Oral Abundance of *Actinomyces* spp. in Breast Cancer Patients

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Keywords

Actinomyces spp. · Medication-related osteonecrosis of the jaw · Breast cancer

Abstract

Objectives: Pathophysiology of medication-related osteonecrosis of the jaw (MRONJ) is still unclear, and disease development is associated with adverse reaction of bisphosphonates and denosumab, and *Actinomyces* spp. as well. In this study, we evaluated the abundance of *Actinomyces* spp. in breast cancer patients undergoing chemotherapy compared to healthy controls. **Methods:** Oropharyngeal samples were collected from treatment-naïve early-stage breast cancer patients, who were scheduled for standard of care therapy (eight samples throughout chemotherapy, one prior to radiotherapy and one after a year of start), as well as from healthy controls at matched timepoints. We quantified *Actinomyces* spp. in the samples with a highly sensitive and specific quantitative polymerase chain reaction. **Results:** Twenty-one patients and 16 healthy subjects were enrolled. Forty-eight percent of patients suffered from estrogen receptor-positive/progesterone receptor-positive or -negative/human epidermal growth factor receptor 2 (HER2)-neg-

ative disease, 38% were HER2-positive, and 14% were triple-negative. Comparison of *Actinomyces* spp. loads in cancer patients and healthy controls did not reveal significant difference. Fluctuations on bacterial quantity were observed in both groups over time. Tumor receptor status or different chemotherapy schemes of patients were not correlated with a particular pattern on abundance of *Actinomyces* spp. **Conclusions:** We suggest that *Actinomyces* spp. are not the initiative factors in MRONJ development. These bacteria are not altered in abundance during chemotherapy, but they behave opportunistic when there is a bone disruption in the oropharynx in the first place caused by antiresorptive drugs or dental trauma and proliferate in their new niche. Thus, *Actinomyces* spp. plays a latter role in MRONJ development, rather than a primary causative one.

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Introduction

Medication-related osteonecrosis of the jaw (MRONJ) was first described in 2003, shortly after approval of intravenous treatment with bisphosphonates, in patients with malignant diseases [1]. Several case series and observa-

tional studies have revealed that development of MRONJ is associated with serious adverse drug reaction of bisphosphonates and the receptor activator of nuclear factor kappa B ligand inhibitor, denosumab [2]. Incidence of this severe debilitating condition ranges between 0.7 and 18% in cancer patients according to recent studies [2–5].

Bisphosphonates and denosumab are two different classes of antiresorptives that are prescribed as the most effective antiresorptive drugs to treat (malignant) hypercalcemia and to reduce the incidence of skeletal-related events in patients with bone metastases [6–9]. Despite the benefits of these drugs in oncology, they were identified as significant risk factors for MRONJ when given long term and in high dosage [10, 11]. Antiangiogenic drugs were associated with MRONJ development as well [12].

The pathophysiology of MRONJ cannot be fully explained by a single model. Main characteristics of MRONJ lesions are scattered areas of exposed or nonhealing necrotic bone in the maxillofacial region persisting for more than 8 weeks and is often accompanied by local infection of the necrotic bone [13, 14]. The unique localization of the jaw in MRONJ is explained by the higher susceptibility of jawbone to infections compared to other bones in the body, considering exposure to millions of bacteria in the oral cavity [15]. In case the mucosal barrier is breached due to trauma or surgical procedures during or after bisphosphonates or denosumab treatment, emerging infection causes lower pH in that area, which ultimately leads to suppression of the healing process and thereof bone necrosis [9, 16]. The most common precipitating factor for osteonecrosis is tooth extraction, suggested by 52–61% of MRONJ patients [15, 17].

In recent years, small case series and reviews suggested a link between evolution of MRONJ and *Actinomyces* species [18–22]. *Actinomyces* spp. were found in 89% and 82% of bone samples from MRONJ patients in two recent retrospective studies by Russmüller et al. [16] and Cerato et al. [23], respectively. Clinical relevance of the family of Actinomycetaceae is progressively recognized, and more than 40 species identified [24]. *Actinomyces* spp. are ubiquitous Gram-positive, facultative anaerobic (except *A. meyeri* and *A. israelii* that are obligate anaerobes), non-spore-forming bacteria, and they are commensals in the skin, oral, gut, and vaginal flora [16].

Oral microbiota is altered during chemotherapy in patients with cancer, and the alterations may lead to severe local or systemic pathologies [25]. Mucositis and oral candidiasis are common nonhematological complications of cancer chemotherapy, range from 40–80% to 7–52% in cancer patients, respectively [26, 27]. In a recent

study by Hong et al. [28], oral bacteriome disruption associated with mucositis during chemotherapy was investigated, and they represented a negative correlation between severity of mucositis and salivary bacterial abundance of *Streptococcus*, *Veillonella*, and *Actinomyces* species.

We revealed that the largest group within MRONJ patients consists of breast and prostate cancer patients, in a previous retrospective study [16]. Thereof we chose early-stage breast cancer patients, who were treatment-naïve and did not receive bisphosphonates or denosumab, and collected oropharyngeal samples from them for a year throughout their therapies. It was the aim of this study to determine the fluctuations of *Actinomyces* spp. quantity during chemotherapy and compare the abundance of *Actinomyces* spp. in early-stage breast cancer patients to healthy individuals at matched timepoints.

Materials and Methods

Patients and Samples

This study was conducted with a total of 37 female patients who were older than 18 years. Of the 37 patients, two groups were formed with 21 early-stage treatment-naïve breast cancer patients (mean age 51.7 ± 11 years) scheduled for neoadjuvant chemotherapy and 16 healthy controls (mean age 50.2 ± 12.5 years). The patients were excluded from the study if they (a) had a metastatic disease and/or received chemotherapy previously; (b) received antimicrobial drugs within 1 month prior to enrollment; (c) received bisphosphonates or denosumab, oral hormone contraceptives, and immunosuppressive treatments within 3 months prior to enrollment; (d) had invasive dental procedures within 3 months prior to enrollment; and (e) had known endocrine disorder requiring treatment.

Clinical data collection included patients' age, ECOG performance status, medical history, body mass index, clinical stage, histological subtype of carcinoma, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status of tumor, chemotherapy cycles administered, and adverse events during chemotherapy such as mucositis or oral candidiasis. Oropharyngeal lavage samples were collected at 10 timepoints from each patient. These were the days of each chemotherapy cycle with approximately 3 weeks of intervals (starting from day 1 and continues with cycles 2–8), prior to radiotherapy (pre-Radio; or on a matching day if the patient did not need radiotherapy), and at the end of a year (week 52) for early-stage breast cancer patients. Samples were collected from healthy individuals as well at matched timepoints. The samples were stored at -80°C until the microbial evaluation, and all samples from the same patient were thawed and processed further in the same batch.

Microbial Evaluation

An *Actinomyces* spp.-specific quantitative polymerase chain reaction (qPCR) was developed by using the DNA isolated from pure bacterial cultures of the six most abundant species of *Actino-*

Table 1. Clinical data of breast cancer patients enrolled in the study

Patient name	Age	BMI	Histological subtype	ER status	PR status	HER2 status	Neoadjuvant chemotherapy
BCA1	49	25.4	Ductal	Positive	Positive	3+	EC → DTP
BCA2	65	26.5	Ductal	Positive	Positive	3+	EC → DTP
BCA3	42	21.5	Ductal	Positive	Positive	3+	EC → DTP
BCA4	60	24.9	Ductal	Positive	Negative	3+	EC → DTP
BCA5	44	30.1	Ductal	Negative	Negative	3+	EC → DTP
BCA6	60	27.7	Ductal	Positive	Positive	3+	EC → DTP
BCA7	65	41.4	Ductal	Negative	Negative	3+	EC → DTP
BCA8	43	26.6	Ductal	Positive	Positive	Negative	EC → Dtx
BCA9	52	28.3	Ductal	Negative	Negative	Negative	EC → Dtx
BCA10	36	39.4	Ductal	Positive	Negative	Negative	EC → Dtx
BCA11	63	35.8	Ductal	Positive	Negative	Negative	EC → Dtx
BCA12	64	24.2	Ductal	Positive	Positive	Negative	EC → Dtx
BCA13	64	41.5	Ductal	Positive	Positive	Negative	EC → Dtx
BCA14	58	31.2	Ductal	Positive	Negative	Negative	EC → Dtx
BCA15	33	22.7	Ductal	Positive	Positive	Negative	EC → Dtx
BCA16	62	29.7	Ductal	Positive	Positive	Negative	EC → Dtx
BCA17	55	29.4	Ductal	Positive	Positive	Negative	EC → Dtx
BCA18	41	19.7	Ductal	Negative	Negative	Negative	Dtx → EC
BCA19	31	31.0	Ductal	Positive	Positive	Negative	Dtx → EC
BCA20	52	23.9	Ductal	Negative	Negative	Negative	Dtx → EC
BCA21	46	22.4	Ductal	Negative	Negative	3+	DTP → EC

Neoadjuvant chemotherapy was scheduled for 8 cycles (4 + 4). BMI, body mass index; ER, estrogen receptor; PR, progesterone receptor; EC, epirubicin + cyclophosphamide; DTP, docetaxel + trastuzumab + pertuzumab; Dtx, docetaxel.

myces in the oral cavity; *A. turicensis*, *A. meyeri*, *A. neuui*, *A. odontolyticus*, *A. europaeus*, and *A. naeslundii*. Sensitivity of the assay was assessed with spiking experiments, in which different pre-defined concentrations of bacteria were introduced to naive samples. The primers (adapted from Bizhang et al. [29]) and the probe used 16S rRNA gene amplification and was designed as following: Act-fw: 5'-GGTCTCTGGGCGCTTACTGA-3', Act-rv: 5'-CCCCACACCTAGTGCCC-3', and Act-probe: 5'-Fam-CGTGGGGAGCGAACAGGATTAGATACC-Tamra-3'.

Genomic DNA (gDNA) was isolated from the oropharyngeal lavage samples of 21 breast cancer patients and 16 healthy individuals with the use of QIAasymphony DSP Virus/Pathogen Kit (QIAGEN, Hilden, Germany), following the manufacturer's recommendations. The quality and concentration of each gDNA sample were measured by the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and to normalize the amount of gDNA used in qPCR, the samples were diluted to an end concentration of 10 ng/μL gDNA. The reaction mixture consisted of 10 μL iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA, USA), 1.1 μM of both forward and reverse primers, 0.25 μM of the probe, and 1 μL of gDNA extract made up to 20 μL with water. The cycling protocol was designed according to manufacturer's recommendations that follows as polymerase activation and initial DNA denaturation at 95°C for 2 min, and 40 cycles of denaturation at 95°C for 15 s and primer annealing and extension at 65°C for 1 min. As positive control and negative controls in qPCR, *A. odontolyticus* DNA that was previously isolated from pure bacterial culture and distilled water was added to the master mix, respectively. All samples were run in duplicates.

All samples from the same patient were run in the same *Actinomyces* spp.-specific qPCR, and the Ct value of the positive control was noted. Considering that Ct value of positive control remained equal in each qPCR run, we calculated the ΔCt values and fold change by $2^{-\Delta\Delta C_t}$ method as described before [30]. Abundance of bacteria is inversely correlated with ΔCt and positively correlated with fold change.

Statistical Analysis

We assessed statistical analysis of our *Actinomyces* spp.-specific qPCR results with the use of GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA) and ran 2-tailed tests or 2-way ANOVA multiple comparison tests, as appropriate. Significance was determined by *p* values <0.05.

Results

Clinical data of the 21 breast cancer patients were included in this study, and immunohistochemistry results of their biopsy samples are described in Table 1. Patients were divided into three groups based on tumor receptor status; (i) ER+/PR+/HER2- or ER+/PR-/HER2- (10/21 patients), (ii) ER+/PR+/HER2+, ER-/PR-/HER2+ or ER+/PR-/HER2+ (8/21 patients), and (iii) triple-negative: ER-/PR-/HER2- (3/21 patients). Different chemotherapy regimens

Fig. 1. Quantification of *Actinomyces* spp. in oropharyngeal samples of breast cancer patients during chemotherapy and healthy individuals at matched timepoints. Columns represent the mean of the group at a given timepoint, and standard deviations are in-

dicated by error bars. Abundance of bacteria is inversely correlated with ΔCt (a) and positively correlated with fold change (b). No significant difference was observed between two groups at any time.

Fig. 2. Bacterial loads of *Actinomyces* spp. in oropharyngeal samples of healthy individuals (a) and breast cancer patients (b) on a single patient level. Each column represents a timepoint of collection, respectively. In the chart of breast cancer patients, the chemotherapy schemes of the patients are indicated below. Chemo-

therapeutic drugs were prescribed in different schemes for patients for their first four and last four cycles in therapy. EC, epirubicin + cyclophosphamide; DTP, docetaxel + trastuzumab + pertuzumab; Dtx, docetaxel; D1, day 1; C2–8, cycles 2–8, prior to radiotherapy; W52, week 52; preRadio, prior to radiotherapy.

were prescribed to breast cancer patients according to their tumor biology for routine purposes; 17/21 patients received epirubicin + cyclophosphamide in the first four cycles and continued with either only docetaxel (Dtx) or Dtx + trastuzumab + pertuzumab ($n = 10$ and $n = 7$, respectively) in the next four cycles, while 4/21 patients received these drugs the other way round ($n = 3$ and $n = 1$, respectively). Surprisingly, none of the cancer patients develop mucositis or oral candidiasis during chemotherapy. Neither breast cancer patients nor healthy controls used antibiotics or visited a dentist during the time period of oropharyngeal sample collection for a year.

Amplification of *Actinomyces* spp.-specific genes from gDNA extracted from oropharyngeal samples of breast cancer patients and healthy controls at different timepoints is shown in Figure 1. *Actinomyces* spp. were more abundant in the samples of healthy individuals at all timepoints, with an insignificant difference to breast cancer patients ($p > 0.05$). We observed variations of the bacterial loads of *Actinomyces* spp. during chemotherapy cycles in breast cancer patients; however, these differences were also not significant. A similar fluctuation of bacterial loads was monitored in healthy controls as well.

The levels of *Actinomyces* spp. in samples collected from all patients enrolled in this study are represented in Figure 2, on a timeline. Bacterial loads were not correlated with age ($p = 0.852$), body mass index ($p = 0.242$), or tumor receptor status ($p = 0.093$) at day 1. Over the period of a year, changes on bacterial loads of *Actinomyces* spp. were observed in both groups. Since only one sample per timepoint was collected from the patients and gDNA was extracted once, it is not reliable to make statistical analysis on abundance of *Actinomyces* spp. compared between timepoints on a patient level. However, when all patients in the same group observed together, a trend could be seen. In our study, we did not observe a specific trend on abundance of *Actinomyces* spp. in early-stage breast cancer patients over their chemotherapies. Considering the different chemotherapy schemes prescribed to breast cancer patients, we also did not record a specific pattern with any treatment option (Fig. 2b).

Discussion

Increasing cases of MRONJ have been reported in the last years; nevertheless, we still could not decipher the underlying mechanism clearly. It is urgently necessary to develop competent prophylactic and therapeutic strategies since only 30% of MRONJ patients may be treated by cur-

rently applications [31]. Data on MRONJ published so far suggest three different, successive factors for the development of the disease: (a) changes in bone metabolism leading to a reduced tendency of spontaneous healing of bone and mucosa; these changes may be associated with antiresorptive drugs but also underlying malignant disease, hormone disorders or treatments, genetic polymorphisms, etc.; (b) exposure of the bone; for instance, by mucositis induced by chemotherapy, poor oral hygiene, or tooth extraction; and (c) the presence of *Actinomyces* spp. in high quantities in the oropharynx that allows the bacteria to enter the exposed bone in significant numbers to overwhelm host immunity and cause local infection [13–15, 17]. In this study, our focus was on the latter factor so that we investigated the abundance of *Actinomyces* spp. in early-stage breast cancer patients as a leading patient group in MRONJ development.

We monitored the bacterial loads of *Actinomyces* spp. throughout the chemotherapy of breast cancer patients, compared to healthy controls, in order to investigate if these bacteria have an altered abundance in cancer patients in the first place, or if the chemotherapeutic drugs have an effect on *Actinomyces* spp. in quantity. In order to eliminate biases of that, use of bisphosphonates and denosumab has been associated with MRONJ development, together with the presence of *Actinomyces* spp. in the oral cavity, and we only chose the breast cancer patients who did not receive any of these drugs in their treatment. Patients enrolled in this study, either to the breast cancer or control group, did not use antimicrobial drugs during the time of sample collection for a year. Since early-stage breast cancer patients received 3-weekly chemotherapy and an oropharyngeal sample was collected before each cycle, the healthy individuals were invited to donate samples every 3 weeks, as well. A final sample was collected from both groups at the end of the year to indicate if there is a consistent effect of chemotherapeutic drugs on *Actinomyces* spp. levels.

Abundance of *Actinomyces* spp. in the oral cavity fluctuated over time both in breast cancer patients and healthy controls, without any specific pattern. We did not observe an influence of chemotherapeutic drugs (epirubicin, cyclophosphamide, docetaxel, trastuzumab, and pertuzumab) on bacterial loads as well. Yet, these findings should be interpreted with caution since none of the cancer patients included in this study developed mucositis during chemotherapy. Oral mucositis has been associated with alterations in oral microbiome so that due to lack of mucositis and small cohort size in this study, there might be biases.

When bacterial loads of *Actinomyces* spp. in oropharyngeal samples of breast cancer patients and healthy controls were compared, there was no significant differ-

ence. Despite not being significant, *Actinomyces* spp. were more abundantly found in healthy donors' samples at all timepoints. Thus, it is tempting to speculate that early-stage breast cancer patients might have a dysbiotic gut microbiota, and some other types of commensal bacteria could have surpassed *Actinomyces* spp.. Multiple studies published in recent years showed an association between dysbiosis of gut microbiota leading to changes in estrogen mechanism and hormone-related diseases such as breast cancer [32, 33]. The phyla Proteobacteria, Firmicutes, and Bacteroidetes are the most abundant bacteria in samples of breast cancer patients [34–36].

We suggest that *Actinomyces* spp. are not the initiative factors in MRONJ development, and they are rather the opportunistic bacteria that proliferate when they find a new niche. Only after a complication such as disruption of the bone, *Actinomyces* spp. may invade the maxilla or mandible and facilitate an infection by overwhelming host immunity with increased quantity in the oropharynx, as also suggested by Naik and Russo [37]. As a future aspect, abundance of *Actinomyces* spp. may be investigated in cancer patients who received antiresorptive drugs throughout the chemotherapy. Thereof, the phases where bacterial loads of *Actinomyces* spp. are increased may be determined, and with this knowledge, we may suggest antimicrobial treatment at particular stages in chemotherapy to prevent development of MRONJ.

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Statement of Ethics

This study protocol was reviewed and approved by the local Ethics Committee at Medical University of Vienna, approval number EK1539/2016. The study was performed in accordance with the Declaration of Helsinki and good practical guidelines. A written informed consent was obtained from all patients enrolled in the study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

C.B. performed experiments and data analysis and wrote the manuscript. T.F. contributed to guidance and critically revised the manuscript. M.-T.K., Z.V., I.B., and H.S. provided technical assistance and collected clinical samples. C.F.S. contributed to guidance. C.S. designed and supervised the study.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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