Gene Expression of Inflammation and Bone Healing in Peri-Implant Crevicular Fluid after Placement and Loading of Dental Implants. A Kinetic Clinical Pilot Study Using Quantitative Real-Time PCR

Christer Slotte, DDS, PhD;* Maria Lennerås, MSc;† Catharina Göthberg, DDS;‡ Felicia Suska, DDS, PhD;§ Neven Zoric, MSc;¶ Peter Thomsen, MD, PhD;†† Ulf Nannmark, DDS, PhD‡‡

ABSTRACT

Purpose: Early detection of healing complications after placement of dental implants is a pressing but elusive goal. This paper proposes a non-invasive diagnostic tool for monitoring healing- and peri-implant disease specific genes, complementary to clinical evaluations.

Material and Methods: Eighteen partially edentulous patients were recruited to this pilot study. Three Brånemark TiUnite® implants/patient (Nobel Biocare) were placed in a one-stage procedure. Abutments with smooth or rough (TiUnite®) surface were placed. The test group (n = 9) received fixed bridges (immediate loading), whereas the control group (n = 9) implants were loaded 3 months after surgery. In addition to clinical measurements, crevicular fluid was collected using paper strips at the implant abutments 2, 14, 28, and 90 days postoperative. mRNA was extracted, purified, and converted to cDNA. Quantitative PCR assays for IL-1β, TNF-α, Osteocalcin (OC), Alkaline Phosphatase (ALP), Cathepsin K, Tartrate Resistant Acid Phosphatase, and 18S ribosomal RNA were designed and validated. Relative gene expression levels were calculated.

Results: One implant was lost in the control group and three in the test group. In one test patient, one implant showed lowered stability after 2 to 4 weeks and was unloaded. Later implant stability improved which allowed for loading after 3 to 4 months. TNF-α and ALP most commonly showed correlation with clinical parameters followed by IL-1β and OC. The strongest correlation was found for TNF-α with clinical complications at 2 and 14 days (p = .01/r = −.048, and p = .0004/r = −.56, respectively; test and control groups together). In some cases, gene expression predicted clinical complications (TNF-α, ALP, CK).

Conclusion: This study is based on samples from few individuals; still, some genes showed correlation with clinical findings. Further studies are needed to refine and optimize the sampling process, to find the appropriate panel, and to validate gene expression for monitoring implant healing.

KEY WORDS: implant abutments, paper strips, quantitative polymerase chain reaction, rough surface, smooth surface

*Senior consultant in periodontology, The Institute for Postgraduate Dental Education, Jönköping, Sweden and Department of Biomaterials, Institute for Clinical Sciences, Sahlgrenska Academy, Gothenburg University, Göteborg, Sweden; †research scientist, TATAA Biocenter AB Göteborg, Sweden; ‡senior consultant in prosthodontics, The Institute for Postgraduate Dental Education, Jönköping, Sweden and Department of Biomaterials, Institute for Clinical Sciences, Sahlgrenska Academy, Gothenburg University, Göteborg, Sweden; §Department of Biomaterials, Institute for Clinical Sciences, Sahlgrenska Academy, Gothenburg University, Göteborg, Sweden; ¶general manager, TATAA Biocenter AB, Göteborg, Sweden; ††professor, Department of Biomaterials, Institute for Clinical Sciences, Sahlgrenska Academy, Gothenburg University, Göteborg, Sweden; ‡‡associate professor, Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, Gothenburg University, Göteborg, Sweden

Reprint requests: Dr. Christer Slotte, The Institute for Postgraduate Dental Education, Jönköping, P.O. Box 1030, SE 55111 Jönköping, Sweden; e-mail: christer.slotte@lj.se

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INTRODUCTION

Early detection of healing complications after placement of dental implants is a pressing but elusive goal. At present, clinical diagnostic parameters include probing of the peri-implant mucosa. In addition, implant stability is most commonly assessed by percussion although some more objective stability tests are available, for example, Periotest™1,2 and Oss-Tell™ (resonance frequency analysis [RFA]).3 Of these, RFA has been thoroughly studied and validated to removal torque testing in in vitro and animal models.4–9 In addition, clinical reports have demonstrated the benefits of this technique especially in compromised implant cases or when immediate or early implant loading is performed.10,11

Various techniques have been used to analyze the molecular activities in the peri-implant crevicular fluid (PICF) as well as in the gingival crevicular fluid (GCF). In PICF, on the protein level; enzyme-linked immunosorbent assays (ELISA),12–17 Periocheck®, a colorimetric technique assessing neutral proteolytic enzyme (NPE) activity,18 immunoblotting,19–22 radioimmunoassay,23 and spectrophotometric techniques24 have been used. On the gene expression level, semi-quantitative RT-PCR has been used.25

Quantitative polymerase chain reaction (qPCR) represents a promising new tool to analyze and quantify spatially and temporally the biological processes in bone at a high level of precision and accuracy.25 It has been used in many in vitro studies in relation to bone cells, for example,26–30 and also in recent in vivo studies.31–33 However, no studies using qPCR are available on analysis of neither PICF or GCF in general, or of inflammation or healing-in of implants.

The aim of the present study was to test qPCR as a non-invasive diagnostic tool for the monitoring of healing-specific and peri-implant disease specific genes as a complement to clinical evaluations.

MATERIALS AND METHODS

Patients

Eighteen partially edentulous patients, selected to take part in a prospective randomized controlled trial on immediate and delayed loading of dental implants,34 were consecutively recruited to this pilot study. Briefly, the patients, in need of implant-supported fixed constructions, were referred to the Department of Prosthetic Dentistry at the Institute for Postgraduate Dental Education, Jönköping, Sweden. At the examination before implant treatment, the patients were thoroughly informed orally and in writing about the study’s purpose and procedures and about possible risks, and thereafter signed an informed consent. All patients had been dentally pretreated before participation in the study. The study was approved by the Ethics Committee for Research at Linköping University, Sweden (Dnr. M102-05). The study outline is showed in Table 1.

Single-Stage Implant Surgery and Prosthetic Treatment

Local anesthesia was administered using Xylocain Dental Adrenalin 2%, 12 μg/mL (Dentsply, Skarpnäck, Sweden). A crestal incision was followed by elevation of mucoperiosteal flaps buccally and lingually. Preparation of implant sites were done under thorough rinsing with sterile saline. Three implants (Brånemark System MkIII TiUnite; Nobel Biocare, Gothenburg, Sweden) were...
placed in each patient. After randomization, two implants were fitted with titanium abutments (MUA™, Nobel Biocare): one abutment with a roughened surface (TiUnite®) and one abutment with a conventional smooth surface. Additionally, one implant received a healing abutment. Flaps were relocated using Vicryl® sutures (Johnson & Johnson, Solna, Sweden). Antibiotics were prescribed postoperatively (either Kåvepenin 2g ¥ 2; AstraZeneca AB, Södertälje, Sweden or Dalacin 300 mg ¥ 2; Pfizer AB, Täby, Sweden). The patients were instructed to refrain from mechanical brushing in the operated area and instead rinse with chlorhexidine 0.1% (Hexident, Ipex Medical AB, Solna, Sweden) for 4–6 weeks. At the randomization, nine patients were allocated to immediate loading (test group) and the other nine patients to delayed loading (control group). In the test patients, an impression was carried out immediately after surgery and 2 days postoperatively, a fixed dental prosthesis (FDP) was placed onto the implants. The implants in the control patients were not loaded until 3 months after surgery.

**Sampling Procedure**
Crevicular fluid was collected using standardized paper strips (Periopaper™, Proflow, Amityville, NY) at the implants provided with abutments from each patient 2 days, 14 days, 28 days, and 90 days after surgery. Healing caps and FDPs were removed and cotton rolls were applied to avoid saliva contamination in the sampling area. One strip at each site was inserted in the crevice at the mesial side of the abutment for 1 minute and thereafter placed in RNALater (Ambion, Applied Biosystems, Austin, TX, USA). After a wash-out period of 2 minutes, the sampling procedure was repeated. The sampling procedure had previously been tested in different clinical settings according to the literature.

**qPCR**
Figure 1 is a flow chart of the preparation for qPCR. RNA from cells attached to the strips was extracted and purified using Qiagen RNeasy Micro kit (Qiagen AB, Hilden, Germany) according to the manufacturer’s instructions. Carrier RNA included in the kit was used to minimize losses of RNA during extraction. RNA was converted to cDNA using Bio-Rad iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer’s instructions using 7.5 µL of the RNA. The cDNA was diluted to 100 µL in UltraPure water (Invitrogen Corp., Carlsbad, CA, USA). Quantitative PCR assays for IL-1β, TNF-α, Osteocalcin (OC), Alkaline Phosphatase (ALP), Cathepsin K (CK), TRAP, and 185 ribosomal RNA were designed and validated. Primers were designed using the Primer3 website (http://fokker.wi.mit.edu/primer3/input.htm). The PCR product was verified on a 1.5% agarose gel and PCR efficiency was validated using dilution series. Bio-Rad SYBR Supermix (Bio-Rad Laboratories Inc.) and 2 µL of cDNA template together with 0.4 µM of forward and reverse primer was used in the quantitative PCR. Each cDNA sample was quantified in duplicate. The temperature protocol for each assay was: enzyme activation 3 minutes at 98°C, followed by 45 cycles of 20 seconds at 98°C, 20 seconds at 60°C, and 20 seconds at 72°C. Fluorescence detection was performed in the FAM/SYBR channel in the 72°C step. Experiments were performed on the Light Cycler® 480 (Roche, Penzberg, Germany). After amplification, a dissociation/melting curve was generated to verify that specific products were generated. Relative gene expression levels were calculated by normalizing gene expression of each gene using 185 ribosomal RNA and the ΔΔCt method using 90% efficiency for each assay.

**Microscopic Analyses.** In three test and two control patients, additional strips were taken at 2 days and 14 days for light (LM) and scanning electron (SEM) microscopy. In brief, strips were put into 4% paraformaldehyde immediately after sampling. The specimens were then dehydrated and either embedded in epoxy resin (Agar 100, Agar Scientific Ltd., Stanstead, UK), followed by sectioning (0.5 µm) and examined under a light microscope or treated with the OTOTO method,37 dried with hexamethyldisilazane and examined in a Zeiss 982 SEM (Oberkochen, Germany).

**Clinical Assessments**
Parallel to these analyses, clinical measurements were done during the study period. Among these, the following parameters were chosen for comparison with qPCR gene expressions:

- Bone quality (BQ) acc to Lekholm and Zarb.38
- Wound healing index (WHI) according to Wachtel and colleagues as follows: 1: complete flap closure – no fibrin line in the interproximal area; 2:
complete flap closure – fine fibrin line in the interproximal area; 3: complete flap closure – fibrin clot in the interproximal area; 4: incomplete flap closure – partial necrosis of the interproximal tissue; 5: incomplete flap closure – complete necrosis of the interproximal tissue.

- Sulcular bleeding (SB; peri-implant mucositis). Score 0: No bleeding when a periodontal probe is passed along the mucosal margin adjacent to the implant. Score 1: Isolated bleeding spots visible. Score 2: Blood forming a confluent red line on margin. Score 3: Heavy or profuse bleeding.

- Bleeding on probing to the bottom of the pocket (BoP) was recorded. A mm-graded pocket probe (PCP UNC-15 Hu-Friedy, Chicago, IL, USA) was used. Bleeding was assessed as follows: 0 = no bleeding, 1 = minute (slight) bleeding from the pocket, 2 = abundant bleeding.

- RFA as expressed as ISQ (Implant Stability Quotient) values.

- Implant complications–COMPL (ie, bone dehiscence at the implant surface at surgery, rotation instability of implant, loose implant or implant removal).

**Statistical Analyses**

Data were analyzed using a calculation software (Microsoft Excel, Microsoft Corp., Redmond, WA, USA) and a statistics software (SPSS 13.0, SPSS inc. Chicago, IL, USA). The Mann-Whitney test was carried out for comparison of gene expression between abutments with rough or smooth surfaces and between treatment with immediate or delayed loading of implants. Spearman rank correlation test was executed to find correlation between gene expressions and clinical parameters. Here, test and control groups were analyzed together.
RESULTS
In all, four implants were lost during the study. One implant was lost in the control group after 14 days. One patient in the test group lost one implant after 28 days. Another patient in the test group showed two unstable implants (both with abutments) that were removed after 90 days. Further, in one test patient, one implant showed lowered stability after 14–28 days and was, therefore, left unloaded. After this intervention the implant stability improved which later allowed for full loading after 3–4 months.

At sampling two days postoperatively, a considerable flow of PICF was seen at most sites. Gradually, this flow was reduced at the following sampling time-points. At day 90, hardly any fluid could be clinically detected.

Microscopic Findings
Sectioned strips analyzed by LM showed relatively large quantities of cells at the rim of the strips (Figure 2A) but some of the cells had also penetrated into the cellulose mesh. Most of these cells were granulocytes/monocytes, but occasional cells with mesenchymal morphology were also recorded (Figure 2B). SEM analysis showed the same appearance with cells entrapped in the cellulose fiber network of the strips. The most common cells were erythrocytes followed by platelets. However, granulocytes and larger cells were also observed and surrounded by fibrin (Figure 2C). No attempts were made to elucidate the number of cells at different time points.

qPCR Analysis
No difference was found between the duplicate measurements at each interval. The relative expression of the different genes in the panel are shown in Table 2 and Figure 3. In general, gene expressions for IL-1β, TNF-α, and ALP were found to be much higher than for OC, CK, and TRAP (Table 2). Initially, TNF-α was highly expressed and thereafter gradually decreased while OC and ALP slightly increased over time. No clear picture of the spatial changes were found for IL-1β, ALP, CK, and TRAP. The following is a short summary of findings for the particular genes:

**IL-1β.** Two days postoperatively, significantly higher expression was found at smooth abutments in the unloaded group, while no other significant differences were found at any time point.

*Figure 2* (A) LM image of filter strips after insertion in a peri-implant crevice for 60 seconds. Cells penetrating the rim of the cellulose filter strip. (B) Higher magnification of Figure 3A. Most of the cells are granulocytes/monocytes but occasional cells with mesenchymal morphology are also found. (C) SEM images of filter strips after insertion in a peri-implant crevice for 60 seconds. An erythrocyte and a thrombocyte are shown attached to the fibrin mesh. Magnification × 7250.
No significant differences were found, neither between smooth and rough abutments nor between immediately loaded and unloaded implants. In the test patient where two implants were found unstable at 90 days, TNF-α expression showed a sharp increase at this time point, as shown in Figure 4.

OC. At rough abutments, unloaded implants showed significantly higher OC expression than immediately loaded implants at 14 days. In the unloaded implants, OC expression was significantly higher at rough than at smooth abutments at 14 days.

ALP. At rough abutments, ALP was significantly higher expressed after 90 days at unloaded implants. No other differences were found significant for this gene.

Cathepsin K. Significantly higher expression for delayed than for immediate loading was found at rough abutments at 14 days, while no other differences were found.

TRAP. At unloaded implants, rough abutments showed significantly higher expression than smooth abutments at 14 days.

Correlation Analyses – Significant Findings

IL-1β. RFA correlated with IL-1β at 2 days \( (p = 0.013/r = 0.47) \). COMPL correlated with IL-1β at 14 days \( (p = 0.025/r = 0.47) \), 28 days \( (p = 0.039/r = 0.42) \).

TNF-α. Correlation was found with COMPL at 90 days \( (p = 0.05/r = -0.43) \). In addition, correlations were found with TNF-α at 2 and 14 days with COMPL at 90 days \( (p = 0.01/r = -0.48 \) and \( p = 0.004/r = -0.56 \), respectively), at 28 days with WHI at 2 days \( (p = 0.033/r = -0.43) \), and at 90 days with RFA at surgery \( (p = 0.038/r = -0.46) \).

OC. OC correlated with BQ at 28 days \( (p = 0.038/r = 0.51) \). Also, correlation was found with RFA at 14 days \( (p = 0.041/r = 0.50) \).

ALP. Correlations were found with WHI at 2 days \( (p = 0.029/r = -0.42) \). In addition, correlations were found at 90 days with WHI at 2 days \( (p = 0.046/r = -0.43) \) and at 28 days with RFA at surgery \( (p = 0.034/r = 0.43) \), RFA at 2 days \( (p = 0.023/r = 0.45) \), and at 28 days \( (p = 0.030/r = 0.44) \). At 14 days, correlation was found with COMPL at 90 days \( (p = 0.022/r = -0.46) \).
Figure 3A–F Graphical illustrations of gene expression at rough/smooth abutments and immediate/delayed loading. (A) interleukin-1β (IL-1β), (B) tumor necrosis factor-α (TNF-α), (C) osteocalcin (OC), (D) alkaline phosphatase (ALP), (E) cathepsin K (CK), (F) tartrate resistant acid phosphatase (TRAP). Bars = SEM.
CK. At 2 days, correlation was found with COMPL ($p = .038/r = -0.56$). At 90 days, correlation was found with BoP ($p = .036/r = -0.51$). Correlations were found at 14 days with COMPL at 90 days ($p = .016/r = -0.63$), and at 28 days with WHI at 2 days ($p = .007/r = -0.64$).

TRAP. Correlations were found at 90 days with BQ ($p = .014/r = 0.54$), and BoP ($p = .025/r = -0.50$). In addition, correlations were found at 2 days with COMPL at 90 days ($p = .052/r = 0.43$), and at 28 days with RFA at surgery ($p = .049/r = -0.76$).
DISCUSSION

In general, gene expression for IL-1β, TNF-α, and ALP were found much higher than for OC, CK, and TRAP (Table 1). Initially, TNF-α was highly expressed, and thereafter gradually decreased, while OC and ALP slightly increased over time. No clear picture of the spatial changes were found for IL-1β, CK, and TRAP. Albeit significant differences of gene expression were found at some occasions (higher OC at 14 days at rough than at smooth abutments, higher ALP at 90 days at unloaded than immediately loaded rough abutments, higher TRAP at unloaded rough than at unloaded smooth abutments at 14 days), no pattern was revealed comparing rough and smooth abutments or immediately loaded and unloaded (non-submerged) implants. Most likely, this can be explained by the relatively few number of samples at each time point, as well as large variations. Significantly higher expression was found for IL-1β at 2 days between test and control group for smooth abutments. This finding is difficult to explain because no loading had been instituted before sampling at day two. Whether this is related to shortcomings in the sampling procedure, the further analysis or by chance cannot, at present, be determined.

In one patient exhibiting unstable implants at 90 days, TNF-α was much higher expressed at 90 days than at the earlier assessments (Figure 4). However, the time gap between the two last assessments is considerable;
why it cannot be ruled out that this high expression might have been found earlier and, therefore, could have predicted the clinical complication. Interestingly, the initial values for this patient were much lower than the mean TNF-α-values and, conversely, the 90-day value was much higher than average.

Correlation was revealed with gene expression and clinical parameters at different time points. Of these, WHI, RFA, and COMPL displayed more correlations with gene expression than the other parameters and showed frequent correlations with ALP, TNF-α, and IL-1β. COMPL showed correlations with all studied genes at at least one time point. ALP, TNF-α, and IL-1β were most commonly correlated with clinical parameters. The strongest correlations were found for TNF-α at 2 and 14 days with COMPL at 90 days. Hence, in the present study, some gene expressions even predicted complications (TNF-α at 2 and 14 days, and ALP and CK at 14 days).

To our knowledge, this pilot study is the first attempt to apply qPCR gene expression analysis of peri-implant crevicular fluid in a clinical prospective implant investigation.

The perio paper strips were originally designed to collect crevicular fluid for further analysis of polypeptides, proteins and other molecules by, e.g. ELISA tests. In the present study, they appeared feasible also to collect cells in the crevicular area of dental implants.

Figure 3A–F Continued
Light microscopic and SEM analyses detected leukocytes, erythrocytes, and thrombocytes at the surface of the strips. However, the amount of cells was mostly low at all collection time points. Besides this low amount of cells, gene expression was possible to detect by qPCR, confirming the high sensitivity of the method.\(^{42-45}\) As a rule, a larger amount of cells will generate a higher gene expression,\(^{46}\) however, as found in the present study, even extremely small cell samples can give rise to detectable gene expression. Also, the intensity of the specific expression, not only the number of cells, are crucial for an accurate result. In general, a larger variation is gained with few target mRNA copies, and it is the number of these copies that determines the result. To have a precise answer to this, a fluorescence-activated cell sorting analysis must be carried out exploring the relative amount of expression of, for example, TNF-\(\alpha\) that can be found in an osteoclast. Questions may be raised that the number of observations at some time-points were too few to allow any conclusions, however one explanation could be that none of the specific cells were present, and, accordingly no gene expression was accessible. Hence, the various amounts of observations may represent a natural biological variation of gene expression during wound healing among individuals, and hence, the described sampling technique may represent the lower limit of what can be measured. Further studies must be carried out to determine the precise detection
limit for gene expression by this technique and should be followed by systematic studies mapping up- and down-regulation of genes during peri-implant wound healing and/or peri-implant tissue destruction. In the longer perspective, qPCR monitoring may be also useful in the clinic for the early detection and prevention of implant failure or implant-related diseases.

CONCLUSION

This pilot study is based on samples on relatively few individuals. Some genes showed correlation with clinical findings. However, further studies are needed to refine and optimize the sampling process to find the appropriate panel and to validate gene expression for monitoring implant healing.

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