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Metal elements in tissue with dental peri-implantitis: a pilot study

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Abstract

Objectives: Dental peri-implantitis is characterized by a multifactorial etiology. The role of metal elements as an etiological factor for peri-implantitis is still unclear. The aim of this study was to investigate the incidence of metal elements in bone and mucosal tissues around dental Grade 4 CP titanium implants with signs of peri-implantitis in human patients.

Methods: In this prospective pilot study, all patients were enrolled consecutively in two study centers. Bone and soft tissue samples of patients with peri-implantitis with indication for explantation were analyzed for the incidence of different elements (Ca, P, Ti, Fe) by means of synchrotron radiation X-ray fluorescence spectroscopy (SRXRF) and polarized light microscopy (PLM). The existence of macrophages and lymphocytes in the histologic specimens was analyzed.

Results: Biopsies of 12 patients (seven bone samples, five mucosal samples) were included and analyzed. In nine of the 12 samples (75%), the SRXRF examination revealed the existence of titanium (Ti) and an associated occurrence with Iron (Fe). Metal particles were detected in peri-implant soft tissue using PLM. In samples with increased titanium concentration, lymphocytes were detected, whereas M1 macrophages were predominantly seen in samples with metal particles.

Conclusion: Titanium and Iron elements were found in soft and hard tissue biopsies retrieved from peri-implantitis sites. Further histologic and immunohistochemical studies need to clarify which specific immune reaction metal elements/particles induce in dental peri-implant tissue.

Dental peri-implantitis as an acute and chronic form of an inflammatory disease is currently discussed to have a multifactorial etiology and is characterized by peri-implant bone loss eventually leading to the loss of the dental implant (Heitz-Mayfield 2008; Zitzmann & Berglundh 2008; McCrea 2014). Plasma cells and lymphocytes have been identified to dominate the immunoreaction in peri-implantitis. However, neutrophil granulocytes and/or macrophages are found in peri-implantitis lesions as well (Berglundh et al. 2011). The different composition of the inflammatory infiltrates raises the question, whether other factors besides bacteria induce such an immune response.

Dental implants are commonly screwed joints being composed of the implant body fixated in the bone and the abutment screwed into the implant body. Usually, the abutment consists of TiAl alloy or Zirconia and the implant body is made from CP titanium Grade 4. This screwed joint is known to exhibit micromovement, and therefore, wear and fretting occurs (Rack et al. 2013). It has been shown that metal wear particles are formed at the implant–abutment interface (Klotz et al. 2011; Stimmelmayr et al. 2012). A current study demonstrated that micromotion of the abutment during cyclic loading can induce wear particles ranging between 2 and 80 μm in conical dental implant systems (Blum et al. 2015). A permanent and during-loading-expanding microgap between the abutment and implant allows the release of wear debris into the peri-implant tissue.

Wear debris have been described to be one of the responsible factors for aseptic loosening of orthopedic implants besides biocorrosion, material fatigue, and inappropriate implant geometry (Sundfeldt et al. 2006). These factors remain unexplored for dental implants. Studies concerning orthopedic

implants have shown that aseptic loosening has an incidence of 5–20% (Burton et al. 2013). Bone loss in absence of bacteria is the most common cause of revision of total hip replacement (O'Neill et al. 2013). Wear debris consists either of titanium and/or zirconium particles which are known to induce a macrophage response (Nich et al. 2013). The metal content, the wear profile, and the corrosion properties may have an influence on the cellular pathway of the immune reaction (Lohmann et al. 2014). Many studies showed that wear debris phagocytized by macrophages induces the expression of messenger ribonucleic acid (mRNA) for the formation of pro-inflammatory parameters such as tumor necrosis factor (TNF) alpha (Tsutsui et al. 1999; Hatton et al. 2003; Obando-Pereda et al. 2014; Wang et al. 2013). It is assumed that TNF alpha influences the expression of RANKL and M-CSF on osteoblasts promoting the maturation of osteoclasts leading to an increased bone resorption (Ingham & Fisher 2005; Burton et al. 2013). Titanium is considered to be a bioinert metal, with high corrosion resistance and excellent biocompatibility (Tschernitschek et al. 2005; Nebe et al. 2008). It has been assumed that during the insertion of implants in rabbit tibia, titanium, aluminum, and/or vanadium elements are released as they were detected by way of graphite furnace atomic absorption spectrophotometer or synchrotron radiation X-ray fluorescence spectroscopy (SRXRF) systemically and locally (Schliephake et al. 1993; Wennerberg et al. 2004; Meyer et al. 2006; de Morais et al. 2009). But the immunogenic impact remains to be elucidated.

In SRXRF, the sample material is excited either by polychromatic X-rays, gamma-rays, or ion beams. The released element-specific fluorescent radiation is evaluated with an appropriate detector and analyzed. SRXRF analysis allows the detection of multiple elements such as Ca, Si, P, Ti, Va, Zn, and Fe in hard tissue and soft tissue independent of the light microscopic visibility (Riesemeier et al. 2005).

A recently published human study demonstrated the existence of foreign bodies consisting of titanium and dental cement components only in soft tissue with peri-implantitis using scanning electron microscopy (SEM) and energy dispersive X-ray spectrometer (EDX) (Wilson et al. 2015). The existence of metal elements has not been well investigated in human peri-implantitis tissue, and it still remains unclear whether titanium or titanium particles are released from loaded dental implants having local and/or systemic impact (Olmedo et al. 2009; Wilson et al. 2015). However, metal wear particles could be an explanation for the clinical observation that in a line of several implants in one patient, only one implant is affected by peri-implantitis, while the others are healthy.

The aim of this pilot study was to investigate the existence of metal elements in surrounding bone and soft tissue of dental implants with signs of peri-implantitis in human patients.

Materials and methods

The study was approved by the ethics committee of the Charité Berlin, Germany (Ethikkommission Ethikausschuss 4 am Campus Benjamin Franklin) No EA4/050/13 and by the ethics committee of University Medical Center Freiburg, Germany (Ethik-Kommission Albert-Ludwigs-Universität, Freiburg) No 268/13. This study was performed in accordance with the Helsinki Declaration of 1964, as revised in 2013.

Population

Bone and soft tissue samples were taken from patients with severe peri-implant disease with indication for explantation, diagnosed by clinical investigation (probing with a periodontal probe, mobility testing) and radiographic bone destruction. Patients were enrolled consecutively in two study centers (Department of Oral and Craniomaxillofacial Surgery, University Medical Center Freiburg, and Department of Oral and Maxillofacial Surgery, Charité Campus Virchow Berlin). After determination for implant explantation, all patients were informed about the study conditions, were asked for their participation, and had to give written consent for the surgical procedure. A negative control was performed in this study harvested from a patient during explantation of a ceramic implant with peri-implantitis.

Inclusion criteria

Severe peri-implant disease with indication for explantation included radiographic bone loss of more than two-third of the implant length, suppuration, mobility, or cortical bone perforations (Lang et al. 2000; Schwarz & Becker 2009).

Exclusion criteria

Patients who were younger than 18 years of age, patients with a periodontitis, and patients who had previous surgical or periodontal therapy of the dental implant (to avoid iatrogenic causes of titanium in the tissues) were excluded as well as immunosuppressed and irradiated patients and patients who received chemotherapy or participated in other studies during the retrieval of the biopsy. Patients were excluded when a debridement by curette or ultrasound of the implants had been performed within 1 year prior to explantation.

Retrieving and processing of the biopsies

The tissue samples were obtained at the time of surgical removal of the dental implant under local anesthesia with Ultracain forte (Sanofi Aventis, Frankfurt, Germany). A circular incision with releasing incisions mesial and distal of the implant in a distance of 2 mm from the implant was performed in the soft tissue using a scalpel (15c) (KLS Martin Group, Tuttlingen, Germany). Meticulous care was taken to prevent accidental release of titanium by scraping of the implant surface. A mucoperiosteal flap was mobilized and the remaining inflamed peri-implant tissue was removed using a clamp and scalpel. The biopsies were placed in 3.5% neutral-buffered formalin solution (Otto Fischer GmbH, Saarbrücken, Germany) until further processing. The samples were embedded in paraffin (Engelbrecht Medizin- und Labortechnik GmbH, Edermünde, Germany) according to the manufacturer's guidelines. After complete embedding and cooling, the specimens were cut using a rotary microtome with a glass diameter $400 \times 25 \times 100$ mm (Leica, Nussloch, Germany) to a thickness of 17 μm required for SRXRF analysis on the one hand. The samples were fixed on a kiesol foil (Kettenbach GmbH, Eschenburg, Germany) and analyzed using SRXRF. On the other hand, the samples were cut to a thickness of 5 μm required for histologic examination. The samples were applied on poly-L-lysine (Sigma-Aldrich, Steinheim, Germany). All the experimental equipment and supplies (microtome blade, all solutions, specimen holders, etc.) were metal-free. The scalpel was made of stainless steel (iron- and titanium-free).

Synchrotron radiation X-ray fluorescence spectroscopy and polarized light microscopy

The SRXRF measurements were carried out at the BAMline at the Berlin electron storage ring for synchrotron radiation (BESSY) in Berlin. A detailed description of this beamline and its optical elements is given in a publication by Riesemeier et al. (2005). The double multilayer monochromator (DMM) was used, as it provides an about 100 times higher flux
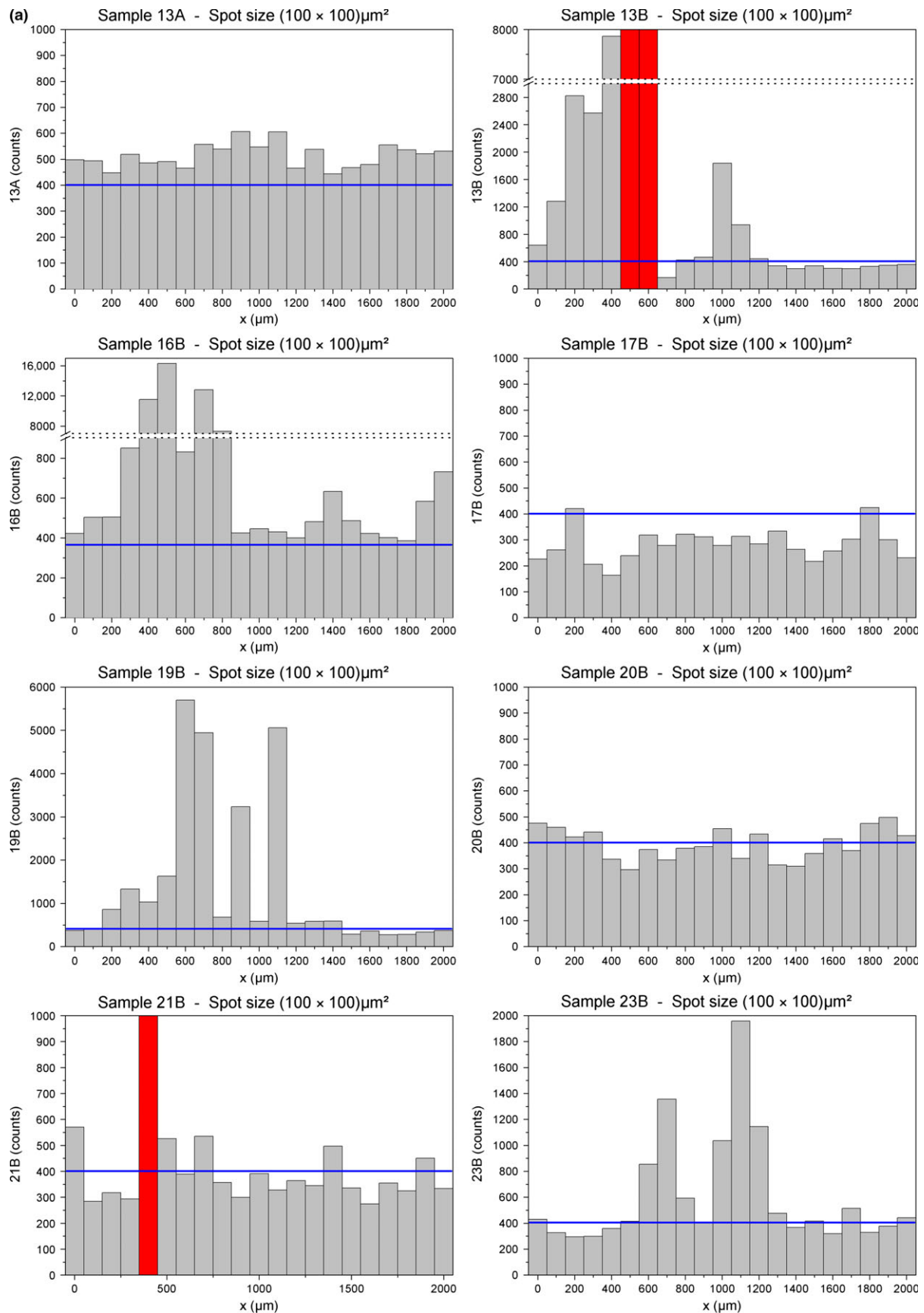
Fig. 1. (a,b) SRXRF line scans for the element Ti of each sample. The spot size (resolution) is 100.0 × 100.0 μm². The y-axis shows different gradings on each image (Counts norm). The blue line is marking peaks above 400 counts norm. 75% of the samples show clear signs of titanium. Red bars indicate a detector overflow mostly due to very strong Ca signal.
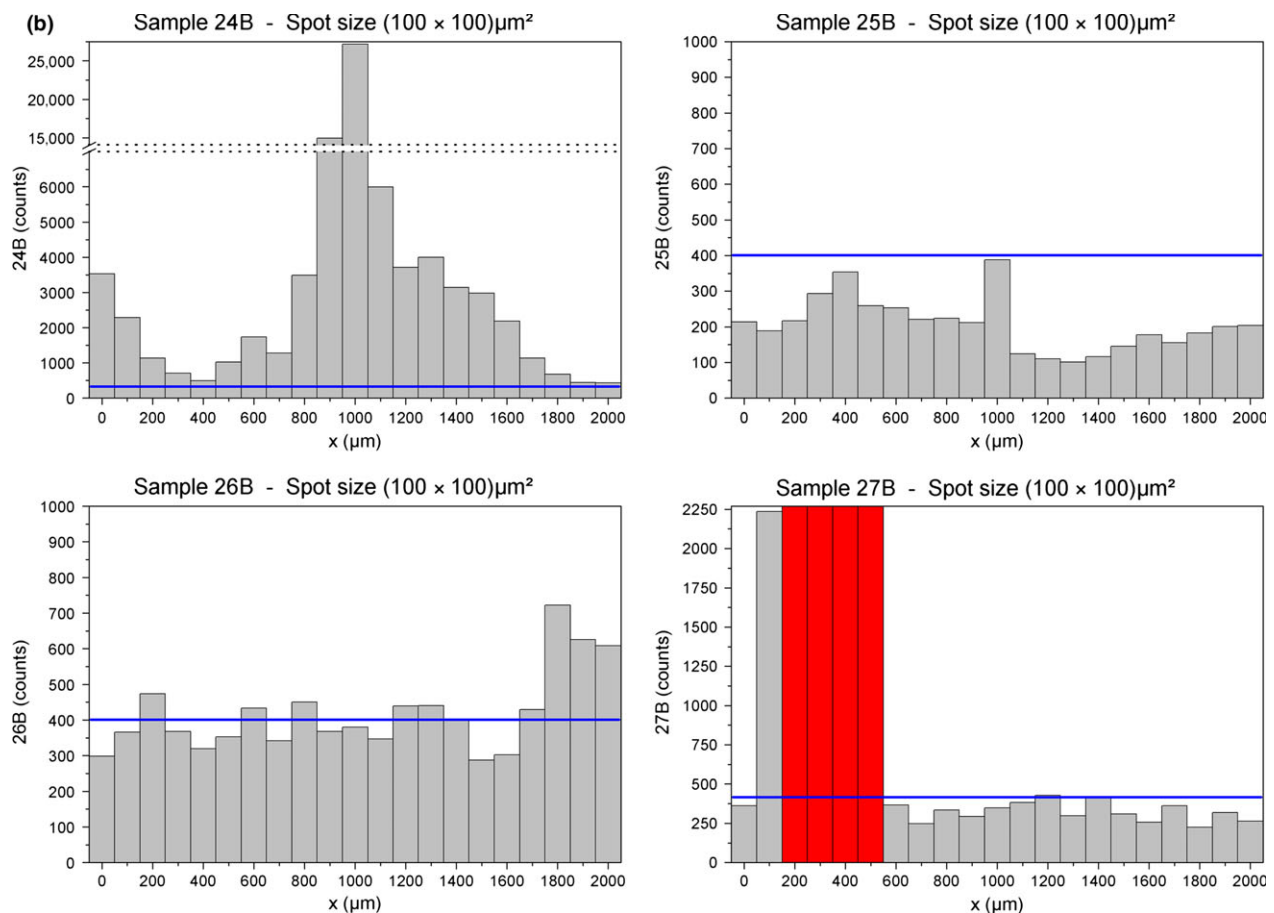
Fig. 1. Continued.

than the double-crystal monochromator (DCM) and therefore better detection limits. The fluorescence signal was detected with a silicon drift detector, and all measurements were performed in air.

The samples were applied on a mounting tape and mounted on a motorized xyz stage to conduct the scans.

First line scans of the specimens with a 100 μm × 100 μm beam size were carried out, using the DMM with an energy setting of 7 keV and a measuring time of 60 s per

point. The starting points of the scans were randomly chosen on the samples, and from there, a series of 20 points in a horizontal line with 100 μm spacing were measured (Fig. 1a,b).

When higher concentrations of the examined elements was evident, area scans with a 30 μm × 30 μm resolution were performed for these samples. Accordingly, the energy of the primary beam was increased to 10 keV to be able to excite heavier elements like iron, copper, and zinc. The measuring time per

point was 10 s, and the areas analyzed were of different size.

The samples were screened using polarized light microscopy (PLM) (Olympus-Vanox AH2, crossed polarized light, magnification 100×) after immunohistologic preparation.

Histologic investigation

Overview (HE) and immunohistochemical staining was performed using a monoclonal antibody directed against CD68 (clone PGM1, dilution 1 : 50; Abcam, Cambridge, UK) glycoprotein. Antigen retrieval was performed by microwave heating slides in target retrieval buffer (10× concentrated; DAKO, Carpinteria, CA, USA) for 20 min. The avidin–biotin complex (ABC) method was used for immunohistochemical staining (Vector Laboratories, Burlingame, CA, USA), followed by antibody detection using 3,3'-diaminobenzidine as a chromogen. The slides were counterstained with hematoxylin and coverslipped. To confirm the specificity of the antibody sections with human, tonsils were used as appropriate positive controls. The samples were fixed in Technovit 7200 (Carl Roth, Karlsruhe, Germany).

Table 1. **Sample number, gender, affected implant regions, implant types, implant and abutment material**

Sample	Gender	Region	Impl.-Type	Impl. material	Abutment material
13A	w	11	Camlog	Titanium Grade 4	Titanium Grade 5 (Ti6AI4V)
13B	w	11	Camlog	Titanium Grade 4	Titanium Grade 5 (Ti6AI4V)
16B	m	46	Camlog	Titanium Grade 4	Titanium Grade 5 (Ti6AI4V)
17B	m	46	Camlog	Titanium Grade 4	Titanium Grade 5 (Ti6AI4V)
19B	w	32	Straumann	Titanium Grade 4	Titanium Grade 4
20B	m	32	SteriOss	Titanium Grade 4	Titanium Grade 5 (Ti6AI4V)
21B	m	42	SteriOss	Titanium Grade 4	Titanium Grade 5 (Ti6AI4V)
23B	m	34	SteriOss	Titanium Grade 4	Titanium Grade 5 (Ti6AI4V)
24B	w	17	Nobel Speedy	Titanium Grade 4	Titanium Grade 5 (Ti6AI4V)
25B	w	17	Nobel Speedy	Titanium Grade 4	Titanium Grade 5 (Ti6AI4V)
26B	w	26	Nobel Speedy	Titanium Grade 4	Titanium Grade 5 (Ti6AI4V)
27B	w	26	Nobel Speedy	Titanium Grade 4	Titanium Grade 5 (Ti6AI4V)

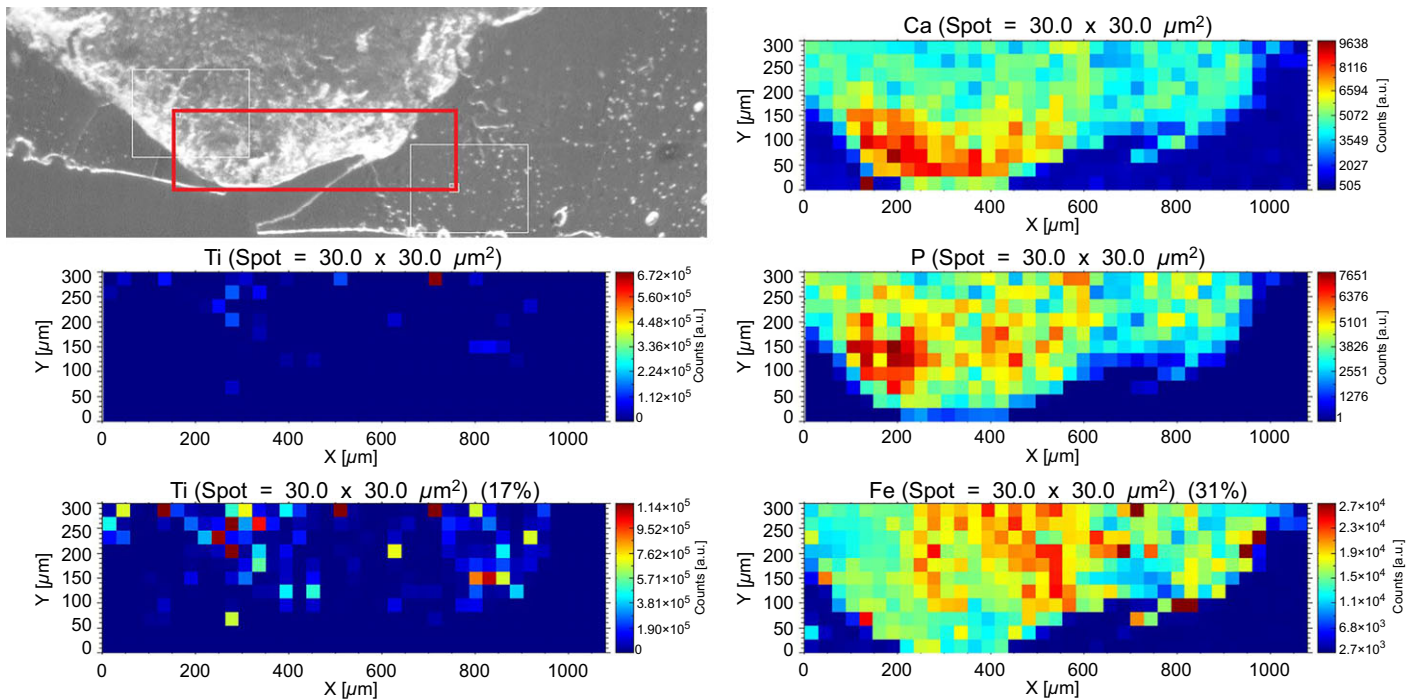*Fig. 2.* SRXRF area scan for the elements Ca, P, Ti, and Fe of soft tissue (16 B). The spot size is 30.0 × 30.0 μm². High concentrations are illustrated as dark red and low concentration as dark blue. The y-axis shows different gradings on each image (Counts norm). The red square demonstrates the synchrotron-analyzed region.

Results

The biopsies were retrieved from 12 patients with the mean age of 58.8 years (range: 54–65 years). Six samples were harvested from female patients and six from male. Half of the samples were taken from the mandible and the other half from the maxilla. The affected implant regions, implant types, implant and abutment material of manufactures Camlog, Straumann, and Nobel Biocare are shown in Table 1.

The results of the line scans are presented in Fig. 1a,b. Six of the twelve samples showed high levels of titanium (Ti) (13B, 16B, 19B, 23B, 24B, 27B) and three samples revealed the existence of Ti in lower concentration (13A, 21B, 26B). The red bars in Fig. 1a,b indicated a detector overflow mostly due to a very strong Ca++ signal. In these points, no spectra could be acquired and therefore, the Ti signal is unknown.

In four of the samples (16B, 19B, 20B, 25B), area scans were performed and the distribution of the elements calcium, phosphorus, titanium, and iron was determined (Fig. 2). The figure depicts the region of the area scan within the specimens (Inset photo) taken at the beamline (red square). Fig. 2 demonstrates the scan of a soft tissue sample (16B). The maximum concentrations of calcium and titanium are shown in Table 2. Increased

Table 2. **Maximum concentration of calcium, titanium, and iron in soft tissue and hard tissue (area scans)**

	Ca	Ti	Fe
Bone			
Sample 25B	2×10^6	3.14×10^5	1.28×10^4
Sample 19B	3.30×10^4	4188	2.4×10^5
Soft tissue			
Sample 16B	9638	6.72×10^5	2.7×10^4
Sample 20B	9.32×10^5	7.53×10^5	6.06×10^4

concentrations of Iron (Fe) were also found in areas with increased titanium concentration.

The negative control (ceramic implant) demonstrated no presence of titanium or iron in the peri-implant tissue.

Within the specimens (soft tissue and hard tissue), the overall existence of M1 macrophages was low and immunohistologic investigation showed an accumulation of M1 macrophages (PGM-1) in the area of increased titanium and iron concentration (Fig. 3a).

But a correlation between the amount of metal elements and the quantity of macrophages was not detectable in the tissue samples. The bone samples showed a minor amount of M1 macrophages in demineralized areas. The predominant cell type in the soft tissue of the biopsies was lymphocytes (Fig. 3b).

Micrometer-sized metal elements can be detected in CD68⁺ macrophages in peri-implant soft tissue samples using PLM (Fig. 4a, b).

Discussion

In this pilot study, biopsies of patients with peri-implantitis were analyzed for the incidence of different elements by means of SRXRF. In 75% of the samples, a higher amount of titanium was detected. In some biopsies, this was accompanied with an increased iron concentration (up to 2.4×10^5 counts); this might possibly be due to the composition of the (unalloyed) commercially pure titanium (Grade 4) according to the American Society for Testing and Materials (ASTM) International Standard F67, respectively, DIN EN ISO 5832-2 with the following elements: O_2, N, H, C, and Fe. The content of Fe may vary up to maximal 0.35 weight percentage depending on the manufacturer's requirements. CP titanium with a higher concentration of Fe is known to enhance the stability, whereas it makes the material more brittle. Therefore, the congruent localization of titanium and iron elements may be substantiated by this. Localized or aerial Ca peaks show the exis-

tence of bone. In some soft tissue biopsies, localized Ca peaks show residual bone fragments, which are in the process of being resorbed. The negative control (ceramic implant) demonstrated no presence of titanium or iron in the peri-implant tissue.

The abutments used within these patients were made of CP-Ti Gr. 5 with the exception of the Straumann abutment being CP-Ti Gr. 4: as no aluminum was found in the tissue, it could be assumed that the titanium or wear particles probably originated from the damage of the implant body. Furthermore, CP-Ti Gr. 4 shows a lower hardness than CP-Ti Gr. 5 suggesting a higher wear of the implant body (Guilherme et al. 2005). Implant and implant surface properties may influence the amount and consistence of implant-derived elements within the peri-implant tissue.

Definite reasons for the emission of titanium and iron into the peri-implant tissue are unknown. The micromovement between the implant and the abutment and the existence of a microgap could be discussed as the source for wear debris and extrusion of titanium particles (Klotz et al. 2011; Stimmelmayr et al. 2012; Rack et al. 2013; Blum et al. 2015). This hypothesis is supported by the fact that the composition of wear debris found inside the implant in a previous study using EDX analysis shows a similar composition as the wear particles detected in this study in the peri-implant tissue analyzed with SXRF (Blum et al. 2015). Further conceivable causes are surface abrasion by bone contact during implant placement, prosthetic restoration, biocorrosion of the metal, or manipulation of the implant during cleaning.

In the present study, an iatrogenic contamination of metal elements into the peri-implant tissue is not probable: Meticulous care was taken during harvesting of the biopsy to prevent accidental release of titanium. No debridement of implants had been performed within 1 year prior to explantation, and a fast turnover rate of the gingival epithelium has been described varying between 6 and 40 days (Wolf et al. 2004). A positive control was not feasible as the explantation of well osseointegrated implants in humans is a rarity to happen and definite exception (Schminke et al. 2015). Besides this fact, it is surgically impossible to retrieve peri-implant bone without contamination of metal from the implant body in a healthy osseointegrated implant, as there is intimate bone-to-implant contact without any defects or gaps surrounding the implant.

As reason for revision of orthopedic implants, the particle-induced osteolysis has

been described (Sundfeldt et al. 2006; Burton et al. 2013; Wang et al. 2013). Possible reasons for orthopedic aseptic loosening are abrasion debris, crevice and biocorrosion, inappropriate mechanical load and micromotion, cellular digestion mechanisms, high fluid pressure, and stress shielding (Sundfeldt et al. 2006; Grosse et al. 2015). The mechanisms in orthopedic implants are not directly comparable with dental implants. Dental implants are part of an open system communicating with the oral cavity, surrounded by peri-implant tissue with a high turnover rate, so the wear emission and its impact will probably proceed differently. Further studies are needed in this field considering different mechanisms besides bacterial inflammation.

Recent studies show various modulations of the immune response induced by metal particles. Wear debris of orthopedic implants
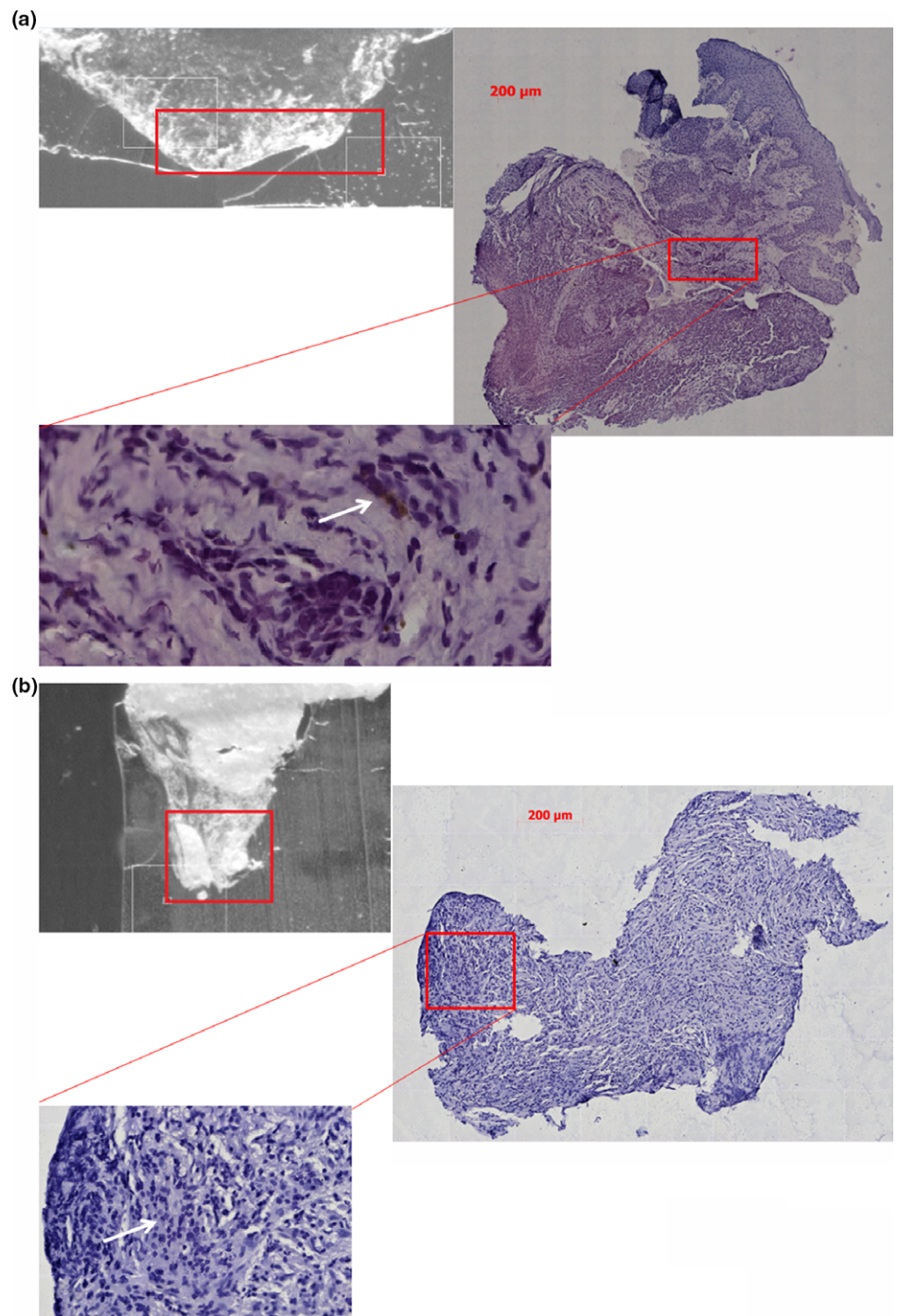

Fig. 3. (a) Histologic detection of M1 macrophages (arrow) with PGM-1-stain in soft tissue (sample 16 B). (b) Lymphocytes in hard tissue samples (arrow) (sample 25 b).
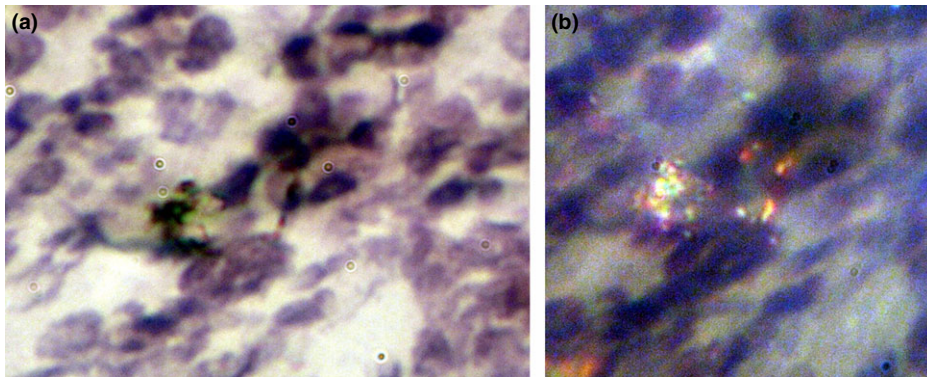
Fig. 4. (a) Metal element in a soft tissue sample stained with PGM-1 (sample 16 B). (b) PLM detection of the metal element in the correlated region of the same sample, stained with PGM-1 (sample 16 B).

influences macrophages, metalloproteinases, fibroblasts, osteoclasts, as well as osteoblasts (Vermes et al. 2000; Fritz et al. 2006; Jiang et al. 2013; O'Neill et al. 2013; Obando-Pereda et al. 2014). In orthopedics, macrophage- and lymphocyte-dominated and mixed reactions (co-existing pathways) of aseptic loosening can be differentiated (Lohmann et al. 2014). In orthopedics, the release of metal ions from the prosthesis can induce an acute phase of a chronic inflammatory disease with infiltrates of monocytes, T cells, and osteoclasts (Dapunt et al. 2014a,b). An aseptic lymphocyte-dominated vasculitis-associated lesion may occur (Dapunt et al. 2014a,b). The metallosis which leads to a local host defense mechanism and bone loss has been described in this context, but is still poorly explored (Watters et al. 2010).

To the authors' knowledge, one human study demonstrated the existence of foreign bodies consisting titanium and dental cement components in soft tissue with peri-implantitis, but a further characteristic of the cells is missing (Wilson et al. 2015). In the current study, pro-inflammatory M1 macrophages and high amounts of lymphocytes in tissue with increased titanium concentration could be detected. The plasticity and polarization of macrophages have hardly been studied in the context of peri-implantitis (Sica & Mantovani 2012; Ben-Mordechai et al. 2013). From the field of orthopedic surgery, it is known that metal particles are phagocytized size-dependent by macrophages and multinucleated giant cells (fused macrophages) (Guilherme et al. 2005; Revell 2008). Grosse et al. (2015) showed in an orthopedic study that the histologic grade of tissue response correlates with the total number of particles. Larger cohorts are necessary to evaluate this issue in dental peri-implantitis in detail.

A current study showed the formation of wear particles within the implant–abutment connection due to micromovement of the abutment with a particle size up to 80 µm (Blum et al. 2015). Metal debris was also found in the soft tissue covering maxillar and mandibular titanium miniplates used for osteosynthesis varying in size between 1 and 200 µm (Langford & Frame 2002). The metal wear particle size from titanium-based hip prostheses ranges between 0.1 and 6.5 µm with >50% of the particles being <0.4 µm (Sundfeldt et al. 2006; Abu-Amer et al. 2007; Grosse et al. 2015).

Lohmann et al. (2014) showed that the mean metal content/wear profile and the corrosion properties may have an influence on the cellular pathway. Possibly, the metal particle configuration could play a greater role in the pathogenesis of dental peri-implantitis than the amount. Some authors subsume in the field of orthopedics that the local periprosthetic tissue reaction is influenced by particle load, inorganic metal salt, metal ions, and chemical reactivity and composition (Matusiewicz 2014; Grosse et al. 2015). A recent review underlines the importance of physico-chemical surface parameters. For example, particle size, geometry and charge, particle surface area distribution, surface patterning, morphology, surface composition, particle surface-to-cell surface area ratio, particle mass dosage, direct toxicity through cellular phagocytosis ion exposure, and hydrophobicity/hydrophilicity may influence the biological response (Prokopovich 2014).

Animal studies have shown that metal ions can be detected in the vicinity of freshly placed dental implants with a titanium ion concentration of 213 ng/implant suggesting a release of metal during the placement of the implant (Schliephake et al. 1993; Wennerberg et al. 2004; Meyer et al. 2006; de Morais

et al. 2009). The existence of metal ions within the peri-implant tissue of dental implants needs to be clarified.

Metal elements were not found in every biopsy examined; however, this does not allow the conclusion that they are not always present as the biopsies retrieved were not from the complete peri-implantitis tissue. The histologic preparation of the specimens includes embedding of the tissue in resin, which makes a possible displacement of the particle from its original position unlikely due to cutting. The full size of the metal particles cannot be visualized, as they might have been modified by the applied microtome cutting technique as this technique limits the sample thickness.

Peri-implantitis is a complex system with multifactorial components which is not understood in detail. The metal particles might not induce the disease independently, but the summation of components might lead to disease progression. The question remains whether peri-implantitis is triggered or amplified by the existence of metal elements and whether an interaction or a synergistic effect exists in the presence of periodontal pathogens. It was shown in a murine model that titanium ions enhance cytokine production of periodontopathic bacterial lipopolysaccharide (Nishimura et al. 2014). The localization of the particles in periimplant tissue is within a different environment compared with those found in the vicinity of hip or knee implants resulting in a different disease mechanism. This study revealed the existence of metal ions and particles in peri-implantitis tissue. Further studies are required to demonstrate how pro-inflammatory parameters are influenced by metallic particles/elements in the context of dental peri-implantitis.

Conclusion

Titanium and iron elements were found in soft tissue and hard tissue retrieved adjacent to implants showing peri-implantitis. Not only macrophages (M1 macrophages) were detected in the tissue with increased titanium concentration, but also lymphocytes could be found. Further studies need to clarify whether there is a correlation between the amount of metal elements and the specificity of the immune reaction induced in dental peri-implant tissue. Understanding the molecular pathogeneses of peri-implantitis may lead to adjuvant drug therapy.

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