

A prospective assessment of peri-prosthetic bone mineral density and osteoimmunological biomarkers variations after total knee replacement surgery

Davide Cucchi , Alessandra Menon , Emanuela Galliera , Carmelo Messina , Beatrice Zanini , Monica Gioia Marazzi , Luca Massaccesi , Riccardo Compagnoni , Massimiliano M. Corsi Romanelli , Pietro Randelli

PII: S1094-6950(18)30052-0
DOI: [10.1016/j.jocd.2018.05.039](https://doi.org/10.1016/j.jocd.2018.05.039)
Reference: JOCD 1042

To appear in: *Journal of Clinical Densitometry*

Received date: 2 April 2018
Revised date: 21 May 2018
Accepted date: 21 May 2018

Please cite this article as: Davide Cucchi , Alessandra Menon , Emanuela Galliera , Carmelo Messina , Beatrice Zanini , Monica Gioia Marazzi , Luca Massaccesi , Riccardo Compagnoni , Massimiliano M. Corsi Romanelli , Pietro Randelli , A prospective assessment of peri-prosthetic bone mineral density and osteoimmunological biomarkers variations after total knee replacement surgery, *Journal of Clinical Densitometry* (2018), doi: [10.1016/j.jocd.2018.05.039](https://doi.org/10.1016/j.jocd.2018.05.039)



This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

A prospective assessment of peri-prosthetic bone mineral density and osteoimmunological biomarkers variations after total knee replacement surgery

Davide Cucchi ^{a,b,*}, Alessandra Menon ^{b,c,*}, Emanuela Galliera ^{b,d}, Carmelo Messina ^e, Beatrice Zanini ^c, Monica Gioia Marazzi ^b, Luca Massaccesi ^f, Riccardo Compagnoni ^{b,c}, Massimiliano M. Corsi Romanelli ^{b,g}, and Pietro Randelli ^{b,c}

* Davide Cucchi and Alessandra Menon contributed equally to this work.

^aDepartment of Orthopaedics and Trauma Surgery, Universitätsklinikum Bonn, Sigmund-Freud-Str. 25, 53127 Bonn, Germany;

^bDepartment of Biomedical Sciences for Health, Università degli Studi di Milano, Via Mangiagalli 31, 20133 Milan, Italy;

^c 1° Clinica Ortopedica, ASST Centro Specialistico Ortopedico Traumatologico Gaetano Pini-CTO, Piazza Cardinal Ferrari 1, 20122 Milan, Italy;

^dIRCCS Galeazzi Orthopaedic Institute, Via Riccardo Galeazzi 4, 20161 Milan, Italy;

^eDepartment of Diagnostic and Interventional Radiology, IRCCS Istituto Ortopedico Galeazzi, Via Riccardo Galeazzi 4, 20161 Milan, Italy;

^fDepartment of Biomedical, Surgical and Oral Science, Università degli Studi di Milano, Via Mangiagalli 31, 20133 Milan, Italy;

^gU.O.C SMEL-1 Patologia Clinica IRCCS Policlinico San Donato, Piazza Malan 1, 20097 San Donato Milanese, Milan, Italy.

Study performed at IRCCS Policlinico San Donato, San Donato Milanese, Milan, Italy.

Corresponding Author

Davide Cucchi, MD - ORCID-ID: 0000-0001-6284-7977

Department of Orthopaedics and Trauma Surgery, University of Bonn, Bonn, Germany.

Universitätsklinikum Bonn, Sigmund-Freud-Straße 25, D-53127 Bonn, Germany.

d.cucchi@gmail.com

Authors contributions:

DC: study design, patient recruitment and data collection, original draft preparation; AM: statistical analysis, figures and tables, draft revision; EG, MGM, LM, MMCR: study design, laboratory analysis; CM: radiological imaging, and analysis of DXA results; BZ: patient recruitment and data collection; RC: discussion, manuscript correction; PR: study design, surgical procedures, manuscript correction.

Compliance with Ethical Standards

Funding: This study was not funded

Conflict of interest: Author DC declares that he has no conflict of interest. Author AM declares that she has no conflict of interest. Author EG declares that she has no conflict of interest. Author CM declares that he has no conflict of interest. Author BZ declares that she has no conflict of interest. Author MGM declares that she has no conflict of interest. Author LM declares that he has no conflict of interest. Author RC declares that he has no conflict of interest. Author MMCR declares that he has no conflict of interest. Author PR declares personal fees from Arthrex and Depuy (Johnson&Johnson), outside the submitted work.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The study protocol was approved by the Regional Ethical Committee (authorization number 2787).

ClinicalTrials.gov Identifier: NCT01971931

Informed consent: Informed consent was obtained from all individual participants included in the study.

Abstract

Purpose: Aseptic loosening is a major cause of premature failure of total knee arthroplasty (TKA). Variations in peri-prosthetic bone mineral density (BMD) and osteoimmunological biomarkers levels could help to quantify prosthesis osteointegration and predict early aseptic loosening.

Methods: The gene expression of five selected osteoimmunological biomarkers was evaluated in tibial plateau bone biopsies by Real Time PCR and changes in their serum levels after TKA were prospectively evaluated with enzyme-linked immunosorbent assays for one year after surgery. These variations were correlated to changes in peri-prosthetic bone mineral density (BMD).

Results: Sixteen patients were evaluated. A statistically significant decrease in serum levels of Sclerostin ($p=0.0135$) was observed immediately after surgery. A specular pattern was observed between DKK-1 and OPG expression. No statistically significant changes were detectable in the other study biomarkers. Peri-prosthetic BMD did not change significantly across the duration of the follow-up.

Conclusion: Prosthetic knee surgery has an impact on bone remodelling, in particular on SOST expression. Although not showing statistically significant changes, in the patterns of DKK-1, OPG and RANKL symmetries and correspondences related to the biological activities of these proteins could be identified. Variation in osteoimmunological biomarkers after TKA surgery can help in quantifying prosthesis osteointegration.

Keywords

Osteoimmunology; Biomarker; Bone mineral density; Total knee replacement; Sclerostin; Osteointegration

Level of Evidence: Prospective cohort study, level II.

1. Introduction

Total knee arthroplasty (TKA) has proven to be a safe and effective surgical treatment for patients with advanced knee osteoarthritis. Despite improvements in prosthetic design and surgical techniques, aseptic loosening resulting from osteolysis or peri-prosthetic bone loss is a major cause of premature failure of prosthetic knee surgery. The diagnosis of early aseptic loosening is based on clinical and radiographic evaluation, but biomarkers are currently gaining interest for the preoperative differentiation between septic and aseptic loosening (1).

Numerous evidences in the literature confirmed differences in biomarker levels in patients undergoing prosthetic revision surgery when compared to levels of the healthy population or patients with stable implants. Only a small number of clinical studies have assessed the pre-operative status of bone metabolism or evaluated post-operative changes in biomarker levels. Collecting these data could help to estimate the risk of aseptic loosening even before implantation of the first prosthesis (1–3).

In the past decade, a growing attention has been directed to osteoimmunological factors, which are soluble mediators linking bone tissue to immune system (4,5). However, no studies described the changes in these proteins after implantation of a TKA and related them to densitometric studies in the attempt to provide a predictive information on osteointegration.

The ligand of the receptor activator of nuclear factor kappa-B (RANKL) is considered the main osteoimmunological marker, together with its receptor and the decoy receptor Osteoprotegerin (OPG) (6). RANK is a membrane-bound receptor, but it can be found also in the serum. Osteoblasts produce RANKL and OPG to regulate the differentiation of the osteoclasts, which express RANK (7). Sclerostin (SOST) and Dickkopf-related protein 1 (DKK-1) are two inhibitors of the Wnt pathway, which plays a crucial role in bone remodelling. By inhibiting Wnt signalling, these molecules reduce osteoblast activity (8–10).

The aim of this study was to analyse the variation in osteoimmunological biomarkers after TKA surgery and compare them with radiological and clinical signs of mobilisation, in order to quantify prosthesis osteointegration and predict early aseptic loosening.

2. Materials and methods

The primary goal of this prospective observational clinical trial was to measure changes in expression of five selected osteoimmunological biomarkers after implantation of a TKA: Dickkopf-related protein 1 (DKK-1), Sclerostin (SOST), Osteoprotegerin (OPG), Receptor activator of nuclear factor kappa-B (RANK) and its ligand (RANKL). Secondary goal was to relate these changes with variations in peri-prosthetic bone mineral density (BMD) and clinical short-term results. The study protocol was approved by the Regional Ethical Committee (authorization number 2787) and registered at ClinicalTrials.gov (NCT01971931).

Patients were prospectively enrolled by two investigators, according to inclusion and exclusion criteria listed in *Table 1*.

All patients underwent clinical examination, long-leg and standard knee radiographs. Prior to the surgery (T0), blood samples were collected for determination of the serum protein levels of the aforementioned osteoimmunological markers and Interleukin-6 (IL-6), and each patient was asked to fill out the Oxford Knee Score (OKS) questionnaire and the Visual Analogue Scale (VAS) evaluation tool.

A cemented, posterior stabilised, mobile bearing, prosthesis with patellar resurfacing (P.F.C. Sigma, DePuy International, England) was implanted using a medial parapatellar approach by the same senior surgeon in all patients. During surgery, a bone biopsy of the tibial plateau, as close as possible to the final prosthetic site, was collected for determination of DKK-1, SOST, OPG, RANK, RANKL and IL-6 mRNA expression levels. The retro transcription phase was performed using 1 µg of total RNA, according to the manufacturer's instruction of the ProtoScript® First Strand cDNA Synthesis Kit for RT-qPCR system (New England BioLabs Inc.). All samples were retrotranscribed 3-5 min using Thermo cycler (Perkin Elmer, 2400 Gene Amp PCR System, Germany) Quantitative PCR was performed using commercial TaqMan® Gene Expression Assay (Life Technologies Italia, Monza), according to the manufacturer's instruction; The fold change in expression of the different genes in patients was normalized to the expression of 18s. The accuracy was monitored by the analysis of the melting curves.

IL-6, DKK-1 and SOST were measured in serum using ELISA sandwich Quantikine Assays, while OPG, RANK and RANKL were measured using an ELISA Duo Set Assay, (R&D Systems, Minneapolis, MN, USA), according to manufacturer's protocols.

No metallic staples were used for wound closure to avoid interference with peri-prosthetic Dual Energy X-ray Absorptiometry (DXA) measurements. Anaesthetic, pain-control medications,

antithrombotic and antibiotic prophylaxes and rehabilitation procedures were standardised according to the institution's internal protocols (*Appendix 1*).

A peri-prosthetic DXA of the operated knee were obtained on the 4th post-operative day (T1) and three (T2), six (T3) and twelve months (T4) after surgery. Peri-prosthetic DXA protocol and determination of the regions of interest (ROI, *figure 1*) are presented in *Appendix 2 (11,12)*. All imaging studies were evaluated by a single clinical radiologist. Blood sample for determination of the serum protein levels of the aforementioned markers and IL-6 were collected at T1, T2 and T4. OKS and VAS were prospectively collected at T2, T3 and T4 and the Forgotten Joint Score (FJS) at final follow-up (T4) (*13*). All protocols for biomarkers identifications are described in the *Appendix 3*.

Statistical analysis was performed using GraphPad Prism v 6.0 software (GraphPad Software Inc.). Continuous variables were expressed as the mean \pm standard deviation (SD), while the dichotomous variables are expressed in numbers of cases and frequencies. The Shapiro-Wilk normality test was used to evaluate the normal distribution of the sample. Evaluation of the differences between and within the groups was performed using one-way analysis of variance (ANOVA) followed by a two tailed, paired Student's t-test or Mann-Whitney test according to the characteristics of the data distribution.

3. Results

Nineteen patients were enrolled. One patient received non-simultaneous bilateral TKA, with a one-year delay between interventions. Complete data were collected for 16 patients, with a large female dominance (4 males, 12 females); all women were in postmenopausal status. Three patients were excluded from due to implantation of a different prosthesis model or need for additional osteosynthesis of an intra-operative periprosthetic fracture. Demographic data are shown in *Table 2*.

3.1 Evaluation of mRNA expression in bone biopsies (T0) (Table 3)

The biomarker gene expression displayed a great variability among patients, as shown by a quite high SD, but, as a general trend, the expression level was lower than the 18S expression. Among the biomarkers analysed, the lowest expression was observed for SOST gene and the highest for DKK-1 gene. Mean OPG expression was higher than mean RANKL expression, and the resulting RANKL/OPG ratio was 0.818 ± 0.293 .

3.2 Evaluation of serum protein levels (T0, T1, T2, T4) (Table 4, Figure 2)

In order to estimate pre-and post-operative inflammatory status, IL-6 level was measured: pre-operative IL-6 level was always in the reference range (14). A statistically significant increase ($p=0.0008$) was displayed at T1, followed by return to physiological levels at subsequent follow-up points, comparable to T0 level.

SOST protein level was in the reference range (15) at T0. A little but statistically significant decrease ($p=0.0135$) was observed at T1, followed by return to levels comparable to T0. Since a difference between SOST levels between genders was previously reported (16,17), the different level of serum SOST was evaluated in men and women: men displayed a modestly but non-significantly higher SOST level at T1 and T2. Whereas the level modestly but non-significantly higher level at T4 for women.

The levels of DKK-1 and of the bone-protective marker OPG protein level were comparable to the reference range (18,19) at T0 and, showed non-significant oscillations in the subsequent follow-up points. A specular pattern of was observed between the oscillating expression of these two biomarkers at all time points.

RANK protein level was in the reference range at T0 and displayed a statistically non-significant decrease at T1, followed by an increase in the subsequent follow-up points.

RANKL protein level could be detected only in seven patients and was always in the reference range (19) at T0. At T1, it showed a little and non-significant decrease, whereas at T2 and T4 it displayed a little and non-significant increase. The fact that some subjects can have free RANKL

levels below the limit of detection is described (20). This is due to the fact the majority of commercial kits available measure free RANKL, which represent 1/1000th of the total RANKL (21).

In order to better define the bone resorption status, the RANKL/OPG ratio was evaluated. At T0 RANKL/OPG ratio was 0.45 (\pm 0.57). An increase, even though not statistically significant, was observed in the subsequent follow-up points.

3.3 Evaluation of BMD (T1, T2, T3, T4)

Pre-operative DXA revealed normal BMD in 12.5% of the patients and reduced BMD values in the remaining study population (T-scores: lumbar: -0.5 ± 2.5 ; femur, right: -0.8 ± 1.1 ; femur, left: -0.9 ± 1.1). Average BMD in the different regions investigated did not show any significant reduction across the duration of the follow-up (*Figure 3*). A trend towards increasing BMD was registered in the R1 and R3 zones, whereas a moderate decrease was registered in R2 and R5. Average BMD variation in R4 showed a decrease $> 1\%$ across the study period. None of the recorded variations appeared to reach statistical significance. We found a comparable rate of variation in BMD values during time between periprosthetic cortical areas (ROI 1 to 4) and periprosthetic trabecular area (ROI 5), as well as between males and females. Considering all the ROI, women had a minimal reduction of BMD compared to baseline (overall reduction = -0.66%), while this difference was almost negligible with males (overall reduction = -0.09%).

3.4 Clinical and functional evaluation (T1, T2, T3, T4)

Pre-operative OKS was 19.44 (\pm 8.11) points and VAS 6.5 (\pm 2.92) points. A statistically significant increase in OKS ($p < 0.0001$), associated to decrease in VAS ($p < 0.0001$) was registered (*Figure 4*). At final follow-up average FJS was 61.15 (\pm 23.49) points. No cases of implant failure or prosthetic loosening were recorded.

4. Discussion

The main finding of this study is that a statistically significant decrease in SOST expression occurs shortly after TKA, followed by a steadily increase. This innovative finding confirms the hypothesis that prosthetic knee surgery has an impact on bone remodelling and osteointegration. No statistically significant changes occurred in the expression of the other biomarkers following TKA implantation, but symmetries and correspondences in the patterns of DKK-1, OPG and RANKL could be identified.

DXA was not capable to detect BMD changes on both sides of the prosthesis and no significant correlation between biomarkers levels and clinical or radiological outcomes could be identified, indicating that bone remodelling occurs without visibly affecting the postoperative course after TKA.

Bone quality and prosthesis osteointegration are relevant for long-term success of primary TKA. Lack of integration may lead to aseptic loosening. For this reason, previous research was directed to the clinical relevance of biomarkers evaluating bone remodelling, being placed in relation to the clinical outcomes in orthopaedic surgery (22–24).

Using these bone remodelling biomarkers permits to monitor bone turnover with high sensitivity and specificity, since the serum concentration of these molecules is proportional to their activity in bone tissue. Moreover, serum investigation has minimal discomfort for the patient and does not expose to radiation. For these reasons, the possibility to quantify prosthesis osteointegration and to predict early aseptic loosening the evaluation of these biomarkers is appealing.

This study focuses on the role of osteoimmunological markers in bone remodelling after TKA implantation and highlights a characteristic trend in SOST level not described yet. In our population, a general low state of gene expression was encountered: this is documented in tissue biopsies, where all analysed genes displayed expression levels lower than the 18S, chosen as reference housekeeping gene. The 18S expression was stable and comparable among patients, therefore the low gene expression of all the biomarkers should be referred to a low osteoimmunological response of the bone tissue. This result is in agreement with the absence of significant alteration in BMD in the follow-up period. This implies that DXA was not capable to detect changes in BMD values that occurred on both sides of the prosthesis and suggests that in our patients surgery produced only minor changes to the bone metabolism, without inducing significant bone loss. This was clinically confirmed by the fact that no cases of aseptic loosening were observed in the study population. Other authors have documented changes in the peri-prosthetic BMD, however being not associated with any negative clinical outcome: specifically, Soininvaara,

Järvenpää et al. indicated that the highest bone-loss rates occurred during the first three post-operative months after TKA, and continued around the femoral component for up to 7 years. The clinical outcome, as quantified by the improvement in a functional score, was not associated with peri-prosthetic BMD change (11,12,25). A variable prosthesis-related stress-shielding and different patient's muscle mass could be possible explanations to these differences, as proposed Mau-Moeller et al. after comparing DXA, muscle mass and strength ten days and three months after surgery in 23 patients undergoing TKA (26).

Tissue gene expression gives only a partial information about bone metabolism, since osteoimmunological markers can be regulated at post-translational level. For this reason, the circulating concentration of the osteoimmunological markers was evaluated (Table 3).

Our study demonstrated that typical serum expression patterns could be identified after TKA for SOST, which showed an initial decrease, followed by a steadily increase at later follow-up points.

However, the pre-operative values appeared not to be predictive of post-operative changes. RANK receptor displayed a stable level before and after surgery, suggesting that prosthetic procedures did not alter neither the expression nor the production of this molecule.

SOST promotes the RANKL-dependent osteoclast formation and osteocyte activity (27), while DKK-1 induce the production of RANKL, thereby shifting the balance of RANKL/OPG in favour of RANKL and promoting osteoclastogenesis and bone resorption (28,29). In our series, DKK-1 displayed no significant changes; consistently, a parallelism in the RANKL trend was observed, which could be promoted by the early stimulus of DKK-1. Being DKK-1 a negative regulator of OPG (30), the expression of DKK-1 could explain the specular pattern observed for OPG, resulting in a net increase of RANKL/OPG ratio.

A growing attention has been focused on the relationship between the serum levels and corresponding levels in bone for these important osteoimmunological molecules, OPG and RANKL (21). The first reason for this interest is that the ratio of RANKL mRNA to OPG mRNA (RANKL/OPG ratio) has been shown to correlate with osteoclast surface, a reliable measure of bone resorption (31). The second reason is that circulating OPG and RANKL could have different cellular sources, as part of a negative feedback system. In particular, an inverse relationship was observed between RANKL mRNA and serum RANKL, suggesting that the shedding activity of RANKL into the circulation is decreased with increasing expression of RANKL mRNA (21).

Soluble RANKL has been described as a predictor of non-traumatic fracture risk, in particular with low levels of serum RANKL having been associated to a lower degree of bone remodelling (32). In our patients, RANKL showed a light trend of increase from T1 to following time points, suggesting that bone recovery after surgery is occurring, and potentially reducing non-traumatic fracture risk.

The fact that no correlation could be found between BMD and RANKL in our series is in agreement with previous reports showing that serum RANKL could be a fracture risk predictor unrelated to bone mass assessment (32).

The interpretation of serum OPG is debated: some authors indicate high serum OPG as an indicator of increased osteoblast activity, therefore bone production, whereas some others OPG increase as a response to elevated bone turnover in order to counter excessive bone resorption (33). Among our patients, a single case of significant OPG decrease after surgery was observed; interestingly, this was the only patient who also presented a more pronounced reduction of peri-prosthetic BMD, suggesting a relevant role of OPG in the bone formation process.

The RANKL/OPG ratio is considered a better indicator of bone turnover status than RANKL and OPG taken separately. RANKL/OPG ratio has been observed to correlate with BMD changes, suggesting a possible predictive role for osteolysis and fracture risk (34). In our patients, an increasing trend from T0 onwards was observed in RANKL/OPG ratio, indicating a shift of the bone formation/resorption balance towards an increase in resorption of new bone. This trend could not be confirmed by variations in peri-prosthetic BMD.

Since osteoimmunological markers are influenced by inflammatory response, the inflammatory cytokine IL-6 was chosen to be analysed, having its expression already been related to changes in osteoimmunological biomarkers levels. As expected, IL-6 displayed a strong up-regulation at the earliest time point after surgery, confirming the physiological inflammatory response occurring after a surgery procedures (35). At later follow-up points, IL-6 returned to expression levels comparable to T0, confirming that the inflammatory response is transient and limited to the early post-operative period (5).

This study has a number of limitations: the population was composed of a relatively small number of Caucasian, elderly patients, with a large female dominance; this could limit the statistical power of our conclusion and should be considered prior to extrapolating the significant findings to the general population. Follow-up was limited to twelve months, which is insufficient to determine the rate of aseptic loosening at long-term follow-up. For ethical reason, we decided not to enrol a healthy control group; however, biomarkers levels in analysed patients were compared to reference ranges already reported in literature. Finally, a single type prosthesis was used; other systems may affect differently the peri-prosthetic bone and these results may then not be representative for all different implants available.

5. Conclusion

This study evaluated changes in expression of selected osteoimmunological biomarkers after TKA implantation, correlating them with variations in peri-prosthetic BMD and clinical short-term results. A decrease in SOST expression occurs shortly after TKA followed by a steadily increase at later follow-up points. Moreover, a specular pattern was observed between DKK-1 and OPG expression. DXA was not capable to detect BMD changes on both sides of the prosthesis.

Variation in osteoimmunological biomarkers after TKA surgery can help in quantifying prosthesis osteointegration.

ACCEPTED MANUSCRIPT

Compliance with Ethical Standards

Funding: This study was not funded

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The study protocol was approved by the Regional Ethical Committee (authorization number 2787).

ClinicalTrials.gov Identifier: NCT01971931

Informed consent: Informed consent was obtained from all individual participants included in the study.

Appendix 1 : standardized peri-operative procedures

Antibiotic prophylaxis

Perioperative antibiotic prophylaxis was routinely administered to all patients undergoing TKA with Cefazolin (2g intravenous, 30 minutes before the skin incision and repeated 1g x 2 8 and 16 hours after surgery) or Vancomycin in patients with beta lactam allergy (1g intravenous 60 minutes before the skin incision). Prophylaxis for urinary tract infections was administered only if catheterization was necessary (Gentamycin, dosing adapted to patient's weight).

Antitromboembolic prophylaxis

Enoxaparin sodium 4000 IU / day, from 12 hours before surgery and, thereafter, for 35 days after surgery. In presence of long-term oral anticoagulation, interruption of oral anticoagulation and

bridging with Enoxaparin sodium after cardiologic consultation. The standard prophylactic dosage was adjusted for cardiac pre-existing diagnoses and adapted for weight and kidney function if necessary, after cardiologic or internistic consult. Bilateral compression stockings (mediven® thrombexin® 18, medi GmbH & Co. KG, 95448 Bayreuth, Germany) from the first day after surgery up to 2 months, with progressive discontinuation after 35 days after surgery.

Anesthesiological protocol

Combined spinal-epidural anesthesia with Bupivacaine 0.5% 10-12.5 mg (or Levobupivacaine 0.5% 10-12.5 mg) + sedation or general anesthesia. No local intra-articular injection of anesthetics was performed. Monitoring: ECG, non-invasive monitoring of arterial pressure, Δ ST tract, O₂ saturation, diuresis (catheterization of patients according to age and after clinical evaluation).

Blood management procedures

In all patients a tourniquet was inflated before the skin incision and released before inspection of posterior capsular bleeding and insert placement; careful hemostasis with bipolar electrocautery was routinely performed after tourniquet release. One deep intra-capsular Redon type suction drain was used in all patients and was removed on the first or second post-operative day. One gram of tranexamic acid was administrated intra-venously approximately 30 min prior to tourniquet release and 1 g intra-articularly after fascial closure. The same compression bandage from the ankle to the thigh was used in all patients and substituted with compression stockings (mediven® thrombexin® 18, medi GmbH & Co. KG, 95448 Bayreuth, Germany) the first day after surgery.

Rehabilitation procedures

The rehabilitation procedures were standardized and included early mobilization on the first post-operative day, after removal of the surgical drain, followed by continuous passive motion (2 + 2 hours per day with Kinetec®, Kinetec Ltd., GU12 4RH, Hants, United Kingdom) and assisted isometric strengthening exercises with a physiotherapist until reaching of a minimum active knee range of motion of 0-90°. Deambulation with 2 crutches and full weight-bearing was allowed on the first post-operative day. As a common practice in the country where the study was performed, patients started rehabilitation in the surgical centre, and were discharged to a specialized rehabilitation centre five to seven days after surgery.

Appendix 2

Peri-prosthetic DXA - (T1, T2, T3, T4)

To evaluate post-operative variations in the peri-prosthetic region, a DXA of the knee was performed at T1, T2, T3 and T4 (C.M.). All DXA measurements were performed using a Hologic QDR-Discovery A densitometer (Hologic Inc., Bedford, MA, USA).

All knee joints were scanned in the antero-posterior projection. Five different regions of interest (ROI) were defined to analyse peri-prosthetic BMD using metal removal software provided by the manufacturer, as previously described by Soininvaara et al. [11]. Two squared regions (ROI 1 and 2) were placed in the distal metaphysis of the femur, just above the upper limit of the femoral component, with equal width and length. Similarly, two equal rectangular-shaped regions (ROI 3 and 4) were placed in the proximal tibia and fibula, just below the lower limit of the tibial component. A fifth region (ROI 5) was placed just below the 3 and 4, to analyse tibial and fibular diaphysis. *Figure 1* shows an example of the ROI location. Differences in the net average value (NET), which represents the sum of BMD values of each ROI, were also analysed. Once all ROI were defined, they were automatically copied onto the follow-up acquisitions using the compare utility, in order to reduce the variability from manual correction on data analysis.

Appendix 3

RNA extraction using TRI Reagent method - (T0)

Total RNA was extracted from tibial plateau biopsies. The bone samples were transferred in a suitable container and the preservative reagent RNAlater[®] (Qiagen, Düsseldorf, Germany) was removed. The samples were centrifuged in PBS 1X at 3000 rpm (1008 rcf) 5 min at 4 °C and the supernatant was discarded. To obtain nucleic acid RNA, TRI reagent solution (Sigma Aldrich, Missouri, USA) (1 mL) was added and the biopsies were fragmented by a sonicator with a blade, preserving the solution in ice, then centrifuged at 12000 rpm (16128 rcf) for 10 min at 4 °C. The supernatant phase containing RNA was transferred in a new tube and incubated for 5 min at room temperature (RT). Chloroform (200 µL) was added and incubation performed for 15 min at RT to achieve effective separation. A new centrifugation was performed at 12000 rpm (16128 rcf) for 15 min at 4 °C and the resulting supernatant containing RNA was transferred in a new micro tube. After isopropanol addition (500 µL), the solution was gently agitated and then maintained for 10 min at RT. Later, the samples were centrifuged at 12000 rpm (16128 rcf) for 10 min at 4 °C to obtain a RNA pellet from each sample. The supernatant was discarded and ethanol (75%, 1 mL) was added to purify the nucleic acid. The pellet was vortexed in ethanol and then centrifuged at 14000 rpm (21952 rcf) for 5 min at 4 °C to discard the residual supernatant. The dried pellet was dissolved in 20 µL of sterile deionized water and the samples were analysed by spectrophotometry.

RNA quality and yield: spectrophotometric quantification - (T0)

The samples purity and concentration were quantified using the spectrophotometer instrument “NanoDrop 2000” (Thermo Scientific, Massachusetts, USA). The ratios 260/280 “nucleic acid absorbance/protein absorbance” and 260/230 “nucleic acid absorbance/organic absorbance” were evaluated. In particular, in order to have a good quality RNA, the threshold for the 260/280 ratio was set at 1.7 and for the 260/230 ratio at 2.

Gene expression: reverse transcription and Real Time PCR - (T0)

The retro transcription phase was performed using 1 µg of total RNA, according to the manufacturer’s instruction of the ProtoScript® First Strand cDNA Synthesis Kit for RT-qPCR system (New England BioLabs Inc.). All samples were retrotranscribed 3-5 min using Thermo cycler (Perkin Elmer, 2400 Gene Amp PCR System, Germany) by means of the following program: amplification and real-time data acquisition were performed using the followed cycle conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. Quantitative PCR was performed using commercial TaqMan® Gene Expression Assay (Life Technologies Italia, Monza), according to the manufacturer’s instruction; mRNA was amplified using the following primers: DKK-1 (Hs00183740_m1 Life Technologies California, USA), SOST (Hs00383602-m1 Life Technologies California, USA), OPG (Hs00900358_m1 Life Technologies, California, USA), RANK (Hs00187192_m1, Life Technologies California, USA), RANKL (Hs00243522_m1, Life Technologies California, USA), and IL-6 (Hs00985639 _m1 Life Technologies California, USA). The 18s (Hs03928985_g1 Life Technologies, California, USA) housekeeping gene was used.

The fold change in expression of the different genes in patients was normalized to the expression of 18s. The results obtained are the increase (or decrease) of the target gene in the bone tissue. Therefore, the results for upregulated and downregulated genes compared to 18s expression are > 0 and < 0 , respectively. 18s expression did not change significantly among patients. The accuracy was monitored by the analysis of the melting curves.

Enzyme-linked immunosorbent assays (ELISA) - (T0, T1, T2, T4)

IL-6, DKK-1 and SOST were measured in serum using ELISA sandwich Quantikine Assays, while OPG, RANK and RANKL were measured using an ELISA Duo Set Assay, (R&D Systems, Minneapolis, MN, USA), according to manufacturer’s protocols. For IL-6, the sensitivity was 0.7 pg/mL, and intra- and inter-assay coefficients of variation were 4.2% and 3.3%. For DKK-1 assay, the sensitivity was 4.2 pg/mL, with intra- and inter-assay coefficients of variation 4.2% and 6.0%.

For SOST, the sensitivity was 1.74 pg/mL, and intra- and inter-assay coefficients of variation were 1.8% and 8.2%.

References

1. Friedrich MJ, Wimmer MD, Schmolders J, Strauss AC, Ploeger MM, Kohlhof H, et al. 2017 RANK-ligand and osteoprotegerin as biomarkers in the differentiation between periprosthetic joint infection and aseptic prosthesis loosening Clinical Trials Study. *World J. Orthop.* 8(4):342.
2. Granchi D, Pellacani A, Spina M, Cenni E, Savarino LM, Baldini N, et al. 2006 Serum levels of osteoprotegerin and receptor activator of nuclear factor-kappaB ligand as markers of periprosthetic osteolysis. *J. Bone Joint Surg. Am.* 88(7):1501–9.
3. He T, Wu W, Huang Y, Zhang X, Tang T, Dai K. 2013 Multiple biomarkers analysis for the early detection of prosthetic aseptic loosening of hip arthroplasty. *Int. Orthop.* 37(6):1025–31.
4. Liu H, Luo T, Tan J, Li M, Guo J. 2017 Osteoimmunology' Offers New Perspectives for the Treatment of Pathological Bone Loss. *Curr. Pharm. Des.* 23(999).
5. Cassuto J, Folestad A, Göthlin J, Malchau H, Kärrholm J. 2018 The key role of proinflammatory cytokines, matrix proteins, RANKL/OPG and Wnt/ β -catenin in bone healing of hip arthroplasty patients. *Bone* 107:66–77.
6. Hu Y, Liu W, Liu Z, Kuang W, He H. 2015 Receptor activator of nuclear factor-kappa ligand, OPG, and IGF-I expression during orthodontically induced inflammatory root resorption in the recombinant human growth hormone-treated rats. *Angle Orthod.* 85(4):562–9.
7. Martin TJ, Sims NA. 2015 RANKL/OPG; Critical role in bone physiology. *Rev. Endocr. Metab. Disord.* 16(2):131–9.
8. Mariz K, Ingolf J-B, Daniel H, Teresa NJ, Erich-Franz S. 2015 The Wnt inhibitor dickkopf-1: a link between breast cancer and bone metastases. *Clin. Exp. Metastasis* 32(8):857–66.
9. Appelman-Dijkstra NM, Papapoulos SE. 2016 Sclerostin Inhibition in the Management of Osteoporosis. *Calcif. Tissue Int.* 98(4):370–80.
10. van Lierop AH, Appelman-Dijkstra NM, Papapoulos SE. 2017 Sclerostin deficiency in humans. *Bone* 96:51–62.

11. Soininvaara T, Nikola T, Vanninen E, Miettinen H, Kröger H. 2008 Bone mineral density and single photon emission computed tomography changes after total knee arthroplasty: a 2-year follow-up study. *Clin. Physiol. Funct. Imaging* 28(2):101–6.
12. Soininvaara TA, Miettinen HJA, Jurvelin JS, Alhava EM, Kröger HPJ. 2004 Bone mineral density in the proximal femur and contralateral knee after total knee arthroplasty. *J. Clin. Densitom.* 7(4):424–31.
13. Behrend H, Giesinger K, Giesinger JM, Kuster MS. 2012 The “forgotten joint” as the ultimate goal in joint arthroplasty: validation of a new patient-reported outcome measure. *J. Arthroplasty* 27(3):430–436.e1.
14. Arican O, Aral M, Sasmaz S, Ciragil P. 2005 Serum levels of TNF-alpha, IFN-gamma, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. *Mediators Inflamm.* 2005(5):273–9.
15. Cheng Q, Tang W, Sheu T-J, Du Y, Gan J, Li H, et al. 2016 Circulating TGF- β 1 levels are negatively correlated with sclerostin levels in early postmenopausal women. *Clin. Chim. Acta.* 455:87–92.
16. Clarke BL, Drake MT. 2013 Clinical utility of serum sclerostin measurements. *Bonekey Rep.* 2:361.
17. Mödder UI, Hoey KA, Amin S, McCready LK, Achenbach SJ, Riggs BL, et al. 2011 Relation of age, gender, and bone mass to circulating sclerostin levels in women and men. *J. Bone Miner. Res.* 26(2):373–379.
18. Qiu F, He J, Zhou Y, Bai X, Wu G, Wang X, et al. 2014 Plasma and vitreous fluid levels of Dickkopf-1 in patients with diabetic retinopathy. *Eye (Lond).* 28(4):402–9.
19. Dovio A, Generali D, Tampellini M, Berruti A, Tedoldi S, Torta M, et al. 2008 Variations along the 24-hour cycle of circulating osteoprotegerin and soluble RANKL: a rhythmometric analysis. *Osteoporos. Int.* 19(1):113–7.
20. Mezquita-Raya P, de la Higuera M, García DF, Alonso G, Ruiz-Requena ME, de Dios Luna J, et al. 2005 The contribution of serum osteoprotegerin to bone mass and vertebral fractures in postmenopausal women. *Osteoporos. Int.* 16(11):1368–74.
21. Findlay DM, Atkins GJ. 2011 Relationship between serum RANKL and RANKL in bone. *Osteoporos. Int.* 22(10):2597–602.
22. Kuo T-R, Chen C-H. 2017 Bone biomarker for the clinical assessment of osteoporosis: recent developments and future perspectives. *Biomark. Res.* 5(1):18.
23. Niu C-C, Lin S-S, Yuan L-J, Chen L-H, Yang C-Y, Chung A-N, et al. 2017 Correlation of blood bone turnover biomarkers and Wnt signaling antagonists with AS, DISH, OPLL, and

- OYL. *BMC Musculoskelet. Disord.* 18(1):61.
24. Monjo M, Lamolle SF, Lyngstadaas SP, Rønold HJ, Ellingsen JE. 2008 In vivo expression of osteogenic markers and bone mineral density at the surface of fluoride-modified titanium implants. *Biomaterials* 29(28):3771–80.
 25. Järvenpää J, Soininvaara T, Kettunen J, Miettinen H, Kröger H. 2014 Changes in bone mineral density of the distal femur after total knee arthroplasty: a 7-year DEXA follow-up comparing results between obese and nonobese patients. *Knee* 21(1):232–5.
 26. Mau-Moeller A, Behrens M, Felser S, Bruhn S, Mittelmeier W, Bader R, et al. 2015 Modulation and predictors of periprosthetic bone mineral density following total knee arthroplasty. *Biomed Res. Int.* 2015:418168.
 27. Wijenayaka AR, Kogawa M, Lim HP, Bonewald LF, Findlay DM, Atkins GJ. 2011 Sclerostin Stimulates Osteocyte Support of Osteoclast Activity by a RANKL-Dependent Pathway. *PLoS One* 6(10):e25900.
 28. Idolazzi L, Fassio A, Tripi G, Braga V, Viapiana O, Adami G, et al. 2017 Circulating Dickkopf-1 and sclerostin in patients with Paget's disease of bone. *Clin. Rheumatol.* 36(4):925–928.
 29. Fujita K, Janz S. 2007 Attenuation of WNT signaling by DKK-1 and -2 regulates BMP2-induced osteoblast differentiation and expression of OPG, RANKL and M-CSF. *Mol. Cancer* 6(1):71.
 30. Wang J-H, Zhang Y, Li H-Y, Liu Y-Y, Sun T. 2016 Dickkopf-1 negatively regulates the expression of osteoprotegerin, a key osteoclastogenesis inhibitor, by sequestering Lrp6 in primary and metastatic lytic bone lesions. *Medicine (Baltimore)*. 95(24):e3767.
 31. Fazzalari NL, Kuliwaba JS, Atkins GJ, Forwood MR, Findlay DM. 2001 The ratio of messenger RNA levels of receptor activator of nuclear factor kappaB ligand to osteoprotegerin correlates with bone remodeling indices in normal human cancellous bone but not in osteoarthritis. *J. Bone Miner. Res.* 16(6):1015–27.
 32. Schett G, Kiechl S, Redlich K, Oberhollenzer F, Weger S, Egger G, et al. 2004 Soluble RANKL and risk of nontraumatic fracture. *JAMA* 291(9):1108–13.
 33. Wagner D, Fahrleitner-Pammer A. 2010 Levels of osteoprotegerin (OPG) and receptor activator for nuclear factor kappa B ligand (RANKL) in serum: are they of any help? *Wien. Med. Wochenschr.* 160(17–18):452–7.
 34. Grimaud E, Soubigou L, Couillaud S, Coipeau P, Moreau A, Passuti N, et al. 2003 Receptor activator of nuclear factor kappaB ligand (RANKL)/osteoprotegerin (OPG) ratio is increased in severe osteolysis. *Am. J. Pathol.* 163(5):2021–31.

35. Cruickshank AM, Fraser WD, Burns HJ, Van Damme J, Shenkin A. 1990 Response of serum interleukin-6 in patients undergoing elective surgery of varying severity. *Clin. Sci. (Lond)*. 79(2):161–5.

ACCEPTED MANUSCRIPT

7. Figure legends

Figure 1: Regions of interest (ROI) defined to analyse the peri-prosthetic bone mineral density using a metal removal software: distal metaphysis of the femur (R1 and R2), proximal tibia and fibula (R3 and R4), and tibial and fibular diaphysis (R5).

*Figure 2: Comparison of serum levels of the selected osteoimmunological biomarkers before surgery (T0) and four days (T1), three (T2) and twelve months (T4) after surgery. Data are expressed as means \pm SD. Only p-values <0.05 are indicated: ***, $p<0.001$ as compared to T0; *, $p<0.05$ as compared to T0.*

Figure 3: Comparison of post-operative variations in the peri-prosthetic bone mineral density (BMD) four days (T1) and three (T2), six (T3) and twelve months (T4) after surgery: BMD variation in the femoral ROI (blue); BMD variation in the tibial ROI (red). Data are expressed as means \pm SD.

*Figure 4: Comparison of clinical results before surgery (T0), and three (T2), six (T3) and twelve months (T4) after surgery: a) OKS variation from pre-operative level; B) VAS variation from pre-operative level. Data are expressed as means \pm SD. Only p-values <0.05 are indicated: ***, $p<0.001$; **** $p<0.0001$ as compared to T0. OKS: Oxford Knee Score, in points; VAS: Visual Analogue Scale, in mm.*

8. Tables

Table 1. Eligibility criteria.

Table 1. Eligibility criteria.

Inclusion criteria:	
-	Patients eligible to receive a primary TKA;
-	Age < 80 years;
-	Informed consent to participation in the study.
Exclusion criteria:	
-	Knee deformity > 15° in varus, valgus, femoral flexion or tibial slope;
-	Pre-operative diagnosis of osteoporosis;
-	Concomitant treatment with bisphosphonates, strontium ranelate, selective estrogen receptor modulators, parathyroid hormone or its analogues, calcitonine or denosumab;
-	Pre-operative diagnosis of Paget disease;
-	Thyroid and Parathyroid disorders;
-	Patients with metabolic disorders, and serious comorbid conditions that could limit the follow-up (e.g. neoplastic diseases, immune deficiencies, hepatitis).

Table 2. Patients' demographics Data are reported as mean (\pm SD) or frequency/ratio. BMI: body mass index; F/M: female/male; R/L: right/left; n.s.: not significant.

Age	70.44 (\pm 5.69)
BMI	29.50 (\pm 5.43)
F/M ratio	0.75/0.25
R/L ratio	0.44/0.56
Smokers/Non-smokers	0/1

Table 3. mRNA expression (relative quantification/18S). Data are reported as mean (\pm SD).

IL-6	0.245 (\pm 0.175)
RANK	0.503 (\pm 0.177)

RANKL	0.478 (\pm 0.161)
OPG	0.558 (\pm 0.302)
SOST	0.185 (\pm 0.106)
DKK-1	0.707 (\pm 0,145)
RANKL/OPG	0.818 (\pm 0.293)

Table 4. Serum concentration (pg/mL) of the analyzed biomarkers. Data are reported as mean (\pm SD)

	No. patients	T0	T1	T2	T4
IL-6	11	3.371 (\pm 3.643)	43.16 (\pm 25.32)	4.258 (\pm 5.308)	3.256 (\pm 2.174)
RANK	14	587.6 (\pm 214.3)	403.1 (\pm 167.9)	480.9 (\pm 167.2)	497.2 (\pm 144.5)
RANKL	6	704.5 (\pm 337)	681.5 (\pm 467.8)	752.4 (\pm 488.2)	796 (\pm 478.9)
OPG	16	2225 (\pm 613.7)	2275 (\pm 664.5)	2178 (\pm 623.5)	2311 (\pm 366.2)
SOST	14	258.1 (\pm 134.1)	194.4 (\pm 77.18)	240.5 (\pm 83.59)	267.7 (\pm 95.35)
<i>men</i>	3	202.1 (\pm 209.3)	195.3 (\pm 113.3)	217.9 (\pm 119.7)	208.8 (\pm 129.9)
<i>women</i>	11	273.3 (\pm 115.8)	194.2 (\pm 71.95)	246.6 (\pm 77.61)	283.7 (\pm 84.38)
DKK-1	13	4606 (\pm 1580)	4288 (\pm 1436)	5301 (\pm 3575)	3919 (\pm 1485)
RANKL/OPG	6	0.3444 (\pm 0.1736)	0.4983 (\pm 0.7008)	0.7533 (\pm 1.109)	0.3987 (\pm 0.2589)