l) in fluorescence-labeled, viable bone was unaltered compared to fluorescence-negative bone areas ($p = 0.793$). Furthermore, bone samples taken from areas presenting with clinically adequate perfusion turned out to have comparable RANK(L) expression levels compared to the other groups and showing no statistical differences between the groups ($p = 0.996$ and $p = 0.740$) [RANK(L)-positive cells per mm^2 : 8.97 ± 7.85 (fluorescence positive) vs. 7.76 ± 6.41 (fluorescence negative) vs. 7.74 ± 8.00 (clinically viable)] (Fig. 6).

Likewise, TRAP expression (Fig. 2m–o) analysis did not show significant differences between the investigated areas ($p = 0.887$ and $p = 0.424$) [TRAP-positive cells per mm^2 : 0.36 ± 0.38 (fluorescence positive) vs. 0.33 ± 0.41 (fluorescence negative) vs. 0.54 ± 0.50 (clinically viable)] (Fig. 7).

Doxycycline fluorescence of the investigated bone areas was also confirmed under the laboratory microscope, illuminating the bone section with 485 nm light and detecting the emission under a 514-nm filter (Fig. 8).

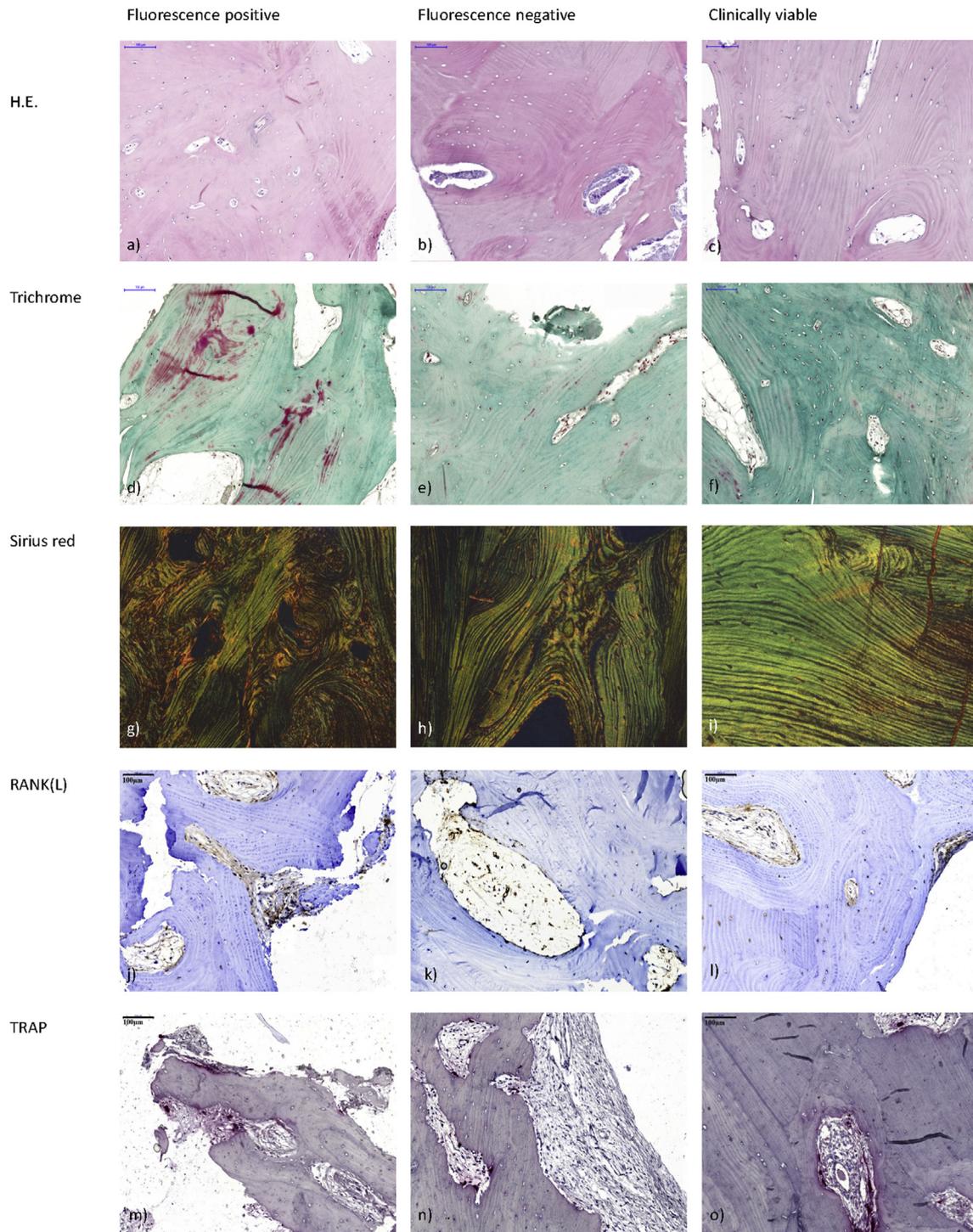


Fig. 2. Histochemical stainings with hematoxylin and eosin. (a–c) Goldner's Masson trichrome (Trichrome). (d–f) Sirius red in polarization microscopy (g–i) and tartrate-resistant acid phosphatase (TRAP) (m–o) as well as immunohistochemical sections visualizing receptor activator of NF- κ B ligand (RANK(L) (j–l) in jaw bone detected as fluorescence positive, negative or clinically viable.

4. Discussion

There is an ongoing debate regarding the treatment options, namely, conservative vs. surgical, in cases of MRONJ. As the number of diagnosed osteonecroses of the jaw (ONJ) have increased over recent years, successful therapeutic options, especially with regard to operative bony resections in cases of extended necrosis stage III, might become more important (Rupel et al., 2014).

Intraoperative fluorescence-guided bone resection in cases of ONJ represents a promising tool with regard to the identification and complete resection of necrotic lesions (Pautke et al., 2011). The possibility to landmark vital bone and therefore achieve a contrast to necrotic, avascularized bone, caused by different fluorescence emission due to different tetracycline uptake, has already been described by others (Olmedo-Garcia and Lopez-Prats, 2002). The clinical application of this technique in cranio-maxillo-facial

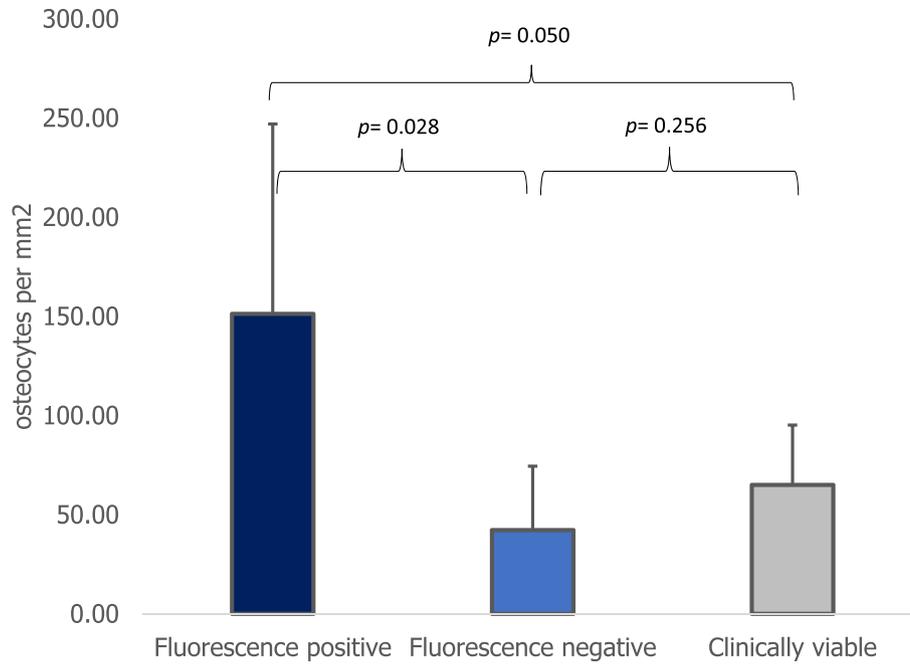


Fig. 3. Semiquantitative analysis of hematoxylin and eosin stainings showing significantly more osteocytes per mm² in fluorescence-positive jaw bone compared to fluorescence-negative ($p = 0.028$) and clinically viable areas ($p = 0.050$).

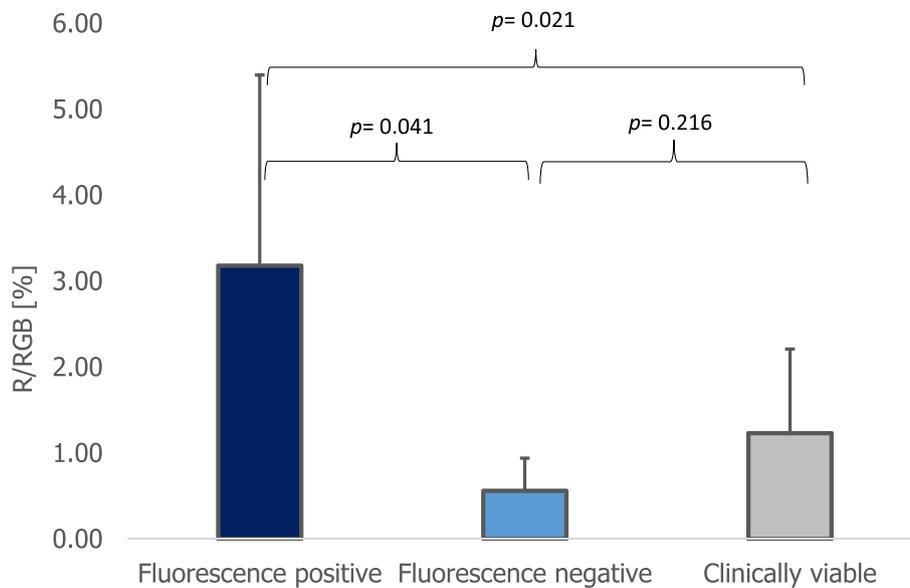


Fig. 4. Quantification of collagen type-I fiber content in polarized microscopy via R/RGB ratio [%] presenting a significantly higher amount of fibers in fluorescence-positive jaw bone compared to fluorescence-negative ($p = 0.041$) and clinically viable areas ($p = 0.021$).

surgery was initially published by Pautke et al. some years ago and was later verified by others (Pautke et al., 2006; Assaf et al., 2014). Although most authors assume a beneficial effect when applying fluorescence-guided surgery together with the clinical signs of vital bone (bleeding bone surface and homogenous bony structures), the clinical benefit still has to be further evaluated and investigated. This is the first study analyzing the intraoperative situation with the corresponding fluorescing and non-fluorescing as well as vital bone areas by immunohistochemical staining focusing on collagen content, osteocyte density as well as RANK(L) expression and osteoclast activity. Our results show a significantly increased level of detected osteocytes and collagen type-I fibers in fluorescing, vital

bone compared to non-fluorescing bone. Interestingly, there was also a significant difference compared to clinically viable areas, indicating that the doxycycline fluorescence signal might be of advantage to distinguish between nonvital and vital bone compared to the conventional methods.

On the other hand, RANK(L) and TRAP expression, resembling the osteocyte and osteoclast activity, did not show any substantial differences between the investigated locations, and the calculated labeling index of RANK(L) was even lower than previously published by our group (Wehrhan et al., 2011). At this stage, we assume that the unaltered levels of the investigated osteocyte and osteoclast markers might be caused by a generalized depressing

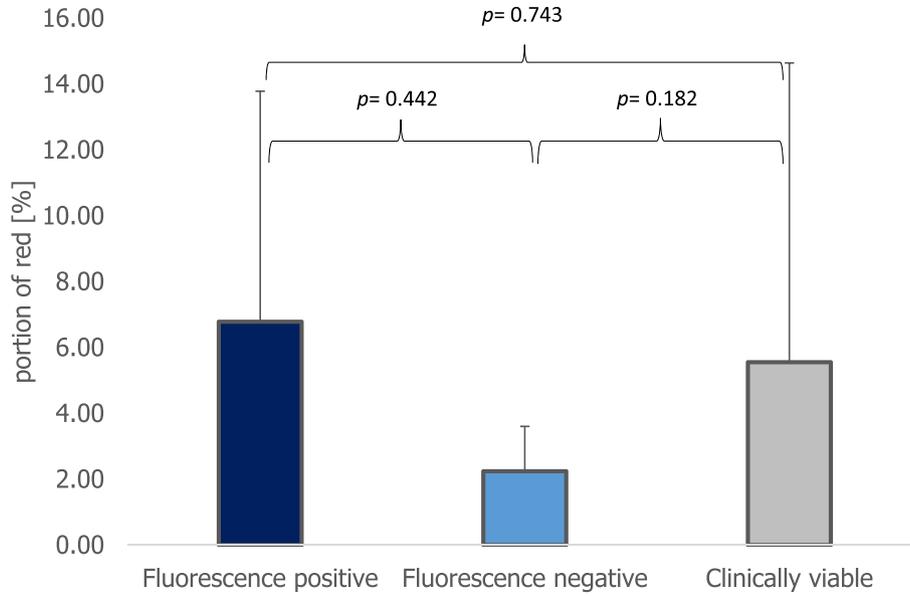


Fig. 5. Analysis of Goldner's Masson trichrome sections showing an increase in labeled in red, immature bone (proportion of red [%]) in fluorescence-positive jaw bone compared to fluorescence-negative ($p = 0.442$) and clinically viable areas ($p = 0.743$).

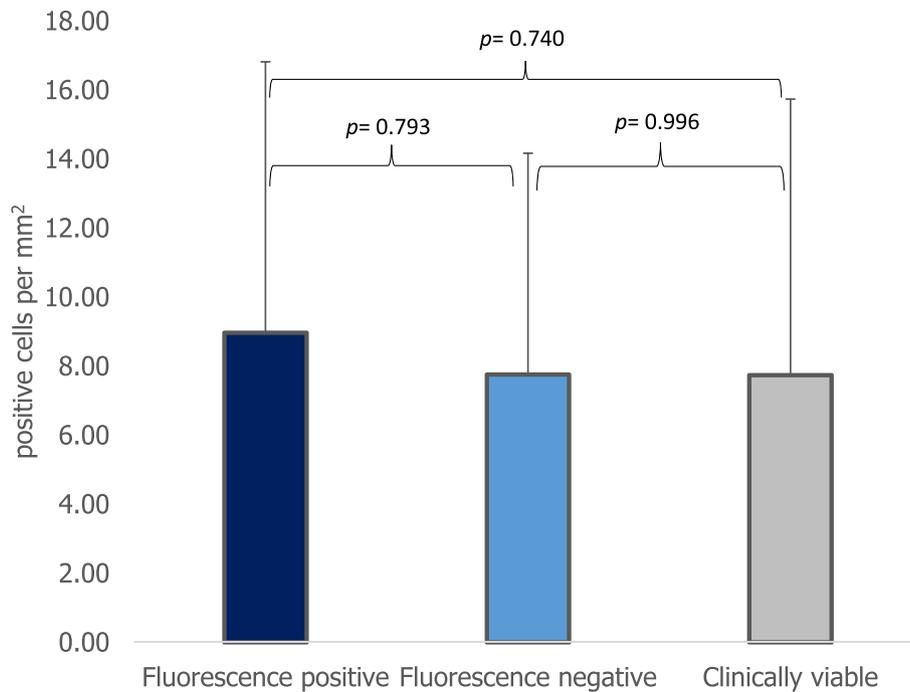


Fig. 6. Semiquantitative analysis of RANK(L) expression (positive cells per mm²) revealed unaltered expression levels between fluorescence-positive and fluorescence-negative jaw bone ($p = 0.793$). Clinically viable areas did not differ compared to those in the other two groups ($p = 0.996$ and $p = 0.740$).

influence of bisphosphonates on jaw bone viability, not only in distinct, necrotic areas.

Labeling fluorochromes in combination with spectral imaging, detecting bone mineralization and formation, has already been established in histological analyses in the past (Milch et al., 1958). Doxycycline, a safe and useful antibiotic applied for decades to treat numerous diseases, was found to be clinically useful in terms of detecting bone mineralization (Pautke et al., 2005). Blue light (wavelength: 390–425 nm) induces the emission of a greenish light with a maximum lying between 520 and 560 nm of the bony

incorporated doxycycline. Interestingly, to date there have been no clinical studies investigating the amount and duration of doxycycline application with regard to intraoperative fluorescence intensity. Published studies in the current literature mainly report oral doxycycline therapy with 100 mg once or twice a day for 7–10 days (Fleisher et al., 2008; Otto et al., 2013; Yoshiga et al., 2015). However, others observed intraoperative fluorescence already after one 100-mg doxycycline infusion 1 hour preoperatively (Assaf et al., 2014). This leads to the assumption that the fluorescing signal detected through the filter integrated in the technical

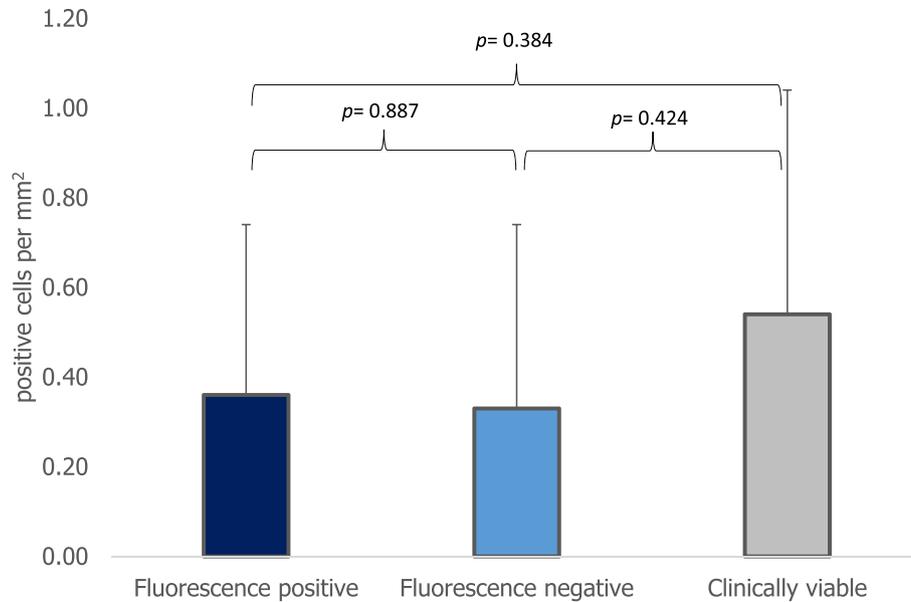


Fig. 7. Semiquantitative analysis of TRAP expression (positive cells per mm²) showed no significant changes in fluorescing versus non-fluorescing bone ($p = 0.887$) and clinically viable areas ($p = 0.384$).

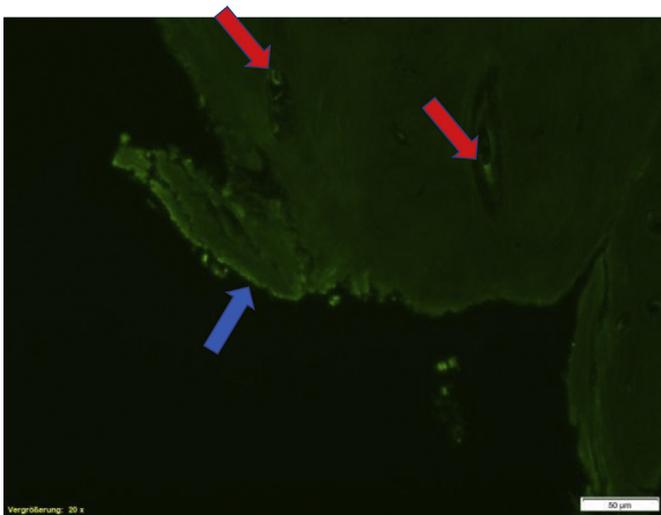


Fig. 8. Bone sample detected by doxycycline fluorescence signal showing luminous areas under the microscope when illuminated with light at a wavelength of 485 nm and observed with a 514-nm filter. Areas are located near osteocytes (red arrows) and at the surface (blue arrows).

systems used may also be caused by auto-fluorescence of the viable bone, presumably caused by the calcified extracellular matrix (Ristow and Pautke, 2014). A technical device frequently used in the context of intraoperative fluorescence-guided bone resection due to the doxycycline-specific spectrum is the VELscope system (Pautke et al., 2011). According to our results, this device distinguishes between necrotic, non-doxycycline-loaded areas and vital bone. However, larger cohorts have to be investigated in order to determine the sensitivity and specificity of this system before standard clinical use.

From a clinical perspective, this system allows only an intermittent rather than a continuous fluorescence detection during osteotomy due to the intraoperative handling with a handheld system and a small, monocular optic. Therefore, new technical devices fulfilling the physical requirements for emitting the

appropriate light and detecting doxycycline fluorescence with adequate filters, for example in an operative microscope, have to be developed in order to routinely and continuously monitor fluorescence signals in the oral cavity during osteotomy.

Another limitation of fluorescence-guided tissue resection is caused by a quick extinction of the fluorescence signal during operative intervention, for example by blood emerging from vital bone areas. Already a thin blood layer covering the bony surface decreased or even extinguished the emitted fluorescence signals. Considering the fact that viable bone is frequently characterized by active bleeding, this aspect might influence the extent of intraoperative resection and has to be taken into consideration during osteotomy and fluorescence detection.

5. Conclusion

Fluorescence-guided bone resection represents a promising and useful tool during the surgical treatment of MRONJ lesions. According to our histopathological findings, the doxycycline fluorescence signal might even be a more accurate and distinctive criterion to determine resection margins during osteotomy compared to conventional clinical indicators used today, such as bleeding bone areas. However, this tool has to be further investigated in larger clinical trials, and further technical developments are needed to make this concept better applicable.

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Author contributions

R.H.M.P., F.N. and F.W. designed the study, R.H.M.P., C.G. and M.W. collected the data, R.H.M.P., F.W., M.K. and F.N. drafted the article and all authors finally approved the version.

Conflicts of interest

The authors of this manuscript have no conflict of interest to disclose.

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