International Wound Infection Institute

Slough: Composition, analysis and effect on healing

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S lough can be defined as "non-viable tissue of varying colour (e.g. cream, yellow, greyish or tan) that may be loose or firmly attached, slimy, stringy or fibrinous" (Haesler et al, 2022; International Wound Infection Institute [IWII], 2022). Slough consistency is determined by the tissue's hydration status and interaction with the dressing material, as well as the depth and type of non-viable tissue (Wounds UK, 2013; Atkin, 2014).

Slough can be found in both acute wounds, such as dehisced surgical wounds, skin tears and other traumatic wounds and skin grafts, as well as in chronic wounds, such as diabetic foot ulcerse (DFUs) pressure ulcers and venous leg ulcers (Percival and Suleman, 2015).

A simple explanation of slough for patients is generally a "yellow/white layer of dead skin [tissue] in the wound, that can prevent or slow down healing" (Harding et al, 2020). Slough can be unpleasant and disturbing for the patient, and difficult to manage clinically as it prevents dressings and topical treatments from supporting the underlying viable tissues (Pritchard and Brown, 2013). Slough or necrotic tissue can promote bacterial growth and biofilm formation, inhibit the penetration of antibiotics, prevent the formation of granulation tissue, and subsequent re-epithelialisation, and interfere with wound contraction (Steed, 2004; Lewis et al, 2008; Ramundo and Gray, 2008).

THE SCALE OF THE PROBLEM

While slough is under-reported in the literature, it is known to be a feature of non-healing wounds (Pritchard and Brown, 2013). In the United Kingdom, Guest et al (2020) reported that there were an estimated 3.8 million patients with a wound managed by the NHS in 2017/18, of which 70% healed in the study year (89% and 49% of acute and chronic wounds healed, respectively); an estimated 59% of chronic wounds healed if there was no evidence of infection, compared to 45% if there was a definite or suspected infection.

In the United States, Medicare cost estimates for acute and chronic wound treatments range from $28.1 to $96.8 billion. The highest expenses are associated with surgical wounds, followed by DFUs, with a higher trend toward costs associated with outpatient wound care compared with inpatient (Sen, 2019). In Australia, DFUs approximately affect 50,000 people, costing healthcare systems an estimated $1.6 billion, and resulting in 28,000 hospital admissions and 5000 amputations each year (Chen et al, 2022). Increasing health care costs, an ageing population, recognition of difficult-to-treat infection threats, such as biofilms, and the continued threat of diabetes and obesity worldwide make chronic wounds a substantial clinical, social and economic challenge (Sen, 2019).

The impact of living with a wound carries a substantial personal cost and can have a significant effect on daily life and overall wellbeing (Moore et al, 2016). Individuals living with wounds report feeling unsupported and uninvolved in decisions about their care (Harding et al, 2020). This can lead to psychological issues, such as anxiety and depression (Wounds International, 2012).

MANAGEMENT OF SLOUGH IN PRACTICE

Debridement is regarded as an essential component of wound preparation and management (IWII, 2022) and is defined as "the removal of devitalised (non-viable) tissue from or adjacent to a wound". Debridement promotes a stimulatory environment by removing and managing exudate, while also creating a window of opportunity where biofilm defences are temporarily interrupted, allowing for increased efficacy of topical and systemic management strategies (Wolcott et al, 2010; World Union of Wound Healing Societies [WUWHS] 2019; IWII, 2022).

Debridement should not be confused with wound cleansing, which is "actively removing surface contaminants, loose debris, non-attached non-viable tissue, microorganisms or remnants of previous dressings from the wound surface and its surrounding skin" (Haesler et al, 2022).

See Table 1 for the different types of debridement that may be used. When performing wound debridement, clinicians should always work within their scope of practice, and local policy and procedures (IWII, 2022).

Table 1. Types of debridement methods and its effect on biofilm *(adapted from IWII, 2022)*		
Type of debridement	**Method***	**Effect on biofilm***
Surgical	Performed in the operating room or specialised clinic by qualified and competent practitioners using sterile scalpel, scissors or a hydrosurgical device (Haesler, 2019)	■ Disrupts biofilm and removes foci of infection (IWII, 2022) ■ If all tissue is removed, deeper biofilm can be disrupted (White and Asimus, 2014)
Conservative-sharp	Performed using aseptic technique with a sterile curette, scalpel and scissors (Haesler and Carville, 2022)	Removes and disrupts superficial biofilm *in vitro* (White and Asimus, 2014)
Autolytic	Autolytic debridement occurs naturally in a moist wound environment and can be aided by using topical agents and contemporary wound dressings that promote autolysis, including (White and Asimus, 2014): ■ Cadexomer iodine ■ Honey ■ Fibre gelling wound dressings ■ Alginate wound dressings ■ Polyhexamethylene biguanide ■ Hydrogel wound dressings	Varying effectiveness in controlling biofilm *in vitro*
Mechanical	Non-selective debridement performed using (Haesler and Carville, 2022): ■ Therapeutic irrigation (4 to 15 psi) ■ Monofilament fibre debridement pads ■ Low-frequency ultrasound ■ Hydrosurgery	Some levels of disruption and removal of biofilm *in vitro* (White and Asimus, 2014)
Enzymatic/chemical/surfactant	Application of exogenous enzymes or chemicals to the wound surface, including: ■ Enzymatic debriders ■ Wound cleaners and gels with high or low concentrations of surfactant	Some levels of disruption and removal of biofilm *in vitro* (White and Asimus, 2014)
Biosurgical/larval therapy	Sterile fly larvae that produce a mixture of proteolytic enzymes (Haesler and Carville, 2022)	Some evidence of reducing bacterial bioburden *in vitro* and in clinical studies (Watts, 2016)
* based on *in vitro* research		

We present a series of case studies focusing on the characterisation of slough collected from various different wound aetiologies. The primary goal of this research project was to identify and classify slough at molecular and microbiological levels. The secondary goal of this research was to determine if the composition of slough has the potential to be used as a biomarker for wound healing outcomes. To achieve this, slough samples were collected and analysed for microbial bioburden and composition using both culture and DNA sequencing-based methods. The protein composition of each sample was then determined using untargeted proteomics analysis and the presence of microbial aggregates or biofilm was evaluated using confocal and scanning electron microscopy.

SUBJECT IDENTIFICATION AND ENROLMENT

For Cases 1–10 (starting on page 8), adults 18 years or older with chronic wounds were recruited from UW-Health Wound Care Clinics (Study ID: 2020-1002). Examples of chronic wounds identified for possible inclusion included and were not limited to: chronic or non-healing diabetic ulcers, pressure ulcers, venous ulcers, surgical or procedural wounds, trauma wounds, burn wounds and wounds of unknown or other aetiology.

For additional Cases 11–14 (starting on page 35), adults 18 years or older presenting with a diabetes-related foot ulcer with visible signs of slough were recruited for the study. The collection of samples and their corresponding patients was undertaken as a sub-analysis of a larger clinical study, with samples being obtained following written consent. Ethics approval for the larger clinical study and the slough sub-analysis was approved by South Western Sydney LHD Research and Ethics Committee.

SAMPLE COLLECTION

For Cases 1–10 (starting on page 8), swabs of the wound edge and centre were collected using Levine's technique into 300µl of DNA/RNA Shield (Zymo Research, Irvine, CA) and stored at -80°C until further processing. To avoid disrupting the slough microbiome, swabs were collected before cleaning the wound for debridement. Swabs were also intentionally taken from over the slough in the center of the wound to capture slough-associated microbes. Swabs were then spun down using DNA IQ Spin Baskets (Promega, Madison, WI) and DNA was extracted. Swabs designated for microbial culture were taken from the wound center using Levine's technique into 1ml of liquid Amies (Copan Diagnostics Inc, Murrieta, CA). Swabs were stored at 4°C for less than 2 hours before being processed for microbial culture.

To promote wound healing, all subjects' wounds were sharply debrided to remove non-viable tissue, surface biofilm and other surface contaminants. Before debridement, wounds were cleansed with soap and sterile water. Debridement was performed by a skilled practitioner using sterile surgical instruments such as a scalpel, curette, scissors, rongeur, and/or forceps. Removed slough material was collected into 1ml of DNA/RNA Shield (Zymo Research, Irvine, CA) and stored at 4°C before being portioned out for scanning electron microscopy (SEM), fluorescence in situ hybridisation (FISH) and proteomics. The remaining slough material was stored at -80°C.

For additional Cases 11–14 (starting on page 35), the patient's wound slough was removed from the ulcer base with a dermal curette and immediately stored in RNA Shield (Zymo Research, Irvine, CA) at 4°C for 24 hours before being frozen at -80°C until further processing.

PHOTO IMAGING

Digital photos of the wound were taken before and after the debridement procedure.

MICROBIAL CULTURE AND BACTERIAL ISOLATE IDENTIFICATION

Swabs designated for microbial culture were spun down using DNA IQ Spin Baskets (Promega, Madison, WI). A portion of each sample was serially diluted with 1X phosphate buffered saline and plated onto Tryptic Soy Agar (TSA) with 5% sheep blood (BBL, Sparks, MD) for quantitative bacterial culture. Plates were incubated at 35°C overnight. To isolate culturable bacteria, colonies with distinct morphology were isolated and incubated overnight at 35°C on TSA with 5% sheep blood. Single colonies were then inoculated into liquid Tryptic Soy Broth (TSB) and incubated overnight. To identify each bacterial isolate, a portion of the overnight TSB culture underwent DNA extraction and sanger sequencing (Functional Biosciences, Madison, WI) of the bacterial 16S ribosomal RNA gene. The remaining portion of the isolated culture was stored in glycerol at -80°C.

DNA EXTRACTION, LIBRARY CONSTRUCTION, SEQUENCING

With minor modifications, DNA extraction was carried out as previously described (Cheong et al, 2022). Briefly, 300µl of yeast cell lysis solution (from Epicentre MasterPure Yeast DNA Purification kit), 0.3µl of 31,500U/µl ReadyLyse Lysozyme solution (Epicentre, Lucigen, Middleton, WI), 5µl of 1mg/ml mutanolysin (M9901, Sigma-Aldrich, St. Louis, MO), and 1.5µl of 5mg/ml lysostaphin (L7386, Sigma-Aldrich, St. Louis, MO) was added to 150µl of swab liquid before incubation for one hour at 37°C with shaking. Samples were transferred to a 2ml tube containing 0.5mm glass beads (Qiagen, Germantown, Maryland) and bead beat for 10 minutes at maximum speed on a Vortex-Genie 2 (Scientific Industries, Bohemia, NY), followed by a 30-minute incubation at 65°C with shaking and a 5-minute incubation on ice. The sample was spun down at 10,000rcf for 1 minute and the supernatant was added to 150µl of protein precipitation reagent (Epicentre, Lucigen, Middleton, WI) and vortexed for 10 seconds. Samples were spun down at maximum speed (~21,000rcf) and incubated at room temperature for 5 minutes. The resulting supernatant was mixed with 500µl isopropanol and applied to a PureLink Genomic DNA Mini Kit (Invitrogen, Waltham, MA) column 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 2x250bp run format (Illumina, San Diego, CA). Reagent-only negative controls were carried through the DNA extraction and sequencing process.

SEQUENCE ANALYSIS

The QIIME2 environment 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. Taxonomy was assigned to ASVs using a naive Bayes classifier pre-trained on full length 16S rRNA gene 99% OTU reference sequences from the Greengenes database (version 13_8). Using the qiime2R package, data was imported into RStudio (version 1.4.1106) running R (version 4.1.0) for further analysis using the phyloseq package. Negative DNA extraction and sequencing controls were evaluated based on absolute read count and ASV distribution in true patient samples. Abundances were normalised in proportion to the total number of reads per sample. Relative abundance plots were produced using the package ggplot2, where taxa below 1% relative abundance were pooled into an 'other' category.

PROTEOMICS

Debrided slough tissue samples were weighed and placed in PowerBead Tubes containing 1.4mm ceramic beads (Qiagen, Germantown, Maryland) for tissue homogenisation, proteomic processing and analysis at the University of Wisconsin Mass Spectrometry and Proteomics Core Facility. In brief, samples were labelled and pooled for multiplex relative mass spectrometry (MS) quantification using the TMTpro 16plex labelling kit (ThermoFisher Scientific, Waltham, MA) and underwent Liquid Chromatography with tandem mass spectrometry on an Orbitrap Elite mass spectrometer (ThermoFisher Scientific). Protein sequences were matched to known human and bacterial proteins. Functions associated with each protein were gathered from KEGG Pathways, Reactome Pathways, and WikiPathways databases. Data was imported into RStudio (version 1.4.1106) running R (version 4.1.0) for further analysis.

FLUORESCENCE IN SITU HYBRIDISATION (FISH)

Formalin-Fixed Paraffin-Embedded (FFPE) histological sections were deparaffinised in xylene and rehydrated in a series of ethanol washes (100%, 99%, 95% and 0%). Subsequently, the samples were allowed to hybridise at 46°C for 4 hours in hybridisation solution (900mM NaCl, 20mM Tris pH 7.5, 0.01% SDS, 20% formamide, 2 μm FISH probe). The FISH probe used was a DNA oligonucleotide (EUB388 sequence) with a 3'-conjugated TEX615 fluorophore (Integrated DNA Technologies, Coralville, IA, USA). Samples were washed in excess wash buffer (215mM NaCl, 20mM Tris pH7.5, 5mM EDTA) at 48°C for 15 minutes, dipped into ice cold water and 100% ethanol, then drained and air-dried. Slides were mounted with Prolong Glass antifade mounting medium with NucBlue counterstain (Thermo Fisher Scientific, Waltham, MA, USA) and a glass coverslip of #1.5 thickness and stored flat to cure overnight in the dark. Micrographs were taken at 5x and 63x magnification using a Zeiss 780 confocal laser scanning microscope (CLSM) on the red TEX615, blue Hoescht and green GFP (tissue autofluorescence) channels. Zeiss Zen software was used to analyse tiled images, z-stacks and generate maximum intensity projections.

PEPTIDE NUCLEIC ACID–FLUORESCENCE IN SITU HYBRIDISATION (PNA-FISH)

FFPE samples were cut, deparaffinised and rehydrated following standard procedures. Subsequently, the samples were stained with a PNA-FISH-TexasRed-5-conjugated universal bacterial (BacUni) 16s rRNA probe (AdvanDx, Woburn, MA, US), incubated and counterstained with 3μM 4',6-diamidino-2-phenylindole (DAPI; life Technologies, Eugene, OR, USA). The samples were then mounted (ProLong™ Gold Antifade Mountant; Life Technologies) and a coverslip was added (Marienfield, Lauda-Königshoffen, Germany). Slides were evaluated using a CLSM (Axio Imager.Z2, LSM880 CLSM; Zeiss, Jena, Germany). Images were taken using 405nm (DAPI) and 561nm (TexasRed-5) lasers, as well as a 488nm laser for visualising the green autofluorescence of the surrounding tissue. Images were subsequently processed with IMARIS 9.2 (Bitplane, Zurich, Switzerland) and displayed in 'Easy 3D' mode. Bacterial aggregates were determined as biofilms if larger than 5μm in diameter.

SCANNING ELECTRON MICROSCOPY (SEM)

Wound slough specimens were rinsed with PBS and fixed overnight in 5mL of 1.5% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.2) at 4°C. Samples were rinsed, treated with 1% osmium tetroxide for 1 hour and then washed again in buffer. Samples were dehydrated through a series of ethanol washes (30–100%) followed by critical point drying (14 exchanges on low speed) and were subsequently mounted on aluminum stubs with a carbon adhesive tab and carbon paint. Samples were left to dry in a desiccator overnight. Following sputter coating with platinum to a thickness of 20nm, samples were imaged in a scanning electron microscope (Zeiss LEO 1530-VP) at 3 kV.

Case studies

RELEVANT MEDICAL HISTORY

- Type II diabetes
- Obesity (BMI = 48.1)
- Atrial fibrillation
- Hypertension
- Hyperlipidaemia
- Chronic anticoagulation
- Interstitial lung disease
- Chronic and recurring venous stasis ulcers
- Recurrent cellulitis.

Table 1. Wound dimensions	
Wound length	8.5cm
Wound width	4cm
Wound depth	0.5cm
Wound surface area	34cm^2
Wound volume	17cm^3
Wound shape	Irregular; round oval

CASE 1

The patient is a 70-year-old white male with a history of multiple non-healing ulcers and bilateral lower extremity oedema secondary to lymphoedema and venous stasis. The wound on his left medial ankle sampled for this study had been present for 15 years. Several years ago, wound samples were sent for culture to test for infection and returned positive for *Pseudomonas* bacteria. Antibiotics were used to treat the infection, but the wound has since been considered chronically colonised with *Pseudomonas*.

At the time of sample collection (summer 2021), the patient reported increased pain and drainage from the wound over the past 24 hours. His wound care included nystatin cream applied to intact skin, silver sulfadiazine and gauze with nystatin paste applied to the open wound and a multilayer compression wrap changed twice a week. In clinic, the patient was treated for 30 minutes with 0.25% sodium hypochlorite soak followed by 15 minutes of air drying before applying new dressings.

In comparison to previous measurements, the wound was 2.2% smaller in volume. Three months following this sample collection, the wound was still present but stable in size.

Figure 1 | Image of subject 1's left medial ankle wound before the debridement procedure

Table 2. Wound information	
Wound duration	15 years
Wound aetiology	Lymphoedema, venous stasis and *Pseudomonas* wound infection
Bates-Jensen wound assessment score	40/65
Wound edges	Well-defined, not attached to base, rolled and thickened
Necrotic tissue	Loosely adherent yellow slough
Exudate type	Serosanguineous (thin, watery and pale red/pink in colour)
Exudate level	Moderate
Periwound colour	White/grey pallor and hypopigmented
Oedema	Non-pitting oedema extending <4cm around wound
Induration	2–4cm induration extending >50% around wound
Granulation	Pink/dull/dusky red in colour and fills <25% of wound
Epithelialisation	<25% of wound covered
Surface area wound healing rate (% change per week)	2.2%
Wound volume healing rate (% change per week)	2.2%

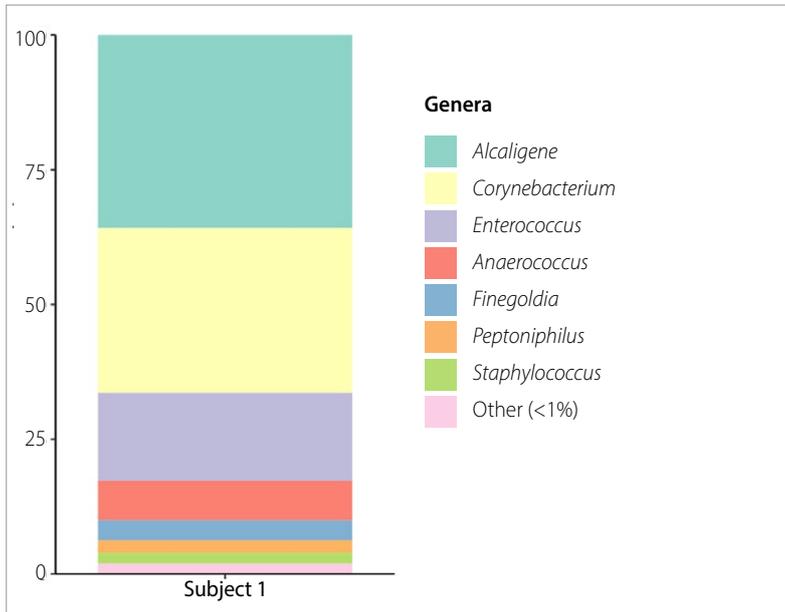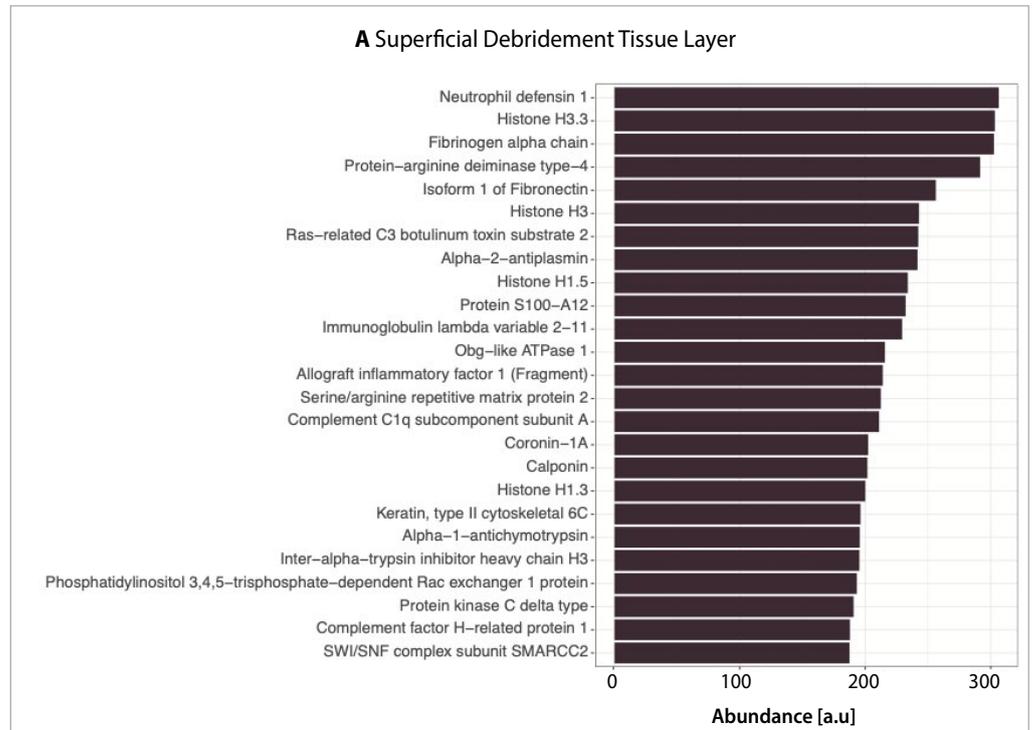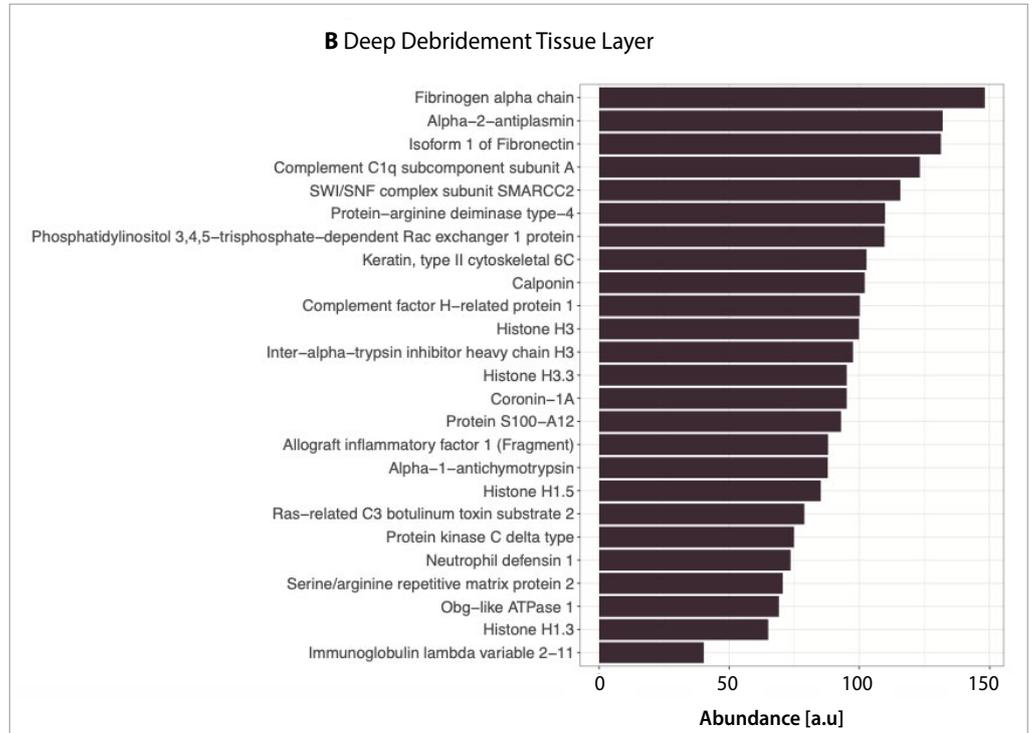| Table 3. Microbiology	
Bacterial-colony forming units (CFU) per inch^2 wound	Isolated bacteria
1.30 x 10^7 CFU	*Alcaligenes faecalis Corynebacterium sp. Corynebacterium striatum Enterococcus faecalis Staphylococcus simulans Staphylococcus sp.*

Figure 2 | Relative abundance of bacterial genera on the surface of subject 1's wound based on high-throughput bacterial 16S ribosomal gene sequencing. Molecular detection of wound bacteria identified 3 new species of anaerobic bacteria that were not detected by culture-based methods.

Figure 3 | Top 25 most abundant proteins in superficial (A) and deep (B) debridement tissue layers of wound slough in subject 1. The most abundant proteins in both the superficial and deep slough layers are all of human origin and related to extracellular matrix formation (i.e. collagen biosynthesis), blood clot formation (i.e. platelet activation and the coagulation cascade) and antibacterial immune responses (i.e. neutrophil degranulation, chemokine signalling and the complement cascade).

A Superficial Debridement Tissue Layer

Figure 3 (continued) | Top 25 most abundant proteins in superficial (A) and deep (B) debridement tissue layers of wound slough in subject 1. The most abundant proteins in both the superficial and deep slough layers are all of human origin and related to extracellular matrix formation (i.e. collagen biosynthesis), blood clot formation (i.e. platelet activation and the coagulation cascade) and antibacterial immune responses (i.e. neutrophil degranulation, chemokine signalling and the complement cascade).

B Deep Debridement Tissue Layer

RELEVANT MEDICAL HISTORY

■ Former smoker
■ Peripheral vascular disease
■ Coronary artery disease
■ Varicose veins of lower extremities
■ Obesity (BMI = 43.2)
■ Osteoarthritis of the hip and knee
■ Obstructive sleep apnoea.

Table 1. Wound dimensions

Wound length	7.9cm
Wound width	7cm
Wound depth	0.3cm
Wound surface area	55.3cm^2
Wound volume	16.59cm^3
Wound shape	Irregular; round oval

CASE 2

The patient is a 74-year-old white male with a history of a non-healing ulcer on his left lower leg secondary to venous stasis. The wound on his left anterior shin sampled for this study had been present for 3 years since an episode of left leg cellulitis that required hospitalisation and IV antibiotics in October 2019. A year later, the wound was healing until green drainage was noted, at which point he was admitted for a second round of IV antibiotics and aggressive wound care. Before this study, the patient was using 0.9% cadexomer iodine ointment and gauze at home every other day, followed by multilayer compression wraps.

At the time of sample collection, the wound was approximately 30% smaller in volume when compared to measurements taken a week before. Three months following sample collection, the wound was still present, yet clinically improving and had decreased in size.

Figure 1 | Image of subject 2's left anterior shin wound before the debridement procedure

| Table 2. Wound information	
Wound duration	3 years
Wound aetiology	Venous stasis
Bates-Jensen wound assessment score	35
Wound edges	Well-defined and not attached to base
Necrotic tissue	Loosely adherent yellow slough
Exudate type	Serosanguineous (thin, watery and pale red/pink in colour)
Exudate level	Low
Periwound colour	Pink
Oedema	Non-pitting oedema extending <4cm around wound
Induration	None present
Granulation	Pink/dull/dusky red in colour and fills <25% of wound
Epithelialisation	<25% of wound covered
Surface area wound healing rate (% change per week)	5.5%
Wound volume healing rate (% change per week)	29.1%
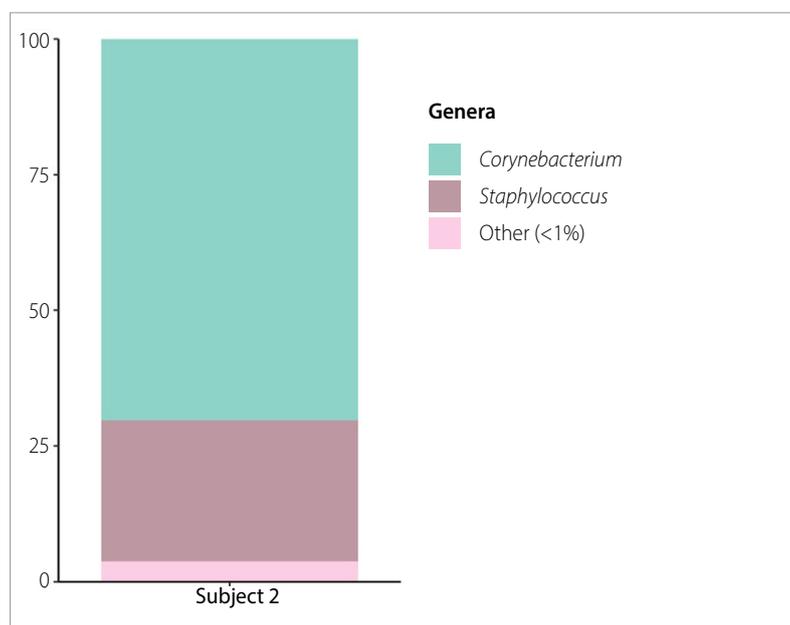
Table 3. Microbiology	
Bacterial-colony forming units (CFU) per inch² wound	**Isolated bacteria**
1.40×10^5 CFU	*Corynebacterium sp.* *Staphylococcus sp.* *Pseudomonas sp.*

Genera
- *Corynebacterium*
- *Staphylococcus*
- Other (<1%)

Subject 2

Figure 2 | Relative abundance of bacterial genera on the surface of subject 2's wound based on high-throughput bacterial 16S ribosomal gene sequencing. Molecular detection of wound closely aligned with those identified via culture-based methods. *Pseudomonas* grows quickly and easily in lab culturing conditions. This likely explains why we were able to culture and isolate *Pseudomonas*, yet molecular detection found *Pseudomonas* to be <1% of the bacteria present in the wound slough.

Figure 3 | Top 25 most abundant proteins in subject 2's wound slough. The most abundant proteins were all of human origin and were associated with extracellular matrix formation (i.e. collagen biosynthesis, elastic fibre associated proteins), blood clot formation (i.e. platelet activation) and immune responses (i.e. cytokine signaling).
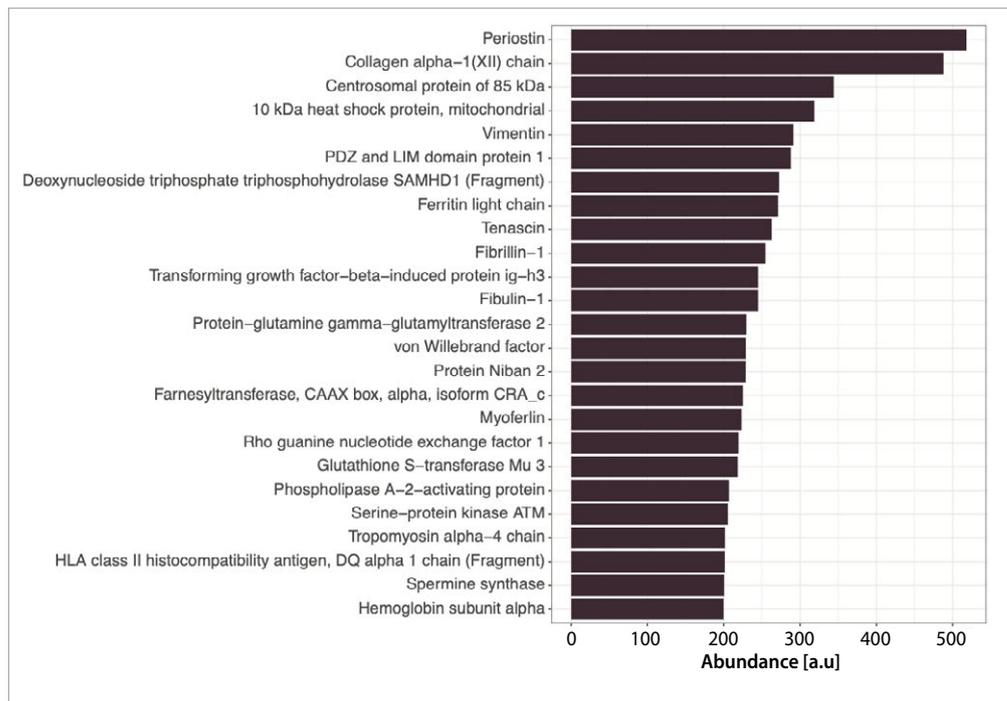
RELEVANT MEDICAL HISTORY

- Type II diabetes
- Hypertension
- Rheumatoid arthritis
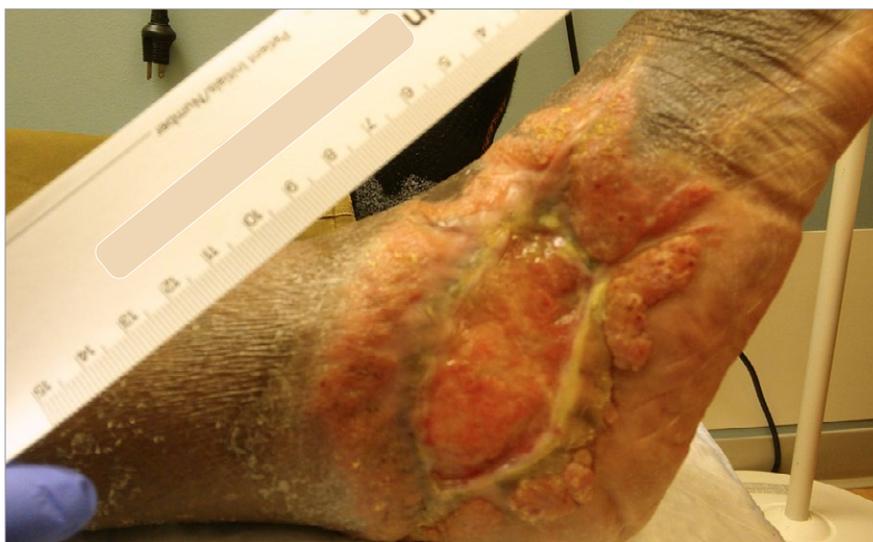- Lumbar spinal stenosis
- Necrotising soft tissue infection.

Table 1. Wound dimensions	
Wound length	3.6cm
Wound width	7.3cm
Wound depth	0.3cm
Wound surface area	26.3cm²
Wound volume	7.8cm³
Wound shape	Round oval

CASE 3

The patient is a 72-year-old black male, with a history of a non-healing wound secondary to necrotising soft tissue infection. In 2019, he presented with a rapid onset, necrotising soft tissue infection of his right ankle complicated by septicemia. He was admitted and taken for emergency operative wound debridement and negative pressure wound dressing placement. Although wound healing had stalled, the patient's wound care consisted of daily application of active Leptospermum (Manuka) honey gel and gauze.

At the time of sample collection, the wound was approximately 5% larger in volume compared to measurements taken the week before. The wound was still present, but clinically improving and had decreased in size, three months following sample collection.

Figure 1 | Image of subject 3's right ankle wound before the debridement procedure

| Table 2. Wound information	
Wound duration	2 years
Wound aetiology	Necrotising soft tissue infection
Bates-Jensen wound assessment score	34
Wound edges	Well-defined, not attached to base, rolled under and thickened
Necrotic tissue	White/grey non-viable tissue and non-adherent yellow slough
Exudate type	Serosanguineous (thin, watery and pale red/pink in colour)
Exudate level	Low
Periwound colour	Pink
Oedema	Non-pitting oedema extending <4cm around wound
Induration	None present
Granulation	Pink/dull/dusky red in colour and fills <25% of wound
Epithelialisation	25% to 50% of wound covered
Surface area wound healing rate (% change per week)	5.3% larger
Wound volume healing rate (% change per week)	5.3% larger
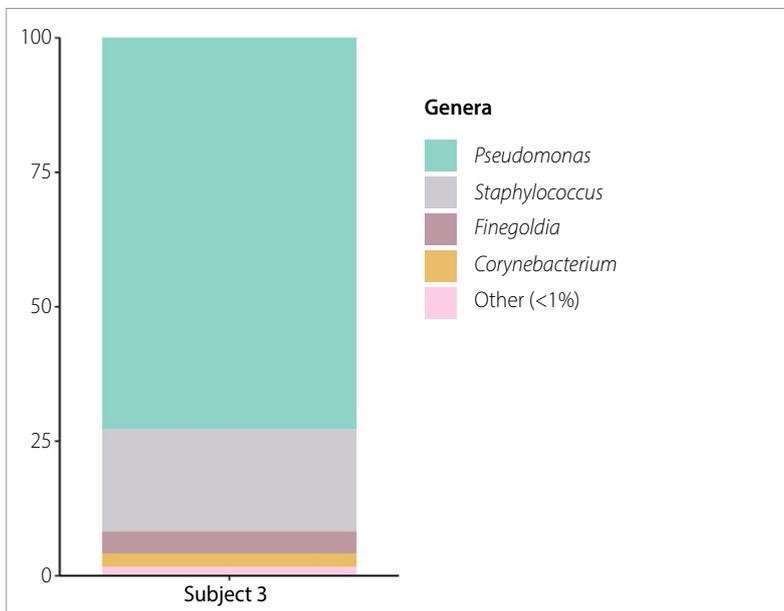
Genera
- *Pseudomonas*
- *Staphylococcus*
- *Finegoldia*
- *Corynebacterium*
- Other (<1%)

Table 3. Microbiology	
Bacterial-colony forming units (CFU) per inch² wound	**Isolated bacteria**
2.00 x 10⁵ CFU	*Pseudomonas aeruginosa* *Staphylococcus epidermidis* *Corynebacterium striatum*

Figure 2 | **Relative abundance of bacterial genera on the surface of subject 3's wound based on high-throughput bacterial 16S ribosomal gene sequencing.** Molecular detection of wound bacteria identified *Finegoldia*, an anaerobic bacteria that was not detected by culture-based methods.

Figure 3 | Top 25 most abundant proteins in subject 3's wound slough. Most proteins were of human origin and related to blood clot formation (i.e. platelet activation) and immune responses (i.e. cytokine signalling, complement cascade and neutrophil degranulation). One core protein associated with the metabolism of *Pseudomonas* was also identified.

Species of protein origin
- Homo sapiens
- Pseudomonas aeruginosa
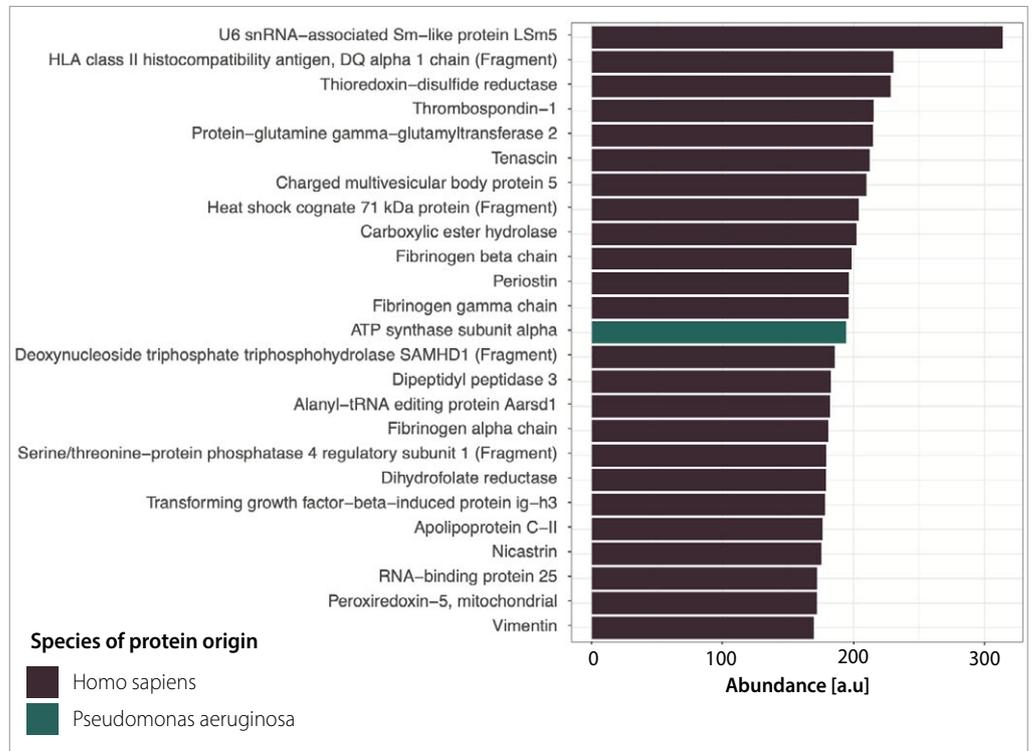
RELEVANT MEDICAL HISTORY
- Atrial fibrillation
- Congestive right-sided heart failure
- Hypertension
- Obesity (BMI = 32.5)
- Lower extremity oedema.

Table 1. Wound dimensions

Wound length	8.0cm
Wound width	4.1cm
Wound depth	0.2cm
Wound surface area	32.8cm^2
Wound volume	6.56cm^3
Wound shape	Irregular

CASE 4

The patient is a 77-year-old white female who developed a haematoma that developed into a 2-month non-healing wound on her right lateral shin following a fall. After the wound occurred, she was unable to bear weight on the leg and was admitted to the hospital for pain management, wound debridement and compression wraps, which significantly reduced her leg pain and allowed her to walk on it. In clinic, the wound was treated for 30 minutes with 0.25% sodium hypochlorite soak, followed by 15 minutes of air drying before applying new dressings. For the dressings, ¼-inch antimicrobial packing was placed in both wound tunnels, antimicrobial gauze was placed over the wound bed and absorbent foam padding was placed on the posterior ankle. Following the application of dressings, a multilayer compression wrap was used.

At the time of sample collection, the wound was 68% smaller in volume compared to previous measurements. Three months following sample collection, the wound had closed.

Figure 1 | Image of subject 4's right lateral shin wound before the debridement procedure

| Table 2. Wound information	
Wound duration	2 months
Wound aetiology	Trauma/haematoma (from fall)
Bates-Jensen wound assessment score	34
Wound edges	Distinct, outline is clearly visible, attached and even with wound base
Necrotic tissue	Loosely adherent yellow slough
Exudate type	Bloody
Exudate level	Moderate
Periwound colour	Pink
Oedema	No swelling or oedema
Induration	None present
Granulation	Healthy/bright/beefy red in colour and fills 75% to 100% of wound
Epithelialisation	<25% of wound covered
Surface area wound healing rate (% change per week)	20% smaller
Wound volume healing rate (% change per week)	68% smaller
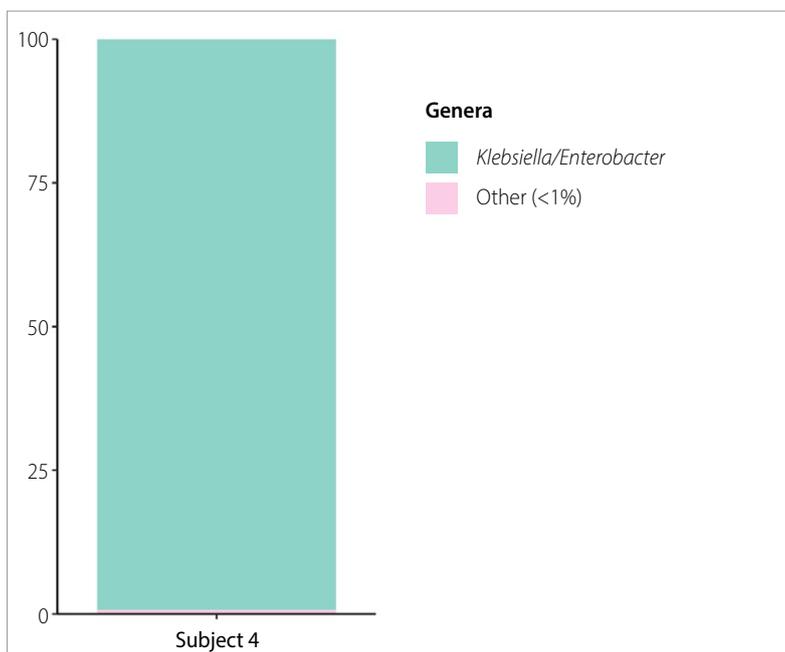
Genera

- Klebsiella/Enterobacter
- Other (<1%)

Table 3. Microbiology	
Bacterial-colony forming units (CFU) per inch² wound	**Isolated bacteria**
2.70 x 10⁶ CFU	Enterobacter sp. Klebsiella sp.

Figure 2 | Relative abundance of bacterial genera on the surface of subject 4's wound based on high-throughput bacterial 16S ribosomal gene sequencing. Molecular identification and culture-based methods identified *Klebsiella/Enterobacter* species as the prominent microorganism.

Figure 3 | Top 25 most abundant proteins in subject 4's wound slough. Most of these proteins were human in origin and related to blood clot formation (i.e. platelet formation), antibacterial immune responses (i.e. neutrophil degranulation) and skin layer formation.
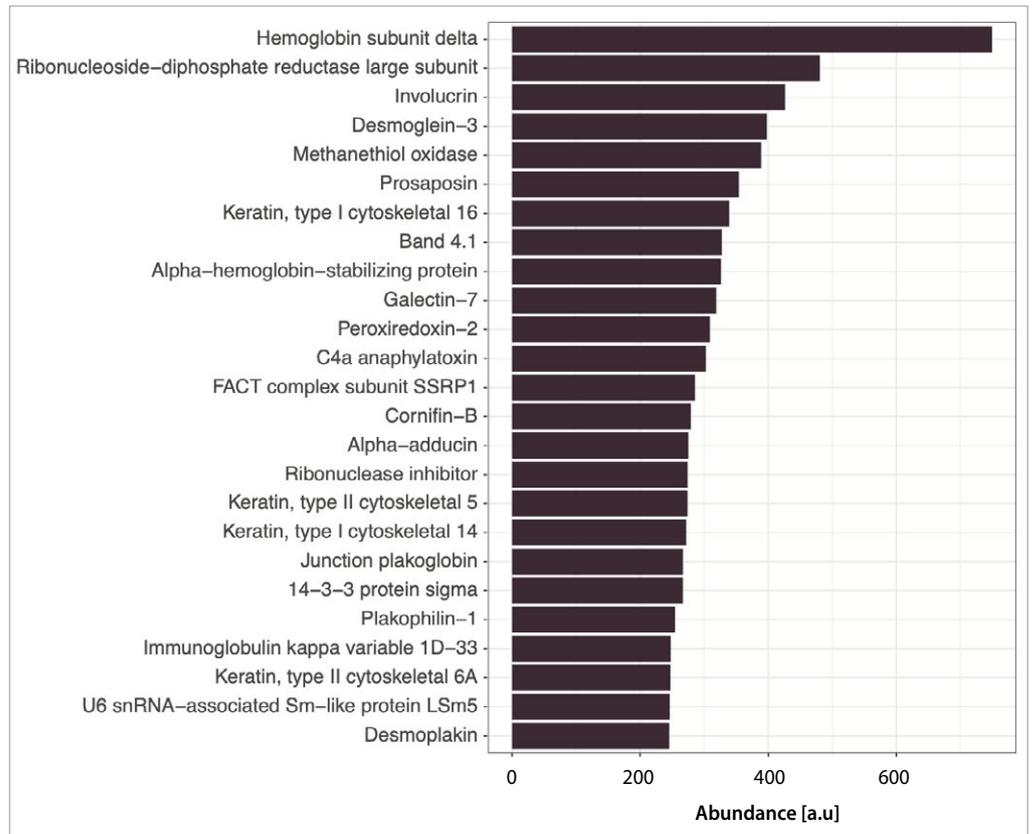
RELEVANT MEDICAL HISTORY

- Hypertension
- Obesity (BMI = 62.7)
- Motor vehicle collision.

Table 1. Wound dimensions	
Wound length	1.1cm
Wound width	4.0cm
Wound depth	1.0cm
Wound surface area	4.4cm^2
Wound volume	4.4cm^3
Wound shape	Bowl; boat

CASE 5

The patient is a 62-year-old white female who sustained a non-healing laceration to her left anterior shin during a motor vehicle crash 6 weeks before sample collection. At the time of sample collection, there was increased granulation tissue, decreased necrotic fat and the wound was 17% smaller than her previous visit. Her wound care consisted of dressing changes three times per week with silver sulfadiazine cream and gauze application, followed by a multilayer compression wrap.

The wound had closed three months following sample collection.

Figure 1 | Image of subject 5's left anterior shin wound before the debridement procedure

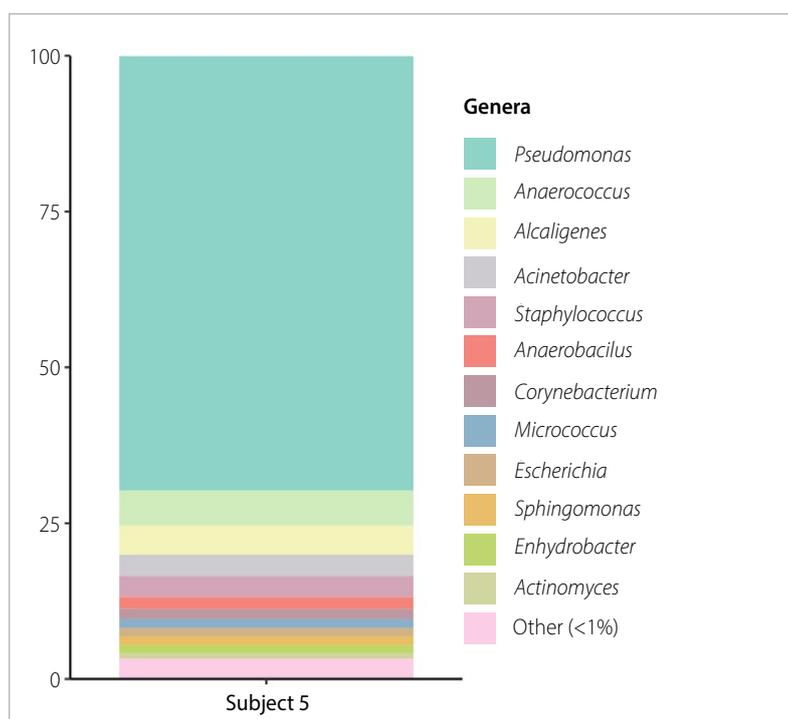| Table 2. Wound information	
Wound duration	6 weeks
Wound aetiology	Trauma/motor vehicle crash
Bates-Jensen wound assessment score	35
Wound ddges	Well-defined, not attached to base, rolled under and thickened
Necrotic tissue	Loosely adherent yellow slough
Exudate type	Serosanguineous (thin, watery and pale red/pink in colour)
Exudate level	Low
Periwound colour	Pink
Oedema	Non-pitting oedema extends >4 cm around wound
Induration	None present
Granulation	Healthy/bright/beefy red in colour and fills <75% of wound
Epithelialisation	<25% of wound covered
Surface area wound healing rate (% change per week)	13% smaller

Genera

- *Pseudomonas*
- *Anaerococcus*
- *Alcaligenes*
- *Acinetobacter*
- *Staphylococcus*
- *Anaerobacilus*
- *Corynebacterium*
- *Micrococcus*
- *Escherichia*
- *Sphingomonas*
- *Enhydrobacter*
- *Actinomyces*
- Other (<1%)

Table 3. Microbiology	
Bacterial-colony forming units (CFU) per inch² wound	**Isolated bacteria**
1.00 x 10² CFU	*Staphlylococcus warneri*

Figure 2 | Relative abundance of bacterial genera on the surface of subject 5's wound based on high-throughput bacterial 16S ribosomal gene sequencing. Molecular detection identified several anaerobic and aerobic bacteria that were not detected via microbial culture.

Figure 3 | **Top 25 most abundant proteins in subject 5's wound slough.** Most of these proteins were human in origin and related to blood clot formation (i.e. platelet formation), antibacterial immune responses (i.e. neutrophil degranulation) and skin layer formation. One protein of *Pseudomonas* origin, which is involved in virulence, was detected.
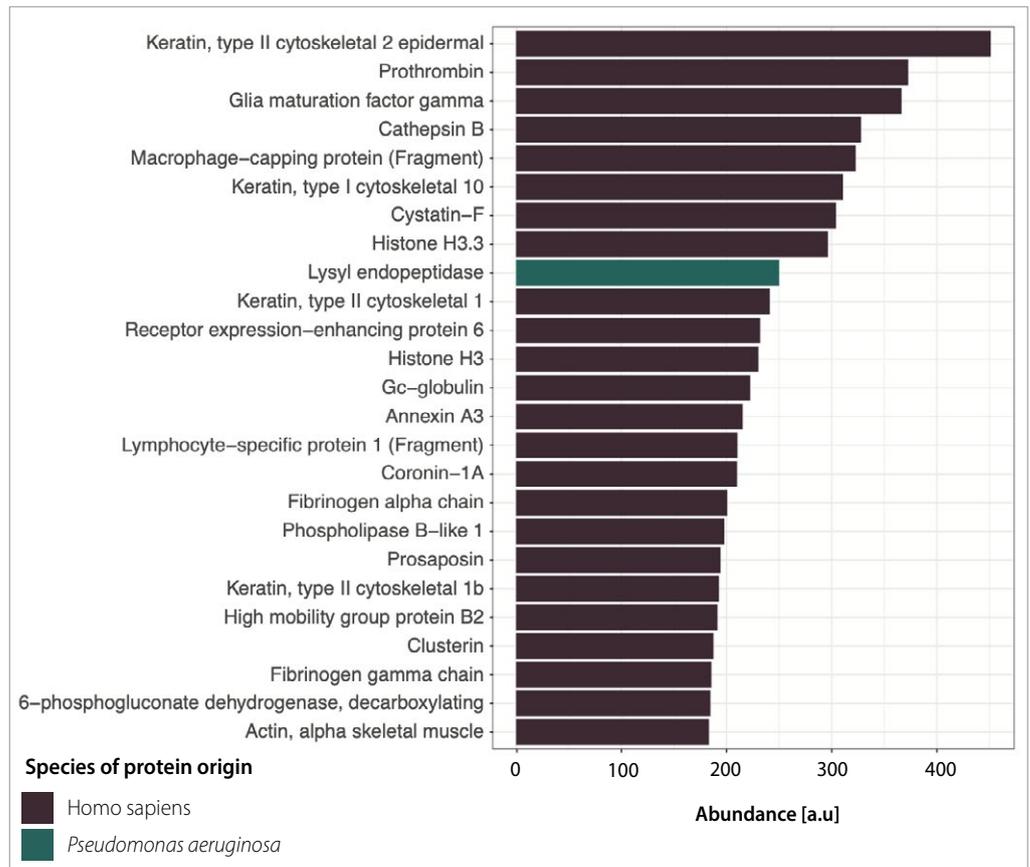
RELEVANT MEDICAL HISTORY
- Obesity (BMI > 85)
- Bilateral lower extremity lymphoedema
- Bilateral lower extremity oedema
- Recurrent cellulitis of lower extremities
- Recurrent lower extremity ulcers.

Table 1. Wound dimensions	
Wound length	1.0cm
Wound width	1.0cm
Wound depth	0.5cm
Wound surface area	1.0cm^2
Wound volume	0.5cm^3
Wound shape	Round oval

CASE 6

The patient is a 43-year-old white male with a history of recurrent, non-healing wounds secondary to lower extremity lymphoedema and venous stasis. The wounds on his right posterior ankle had been present for 5 weeks. The patient believes his wounds occurred from scratching the back of his leg against a bedpost. The wound with the largest surface area and greatest depth on the medial side of his posterior ankle (top of figure) was sampled for this study. The wound was noted to have approximately 10cm x 10cm of periwound redness and inflammation, as well as copious drainage and weeping from the area. His wound care included daily dressing changes with nystatin powder applied to intact skin, silver antimicrobial dressings and gauze applied to the wound. Following the application of the dressings, multilayer compression wraps were applied.

At the time of sample collection, his bilateral lower extremity oedema was still present but reduced and the periwound inflammation and drainage had also largely resolved. The wound was 75% smaller in volume compared to previous measurements. Three months following sample collection, the wound had completely healed.

Figure 1 | **Image of subject 6's wounds on the right posterior ankle before the debridement procedure**

| Table 2. Wound information	
Wound duration	5 weeks
Wound aetiology	Lymphoedema, venous stasis ulcer
Bates-Jensen wound assessment score	26
Wound edges	Distinct, outline clearly visible, attached and even with wound base
Necrotic tissue	White/grey non-viable tissue and non-adherent yellow slough
Exudate type	Serosanguineous (thin, watery and pale red/pink in colour)
Exudate level	Low
Periwound colour	Red/pink
Oedema	Oedema present
Induration	None present
Granulation	Skin intact or partial thickness wound
Epithelialisation	<25% of wound covered
Surface area wound healing rate (% change per week)	75% smaller
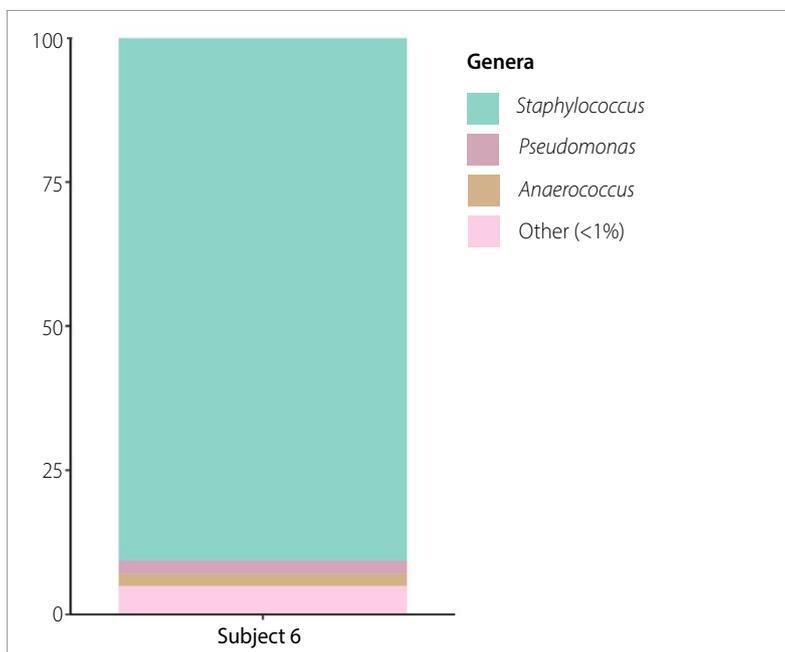
Genera
- *Staphylococcus*
- *Pseudomonas*
- *Anaerococcus*
- Other (<1%)

Table 3. Microbiology	
Bacterial-colony forming units (CFU) per inch² wound	**Isolated bacteria**
7.00 x 10³ CFU	No bacteria isolated via microbial culture

Figure 2 | Relative abundance of bacterial genera on the surface of subject 6's wound based on high-throughput bacterial 16S ribosomal gene sequencing. Molecular detection identified several anaerobic and aerobic bacteria that were not detected via microbial culture.
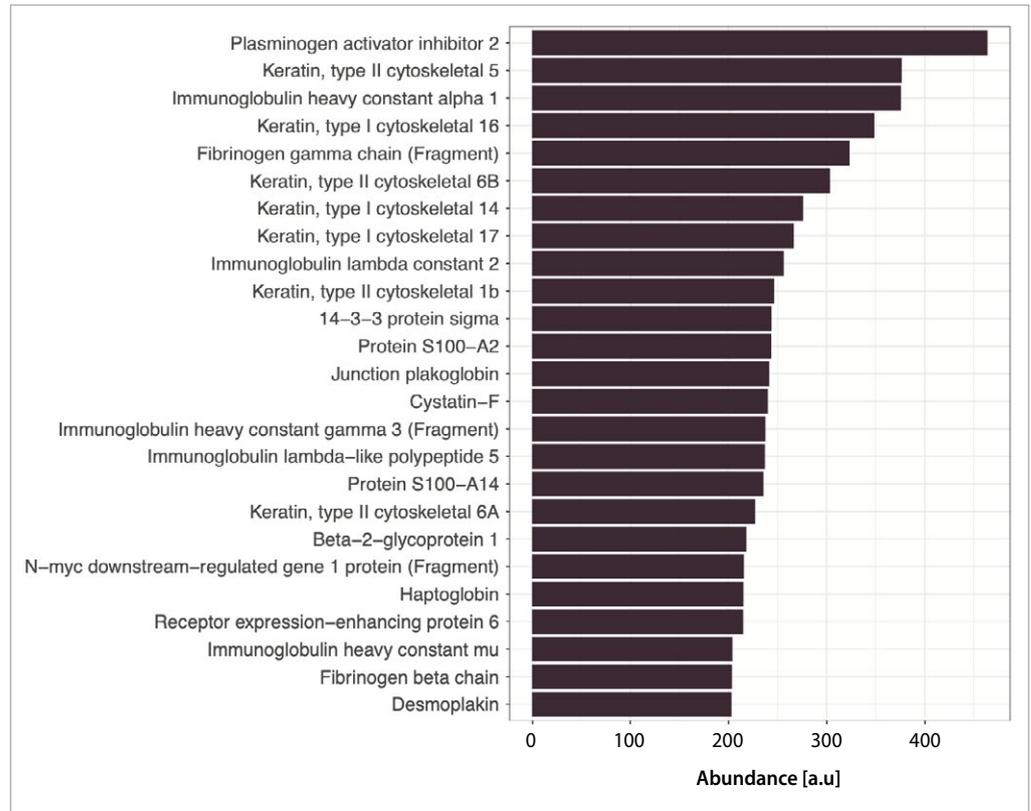
RELEVANT MEDICAL HISTORY
- Former smoker
- Coronary artery disease
- Hypertension
- Myocardial infarction
- Osteoarthritis of the right knee
- Osteopenia.

Table 1. Wound dimensions	
Wound length	12.0cm
Wound width	9.0cm
Wound depth	0.9cm
Wound surface area	108.0cm²
Wound volume	97.2cm³
Wound shape	Round oval

CASE 7

The patient is a 77-year-old white female with a non-healing surgical wound following open reduction and internal fixation of the right ankle a year before sample collection. For the past year, the patient had been managing wound care herself and had not sought medical care for her wound. The patient looked after her wound at home by washing the wound with 4% chlorhexidine gluconate soap and using dry gauze with dressing changes twice daily. One week before sample collection she was seen by her primary provider and was subsequently referred to the wound clinic. At that visit, significant lower extremity oedema and reduced pulses in the right lower leg were observed.

At the time of sample collection, the wound was 7% smaller in volume compared to the previous measurements taken by her primary provider. Three months following sample collection, the wound was still present and worsening. The patient had been reviewed by the vascular and orthopaedic surgery teams and there was concern for continued infection of the underlying hardware in the ankle.

Figure 1 | Image of subject 7's right ankle wound before the debridement procedure

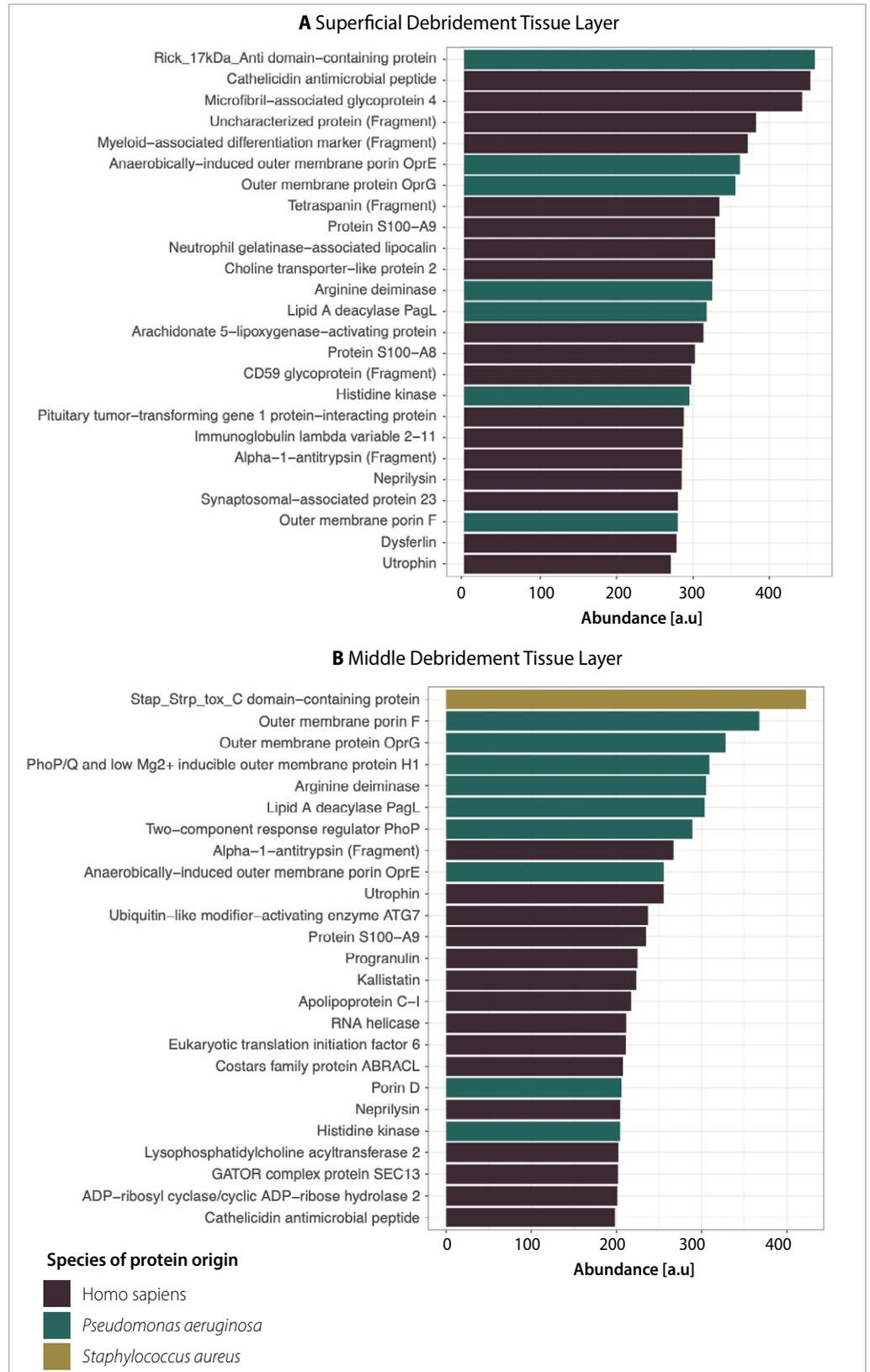| Table 2. Wound information	
Wound duration	1 year and 2 months
Wound aetiology	Non-healing surgical wound
Bates-Jensen wound assessment score	46
Wound edges	Well-defined and not attached to wound base
Necrotic tissue	Adherent and soft black eschar visible
Exudate type	Purulent drainage that is thick, opaque and tan/yellow in colour
Exudate level	Moderate
Periwound colour	Pink
Oedema	Pitting oedema extending <4cm around the wound
Induration	None present
Granulation	Pink/dull/dusky red in colour and fills <25% of wound
Epithelialisation	<25% of wound covered
Surface area wound healing rate (% change per week)	No change
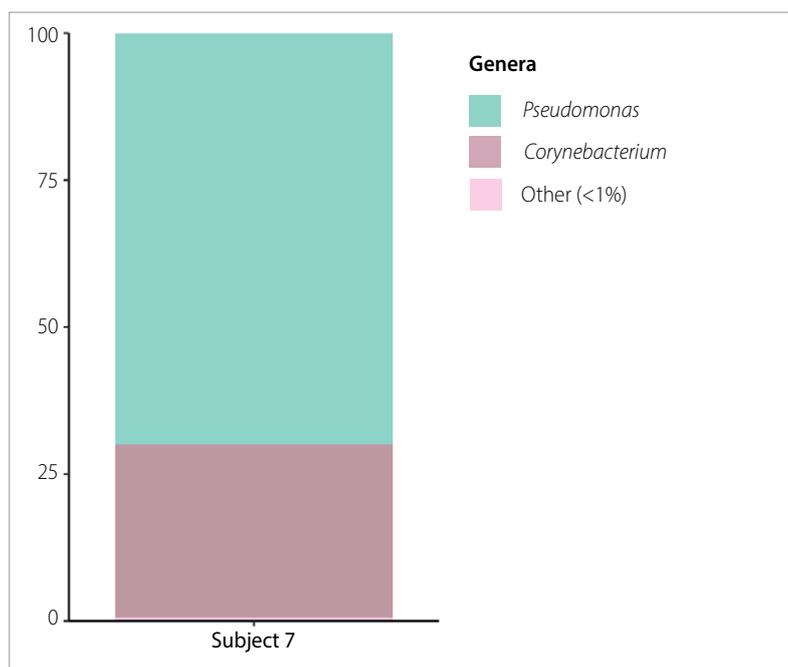
Genera
- Pseudomonas
- Corynebacterium
- Other (<1%)

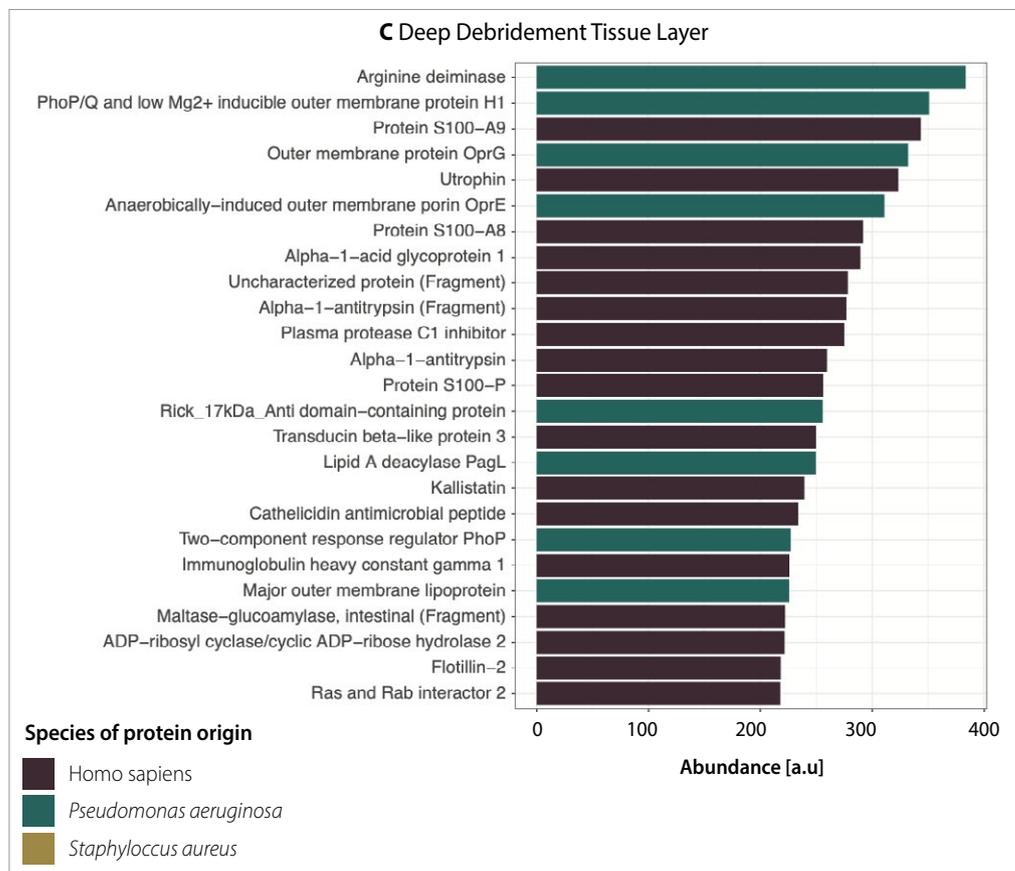| Table 3. Microbiology	
Bacterial-colony forming units (CFU) per inch^2 wound	**Isolated bacteria**
8.00 x 10^7 CFU	Pseudomonas aeruginosa

Figure 2 | Relative abundance of bacterial genera on the surface of subject 7's wound based on high-throughput bacterial 16S ribosomal gene sequencing.
In addition to Pseudomonas detected by microbial culture, molecular detection revealed the presence of Corynebacterium sp. in the wound. Corynebacterium can be found on human skin. However, Corynebacterium is slow to grow in microbial culture conditions, especially when compared to Pseudomonas, which could explain why they were not cultured from this patient.

Figure 3 | Top 25 most abundant proteins in superficial (A), middle (B) and deep (C) debridement tissue layers of subject 7's wound slough. Most proteins were human in origin and related to blood clot formation, immune responses (such as neutrophil degranulation, cytokine signalling and the complement cascade) and extracellular matrix formation. Multiple bacterial proteins, primarily structural proteins, as well as those involved in *Pseudomonas* and *Staphylococcus* nutrient uptake and metabolism were identified.

A Superficial Debridement Tissue Layer

B Middle Debridement Tissue Layer

Species of protein origin
- Homo sapiens
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*

Figure 3 (continued) | Top 25 most abundant proteins in superficial (A), middle (B) and deep (C) debridement tissue layers of subject 7's wound slough. Most proteins were human in origin and related to blood clot formation, immune responses (such as neutrophil degranulation, cytokine signalling and the complement cascade) and extracellular matrix formation. Multiple bacterial proteins, primarily structural proteins, as well as those involved in *Pseudomonas* and *Staphylococcus* nutrient uptake and metabolism were identified.

C Deep Debridement Tissue Layer

Species of protein origin
- Homo sapiens
- *Pseudomonas aeruginosa*
- *Staphyloccus aureus*

Figure 4 | Microscopy image of subject 7's wound slough. Confocal laser scanning microscopy (CLSM) at low magnification (5x objective) of a histological cross-section of Formalin-Fixed Paraffin-Embedded (FFPE) slough samples. (A) Tissue and matrix autofluorescence appear green, nucleic acids appear blue, and bacteria appear red. In a high magnification (63x objective) micrograph, bacterial aggregate is visible. (B) The red outlines represent the magnified region.
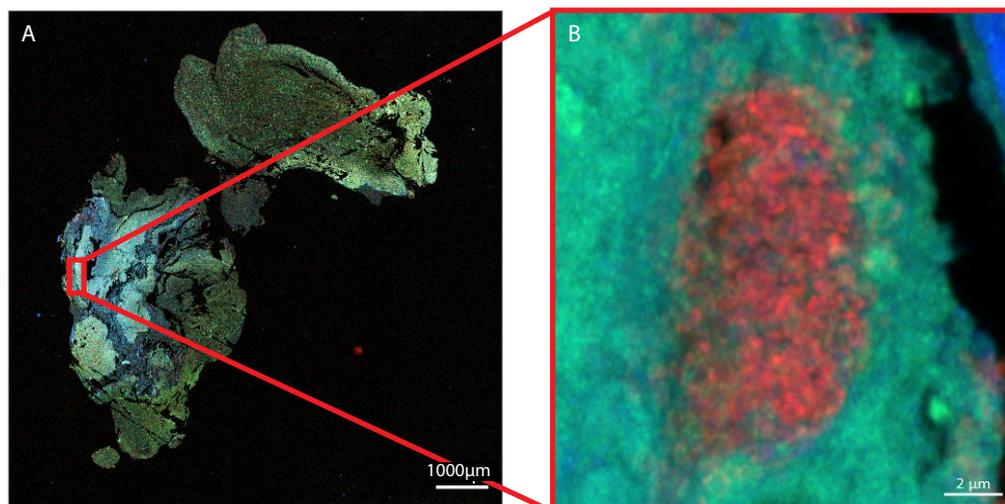
RELEVANT MEDICAL HISTORY

- Former smoker
- Alcohol dependence
- Hypertension
- Arterial insufficiency of lower extremities
- Lower extremity oedema
- Chronic obstructive pulmonary disease
- Type II diabetes
- Obesity (BMI = 36.9)
- Focal segmental glomerulosclerosis
- Osteoarthritis
- Normocytic anaemia.

Table 1. Wound dimensions	
Wound length	9.0cm
Wound width	10.4cm
Wound depth	1.1cm
Wound surface area	93.6cm^2
Wound volume	102.96cm^3
Wound shape	Irregular

CASE 8

The patient is a 73-year-old white female with recurrent non-healing wounds on her lower extremities that had been present for one year, secondary to bilateral lower extremity oedema and peripheral vascular disease. Wound healing was complicated by renal disease and a history of alcohol and tobacco use. The patient's wound care included daily washing with soap and water, drying the wound and covering it with absorbent foam padding, gauze and compression wraps.

At the time of sample collection, the patient reported that she needed to change her wound dressing 2-3 times per day due to wound drainage and subsequent bandage slipping, as well as increased pain at the wound. The patient reported no redness or warmth in the area. The wound was also approximately 15% larger in volume compared to measurements taken at the previous visit. Three months following sample collection the wound was still present, but clinically improving.

Figure 1 | Image of subject 8's lower leg wound before the debridement procedure

| Table 2. Wound information	
Wound duration	1 year and 3 months
Wound aetiology	Peripheral vascular disease and venous stasis ulcers
Bates-Jensen wound assessment score	40
Wound edges	Well-defined and not attached to wound base
Necrotic tissue	Loosely adherent yellow slough
Exudate type	Serosanguineous (thin, watery and pale red/pink in colour)
Exudate level	Low
Periwound colour	Pink
Oedema	Pitting oedema extending >4cm around the wound
Induration	Induration <2cm around wound
Granulation	Healthy/bright/beefy red in colour and fills 75% to 100% of wound
Epithelialisation	<25% of wound covered
Surface area wound healing rate (% change per week)	0.5% smaller
Wound volume healing rate (% change per week)	15.75% larger
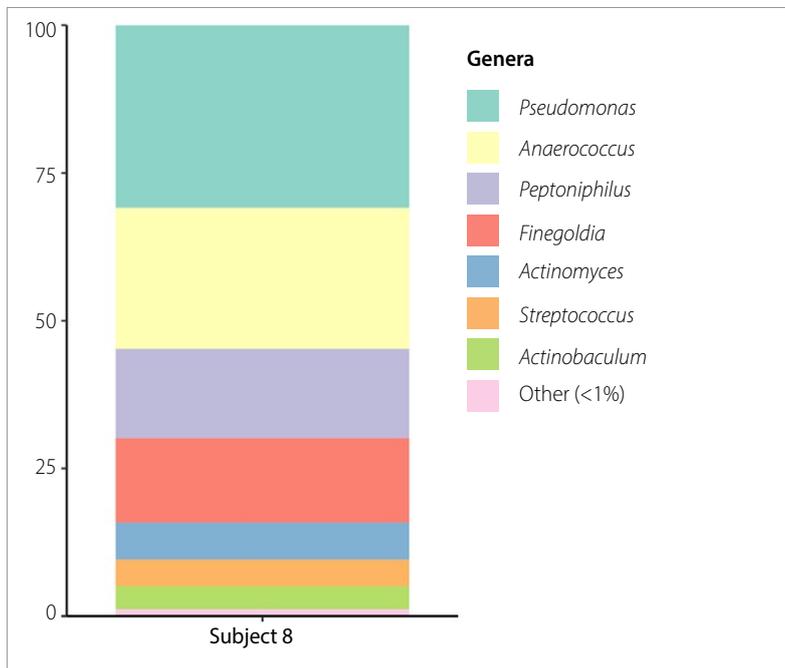
Figure 2 | **Relative abundance of bacterial genera on the surface of subject's 8 wound based on high-throughput bacterial 16S ribosomal gene sequencing.** Molecular detection revealed the presence of multiple aerobic and anaerobic bacterial species at the wound surface, in addition to the *Pseudomonas.*

Table 3. Microbiology	
Bacterial-colony forming units (CFU) per inch^2 wound	Isolated bacteria
2.30 x 10^7 CFU	*Pseudomonas aeruginosa*

Figure 3 | **Top 25 most abundant proteins in subject 8's wound slough.** Most of these proteins were human in origin and related to blood clot formation (i.e. platelet formation), antibacterial immune responses (i.e. neutrophil degranulation) and skin layer formation. Numerous bacterial proteins, primarily structural proteins, as well as those involved in *Pseudomonas* and *Staphylococcus* nutrient uptake and metabolism, were identified.
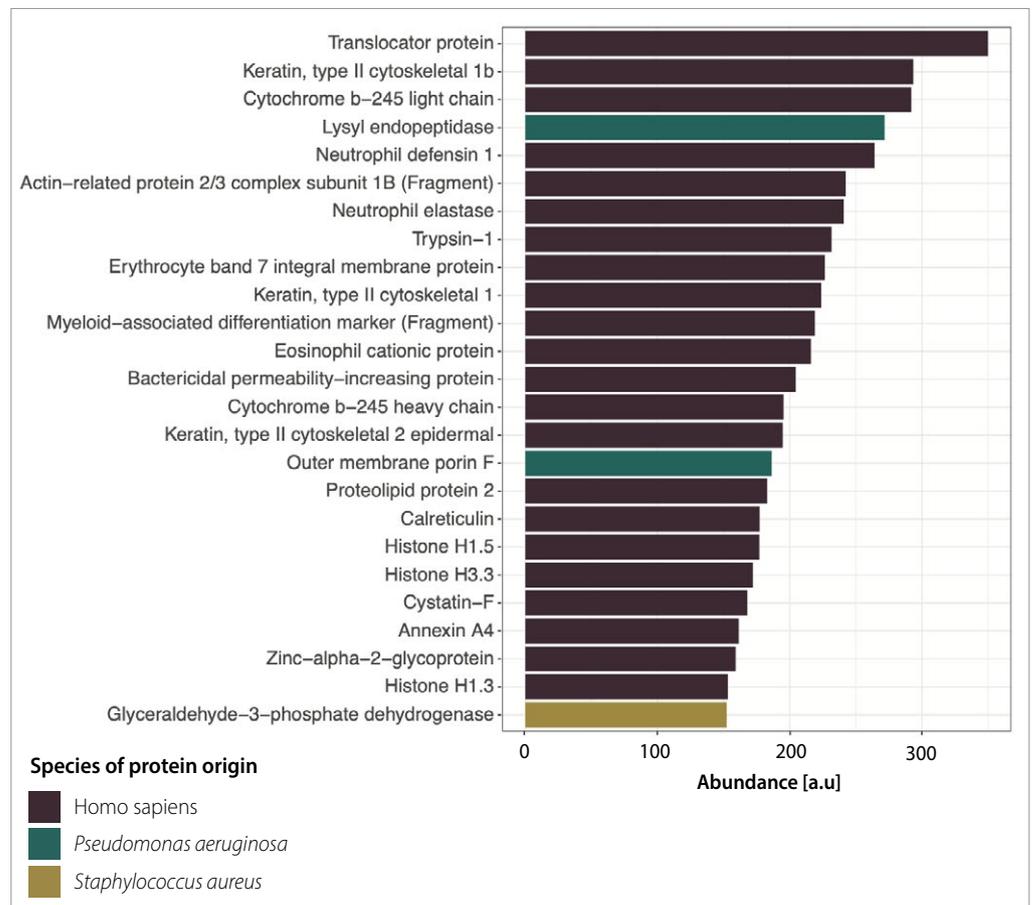
Figure 4 | Microscopy image of subject 8's wound slough.
Confocal laser scanning microscopy (CLSM) at low magnification (5x objective) of a histological cross-section of Formalin-Fixed Paraffin-Embedded (FFPE) slough samples (A). Tissue and matrix autofluorescence appear green, nucleic acids appear blue, and bacteria appear red. In a high magnification (63x objective) micrograph, a bacterial aggregate is visible (B). The red outlines represent the magnified region.
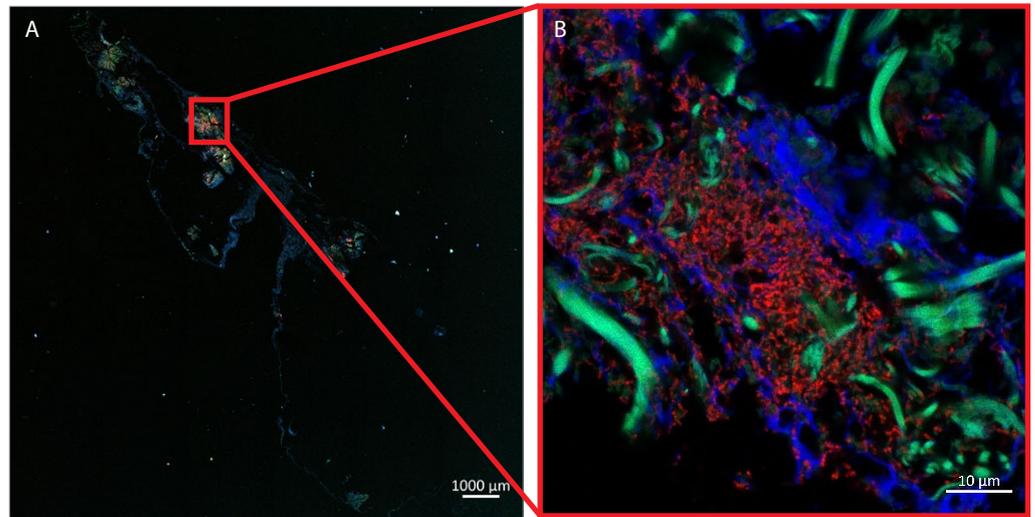
RELEVANT MEDICAL HISTORY

- L1 spinal cord injury/ paraplegia secondary to motor vehicle crash
- Multiple pressure ulcers
- Neurogenic bladder
- Neurogenic bowel
- Obesity (BMI = 30).

Table 1. Wound dimensions	
Wound length	2.2cm
Wound width	1.8cm
Wound depth	0.5cm
Wound surface area	3.96cm^2
Wound volume	1.98cm^3
Wound shape	Round oval

CASE 9

The patient is a 57-year-old white female with a history of paraplegia following a motor vehicle crash in 1990. She had no sensation in the area of the wound or below due to her spinal injury. She had a history of pressure ulcers, the most recent of which healed in 2020. The wound sampled for this study was a pressure ulcer that developed near her anus four months earlier. The patient's wound care consisted of 0.25% sodium hypochlorite solution and moistened gauze wet-to-dry dressing twice daily. During the patient's second dressing change, the wound was packed with absorbent foam pads for the border. The patient reported spending a total of 5 hours or more sitting per day and changing positions every 2 hours, except for at night while sleeping.

At the time of sample collection, the wound appeared worse than in previous visits, with increased wound drainage, odour and devitalised tissue, as well as new significant undermining. Three months following sample collection, the wound was still present, but clinically improving.

Figure 1 | Image of subject 9's pressure ulcer before the debridement procedure

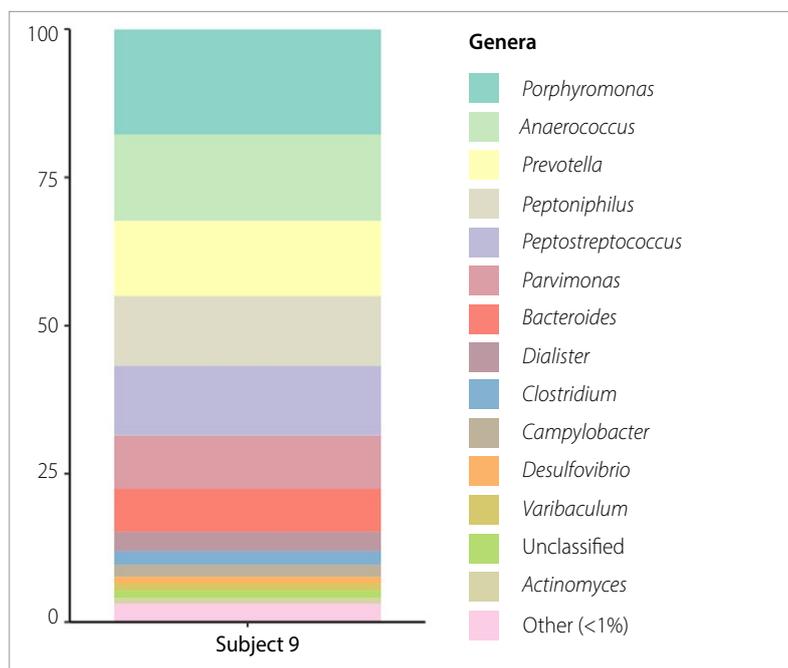| Table 2. Wound information	
Wound duration	4 months
Wound aetiology	Stage 3 pressure ulcer and full thickness skin loss
Bates-Jensen wound assessment score	42
Wound edges	Well-defined and not attached to wound base with undermining
Necrotic tissue	Adherent with soft black eschar
Exudate type	Serous, thin, watery and clear
Exudate level	Moderate
Periwound colour	Deep red/purple and non-blanchable
Oedema	No swelling or oedema
Induration	Induration 2–4cm extending <50% around wound
Granulation	Pink/dull/dusky red in colour and fills <25% of wound
Epithelialisation	<25% of wound covered
Surface area wound healing rate (% change per week)	4% larger

Genera

- *Porphyromonas*
- *Anaerococcus*
- *Prevotella*
- *Peptoniphilus*
- *Peptostreptococcus*
- *Parvimonas*
- *Bacteroides*
- *Dialister*
- *Clostridium*
- *Campylobacter*
- *Desulfovibrio*
- *Varibaculum*
- Unclassified
- *Actinomyces*
- Other (<1%)

Table 3. Microbiology	
Bacterial-colony forming units (CFU) per inch² wound	**Isolated bacteria**
8.30 x 10⁴ CFU	*Bacteroides sp.* *Eikenella sp.* *Peptostreptococcus sp.* *Porphyromonas sp.* *Staphylococcus epidermidis* *Streptococcus agalactiae*

Figure 2 | Relative abundance of bacterial genera on the surface of subject 9's wound based on high-throughput bacterial 16S ribosomal gene sequencing. Molecular detection indicates the presence of a variety of bacteria at the wound surface, which is consistent with the variety of bacteria isolated from bacterial culture.

Figure 3 | Top 25 most abundant proteins in subject 9's wound slough. The majority of these proteins were of human origin and were involved in blood clot and extracellular matrix formation, as well as immune responses (i.e. neutrophil degranulation, B cell receptor activation and the complement cascade). *Pseudomonas*, which is involved in metabolism, was also identified.
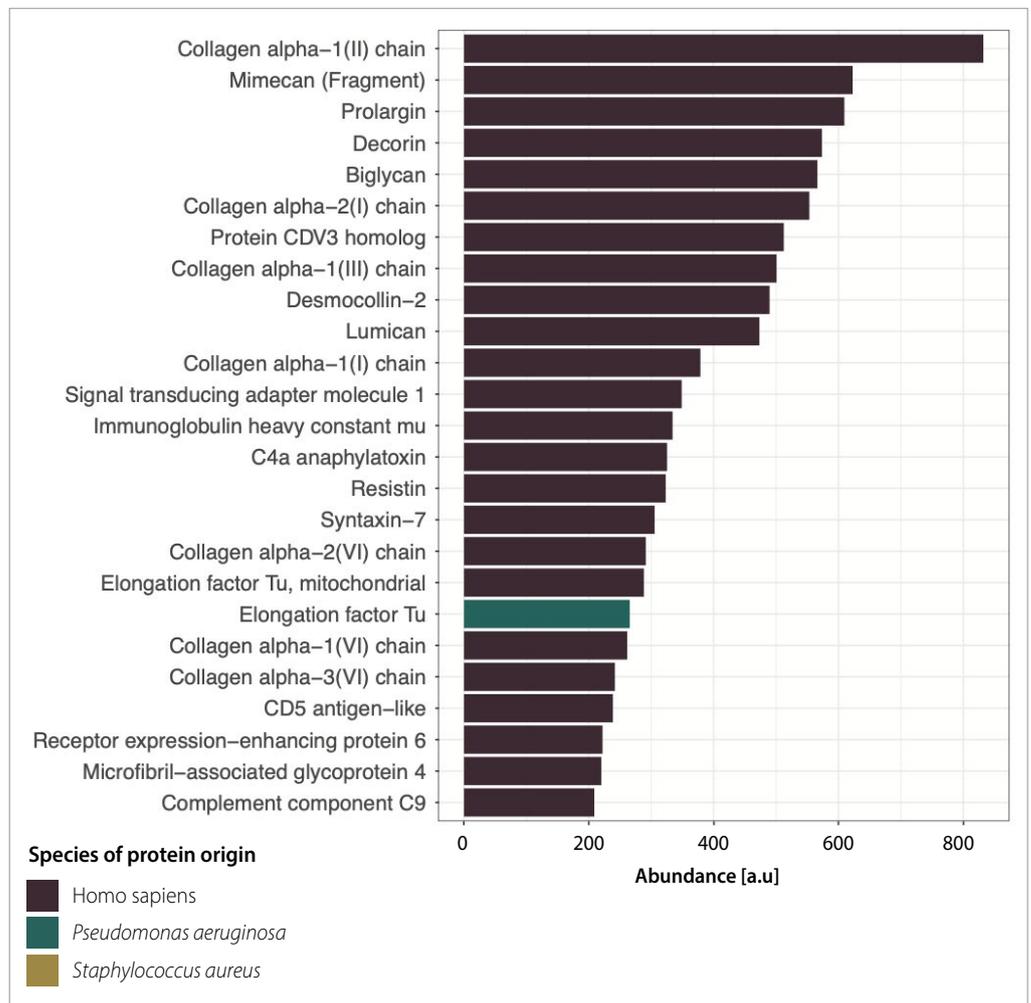

Figure 4 | Microscopy image of subject 9's wound slough. Confocal laser scanning microscopy (CLSM) at low magnification (5x objective) of a histological cross-section of Formalin-Fixed Paraffin-Embedded (FFPE) slough samples (A). Tissue and matrix autofluorescence appear green, nucleic acids appear blue, and bacteria appear red. In a high magnification (63x objective) micrograph, a bacterial aggregate is visible (B). The red outlines represent the magnified region.

100X

500X

2000X

5000X
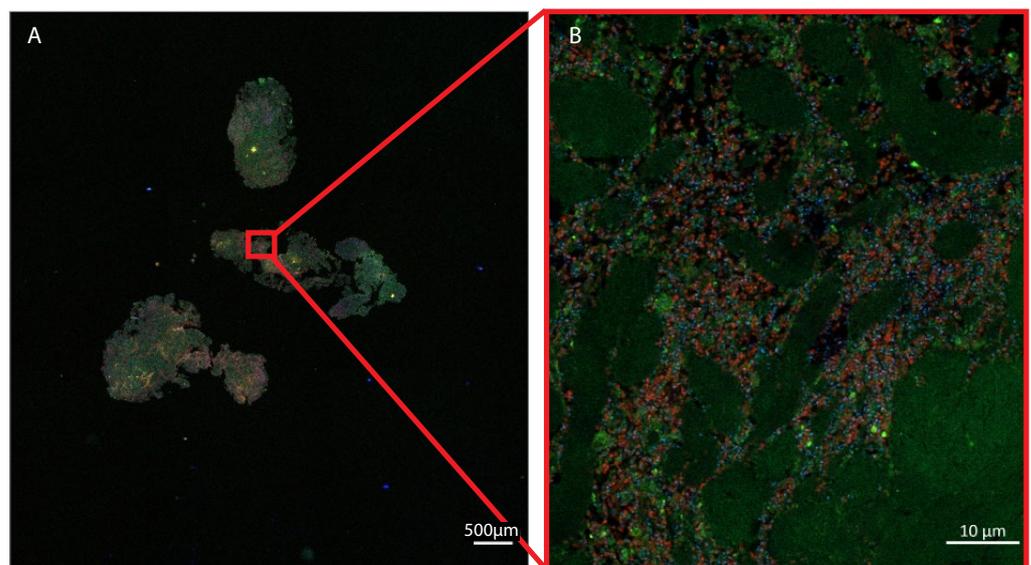
Figure 5| Microscopy image of subject 9's wound slough. Scanning electron microscopy (SEM) of a single region of interest on the patient slough sample at four different magnifications (100X, ×500, ×2000 and ×5000) showing putative mixed cocci and bacilli surrounded by the extracellular matrix on collagen fibers. Magnified regions are represented by solid outlines.
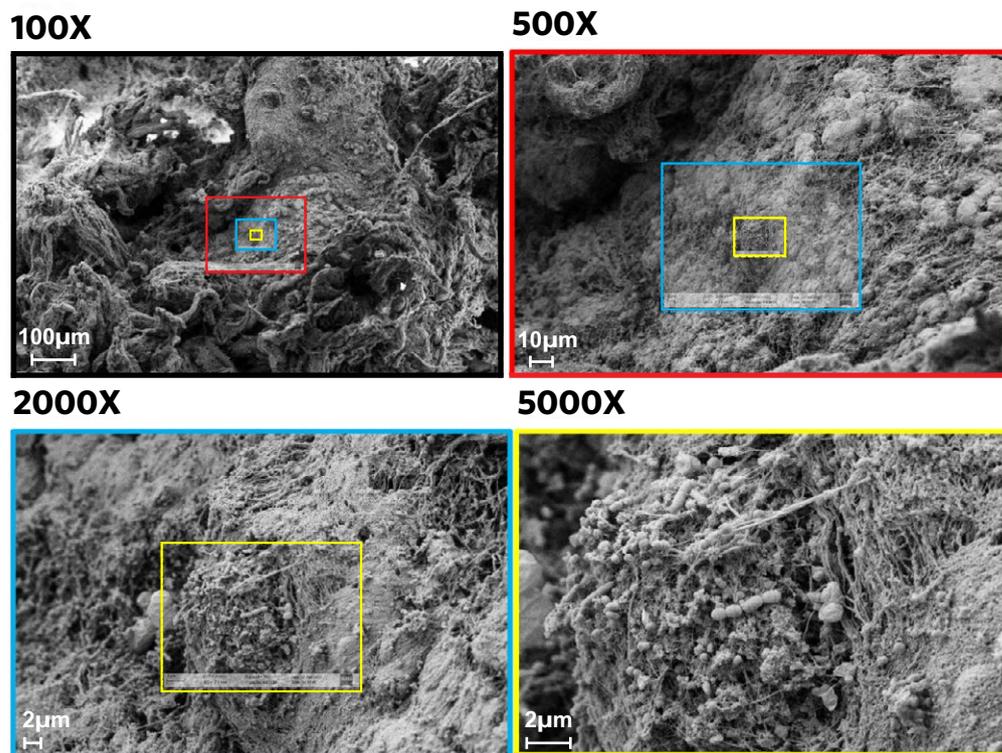
RELEVANT MEDICAL HISTORY

- Thoracic spinal cord injury/ paraplegia secondary to motor vehicle crash in 2020
- Neurogenic bowel
- Neurogenic bladder
- Chronic obstructive pulmonary disease
- Type II diabetes
- Obesity (BMI = 33.6)
- Malnutrition
- Zinc deficiency.

Table 1. Wound dimensions	
Wound length	2.2cm
Wound width	1.8cm
Wound depth	0.5cm
Wound surface area	3.96cm^2
Wound volume	1.98cm^3
Wound shape	Round oval

CASE 10

The patient is a 59-year-old white female with a history of paraplegia following a motor vehicle crash in September 2020. Due to her spinal injury, she had no sensation below her umbilicus. A few weeks after her spinal injury, she developed pressure ulcers on her coccyx and bilateral ischium. In February 2021, she was hospitalised for infection management with broad-spectrum antibiotics and surgical debridement of the wound. She was hospitalised again in April 2021 for infection management and surgical debridement. During this debridement session, a gluteal abscess underlying her sacral pressure ulcer, as well as a subcutaneous fistula connecting the coccygeal and right gluteal ulcers was discovered. Bone was also visible at the base of each of the wounds. The patient's wound care consisted of twice daily dressing changes, with 0.25% sodium hypochlorite solution moistened gauze wet-to-dry dressing for the first dressing change and dry packing gauze for the second dressing change to better control drainage. She was spending 2 to 4 hours per day in her wheelchair, for no more than 2 hours at a time, to offload her wound.

For this study, a pressure ulcer on her sacrum/coccyx was sampled. Three months following sample collection, the wound was still present and worsening, with ongoing infection.

Figure 1 | Image of subject 10's pressure ulcer before the debridement procedure

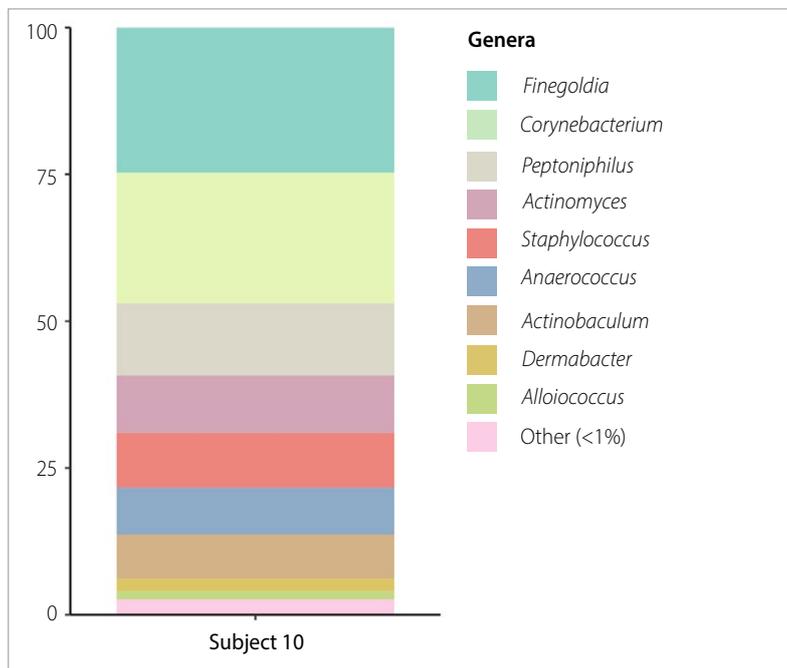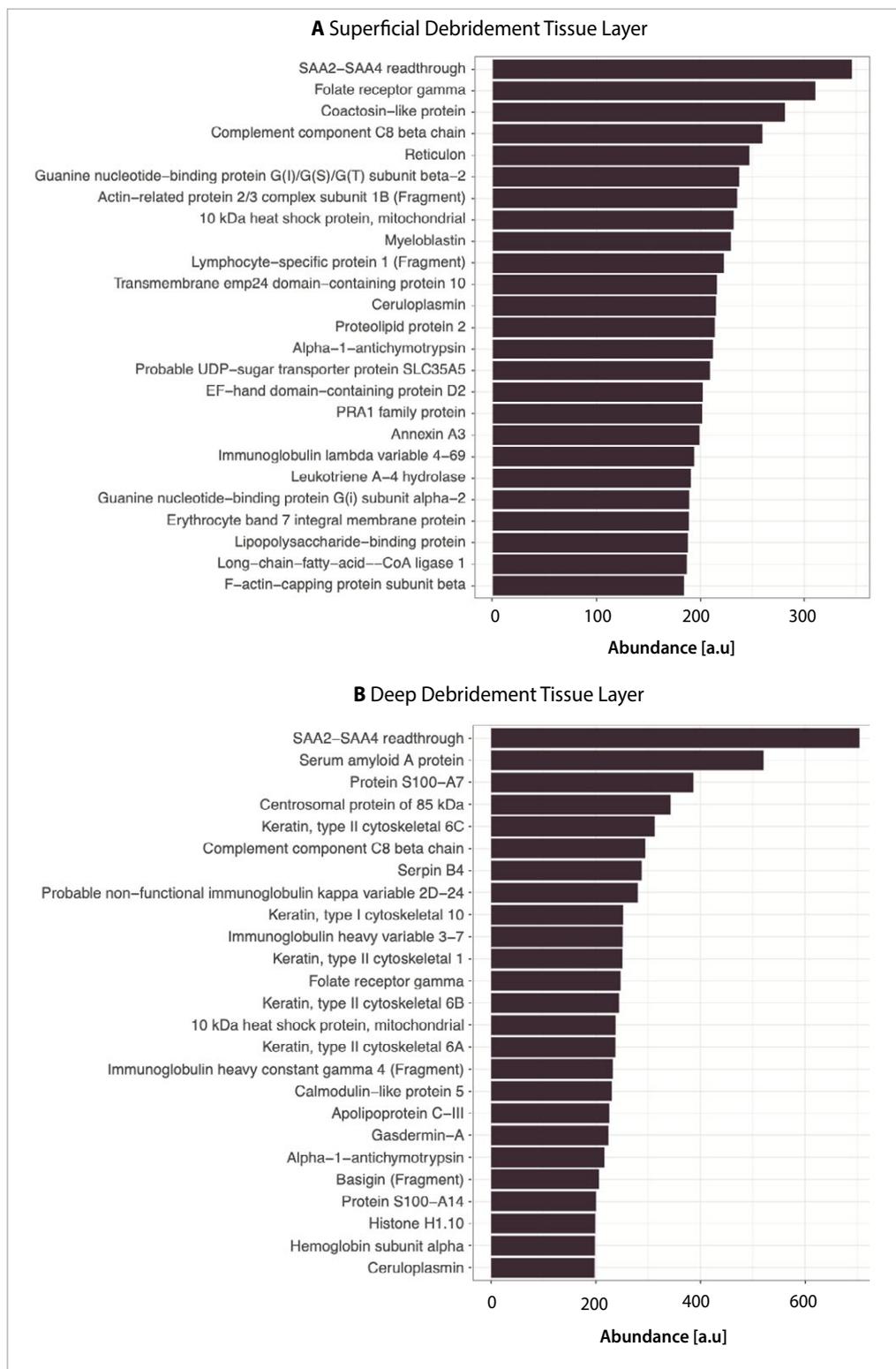| Table 2. Wound information	
Wound duration	1 year
Wound aetiology	Stage 4 pressure ulcer with full thickness tissue loss
Bates-Jensen wound assessment score	42
Wound edges	Well-defined, not attached to base, rolled under and thickened
Necrotic tissue	White/grey non-viable tissue and non-adherent yellow slough
Exudate type	Serosanguineous (thin, watery and pale red/pink in colour)
Exudate level	High
Periwound colour	Deep red/purple and non-blanchable
Oedema	No swelling or oedema
Induration	None present
Granulation	Pink/dull/dusky red in colour and fills <25% of wound
Epithelialisation	<25% of wound covered
Surface area wound healing rate (% change per week)	1.3% smaller

Genera
- *Finegoldia*
- *Corynebacterium*
- *Peptoniphilus*
- *Actinomyces*
- *Staphylococcus*
- *Anaerococcus*
- *Actinobaculum*
- *Dermabacter*
- *Alloiococcus*
- Other (<1%)

Table 3. Microbiology	
Bacterial-colony forming units (CFU) per inch² wound	**Isolated bacteria**
1.20 x 10⁶ CFU	*Corynebacterium striatum. Peptoniphilus sp.*

Figure 2 | Relative abundance of bacterial genera on the surface of subject 10's wound based on high-throughput bacterial 16S ribosomal gene sequencing. Molecular detection revealed the presence of multiple anaerobic and aerobic bacterial species in the wound that could not be isolated from bacterial culture.

Figure 3 | Top 25 most abundant proteins in superficial (A) and deep (B) debridement tissue layers of subject 10's wound slough. Most of these proteins were human in origin and related to blood clot formation (i.e. platelet formation), antibacterial immune responses (i.e. neutrophil degranulation) and skin layer formation.

A Superficial Debridement Tissue Layer

B Deep Debridement Tissue Layer

Discussion

Slough is a heterogeneous material associated with the surface of wounds that, in some cases, may provide a substrate for microbial colonisation and proliferation, including the formation of biofilm (Percival and Suleman, 2015). It is hypothesised that slough subsequently limits penetration and thus the efficacy of antibiotics, as well as host immune cell infiltration (Percival and Suleman, 2015). Due to this heterogeneity in colour, consistency and microbial bioburden, a detailed understanding of slough composition and its association with impaired wound healing remains vastly understudied. This is despite debridement being considered the gold standard of care and an increasing emphasis on biofilm-based wound care (Powers et al, 2016; Evans and Kim, 2020). Understanding the composition of slough is critical for clinicians and researchers to improve clinical practice and develop targeted therapies to manage or prevent the development of slough. Improving the wound environment to an optimal state of a warm moist wound bed with cellular and microbial balance, as well as advancing wound edges and periwound health, should be considered the goal of care to enhance healing.

> **Understanding slough allows us to target therapies and invest in wound cleansing and debridement to promote healing.**

Current understanding and identification of the wound condition consist of:
- Necrotic/non-viable tissue
- Slough
- Eschar
- Fibrinous surface substance
- Granulation tissue and the distinction between healthy and unhealthy tissue
- Epithelial tissue.

However, there is still a lack of consensus in terminology, practice and molecular level of knowledge regarding different tissue types (McGuire and Nasser, 2021). Here we sought to collect samples of 'yellow' tissue and characterise the host and microbial composition of this material from mixed wound aetiology. In this pilot study, we present data summarising the appearance of slough, microbial burden, microbial taxonomic composition and host protein composition. One of the primary goals of this pilot project is to inform us of the most relevant assays for determining the composition of slough and to determine whether our study protocols are adequate for obtaining this information in a reproducible manner across clinical and laboratory sites around the world.

> **Given the microbial density in all samples, the need for therapeutic wound and periwound cleansing and debridement is reinforced.**

Samples were collected from a diverse set of wound types in Australia and the USA. Previously, wounds with a microbial burden exceeding 1×10^5 colony-forming units (CFUs) were considered at a high risk of developing infection (Georgiade,1983; Bowler, 2003), which impedes healing (Anderson and Hamm, 2021). This cut-off (Georgiade,1983; Bowler, 2003) is based on outdated methods. Instead, in our comprehensive analysis of 23 slough samples from different countries, we used molecular methods to determine bacterial bioburden that generally exceeded this threshold (median = 2.13×10^5), regardless of healing outcome.

Quantitative microbiological culturing agreed with the molecular results, with many slough samples containing as many as 8×10^7 CFUs. This was an unexpected finding, particularly in healing wounds. We know that the common practice of wound culturing and using standard microscopy is significantly flawed and outdated. Further research is required to understand and determine how and what microorganisms are pathogenic and in what density. For example, new technologies that can visualise microbial density are being developed for use in everyday practice, such as smart dressings (Shou et al, 2018) and infrared cameras (Peng et al, 2017; Dacy et al, 2019). However, even these technologies have limitations in the diversity of bacterial species they can detect.

> **Wound healing isn't always guaranteed. Even wounds with low bacterial counts can fail to heal, while wounds with high bacterial counts can improve and progress to heal.**

PRIMARY FINDINGS OF MICROBIAL COMMUNITIES THROUGH DNA SEQUENCING

Consistent with previous studies employing DNA-based typing methods, we found the species of bacteria isolated from slough samples were varied and diverse (Loesche et al, 2017; Kalan et al, 2019). Each sample contained multiple species of bacteria, some of which had not been isolated or identified using traditional culture-based approaches. This supports the notion that slough is polymicrobial in nature. In addition to molecular analysis, quantitative culturing was performed on a variety of microbiological media types in order to capture the greatest diversity of microbes from each sample. Typical wound pathogens such as *Pseudomonas aeruginosa*, *Corynebacterium sp.*, *Staphylococcus sp.*, *Enterobacter sp.* and *Streptococcus sp.* were frequently isolated. We also had single isolates of *Alcaligenes sp.*, *Eikenella sp.*, *Enterococcus sp.*, *Klebsiella sp.*, and anaerobic taxa such

as *Bacteroides sp.*, *Peptoniphilus sp.*, *Peptostreptococcus sp.*, and *Porphyromonas sp*. Future research should focus on determining the role of different species and their interactions within polymicrobial communities on wound healing outcomes.

BIOFILM AND SLOUGH: WHAT DID WE SEE THROUGH MICROSCOPY?

Historically, specialised microscopy techniques were used to detect the presence of bacteria that form biofilms in slough samples. Using a combination of CLSM and SEM, we found that less than 30% of samples had visible bacterial cells or biofilm. This is not surprising given our understanding that biofilm is not homogeneous in the wound and reinforces the notion that 'yellow' does not equal slough. It has previously been reported that biofilm is found in more than 70% of chronic/non-healing wounds (Mendosa et al, 2019) but is not visible to the naked eye. Biofilm has been reported in deeper tissues, wound edges, wound dressings and aggregates in and around wounds (Fasli et al, 2009; Phillips et al, 2016; Thaarup et al, 2022). There are numerous reasons why our microscopy results detected a much lower percentage of biofilm. Bacterial aggregates are unlikely to be uniform across the wound bed or within tissue, and thus may have been missed in the section of slough examined. As a result, the generalised use of microscopy to detect bacterial aggregates is not recommended and is subject to high levels of uncertainty. However, by utilising DNA-sequencing-based methods that did not require bacterial culturing, we determined that all slough samples were polymicrobial in nature. Each sample contained between 6 and 42 different bacterial genera.

PRIMARY FINDINGS OF PROTEOMICS: WHAT'S IN SLOUGH?

The most prevalent proteins detected in slough samples were associated with skin structure, wound healing and immune responses. Proteins involved in maintaining the skin's structure and wound healing primarily included proteins for extracellular matrix formation (e.g. keratins and collagens), skin cell adhesion (e.g. desmogleins) and blood clot formation (e.g. fibrinogen, thrombin and other coagulation factors. Immune response proteins included those associated with immune activation and signalling (e.g. cytokines and immunoglobulins), particularly those involving neutrophils (e.g. myeloperoxidase, neutrophil defensins and neutrophil extracellular trap formation), as well as those involved in innate antimicrobial responses (e.g. cathelicidin antimicrobial peptide, bactericidal permeability-increasing protein and the complement cascade). Proteins involved in general cellular metabolism, intracellular signalling and cell structure were also identified.

All subjects, particularly subject 7, had detectable bacterial proteins within their wound slough. These include bacterial structural proteins and proteins involved in bacterial metabolism and toxin production (e.g. enterotoxin). It should be noted that microbial proteomics databases are still limited. In this analysis, the database only contained proteins for *Staphylococcus aureus* and *Pseudomonas aeruginosa*, thus, we expect the number of bacterial proteins present in the slough to be underestimated. However, even with this limitation, our findings are consistent with other studies reported in the literature showing that proteins associated with *Pseudomonas aeruginosa* are found deeper within wound tissue, whereas *Staphylococcus aureus* proteins are found closer to the surface in more superficial material (Fasli et al, 2009).

Compared to wounds that worsened over time, wounds that went on to heal contained higher levels of proteins involved in wound healing (e.g. coagulation factors), skin structure and cell adhesion (e.g. keratin and tight junction proteins), as well as proteins that reduce inflammation (e.g. cystatin F, which attenuates immune cell killing, and peroxiredoxin 2, which removes reactive oxygen radicals).

While the number of samples in our proteomics analysis was small and underpowered, we discovered that samples clustered based on wound healing outcome, with older persistent wounds clustering based on their protein profiles. This data adds to our understanding that older/hard-to-heal wounds may be suffering from senescence or a significant imbalance in the wound environment.

Biofilm is not homogenous in the wound and not all 'yellow' equals slough. Microscopy techniques alone will not detect all bacterial biofilms.

The wound environment is complex, and it is difficult to know what microbes are present at the superficial and deeper layers without modern therapeutics and diagnostics.

In this study, slough samples contained a highly complex and diverse set of proteins.

Wounds that had been present for less than a year had higher levels of wound healing proteins (e.g. coagulation factors and cell proliferation), as well as immune responses (e.g. immunoglobulins).

Conclusion and future outlook

> Histology and biological and bacterial status of the wound contributes to a better understanding of how the body and wound respond to the presence of planktonic and biofilm microbes via a common chronic inflammatory response.

This study met our goals of improving our understanding of slough composition and laid the groundwork for future research. Our findings support the idea that slough is not the same as biofilm, but it does harbour multiple bacterial species. Non-healing wounds continue to pose significant management challenges, necessitating proactive care.

The wound history, clinical, molecular, microbiological, imaging and proteomic data will be combined in a future scientific publication to determine relationships between each of these factors. This highly unique and important dataset adds foundational knowledge to the question that prompted the start of this project 'what is slough?'. It also provides exciting preliminary evidence that the composition of slough itself may serve as a marker for wound healing outcomes. The next phase of this project will be larger in scope and will aim to strengthen the associations observed here between slough composition and clinical outcome.

RELEVANT MEDICAL HISTORY

- Type II diabetes
- Obesity
- Hypertension
- High cholesterol
- Peripheral neuropathy
- ABI 0.9 bi-laterally
- Palpable foot pulses.

Table 1. Wound dimensions

Wound length	4.5cm
Wound width	4.0cm
Wound depth	1.0cm
Wound shape	Round and oval

CASE 11

The patient is a 62-year-old male with type 2 diabetes. Due to an acutely infected DFU that developed osteomyelitis, he had his fifth toe amputated. The patient developed a post-operative infection at the debridement site, necessitating surgical debridement of the surrounding soft tissue. Three months later, this developed into a non-healing surgical wound, necessitating another revision debridement (3 debridements in total). This wound history strongly indicated a chronic biofilm infection.

At the time of sample collection, the patient was awaiting their elective surgery for the final revision debridement. The wound had fibrin buildup in the wound bed, which would quickly reform following debridement.

Figure 1 | Image of subject 11's wound before the debridement procedure

Table 2. Wound information

|	
Wound duration	16 weeks
Wound aetiology	Neuropathic DFU
Wound edges	Well-defined, not attached to base, rolled under and thickened
Necrotic tissue	Fibrin slough
Exudate type	Sanguineous and viscous in consistency
Exudate level	Very high
Granulation	Hypergranulation
Epithelialisation	0%

Figure 2 | Relative abundance of bacterial genera on the surface of the wound based on high-throughput bacterial 16S ribosomal gene sequencing. Molecular detection of wound bacteria identified the presence of several bacterial genera, including *Morganella sp.*, *Enterobacteriaceae sp*. and *Finegoldia sp*.
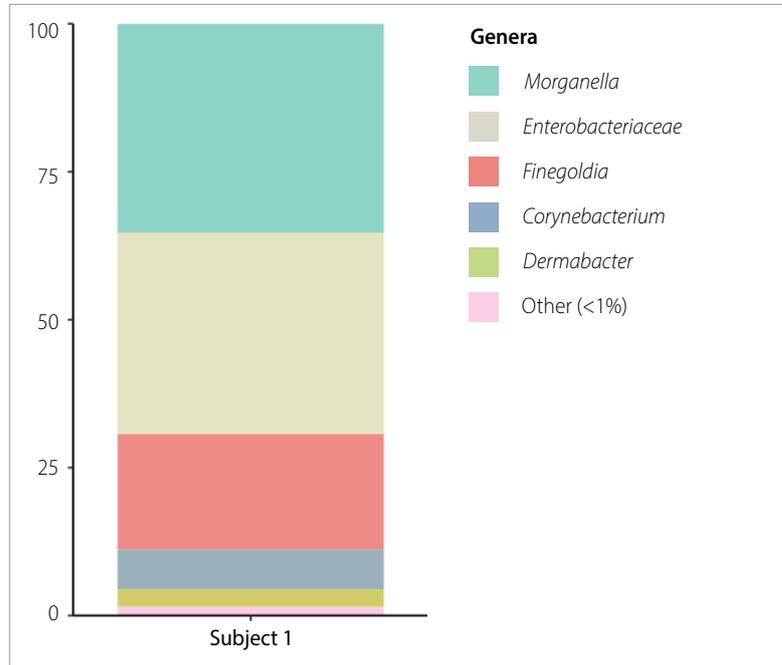
Figure 3 | Microscopy image of wound slough. Scanning electron microscopy (SEM) at 15.79KX magnification. The green arrow indicates an immune cell, while the yellow arrow indicates a small biofilm aggregate of coccoid cells.
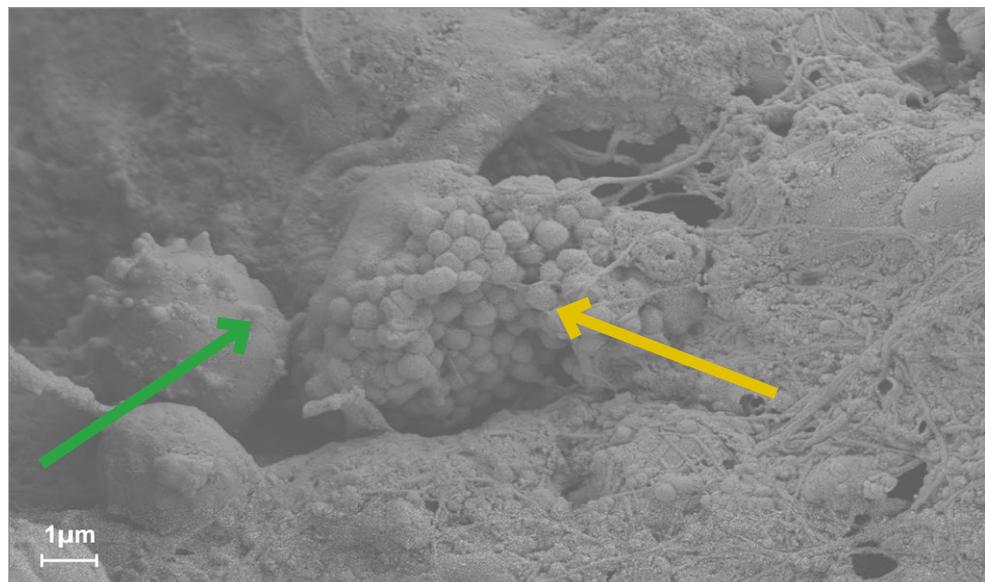
RELEVANT MEDICAL HISTORY

- Type II diabetes
- Obesity
- Hypertension
- High cholesterol
- Peripheral neuropathy
- Retinopathy
- ABI 0.7
- Non-palpable foot pulses, weak with monophasic Doppler waveforms.

Table 1. Wound dimensions

Wound length	6.0cm
Wound width	4.0cm
Wound depth	0cm
Wound shape	Elliptical

CASE 12

The patient is a 68-year-old male with type 2 diabetes and stage 5 chronic kidney disease and is on haemodialysis. He had a surgical wound that was healing slowly, his fourth and fifth toes on his left foot amputated and the surrounding infected tissue debrided. Figure 1 was taken three months after surgery and shows firmly adhered fibrin slough that reformed fully within two days of debridement. The patient also had fluctuating leg and foot oedema due to renal impairment.
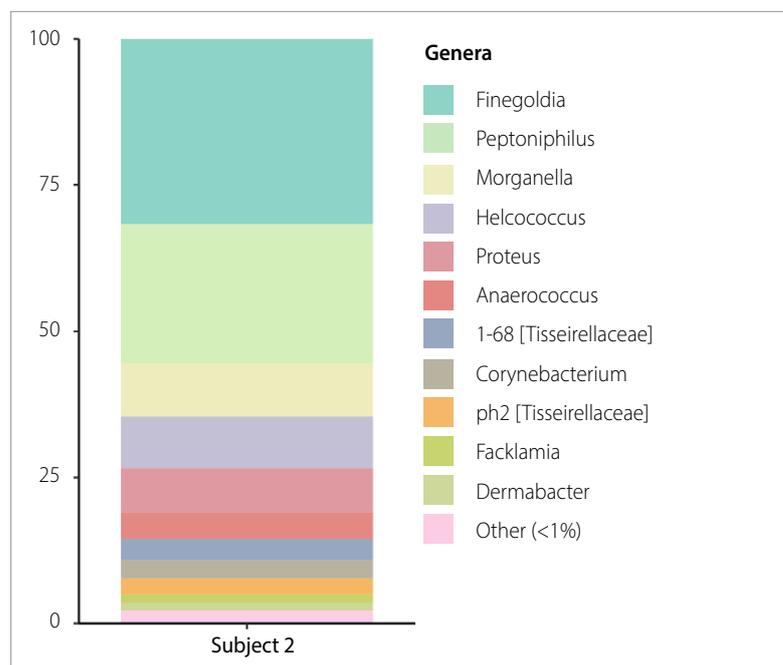

Figure 1 | Image of subject 12's foot wound 3 months after surgery and debridement procedure

Table 2. Wound information

Wound duration	12 weeks
Wound aetiology	Neuroischemic DFU
Necrotic tissue	Firmly bound fibrin
Exudate type	Serous and non-viscous in consistency
Exudate level	Very high
Granulation	Fibrous

Figure 2 | Relative abundance of bacterial genera on the surface of the wound based on high-throughput bacterial 16S ribosomal gene sequencing.
Molecular detection of wound bacteria identified fastidious anaerobes including *Finegoldia*, *Peptoniphilus* and *Anaerococcus*. Among the others were the gram-negative rods *Morganella*, *Bacilli*, and *Proteus*, as well as the gram-positive facultative anaerobe *Helcococcus*.

Genera
- Finegoldia
- Peptoniphilus
- Morganella
- Helcococcus
- Proteus
- Anaerococcus
- 1-68 [Tisseirellaceae]
- Corynebacterium
- ph2 [Tisseirellaceae]
- Facklamia
- Dermabacter
- Other (<1%)

Subject 2

Figure 3 | Microscopy image of wound slough. Scanning electron microscopy (SEM) at 13.94KX magnification. A large, dense aggregate of coccoid cells is seen attaching directly beneath the slough layer.
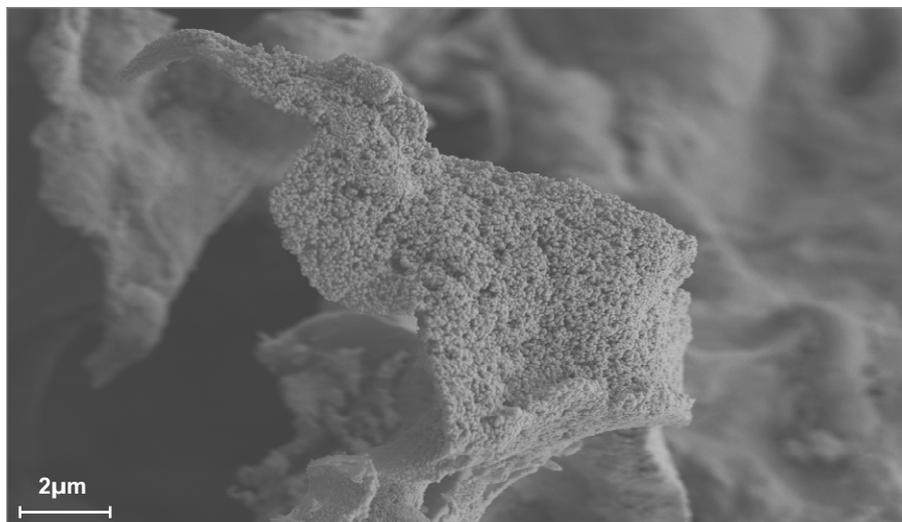
RELEVANT MEDICAL HISTORY

■ Type II diabetes
■ Peripheral neuropathy
■ Palpable foot pulses
■ ABI 0.9 bi-laterally.

Table 1. Wound dimensions

Wound length	2.8cm
Wound width	4.1cm
Wound depth	1.0cm
Wound shape	Irregular

CASE 13

The patient is 46-years-old with underlying peripheral neuropathy and type 2 diabetes. The patient developed this ulcer as a result of significant daily activities while wearing inappropriate footwear. At the time Figure 1 was taken, the ulcer had been healing on a slow trajectory but frequently presented with a thick gelatin-like slough.


Figure 1 | Image of subject 13's wound before the debridement procedure

Table 2. Wound information

Wound duration	13 weeks
Wound aetiology	Neuropathic DFU. Non-adherent to using offloading devices
Wound edges	Well-defined and not attached to base
Necrotic tissue	Gelatin slough
Exudate type	Serous
Exudate level	Moderate
Granulation	Hypergranulation
Epithelialisation	0%

Figure 2 | Relative abundance of bacterial genera on the surface of the wound based on high-throughput bacterial 16S ribosomal gene sequencing. Molecular detection of wound bacteria identified the presence of several bacterial genera, including *Corynebacterium sp.*, *Streptococcus sp.* and *Acinetobacter sp*.
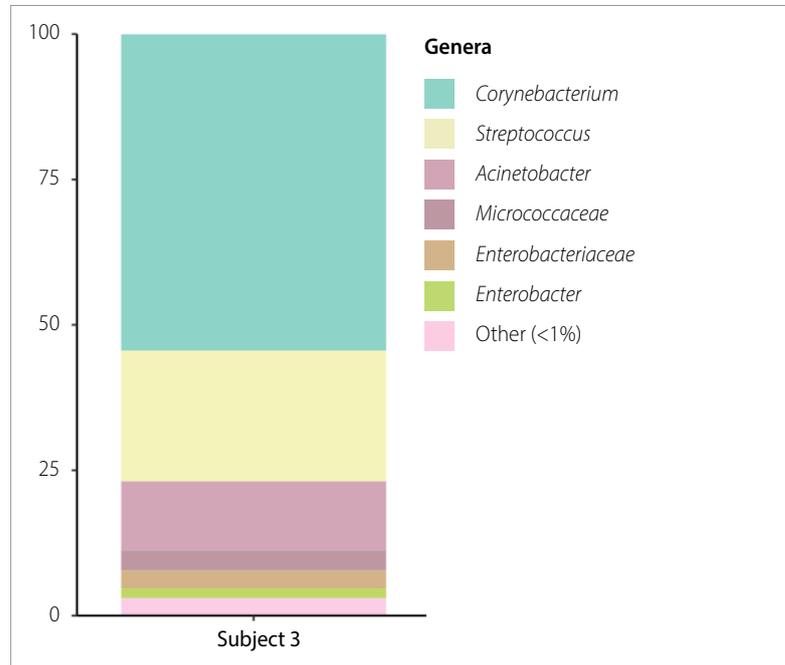
Figure 3 | Microscopy image of wound slough. Scanning electron microscopy (SEM) at 14.16KX magnification. An aggregate of microbial cells is visible.
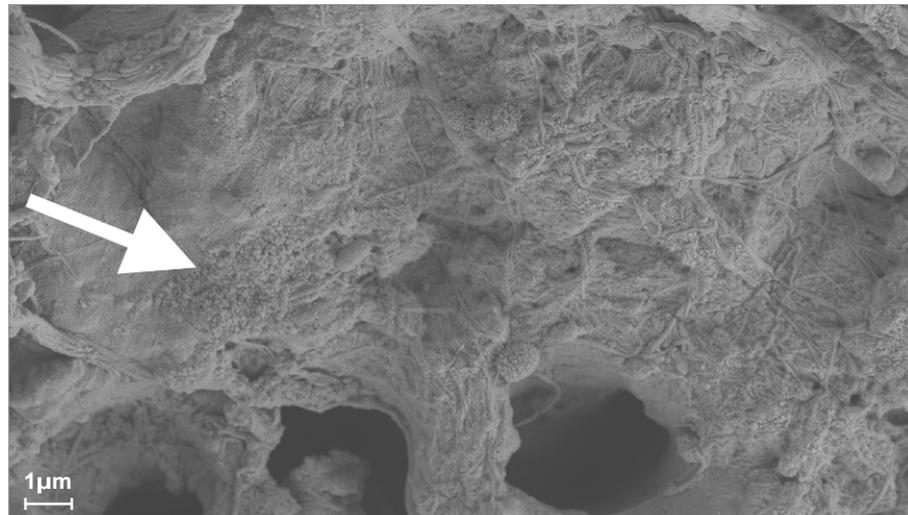
RELEVANT MEDICAL HISTORY

- Type II diabetes
- Obesity
- Hypertension
- High cholesterol
- Peripheral neuropathy
- Non-palpable foot pulses
- Chronic venous insufficiency.

Table 1. Wound dimensions	
Wound length	4.5cm
Wound width	4.0cm
Wound depth	1.0cm
Wound shape	Round and oval

CASE 14

The patient is a 66-year-old male with type 2 diabetes and pitting oedema. The patient was referred after developing a hospital-acquired pressure ulcer while being treated for a femur fracture caused by a fall. The wound was a slow-healing ulcer with a copious amount of slough. After debridement, the slough quickly rebuilt itself.


Figure 1 | Image of subject 14's wound before the debridement procedure

| Table 2. Wound information	
Wound duration	18 weeks
Wound aetiology	Stage 2 hospital-acquired pressure ulcer
Necrotic tissue	Gelatin-like slough
Exudate type	Serous and viscous in consistency
Exudate level	Moderate
Granulation	Poor, devitalised granular tissue
Epithelialisation	0%

Figure 2 | Relative abundance of bacterial genera on the surface of the wound based on high-throughput bacterial 16S ribosomal gene sequencing. Molecular detection of wound bacteria revealed a predominance of *Pseudomonas sp*.
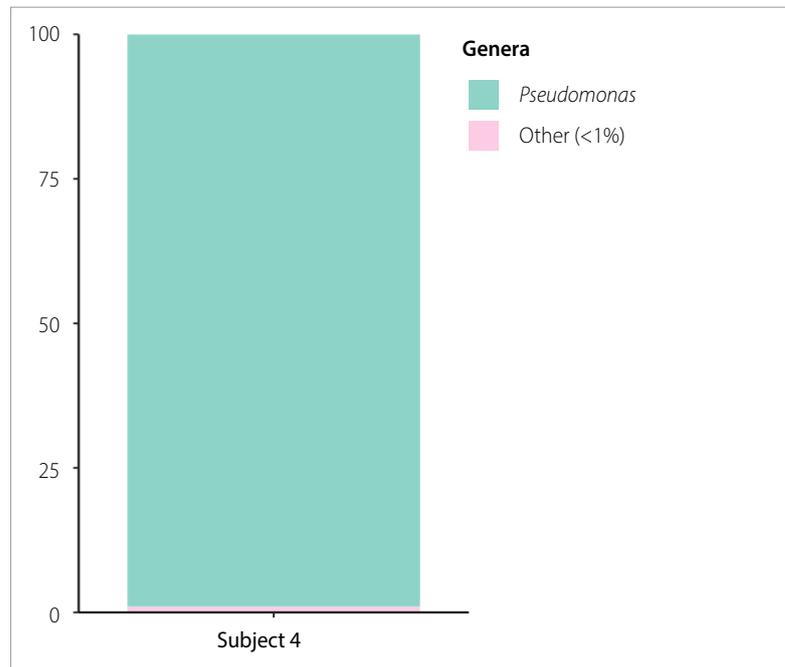
Figure 3 | Microscopy image of wound slough. Scanning electron microscopy (SEM) at magnifications of 4.42KX (A) and 36.86KX (B). There are small aggregates scattered throughout, but the vast majority are hidden by host material or bacterial-derived exopolysaccharides. The red outlines represent the magnified region.
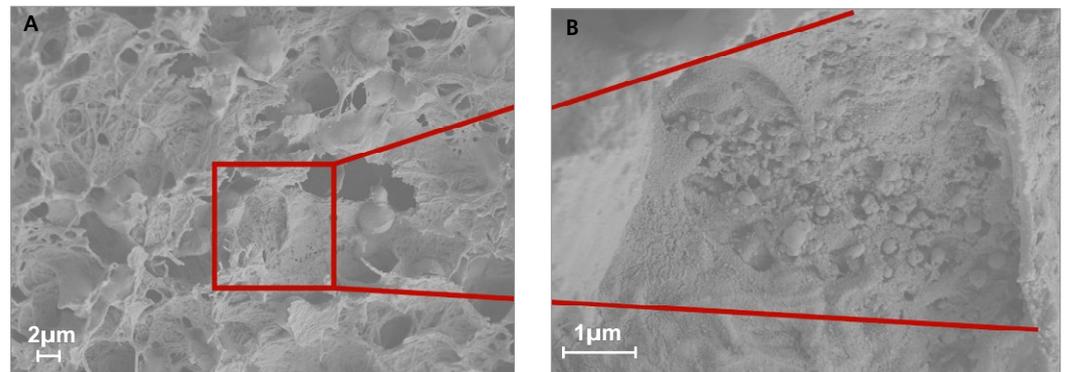
Literature search

S lough is, in general, an under-researched area of wound care with no clear definition. We conducted a literature search for slough, in order to ascertain the current knowledge and evidence around slough and how this affects wound healing. We searched the PubMed database for papers published between 2015 and the end of 2020 using a combination of search terms:

- Slough
- Slough + exudate
- Slough + exudate + wound
- Slough + exudate + wound + chronic wound.

See Table 2 for a summary of the literature, and Appendix 1 for the full literature search results.

Table 2. A summary of the literature search results	
Search terms	**Number of papers (excluding those unrelated to wounds)**
Slough	115
Slough + exudate	20
Slough + exudate + wound	12
Slough + exudate + wound + chronic wound	9

The majority of papers identified solely using the search term 'slough' did not relate to wound healing. In all search categories, most of the papers were simply reports of wound dressings that manage slough or biofilm, or focused more widely on wound bed assessment. There were very few papers that clearly reflected definitions and effects of slough, or the components of slough.

Only one paper (Pritchard and Brown, 2013) focused on understanding the components of slough through probing slough tissue using Western blotting. They were investigating for the presence of proteins with the capacity to engage microbial surface components. They detected adhesive matrix macromolecules; fibrinogen, fibronectin, immunoglobulin G, collagen, human serum albumin and matrix metalloproteinase-9 within the samples. The authors concluded that further research in this area is needed.

One paper (Petrlova et al, 2017) were identified through hand-searching. Petrlova et al (2017) observed that the addition of lipopolysaccharide or bacteria to human wound fluids leads to precipitation of protein aggregates, a phenomenon not observed in plasma.

A further study by Jung and Lee (2020) used a punch instrument, to perform 34 viable subeschar tissue cultures with a secure junction between the eschar and the normal skin. The authors noted that the patient's overall condition does not always allow for the complete removal of a dead eschar or injured slough in cases involving a pressure-ulcer skin lesion, particularly in bedridden and older patients. However, when a mature or immature eschar is treated without proper debridement, liquefaction necrosis underneath the eschar or slough tends to reveal a large, open wound with infectious exudate. The authors hypothesised that if the presence of any bacteria under the eschar could be evaluated and the progression of the presumed infection of the subeschar could be halted or delayed without creating an open wound, the final wound may be small, shallow and uninfected. The bacterial study had 29 positive results; based on these results and the patient's individual status, appropriate antibiotics could be selected and administered. The use of suitable antibiotics led to relatively shallow and small exposed wounds. Therefore, these results indicate that the early detection of infection and the use of appropriate antibiotics is beneficial to patients.

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