# Microbiome and Innate Immunity with Percutaneous Osseointegrated Prostheses

## Protocol Summary

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| **IRB Approval Date of Current Version:** | 7/12/2019 |
| **University of Utah IRB #:** | IRB_00073178 |
| **Sponsor:** | VA REHABILITATION RESEARCH AND DVLPMNT |
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## Background and Introduction

Percutaneous osseointegrated prosthetic attachment (i.e., the direct skeletal attachment of artificial limbs) is a rapidly evolving technology that is now ready to be introduced into the United States. This follows over a decade of European trials that largely involved transfemoral amputees. Translational animal research carried out over the past seven years at the VA , Salt Lake City, Utah, has clarified principles of osseointegration, and bone and skin interface responses to chronic weight bearing on a skeletally attached artificial limb. This information has produced new prosthetic designs that make the technique safer and more likely to succeed over the long-term. This translational animal data, gathered in parallel to the European human data, has made it possible to commence an Early Feasibility Device Exemption (IDE) Pilot Program under the direction of the FDA. This trial is anticipated to begin October 19, 2015, at the VAH in Salt Lake City, Utah, and is funded by VA RR&D grant (RX001208-01). Ten transfemoral amputees, selected from the veteran and active military populations will receive a PODS device.

The critical limiting factor to the prior U.S. introduction of this technology has been an up to 30% infection rate at the implant/skin interface in European patients. Evolving designs and surgical techniques have lowered this incidence, but it remains remarkable that 70% to perhaps 95% of European patients now remain infection free by simply using mild soap and shower hygiene at the stoma, and avoiding the use of antibiotics. In fact antibiotics and disinfectants have been found to be counter productive and lead to antibiotic resistance. It is increasingly evident that the key to this improbable success is the biology and presence of a mutualistic-commensal microbiota at the skin/implant interface. These “good bacteria” are the “guard dogs at the gate”.

**Microbiome studies:** Traditionally, wound microbiotas have been characterized by culture based assays. Cultures, however, account for only 3% of the bacterial strains actually present. A recent study, reported that, on average, culturing techniques underestimated microbial load by 2.34 logs ($P<0.0001$), and in some cases greater than 6 logs. Bacterial cultures, because they are designed to select for specific pathogens, are unable to grow and recognize most of the other resident skin microbiota. These non-molecular techniques do not allow a full understanding of the complexity of stomal colonization in both the diseased and healthy states.

The relationships between the bacterial subsets within an ecological community are complex. There is strong evidence to suggest that microbes that are commonly thought of as pathogens can live commensally on the skin. In fact, bacteria from the genera Staphylococcus, Corynebacterium, Propionibacterium, Streptococcus and Pseudomonas, may directly benefit the host (mutualism) and only rarely will they become pathogens. Recent developments in molecular based technology have now paved the way to more advanced genome based techniques for identifying bacterial ecologies (microbiome). Techniques based upon distinct bacterial molecular markers, such as 16S rRNA are now being employed to truly understand the complexity and complicity of the human microbiome.

**Inflammatory biomarkers:** Wound healing involves a complex process with many overlapping stages: coagulation, inflammation, cell proliferation and migration, and finally tissue remodeling with wound closure within 3-14 days. However, as a result of our percutaneously implanted device, we expect the area around the stoma of our patients to

evidence a chronic/continuous wound healing process. Understanding the relationship between the microbiome and modulating the cycle of inflammation produced by microbial colonization and/or infection is a critical step towards improving treatment strategies.

We postulate that comparing the profiles of local and systemic inflammatory biomarkers can provide valuable information regarding the state of health of the skin surrounding the implant. Because different cytokines can have overlapping biological functions, as well as regulate production of other cytokines, the ability to simultaneously analyze a large set of cytokines within a localized environment (such as a site of inflammation) can be more valuable than detecting the levels of individual cytokines (particularly if only small volume test samples are obtained). Collecting multiple samples over time will also allow for longitudinal studies of cytokine profiles within a single patient.

Biomarkers (e.g., cytokines and chemokines) are proteins that act as effectors or modulators of the inflammatory response. Custom TaqMan microfluid array cards (assaying 192 genes) will be able to detect mRNA expression patterns of systemic inflammatory proteins. These can then be compared to native secreted proteins locally present at the implant site: the latter detected using the Bio-Plex multiplex immunoassay system measuring 27 cytokines.

The biomarkers detected in this study (using the Bio-Plex system) outperformed the erythrocyte sedimentation rate (ESR) and the C-reactive protein (CRP); tests that are commonly used in standard clinical practice to detect the presence of infection. We therefore believe that in addition to having a better understanding of the cytokine profile expected of a chronic wound in a stable state (when compared to an infected state) it is possible that a diagnostic cytokine profile may be determined that will predict the onset or detect the presence of a subclinical infection.

## Purpose and Objectives

### Purpose

The purpose of this study is to investigate the clinical implementation of a new percutaneous prosthetic attachment system (Safety Study of Percutaneous Osseointegrated Implants for Prosthetic Attachment; separate IRB # 00082763 ) by determining the resident microbial ecology of the implant exit site and to simultaneously study the systemic and local stomal immune responses. We will use two, state-of-the-art, pre- and post-surgery bacterial monitoring technologies: these procedures are intended to facilitate the early prediction, detection, and treatment of infection, as well as to provide follow-up data that can potentially be used to advantageously manipulate the stomal microbial environment in future clinical trials.

### Objectives

The objective of this study is to follow 10 patients implanted with a percutaneous osseointegrated exoprosthetic docking system (PODS) for a period of one year. This PODS

technology requires invasive surgery to skeletally attach a metal implant, which protrudes from the long bone and exits the stump skin of the amputee patient (IRB# 00082763, as above). All stomas are colonized by local skin bacteria; colonization does not necessarily result in infection. Over time, the presence of this skin penetrating foreign object (implant) will cause measurable changes in the bacterial population (microbiota) at and around the PODS exit site.

It is anticipated that the evolving microbiota, in concert with measurable changes in the local and systemic cytokine responses, will reveal patterns associated with mutualistic-commensal bacteria and/or pathogenic bacteria related to the stages of chronic wound healing. These patterns could be used to determine the presence of a stable uninfected stoma or the progression of a stomal infection. Hopefully, this information will allow timely intervention to prevent infection, i.e. by detecting early stages of infection or discerning common patterns of stable mutualistic-commensal bacterial strains, effective intervention protocols (antibiotics, probiotics or manipulation of the stomal and skin microbiota) may be developed to avoid patient morbidity and assure implant survival.

The study aims will test the following:

> **Aim 1:** Determine and characterize the microbiota in the region surrounding the skin/implant interface. Sampling will take place over all stages of wound healing and stomal maturation and will begin with Stage 1 and Stage 2 surgeries, as well as at defined time points, and collection sites (i.e., the stoma, ipsilateral and contralateral thigh skin) for up to one year post surgery. This will be carried-out by using a specific swabbing technique to collect bacterial DNA and to amplify and sequence bacterial 16S rRNA genes.
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> **AIM 2**: Compare the expression patterns of the local and systemic inflammatory biomarkers over time and determine if there is a correlation with the microbiota pattern to diagnose the state of wound healing at the skin/implant interface and the systemic response to a potentially life-long chronic wound. The measurements of the pro-inflammatory cytokines, found in the stomal exudate (local biomarkers) and blood serum (systemic biomarkers), along with evolving microbiota profiles (Aim #1) will help to better characterize the homeostatic state of the stoma and subsequent optimum wound care therapies.

The ability to predict infection and to avoid it without the use of antibiotics would be of great value to future clinical trials. Assuming the success of this feasibility pilot trial, it is anticipated that the trial will be expanded to include 200 patients.

## Study Population

**Age of Participants:** 18-70 years old

**Sample Size:**

At Utah: 10
All Centers: 10

**Inclusion Criteria:**

Only participants enrolled in IRB #00082763 study will be enrolled in this study. For the IRB study #00082763 study, participants come from a pool of subjects who are veterans and have transfemoral limb loss that is not a result of dysvascular disease. These patients are considered to be capable of functioning with socket suspension transfemoral prostheses but are also suitable for fitting with a percutaneous implant system.

Inclusion criteria for # IRB 00082763 are:

A potential subject will be included in the study if he or she meets all of the following inclusion criteria:

1. Is a US military veteran with transfemoral limb loss, that occurred at least 6 months prior to consent, and that the amputation is not a result of dysvascular disease.

2. Is at least 18 years of age or older.

3. Has previously used or is currently using a "socket suspension technology" prosthesis

4. Has, in the opinion of the investigator, normal cognitive function and no physical limitations, addictive diseases, or underlying medical conditions including tobacco use (continued testing for tobacco use will be performed at screening) that may prevent the subject from being an appropriate study candidate.

5. Is willing, able, and committed to participation in baseline and follow-up evaluations for the entire duration of the study.

6. Can provide written informed consent to participate.

**Exclusion Criteria:**

A potential subject will be excluded from study participation if he/she meets any of the following criteria (the IRB 00082763 study criteria)

1. Is currently on active or reserve military duty
2. Has experienced systemic bacterial infection or localized infection at the stump site within the previous 6 months
3. Has had more than 1 limb amputated
4. Has a body mass index (BMI) $\geq 30$
5. Has insulin dependent diabetes mellitus (IDDM) or has adult onset DM with a glycated hemoglobin ($HbA_{1c)} > 53$ mmol/mol (7.0%) at screening

6. Has residual femur bone length of less than 25% of the length of the contralateral femur.
7. Has clinically diagnosed vascular compromise proximal to the surgical site
8. Is pregnant at the time of surgery or plans to become pregnant within the first year of follow-up
9. Has evidence of recent tobacco use (urine cotinine test > 300 ng/mL [1703 nmol/L]) and is not committed to a smoking-cessation program
10. Has renal insufficiency (defined as serum creatinine of ≥ 1.8 mg/dL) or is currently receiving renal dialysis
11. Is currently involved in or plans to be involved in high levels of physical activity (competitive sports, heavy physical labor, etc) during the first 12 months of the rehabilitation stage
12. Has muscular, neurologic or vascular deficiencies that may compromise the bone or soft tissue healing of the affected extremity
13. Has anemia characterized by a hemoglobin of ≤ 11 g/dL at the time of surgery
14. Is currently on oral anticoagulation (excluding low-dose aspirin for cardiac prophylaxis)

## Design

Other

Retrospective sample analyses

## Study Procedures

**Recruitment/Participant Identification Process:**

Only patients, who will be recruited and consented to participate in IRB#00082763 study, will participate in this "Microbiome" study to retrospectively assess the progression of wound and stomal healing and/or the occurrence of infection, as a safety survey and previously described.

**Informed Consent:**

**Description of location(s) where consent will be obtained:**

George E Wahlen Department of Veterans Affairs Hospital, Salt Lake City Health Care System.

**Description of the consent process(es), including the timing of consent:**

Consent process is now part of the IRB study # 00082763. No separate consent process will be used.

**Requested Waivers/Alterations of Consent:**

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| Waiver of Informed Consent | VA Waiver of Consent for Recruitment Purposes Only |

**Procedures:**

-Patient selection

All patients selected to participate in the "Safety Study of Percutaneous Osseointegrated Implants for Prosthetic Attachment (IRB #00082763)" will participate in this study. As this is safety evaluation study, subjects will not be randomized. Follow-up end point will be 1 year post 2nd stage surgery.

Patients will be consented through IRB study # 00082763.

- Obtaining consent

Patients are consented as part of the IRB #00082763 consenting process

- Sample collection

Samples (skin swabs, tissue and blood) for this study will come from the samples collected and tissue-banked as part of the IRB # 00082763.

Collection process is described in IRB # 00082763. Briefly, samples will be collected from each patients at different pre- and post-operative time points (see below).

Pre-op stage 1/day of the procedure, 3+/-7 days of stage 1 post-op, pre-op of stage 2/day of procedure, day 7 +/- 7 days of stage 2, 2 wk +/- 14 days of Stage 2, and 6wk +/- 14 days, 3, 6,9, 12 mo +/- 30 Days of stage 2.

-Analysis

-Reporting

**Procedures performed for research purposes only:**

## Statistical Methods, Data Analysis and Interpretation

Statistical analysis: A sample size of n=10 patients is planned to be used.

Sample Size Justification. A sample size of n=10 patients will be used. Even if the specific comparisons, which involve a single predictor variable, described for Aims 1 to 3 reveal trends that are not statistically significant, it would be sufficient to show that overall model fit is significant. That is, a significant multivariable curvilinear correlation with pro-inflammatory cytokines, for example, would demonstrate that Bio-Plex array is potentially a sensitive indicator of inflammation and/or infection, provided that a visible shift in the graphed growth curve occurs

with the onset of inflammation and/or infection. With a sample size of 10, there is 80% power using a two-sided alpha 0.05 comparison, to detect a correlation of r=0.71 or greater. Converting this to a coefficient of determination, r2 = 0.50, the multivariable model would have to account for 50% of the variability in pro-inflammatory cytokines concentration. Since cytokines is measurable, going up or down across healing time in an anticipated fashion, with time being one of the predictor variables making up the multivariable curvilinear correlation, it is reasonable to assume that this magnitude of correlation will be observed in this study. Furthermore, the repeated measurements increase the statistical power somewhat. The study, then, is adequately powered to demonstrate the feasibility of microbiota, and pro-inflammatory cytokines/markers as indicators of inflammation and/or infection.

Microbiota analysis

In this microbiota study, the temporal dynamics of the stomal bacterial ecology of these ten patients were investigated over the duration of one-year following the second surgery. skin swab samples were collected at regular intervals using swabs obtained from 3 different sites. Pre-surgery data was used as a baseline, thus, each patient had their internal control, and the microbiota changes to the individual patient's per- and post- skin swab samples were compared. The DNA extraction were performed using the standard procedure. The 16S ribosomal RNA genes were amplified using broad range PCR primers targeted to the V1-V3 hypervariable region (V1V3 Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and V1V3 Reverse primer 5-ATTACCGCGGCTGCTGG-3'), with barcodes unique to each sample. PCR reactions were carried out in quadruplicate using high fidelity Accuprime Taq. The resulting 16S rDNA amplicons were purified using a 1:1 volume of SPRI beads, quantified using PicoGreen, pooled in equal amounts, and sequenced on the Illumina MiSeq using 2x300 bp chemistry. Extraction blanks and DNA-free water were subjected to the same amplification and purification procedure to allow for empirical assessment of environmental and reagent contamination. Sequencing was performed by the PennCHOP Microbiome Center on the Illumina MiSeq instrument. Sequencing results were then processed using the hmmUFOtu workflow. Reads were demultiplexed using the prior mentioned barcodes. Individual reads (with ≥97% identity) were treated as same species sequences, and were clustered and assigned to operational taxonomic units (OTUs) using the Greengenes 13_8 database. OTUs that were found to represent just one sequence in one sample were also removed. Any reads assigned to Cyanobacteria, a common contaminant, were also removed. OTU tables and phylogenetic trees output from hmmUFOtu were then further analyzed using the phyloseq package in R. This analysis included rarefaction of samples down to an even depth of ~1000 sequences per sample. Alpha and beta diversity metrics were computed using the vegan package.

All statistical analysis was performed in R. For testing statistically significant differences between populations, the nonparametric Wilcoxon rank-sum test was used (wilcox.test function in R). Due to the sample losses from rarefaction, the

unpaired test was used. Multiple testing correction was also applied with the p.adjust function, using the false discovery rate correction. For differential abundance testing, the DESeq2 and phyloseq packages were used with default settings. Sample similarity was calculated using the Weighted UniFrac, Unweighted UniFrac, and Bray Curtis Beta diversity metrics, and/or statistical comparisons of community composition. The Adonis function (PERMANOVA test), from the vegan package was applied to each of the aforementioned metrics. Dirichlet multinomial mixture modelling was performed using the DirichletMultinomial package. Ordination was performed using the labdsv package.

Whole Blood RNA expression data

Whole-blood samples were collected from these patients at the following time points: pre-surgery 1 (PrS1), post-surgery 1 (PoS1), pre-surgery 2 (PrS2), post-surgery 2 week 1 (PoS2-W1), post-surgery 2 week 2 (PoS2-W2), post-surgery 2 month 1 (PoS2-M1), post-surgery 2 month 3 (PoS2-M3), post-surgery 2 month 6 (PoS2-M6), post-surgery 2 month 9 (PoS2-M9), and post-surgery 2 month 12 (PoS2-M12). Whole-blood RNAs were isolated and undergone complete RNA sequencing, where single-end base pair sequencing was performed at the University of Utah Core genomic facility. Gene read counts were quantified via featureCounts and differential gene expression (DEG) analysis was performed using DESeq2.

For the DEG analysis, pair-wise comparisons were made in two sets: first, between PrS1 and all subsequent time points, second between PrS2 and all subsequent time points. Results from the DEG analysis were used to perform an enrichment analysis, which implemented topGO's elim graph algorithm paired with the Kolmogorov-Smirnov test for significance. Enrichment analysis was run on each of the Gene Ontology's (GO) sub ontologies: biological process (bp), cellular component (cc) and molecular function (mf).

References:

1. Bland, J.M. and D.G. Altman, Calculating correlation coefficients with repeated observations: Part 1--Correlation within subjects. BMJ, 1995. 310(6977): p. 446.

2. Bland, J.M. and D.G. Altman, Calculating correlation coefficients with repeated observations: Part 2--Correlation between subjects. BMJ, 1995. 310(6980): p. 633.