Immunohistological Composition of Peri-implantitis affected tissue around Ceramic Implants – A pilot study

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Running Title: Peri-implantitis around Ceramic Implants

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Key findings: Peri-implantitis around ceramic implants in comparison with titanium implants seems to have a similar histological appearance, differences in cellular composition of peri-implantitis lesions might also depend on the patients specific immune status and not only on the material used.

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Abstract

Background

Aim of the pilot study was the histologic classification of the inflamed peri-implant soft tissue around ceramic implants (CI) in comparison to titanium implants (TI).

Methods

Peri-implant tissue were retrieved from 15 patients (age 34-88 years, 7 male/8 female) with severe peri-implantitis [8 CI, 7 TI]. The peri-implant soft tissue samples were retrieved from the sites during scheduled removal of the implant and prepared for immunohistochemical analysis. Monoclonal antibodies (targeting CD3, CD20, CD138, CD68) were used to identify T- and B-cells, plasma cells and macrophages. Quantitative assessment was performed by one histologically trained investigator. Linear mixed regression models were used.

Results

A similar numerical distribution of the cell population was found in peri-implantitis around CI compared to TI. CD3 (TI 17 – 85 % vs CI 20 - 70 % of total cell number) and CD138 (TI 1 -73 % vs CI 12-69 % of total cell number) were predominantly expressed. Notably, patient-individual differences of numerical cell distribution were detected. Co-localization of B- and T-lymphocytes was observed.

Conclusion

Peri-implantitis around CI in comparison with TI seems to have a similar histological appearance. Differences in cellular composition of peri-implantitis lesions might also depend on the patients specific immune status and not only on the material used.

Word count: 206 words

MeSH term key words: Peri-implantitis; titanium; ceramic; zirconia; implantology; immunohistology;

Introduction

Oral implants enlarge the treatment options to replace missing teeth and have been proven to be successful as shown in systematic reviews with long-term follow-up[1,2]. Although survival rates appear convincing, peri-implantitis around dental implants is a challenge in daily practice, with a prevalence around 20 %[3]. The prevalence rate of peri-implantitis is highly variable and seems to be affected by clinical case definition and local factors such as implant characteristics[4]. Peri-implantitis is defined by the Consensus Conference of the American Academy of Periodontology and the European Federation of Periodontology as a "plaque-associated pathological condition occurring in tissues around dental implants, characterized by inflammation in the peri-implant mucosa and subsequent progressive loss of supporting bone"[5]. However, current articles with varying evidence consider additional trigger mechanisms for peri-implant bone loss, such as prosthetic, surgical and biomechanical factors[6-9]. For the identification of possible etiologic factors of a disease it is necessary to study the immunohistological composition as known from studies regarding aseptic loosening of orthopedic implants[10]. The cellular composition of peri-implantitis around titanium implants is characterized by the existence of neutrophils, macrophages, and/or T- and B-cells[11]. The infiltrated connective tissue (ICT) in peri-implantitis is more than twice as large as the ICT of periodontitis and presents a significantly higher number of CD68 and

myeloperoxidase (MPO)-positive cells[11]. Further, current histological studies showed a distinct macrophage M1 polarization compared with periodontitis[12,13].

Even though titanium and its alloys are the most commonly used materials for oral implants and its components, ceramic oral implants are increasingly being placed[14]. Three zirconia-containing ceramic systems are established in implant dentistry: yttrium-stabilized tetragonal zirconia polycrystals (Y-TZP), alumina-toughened zirconia (ATZ) and zirconia-toughened alumina (ZTA)[15]. Currently, there are mainly one-piece implants on the dental market, however more and more two-piece ceramic implants are available[16-18]. To produce a roughened zirconia surface, sintering particles onto the implant surface, nanotechnology, lasertechnology, sandblasting or sandblasting and acid etching with in a mixture of hydrofluoric and sulfuric acid and have been used leading to different manufacturer dependent microtopographys[19]. Y-TZP has been described to exhibit various benefits including excellent biocompatibility[20]. Since zirconia implant surfaces show a difference in biofilm formation in comparison to titanium implant surfaces, the peri-implant immunological cellular response might be different between titanium und zirconia implants[18]. Peri-implantitis also occurs around ceramic implants but due to missing data the prevalence is unknown[3,18]. To date there is no histological analysis of the tissues around ceramic implants with signs of peri-implantitis. Although, there might be some similarities between the clinical appearance of peri-implantitis around titanium and ceramic implants, a congruence between these conditions was never shown on a histological level so far.

The aim of the pilot study was the histologic classification of the inflamed peri-implant soft tissue around ceramic implants in comparison to titanium implants.

Material and Methods

The study was approved by the ethics committee of the University Medical Center Freiburg, Germany (Ethik-Kommission Albert-Ludwigs-Universität, Freiburg) No 337/04. This study was performed in accordance with the Helsinki Declaration of 1964, as revised in 2013 and with EQUATOR guidelines[21]. Before enrollment, the patients received information regarding the purpose of the study and signed an informed consent. All patients were consecutively enrolled in two study centers (Department of Oral and Craniomaxillofacial Surgery/Translational Implantology and the Department of Prosthetic Dentistry, University Medical Center Freiburg, Germany). Screw-retained and cemented restorations were included. None of the patients had a known systemic disorder (e.g. diabetes mellitus) that could have affected the periodontal and peri-implant tissue conditions.

Inclusion criteria

Soft tissue samples were taken from patients with severe peri-implant disease around ceramic and titanium rough implants with indication for explantation, diagnosed by clinical investigation and radiographic bone destruction (bone loss of more than 2/3 of the implant length or mobility with or without suppuration) according to the definition by Lang et al.[22]. Prosthetically restored implants were included which had received the restoration more than 12 months ago.

Exclusion criteria

Immunosuppressed, irradiated patients or patients with current chemotherapy were excluded as well as patients under the age of 18. Nicotine users and patients with generalized active periodontal disease were excluded in the present study.

Retrieval and processing of biopsies

The retrieval of biopsies was performed as described earlier[7]. Briefly, the biopsies were obtained at the time of surgical removal of the implant. The intervention was performed under local anesthesia with Ultracain DS forte**. A circular incision and two releasing incisions mesial and distal of the implant were performed with a scalpel (15c)[††]. A mucoperiosteal flap was mobilized and a clamp and a scalpel were used to remove the inflammatory tissue as a biopsy which was histologically processed. For further processing the biopsies were fixed in 3,5 % neutral buffered Formalin[‡‡]. Subsequently, the biopsies were embedded in paraffin[§§] and cut with a rotary microtome into serial sections of 2 µm thickness using the Leica microtome[##].

Immunohistochemical Analysis

Sections were de-waxed and incubated in DIVA antigen retrieval solution*** at 60°C over night. The sections were incubated with a primary antibody for 30 min followed by incubation with Envision HRP labeled polymer[†††] for 30 min. Positive cells were detected using DAB substrate[†††]. The chosen antibodies[†††] were CD3 1:200 (T-lymphocyte), CD20 1:400 (B-lymphocyte), CD138 1:50 (plasma cell, clone MI15), CD68 1:200 (macrophage, clone PG-M1). Counterstaining was performed with hematoxylin[†††].

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Histological Analysis

The prepared serial sections were digitized via the Panoramic SCAN® device and investigated by a Panoramic Viewer (3DHISTECH, Budapest, Hungary). Every sample was filled covered with 20 randomly selected ROI's (Figure 1). The ROI's comprised a size of 500 µm x 800 µm at a magnification of 15.5x. Pictures of the ROI's were taken and antibody positive cells in the ROI's were counted using ImageJ (NIH, Bethesda, USA). The results were assessed by one for this evaluation trained investigator (JM). The investigator was blinded regarding clinical patient characteristics.

Statistical Analysis

For descriptive analyses, the mean, median, and standard deviation were computed. Box and stacked plots were used for graphical presentations.

Linear mixed regression models were used to check for differences among the log-transformed cell counts with regard to implant material (ceramic and titan), as well as differences among the cell counts with regard to cell type (CD3, CD20, CD68 and CD138) within each material. All of the aforementioned linear mixed models were adjusted for region of interest. To correct for the multiple testing problem, the results

of pairwise comparisons were adjusted by the method of Scheffe. The calculations were performed with the statistical software STATA 15.1.

Results

Biopsies of the peri-implant tissue were retrieved from 15 patients (age 34-88 years, 6 male/9 female) with severe peri-implantitis [8 ceramic implants (CI), 7 titanium implants (TI)] (see Table S1 in online Journal of Periodontology). The presence of macrophages, B-Lymphocytes, T-Lymphocytes and plasma cells was identified in all samples. Micrographs illustrating peri-implantitis lesions for both implant materials are presented in Figure 2. The predominant cell-type in peri-implantitis lesions around ceramic implants were plasma cells CD138 (mean 53 %), followed by T-lymphocytes CD3 (mean 32 %), B-lymphocytes CD20 (mean 10 %) and macrophages CD68 (mean 5 %) (Table 1). There was no significant difference regarding the total number of stained cells in peri-implantitis lesions around ceramic implants in comparison to titanium implants ($p > 0.05$) (Figure 3, see Table S2 in online Journal of Periodontology). In 249 of 300 (83 %) ROI, there was a co-appearance of CD3 and CD20 (see Figure S1 in online Journal of Periodontology). Interestingly, a high interindividual variation regarding the prevalence of the cell-type was observed in both materials. The range in the ceramic group was for CD3-positive cells 20-70 %, CD20-positive cells 3-22 %, CD68-positive cells 2-10 % and CD138-positive cells 12-69 % of all cells, whereas in the titanium group: CD3 17-85 %, CD20 3 -18 %, CD68 1- 26 %, CD138 1-73 % (see Table S2 in online Journal of Periodontology). Interindividual differences in the immunohistological cellular

composition of the biopsies evaluated might also hint to an influence of the patient-specific immune status rather than only the implant material used (Figure 4).

Discussion

The cellular composition of tissue with peri-implantitis around zirconia-based implants has never been explored before. The specific interaction of inflammatory cells in tissue with peri-implantitis and their impact on peri-implant osseous breakdown with regard to the implant material is still unknown[11,23]. The present pilot study demonstrated that there is a similar histological appearance of peri-implantitis lesions around ceramic and titanium implants in the soft tissue. The most predominant cells found for both materials were plasma cells (CD138) followed by T-lymphocytes (CD3). The mean distribution of cells observed in this study is in accordance with the results Carcuac and Berglundh presented for peri-implantitis around titanium implants (mean CD138: 33 %, CD 3: 21 %, CD 68: 11 %, CD 20: 7 %)[11]. However, the latter authors provided mean values and analyzed solely titanium samples. Information regarding individual variations of the patients' immune response was not reported. In the present study, an interindividual distinction regarding cell-type frequency on the patient level was seen irrespective of the implant material. The current results may suggest that an immune response is associated with patient-specific parameters rather than with implant materials.

The present immunohistological data display the late phase of peri-implantitis (with indication of implant removal) with the differentiation of B cells and the existence of Antibody-secreting cells (plasma cells) verified by CD138 staining[24,25]. Antibody-secreting cells are fundamental in humoral immunity by secreting antibodies which

prevent bacterial adherence, promote phagocytosis and activate the complement system[26]. Interestingly, the T-cell arm of the immune response is activated in this stadium of peri-implantitis as well, demonstrated by the amount of expressed CD3-positive T-cells. T cells are able to differentiate into various different subsets. Basically, CD8-positive T cells showed a cytotoxic potential acting to kill cells that have been infected with intracellular microbes and tumors. CD4-positive T cells are designated as helper cells as they are responsible to regulate the cellular (B cell help) and humoral immune responses (delayed-type hypersensitivity)[27]. Since the aim of the present study was a general overview of inflammatory cell appearance in peri-implant tissue around two implant materials, a T-cell subset characterization was not performed. A detailed lymphocyte characterization should be performed in following studies to discriminate this lymphocyte-driven inflammation process.

Since variations regarding cell-type distribution on the patient level were detected, the present results may suggest an immune response associated with patient-specific parameters like implant biofilm/oral microbiome composition, different implant surface characteristics, different anatomical features like bone quality and soft tissue condition, different and/or combined etiology pathways and individual genetic and epigenetic immunological conditions [5,9,28]. Further, the histological samples may have been taken at a different stages of peri-implant inflammation, a classification between clinical diagnostic parameters and histological appearance seems difficult. In the field of periodontology it is well accepted that patient individual genetic and epigenetic patterns lead to a different immunological potential based on histone modification and DNA methylation with varying expression levels of cytokines, chemokines and toll-like receptors of the oral epithelia[28]. For peri-implantitis similar mechanisms are suspected but not sufficiently examined[29].

Orthopedic studies revealed typical histological patterns in low- and high-grade infections around orthopedic prostheses consisting of neutrophil granulocytes, plasma cells as well as small lymphocyte aggregates, whereas in particle-induced aseptic lesions macrophages and multinucleated giant cells can occupy more than 20 % of the lesion depending on particle size and configuration[10]. High amounts of macrophages (up to 26 %) in some patients of the present study may suggest a particle/ion related etiology for this patient subgroup. Tissue retrieval studies in orthopedics found a predominance of M1 macrophages in response to wear particles and an in-vitro study based on genome-wide microarray and a multiplex cytokine assay demonstrated that the response to titanium particles is determined by the state of macrophage polarization[30,31]. Recent studies demonstrated a specific immunological macrophage polarization pattern comparing periodontitis and peri-implantitis lesions whereas peri-implantitis lesions display a higher number of macrophages coupled with a distinct macrophage pro-inflammatory M1 polarization signature[12,13]. A particle-triggered mechanism involved in peri-implantitis lesions around titanium implants is being discussed, but an unidirectional causal relationship between titanium particles in the peri-implantitis lesions and onset or progression of peri-implantitis disease has not been proven[32,33]. Influence of metal particles and ions on peri-implant biofilm and their possible role in the development, formation and production of extracellular polysaccharides is discussed in current research[34]. In the peri-implant mucosa of zirconia ceramic implants, zirconia elements have been detected as well, however the origin and influence of metal or ceramic ions or particles on peri-implantitis remains unclear[17]. Furthermore, the host-response and interaction between lymphocyte- and monocyte-macrophage lineage as well as the influence of nano- and microparticles on the microbial biofilm and cytokine release in

peri-implant inflammation is not elucidated and future studies could shed more light on the etiological discussion[9].

Within this study, the first histological comparison of human peri-implantitis lesions around ceramic and titanium implants was performed. However, we have to acknowledge several limitations. First, this pilot study has only a small number of samples. Due to the limited sample size conclusions have to be drawn carefully, but could help optimize the study protocol in future studies with a larger sample size. In the current study Y-TZP implants of a single manufacturer and type (one-piece implants) were examined. To validate the present findings, peri-implantitis around different ceramic implant systems with different surface treatment like sandblasting, etching and special coatings of the implant as well as different modification of the implant material like alumina toughened zirconia (ATZ) should be considered in future clinical studies[35]. All implant samples for this investigation were obtained from patients with severe peri-implantitis (late phase of peri-implantitis) with the indication for removal of the implant. A comparison between clinical conditions at explantation and histological appearance cannot be performed, since the definition of Lang et al. was used as general endpoint in the present study[22]. In following studies the clinical conditions (e.g. attached Gingiva, prosthetic restoration, grafting procedures) and histological appearance could be considered as well as the clinical conditions at implant placement and at the time of implant removal compared. The complete clinical history of implants from placement to removal would be preferential but can only be acquired if the patients have not been referred solely for the surgical removal of the implant.

A conclusion concerning the etiology of peri-implantitis, conversion of mucositis into peri-implantitis, early peri-implantitis, histological progression pattern of peri-

implantitis cannot be derived from the available samples, mainly due to ethical concerns of sample harvesting.

Within this investigation, it could be demonstrated that the immunohistological cellular composition seems to exhibit interindividual differences and is not only associated with the implant material. Profound research with a higher patient sample size is, therefore, needed for thorough understanding of the pathogenesis of peri-implantitis in general and of peri-implantitis around ceramic implants in particular. With such knowledge, the clinicians might find an optimal treatment modality to manage this difficult-to-treat disease.

Conclusion

Soft tissues obtained from peri-implantitis lesions around ceramic implants in comparison with titanium implants seem to have a similar histological appearance at time of implant removal. Differences in the immunohistological cellular composition of the biopsies evaluated might also hint to an influence of the patient-specific immune status rather than only the implant material used.

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Immunohistological analysis. Review & Editing. RC: Review & Editing. DH: Statistics. All authors corrected and approved the final manuscript.

Conflict of interests

The authors declared no conflicts of interest concerning the research, authorship, and/or publication of this article. The study was supported by an Osteology Young Researcher Grant (16-133) of the Osteology Foundation, Lucerne, Switzerland. The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the paper. The authors do not have any financial interests, either directly or indirectly, related to the products or information discussed in the paper.

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Figure legends

Figure 1 – Histomorphometric analysis was performed in 20 randomly selected ROI's. The ROI's comprised a size of 500 µm x 800 µm at magnification of 15,5x. Pictures of the ROI's were taken and positive cells in the ROI's were counted using ImageJ. The results were evaluated by one histological trained investigator (JM).

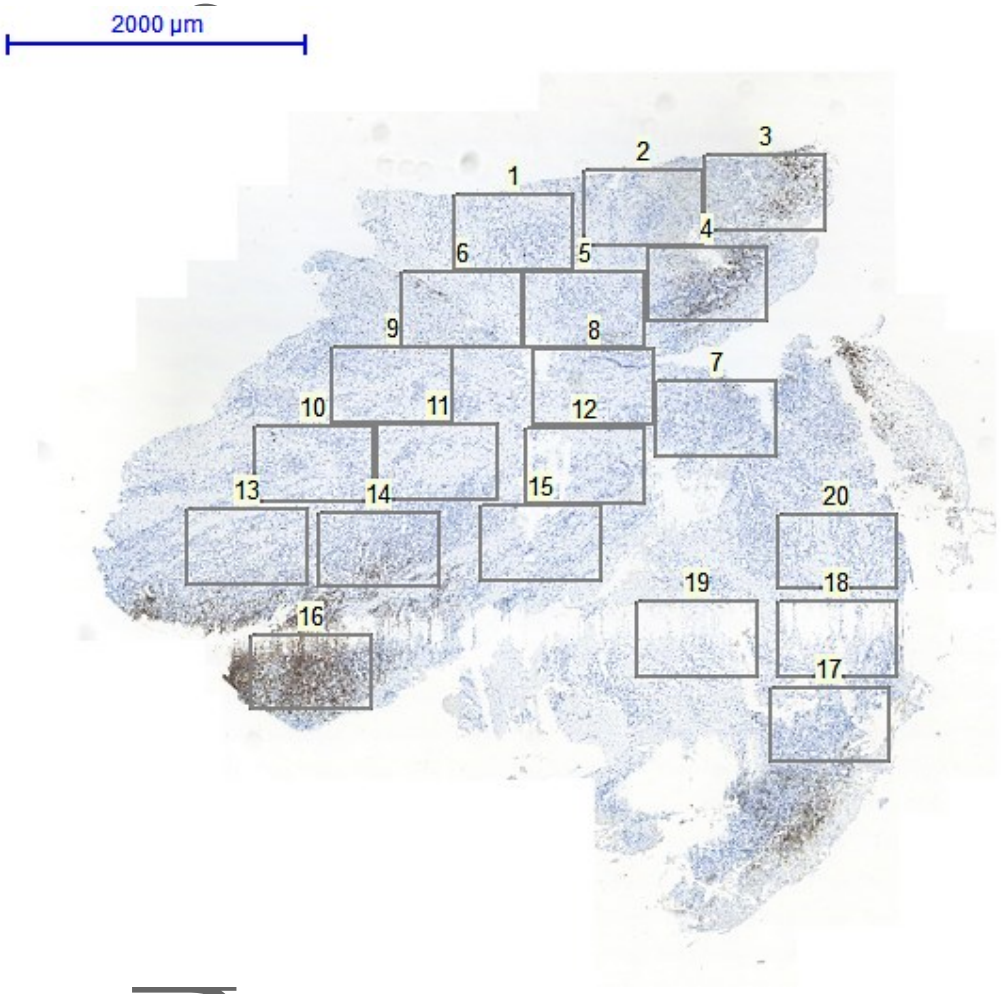


Figure 2 – Biopsies retrieved from peri-implantitis tissue around titanium and ceramic implants. Staining was performed using hematoxylin and immunostaining against CD3, CD20, CD68 and CD138. Magnification 5x and 40x.

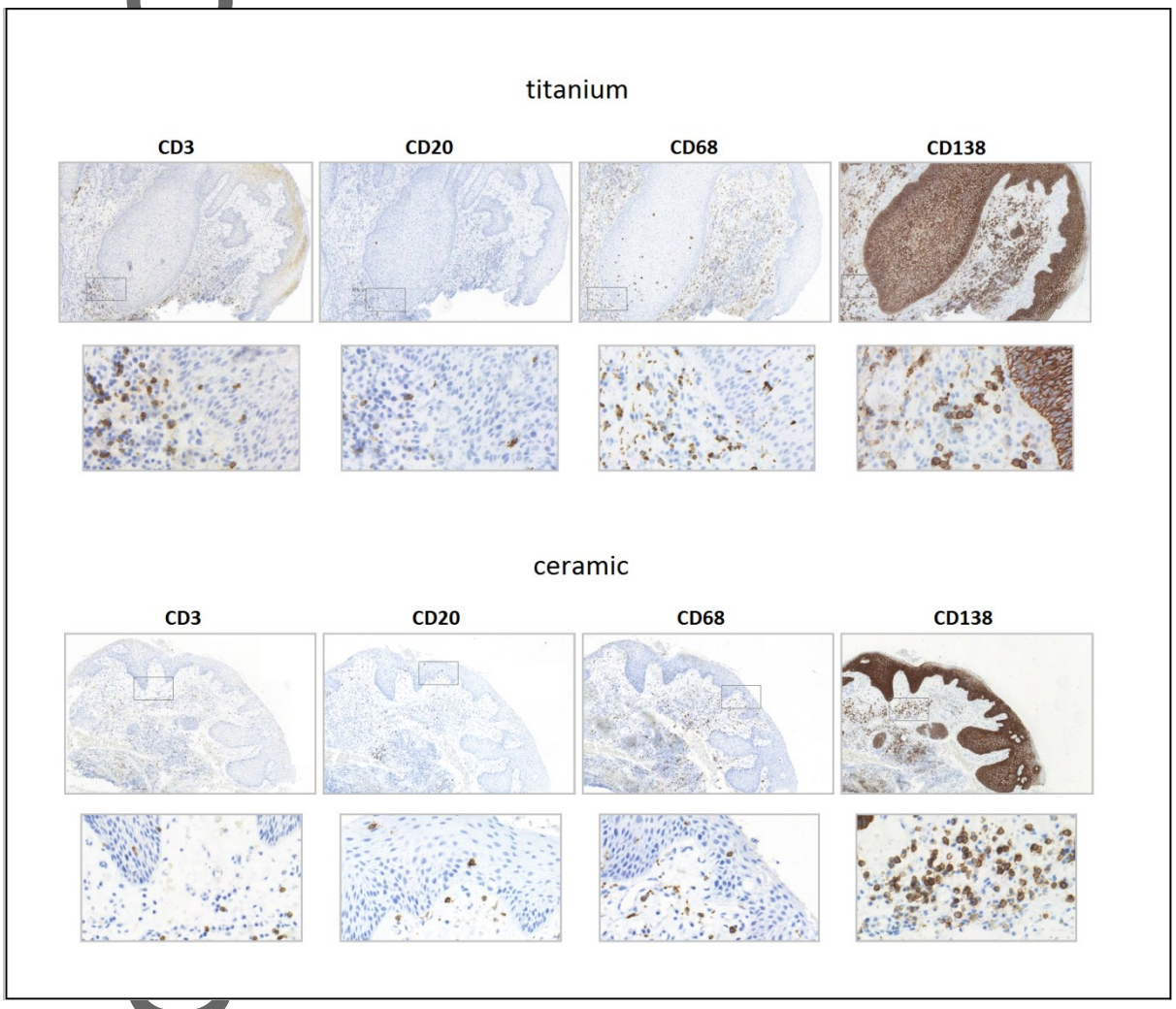


Figure 3 – Biopsies derived from tissue around ceramic and titanium implants demonstrated the same numerical distribution of cell population (CD 3, CD 20, CD 68, CD 138). Cell count of each antibody in peri-implantitis around ceramic (blue) and titanium (green) implants.

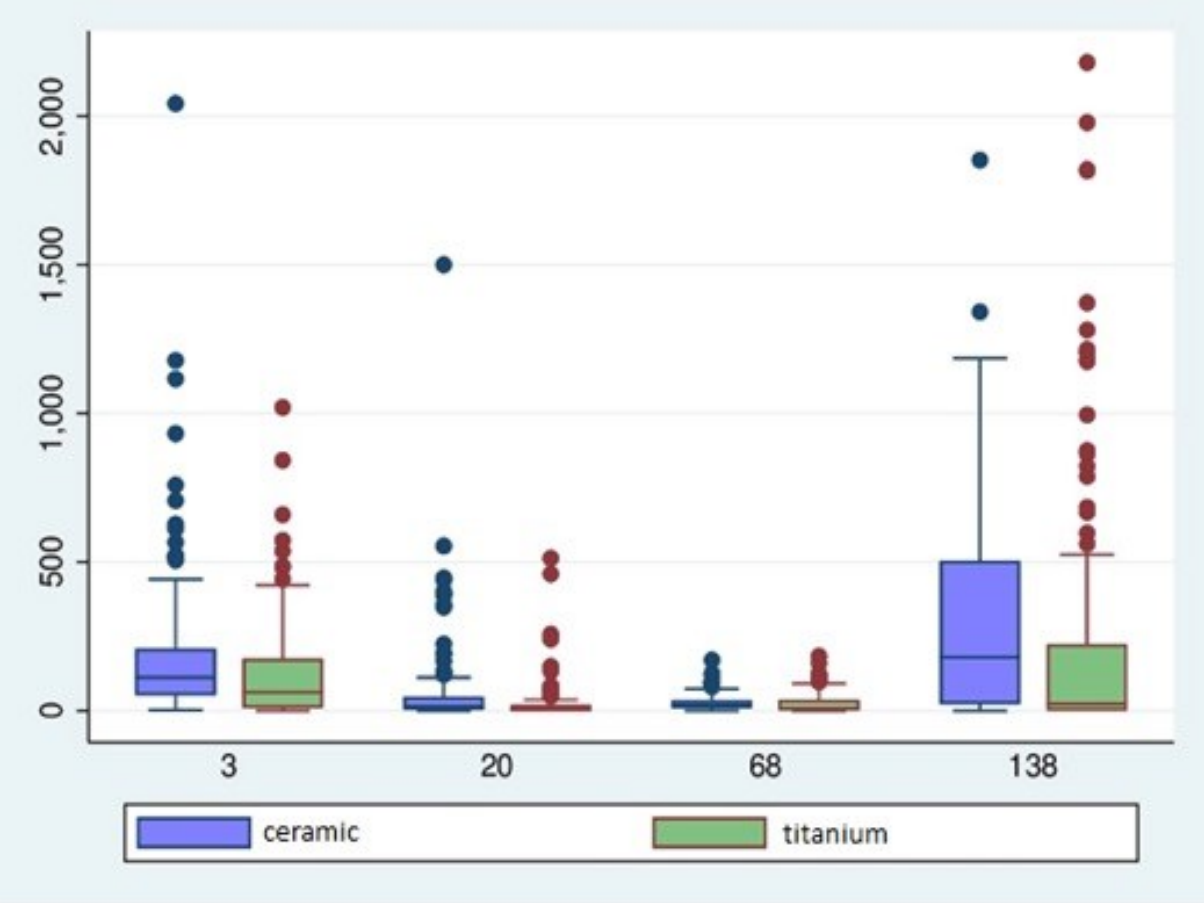
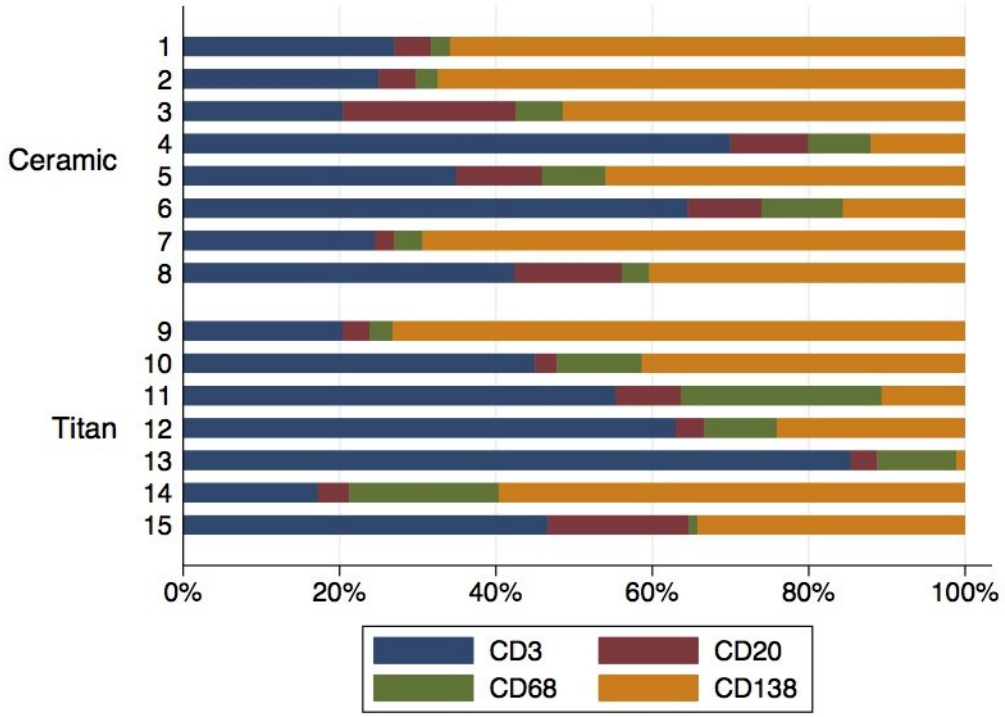


Figure 4 – Stacked plot demonstrated a patient-specific immune response. Percentage distribution of cells stained with CD3, CD20, CD68 and CD138 for each patient (Patient 1-15).



	N	p50	mean	sd	min	max
titanium						
CD3	7	928	2483	2395	34	6215
CD20	7	179	477	634	2	1663
CD68	7	431	489	435	5	1032
CD138	7	2663	4612	7985	11	22340
ceramic						
CD3	8	2915	3755	2241	2324	8980
CD20	8	533	1165	1212	340	3283
CD68	8	519	529	252	238	907
CD138	8	7111	6128	4397	419	13477

Table 1 –Total cell count for each antibody used: p50, standard deviation (sd), mininimum (min) and maximum (max) cell count.