

Ankle brachial indices and anaerobes: is peripheral arterial disease associated with anaerobic bacteria in diabetic foot ulcers?

J.Z. Alex Cheong, Jessica M. Irvine, Shane Roesemann, Anna Nora, Courtney E. Morgan, Christopher Daniele, Lindsay R. Kalan and Meghan B. Brennan 

Abstract

Background: Lower extremity amputations from diabetic foot ulcers (DFUs) are rebounding, and new biomarkers that predict wound healing are urgently needed. Anaerobic bacteria have been associated with persistent ulcers and may be a promising biomarker beyond currently recommended vascular assessments. It is unknown whether anaerobic markers are simply a downstream outcome of peripheral arterial disease (PAD) and ischemia, however. Here, we evaluate associations between two measures of anaerobic bacteria—abundance and metabolic activity—and PAD.

Methods: We built a prospective cohort of 37 patients with baseline ankle brachial index (ABI) results. Anaerobic bacteria were measured in two ways: DNA-based total anaerobic abundance using 16S rRNA gene amplicon sequencing and resulting summed relative abundance, and RNA-based metabolic activity based on bacterial read annotation of metatranscriptomic sequencing. PAD was defined three ways: PAD diagnosis, ABI results, and a dichotomous definition of mild ischemia (*versus* normal) based on ABI values. Statistical associations between anaerobes and PAD were evaluated using univariate odds ratios (ORs) or Spearman's correlations.

Results: Total anaerobe abundance was not significantly associated with PAD diagnosis, ABI results, or mild ischemia ($OR_{PAD} = 0.47$, 95% CI = 0.023–7.23, $p = 0.60$; Spearman's correlation coefficient $_{ABI} = 0.24$, $p = 0.17$; $OR_{mild\ ischemia} = 0.25$, 95% CI = 0.005–5.86, $p = 0.42$). Anaerobic metabolic activity was not significantly associated with PAD diagnosis, ABI results, or mild ischemia ($OR_{PAD} = 1.99$, 95% CI = 0.17–21.44, $p = 0.57$; Spearman's correlation coefficient $_{ABI} = 0.12$, $p = 0.52$; $OR_{mild\ ischemia} = 0.90$, 95% CI = 0.03–15.16, $p = 0.94$).

Conclusion: Neither anaerobic abundance nor metabolic activity was strongly associated with our three definitions of PAD. Therefore, anaerobic bacteria may offer additional prognostic value when assessing wound healing potential and should be investigated as potential molecular biomarkers for DFU outcomes.

Keywords: ankle brachial index, biomarker, ischemia, microbiome

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Introduction

Despite the plethora of wound care options currently available to treat diabetic foot ulcers, lower extremity amputations have increased 63% since 2009.¹ This is the first resurgence in over 20 years.² An objective biomarker that could monitor response

to therapy and predict healing, especially early in the disease course, would be a major addition to the armamentarium of limb salvage teams.^{3,4} It would allow providers to rapidly identify and employ the most effective wound care strategy for a given patient's ulcer, minimizing the risk of

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Correspondence to:
Meghan B. Brennan

Department of Medicine,
University of Wisconsin–
Madison, 1685 Highland
Avenue, Madison, WI
53583, USA.

Department of Medicine,
William S. Middleton
Memorial Veterans
Hospital, Madison, WI, USA
mbbrennan@medicine.wisc.edu

Lindsay R. Kalan
Department of Medical
Microbiology and
Immunology, University of
Wisconsin–Madison, 6325
Microbial Science Building,
Madison, WI 53583, USA

Department of Medicine,
University of Wisconsin–
Madison, Madison, WI, USA
lkalan@wisc.edu; lrkalan@wisc.edu

J.Z. Alex Cheong
Shane Roesemann
Department of Medical
Microbiology and
Immunology, University of
Wisconsin–Madison,
Madison, WI, USA

Microbiology Doctoral
Training Program,
University of Wisconsin–
Madison, Madison, WI, USA

Jessica M. Irvine
Anna Nora
Department of Medicine,
University of Wisconsin–
Madison, Madison, WI, USA

Department of Medicine,
William S. Middleton
Memorial Veterans
Hospital, Madison, WI, USA

Courtney E. Morgan
Department of Vascular
Surgery, University of
Wisconsin–Madison,
Madison, WI, USA

Department of Vascular
Surgery, William S.
Middleton Memorial
Veterans Hospital,
Madison, WI, USA

Christopher Daniele
Department of Podiatry,
William S. Middleton
Memorial Veterans
Hospital, Madison, WI, USA

amputation. Without such a marker, providers are currently left with subjective wound assessments sometimes spanning weeks before being able to tailor therapy.^{3–8} The Food and Drug Administration recently addressed this gap by redefining clinical endpoints in trials of new wound therapies.⁹ Concurrently, the National Institutes of Health cited the lack of biomarkers that predict wound healing as a major hurdle to developing effective therapies and earmarked specific funds for their development.^{10,11}

Peripheral arterial disease (PAD) is one of the strongest risk factors for non-healing wounds and amputation among those with diabetic foot ulcers.¹² PAD may also increase the abundance of anaerobic bacteria in the ulcer because of downstream ischemia. It is unclear whether high anaerobic abundance and metabolic activity is simply a sequela of PAD, however. The introduction of culture-independent high-throughput sequencing technologies to characterize ulcer microbiomes has revealed that ulcers have higher microbial diversity and colonization by anaerobic bacteria than detection by traditional culture-based laboratory methods. Studies employing these methods have reported associations between the abundance of anaerobes and healing outcomes.^{13–16} Thus, the anaerobic component of an ulcer's microbiome is emerging as a potential biomarker that may be useful for monitoring therapeutic response and predicting wound healing.^{16–19} If anaerobes are solely a microbiologic manifestation of PAD, however, they may not offer additional prognostic value beyond currently recommended vascular assessments.^{20,21}

The aim of this study is to determine whether anaerobic abundance and metabolic activity within the microbiome of diabetic foot ulcers are strongly associated with PAD. If not, efforts to develop an anaerobic biomarker may be more likely to offer new, valuable information for the more than two million Americans who develop a foot ulcer annually.²²

Methods

Prospective cohort

We constructed a prospective cohort of patients with diabetic foot ulcers presenting to the William S. Middleton Memorial Veterans Hospital

podiatric clinic in Madison, WI, USA, between 12 March 2019 and 4 February 2020. We included patients who were: (1) able to provide informed consent, (2) older than 18 years, (3) diagnosed with diabetes based on medical records review, and (4) being seen for a foot ulcer. We excluded patients who were not undergoing sharp debridement or who were scheduled to have a skin substitute applied. Patients receiving a skin substitute were excluded because this therapy would have prevented longitudinal sampling of the ulcer, a goal of the overall cohort study independent of the current analysis. When patients presented with more than one ulcer at the time of enrollment, the treating clinician identified the ulcer that he or she thought would be the most challenging to heal, and this was selected for sampling. Patients who were initially enrolled in the study and later developed a second foot ulcer were excluded from participating again to preserve independence between observations. For this analysis, the cohort was further restricted to patients with baseline ankle brachial index (ABI) testing (Figure 1).

At baseline, patients reported their age, sex, race, ethnicity, and smoking status (categorized as never, former, or current). We solicited information on systemic antibiotics taken within 30 days of sample collection, including those prescribed outside the Veterans Administration (VA) system. We abstracted the presence of the following baseline comorbidities from the VA medical chart: peripheral neuropathy, neuroarthropathy, PAD, coronary artery disease, hypertension, and hyperlipidemia. Patients were classified as having peripheral neuropathy if either one of two criteria was met: peripheral neuropathy was included in their problem list, or patients had a documented abnormal three-site Semmes–Weinstein monofilament test within a podiatry clinic note.²³ Patients undergo routine monofilament testing when they present with a foot ulcer to the podiatry clinic, and documentation is standardized using a note template. Patients were classified as having PAD if any one of the following three criteria were present: (1) PAD was included in their problem list, (2) a vascular surgeon diagnosed the patient with PAD in a clinic note or within their interpretation of the ABI testing, or (3) the patient had undergone a lower extremity revascularization. The ulcer laterality, largest dimension, Wagner grade, and location (categorized as digit, metatarsal,

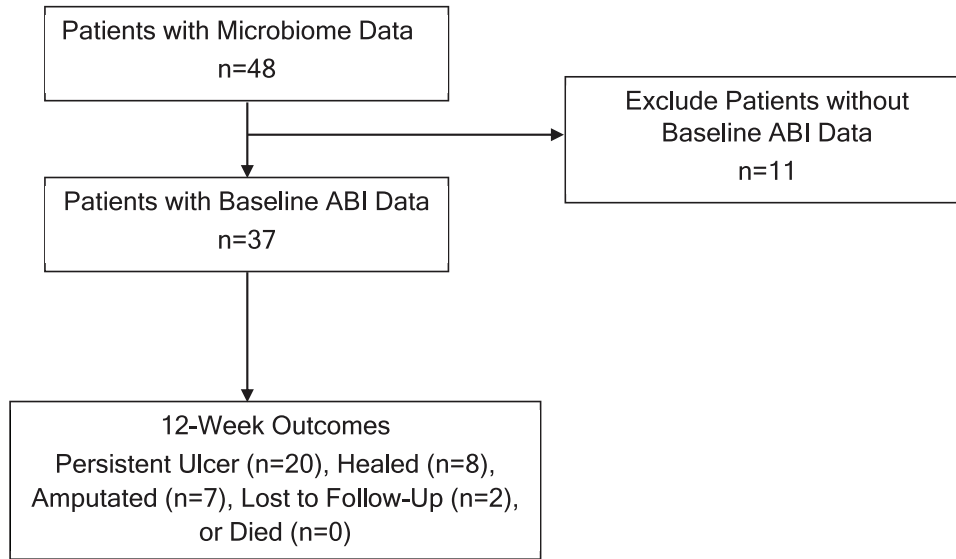


Figure 1. Flow chart for patient cohort selection criteria.

tarsal, or calcaneus) were recorded.²⁴ The Wagner grade was determined by the treating clinician, a podiatrist or infectious disease physician who participated in a multidisciplinary team within the podiatry clinic. Clinicians used Infectious Disease Society of America criteria to diagnose soft tissue infection: at least two classic symptoms or signs of inflammation (non-dependent erythema, warmth, tenderness, pain, or induration) or purulent secretions.²⁵ They also used these guidelines to diagnose ulcers complicated by osteomyelitis, typically based on a probe-to-bone test or positive culture/histopathology from a bone sample. Finally, we abstracted baseline ABI results, focusing on the ipsilateral leg and noting whether vessels were non-compressible. We swabbed the ulcer base following baseline sharp debridement and used this specimen in our microbiome analysis detailed below. We followed patients for 3 months with in-person study assessments during their podiatric appointments and chart reviews to determine whether the ulcer healed, persisted, or required amputation.

Microbiome analysis

Sample collection. Deep wound swabs were collected using Levine's technique into 300 μ l of DNA/RNA Shield (Zymo Research, Irvine, CA, USA) and stored at -80°C until further processing. Swabs were spun down using DNA IQ Spin Baskets (Promega, Madison, WI, USA), and the

liquid was split in half for parallel DNA and RNA extractions from the same sample.

DNA/RNA extraction, library construction, sequencing. DNA extraction was performed as previously described with minor modifications.^{16,17,26,27} Briefly, 300 μ l of yeast cell lysis solution (from Epicentre MasterPure Yeast DNA Purification kit, Madison, WI, USA), 0.3 μ l of 31,500 U/ μ l Ready-Lyse Lysozyme solution (Epicentre, Lucigen, Middleton, WI, USA), 5 μ l of 1 mg/ml mutanolysin (M9901, Sigma-Aldrich, St. Louis, MO, USA), and 1.5 μ l of 5 mg/ml lyso-staphin (L7386, Sigma-Aldrich, St. Louis, MO, USA) were added to 150 μ l of swab liquid before incubation for 1 h at 37°C with shaking. Samples were transferred to a 2-ml tube with 0.5 mm glass beads (MoBio, Carlsbad, CA, USA) and bead beat for 10 min at maximum speed on a Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA), followed by a 30-min incubation at 65°C with shaking, 5-min incubation on ice. The sample was spun down at 10,000 rcf for 1 min, and the supernatant was added to 150 μ l of protein precipitation reagent (Epicentre, Lucigen) and vortexed for 10 s. Samples were spun down at maximum speed ($\sim 21,000$ rcf) and allowed to incubate at room temperature for 5 min. The resulting supernatant was mixed with 500 μ l isopropanol and applied to a column from the Pure-Link Genomic DNA Mini Kit (Invitrogen, Waltham, MA, USA) for DNA purification using

the recommended protocol. 16S rRNA gene V4 region amplicon libraries were constructed using a dual-indexing method at the University of Minnesota Genomics Center and sequenced on a MiSeq with a 2×250 bp run format (Illumina, San Diego, CA, USA). Reagent-only negative controls were carried through the DNA extraction and sequencing process.

RNA extraction was performed using 1 ml of chilled (4°C) TRIzol (Invitrogen) with bead beating (single 5 mm steel bead) using a FastPrep-24 Classic tissue homogenizer (MP Biomedicals, Irvine, CA, USA) for five cycles of 4 s at 4.0 m/s with samples resting on ice for 5 min between cycles. 200 μl of chloroform was added to each sample, mixed, and allowed to incubate for 3 min at room temperature. Samples were spun for 15 min at 12,000 rcf at 4°C . The aqueous phase was transferred to the gDNA eliminator column from the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany) and processed using the kit's instruction thereafter. Libraries were constructed using the Smarter Stranded Total RNA-Seq Kit v2 – Pico Input (Takara Bio, Mountain View, CA, USA) with mammalian rRNA depletion at a concentration of 10 ng in 8 μl . Libraries were prepared without fragmentation because of low-quality input RNA. Samples were sequenced on a NovaSeq 6000 with a 2×100 bp run format (Illumina) at the University of Wisconsin Biotechnology Center.

Sequence analysis. The QIIME2 environment²⁸ was used to process DNA-based 16S rRNA gene amplicon data. Paired end reads were trimmed, quality filtered, and merged into amplicon sequence variants (ASVs) using DADA2.²⁹ Taxonomy was assigned to ASVs using a naive Bayes classifier pre-trained on full length 16S rRNA gene 99% operational taxonomic unit reference sequences from the Greengenes database (version 13_8).³⁰ ASVs classified as *Propionibacterium acnes* were manually renamed to *Cutibacterium acnes* to reflect the reclassification and renaming of this organism.³¹ Using the qiime2R package, data were imported into RStudio (version 1.4.1106)³² running R (version 4.1.0)³³ for further analysis using the phyloseq package.³⁴ The data set was decontaminated with the decontam package³⁵ using prevalence-based contamination at a 0.5 threshold to remove ASVs that were more prevalent in negative DNA extraction and sequencing controls than in true samples. Abundances were

normalized proportionally to total reads per sample.

Transcriptomic reads were trimmed of adapter sequences and quality filtered with fastp (version 0.20.0)³⁶ using default parameters. Kraken2 (version 2.0.8-beta)³⁷ was used to assign reads to taxonomic labels based on exact kmer matches to the National Center for Biotechnology Information (NCBI) Refseq indexes that included Standard (archaeal, bacterial, human, UniVec_Core, and viral genomes, along with plasmid sequences), protozoan, and fungal genomes (PlusPF collection). The database was curated to classify reads assigned to plasmid sequences as 'plasmid' regardless of the bacterial taxonomy of the plasmid source. Reads were re-estimated to species level abundances using Bracken.³⁸ Bracken outputs were imported into R and reads annotated within the kingdom Bacteria were analyzed with phyloseq. Abundances were normalized proportionally to total bacterial reads per sample.

Custom R scripts were used to merge 16S rRNA gene amplicon and transcriptomic abundance data sets for each sample at the genus level. Anaerobic bacteria in the genera *Cutibacterium*, *Propionibacterium*, *Anaerococcus*, *Finegoldia*, *Peptoniphilus*, *Clostridium*, *Peptoclostridium*, *Peptostreptococcus*, *Peptococcus*, *Helcococcus*, *Bacteroides*, *Parabacteroides*, *Porphyromonas*, *Prevotella*, *Fusobacterium*, and *Veillonella* were summed for analysis. Figures were produced using the package ggplot2.³⁹

Statistical analysis

We calculated descriptive statistics for the cohort and built a Kaplan–Meier graph to show the proportion of patients with persistent ulcers over the 12-week follow-up period. We modeled PAD three ways: clinical diagnosis (dichotomous), numeric ABI values (continuous), and normal *versus* mild ischemia based on ABI values (dichotomous). Specifically, we classified patients as having normal vascular status if their ABI values ranged from >0.9 to 1.4. Those with ABI values 0.90–0.50 were classified as having mild ischemia.⁴⁰ We modeled anaerobes two ways, both of which were continuous: the percent of total DNA (abundance) and the percent of total RNA (metabolically active) in the wound microbiome. We began exploring correlations between

arterial disease and anaerobes by plotting scatter graphs. Next, we used Spearman's correlation to compare ABI and anaerobic values (correlation between two continuous variables).⁴¹ We used univariate logistic regression to determine whether there was an association between anaerobic values and (1) the clinical diagnosis of PAD or (2) our classification of normal *versus* mild ischemia (association between dichotomous and continuous variables).⁴² We performed a sensitivity analysis restricting the sample to those who were not on antibiotics with anaerobic activity. Two-tailed *p*-values were calculated in all cases. Statistics were performed using R (version 4.1.0).³³

Results

Our prospective cohort consisted of 48 patients, 37 (77%) of whom had baseline ABI data and were included in the main analysis (Figure 1). Nearly all patients were male and identified as non-Hispanic White. Glycemic control was sub-optimal, with a mean hemoglobin A1C of 8.0%. Comorbidities were prevalent. Over 90% of patients were diagnosed with hypertension, hyperlipidemia, and neuropathy. Only five patients (14%) were lifetime non-smokers (Table 1). Most ulcers were classified as Wagner grade 1 or 2 and were located in the forefoot (Table 1). Patients who were excluded from this study owing to missing ABI values had less comorbidities and less severe, forefoot ulcers compared with included patients (Table 1). Seven patients received antibiotics with anaerobic activity within 30 days of sample collection, including: amoxicillin-clavulanate acid ($n=3$), clindamycin ($n=2$), and piperacillin-tazobactam ($n=2$). Our sensitivity analysis excluded these seven patients. Twenty (54%) patients had persistent ulcers at the end of the 12-week follow-up period (Figure 2). Only two (5%) were lost to follow-up.

Regarding PAD, 11 (30%) patients carried this diagnosis. ABI values ranged from 1.38 to 0.65 among the 34 patients with compressible vessels. Eight patients, or 24% of those with ABI values, met our definition of having mild ischemia. Regarding anaerobic results from the baseline wound sample, the mean percentage of bacterial DNA that was attributable to anaerobes was 27.9%. The mean percentage of bacterial RNA that was attributable to anaerobes was 22.7%. Both metrics had large variance across the cohort samples, reflected in their standard deviations

(Table 1). Anaerobe relative abundances were strongly correlated within samples across the DNA and RNA data sets (Spearman's $\rho=0.84$, $p<1E-07$; data not shown), supporting the use of either method of detection for determining the proportion of anaerobic bacteria in wound samples.

Univariate logistic regression did not demonstrate a statistically significant association between the clinical diagnosis of PAD and the percent of anaerobic DNA or RNA in the baseline sample (odds ratio (OR)_{DNA}=0.47, 95% CI=0.023–7.23, $p=0.60$; OR_{RNA}=1.99, 95% CI=0.17–21.44, $p=0.57$; Figure 3(a)). Similarly, no statistical associations between mild ischemia and anaerobic metrics were found (OR_{DNA}=0.25, 95% CI=0.005–5.86, $p=0.42$; OR_{RNA}=0.90, 95% CI=0.03–15.16, $p=0.94$; Figure 3(b)). Spearman's correlation coefficient provided no statistical evidence to suggest a significant correlation between ABI values and the percentage of anaerobic bacterial DNA ($r_s=0.24$, $p=0.17$) and RNA ($r_s=0.12$, $p=0.52$; Figure 3(c)). When restricting the sample to patients who did not receive an antibiotic with anaerobic activity within 30 days of baseline sample collection, results did not substantially deviate from the main analysis.

Discussion

We found no evidence to suggest a positive association between anaerobic abundance and metabolic activity with PAD. Therefore, further pursuit of an anaerobic biomarker to monitor therapeutic response and predict wound healing may be fruitful. Such a biomarker is likely to provide data independent of currently available vascular testing.

Initially, our results may seem counterintuitive. Patients with PAD, by definition, have ischemia. Anaerobic bacteria prefer ischemic environments. Therefore, one might logically assume that patients with PAD have wound microbiomes with higher anaerobic abundance and activity. We did not find evidence to suggest such a relationship, however. One reason for this discrepancy is that the microbiome assessment occurs on a much smaller scale than the vascular assessment. The microbiome can exist as a biofilm at the interface between the patient and environment.⁴³ Oxygen availability varies across a gradient within the biofilm, with a central area of oxygen depletion.^{44–46}

Table 1. Patient characteristics.

Characteristic	Total cohort (n=37)	Patients without ABIs (n=11)
Demographics		
Age, <i>m</i> (range)	67.08 (50–81)	67 (50–82)
Male ^a	36 (97.30)	11 (100)
Race, <i>n</i> (%)		
White	33 (89.19)	9 (81.82)
Black	1 (2.70)	1 (9.09)
Other	3 (8.11) ^b	1 (9.09) ^c
Hispanic ethnicity, <i>n</i> (%)	2 (5.41)	1 (9.09)
Comorbidities, <i>n</i> (%)		
Neuropathy	35 (94.59)	10 (90.91)
Neuroarthropathy	10 (27.03)	2 (18.18)
Peripheral arterial disease	11 (29.73)	1 (9.09)
Coronary artery disease	21 (56.76)	6 (54.55)
Hypertension	35 (94.59)	9 (81.82)
Hyperlipidemia	37 (100)	8 (72.73)
Tobacco use, <i>n</i> (%)		
Never	5 (13.51)	3 (27.27)
Former	25 (67.57)	7 (63.64)
Current	7 (18.92)	1 (9.09)
Baseline A1C, <i>m</i> (range)	8.03 (5.1–12.6)	7.35 (5.2–12.5) ^d
Baseline ulcer characteristics		
Largest dimension, millimeter, <i>m</i> (SD)	14.24 (12.42)	12.89 (9.12)
Wagner grade, <i>n</i> (%)		
1	14 (37.84)	6 (54.55)
2	16 (43.24)	4 (36.36)
3	7 (18.92)	1 (9.09)
4	0 (0)	0 (0)
Location, <i>n</i> (%)		
Digit	12 (32.43)	4 (36.36)
Metatarsal	16 (43.24)	7 (63.64)
Tarsal	3 (8.12)	0 (0)

(Continued)

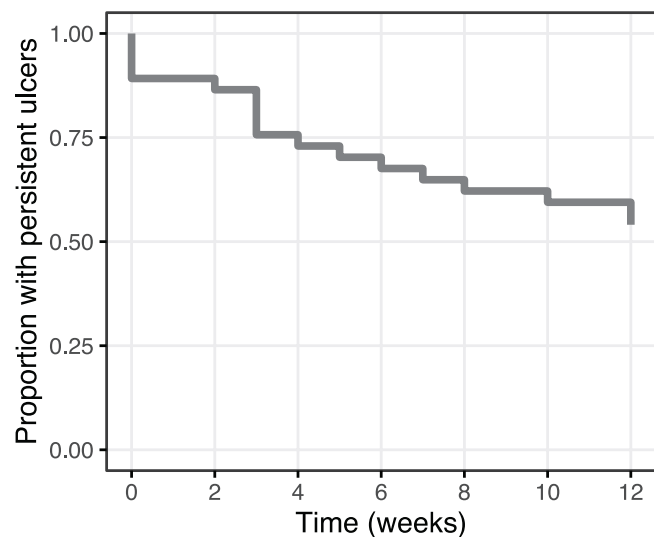
Table 1. (Continued)

Characteristic	Total cohort (<i>n</i> = 37)	Patients without ABIs (<i>n</i> = 11)
Calcaneus	6 (16.21)	0 (0)
Ankle brachial index		
Non-compressible, <i>n</i> (%)	3 (8.12)	–
Calculated index, <i>m</i> (range)	1.07 (0.65–1.38)	–
Meets ischemic definition, <i>n</i> (%) ^e	8 (23.53)	–
Baseline anaerobic characteristics		
Percent total abundance, <i>m</i> (SD)	27.9 (26.3)	23.7 (28.6)
Percent of metabolically active microbes, <i>m</i> (SD)	22.7 (29.3)	20.0 (29.0)

ABI, ankle brachial index; SD: standard deviation.
^aOne (3.33%) patient had an unreported sex.
^bThe other racial category consisted of one individual identifying as Asian, one identifying as Native American or Alaska Native, and one identifying as both Caucasian and Native American or Alaska Native.
^cThe other racial category consisted of one individual identifying as Native Hawaiian or Other Pacific Islander.
^dOne individual did not have an A1C value.
^eDenominator equals 34 patients with ABI values.

Microbiologists hypothesize that this center contains persister cells, which function to impair wound healing and maintain local inflammation.^{47,48} We do not yet know whether anaerobes are key persister cells. Given a microenvironment favorable to anaerobic metabolism and early data linking anaerobes to delayed wound healing, however, this may be true.^{16,49} As we start to explore potential microbiome-based biomarkers, we need to recalibrate our frame of reference. As providers, we are accustomed to thinking on a scale appropriate for human physiology. This served us well during the advent of endovascular surgery, which drove down amputation rates in the early 2000s.^{50–52} Now, infection commonly serves as the tipping point between limb salvage and amputation.⁵³ We need to respond by shifting our thinking to a microbiologic scale. Doing so will allow us to capitalize on microbiome data so that we are better equipped to address infection and rising amputation rates.

Our results are robust. We examined associations between anaerobic bacteria measured in terms of both DNA and RNA, and PAD defined three ways. We found no positive relationship between PAD and anaerobic markers. Results held in the sensitivity analysis that excluded patients whose anaerobic results might have been impacted by

**Figure 2.** Kaplan–Meier curve for proportion of patients with persistent ulcers after 12 weeks (*n* = 37).

prior antibiotic use. Furthermore, wounds displayed a high degree of variation in anaerobic values. Variation is useful when developing biomarkers. Dispersed results allow for easier detection of clustering that may indicate one clinical phenotype (e.g. prone to healing) from another (e.g. prone to persistent ulceration).⁵⁴

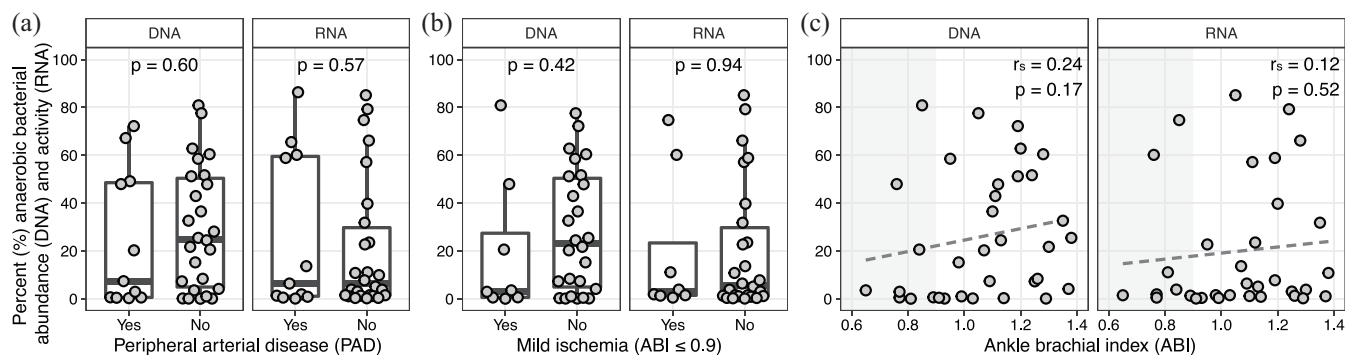


Figure 3. Anaerobic bacterial abundance (DNA) and activity (RNA) in DNA and RNA data sets are not associated with (a) PAD diagnosis, (b) mild ischemia, and (c) ABI. Each datapoint represents a sample from a single patient. For (a) and (b), boxplots show the interquartile range with median while whiskers represent the top and bottom quartiles up to 1.5 times the interquartile range. p -values reported for logistic regressions. For (c), dashed lines represent the (non-significant) linear regression lines, and p -values are reported for Spearman's correlation coefficient (r_s).

As with any study, ours has limitations. Although our cohort was similar to the general population of patients with diabetic foot ulcers, it did not contain patients with moderate-to-severe PAD. Patients with a high degree of vascular disease would not have undergone sharp debridement owing to concerns of exacerbating their ulcers. Without debridement, we could not collect a sample of the wound base for analysis. These patients are already known to be at high risk of amputation, hence; the prognostic utility of an anaerobic-based biomarker in this group may be limited.¹² Because there may be a role for biomarkers to assess conservative response to therapy in patients with moderate-to-severe PAD, we plan to include these patients in future cohorts. This may require changing the sampling technique to swabbing the ulcer without sharp debridement. Assessment of the wound microbiome before and after revascularization procedures may also offer key insights. Few of our patients had toe-brachial index testing or transcutaneous oxygen pressure testing to assess their microvascular status. Therefore, we cannot rule out a correlation between microvascular disease and high anaerobic markers. Microvascular assessments are technically more challenging, less accurate, and more difficult to utilize in clinical practice, however.⁵⁵ Even if there was a correlation between transcutaneous tissue pressure values and anaerobes, there may still be a niche for an anaerobic biomarker if it was more readily available and reliable.

An additional limitation of our cohort, typical of current genomic and transcriptomic studies, is

our small sample size.^{16,56–58} We may have been unable to detect a moderate degree of correlation between vascular disease and anaerobic abundance or activity. Second, our Veteran population was largely composed of males. Further inclusion of women would be welcome to ensure there are no sex-based differences; greater representation of individuals identifying as a racial or ethnic minority would also be welcome and increase generalizability of our findings.

Culture-based methods are the gold standard to identify pathogenic microbes. Standard clinical microbiology workflows, however, have been shown to underestimate the diversity of chronic wounds and diabetic foot ulcers, especially of fastidious bacteria such as obligate anaerobes.^{43,49,59} Molecular-based approaches are able to detect bacteria *via* the nucleic acid content of microbial cells without the need for cultivation.⁶⁰ We used two different high-throughput sequencing methods that together are able to describe both taxonomy and gene expression activity of microbial communities. Using the 16S rRNA gene as a DNA-based marker, we were able to use amplicons of the hypervariable V4 region as a molecular barcode for taxonomical assignment. It is important to note that these amplicon-based methods are limited in their classification beyond the genus level. Thus, we have chosen to limit our analysis to the genus level. Also, DNA-based methods are unable to distinguish live bacteria from dead cells and are similarly unable to discern dormancy *versus* transcriptional activity. Therefore, we used total

RNA sequencing to assess gene expression activity. Dead or dormant cells do not actively transcribe RNA, and therefore do not contribute to the RNA pool. Our DNA and RNA data sets are strongly correlated, indicating that the most abundant species tend to also be the most transcriptionally active. We do note that we are summarizing anaerobe activity in terms of overall contribution to the total RNA pool sequenced. We anticipate that further functional annotation of the sequenced RNA will lead to a better understanding of the microbial lifestyle within wounds and lead to potential biomarkers for patient wound healing outcomes.

Conclusion

Neither anaerobic abundance nor metabolic activity strongly associated with our measures of PAD in our cohort of Veterans with normal vascular status to mild ischemia. Therefore, anaerobic biomarkers may offer unique prognostic information regarding diabetic foot ulcers, independent of peripheral vascular disease.

Declarations

Ethics approval and consent to participate

The study was approved by the University of Wisconsin Health Sciences Institutional Review Board (2018-1372) and the William S. Middleton Memorial Veterans Hospital Research and Development Committee. All patients provided written informed consent at the time of study enrollment.

Consent for publication

Not applicable.

Author contributions

J.Z. Alex Cheong: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; Writing – review & editing.

Jessica M. Irvine: Data curation; Formal analysis; Investigation; Methodology; Project administration; Writing – review & editing.

Shane Roesemann: Data curation; Formal analysis; Methodology; Writing – review & editing.

Anna Nora: Conceptualization; Data curation; Methodology; Project administration; Supervision; Writing – review & editing.

Courtney E. Morgan: Conceptualization; Formal analysis; Writing – review & editing.

Christopher Daniele: Conceptualization; Writing – review & editing.

Lindsay R. Kalan: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Writing – review & editing.

Meghan B. Brennan: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Supervision; Validation; Writing – original draft; Writing – review & editing.

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Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Availability of data and materials

Data will be made available upon reasonable request.

ORCID iD

Meghan B. Brennan  <https://orcid.org/0000-0002-2316-4150>

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