The clinical, radiological, microbiological, and molecular profile of the skin-penetration site of transfemoral amputees treated with bone-anchored prostheses

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Abstract: The breach of the skin barrier is a critical issue associated with the treatment of individuals with transfemoral amputation (TFA) using osseointegrated, percutaneous titanium implants. Thirty TFA patients scheduled for abutment exchange or removal were consecutively enrolled. The aims were to determine the macroscopic skin signs, the presence of bacteria and the gene expression in abutment-adherent cells and to conduct correlative and comparative analyses between the different parameters. Redness and a granulation ring were present in 47% of the patients. Bacteria were detected in 27/30 patients, commonly in the bone canal. *Staphylococcus aureus*, coagulase-negative staphylococci, streptococci, and *Enterococcus faecalis* were the most common. A positive correlation was found between TNF-α expression and the detection of *S. aureus*. *Staphylococcus aureus* together with other bacterial species revealed a positive relationship with MMP-8 expression. A negative correlation was demonstrated between the length of the residual femur bone and the detection of a granulation ring and *E. faecalis*. A positive correlation was revealed between fixture loosening and pain and the radiological detection of endosteal bone resorption. Fixture loosening was also correlated with the reduced expression of interleukin-10 and osteocalcin. It is concluded that several relationships exist between clinical, radiological, microbiological, and molecular assessments of the percutaneous area of TFAs. Further long term studies on larger patient cohorts are required to determine the precise cause-effect relationships and unravel the role of host-bacteria interactions in the skin, bone canal and on the abutment for the longevity of percutaneous implants as treatment of TFA. © 2016 The Authors Journal of Biomedical Materials Research Part A Published by Wiley Periodicals, Inc. J Biomed Mater Res Part A: 105A: 578–589, 2017.

Key Words: gene expression, infection, osseointegration, percutaneous, transfemoral amputees

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How to cite this article: Lennerås M, Tsikandylakis G, Trobos M, Omar O, Vazirisani F, Palmquist A, Berlin Ö, Brånemark R, Thomsen P. 2017. The clinical, radiological, microbiological, and molecular profile of the skin-penetration site of transfemoral amputees treated with bone-anchored prostheses. J Biomed Mater Res Part A 2017:105A:578–589.

INTRODUCTION

Clinical[1-4] and experimental[5] studies suggest that a percutaneous, osseointegrated prosthesis is an interesting and promising alternative for the treatment of amputees. During the past 20 years, a new method for the rehabilitation of individuals with transfemoral amputation (TFA) has evolved. Instead of suspension through a socket, the prosthetic leg is directly anchored to the residual femur by an osseointegrated, percutaneous implant.[6] The implant consists of a titanium screw called the "fixture", an extension called the "abutment" and an abutment screw that keeps the two components together. The fixture is implanted in the medullary cavity in first-stage surgery (S1), while the abutment, which is inserted in-second stage surgery (S2), usually 6 months later, penetrates the skin through the skin-penetration site (SPS). A similar concept is used for the direct anchorage of dental implants in the jaw[7] and hearing aids in the temporal bone[8] before its implementation in transfemoral and arm amputees.[9] The direct skeletal anchorage of the prosthetic leg results in an enhanced quality of life, improved function and fewer out-patient visits for the adjustment of the prosthesis compared with socket prostheses.[1,10] Since roentgen stereo photogrammetric analysis (RSA) of fixtures in TFAs have indicated the stable fixation of the fixture in the residual bone,[11] the scientific interest has converged on the SPS, where an interface of titanium, bone, and soft tissue comes into contact with the outer environment.

There is growing evidence that periprosthetic orthopedic infections have a biofilm origin, are frequently subclinical and culture negative, making the diagnosis and treatment challenging.[12]

Although only a few infections leading to fixture removal have occurred[13] in arm and leg amputees and the fixture has demonstrated a survival rate of 92% at 2 years[9] in transfemoral amputees, it can be expected that a percutaneous implant is at great risk and will become infected and fail. Our knowledge of the bacteriological profile of the SPS is still limited. Moreover, there is an absence of information on the biological mediators, for example, pro- and anti-inflammatory cytokines and markers of cell differentiation and matrix remodeling,[14-16] contributing to inflammation and repair of the tissues which interface the material components in the SPS.

The aims of this study of TFAs with bone-anchored amputation prostheses are first, to determine the frequency of macroscopic signs of inflammation in the SPS, to determine which bacteria are resident in the SPS, abutment and surrounding bone canal and to analyze the gene expression of inflammatory, bone, and soft-tissue markers in abutment-adherent cells and, secondly, to conduct correlative and comparative analyses between clinical signs, radiological findings, gene expressions, and the presence of different bacterial species.

MATERIALS AND METHODS
Implants and abutments

The materials used in this study are commercially available components (Integrum AB, Mölndal, Sweden) [Fig. 1(A,B)].

The fixture is made of commercially pure titanium (cp-Ti), the abutment screw of Ti6Al4V and the abutment of cp-Ti or Ti6Al4V. The topography, oxide thickness, morphology and crystallinity of the implants has been described elsewhere.[17] The surface morphology of the abutment was characterized by scanning electron microscopy and interference microscopy (Supporting Information Fig. 1).

Clinical data

Study design and patient demographics. This study was conducted as a cross-sectional uncontrolled study. During a 2-year period, 30 consecutive TFA patients with a percutaneous, osseointegrated implant who had to exchange (26 patients) or remove (4 patients) their abutment were enrolled. Patient data is found in Table I. Two of the thirty patients were bilateral femoral amputees treated on both sides with the implant system. In one of these patients, the abutment on one side was exchanged. In the other patient, both abutments were exchanged and one side was randomly selected to be included in the study. A total of 30 abutments in 30 patients were therefore included in the study. The reason for abutment exchange was mechanical problems (26 patients) of the abutments (i.e. wear, bending, cracking, or fracture of the abutment). One of these patients (ID 6) received flucloxacillin (1 g x 3) due to a culture verified superficial infection of the SPS. Three patients (ID 4, 12 and 15) were diagnosed with fixture loosening and had their complete implant system removed. One of the three latter patients (ID 4) had completed a course of antibiotics within two weeks prior to implant removal due to a superficial infection of the SPS. One patient (ID 5) had the abutment removed and the SPS closed (leaving only the fixture in place) because of hyperesthesia, phantom pain and pain upon loading.

Observations and sampling at abutment exchange or removal. The abutment exchange or removal was performed in the operating theatre under sterile conditions. The SPS and the visible part of the abutment protruding through the skin (distal of the bone canal) were washed with clorhexidine solution. After obtaining a swab for microbiology from the SPS [Fig. 1(C)], the abutment was removed [Fig. 1(F)]. A second swab was taken from the surface of the part of the abutment that had been located inside the bone canal [Fig. 1(D)]. The removed abutment was then placed in a tube containing RNA*later* Stabilisation Reagent (Qiagen, Germany) [Fig. 1(G)] for the subsequent extraction of RNA and protein. Only the part of the abutment that was inside the bone canal was covered by RNA*later*. Next, a third swab was obtained from the surface of the tissue in the canal between the distal bone and the abutment, here called bone canal [Fig. 1(E)].

The clinical appearance of the SPS was documented by photography and macroscopically observed clinical signs were documented by one observer. Using a modification of the so-called Holgers scoring system[18] for the assessment of soft tissue around bone-anchored hearing aids, a modified classification system was implemented [Fig. 1(H-K), Table
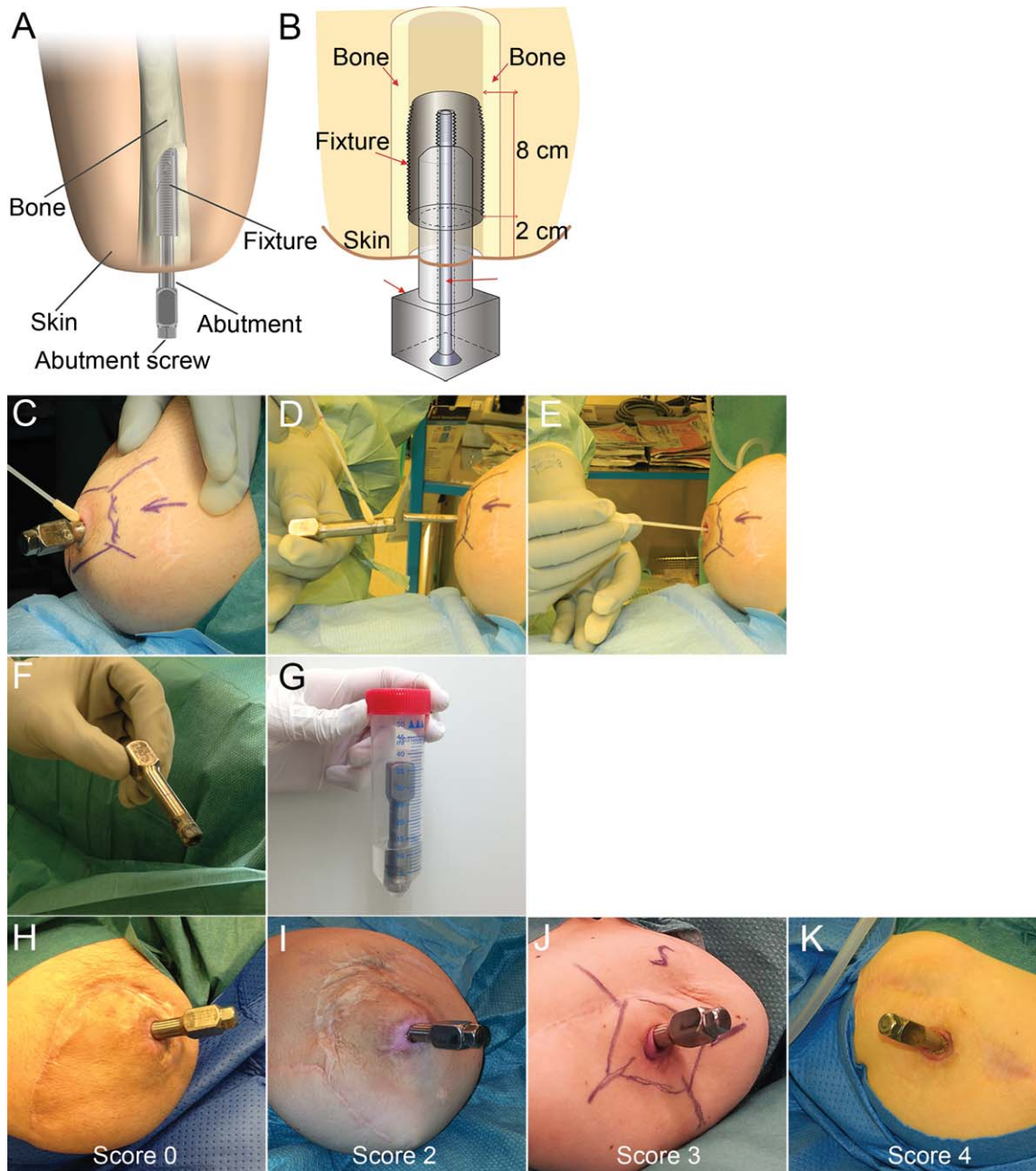
FIGURE 1. An overview of the implant components, the sampling procedures and the clinical assessment of the skin. (A, B) Schematic drawing showing the implant components and surrounding tissue. Abutments with a diameter of 11 mm and a length of 72–77 mm were machined from cp-Ti grade IV ($n = 29$) and titanium alloy (Ti6Al4V, grade V) ($n = 1$), cleaned by ultrasonication followed by sterilisation. (C-E) Bacterial swabs taken from skin-penetration site (C), abutment (D) and bone canal (E). (F, G) Retrieval of abutment for macroscopic inspection (F) and placement in a fixed volume of RNA*later* (G) for subsequent RNA and protein extraction. (H-K) Representative skin-penetration sites for the scoring system: H) Score 0: clean, dry, tight; I) Score 2: purple, serous secretion; J) Score 3: granulation ring, redness; K) Score 4: granulation ring, redness, serous secretion.

II]. The symptoms (e.g. pain) from the stump were also recorded. Moreover, the length of the residual femur (top of the trochanter major to distal femur) was recorded using radiographs. The most recent plain radiographs of the residual femur at the time of abutment exchange were examined and compared with the postoperative (S2) plain X-ray examination. The radiographs were examined qualitatively by one observer for signs of bone remodeling around the fixture (Fig. 2).[11] The mean time interval between the latest plain X-ray and the time of abutment exchange was 5.8 (SD 7.4) months. The study protocol was approved by the Regional Ethical Review Board of Gothenburg (Dnr 110–14; 2014–03-27). All subjects enrolled have responded to an Informed Consent which has been approved by the Ethical Review

TABLE I. Demographic Data on 30 Patients with TFA and Osseointegrated Bone-Anchored Amputation Prostheses Undergoing Exchange or Removal of Their Abutments

Variable	Population	Mean (SD)	Median (min; max)
Patients	30		
Unilateral	28		
Bilateral	2		
Gender (male/female)	24/6		
Age at abutment exchange (years)		51 (13)	53 (25; 76)
Time since first surgery (S1) (years)		6.6 (3.8)	5 (0.6; 16)
Time since last exchange of abutment (years)		4.4 (3.1)	3 (2; 13)
Estimated weight at inclusion (S1) (kg)		77 (18)	76 (48; 116)

Board. The protocol was found acceptable and signed by them.

Molecular analyses

RNA and protein isolation from cells adherent to the abutment. The retrieved abutment was transported in a vertical position and kept at 4°C until further analysis. The cells adhering to the surface of retrieved abutments in the bone canal were homogenised by RP1 lysis buffer and rough vortexing for three minutes. Total RNA and protein were extracted using a NucleoSpin RNA/protein kit (Macherey-Nagel, Germany).

Quantitative polymerase chain reaction (qPCR). Total RNA was normalised to 20 ng/μl before transcribed to cDNA. Primer design was performed using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The sequences are available at TATAA Biocenter AB, Gothenburg, Sweden. The following gene panel was used: calcitonin receptor (CR), cathepsin K (CatK), osteocalcin (OC), osteopontin (OPN) and Runx2 (bone markers); keratin (KRT6A), collagen I (Coll1a1) and collagen IV (Coll4a1), (soft-tissue markers); tumour

TABLE II. Modified Holgers Scoring System With Values Ranging From 0 to 6 Depending on the Appearance of the Skin-Penetration Site (SPS)

Inspection		
Skin color	Normal	0
	Purple	1
	Red	2
Moistness/secretion	No	0
	Serous	1
	Purulent	2
Granulation	No	0
	Yes	1
Fistulas	No	0
	Yes	1
Sum	0 - 6	

necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-1 beta (IL-1β), matrix metalloproteinase-8 (MMP-8), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and leukocyte esterase (ELA2) (markers of inflammation, matrix-degrading enzymes and inhibitor). Quantities of target genes were normalized using the mean of the reference genes 18S ribosomal RNA (18S), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ). The normalized relative quantities were calculated using the delta-delta Cq method and assuming 90% PCR efficiency ($k*1.9^{\Delta\Delta Cq}$).[19] The qPCR analysis was performed with a TATAA SYBR GrandMaster Mix (TATAA Biocenter AB) in 10 μL reactions on the CFX96 platform (Bio-Rad Laboratories, CA). The qPCR procedure was performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.[20]

Western blot. The protein concentration of the abutment adherent cells was determined using a Pierce®BCA protein assay kit (Thermo Fisher Scientific, USA). Equal amounts of proteins (100 μg) were loaded per well separated on a 10% Mini-Protean®TGX Precast Gel (Bio-Rad Laboratories, CA) and transferred to nitrocellulose membranes (Bio-Rad Laboratories). To prevent nonspecific binding, membranes were blocked with 2% nonfat milk powder in 1x Tris-buffered saline (TBS)-Tween at room temperature (RT) for 1.5 h. The membranes were incubated at RT for 1 h with HRP-conjugated protein A antibody (Abcam, Cambridge, UK), diluted 1:2500 in 2% nonfat milk powder in TBS-Tween. The membranes were washed 3 × 20 min and finally developed using an Immun-Star™WesternC™ chemiluminescence detection kit (Bio-Rad Laboratories), according to the instructions of the manufacturer. Digital detection was performed using the ChemiDoc XRS+ system with Image Lab Software (Bio-Rad Laboratories). The specificity of the antibody was confirmed in blot experiments using *S. aureus* (ATCC 25923) (positive control) and *S. epidermidis* (ATCC 35984) (negative control) derived extracellular vesicles.

Bacterial cultures

Samples were taken for bacterial cultures with a sterile cotton swab, transported in a coal-based medium and cultured on general purpose and selective media according to a routine procedure at Sahlgrenska University Hospital (Clinical Microbiology Lab, Sahlgrenska University Hospital, Gothenburg, Sweden). Briefly, swabs were streaked on five different agar plates: Columbia horse blood (incubated under aerobic and anaerobic conditions), Grand Lux (GL) plate (5% CO$_2$), *Streptococcus* plate (5% CO$_2$), chromogenic *Staphylococcus* plate and Drigalski plate, following incubation at 37°C for two to six days. A tube containing thioglycollate medium was inoculated with each swab for enrichment, in order to increase the detection, and incubated for six days at 37°C. All cultures were examined by experienced bacteriological analysts and the susceptibility of the clinical isolates to several antimicrobial agents was evaluated using the disk
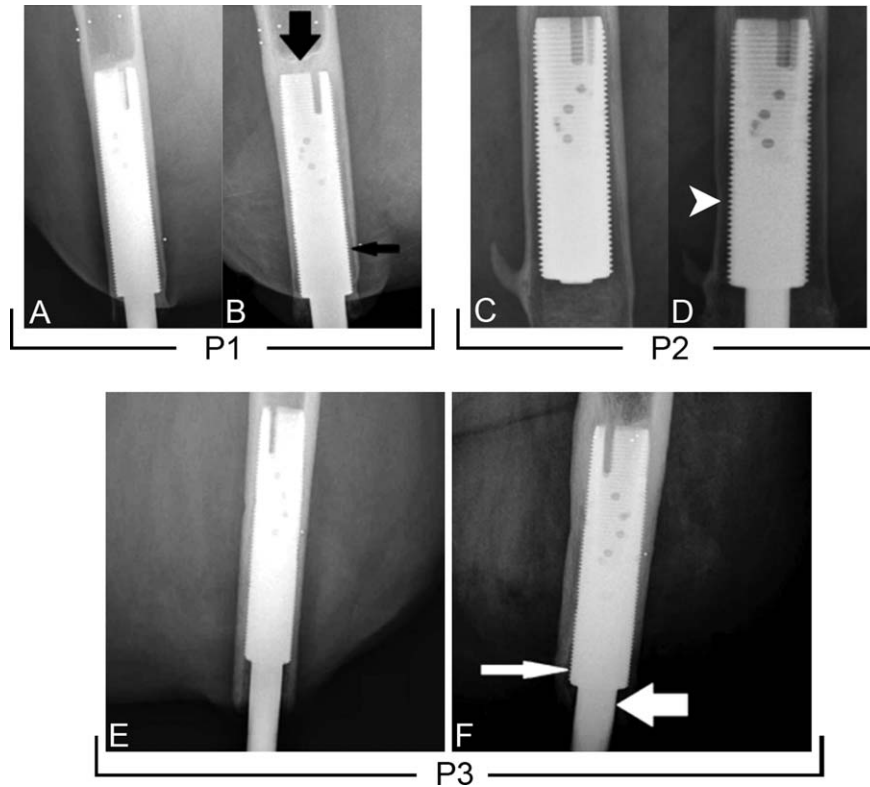
FIGURE 2. Radiological changes (A-F). (A) The early (after 3 years) postoperative X-ray from patient 1 (P1). (B) The corresponding X-ray at the 10-year post-operative follow-up of P1. Proximal trabecular buttressing (an increase of the bone density at the proximal end of the fixture) (thick, black arrow) and cancellisation (an increase in the porosity of the cortex surrounding the fixture) (thin, black arrow) have developed. (C) The early postoperative X-ray (after the first surgery, S1) from patient 2 (P2). (D) At two-years, a cortical thinning (a decrease in the width of the cortex around the fixture) (white arrow-head) is evident. (E) An early postoperative X-ray from patient 3 (P3) after the second surgery, S2. (F). The X-ray from patient 3 after seven years, demonstrates endosteal bone resorption (thin, white arrow) and distal bone resorption (thick, white arrow). Endosteal bone resorption is the resorption of endosteal bone around the threads of the fixture, while distal bone resorption is the resorption of the bone at the distal end of the fixture.

diffusion method. Further identification to species level was performed by MALDI-TOF mass spectrometry.

STATISTICS

For descriptive purposes the mean, standard deviation (SD), median, maximum, and minimum values are presented. The gene expression data were tested for normality, which revealed a general non-normal distribution for all analyzed genes. For this reason, nonparametric tests were considered for the correlational and comparative analyses. A Spearman correlation analysis was performed between the different continuous and categorical (ordinal or nominal) variables. All variables entered in the correlation analysis and the numbers of patients (n) under each variable are described in Supporting Information Table 1. Correlations of nominal variables were excluded if the number of observations within a variable was fewer than 3. Based on the correlation, variables that showed significant correlations with gene expression were further compared with respect to the gene expression of the different biological factors analysed in the study. Statistical analyses were performed using SPSS software (SPSS Statistics, v.15, IBM Corporation) and p-values of <0.05 were considered significant.

RESULTS
Surface analysis

A typical machined surface morphology was revealed by scanning electron microscopy, with scratches and ridges along the machining direction (Supporting Information Fig. 1). A homogenous surface structure was observed along the length of the abutment, with slightly smoother surface structure on flat surfaces, that is the hexagon and the

TABLE III. Clinical Signs of the Skin-Penetration Site (SPS) and Their Frequency

Clinical sign	No of patients (Frequency)	Percentage (%)*
Normal skin color	7	23
Purpleness	9	30
Redness	14	47
Serous secretion	5	17
Purulent secretion	3	10
Granulation ring	14	47
Fistulas	0	0

*$n = 30$.

TABLE IV. Modified Holgers Score

Modified Holgers score	No of patients
0	4
1	7
2	9
3	5
4	3
5	2
6	0
Total	30

square head. The smooth surface roughness was confirmed by the measurements of S_a 313 nm.

Clinical symptoms and macroscopic signs

Pain upon loading was experienced by 8/30 patients, whereas 3/30 patients experienced pain at rest. The most common clinical sign observed in the SPS was redness and a granulation ring, followed by purpleness and serous discharge (Table III). The most frequent modified Holgers score was 2 (on a scale of 0 to 6) with 30% (9/30) of the SPSs having a score of 2 and 67% (20/30) a score of 2 or lower (Table IV). Three of the 30 patients scheduled for abutment change had symptoms and macroscopic signs indicating fixture loosening and underwent removal of the entire implant system. The detailed description of these patients is found in Supporting Information. The clinical, radiological, and bacteriological analyses related to these three patients are provided in Supporting Information Table 2.

Radiology

The most common radiological change in the bone around the fixture was proximal trabecular buttressing (14 patients), followed by endosteal bone resorption (10 patients), cancellisation (2 patients) and cortical thinning (1 patient) (Table V).

Gene expression

The sampling and extraction of RNA from cells adhering to the abutment was successfully performed. The sampling procedure provided good RNA quality and detectable values for all selected genes with the exception of calcitonin receptor.

TABLE V. Radiological Changes

Radiological changes since S2	No of patients (Frequency)	Percentage (%)*
Unchanged	10	33
Endosteal bone resorption	10	33
Distal bone resorption	0	0
Cancellisation	2	7
Cortical thinning	1	3
Proximal trabecular buttressing	14	47

*$n = 30$.

Bacteriology

Positive bacterial cultures from routine microbiological procedures from at least one site were found in 27/30 patients. The most commonly found bacterial species were *Staphylococcus aureus* (14 patients; 47%), followed by streptococci (7 patients), *Enterococcus faecalis* (6 patients), coagulase-negative staphylococci (CoNS) (5 patients, including *Staphylococcus lugdunensis*) followed by a large range of different bacterial species (Table VI). The majority of positive bacterial cultures were obtained from the bone canal (40 positive cultures in 26 patients). Western Blot analysis of protein A on abutments demonstrated that 28 out of 30 abutments were positive for the protein (Figure 3). The two patients who did not show detection of protein A presented negative cultures, irrespective of sampling sites.

Correlation analyses

Several correlations between genes were revealed (Table VII). A positive relationship was demonstrated between inflammatory cytokine genes (TNF-α, IL-1β and IL-6). Furthermore, all analysed inflammatory cytokines revealed a positive correlation with TIMP-1. In addition, both inflammatory cytokines TNF-α and IL-6 showed positive correlations with ELA2. Also, a positive relationship was found between factors denoting tissue regeneration (Runx2 with Coll1a1 and Coll1a1with Coll4a1). Positive correlations were also found between factors denoting tissue regeneration and factors denoting tissue degradation and remodeling, such as Coll1a1 with CatK, OPN with CatK, OPN with MMP-8 and Runx2 with ELA2. A relationship was also detected between tissue degradation and remodelling factors (CatK and MMP-8). On the other hand, negative correlations were shown for the inflammatory cytokines (TNF-α and IL-1β) with tissue regeneration genes (Coll1a1 and Coll4a1).

When correlating the clinical parameters with gene expression activities (Table VIII), a significant negative correlation was found for the loosened fixtures with the expression levels of the bone related gene OC and the anti-inflammatory gene IL-10. Furthermore, a negative relationship was found between the time span since the S1 or placement of the abutment with the expression of OPN, Coll1a1, or Coll4a1.

When the correlation was performed between the different clinical parameters (Table IX), a positive relationship was shown between the loosened fixtures and the radiological detection of endosteal bone resorption. Moreover, a positive association was found between the fixture loosening and the reported pain symptom. The length of the residual femur bone demonstrated a negative correlation with the detection of granulation ring around the abutment.

With respect to bacterial colonisation (Table X), the analysis revealed that the detection of bacteria (irrespective of which species) at the SPS was positively correlated to the time span since the last abutment installation, as well as with the detection of secretion around the abutment, whereas the detection of bacteria in the canal was only positively correlated to the time span since S1 surgery. In addition, the detection of *Enterococcus faecalis* (irrespective of

TABLE VI. Bacterial Species Found Post Chlorhexidine Exposure at the Skin-Penetration Site (SPS), Abutment and Bone Canal of Patients ($n = 30$)

Bacteria	Number of patients (%)	SPS	Abutment	Bone canal
Staphylococcus aureus	14 (47%)	7	5	13
Coagulase-negative staphylococci (CoNS)	3 (10%)	0	1	3
Staphylococcus lugdunensis	2 (7%)	0	0	2
Group B *Streptococcus* (GBS)	4 (13%)	1	1	4
Group G *Streptococcus* (GGS)	1 (3%)	0	1	1
Group A *Streptococcus* (GAS)	1 (3%)	1	0	1
Alpha-haemolytic streptococci	1 (3%)	0	0	1
Enterococcus faecalis	6 (20%)	0	0	6
Enterobacter spp.	1 (3%)	0	1	1
Corynebacterium simulans	1 (3%)	1	1	1
Propionibacterium acnes	1 (3%)	0	0	1
Morganella spp.	1 (3%)	0	0	1
Haemophilus parainfluenzae	1 (3%)	0	0	1
Prevotella spp.	1 (3%)	0	0	1
Anaerobic mixed flora	2 (7%)	0	0	2
Gram-positive skin flora	2 (7%)	1	1	1
No growth	3 (10%)	0	0	0
Total number of positive cultures		11*#	11*#	40*#

*Several patients displayed the growth of ≥ 2 bacterial species at the same site. #Several patients displayed the growth of bacteria in more than one compartment.

site) was positively correlated to the presence of granulation tissue. A negative correlation was shown between the length of femur bone and the detection of *E. faecalis*.

The correlation between bacteria and gene expression (Table XI) revealed a positive correlation between the expression of TNF-α and the detection of *S. aureus* irrespective of site. Moreover, the detection of *S. aureus* together with any other bacterial species showed a positive relationship with the expression of MMP-8. A detection of a polymicrobial flora at the different sites was positively correlated to Runx2, IL-6 and MMP-8.

Comparative gene expression analyses

Based on the results of the correlation analysis, nominal bacterial or clinical variables that revealed significant correlations with gene expression were further evaluated by comparative analysis. When the subjects were divided into osseointegrated ($n = 27$) and loosened fixture ($n = 3$) groups, the comparative gene expression analysis revealed about a 3.5-fold lower expression of the OC in the loosened fixture group compared with the osseointegrated one [Fig.
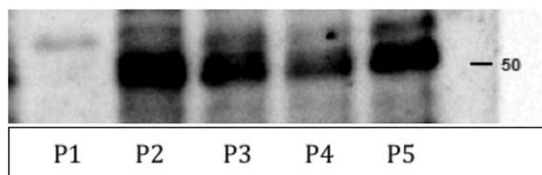

FIGURE 3. Western blot analysis of protein A on the abutment. Examples of positive and negative *Staphylococcus aureus* protein A detection on abutment. P1: One patient of two who did not demonstrate positive detection of protein A. P2-P4: Four patients (P2-P4) with positive bands of *Staphylococcus aureus* protein A (≈ 50 kDa) detected in 28/30 patients.

4(A)]. Furthermore, the loosened fixture group showed a seven-fold lower expression of the anti-inflammatory cytokine IL-10 compared with the osseointegrated group [Fig. 4(B)]. When the patients were divided based on the detection ($n = 14$) or absence ($n = 16$) of *S. aureus*, a two-fold higher expression of TNF-α was registered in the group with viable *S. aureus* detected [Fig. 5(A)]. This was further verified when the patients were divided into three groups:

TABLE VII. Correlation Analysis of Genes Expressed in the Abutment-Adherent Cells

Positive correlations			Negative correlations		
Genes	(r)	(p)	Genes	(r)	(p)
TNF-α/IL-1β	0.85	<0.01	TNF-α/OPN	−0.426	<0.05
TNF-α/IL-6	0.731	<0.01	IL-1β/OPN	−0.46	<0.05
TNF-α/TIMP-1	0.487	<0.01	IL-1β/Coll1a1	−0.39	<0.05
TNF-α/ELA-2	0.365	<0.05	IL-1β/Coll4a1	−0.378	<0.05
IL-1β/IL-6	0.721	<0.01	IL-1β/CatK	−0.547	<0.01
IL-1β/TIMP-1	0.543	<0.01			
IL-6/Runx2	0.452	<0.05			
IL-6/TIMP-1	0.467	<0.01			
IL-6/ELA2	0.446	<0.05			
Coll1a1/Runx2	0.438	<0.05			
Coll1a1/Coll4a1	0.777	<0.01			
Coll1a1/CatK	0.501	<0.01			
OPN/CatK	0.634	<0.01			
OPN/MMP8	0.492	<0.01			
Runx2/KRT6A	0.369	<0.05			
Runx2/ELA2	0.474	<0.01			
CatK/Coll4a1	0.411	<0.05			
CatK/MMP8	0.622	<0.01			
IL-10/ELA2	0.568	<0.01			

The data show genes that revealed significant positive or negative relationships. Spearman correlation coefficients (r) and level of significance (p) are presented.

TABLE VIII. Correlation Analysis Between Clinical Parameters[a] and Genes Expressed in the Abutment-Adherent Cells

Clinical Parameters	Correlated Genes	(r)	(p)
Time since S1 surgery (fixture installation)	OPN	−0.413	<0.05
	Coll4a1	−0.378	<0.05
Time since last abutment installation	Coll4a1	−0.521	<0.01
Fixture loosening	OC	−0.430	<0.05
	IL-10	−0.404	<0.05
Length of residual femur bone	OC	−0.398	<0.05

[a]No significant correlations were detected for other clinical parameters with any of the analyzed genes.

The data show genes that revealed significant correlations with clinical parameters. Spearman correlation coefficients (r) and level of significance (p) are presented.

no detection of viable *S. aureus* ($n = 16$); single detection of *S. aureus* ($n = 6$) and *S. aureus* detected with other bacteria ($n = 8$) [Fig. 5(B)]. Further, a higher expression of TNF-α was found when *S. aureus* was detected alone in comparison with no detection of *S. aureus*. On the other hand, a 4.5- and 2-fold higher expression of MMP-8 was demonstrated for *S. aureus* with other bacteria compared with *S. aureus* detected alone and undetected, respectively [Fig. 5(C)].

DISCUSSION

The permanent penetration of the skin by a nonbiological material represents a major clinical challenge. The breach of the skin barrier and the presence of the material create markedly different conditions compared with a normal skin.[21] The most common clinical signs observed in the SPS in thirty consecutively observed patients were redness and the presence of a granulation ring. A modification of a commonly used scoring system for skin status around bone-anchored hearing aids[18] did not correlate with any clinical diagnosis, radiological, microbiological or proinflammatory markers. The problems most frequently reported by transfemoral amputees are heat/sweating in the prosthetic socket and sores/skin irritation.[22] In a two-year prospective follow-up of uni-lateral transfemoral amputees with this percutaneous bone-anchored system, a considerable improvement in these socket-related problems was observed.[23] Nevertheless, in comparison with percutaneous, bone-anchored hearing aids,[18] a considerably higher frequency of macroscopic inflammatory signs was found in the skin around the percutaneous, bone-anchored amputation prostheses. Several plausible factors may explain this

difference. Firstly, there are distinct differences in anatomy and the mechanical forces imposed on the bone and soft tissue in the two regions. In amputees the implant is weight bearing and the SPS is more mobile during ambulation and therefore presumably more prone to inflammation. Secondly, after amputation, the skin of the stump is considered to be more fragile and prone to skin problems due to pre-existing disease and surgical trauma.[24]

The stump skin of lower-extremity amputees wearing artificial limbs and complying with satisfactory stump hygiene, harbors a bacterial flora that is considerably more abundant and polymicrobial than that of the contralateral leg.[25] In the present study, the majority of the implants were colonised at the skin exit with various pathogenic bacteria, and variably but frequently along the abutment down into the bone canal. Twenty-seven out of 30 patients had positive cultures of potentially pathogenic bacteria such as *Staphylococcus aureus,* CoNS, various groups of streptococci, *Enterococcus faecalis* and *Enterobacter*. However, only one patient had a clinical manifestation of infection at the SPS, septic loosening of the fixture and a definite diagnosis of deep infection at the time of observation. The present bacterial findings are in general agreement with the results of a prospective, bacteriological study of both leg and arm amputees with osseointegrated prostheses.[13] In the present study, the frequency of bacteria in the SPS may be underestimated due to the preoperative disinfection. The vast majority of the positive cultures in our study were derived from the bone canal. Although bacteria were detected in much lower frequency on the abutment compared with the bone canal, it is of importance that protein A, a surface

TABLE IX. Correlation Analysis of Clinical Parameters[a]

Positive correlations	(r)	(p)	Negative correlations	(r)	(p)
Fixture loosening/Endosteal bone resorption	0.471	<0.01	Time since S1 surgery (fixture installation)/ Fixture loosening	−0.392	<0.05
Fixture loosening/Pain symptom	0.438	<0.05	Time since S1 surgery/Pain symptom	−0.412	<0.05
Skin color/Pain symptom	0.392	<0.05	Length of residual femur bone/Granulation ring	−0.479	<0.01

[a]No significant correlations were detected for the other clinical parameters.

The data show clinical parameters that revealed significant positive or negative relationships. Spearman correlation coefficients (r) and level of significance (p) are presented.

TABLE X. Correlation of Bacterial and Clinical Parameters[a]

Bacterial findings	Correlated clinical parameters	(r)	(p)
Detection of any bacterial species at SPS	Time since last abutment installation	0.467	<0.01
	Secretion	0.428	<0.05
Detection of any bacterial species on the abutment	Length of residual femur bone	0.399	<0.05
Detection of any bacterial species in the bone canal	Time since S1 surgery (fixture installation)	0.431	<0.05
Detection of *Enterococcus faecalis* irrespective of site	Length of residual femur bone	−0.398	<0.05
Detection of *Enterococcus faecalis* irrespective of site	Granulation ring	0.367	<0.05

[a]No significant correlations were detected for other clinical parameters with any of the analysed bacterial findings.

The data show clinical parameters that revealed significant correlations with bacterial findings. Spearman correlation coefficients (r) and level of significance (p) are presented.

protein in the cell wall of *Staphylococcus aureus*,[26] was detected on the surface of abutments from 28/30 patients. The difference could reflect a lower sensitivity of swab sampling and culturing compared with Western blot. Further, Western blot might detect even the presence of dead staphylococci that presumably existed on the abutment because of the pre-operative scrubbing of the SPS. The findings of viable bacteria and protein A on the abutment indicate that the bacteria are able to adhere to the surface of the titanium abutment, presumably via a surface coat of proteins and other cell debris. Since the property of forming biofilm constitutes an important virulence factor in bacteria, it is of great importance to determine whether the bacteria isolated from the abutment possess biofilm-forming abilities.

The skin has a great diversity of microorganisms that can potentially induce infection: first, via microbial invasion of the skin and subcutaneous tissues and, second, via the interaction with the host defense.[27] In addition, the lower extremity has been found to be the most common location experiencing soft tissue infections.[28] Although the present and previous observations[13] suggest that the mere colonisation of the skin by bacteria is not equivalent to clinical manifestation of infection, the high frequency of bacterial colonisation indicates that the macroscopical inflammatory signs are associated with a high bacterial burden. Support for this assumption is that the presence of any bacteria in the skin and the presence of *E. faecalis* at any of the three sites were associated with skin secretion and a granulation ring, respectively. Moreover, the length of the femur of the amputated limb was positively correlated to the detection of any bacteria on the abutment and negatively correlated to the presence of *E. faecalis*. Since no significant improvements

in maintaining residual limb hygiene were achieved two years after fitting with the osseointegrated, transcutaneous system,[23] the present findings suggest the need of enforced and specific cleaning tools and instructions to patients. This is supported by the frequent detection of *E. faecalis* in the bone canal, and a positive *Enterococcus* spp. culture from one of the loosened fixtures. Moreover, enterococcal strains, isolated from implant-associated osteomyelitis in the same type of patients, exhibited very high minimum biofilm eradication concentrations and none of the 10 tested antimicrobials was able to eradicate enterococcal biofilms.[29] Taken together, the present findings underscore the necessity of future studies of the relationship between the bacterial species and the particular strain virulence, including biofilm properties and antimicrobial susceptibility when isolated from soft-tissue sites and deep infections. Moreover, the search for antimicrobial strategies for the prevention and control of biomaterial-associated infections has increased during the last years. However, only a few products are currently in clinical use, and device coating strategies are often tested and applied without taking into consideration each unique or specific clinical application.[30] The present finding on the bacterial colonisation of percutaneous devices strongly highlights the need for alternative antimicrobial coatings, such as contact-killing or anti-adhesive surfaces, topical treatments, and cleaning regimens of the SPS that can help reduce the bacterial burden and entrance of potential pathogens into the bone site.

A variety of radiological changes in the fixture-bone interface up to 7 years post-operatively have been described in transfemoral[11] and transhumeral[9] amputees. These changes are considered as signs of stress shielding similar to those observed in uncemented hip stems. Our results are

TABLE XI. Correlation of Bacteria and Genes[a] Expressed in the Abutment-Adherent Cells

Bacterial findings	Correlated genes	(r)	(p)
Detection of *S. aureus* irrespective of site	TNF-α	0.394	<0.05
Detection of coagulase-negative staphylococci irrespective of site	OPN	0.392	<0.05
Detection of *S. aureus* with other bacterial species irrespective of site	MMP-8	0.531	<0.01
Detection of polymicrobial flora	Runx2	0.400	<0.05
	IL-6	0.423	<0.05
	MMP-8	0.369	<0.05

[a]No significant correlations were detected for other genes with any of the analyzed bacterial findings..

The data show genes that revealed significant correlations with bacterial findings. Spearman correlation coefficients (r) and level of significance (p) are presented.
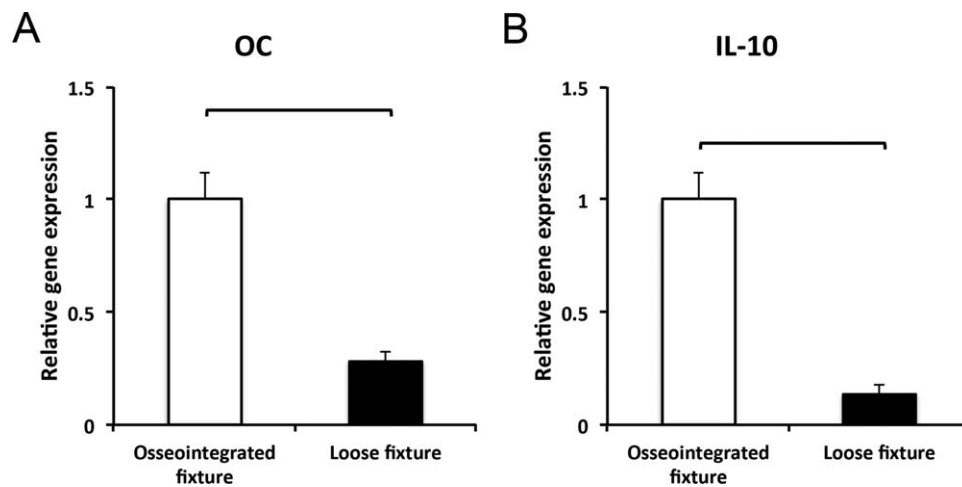
FIGURE 4. Gene expression of (A) osteocalcin (OC) and (B) interleukin-10 (IL-10) in the abutment-adherent cells. The comparison was performed between patients with osseointegrated fixtures ($n = 27$) and patients with loosened fixtures ($n = 3$). Bars indicate statistically significant differences ($p < 0.05$).

consistent with previous radiologic studies with the exception of distal bone resorption which was not observed. The present findings support our assumption that endosteal bone resorption due to stress shielding or inflammation, with or without bacterial activity, could compromise the stability of the fixture. Although longer term studies are needed to understand the impact of endosteal bone resorption on fixture survival, this radiological finding could potentially increase the probability of fixture loosening in years to come.

One important finding was the correlation between fixture loosening and reported pain as well as radiologically verified endosteal bone resorption. On the other hand, no association was found between endosteal bone resorption and bacteria in the SPS, on the abutment and in the bone canal. At present, pain upon loading, stump swelling and purulent secretion are regarded as important symptoms which, together with the results of microbiological cultures and radiologically verified osteolysis (i.e. bone resorption), constitute the criteria for deep infections.[13] However, it is well known that amputees have a wide spectrum of pain sensations and the perception of different types of pain, not related to the actual transcutaneous prosthesis, does not appear to be improved after the two-year follow-up.[23]

An amputee may face serious effects to the prosthesis and the residual limb after e.g. a fall. The currently studied amputation system has been designed to cause a bending of the cp titanium abutment rather than fractures in the bone and/or breakage of the fixture. However, an optimisation of the material properties, biomechanics and antibacterial properties is foreseen. Although the need for exchange or removal of the abutment constituted the inclusion criterion in the present study, the mechanical complications were not the focus. Two-year data from a prospective study reveals a complication rate of 8% (4/51 patients) leading to an exchange of the abutment and/or abutment screw.[1]

Our study has certain limitations. The 30 selected patients constituted a heterogeneous group with a spread in time since fixture installation and time since the last abutment exchange. The common feature in our cohort was the need for abutment exchange or removal, mainly due to the mechanical failure of the abutment. This represents a potential selection bias, as the study could not have been conducted on a randomly selected sample of transfemoral amputees. The bacterial sampling was performed after disinfecting the SPS, which is expected to have resulted in an under-estimation of the bacterial colonisation. Moreover, there was a latency between X-ray and abutment exchange. Additionally, the number of patients with loose fixture was low which makes it difficult to draw solid conclusions in relation to this group.

Importantly, a correlation was found between fixture loosening and a lower expression of osteocalcin and IL-10. IL-10 is generally regarded as an anti-inflammatory cytokine which is implicated in the control of the inflammatory response[15] and a regulator of immunity to infection.[31] IL-10 also induces the expression of TIMPs and OPG, the respective inhibitors of MMPs and RANKL systems,[32] hence implicated in preventing bone resorption. Myeloid-derived suppressor cell secretion of IL-10 promotes the development of macrophage anti-inflammatory phenotype.[33] Interestingly, a monocyte and macrophage proinflammatory gene expression and clearance of *S. aureus* was demonstrated in IL-10 knock-out mice,[33] at least partly explained by inhibition of myeloid-derived suppressor cells. Although a cause-effect relationship has not been established, the present observation of *S. aureus* in high frequency in the bone canal was associated with an increased expression of TNF-α and MMP-8, suggesting a mounted inflammatory response in order to clear the bacteria. On the other hand, no correlation was detected between the presence of bacteria and IL-10 expression. Albeit speculative, the multiple clinical signs of skin inflammation, an abundance of bacteria and an increased pro-inflammatory gene expression provide a scenario, which may ultimately lead to a degradation of the bone holding the fixture. A prerequisite is that pro-inflammatory/degradation signals and inflammatory cells would reach and migrate, respectively, to the actual fixture-
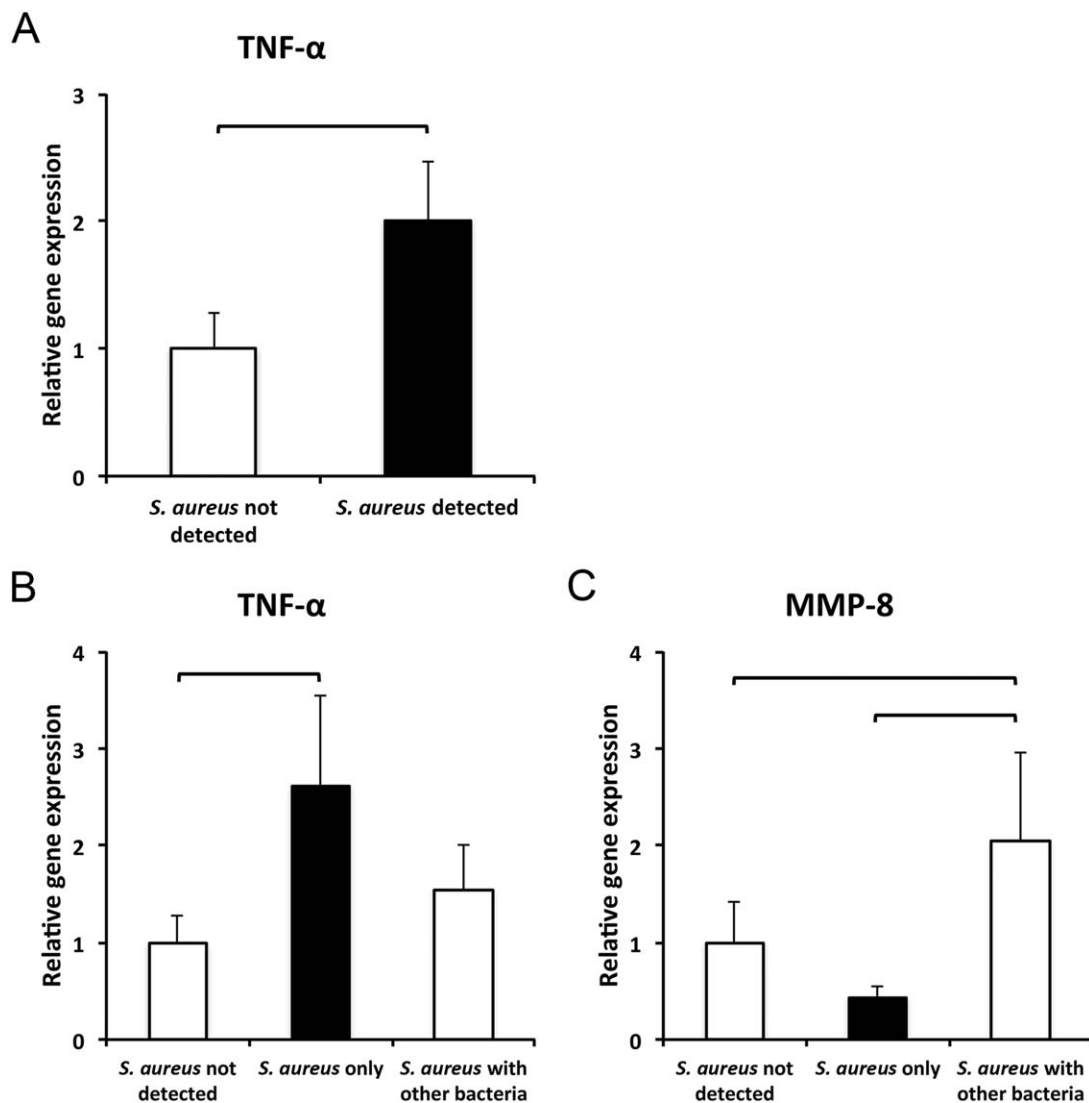
FIGURE 5. Gene expression of tumour necrosis factor-alpha (TNF-α) and matrix metalloproteinase-8 (MMP-8) in the abutment-adherent cells. The comparative analyses were performed as following: (A) TNF-α expression in patients in whom *S. aureus* was not detected ($n = 16$) versus patients in whom *S. aureus* was detected ($n = 14$); (B) TNF-α expression in patients in whom *S. aureus* was not detected ($n = 16$) versus patients in whom *S. aureus* was detected alone ($n = 6$) or detected with other bacteria ($n = 8$); (C) MMP-8 expression in patients in whom *S. aureus* was not detected ($n = 16$) versus patients in whom *S. aureus* was detected alone ($n = 6$) or detected with other bacteria ($n = 8$). The bars indicate statistically significant differences ($p < 0.05$).

bone interface. Such process would be balanced by anti-inflammatory activity and pro-regenerative activities. Interestingly, this hypothesis is at least partly supported by the finding that the three patients with fixture loosening displayed a lower anti-inflammatory IL-10 and pro-osteogenic OC gene expression in abutment-adherent cells.

CONCLUDING REMARKS

- The skin penetrating abutment of amputation prostheses was frequently colonized with pathogens and often independent from traditional clinical signs of infection.
- While bacteria were detected in 27/30 patients, only 10% of the implants required complete removal over the study interval.

- Collected correlations across observational, microbiological and radiographic results indicated that the confirmed presence of bacteria (i) increased the gene expression of markers involved in inflammation and (ii) prompted a potential shift of host bone homeostasis.
- Nearly half of the proximal femurs exhibited proximal buttressing in response to the osseointegrated implant. Endosteal bone resorption could be an alarming radiological sign regarding the fixation and future survival of the fixture especially when combined with pain at loading.
- The current prescribed daily abutment cleaning protocol, while considered adequate to this point to limit commensal bacterial load, may require revision or improved patient compliance.

REFERENCES

1. Branemark R, Berlin O, Hagberg K, Bergh P, Gunterberg B, Rydevik B. A novel osseointegrated percutaneous prosthetic system for the treatment of patients with transfemoral amputation: A prospective study of 51 patients. Bone Joint J 2014;96-b:106–113.
2. Juhnke DL, Beck JP, Jeyapalina S, Aschoff HH. Fifteen years of experience with Integral-Leg-Prosthesis: Cohort study of artificial limb attachment system. J Rehabil Res Dev 2015;52:407–420.
3. Khemka A, FarajAllah CI, Lord SJ, Bosley B, Al Muderis M. Osseointegrated total hip replacement connected to a lower limb prosthesis: A proof-of-concept study with three cases. J Orthop Surg Res 2016;11:13.
4. Schalk SA, Jonkergouw N, van der Meer F, Swaan WM, Aschoff HH, van der Wurff P. The evaluation of daily life activities after application of an osseointegrated prosthesis fixation in a bilateral transfemoral amputee: A case study. Medicine (Baltimore) 2015; 94:e1416.
5. Jeyapalina S, Beck JP, Bachus KN, Bloebaum RD. Cortical bone response to the presence of load-bearing percutaneous osseointegrated prostheses. Anat Rec (Hoboken) 2012;295:1437–1445.
6. Branemark R, Branemark PI, Rydevik B, Myers RR. Osseointegration in skeletal reconstruction and rehabilitation: A review. J Rehabil Res Dev 2001;38:175–181.
7. Esposito M, Hirsch JM, Lekholm U, Thomsen P. Biological factors contributing to failures of osseointegrated oral implants. (I). Success criteria and epidemiology. Eur J Oral Sci 1998;106:527–551.
8. Tjellstrom A, Lindstrom J, Hallen O, Albrektsson T, Branemark PI. Osseointegrated titanium implants in the temporal bone. A clinical study on bone-anchored hearing aids. Am J Otol 1981;2:304–310.
9. Tsikandylakis G, Berlin O, Branemark R. Implant survival, adverse events, and bone remodeling of osseointegrated percutaneous implants for transhumeral amputees. Clin Orthop Relat Res 2014; 472:2947–2956.
10. Hagberg K, Haggstrom E, Uden M, Branemark R. Socket versus bone-anchored trans-femoral prostheses: Hip range of motion and sitting comfort. Prosthet Orthot Int 2005;29:153–163.
11. Nebergall A, Bragdon C, Antonellis A, Karrholm J, Branemark R, Malchau H. Stable fixation of an osseointegated implant system for above-the-knee amputees: Titel RSA and radiographic evaluation of migration and bone remodeling in 55 cases. Acta Orthop 2012;83:121–128.
12. McConoughey SJ, Howlin R, Granger JF, Manring MM, Calhoun JH, Shirtliff M, Kathju S, Stoodley P. Biofilms in periprosthetic orthopedic infections. Future Microbiol 2014;9:987–1007.
13. Tillander J, Hagberg K, Hagberg L, Branemark R. Osseointegrated titanium implants for limb prostheses attachments: Infectious complications. Clin Orthop Relat Res 2010;468:2781–2788.
14. Malik MH, Jury F, Bayat A, Ollier WE, Kay PR. Genetic susceptibility to total hip arthroplasty failure: A preliminary study on the influence of matrix metalloproteinase 1, interleukin 6 polymorphisms and vitamin D receptor. Ann Rheum Dis 2007;66:1116–1120.
15. Pigossi SC, Alvim-Pereira F, Montes CC, Finoti LS, Secolin R, Trevilatto PC, Scarel-Caminaga RM. Genetic association study between Interleukin 10 gene and dental implant loss. Arch Oral Biol 2012;57:1256–1263.
16. Chen Q, Jin M, Yang F, Zhu J, Xiao Q, Zhang L. Matrix metalloproteinases: inflammatory regulators of cell behaviors in vascular formation and remodeling. Mediators Inflamm 2013;2013:928315.
17. Jarmar T, Palmquist A, Branemark R, Hermansson L, Engqvist H, Thomsen P. Technique for preparation and characterization in cross-section of oral titanium implant surfaces using focused ion beam and transmission electron microscopy. J Biomed Mater Res A 2008;87:1003–1009.
18. Holgers KM, Tjellstrom A, Bjursten LM, Erlandsson BE. Soft tissue reactions around percutaneous implants: a clinical study of soft tissue conditions around skin-penetrating titanium implants for bone-anchored hearing aids. Am J Otol 1988;9:56–59.
19. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
20. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611–622.
21. Holgers KM, Källtorp M, Thomsen P. Titanium in soft tissues. In: Brunette DM, Textor M, Thomsen P, editors. Titanium in Medicine. Berlin: Springer Verlag; 2001. p 513–560.
22. Hagberg K, Branemark R. Consequences of non-vascular transfemoral amputation: A survey of quality of life, prosthetic use and problems. Prosthet Orthot Int 2001;25:186–194.
23. Hagberg K, Hansson E, Branemark R. Outcome of percutaneous osseointegrated prostheses for patients with unilateral transfemoral amputation at two-year follow-up. Arch Phys Med Rehabil 2014;95:2120–2127.
24. Buikema KE, Meyerle JH. Amputation stump: Privileged harbor for infections, tumors, and immune disorders. Clin Dermatol 2014;32:670–677.
25. Allende MF, Barnes GH, Levy SW, O'Reilly WJ. The bacterial flora of the skin of amputation stumps. J Invest Dermatol 1961;36:165–166.
26. DeDent AC, McAdow M, Schneewind O. Distribution of protein A on the surface of Staphylococcus aureus. J Bacteriol 2007;189:4473–4484.
27. Eron LJ, Lipsky BA, Low DE, Nathwani D, Tice AD, Volturo GA. Managing skin and soft tissue infections: Expert panel recommendations on key decision points. J Antimicrob Chemother 2003;52:i3–17.
28. Ellis Simonsen SM, van Orman ER, Hatch BE, Jones SS, Gren LH, Hegmann KT, Lyon JL. Cellulitis incidence in a defined population. Epidemiol Infect 2006;134:293–299.
29. Zaborowska M, Tillander J, Brånemark R, Hagberg L, Thomsen P, Trobos M. Biofilm formation and antimicrobial susceptibility of staphylococci and enterococci from osteomyelitis associated with percutaneous orthopaedic implants. J Biomed Mater Res Part B 2016;00B:000–000.
30. Busscher HJ, van der Mei HC, Subbiahdoss G, Jutte PC, van den Dungen JJ, Zaat SA, Schultz MJ, Grainger DW. Biomaterial-associated infection: Locating the finish line in the race for the surface. Sci Transl Med 2012;4:153rv10.
31. Couper KN, Blount DG, Riley EM. IL-10: The master regulator of immunity to infection. J Immunol 2008;180:5771–5777.
32. Garlet GP, Martins W Jr, Fonseca BA, Ferreira BR, Silva JS. Matrix metalloproteinases, their physiological inhibitors and osteoclast factors are differentially regulated by the cytokine profile in human periodontal disease. J Clin Periodontol 2004;31:671–679.
33. Heim CE, Vidlak D, Kielian T. Interleukin-10 production by myeloid-derived suppressor cells contributes to bacterial persistence during Staphylococcus aureus orthopedic biofilm infection. J Leukoc Biol 2015;98:1003–1013.