INSIGHTS Analysis Plan

# Background and Introduction

Sickle cell disease (SCD) is caused by a single point mutation in the 6th codon of the β-globin gene resulting in abnormal β. This produces red blood cells that are sickle-shaped and impaired in their function. In particular, hemolysis and erythrocyte membrane damage are hallmarks of the disease. Furthermore, SCD is characterized primarily by severe anemia and vaso-occlusion. SCD symptoms include severe pain (often referred to as the “painful crisis”), often recurrent and requiring frequent hospitalizations, hemolytic anemia, and end-organ damage that results in premature death.

The term sickle cell disease refers to different genotypes that define this complex clinical syndrome in which HbS is the most abundant species. There are varying degrees of disease severity within the spectrum of SCD. The most severe phenotypes are in individuals that have two HbS genes (HbSS) commonly known as sickle cell anemia or a double heterozygosity for beta thalassemia0 and HbS (HbSB0). The least severe phenotypes comprise individuals that either have sickle cell with hemoglobin C (HbSC) disease or hemoglobin S-β+-thalassemia.

Leg ulcers are commonly the result of chronic and complex conditions such as diabetes and SCD. SCD-associated leg ulcers are breaks in the skin and subcutaneous tissues typically occurring at the ankles, where there is less subcutaneous fat, thin skin, and possibly decreased blood flow but are also infrequently observed on the anterior tibial area, the dorsum of the feet, and the Achilles tendon. Ulcers are extremely painful and sometimes difficult to heal, and recur often. In sickle cell patients, leg ulcers are common. Research studies have documented varying rates according to geographic location from 1.5 to 13.5% in Africa, 2.5% in the U.S. to over 29% in Jamaica. Socioeconomic status has been implicated as one of the contributing factors. Leg ulcers usually appear between the ages of 10 to 50 years and, in some reviews are more prevalent in men than women, others did not, including. Although the pathogenesis of leg ulcer formation remains unclear, investigators speculate that trauma, infection, and inflammation may be responsible for causing SCD leg ulcerations. It is a complex disorder with multiple complications and the etiology of leg ulcers remains unknown warranting further investigation into the underlying mechanism(s) responsible for this condition.

There are several reports from the 1980s that highlight, using culture-based studies, an increase in the abundance of specific bacterial microflora. Interestingly, Staphylococcus, Pseudomonas, and Streptococcus were also commonly identified in leg ulcer cultures. In these studies, individuals with infections responded well to topical antibiotic treatment. This would suggest that microbes, if not responsible, are associated with the progression of this condition and likely impede the healing process similar to the speculated role of microbes in non-healing diabetic foot ulcers. A recent study characterized the microbiome of diabetic foot ulcers in 52 patients using two approaches, high-throughput sequencing and culturing. The most abundant sequenced genera in the diabetic foot ulcers were Staphylococcus (49/52 samples), Streptococcus (15/52), and Lactococcus (38/52). Although culturing was only conducted in the HbSS leg ulcer studies from the 1980s, similar bacterial isolates were also recovered in the diabetic microbial foot survey. Gardner et al.[[1]](#endnote-1) suggest that in order to reveal informative relationships between microbiome changes and disease state (i.e. leg ulcers), microbiome studies must also consider the clinical metadata for each individual patient. To be more specific, the Gardner et al. study found that the microbiome colonizing diabetic foot ulcers (DFUs) is associated with clinical factors. Thus, the 52 participants with foot ulcers were also evaluated for the following clinical factors: hemoglobin A1c, oxygenation, ulcer duration, ulcer depth, ulcer surface area, and necrotic tissue. The final results indicated that the following variables were significantly associated with the microbiome of DFUs: ulcer depth, ulcer duration, and poor glycemic control (i.e. higher hemoglobin A1c). The results indicated that poor glycemic control, higher ulcer duration and deeper ulcers gave higher species levels that were colonizing the DFU.

It is widely known that stress alters the immune system. For example, individuals under a significant amount of stress are more susceptible to infections. In the case of leg ulcers, wound healing is mediated by the immune system requiring cytokines and other immunological factors. Stress and racial discrimination also affects an individual’s mental health status and, perturbs the immune system. SCD patients (and those with leg ulcers) experience stigma because of their condition. We are interested in understanding whether psychological stress, the microbiome, and other factors impair the wound healing process in SCD patients by altering the immune system.

The ultimate goal of the proposed pilot research is to integrate research on microbiome and clinical factors to understand sickle cell disease and the formation and healing process of individuals living with leg ulcers. To our knowledge, this is the first program of research to explore leg ulcers and SCD. The proposed project investigates skin microbiome and clinical factors —to offer an integrative theory as to why SCD patients develop leg ulcers and how to intervene to reduce severity and facilitate healing.

In this study, we will characterize the microbiome of leg ulcers in the recruited cohort of participants while surveying them for clinical triggers, which may put them at an increased risk for developing leg ulcers. We will also compare the leg ulcer microbiome data to published diabetic foot ulcer microbiome data. As a result, comparing these two datasets will likely identify shared or distinct microbial signatures between the two ulcer types improving treatment options, developing new interventions (i.e. utilization of multiple antimicrobials that act in synergy) for those living with this complication while also identifying effective coping strategies that may help patients better manage leg ulcers and the disease.

# Proposed Methods

## General Methods

We propose to follow the methods used by Gardner et al.i to allow for maximal comparability with the authors’ diabetes cohort. Briefly, k-medoid clustering analysis will be used to determine the structure of our sample based on the Euclidean distance matrix of normalized species-level operational taxonomic unit (OTU) counts; this procedure will be validated by comparison with the average silhouette score for each provisional cluster. Covariation matrices of abundance of microbial species will be established using Spearman correlation procedures and significance levels will be corrected using FDR.

Preliminary analyses will determine patterns in the microbiome, clinical, and psychosocial data over the entire population. Overall associations of clinical factors with microbiome community structure and membership will be assessed with the analysis of molecular variance (AMOVA) function in the mother software package. Shannon index values will be calculated for each subject to evaluate microbial diversity. Population clusters will be examined for association with three dimensions of bioburden―microbial load, microbial diversity, and pathogenicity―and clinical and psychosocial factors by Kruskal-Wallis test, followed by pair-wise post hoc comparisons using two-tailed Wilcoxon rank-sum tests.

## Specific Aim #1: Comparison of microbial measures between SCD ulcer groups

To examine differences in structure of microbial populations between SCD subjects currently, previously, or never suffering from leg ulcers, we will conduct principal coordinate analysis on thetayc values (denoting community dissimilarity and calculated using the mother tool), followed by AMOVA to determine mean square differences among and within ulcer groups. Differences in microbial diversity (Shannon index values) between groups will be calculated using Kruskal-Wallis testing, followed by pairwise post-hoc comparisons.

## Specific Aim #2: Comparison of microbial measures between treatment groups

We will use similar methods to those in SA#1 to establish the presence of difference in microbial populations based on presence/absence of treatment with Hydroxyurea.

## Specific Aim #3: Comparison of microbial measures between disease groups

Participant data from current ulcer sufferers in the present study and the population examined in Gardner et al.i will be pooled to create a dual disease dataset. Methods similar to those used in SA#1 and SA#2 will be used to identify potential differences in microbial population structure and diversity based on disease state.

1. Gardner SE, Hillis SL, Heilmann K, Segre JA, & Grice EA. (2013) The Neuropathic Diabetic Foot Ulcer Microbiome Is Associated With Clinical Factors. *Diabetes, 62,* 923-930. doi:10.2337/db12-0771. [↑](#endnote-ref-1)