Int. J. Oral Maxillofac. Surg. 2019; 48: 560–565
https://doi.org/10.1016/j.ijom.2018.10.017, available online at https://www.sciencedirect.com

International Journal of Oral & Maxillofacial Surgery

Clinical Paper
Dental Implants

Experimental peri-implant mucositis around titanium and zirconia implants in comparison to a natural tooth: part 2—clinical and microbiological parameters

K. Clever[1], K. A. Schlegel[1], H. Kniha[2], G. Conrads[3], L. Rink[4], A. Modabber[5], F. Hölzle[5], K. Kniha[5]
[1]Department of Oral and Cranio-Maxillofacial Surgery, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Erlangen, Germany; [2]Private Dental Clinic Drs. Kniha and Gahlert, Munich, Germany; [3]Division of Oral Microbiology and Immunology, Department of Operative Dentistry, Periodontology and Preventive Dentistry, University Hospital RWTH Aachen, Aachen, Germany; [4]Institute of Immunology, University Hospital RWTH Aachen, Aachen, Germany; [5]Department of Oral and Cranio-Maxillofacial Surgery, University Hospital RWTH Aachen, Aachen, Germany

K. Clever, K.A. Schlegel, H. Kniha, G. Conrads, L. Rink, A. Modabber, F. Hölzle, K. Kniha: Experimental peri-implant mucositis around titanium and zirconia implants in comparison to a natural tooth: part 2—clinical and microbiological parameters. Int. J. Oral Maxillofac. Surg. 2019; 48: 560–565.

Abstract. The aim of this study was to assess the clinical and microbiological parameters around dental zirconia and titanium implants compared with natural teeth during experimental plaque accumulation. Clinical parameters were evaluated (gingival index, plaque index, bleeding on probing, and probing pocket depth). Microbiological samples were analyzed for total bacterial cell counts, as well as *Tannerella forsythia* and *Prevotella intermedia* counts. A statistically significant difference over time was observed in the groups in terms of the gingival index ($P < 0.001$), plaque index ($P < 0.001$), and bleeding on probing ($P = 0.039$). The lowest mean total number of bacterial cells was measured around the teeth, followed by the zirconia implants; the highest values were found around the titanium implants. *T. forsythia* and *P. intermedia* values showed significant changes over time and sessions around the titanium implants. Compared to the soft tissues around zirconia implants and the teeth, those around titanium implants developed a stronger inflammatory response to experimental plaque accumulation in terms of the total number of bacterial cells and *T. forsythia* and *P. intermedia* values.

Key words: experimental reversible mucositis; zirconia; host response; plaque accumulation; titanium; dental implant.

Accepted for publication 23 October 2018
Available online 2 November 2018

The prevalence of peri-implant inflammation, which is associated with multiple factors including the level of oral hygiene, history of periodontal disease, smoking, age, and presence of rough surfaces, is as high as 54%[1]. A meta-analysis by Jepsen et al. estimated the prevalence of peri-implant mucositis to be 43% and of peri-implantitis to be 22%, this latter condition potentially leading to implant loss[1]. The development of dental implant materials that alleviate the risk of inflammation has always been desired. Laboratory investigations have shown that zirconia implants may have the ability to withstand oral forces[2]. The integration of magnesium, aluminium, or yttrium ions has led to an extremely stable and reliable product[3]. Obtaining further science-based clinical results on commercially available zirconia dental implants is therefore of great significance.

The primary aim of this prospective cohort study was to assess clinical parameters, i.e. the gingival index (GI), plaque index (PI), bleeding on probing (BOP), and probing pocket depth (PPD), during experimental plaque accumulation. In addition, it was aimed to evaluate the proportions and distribution of bacteria associated with soft tissue inflammation, as well as the resolution of inflammation around zirconia implants in comparison to titanium implants and natural teeth.

Materials and methods

Written informed consent was obtained from all patients included in this study. Examinations and data collection were performed at a single clinic, starting on January 13, 2015 and ending on December 15, 2015. The same examiner performed all assessments for clinical, immunological, and microbiological diagnosis during the entire study period. The study protocol was approved by the local ethics committee in accordance with the principles of the Declaration of Helsinki. Patients who had received one titanium implant and one zirconia implant were included in this prospective cohort study. The study complied with the STROBE statement (Strengthening the Reporting of Observational Studies in Epidemiology Statement)[4].

Inclusion criteria were: (1) patients who voluntarily signed the informed consent and data protection consent form; (2) patients with healthy or treated periodontal conditions; (3) patients who had received both a titanium implant (Standard Plus, tissue level; Straumann AG, Basel, Switzerland) and a zirconia implant (PURE Ceramic, tissue level; Straumann AG); (4) implants in function for at least 1 year, with a maximum of 5 years; (5) implants and control teeth without any clinical or radiological signs of inflammation, such as bone loss, bleeding on probing, or pocket depth >5 mm.

Exclusion criteria were: systemic disease; current irradiation therapy; severe bruxism or clenching habits; patients not motivated to perform adequate oral hygiene; patients who smoked; physical or mental conditions that would interfere with the ability to perform adequate oral hygiene; age <18 years; pregnancy; patients treated chronically (i.e., for 2 weeks or more) with any medication known to affect periodontal status or the immune system (e.g., phenytoin, calcium antagonist, cyclosporine, coumadin, and non-steroidal anti-inflammatory drugs); untreated periodontal condition or implants with a history of peri-implantitis; use of systemic antibiotics within the past 3 months.

Prior to the experimental phase of the study, the patients were treated with oral prophylaxis and given instructions on perfect oral hygiene for both the upper and lower jaws. The patients were also instructed and asked to perform optimal tooth cleaning twice a day for 4 weeks using the method described by Bass in 1954[5] (Table 1). Each patient was assessed for their ability to perform proper plaque control before the experimental phase was commenced. At baseline, the deepest site around each unit (tooth and both implants) was chosen for later analysis. The patients were then asked to refrain from oral hygiene practices in the upper jaw for a period of 2 weeks. Subsequently, they were again treated with oral prophylaxis, given hygiene instructions, and requested to practice optimal tooth cleaning for the next 4 weeks (Table 1). Parameter assessment was performed on one zirconia implant, one titanium implant, and one natural tooth. The same unit was used in each investigation. All crevicular fluid samples were evaluated at a university clinic. Sterile rubber gloves and sterile instruments were used for sample assessment to avoid contamination of the samples.

Clinical parameters

The GI (Löe: score 0 = normal gingiva, 1 = mild inflammation, 2 = moderate inflammation, and 3 = severe inflammation), PI (Löe: score 0 = no plaque, 1 = plaque not visible but verified with a probe, 2 = visible plaque, and 3 = massive plaque), GI and PI adapted for dental implants (mGI, mPI; Mombelli et al.), and the BOP index (Saxer and Mühlemann: score 0 = no bleeding, 1 = isolated bleeding, 2 = confluent linear bleeding, and 3 = severe bleeding) were investigated (sessions 2–8)[6–8]. In addition, the PPD was assessed from the bottom of the sulcus to the gingival/mucosal margin using a plastic Michigan periodontal probe at four points around each unit (sessions 2, 4, and 8).

Table 1. Study design for the experimental gingivitis/mucositis.

Week	0	4	4	5	6	7	8	9	10
Session	1		2	3	4	5	6	7	8
	Oral prophylaxis, oral hygiene instructions	Perfect oral hygiene	Baseline measurements obtained; oral hygiene cessation started	Oral hygiene cessation week 1	Oral hygiene cessation week 2	Reinstitution of perfect oral hygiene, oral prophylaxis, oral hygiene instructions			
			PI GI BOP CF PPD	PI GI BOP CF	PI GI BOP CF PPD	PI GI BOP CF	PI GI BOP CF	PI GI BOP CF	PI GI BOP CF PPD

PI, plaque index; GI, gingival index; BOP, bleeding on probing; CF, crevicular fluid sampling, PPD, probing pocket depth.

Subgingival/submucosal plaque sampling and DNA extraction

The deepest probing pocket depth around each unit (implants and tooth) was sampled for 30 s with sterile paper points (29 mm, ISO 25; VDW, Munich, Germany). The samples were taken from the same unit site at each session (sessions 2–8). No buffer was used for the bacterial samples. The samples were then stored in 1.5-ml Eppendorf tubes (VWR International GmbH, Langenfeld, Germany) at −80 °C.

In the laboratory, the paper point samples were re-hydrated by adding 200 µl sterile double-distilled water. The cells were recovered by adding five glass beads (1.0 mm diameter) and vortexing for 2 min. The paper tips were discarded and the microbial cells were recovered by centrifugation at 7000 rcf for 3 min. The supernatant was discarded and 20 µl of a lysozyme/mutanolysin solution (0.3 mg lysozyme + 10 U mutanolysin) was added to the pellet, with subsequent incubation for 30 min at 37 °C. Subsequent DNA extraction and purification were performed using the QIAmp DNA Mini Kit (tissue protocol; Qiagen, Hilden, Germany) as per the manufacturer's instructions. The DNA concentration (A260) and purity (A260/A280) were calculated using a GeneQuant II photometer (Pharmacia Biotech, Cambridge, UK). DNA was stored at −20 °C until further use.

Determination of the entire bacterial load and quantification of the dysbiosis markers *Tannerella forsythia* and *Prevotella intermedia*

The total bacterial amount, as well as the amounts of *T. forsythia* and *P. intermedia*, which are both oral Gram-negative anaerobic bacteria associated with the first symptoms of periodontal diseases, were determined by real-time quantitative polymerase chain reaction (RTQ-PCR) on a LightCycler 2.0 instrument (Roche Applied Science, Penzberg, Germany) using LightCycler FastStart DNA Masterplus SYBR Green I in a total volume of 20 µl. The final reactions contained 0.5 µM of each primer and 2 µl of template DNA (approximately 50 ng). For the quantification of all bacteria, the broad-range bacterial forward primer 5′-TCCTACGGGAGGCAGCAGT-3′ and reverse primer 5′-GGACTACCAGGGTATCTAATCCTGT-3′ were used[9]. This primer pair encompasses the V3 and V4 regions of the 16S rRNA gene and is proven to cover many oral phyla. The temperature profile for the universal PCR was as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 25 s, as well as a fluorescence read at 80 °C.

The assay primers for the quantification of *T. forsythia* were PF1 5′-AGAGTTTGATCCTGGCTCAG-3′[10] and Tf-1 5′-TGCTTCAGTGTCAGTTATACCT-3′[11,12]; primers for the quantification of *P. intermedia* were PF1, as above, and Pi-1 5′-GTTGCGTGCACTCAAGTCCGCC-3′[13]. The temperature profile used was as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 10 s, 56 °C for 10 s, and 72 °C for 20 s, as well as a fluorescence read at 75 °C.

Melting curve analysis and gel electrophoresis of the amplification products on 1% agarose gel were performed to confirm reaction specificity. In addition, the PCR products were spot-check sequenced to verify identity in the case of the species-specific PCRs. Quantification was performed based on the automated (default) algorithm of the LightCycler software, a strategy that calculates the crossing point (Cp) as the first maximum of the second derivative of the amplification curve. The conversion of Cp values to the initial gene target molecule numbers was based on the dilution series of the target DNA (calibration standards) with defined target molecule amounts. The preparation of calibration standards, the detection range, and amplification efficiency has been described in detail previously[14].

Study size

The sample size was calculated using G*Power version 3.1.9.2 software (G*Power, Düsseldorf, Germany) and analysis of variance (ANOVA) for repeated measures within factors using $\alpha = 0.05$. The time and sampling units were analyzed at the patient level. It was calculated that there was an 85% chance of correctly rejecting the null hypothesis of a significant effect existing for a total of 11 participants for the following question: What is the statistical power for detecting a significant difference in clinical parameter results over time (sessions 2 to 8)?

Statistical analysis

Analyses were performed using GraphPad Prism 6 software for Mac (GraphPad Software Inc., La Jolla, CA, USA) running on Apple OS X. Variables were analyzed using two-way ANOVA, comparing clinical parameters and microbiological values. Time point and tooth/implant type were included in the model, along with an interaction between the two factors, to determine if the response was different over time for the three types of surface. Additionally, a post hoc Tukey multiple comparison test was used to identify any differences between the mean values of the groups. Effects in the statistical model were considered significant if the corresponding *P*-value fell below the 5% margin.

Results

A total of 16 patients with 32 implants (16 zirconia and 16 titanium implants) were investigated. Seven were male and nine were female, and their mean age was 57 years at the time of study assessment (Table 2). The implants had been in function for an average of 2.1 years (minimum 1 year, maximum 5 years).

Clinical parameters

A statistically significant difference over time was observed in the groups in terms of GI ($P < 0.001$), PI ($P < 0.001$), and BOP ($P = 0.039$) (Fig. 1A–C). When the mean PI values were compared between the groups, a statistically significant difference was observed for session 4 (zirconia vs. tooth; $P = 0.001$). A significant difference between groups was found for the PPD in session 2 (zirconia vs. titanium and titanium vs. tooth; both $P = 0.001$) (Fig. 1D). PPD was significantly higher around the titanium implants than around the zirconia implants in session 4 ($P = 0.003$). Furthermore, PPD was significantly increased around the natural teeth between sessions 2 and 4 ($P = 0.003$). In addition, no statistically significant interactions between time and the sampling units for the clinical parameters were observed.

Table 2. Implant and tooth positions. All implants were placed in the upper jaw.

	Position														Total
	17	16	15	14	13	12	11	21	22	23	24	25	26	27	
Zirconia implant			2	1		3	1	1	3	2	1	2			16
Titanium implant		1	1	3	2	1		1	1		1	2	2	1	16
Tooth		1		1	5	1	1	1	3	1	2				16

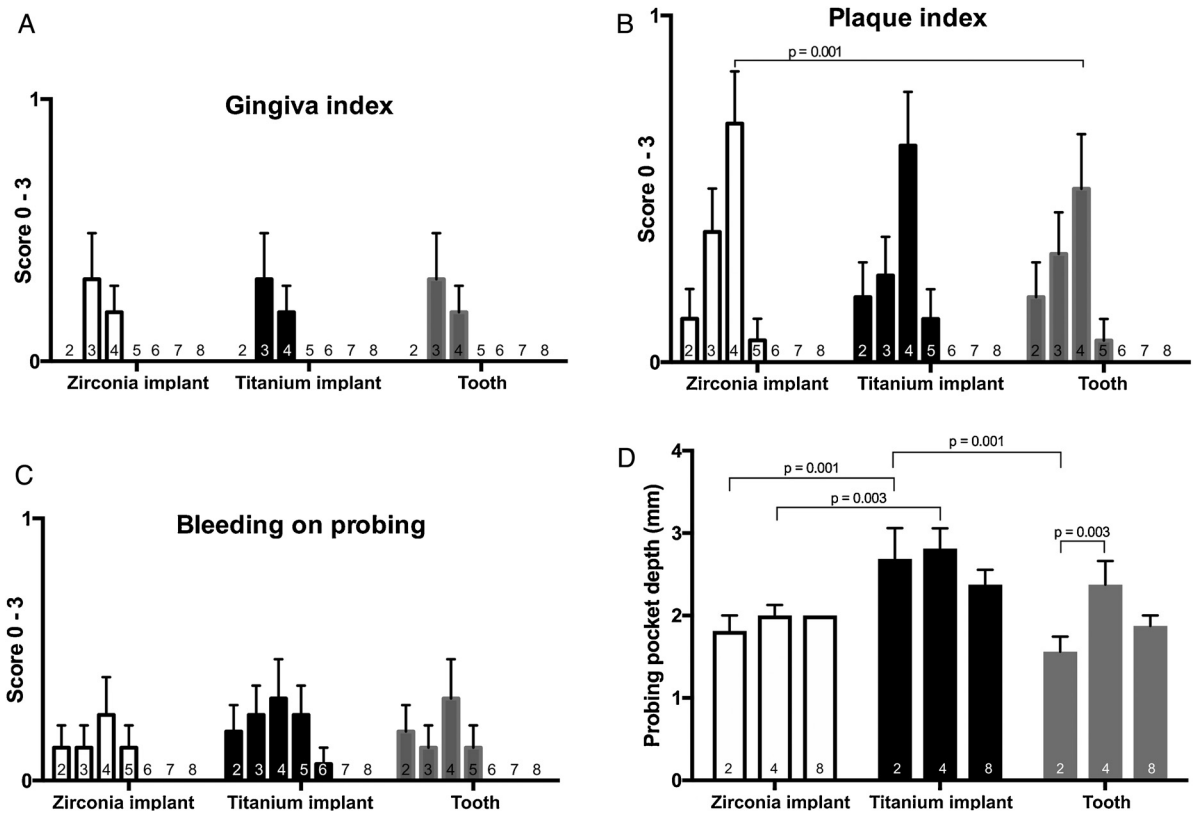

Fig. 1. (A) Assessment of the gingival index (mean values) for each zirconia implant, titanium implant, and natural tooth at sessions 2–8. (B) Assessment of the plaque index (mean values) for each zirconia implant, titanium implant, and natural tooth at sessions 2–8. (C) Assessment of bleeding on probing (mean values) for each zirconia implant, titanium implant, and natural tooth at sessions 2–8. (D) Assessment of the probing pocket depth (mean values) at four sites around each zirconia implant, titanium implant, and natural tooth at sessions 2, 4, and 8.

Microbiological parameters

There was a significant difference in total bacterial count between the groups in session 4: titanium vs. tooth, $P = 0.017$ (Fig. 2A). The lowest mean total number of bacterial cells was measured around the teeth, followed by the zirconia implants; the highest values were found around the titanium implants. With regard to *T. forsythia*-positive samples, significantly lower counts around the zirconia implants ($P = 0.021$) and the teeth ($P = 0.010$) were measured compared to the count around the titanium implants in session 4 (Fig. 2B). However, *T. forsythia* values around the titanium implants decreased significantly after 4 weeks of perfect oral hygiene ($P < 0.05$). With regard to *P. intermedia* counts (Fig. 2C), there were significant differences between the groups in session 4: zirconia vs. titanium ($P = 0.001$) and titanium vs. tooth ($P = 0.015$). Again, titanium implants showed the highest values after 2 weeks of plaque accumulation, with a significant decrease in values after 4 weeks of perfect oral hygiene.

In the case of high test values because of plaque accumulation, the value increase was reversed after 4 weeks of perfect oral hygiene practices for the microbiological parameters. *T. forsythia* and *P. intermedia* values showed significant changes over time and sessions around the titanium implants, whereas there was no significant change in numbers over time and sessions for the zirconia implants or the teeth during the experimental plaque accumulation; these numbers decreased with the resolution of inflammation. A statistically significant difference in *P. intermedia* values over time was evident ($P = 0.002$). No statistically significant interactions between time and the sampling units for microbiological parameters were observed.

Discussion

The objective of this prospective cohort study was to evaluate clinical parameters and the local expression of microbiological factors during soft tissue inflammation and subsequent resolution of the inflammation around teeth and two types of implant (titanium and zirconia). Patients initially ceased to perform oral hygiene practices and then reinstated optimal oral hygiene practices with mechanical plaque control. In this study design, comparisons were made between zirconia and titanium tissue-level implants and a natural tooth.

The process of inflammation around the teeth and implants is very complex and not understood in depth. BOP and PPD have been described as reliable parameters for developing and existing peri-implant infections[15]. Ata-Ali et al. presented data showing that PPD scores in the peri-implant mucositis group were significantly higher than those in the healthy group[16]. In this study, PPD was significantly deeper for titanium implants than for zirconia implants and the teeth, with the latter two showing similar measurements.

The interplay between bacterial colonization/invasion and the host response changes both microbiological and immunological factors significantly[17]. These changes are widely accepted indicators of the initiation and progression of periodontal diseases. With regard to the microbiome, the change from a commensal (friendly)

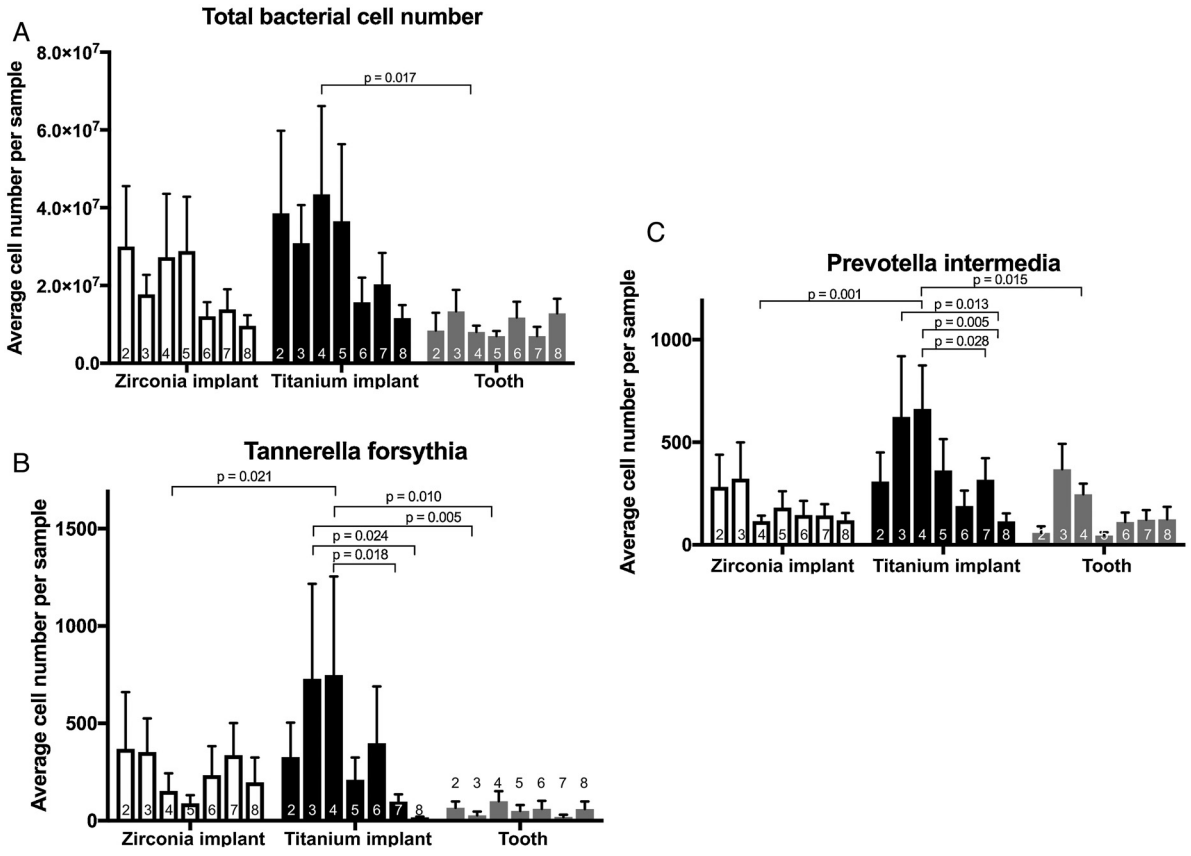

Fig. 2. (A) Assessment of the total number of bacterial cells around each zirconia implant, titanium implant, and natural tooth at sessions 2–8 (mean values). (B) Assessment of the number of *Tannerella forsythia* cells around each zirconia implant, titanium implant, and natural tooth at sessions 2–8 (mean values). (C) Assessment of the number of *Prevotella intermedia* cells around each zirconia implant, titanium implant, and natural tooth at sessions 2–8 (mean values).

group associated with the healthy state to a more periopathogenic (aggressive) group is seen[18]. The term dysbiosis was introduced to describe this shift. The major representatives of the early stage are, among others, *T. forsythia* and *P. intermedia*, which were chosen as the indicators in the present study[17]. The more prominent periopathogenic species, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, were not selected, as they are associated more with later-stage, non-reversible periodontitis and were therefore not likely to be present (except for traces) in the study samples[18,19].

Salvi et al. measured the total number of bacterial cells in a mucositis model comparing implants and the teeth, and they found a greater inflammatory response in the peri-implant mucosa[20]. The results for the total bacterial counts in the present study showed a significant difference between the groups (titanium vs. tooth at session 4). The lowest mean total number of bacterial cells was measured around the teeth, followed by the zirconia implants and the titanium implants. Salvi et al. showed that the frequency of detection of *P. intermedia* did not differ significantly between the implants and the teeth during experimental plaque accumulation[20]. In contrast, the present study revealed significantly lower counts of *P. intermedia* and *T. forsythia* around the teeth and the zirconia implants compared with the titanium implants.

A critical consideration with regard to this study is that the two different implants were not placed at the same appointment. Nevertheless, no implant presented any signs of infection prior to the investigation.

In conclusion, the soft tissues around titanium implants developed a stronger inflammatory response to experimental plaque accumulation than those around zirconia implants and natural teeth in terms of the total number of bacterial cells and numbers of *T. forsythia* and *P. intermedia*. Results for the natural teeth were more favourable than those for both implant types.

Acknowledgements. This work was performed at the Friedrich-Alexander-University Erlangen-Nürnberg (FAU) in fulfilment of the requirements for a Dr. med. dent. degree (Kim Clever).

Funding. The authors declare no sources of funding.

Competing interests. The authors declare no conflicts of interest.

Ethical approval. Ethical approval was obtained (LMU University Munich 164–16).

Patient consent. Written patient consent was obtained.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijom.2018.10.017.

References

1. Jepsen S, Berglundh T, Genco R, Aass AM, Demirel K, Derks J, Figuero E, Giovannoli JL, Goldstein M, Lambert F, Ortiz-Vigon A, Polyzois I, Salvi GE, Schwarz F, Serino G, Tomasi C, Zitzmann NU. Primary prevention of peri-implantitis: managing peri-implant mucositis. *J Clin Periodontol* 2015;**42**(Suppl 16):S152–7.
2. Silva NR, Coelho PG, Fernandes CA, Navarro JM, Dias RA, Thompson VP. Reliability of one-piece ceramic implant. *J Biomed Mater Res B Appl Biomater* 2009;**88**:419–26.
3. Rocchietta I, Fontana F, Addis A, Schupbach P, Simion M. Surface-modified zirconia implants: tissue response in rabbits. *Clin Oral Implants Res* 2009;**20**:844–50.
4. Vandenbroucke JP, von Elm E, Altman DG, Gotzsche PC, Mulrow CD, Pocock SJ, Poole C, Schlesselman JJ, Egger M. Strengthening the Reporting of Observational Studies in Epidemiology (STROBE): explanation and elaboration. *Epidemiology* 2007;**18**:805–35.
5. Bass CC. An effective method of personal oral hygiene. *J La State Med Soc* 1954;**106**:57–73. contd.
6. Mombelli A, van Oosten MA, Schurch Jr E, Land NP. The microbiota associated with successful or failing osseointegrated titanium implants. *Oral Microbiol Immunol* 1987;**2**:145–51.
7. Löe H. The gingival index, the plaque index and the retention index systems. *J Periodontol* 1967;**38**(Suppl):610–6.
8. Saxer UP, Mühlemann HR. [Motivation and education]. *SSO Schweiz Monatsschr Zahnheilkd* 1975;**85**:905–19.
9. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 2002;**148**:257–66.
10. Edwards U, Rogall T, Blocker H, Emde M, Bottger EC. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* 1989;**17**:7843–53.
11. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 1996;**11**:266–73.
12. Slots J, Ashimoto A, Flynn MJ, Li G, Chen C. Detection of putative periodontal pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. *Clin Infect Dis* 1995;**20**(Suppl 2):S304–7.
13. Conrads G, Flemmig TF, Seyfarth I, Lampert F, Lutticken R. Simultaneous detection of *Bacteroides forsythus* and *Prevotella intermedia* by 16S rRNA gene-directed multiplex PCR. *J Clin Microbiol* 1999;**37**:1621–4.
14. Vianna ME, Horz HP, Conrads G, Zaia AA, Souza-Filho FJ, Gomes BP. Effect of root canal procedures on endotoxins and endodontic pathogens. *Oral Microbiol Immunol* 2007;**22**:411–8.
15. Lang NP, Wilson TG, Corbet EF. Biological complications with dental implants: their prevention, diagnosis and treatment. *Clin Oral Implants Res* 2000;**11**(Suppl 1): 146–55.
16. Ata-Ali J, Flichy-Fernandez AJ, Ata-Ali F, Penarrocha-Diago M, Penarrocha-Diago M. Clinical, microbiologic, and host response characteristics in patients with peri-implant mucositis. *Int J Oral Maxillofac Implants* 2013;**28**:883–90.
17. Sekot G, Posch G, Messner P, Matejka M, Rausch-Fan X, Andrukhov O, Schaffer C. Potential of the *Tannerella forsythia* S-layer to delay the immune response. *J Dent Res* 2011;**90**:109–14.
18. Huang S, Li R, Zeng X, He T, Zhao H, Chang A, Bo C, Chen J, Yang F, Knight R, Liu J, Davis C, Xu J. Predictive modeling of gingivitis severity and susceptibility via oral microbiota. *ISME J* 2014;**8**:1768–80.
19. Lie MA, van der Weijden GA, Timmerman MF, Loos BG, van Steenbergen TJ, van der Velden U. Oral microbiota in smokers and non-smokers in natural and experimentally-induced gingivitis. *J Clin Periodontol* 1998;**25**:677–86.
20. Salvi GE, Aglietta M, Eick S, Sculean A, Lang NP, Ramseier CA. Reversibility of experimental peri-implant mucositis compared with experimental gingivitis in humans. *Clin Oral Implants Res* 2012;**23**:182–90.

Address:
Kristian Kniha
Department of Oral and Cranio-Maxillofacial Surgery
University Hospital RWTH Aachen
Pauwelsstraße 30
52074 Aachen
Germany
Tel.:+49 2418088258
E-mail: kkniha@ukaachen.de